Quebec Cooperative Study
of Friedreich's Ataxia

The Neuropathy of Charlevoix-Saguenay Ataxia: An Electrophysiological and Pathological Study

J. M. PEYRONNARD, L. CHARRON AND A. BARBEAU

SUMMARY: *Two female patients aged 30 and 40 years with the Charlevoix-Saguenay ataxia were studied. Both had absent sensory action potentials in upper and lower extremities but, unlike typical cases of Friedreich's ataxia, they displayed a marked slowing of motor conduction velocities. Sural nerve biopsies taken from calf and ankle revealed a severe loss of large myelinated axons contrasting with a normal myelinated fiber density. Evidence for active axonal degeneration was scarce, with no indication of axonal regeneration. Teased myelinated fibers revealed an increased variability of internodal length but no evidence for myelin breakdown. These findings support, as a primary defect, a developmental abnormality of peripheral nerve, namely a lack of maturation of large myelinated axons and possibly a faulty myelination of nerve fibers. We think it is unlikely to represent a progressive axonal atrophic or dystrophic process, as suggested in Friedreich's ataxia.*

RÉSUMÉ: *Deux patientes âgées de 30 et 40 ans furent étudiées. Elles n'avaient aucun potentiel d'action sensitif enregistrable dans les membres supérieurs ou inférieurs mais différaient des cas typiques d'ataxie de Friedreich en raison d'un ralentissement très marqué des vitesses de conduction motrice. Les biopsies du nerf sural prélevées au mollet et à la cheville ont montré une perte sévère des axones myélinisés de gros calibre contrastant avec une densité normale de fibres myélinisées. Les signes de dégénérescence axonale active étaient extrêmement discrets sans évidence de régénérescence. Les études de fibres myélinisées isolées ont montré une variabilité anormale des distances internodales mais aucun signe de dégénérescence myélinique. Ces observations suggèrent l'existence d'une anomalie de développement des nerfs périphériques nommément un défaut de maturation des axones myélinisés de gros calibre et possiblement une myélinisation défectueuse des fibres nerveuses. Nous considérons improbable qu'il s'agisse d'une atrophie ou d'une dystrophie axonale progressive tel que suggéré dans l'ataxie de Friedreich.*

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INTRODUCTION

This paper reports the electrophysiological and pathological abnormalities found in the peripheral nervous system of 2 patients presenting with the autosomal recessive spastic ataxia of Charlevoix-Saguenay (Bouchard et al., 1978).

MATERIALS AND METHODS

Two female patients aged 30 (case I) and 40 (case II) years, with the typical features of the disease, were studied.

Electrophysiological techniques

The technical details have been fully described in a previous paper (Peyronnard et al., 1976). Sensory action potentials (SAP) together with sural nerve action potentials (NAP) were recorded with surface electrodes. Motor conduction velocities (MCV) were determined in the median, ulnar, common peroneal, and posterior tibial nerves, using surface recording electrodes. Electromyographic studies (EMG) of the extensor digitorum brevis, tibialis anterior, and gastrocnemius muscles were done with concentric needle electrodes. Patients were tested in a warm room (23° C). Records obtained from 10 normal subjects aged 25 to 35 years served as controls.

Histological techniques

Sural nerve biopsies were performed at the calf in case I and ankle in case II. In addition, a sample of the gastrocnemius muscle was obtained in case I. Control sural nerves from two normal subjects aged 33 and 35 years were taken at the calf (control I) and ankle (control II) respectively. Upon removal, nerve specimens were divided into three segments. The first was immersed for 2 hours in 2%

TABLE I

Electrophysiology

	Case 1	Case 2	Controls
Sensory Conduction (M/sec)			
Median	0	0	55,8±5,0
Ulnar	0	0	53,4±4,4
Sural	0	0	48,9±4,4
MOTOR CONDUCTION			
Distal latency (msec)			
Median	5,7	5,6	3,8±0,4
Ulnar	3,4	4,2	3,4±0,4
Common peroneal	10	9,4	4,8±1,0
Posterior tibial	—	10,3	5,0±1,0
Conduction velocity (M/sec)			
Median	41,8	41,3	57,8±4,2
Ulnar	39,4	44,6	59,7±5,3
Common peroneal	22,3	30,2	49,0±4,6
Posterior tibial	—	30,4	47,4±4,0

glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Post-fixation in 1% osmic acid was followed by dehydration in alcohol and embedding in epoxy resin. For phase microscopy, semithin sections were cut and stained with paraphenylenediamine. Ultrathin sections were examined with a Philips electron microscope after staining with lead citrate. Quantitative studies of myelinated fibers (MF) were made from photomicrographs of nerve transverse sections enlarged to a final magnification of x1,000. The number and size distribution of MF per nerve and per square millimeter of fascicular area were determined. The same parameters for unmyelinated fibers (UNF) were obtained from montage of nonoverlapping electron micrographs at a 10,000 magnification.

The second segment of the nerve was fixed in glutaraldehyde as already described. Post-fixation in 1% osmic acid for 3 hours was followed by maceration in glycerol for 3 days. Single fibers were isolated under a dissecting microscope for measurement of internodal length and diameter. The remainder of the nerve was frozen for longitudinal sections and stained with the modified Gomori trichrome technique. Frozen sections of the muscles were stained with the following histochemical techniques: modified Gomori trichrome, hematoxylinphloxine, succinic dehydrogenase (SDH), myofibrillar adenosine triphosphatase (ATPase) at pH 9.4 and 4.4, Oil Red 0, and periodic acid Schiff (PAS).

RESULTS

Electrophysiological studies

The same abnormalities were noted in both patients. As shown in Table I, median and ulnar SAP and sural NAP were absent. MCV were reduced markedly in the lower limbs and to a lesser extent in the upper extremities. EMG of the extensor digitorum brevis and tibialis anterior muscles revealed occasional fibrillation and positive sharp waves in case I, together with a mild increase of high voltage or polyphasic motor unit potentials during voluntary effort (cases I and II).

Nerve biopsy specimens

Light microscopy observations. The most obvious abnormality seen in sural nerve transverse sections was a marked reduction in the number of large myelinated fibers (Fig. 1). All the residual fibers had a normal appearance and only one swollen degenerating axon was observed in case II. Longitudinal sections of frozen material were normal with the exception of a single fiber showing a row of myelin ellipsoids in case II. No abnormality of perineurium, endoneurium, or blood vessels was noted.

Electron microscopy findings. The loss of large myelinated fibers was conspicuous. Remaining myelinated axons were normal except for the presence of an occasional degenerating fiber showing condensation of the axoplasm, with accumulation of mitochondria and vesicular bodies and degeneration of the myelin sheath (Fig. 2). Schwann cells associated with myelinated fibers were unremarkable. Unmyelinated axon-Schwann cell complexes appeared normal, although a mild axonal loss could be suspected in view of the increased number of collagen pockets together with

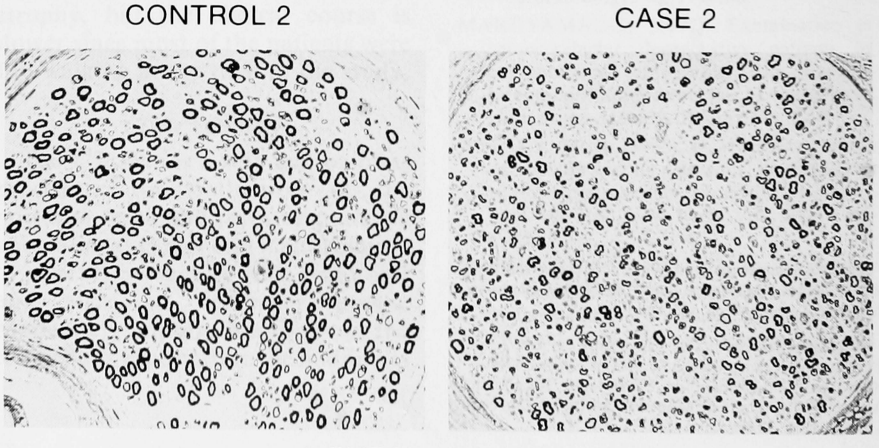

Fig. 1 — Cross section of sural nerve fascicle, taken from ankle in case II and matched control. An obvious loss of large myelinated fibers can be seen (phase micrograph x64).

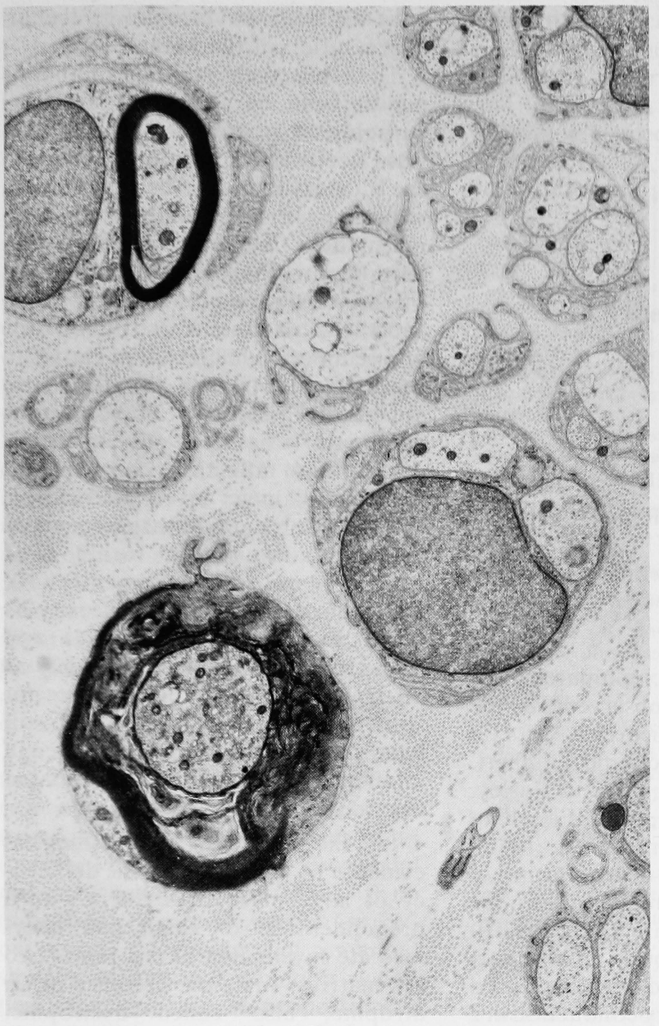

Fig. 2 — Sural nerve biopsy from case II. A degenerated myelinated axon shows clumping of neurotubules and neurofilaments, accumulation of mitochondria and small vesicular bodies and degeneration of myelin (cross sectional electronmicrograph x10,000).

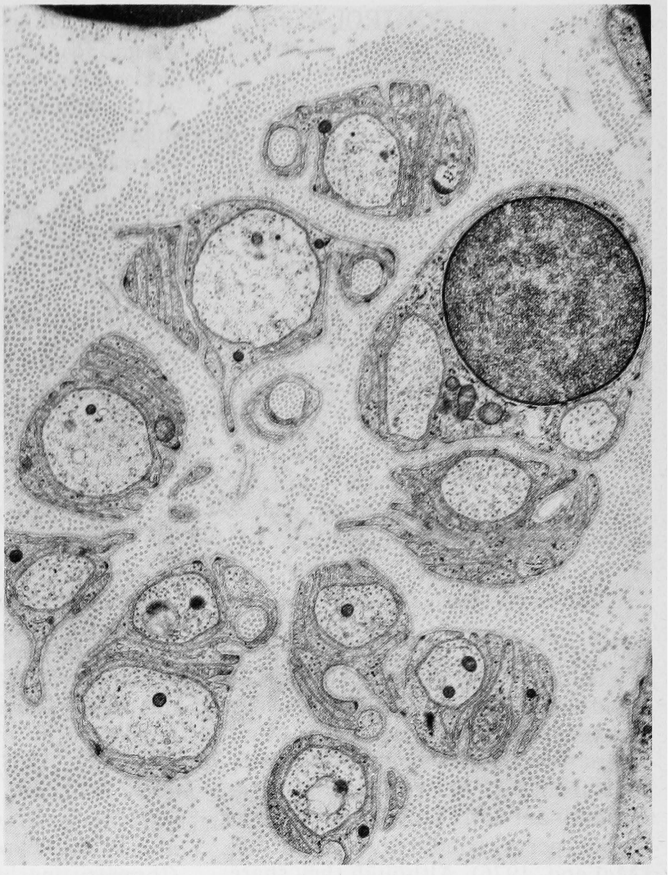

Fig. 3 — Sural nerve biopsy from case I. Unmyelinated axon — Schwann cell complexes showing an increased number of collagen pockets with slight budding of Schwann cell cytoplasm (cross sectional electronmicrograph x 15,600).

excessive layering of Schwann cell cytoplasm (Fig. 3). There was no evidence of regeneration of either myelinated or unmyelinated axons.

Teased nerve fiber studies. Single fibers were teased from the sural nerve of case II. The majority of fibers were of small and intermediate diameter. No fiber showed paranodal or internodal absence of myelin. Measurement of internodal distances and fiber diameters revealed in some fibers a higher than normal variability of internodal length (Fig. 4).

Quantitative results. The density of MF was 12,937/mm^2 in case I and 8,960/mm^2 in case II. The values are comparable to those obtained in control subjects (8,960 and 7,572/mm^2 for control I and control II, respectively). In spite of their normal density, the size distribution of MF was abnormal. As shown by the histograms (Fig. 5) most of the large myelinated fibers were absent. Fibers with a diameter greater than 8μ constituted 16.2% (case I) and 18.3% (case II) of all myelinated fibers, as compared with control values of 31% and 36% at calf and ankle respectively. The density of UNF was within normal range: 36,425/mm^2 in case I and 42,529/mm^2 in case II (Ochoa, 1969). No appreciable difference between patients and controls was noted in the size distribution histograms of UNF (Fig. 6).

Muscle biopsy specimen

In case I, where it was available, the muscle biopsy showed variations in fiber size, with mildly atrophic and hypertrophic fibers. ATPase and SDH reactions revealed moderate type grouping and hypertrophic fibers to be of type I. These observations are consistent with mild chronic denervation.

DISCUSSION

This report suggests that, besides distinct genetic and clinical features, patients with the autosomal recessive spastic ataxia of Charlevoix-Saguenay also have abnormalities of the peripheral nervous system at variance with those described in other forms of spino-cerebellar degeneration.

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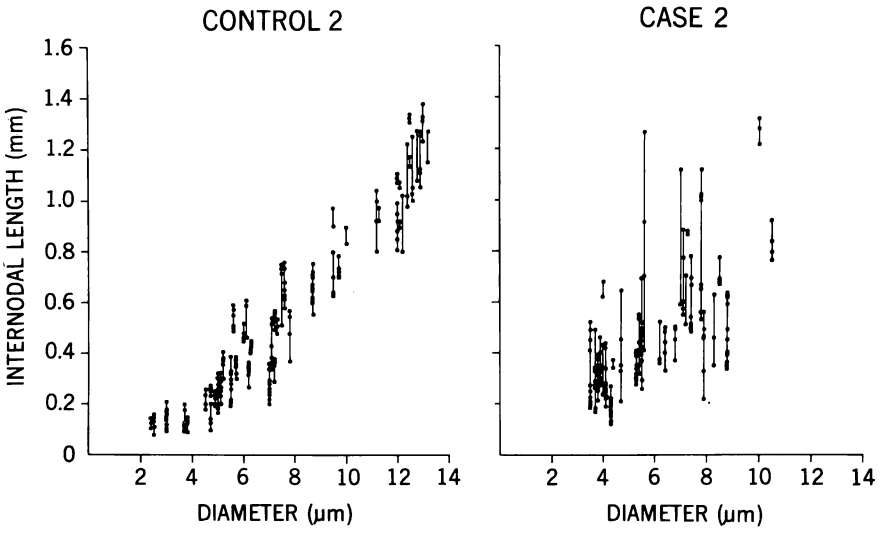


Fig. 4 — Distribution of internodal length in single fibres. Lengths of internodes in individual fibers are plotted against diameters and joined by a vertical line.

From an electrophysiological standpoint, our patients show a marked impairment of sensory and motor functions which distinguishes them from patients with spastic ataxia of dominant or recessive inheritance in whom nerve conduction is normal (McLeod, 1976). Although they share a complete absence of sensory action potentials with patients suffering from Friedreich's ataxia, the marked reduction of motor conduction velocities found in our cases represents a distinctive feature, compared with the mild slowing observed in the typical form of Friedreich's ataxia (Preswick, 1968; Dyck and Lambert, 1968; McLeod, 1971; Oh and Hasley, 1973; Salisachs et al., 1975; Fiaschi et al., 1976; Peyronnard et al., 1976). Comparisons with other forms of spino-cerebellar degeneration is difficult, the literature being insufficient with the exception of a report (McLeod, 1976) describing mild impairment of sensory conduction in 2 patients with olivo-ponto-cerebellar degeneration of the Menzel type.

These electrophysiological abnormalities can be correlated with the pathological changes observed on sural nerve biopsies. In both patients, the most striking feature is a marked reduction in the number of large myelinated fibers. This finding has been reported by several authors in Friedreich's ataxia (Friedreich, 1863; Mott, 1907 Hughes et al., 1968; Dyck and Lambert, 1968; Dyck et al., 1971; McLeod, 1971) and explains the absence of sensory nerve action potentials. It has been attributed to a degeneration affecting, early in life, the large diameter myelinated axons. In Friedreich's ataxia, progression of the disease would lead to a loss of smaller axons and consequently to a marked reduction in fiber density as noted by Dyck et al. (1971) and McLeod (1971) in patients aged 17 to 25. However, this mechanism does not seem to apply in our patients who still have, despite a disease which started in infancy, entirely normal myelinated fiber densities at age 30 and 40.

In the absence of histological evidence of regeneration, the deficiency of large myelinated fibers with a preponderance of small and intermediate myelinated axons raises two alternatives: first, the presence of an atrophic, also called dystrophic,

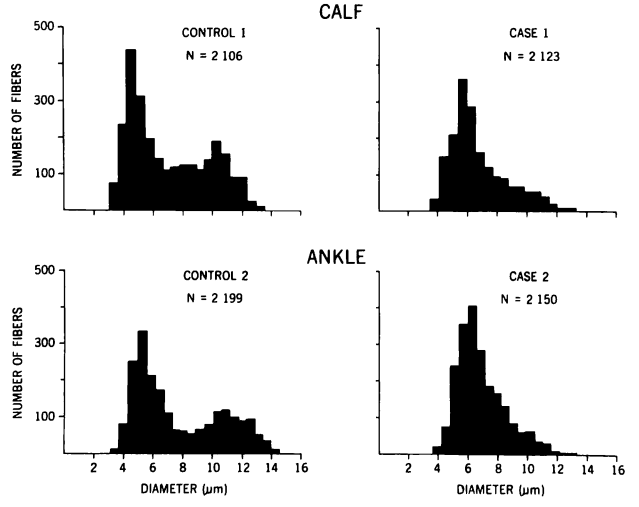
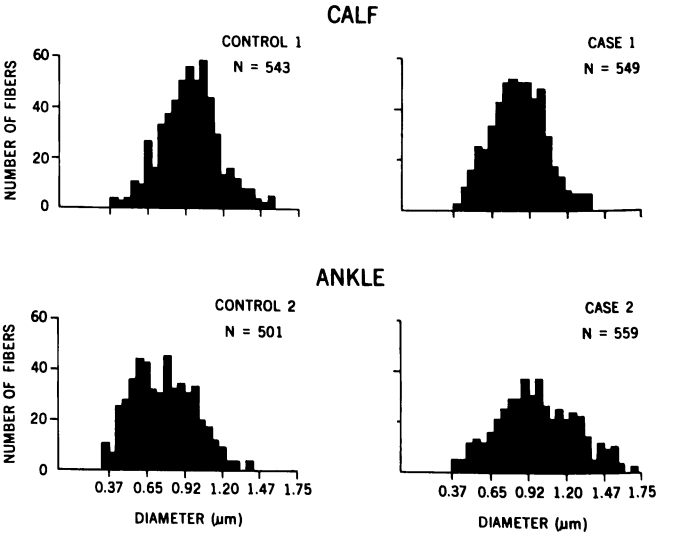


Fig. 5 — Fiber-diameter histograms of myelinated fibers from patients and controls. Note markedly decreased number of myelinated fibers in both patients.



Fib. 6 — Fiber-diameter histograms of unmyelinated fibers. No appreciable difference is noted between patients and controls.

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axonal process of slow progression (Greenfield, 1954; Dyck et al. 1971; Dyck and Ohta, 1975) in which large myelinated fibers shrink in diameter distally and so appear in smaller size categories; second, and this is our hypothesis, the existence of a developmental defect of the peripheral nervous system with lack of maturation of large myelinated fibers. In this instance one would expect, as is the case in our patients, the deficiency in the number of large myelinated axons to be of the same magnitude at proximal and distal nerve levels. This hypothesis should also be taken into consideration to explain the absence, in Friedreich's ataxia, of sensory action potentials in patients as young as 2 years of age (Dunn, 1973). Needless to say, a developmental abnormality does not exclude a superadded degenerative process of peripheral nerves, as documented in our patients by the presence of an occasional degenerating axon.

A developmental defect of myelination can also be raised on the basis of two observations: the existence on teased nerve fiber preparations of an increased variability of internodal distances along the same fiber, with no indication of active myelin breakdown, and the presence of a marked slowing of motor conduction velocities, seemingly out of proportion to what could be expected solely from a loss of large myelinated fibers, as in Friedreich's ataxia. It seems unlikely that these observations could represent merely demyelination secondary to axonal degeneration (Dyck and Lais, 1973), as changes suggestive of active axonal breakdown were very rare in our material.

In conclusion, we think that greater emphasis should be put on precise evaluation of peripheral nerve abnormalities, including morphometric studies, in order to better understand and distinguish the various pathophysiologic mechanisms underlying spino-cerebellar degenerations and related disorders.

ACKNOWLEDGEMENTS

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Quebec Cooperative Study
of Friedreich's Ataxia

A Cluster of Friedreich's Ataxia in Rimouski, Québec

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SUMMARY: *We described a cluster of 8 independent sibships of Friedreich's ataxia in the St-Fabien parish of Rimouski and have shown that they are all related within 6 generations. The study of this geographic and genetic isolate permitted the investigation of certain unusual features of the disease such as constant myopia, delayed reaction times to pain, flexor spasms, and a rapid evolution.*

RÉSUMÉ: *Nous décrivons un groupement de 8 fratries indépendantes d'ataxie de Friedreich dans la région de St-Fabien de Rimouski, et montrons qu'elles sont toutes reliées en dedans de 6 générations. L'étude de cet isolat génétique et géographique permet l'investigation de certains aspects inhabituels de la maladie, tels la présence constante d'une myopie, la lenteur des temps de réaction aux stimuli douloureux, les spasmes de flexion et l'évolution plus rapide.*

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INTRODUCTION

The renewed interest in the study of hereditary ataxias in Quebec in the last five years led first to the characterization of "classical" cases (Geoffroy et al., 1976). In that investigation of 50 patients, almost one third (14) were "atypical" on clinical and electrophysiological grounds (Peyronnard et al., 1976). Later, we identified a large group of patients (almost one hundred patients) presenting with autosomal recessive spastic ataxia in the Charlevoix and Saguenay area (Bouchard et al., 1978) and with electrophysiological findings similar to those of the atypical group in the first study (Peyronnard et al., 1976; Bouchard et al., 1979, this issue).

Recently, we had the opportunity to study a group of nineteen ataxic patients from another region of Quebec, clustered within 8 families of which at least seven are closely related. A few generations prior to the study, the ancestors were living in a small village, founded in 1828, and most of their descendants are still inhabiting Rimouski county on the south shore of the St-Laurent River, two hundred miles below Quebec City. The population of the county was around 65,000 in 1976.

Genealogical Studies

Because church records are complete in the Province of Quebec, it has been possible to trace most of the involved families who all lived within 25 miles of the original village of St. Fabien de Rimouski. As can be seen in Fig. 1, seven present sibships with different surnames can be traced to a single ancestral couple, through direct descent of probable carriers. There are many consanguinous marriages in this family and we are still trying to decipher the other probable carrier lines. The studies, to date, are sufficiently advanced to suggest that we are dealing with both a genetic and a geographical isolate. Such groupings are extremely important to unravel the respective influences of gene pools, environment, and other extrinsic factors.

Clinical Findings

It is known that hereditary ataxias encompass a number of variants, often creating a special form in many kindreds. Besides the numerous "intermediate and atypical forms", large groups of "classical" patients are linked genetically and also demonstrate features that contribute to the spectrum of abnormalities seen in spinocerebellar degenerations (Geoffroy et al., 1976; Tyrer, 1975). The group presented here underlines the fact that geographical and genetic isolation may produce significant differences from the general picture, without amputating the accepted list of symptoms and signs of Friedreich's ataxia. Such groups, with little inter and intra familial differences, may help to a better understanding of certain peculiar clinical aspects too diffuse in other kinships.

We know of and we have examined nineteen patients with the full blown disease in the Rimouski area. (A young girl of six has no symptom of ataxia and is not tabulated with the group. She exhibited areflexia and severe cardiomyopathy. Three other patients were dead at the time of the study.) There are 8 males and 11 females: ages ranges from 14 to 24 (mean 18.62) for males and from 14 to 34 (mean 23.27) for females for a global mean of 21.31. Of this group, five patients under 18 years of age can still walk. Ten patients were admitted to hospital and underwent the protocol investigations of the Quebec

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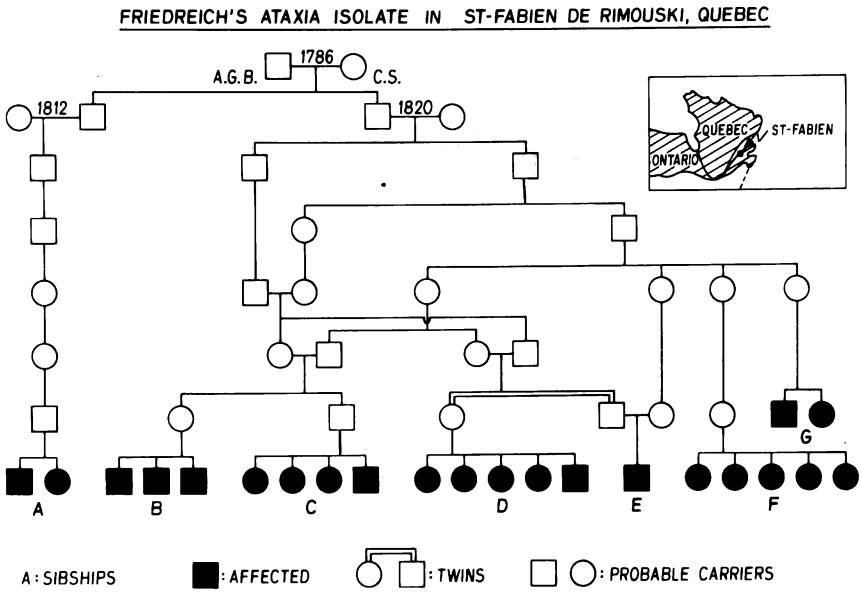

Figure 1 — In this diagram we traced one direct line of probable carriers to a common ancestral couple who married in 1786. Seven sibships can be traced. This diagram is simplified from the more complex one which would trace all possible carrier lines through the various consanguinous marriages. In the affected sibships, for the purpose of clarity, only affected children are indicated.

Cooperative Study. There were four males (18 to 20 year old, mean of 19.5) and 6 females (17 to 28 years of age, mean of 23.0). In this group the onset of ataxia was between 5 and 8 years of age. The disease then progressed steadily and the patients were usually in wheelchairs in their mid-teens. In one family there was some variation in the age of onset from 6 to 12 and less homogeneity in clinical signs. Early onset is not rare in Friedreich's ataxia (Tyrer, 1975; Geoffroy, 1976), but here it shows a strong familial similarity. The aim of this report is not to repeat and check on the long list of accepted symptoms and signs in Friedreich's ataxia. Rather, we would like to underline less common characteristics of this group, also reported occasionally in other kinships with the disease. Data are given for the ten hospitalized patients who completed the protocol. Ataxia of gait was always the first sign, but truncal ataxia soon became so marked that sitting was impossible. None of the 10 patients in hospital could remain sitting without holding the sides of the bed. Dysarthria was variable, often marked, and nystagmus was discrete in half the cases. Increased saccadic pursuit was noted in 2. The only eye problem noted was slight myopia in 9 of 10, symmetrical and varying between — 1 and — 3 diopters. Fundi and color vision were normal.

Spasticity in the lower limbs was exemplified by Babinski signs in 8 of 10 patients and by spontaneous or elicited flexor spasms in 6. Eight subjects complained of cramps in various muscles. The cutaneous abdominal reflexes were present in half the cases while deep tendon reflexes were abolished. The dissociation between the apparent flaccidity of the legs and the marked corticospinal dysfunction leading to flexor spasms has received attention from Tyrer (1975) and seems to be a rare finding.

Seven of the 10 patients presented with bladder problems, either urgency, stress incontinence, or frequent accidents. Dysphagia, especially for liquids, was more frequent affecting 9 of 10 patients. Gastric pain and vomiting, with normal gastrointestinal investigations, were prominent in one case. These symptoms have been reported rarely (Bell and Carmichael, 1939; Tyrer, 1975) and are attributed to autonomic dysfunction.

Most important were the sensory deficits. A definite decrease in proprioception was demonstrated by the absence of vibration sense at the toes and absence or decrease at the ankle, often asymmetrical. Position sense was also impaired in the toes in all cases but one. In two patients it was abnormal at the ankle and finger levels. Superficial sensory deficits were also present in most cases (7/10), consisting mostly of long delays between stimulus and perception of pain (up to 2 seconds) which then often provoked flexor spasms. Temperature perception was clearly impaired in one case, a 19 year old male in a wheelchair for 6 years who showed no perception of vibrations to the waist and marked delays in pain perception.

Radiological Evaluation

Scoliosis was present in all cases, slight in one (<20°), moderate (20-40°) in four, and severe (>40°) in five, up to 105° in the youngest patient admitted to hospital. Pes cavus was seen in most cases, but in some patients with no active distal movements only X-rays could clearly show the deformity. The neuroradiological evaluation is described elsewhere and indicates signs of superior vermis and anterior cerebellar lobe atrophy.

Cardiovascular evaluation

Eight of the ten patients evaluated were asymptomatic. Two patients with known hypertrophic cardiomyopathy were taking regular propanolol because of incapacitating symptoms: angina in one, and marked dyspnea and easy fatigability in the other. Arterial blood pressure was normal in all patients. Cardivascular examination was entirely normal in 3 patients. Two patients presented with an early systolic click at the apex. Two additional patients had a systolic ejection murmur at the left sternal border. One patient had an irregular rhythm which was due to the presence of numerous atrial premature contractions.

Radiological evaluation of the heart was rendered difficult by the presence

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of a marked scoliosis in most of the patients. In all cases where the c/t ratio could be evaluated, it was within normal limits. One patient had radiological evidence of left ventricular dilatation.

The ECG was normal in one patient. The remaining 9 patients presented the following ECG anomalies: infero-lateral ischemia in 4, septal hypertrophy in 2, right ventricular hypertrophy in 2, left axis deviation, atrial premature contraction and diffuse non specific anomalies of the repolarization phase in one case each. Echocardiographic examination was performed in 9 out of the 10 patients. The tracing was normal in two. Pansystolic prolapse of the mitral valve was observed in 3 cases. Left ventricular wall hypertrophy was present in 6 cases; it was of the concentric type in 3 while in the remaining 3 the echocardiogram showed asymmetric septal hypertrophy (ASH).

The 10 patients included in this study presented a wide variety of cardiovascular anomalies. The findings on physical examination were nonspecific in all but one patient who had auscultatory evidence of a sub-aortic obstruction. Ischemia in the infero-lateral region was the most common electrocardiographic findings (4/10); this may be secondary to poor perfusion of the sub-endocardial layers of the myocardium in the presence of left ventricular wall hypertrophy. This finding, either of the asymmetric variety or of the concentric type was the most common echocardiographic anomaly noted in this series. This is in accordance with the findings of Gattiker et al. (1976). None of the patients had signs of left ventricular outflow tract obstruction i.e. systolic anterior motion (SAM) of the anterior mitral leaflet or mid-systolic closure of the aortic valve. Mitral valve prolapse was observed in three patients.

While most patients presented with few if any abnormalities on physical examination, the ECG and the echocardiogram were abnormal in most cases and seem to be better indicators of the evolution of the cardiac involvement than the subjective complaints and the physical examination, as was previously noted in Phase One. Furthermore, our findings are in agreement with what has been reported (Boyer et al., 1962) in that there is no correlation between the degree of neurologic impairment and the severity of the cardiac disease.

Psychometric evaluation

The Ottawa-Wechsler intelligence battery was administered to all ten patients when in hospital. The patients were clinically observed while performing, specifically with respect to the degree of physical handicap and its possible influence on the results of different tests.

The mean z-scores (mean equals 0 and standard deviation equals 1 for the entire population) for each scale are presented in Table 1 with the t-test for paired data between both scales. Our patients had normal scoring for verbal scale (mean I.Q.: 100.80; SD: 11.56) but scored significantly lower ($P > .01$) on the non-verbal scale (mean I.Q.: 81.55; SD: 13.43). Only picture completion with a mean z-score of .03 was normal in that scale. The worst performance was with digit symbol (mean z-score: −2.19) and this is related partly to the clumsiness or the weakness of the patients' hands in a timed test. The lower performance on the other sub-tests could not be accounted for on this basis according to our clinical observations during the tests.

As a group, our patients scored better on both scales than another clinically different ataxic group recently studied (Bouchard et al., 1978), although the former were much more clumsy. This supports the view that physical disability cannot in itself explain all the low scores. If one excludes the digit symbol performance, the non-verbal profile still remains slightly lower than normal, although it now approaches the lower limits of the normal population. A greater number of patients would probably confirm this lower profile and better define its significance. There was no difference for either scale between males and females. Geoffroy et al. (1976) also found in their series a verbal I.Q. in the lower limits of normal, but females scored significantly lower. They did not report the non-verbal results, claiming their patients were too severely disabled to allow conclusions. Davies (1949) found in 18 patients a verbal Wechsler-Bellevue I.Q. close to normal (ranging between 75 and 117); for two other tests (the Mill Hill vocabulary test and a non-verbal test, the Raven's progressive matrices). Davies com-

TABLE 1

Means of each Ottawa-Wechsler Scale adjusted for Age, and T-Test for Paired Data between Verbal and Non-Verbal I.Q. (N: 10)

Verbal Scale	**Mean (z-score)**	
Information	−.50	
Comprehension	.47	VERBAL I.Q.*
Digit Span	.24	MEAN: 100.80
Arithmetic	−.80	S.D.: 11.56
Similarities	.70	
Non-Verbal Scale		
Picture Arrangement	−.35	
Picture Completion	.03	NON-VERBAL I.Q.*
Block Design	−.94	MEAN: 81.55
Object Assembly	−.98	S.D.: 13.43
Digit Symbol	−2.19	

* t: 11.49; $p < .01$

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pared his patients to a group of chronic invalids (non-neurological diseases) and found no significant differences. These studies, like ours, confirm that Friedreich's ataxia patients have an overall average verbal intelligence. Our study suggests that these patients have difficulties with non-verbal materials that are not due to their physical disability alone.

Electrophysiological studies

Detailed results of electroencephalography, electromyography, and nerve conduction studies are given in other papers in this issue. These results were identical to the data previously published on Friedreich's ataxia.

Laboratory results

Hematologic data were normal for all patients, as well as the results of folate and vitamin B_{12} determinations done with the kits of Bio-Rad Laboratories (Quanta-Count Folate Radioassay and Quanta-Count B_{12} Radioassay). Serum cholesterol and triglycerides levels were determined in another laboratory (A.D.) and were normal. Fasting blood glucose levels were normal and intravenous glucose tolerance tests failed to show any significant difference from normal.

Determination of total bilirubin was done on three consecutive days for the patients admitted to hospital, and for most patients of the whole group and their parents in a subsequent local visit in the field. Total bilirubinemia was slightly increased in three patients from 3 families and a similar increase was present in one parent.

Cerebrospinal fluid (CSF) was obtained by lumbar puncture in nine patients and showed normal cellular content. Total CSF proteins were normal and ranged from 13 to 43 mg% (mean 27.8). Electrophoresis did not disclose abnormal patterns in any of the cases.

CONCLUSIONS

The clinical picture of "typical" Friedreich's ataxia has been reviewed by Tyrer (1975) and by Geoffroy et al. (1976) who listed the primary and secondary symptoms and signs of the disease. Our patients conform to all these criteria for Friedreich's ataxia.

The homogeneous group that we have described issued from common ancestors and demonstrated almost universal myopia, flexor spasms, and delay in pain perception. Liquid dysphagia, urinary urgency, and incontinence were frequent and could have been underestimated in other series. Scoliosis, cardiac involvement, and electrophysiological studies confirmed the diagnosis. Psychometric evaluations showed better result than expected in non-verbal tests, despite the severe motor deficit.

Laboratory tests failed to disclose any diabetes mellitus in this group, but familial hyperbilirubinemia was found again in 37% of the families and one parent showed an increased value. Although no direct link can be seen between the dominantly transmitted Gilbert's disease and the autosomal recessive ataxias in Quebec (Friedreich's ARSACS), a 30 to 40% correlation has been found throughout this cooperative study.

The more "spinal" form of Friedreich's ataxia that we have reported here, demonstrated rare manifestations of spasticity (flexor spasm), sensory involvement (delayed pain perception), and a somewhat more severe course. Further investigation of these peculiarities may shed more light on the pathogenesis of the spino-cerebellar degenerative diseases. Such studies on geographical and genetic isolates will be more difficult in Quebec in the future, as in other parts of the world (Refsum, 1978).

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Quebec Cooperative Study
of Friedreich's Ataxia

Measurement of Ataxia and Related Neurological Signs in the Laboratory Rat

F. B. JOLICOEUR, D. B. RONDEAU, E. HAMEL, R. F. BUTTERWORTH AND A. BARBEAU

SUMMARY: *The purpose of the present study was to design a standard battery of tests capable of quantitatively characterizing ataxia and concomitant neurological signs in the rat. In addition to a systematic analysis of the walking gait of animals, tests for activity, catalepsy, rigidity, and various reflexive responses were included in the battery. The standardization and validation of the test system was performed by determining and comparing profiles of neurobehavioral effects produced by 3-acetyl pyridine, acrylamide, pyrithiamine, and thiamine deficiency, four experimental treatments reported to induce ataxia in animals. Results indicate that profiles of neurobehavioral disturbances accompanying ataxia in animals varied distinctively with each experimental treatment.*

RÉSUMÉ: *L'objectif de cette étude était de valider une batterie de tests conçue pour évaluer quantitavement chez le rat l'ataxie et les divers symptômes neurologiques l'accompagnant. En plus d'une analyse détaillée de la démarche spontanée de l'animal, cette batterie comportait l'examen de certains réflexes ainsi que des tests mesurant l'activité motrice, la catalepsie et la rigidité musculaire. Les profiles neurocomportementaux produits par quatre modèles expérimentaux de l'ataxie, soit, l'administration de 3-acetyl pyridine, d'acrylamide, de pyrithiamine et la déficience en thiamine, furent comparés afin de déterminer la validité de l'ensemble de ces mesures. Les résultats indiquent des différences marquées entre les profiles et démontrent l'utilité de cette batterie de tests comme instrument descriminatif dans l'analyse expérimentale des syndromes ataxiques chez l'animal.*

From The Clinical Research Institute of Montreal.

Reprint requests for the complete supplement on Friedreich's Ataxia (Phase Two, Part Two) to:

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INTRODUCTION

Standard methods and procedures for detecting and measuring ataxia in experimental animals are generally lacking. Usually, reports of ataxic symptoms in animals are based on visual observations and are qualitative descriptions of these symptoms. Furthermore, examination of possible concomitant neurological signs in ataxic animals is rarely performed. There is a need for more systematic and quantitative methods for the detection and assessment of ataxia in laboratory animals. Such methods would provide research tools for investigating the differential neurobehavioral symptomatology and potential therapeutic manipulations of various models of ataxia in animals.

The purpose of the present study was to devise a standard battery of tests capable of quantitatively characterizing various signs of ataxia in the laboratory rat. The selection of the various tests was based on their proven ability to measure neurobehavioral changes induced by drugs or other experimental manipulations in animals.

The standardization and validation of the test battery was performed by determining and comparing profiles of neurobehavioral effects produced by four experimental treatments reported to produce ataxia in animals. The following animal models of ataxia were selected:

3-Acetyl Pyridine

A single injection of 3-acetyl pyridine in rats (75 mg/kg) produces within 24 hours signs of cerebellar ataxia and damage to the medulla oblongata and climbing fibers of the cerebellum (Desclin, 1974). Microscopic examination of the CNS reveals lesions as early as 7 hours after injection (Desclin and Escubi, 1974). The ataxia resembles, both histologically and biochemically, the olivocerebellar atrophy originally described by Holmes (1907). Recent studies have demonstrated that the lesions are associated with changes in the levels of certain amino acids in specific regions of the CNS (Butterworth et al., 1978).

Acrylamide

Chronic administration of acrylamide in doses of 10-50 mg/kg in animals results in peripheral neuropathy characterized by proprioception impairments, hindlimb paralysis, and progressive ataxia (Hamblin, 1956; Kuperman, 1957). Histopathological examination reveals distal axonal degeneration of peripheral motor and sensory nerve cells (Spencer and Schaumberg, 1974). The neuropathy is first seen with a cumulative dose of 400 mg/kg and is most prominent with a cumulative dose of 500 mg/kg (Gipon et al. 1977).

Thiamine Deficiency

Chronic deficiency of vitamin B_1 results in pervasive metabolic and biochemical alterations in the nervous system. Thiamine deficiency induces a peripheral neuropathy of the "dying back" type that involves both sensory and motor nerve fibers (Schoental and Cavanagh, 1977). When rats are chronically fed a thiamine-free diet, a variety of disturbances such as anorexia, piloerection, tremors, kypokinesia and ataxia develop at about 30-40 days from the start of the diet (Yoshimura et al., 1976; McCandless et al., 1968).

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Pyrithiamine

Chronic administration of pyrithiamine, an antimetabolite of thiamine, results in central histopathological changes mostly localized in the pons and medulla oblongata (Yoshimura et al., 1976). Pyrithiamine also causes axonal degeneration of peripheral nerves (Yoshimura et al. 1976). Ataxic symptoms have been observed in animals injected daily for 18 days with 0.5 mg/kg pyrithiamine (Gubler et al., 1974).

METHODS

Animals

Fifty four male Sprague Dawley rats, 275-350 g in weight, were used. They were divided into nine groups of six animals each. Food, which consisted of standard Purina Rat Chow, and water were available ad lib except when specified in the procedure.

Procedure

For 3-acetyl pyridine, two groups of animals were used. One group received an acute intraperitoneal injection of 75 mg/kg 3-acetyl pyridine dissolved in physiological saline. Volumes injected were 1 ml/kg. Animals in the control group were injected with an equal volume of saline. Animals were tested for ataxia and other neurological symptoms at 6, 12, 24, 48 and 72 hours following injection.

Two groups of animals were included in the acrylamide model. Animals in the experimental group received ten successive daily injections of 50mg/kg acrylamide. Control animals were injected with saline. Acrylamide was dissolved in physiological saline and administered intraperitoneally in a volume of 1 ml/kg. Starting on the second day of acrylamide administration, tests were carried out 30 minutes following the daily injection procedures.

For thiamine deficiency, one group of rats was given a thiamine-free diet (ICN Life Sciences, Nutritional Biochemical) throughout the experiment. Since this regime results in hypophagia with ensuing body weight loss, animals in the control group received standard rat chow in daily rations equivalent to the amounts consumed by the thiamine deficient animals. Tests were performed on days 7, 14, 21, 27, 30 and 33. Starting on day 35, tests were carried out daily until day 44.

To study the effects of pyrithiamine, three groups of animals were given the thiamine free diet and assigned to one of the following experimental treatments as described by Gubler et al. (1974). Pyrithiamine treated rats were administered 100 μg/kg of thiamine. These treatments were given daily for 18 consecutive days. All substances were dissolved in saline and administered subcutaneously in volumes of 2 ml/kg. Animals were tested for neurological symptoms daily throughout the experiment.

Neurobehavioral tests

The neurobehavioral effects induced by the various treatments of this study were assessed by means of the following tests:

Locomotor activity: Spontaneous locomotor activity was measured for two minutes by means of a photocell activity apparatus (Lehigh Valley Electronics).

Catalepsy: Intensity of catalepsy was determined by a modification of the bar test described by Simon et al. (1969). An animal's front paws were placed on a horizontal wooden bar (1 cm in width) suspended 10 cm above the table. Time spent in that position, up to a maximum of 60 seconds, was recorded.

Rigidity: A rat was suspended by its front paws grasping a metal rod (0.5cm diameter) which was held by the experimenter about 50cm above the table. The time the animal remained on the bar (maximum 60sec) was recorded. A prolonged grasping response has been correlated with direct measures of muscle rigidity (Steg, 1964).

Landing foot spread: After staining the hindpaws with ink, an animal was held horizontally 30 cm above a table covered with absorbent paper. The rat was dropped and the distance between the marks of each hindlimb was recorded. This procedure has proved to be useful in detecting peripheral neuropathy in rats (Lee and Peters, 1976).

Gait analysis: After staining the hindfeet with ink, an animal was walked through an enclosed 90cm long corridor with a paper covered floor. When two consecutive strides were obtained, the stride width, length and angle between consecutive steps on contralateral sides were calculated. An illustration of this measuring procedure is presented in Figure 1.

Righting reflex: The animal was held by the tail and backflipped so that it somersaulted 2 or 3 times into the

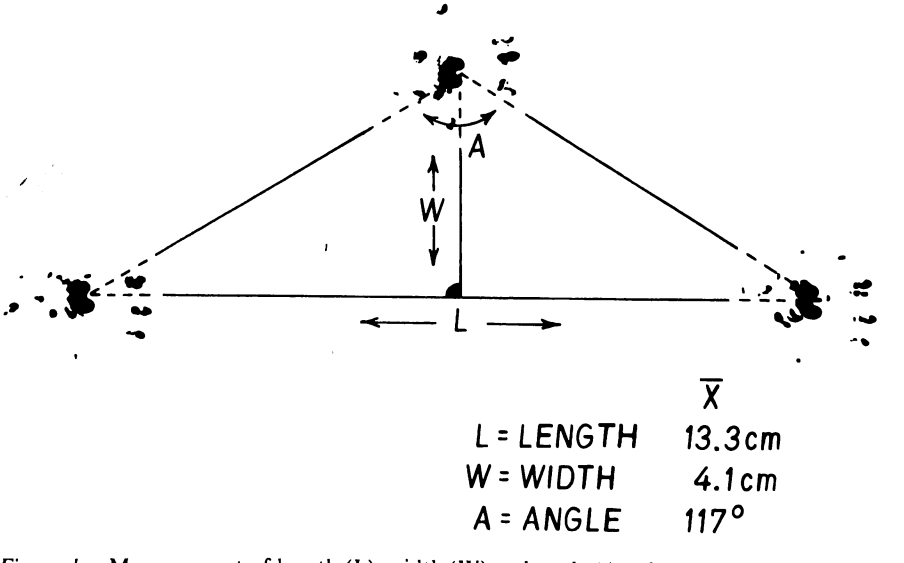

Figure 1 — Measurement of length (L), width (W) and angle (A) of steps in the analysis of rat's walking gait. A triangle is formed by drawing lines between steps. Mean values were obtained in six naive animals.

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air. The presence of normal (landing squarely on all four paws) or of abnormal righting response was recorded (Irwin, 1968).

Corneal reflex: The animal's cornea was touched lightly with the blunt end of a pencil and the presence of a normal eye blink was recorded (Irwin 1968).

Traction: An animal was held by the tail and pulled slowly on a table over a distance of about 60 cm. A normal animal extends is limbs so that the underside of the body is not in contact with the table during the pull.

Forelimb extension: The rat was held by the tail at about 60 cm over a table top and lowered briskly towards the table. The presence or absence of a normal forward extension of the forelimbs during descent was recorded.

Hindlimb extension: An animal, placed on a table, was lifted by the tail so that the hind quarters were suspended at about 15 cm over the surface, with the forelimbs still resting on the table. The presence or absence of a normal extension of the hindlimbs was noted.

Hindlimb position: Animals were vertically suspended under the forelimbs. Postural abnormalities under this condition consist of foot dropping, crossed legs and curled toes (Snyder and Braun, 1977). The presence of any of these signs and of other abnormalities was recorded.

Weight shift: Rats were placed on a wire mesh cylinder. The cylinder was turned in one direction and the presence of a normal opposite weight shift was noted. This procedure was repeated by tilting the cylinder in the other direction.

Tail pinch: Pressure was applied to an animal's tail by means of a pencil. A normal response was recorded if the animal vocalized, jumped, or struggled.

RESULTS

On each model, data obtained on activity, catalepsy, rigidity, foot landing spread, and the three gait components were analysed by individual multifactorial ANOVA's for repeated measures (Winer, 1971). Factors included in each analysis were groups and test periods. When significant groups by test periods interactions were found, simple main effects analyses were carried out at each level of the test period factor to determine when groups differed significantly from each other. When appropriate, significant differences between individual groups were assessed by means of Dunnett and Tukey tests. Results obtained on the various reflex tests constituted non-parametric data and were analysed by means of Fisher Exact Probability tests (Siegel, 1956). For all statistical analyses, a difference between groups was considered significant if it had a probability of random occurrence of less than 5 percent.

3-Acetyl Pyridine

Results obtained at 6, 12, 24, 48 and 72 hours after 3-Acetyl Pyridine were included in the statistical analyses. It was found that 3-Acetyl Pyridine treated animals displayed significantly more catalepsy and muscle rigidity, and had larger landing foot spreads than control animals at each of the five test periods. The treated animals were also found to be significantly less active than controls 6 hours after the injection, but not at the other test periods. For the three components of gait analysis, no significant difference between the groups was found 6 hours after injection. However, starting at 12 hours and for the remainder of the post injection test periods, treated animals were consistently ataxic as revealed by significantly smaller angles and stride lengths as well as by larger widths between steps.

Analyses of the results obtained with the various reflex tests indicated the following significant effects: At 24, 48 and 72 hours, treated animals had lost the righting reflex and the ability to maintain a normal hunched posture during the traction test. Starting at 12 hours and enduring for the remainder of the experiment, treated animals displayed an abnormal hindlimb position characterized by the feet being retracted and held closely to the body. Finally a disturbance in the weight shift response of treated animals was found at the 72 hours post injection test period.

These results are summarized in Table 1 where significant group

TABLE 1

Occurrence of Neurological Symptoms in 3-Acetyl Pyridine (75 mg/kg) treated Rats

	Hours				
	6	12	24	48	72
Gait Analysis					
Length of steps		●↓	●↓	●↓	●↓
Width of steps		●↑	●↑	●↑	●↑
Angle of steps		●↓	●↓	●↓	●↓
General Signs					
Motor activity	●↓				
Catalepsy	●	●	●	●	●
Rigidity	●	●	●	●	●
Landing foot spread	●↑	●↑	●↑	●↑	●↑
Reflexes (Loss of ...)					
Righting reflex			●	●	●
Corneal reflex					
Traction			●	●	●
Forelimb extension					
Hindlimb extension	NT	NT	NT	NT	NT
Hindlimb position		●	●	●	●
Weight shift					●
Tail pinch					

Full circle indicates significant difference from control group ($p<0.05$)
Arrows indicate direction of change
NT = Not tested

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differences are presented for each test period.

Acrylamide

Data obtained in each of the nine daily test periods were included in the statistical analyses. In comparison to controls, acrylamide treated animals manifested significantly higher scores of catalepsy in all nine test periods. They also displayed significantly larger landing foot spreads than control animals in the fourth, sixth, seventh, eighth, and ninth test period. Significant gait disturbances in acrylamide treated animals were found on all test periods including the first test period following acrylamide administration. The ataxia was characterized by significantly smaller stride angle and length and by larger widths between steps. The activity and rigidity scores of acrylamide treated animals did not differ significantly from those of control animals.

Results on the various reflex tests revealed that, in acrylamide treated animals, the righting reflex was absent in the eighth and ninth test period and that the hindlimb extension response was impaired in the ninth test period. An abnormality in hindlimb position manifested by foot dropping and an inability to maintain a hunched position during the traction test were also found on the last test period. All other reflexes were unaffected by acrylamide.

These results are presented in Table 2 where significant group differences are given for each test period.

Thiamine Deficiency

The results were analysed in two parts. First, a statistical analysis was performed on the data obtained on days 7, 14, 21, 27, and 33 of the experiment. This analysis indicated that only transient and sporadic effects were produced during this initial phase of thiamine deficiency. On day 21, thiamine deficient animals displayed significantly less locomotor activity than pair fed controls. A significant decrease in locomotion was also found on day 33 but not on day 27. The gait angles and widths of deficient animals were respectively decreased and increased on day 27 while length of stride was unchanged. On day 33 stride length was significantly decreased in thiamine deficient rats but the other two gait parameters remained unaffected.

The second part of the analysis dealt with the final phase of the experiment, i.e. days 35 to 44. During that phase, all thiamine deficient animals lost the righting reflex, displayed impaired weight shift responses, and eventually died. These effects were not seen in pair fed controls. The time of occurrence of the neurological symptoms and of death in the thiamine deficient group varied from animal to animal. Because of this, and in order to uniformly compare groups, the results obtained on days when individual thiamine deficient animals lost their righting reflex, were retained for analysis. Results collected in yoked pair fed controls on these days were also included in the analysis. No significant group differences were found for activity, catalepsy, rigidity, and landing foot spread. Also, aside from the righting reflex and weight shift response, no other reflexes were affected in thiamine deficient animals. Finally, gait analysis revealed that in thiamine deficient rats the angle and length of strides were significantly smaller than those of pair fed controls.

These results are summarized in Table 3 where all significant group differences are presented for each test period.

Pyrithiamine

Data obtained on days 1, 3, 6, 9, 12, 15, 16, and 17 of pyrithiamine administration were included in the statistical analysis. No significant group differences were detected for activity and rigidity. The landing foot spread of pyrithiamine treated rats was significantly larger than thiamine deficient animals throughout the experiment, but did not differ significantly from controls. For catalepsy, the scores of pyrithiamine animals were significantly higher than controls on day 9. The catalepsy endured until the end of the experiment except for day 16 where differences between the two groups failed to reach statistical significance. Pyrithiamine had a minimal effect on the gait of treated animals. The width of steps in pyrithiamine treated animals was significantly larger than

TABLE 2

Occurrence of Neurological Symptoms in Acrylamide (50 mg/kg) treated Rats

	Days								
	1	2	3	4	5	6	7	8	9
Gait Analysis									
Length of steps	●↓	●↓	●↓	●↓	●↓	●↓	●↓	●↓	●↓
Width of steps	●↑	●↑	●↑	●↑	●↑	●↑	●↑	●↑	●↑
Angle of steps	●↓	●↓	●↓	●↓	●↓	●↓	●↓	●↓	●↓
General Signs									
Motor activity									
Catalepsy	●	●	●	●	●	●	●	●	●
Rigidity									
Landing foot spread				●↑		●↑	●↑	●↑	●↑
Reflexes (Loss of ....)									
Righting reflex								●	●
Corneal reflex									
Traction									●
Forelimb extension									
Hindlimb extension									●
Hindlimb position									●
Weight shift									
Tail pinch									

Full circle indicates significant difference from control group ($p<0.05$)

Arrows indicate direction of change

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controls and this difference did not occur before day 17 of pyrithiamine administration. Similarly, reflexes were not affected until day 17 when pyrithiamine animals lost their righting reflex and their ability to maintain a normal body posture during the traction test.

Following the seventeenth injection the toxic effects of pyrithiamine precipitated. By day 18 two animals had died and the remaining rats were completely debilitated. In addition to the abnormalities found on day 17, these animals were incapable of sustaining locomotion, lost the forelimb extension reflex and could not emit a normal weight shift response.

These results are presented in Table 4 where significant differences between the pyrithiamine treated animals and controls are given for each test day.

An overall summary of the results obtained with all four models is presented in Table 5. Schematic representations of rats' walking patterns obtained under the various treatments of this study are presented in Figure 2.

TABLE 4

Occurrence of Neurological Symptoms Pyrithiamine (0.5 mg/kg) treated Rats

	Days								
	1	3	6	9	12	15	16	17	18
Gait Analysis									
Length of steps									IT
Width of steps								●↑	IT
Angle of steps									IT
General Signs									
Motor activity									
Catalepsy				●	●	●		●	●
Rigidity									
Landing foot spread									
Reflexes (Loss of ....)									
Righting reflex								●	●
Corneal reflex									
Traction								●	●
Forelimb extension									●
Hindlimb extension									
Hindlimb position									
Weight shift									●
Tail pinch									

Full circle indicates significant difference from control group ($p<0.05$)
Arrows indicate direction of change
IT = Impossible to test

TABLE 3

Occurrence of Neurological Symptoms in Thiamine Deficient Rats

	Days					
	7	14	21	27	33	35-44
Gait Analysis						
Length of steps					●↓	●↓
Width of steps				●↑		
Angle of steps				●↓		●↓
General Signs						
Motor activity			●↓		●↓	
Catalepsy						
Rigidity						
Landing foot spread						
Reflexes (Loss of ...)						
Righting reflex						●
Corneal reflex						
Traction						
Forelimb extension						
Hindlimb extension	NT	NT	NT	NT	NT	NT
Hindlimb position						
Weight shift						
Tail pinch						●

Full circle indicates significant difference from control group ($p<0.05$)
Arrows indicate direction of change
NT = Not tested

DISCUSSION

As expected, the four experimental treatments of this study induced ataxia as revealed by the gait analyses. However, the overall pattern of neurological signs accompanying the uncoordinated gait varied from treatment to treatment.

Of all treatments, 3-acetyl pyridine produced the most diversified effects (Table 5). This is not surprising in view of the known pervasive neurotoxic actions of this substance. A decrease in locomotor activity, an increase in landing foot spread, the presence of catalepsy as well as the appearance of distinctive muscle rigidity were found 6 hours after the administration of 3-acetyl pyridine (Table 1). This latency in the effects closely parallels the known time course of neuropathological changes induced by this substance (Desclin and Escubi, 1974).

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TABLE 5

Summary Table: Occurrence of Neurological Symptoms in all Treatments

	Treatments			
	Thiamine Deficient	Pyrithiamine	Acrylamide	3-Acetyl Pyridine
Gait analysis				
Length of steps	●↓		●↓	●↓
Width of steps	●↑	●↑	●↑	●↑
Angle of steps	●↓		●↓	●↓
General Signs				
Motor activity	●↓			●↓
Catalepsy		●	●	●
Rigidity				●
Landing foot spread			●↑	●↑
Reflexes (Loss of ...)				
Righting reflex	●	●	●	●
Corneal reflex				
Traction		●	●	●
Forelimb extension		●		
Hindlimb extension	NT		●	NT
Hindlimb position				●
Weight shift	●	●		●
Tail pinch				

Full circle indicates significant difference from control group ($p<0.05$)
Arrows indicate direction of change
NT=Not tested

However, it is noteworthy that the first evidence of ataxia in this study was not found until the 12 hour test period following injection of 3-acetyl pyridine.

The effects of acrylamide were different in several aspects (Table 5). Ataxia and catalepsy were seen after the first injection of acrylamide while an increase in landing foot spread was not found until the fourth injection (Table 2). The induction of strong and persistent catalepsy by acrylamide was unexpected. This effect of acrylamide has not been reported and it indicates that this substance may have widespread pharmacological effects in the CNS, aside from its documented neuropathological actions in the periphery. The rapid onset of ataxia after a single injection of 50 mg/kg is surprising in view of the known dose related neuropathological effects of acrylamide. As mentioned earlier, evidence of peripheral neuropathy is first detected after cumulative doses of 400 mg/kg (Spencer and Schaumberg, 1974). This suggests that the early ataxic gait of acrylamide treated animals is not due to neuropathological changes but must be related to other unknown effects of acrylamide. The abnormalities observed in the righting, traction, and forelimb extension reflexes as well as in the hindlimb position may be more related to neuropathy since they did not occur until the eighth injection, which corresponds to a cumulative dose of 400 mg/kg of acrylamide. In the hindlimb position test, acrylamide treated animals displayed foot dropping, an effect not seen with the other treatments in this study. This unique symptom may explain the unusual gait of acrylamide treated animals in the final days of injection. As can be seen in Fig. 2, the pattern of gait of acrylamide treated animals is distinctively slanted to the right, suggesting that these animals had more difficulty in bringing back one hindlimb in completing a stride.

Contrary to expectations, thiamine deficiency and pyrithiamine did not

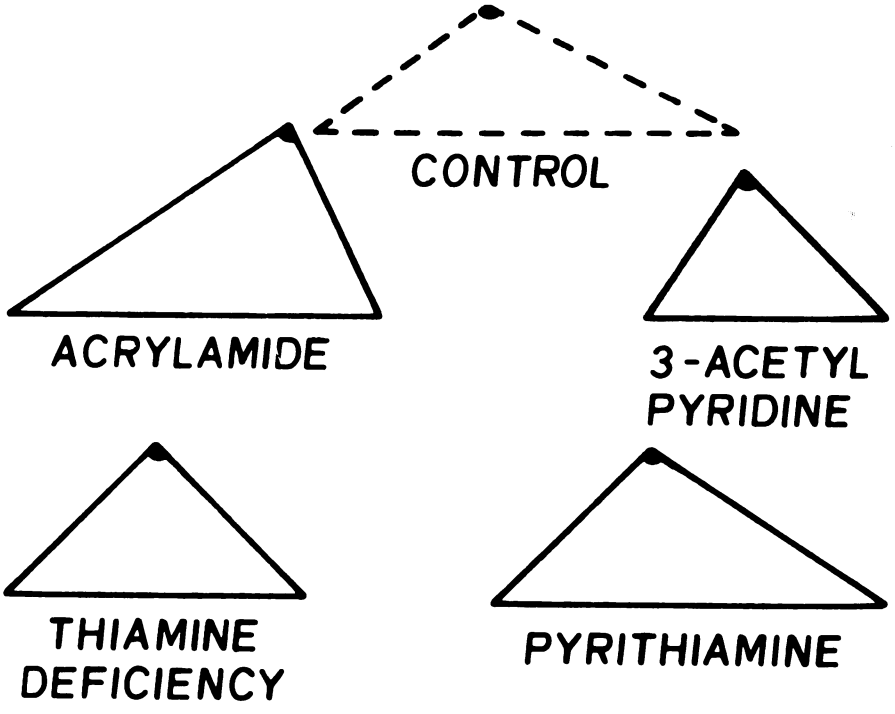

Figure 2—Schematic representations of gait of control and experimental animals in the four models of ataxia. Patterns were obtained in the final phases of each experimental treatment.

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yield similar neurobehavioral effects. Only the righting reflex and weight shift response were simiarly affected by both treatments. However, all three components of gait were disturbed in thiamine deficient animals while only the width of stride was altered in pyrithiamine treated animals. Thiamine deficiency, unlike pyrithiamine, decreased locomotor activity. Catalepsy as well as abnormal traction and forelimb extension reflexes were observed with pyrithiamine but not with thiamine deficiency. These striking discrepancies indicate that thiamine deficiency and administration of pyrithiamine, an antimetabolite of thiamine, affect the nervous system by distinct biochemical and/or neuropathological mechanisms.

Taken together, the results indicate that the battery of neurobehavioral tests used in this study constitutes a sensitive and reliable technique for detecting, quantifying, and differentiating various ataxic syndromes in experimental animals. The use of such a test system should prove to be valuable in future studies using animal models of ataxia.

ACKNOWLEDGEMENTS

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Quebec Cooperative Study
of Friedreich's Ataxia

Amino Acid Changes in Thiamine-Deficient Encephalopathy: Some Implications for the Pathogenesis of Friedreich's Ataxia

R. F. BUTTERWORTH, E. HAMEL, F. LANDREVILLE, AND A. BARBEAU

SUMMARY: *Thiamine-deficient encephalopathy in the rat is characterized by ataxic gait, loss of righting reflex and curvature of the spine. Neurochemical changes include a diminished activity of cerebral pyruvate decarboxylase leading to abnormal pyruvate oxidation. The present study shows that this defective pyruvate oxidation produces a significant depletion of three important amino acid neurotransmitters, namely gamma aminobutyric acid (GABA), glutamic acid, and aspartic acid. Such changes could lead to severe neuronal dysfunction and the observed neurological symptoms of thiamine deficiency. Some implications for the pathogenesis of Friedreich's ataxia are discussed.*

RÉSUMÉ: *L'encéphalopathie produite chez le rat déficient en thiamine se caractérise par une ataxie de la démarche et par la perte du réflexe de redressement (righting reflex). L'activité cérébrale de la pyruvate décarboxylase est diminuée de façon importante et ce changement neurochimique conduit à une oxydation anormale du pyruvate. Le présent travail montre qu'un tel défaut dans l'oxydation du pyruvate produit une diminution significative de GABA, d'acide glutamique et d'acide aspartique, trois acides aminés importants au cerveau. De tels changements seraient probablement à l'origine des symptômes neurologiques observés dans la déficience en thiamine. Nous discuterons des implications possibles dans la pathogénèse de l'ataxie de Friedreich.*

From The Clinical Research Institute of Montreal.

Reprint requests for the complete supplement on Friedreich's ataxia (Phase Two, Part Two) to:

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INTRODUCTION

It has recently been shown that Friedreich's ataxia is associated with certain biochemical abnormalities involving pyruvate metabolism (Barbeau et al., 1976) an abnormally low activity of serum lipoamide dehydrogenase (Melançon et al., 1977; Filla et al., 1978), and diminished activities of the pyruvate and α-ketoglutarate dehydrogenase complexes in various tissues (Blass et al., 1976). The latter finding has, however, not been confirmed by others (Barbeau et al., 1976; Melançon et al., 1978; Stumpf et al., 1978). Abnormalities of amino acid metabolism have also been reported in association with Friedreich's ataxia. These include diminished levels of glutamic and aspartic acids in affected regions of the spinal cord (Robinson et al., 1968), a decreased level of serum aspartic acid (Lemieux et al., 1976), and decreased glutamic acid and gamma aminobutyric acid (GABA) in certain cerebellar nuclei of affected patients (Huxtable et al., 1979, this issue).

Since glutamic and aspartic acids are now considered to be strong candidates as neurotransmitters in the mammalian central nervous system, any changes in their concentrations could lead to severe neuronal dysfunction and resultant neurological abnormalities. To study the possibility of a biochemical link between impaired pyruvate oxidation and the levels of glutamic and aspartic acids in the central nervous system, we have measured the concentrations of several amino acids in regions of the brain, spinal cord and retinae of rats suffering from a thiamine-deficient encephalopathy, a condition in which pyruvate oxidation has been shown to be impaired (Dreyfus and Hauser, 1965; McCandless and Schenker, 1968).

MATERIALS AND METHODS

Thirty six male Sprague-Dawley rats, 190-210 g starting weight, were used for the experiment. From time of arrival all rats received a thiamine-deficient diet (ICN Life Sciences, Nutritional Biochemicals) for a period of three days, after which time they were divided into three treatment groups (as previously described by Gubler et al., 1974):

Group 1. Control rats received 10 μg thiamine in 0.2 ml 0.85% saline per 100 g body weight per day.

Group 2. Thiamine Deficient rats received 0.2 ml 0.85% saline per 100 g body weight per day.

Group 3. Pyrithiamine-treated rats received 10 μg pyrithiamine in 0.2 ml 0.85% saline per 100 g body weight per day.

All rats were maintained throughout the experimental period on the thiamine-deficient diet ad libitum. Injections of saline, thiamine and pyrithiamine were subcutaneous in all cases. Rats were housed individually, had free access to water, and were weighed and fed daily. Conditions of temperature, humidity, and light cycles were kept constant throughout the experiment.

When neurological signs of thiamine deficiency (described in results) were apparent in the pyrithiamine-treated group, a pyrithiamine treated rat was sacrificed along with the appropriate non-symptomatic thiamine-deficient and control animals. Brains were rapidly removed on ice and dissected into the following regions: cerebral cortex, olfactory bulbs, cerebellum, medulla oblongata, hypothalamus, hippocampus, mid-

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brain and striatum. Retinae were also dissected out as were cervical, dorsal, and lumbar-sacral segments of the spinal cord. Nervous tissue was stored in liquid nitrogen until the time of assay.

For amino acid assay, each portion of nervous tissue was separately homogenized in 10 vol. $HC10_4$ (0.48 M) and the amino acids GABA, glutamine, glycine, glutamic acid, aspartic acid, and taurine were measured by the double isotope dansyl microtechnique described by Joseph and Halliday (1975). ^{14}C-radiolabelled amino acids and ^{3}H-dansyl chloride were purchased from New England Nuclear. Pyrithiamine and thiamine were obtained from Calbiochem.

RESULTS

1. Neurological Observations

Figure 1 shows the different weight gains in the three treatment groups during the experimental period. The control group displayed a regular weight increase during the 18 days whereas the thiamine-deficient group gained weight normally for the first week, maintained this for 5 days, then started to lose weight during the final period. In neither the control nor thiamine-deficient groups were there any abnormal neurological signs. The pyrithiamine treated group, on the other hand, showed drastic weight loss during the last 4 or 5 days. This weight loss was accompanied by problems of maintaining equilibrium, an ataxic gait, and in some cases convulsions when manipulated by the tail. A more detailed analysis of the abnormality of gait in these animals is described in an accompanying paper (Jolicoeur et al., this issue).

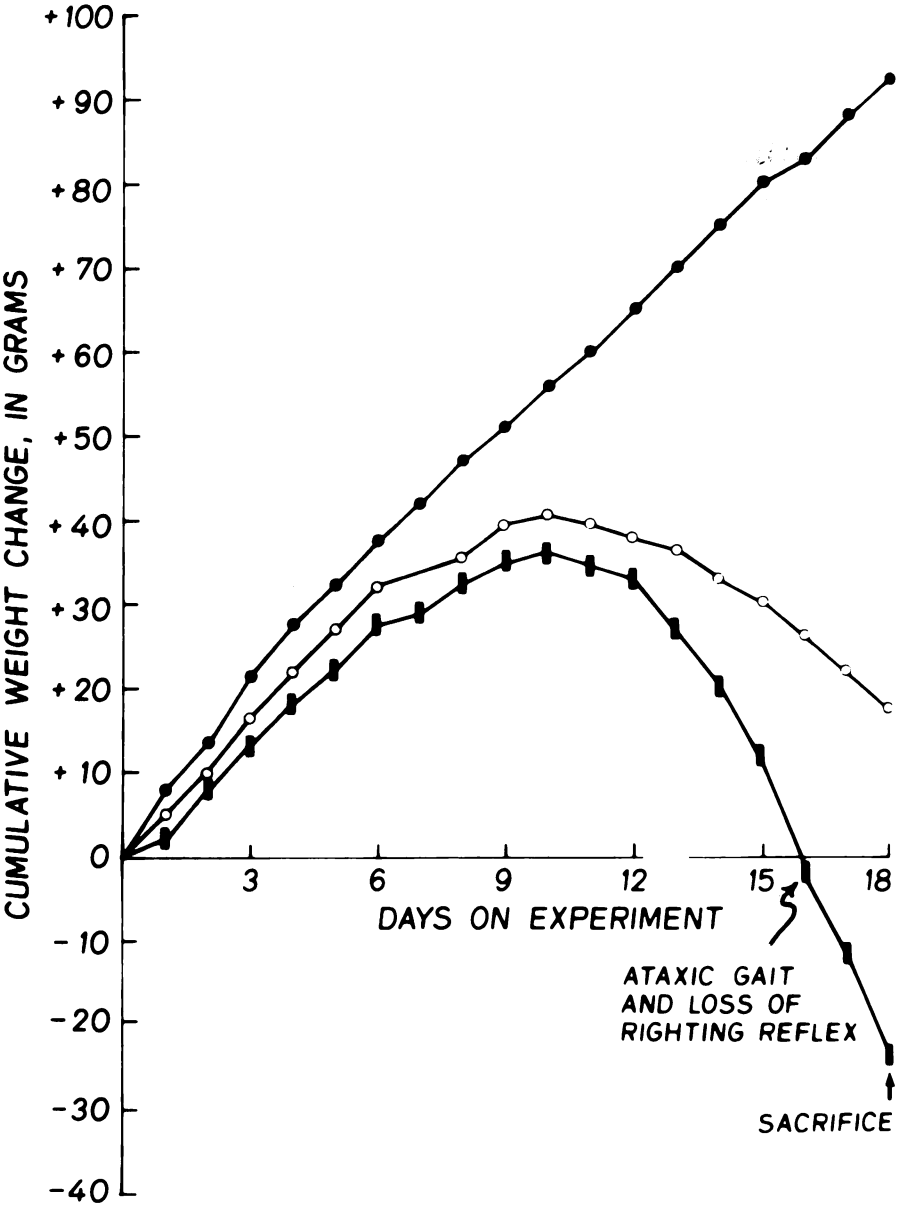

Figure 1 — Mean change in body weight of experimental rats
●——● controls; ○——○ thiamine deficient;
■——■ pyrithiamine-treated

2. Amino Acid Changes

Levels of GABA, aspartic acid, glutamic acid, glutamine, glycine, and taurine in pyrithiamine treated (symptomatic), thiamine deficient (asymptomatic), and control groups of rats are shown in Tables 1-6. Since only the pyrithiamine-treated group are symptomatic, statistically significant differences between amino acid concentrations in pyrithiamine treated rats *vs* both thiamine deficient and controls is most likely to be of importance in the production of neurological symptoms. Such changes include the following: Decreased GABA in medulla oblongata; Decreased aspartic acid in medulla oblongata, cortex and caudate nucleus; Decreased glutamic acid in midbrain, hypothalamus, and hippocampus; Increased glutamine in caudate nucleus.

DISCUSSION

Pyrithiamine is a central thiamine antagonist. It readily crosses the blood-brain barrier and leads to a depletion of brain thiamine and an increased urinary output of the vitamin. The mechanism of action of pyrithiamine is thought to be inhibition of thiamine phosphokinase, the enzyme responsible for the

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TABLE 1

GABA Concentrations

Groups		Brain Region								Spinal Cord			Retina (12)
		Cerebellum (12)	Medulla Oblongata (12)	Midbrain (12)	Cortex (12)	Caudate Nucleus (12)	Hypo-thalamus (12)	Hippo-campus (12)	Olfactory Bulbs (12)	Cervical (8)	Dorsal (8)	Lumbo-sacral (8)	
Pyrithiamine		1.64±0.20	1.44±0.06	3.13±0.31	1.69±0.07	2.60±0.21	3.96±0.10	2.51±0.10	3.22±0.18	1.37±0.05	1.23±0.08	0.81±0.16	2.00±0.09
Thiamine Deficient		1.70±0.16	1.66±0.07	3.35±0.12	1.87±0.11	2.42±0.12	4.48±0.30	2.35±0.18	2.96±0.14	1.15±0.06	1.27±0.07	0.61±0.11	1.86±0.11
Control		2.48±0.14	1.82±0.09	4.81±0.34	2.05±0.05	2.73±0.15	4.52±0.33	2.96±0.16	3.38±0.28	1.40±0.03	1.29±0.12	0.56±0.08	2.20±0.12
p	Pyr./Cont.	*	*	*	*	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Th.Def./Cont.	*	N.S.	*	N.S.	N.S.	N.S.	N.S.	N.S.	*	N.S.	N.S.	N.S.
	Pyr./Th.Def.	N.S.	*	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	*	N.S.	N.S.	N.S.

TABLE 2

Aspartic Acid Concentrations

Groups		Brain Region								Spinal Cord			Retina (12)
		Cerebellum (12)	Medulla Oblongata (12)	Midbrain (11)	Cortex (12)	Caudate Nucleus (12)	Hypo-thalamus (11)	Hippo-campus (12)	Olfactory Bulbs (12)	Cervical (8)	Dorsal (8)	Lumbo-sacral (8)	
Pyrithiamine		2.83±0.16	2.02±0.38	3.08±0.34	2.96±0.16	1.63±0.17	3.03±0.12	2.87±0.26	2.61±0.21	3.26±0.61	2.81±0.40	2.90±0.10	2.25±0.17
Thiamine Deficient		3.17±0.21	3.38±0.27	3.91±0.38	3.84±0.32	2.33±0.23	3.83±0.40	2.69±0.21	3.55±0.23	3.15±0.23	3.35±0.45	2.99±0.21	2.39±0.14
Control		3.55±0.20	3.84±0.18	6.00±0.35	4.04±0.13	2.21±0.13	5.34±0.39	3.62±0.37	3.17±0.33	2.79±0.23	3.96±0.52	2.45±0.17	2.52±0.12
p	Pyr./Cont.	N.S.	*	**	**	*	**	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Th.Def./Cont.	N.S.	N.S.	*	N.S.	N.S.	**	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Control	N.S.	*	N.S.	*	*	N.S.	N.S.	*	N.S.	N.S.	N.S.	N.S.

TABLE 3

Glutamic Acid Concentrations

Groups		Brain Region								Spinal Cord			Retina (12)
		Cerebellum (12)	Medulla Oblongata (12)	Midbrain (12)	Cortex (12)	Caudate Nucleus (12)	Hypo-thalamus (12)	Hippo-campus (12)	Olfactory Bulbs (12)	Cervical (8)	Dorsal (8)	Lumbo-sacral (8)	
Pyrithiamine		9.09±0.22	4.07±0.44	6.16±0.22	10.12±0.30	8.33±0.50	5.08±0.33	9.74±0.25	6.18±0.33	3.80±0.19	2.86±0.20	4.20±0.24	3.84±0.41
Thiamine Deficient		10.08±0.31	4.65±0.46	7.92±0.29	10.32±0.43	9.44±0.26	7.62±0.18	11.52±0.45	7.09±0.33	4.33±0.51	3.40±0.30	4.56±0.19	3.37±0.27
Control		9.86±0.20	5.03±0.50	8.27±0.37	11.76±0.38	8.81±0.37	7.10±0.29	10.97±0.25	6.91±0.35	4.13±0.08	3.44±0.47	4.22±0.26	4.24±0.38
p	Pyr./Cont.	N.S.	N.S.	*	*	N.S.	**	*	N.S.	N.S.	N.S.	N.S.	N.S.
	Th.Def./Cont.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Pyr./Th.Def.	*	N.S.	**	N.S.	N.S.	**	**	N.S.	N.S.	N.S.	N.S.	N.S.

TABLE 4

Glutamine Concentrations

Groups		Brain Region								Spinal Cord			
		Cerebellum (12)	Medulla Oblongata (12)	Midbrain (12)	Cortex (12)	Caudate Nucleus (12)	Hypo-thalamus (12)	Hippo-campus (12)	Olfactory Bulbs (12)	Cervical (8)	Dorsal (8)	Lumbo-sacral (8)	Retina (12)
Pyrithiamine		6.12±0.19	2.82±0.26	6.72±0.20	5.30±0.17	6.41±0.17	5.86±0.17	5.73±0.20	4.90±0.24	3.31±0.16	2.87±0.16	2.60±0.16	1.98±0.12
Thiamine Deficient		5.62±0.19	2.55±0.21	5.21±0.21	4.95±0.24	5.32±0.17	5.57±0.36	5.20±0.17	4.94±0.21	2.94±0.09	3.06±0.27	2.11±0.08	1.61±0.07
Control		5.47±0.11	2.57±0.23	5.98±0.17	4.89±0.28	5.44±0.10	5.35±0.22	5.02±0.18	5.03±0.35	3.17±0.10	3.97±0.50	2.36±0.14	2.07±0.13
p	Pyr./Cont.	N.S.	N.S.	N.S.	N.S.	**	N.S.	*	N.S.	N.S.	N.S.	N.S.	N.S.
	Th.Def./Cont.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	**
	Pyr./Th.Def.	N.S.	N.S.	**	N.S.	**	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	**

TABLE 5

Glycine Concentrations

Groups		Brain Region								Spinal Cord			
		Cerebellum (12)	Medulla Oblongata (12)	Midbrain (12)	Cortex (12)	Caudate Nucleus (12)	Hypo-thalamus (12)	Hippo-campus (12)	Olfactory Bulbs (12)	Cervical (8)	Dorsal (8)	Lumbo-sacral (8)	Retina (12)
Pyrithiamine		1.00±0.08	2.62±0.28	1.71±0.09	1.23±0.05	1.26±0.09	1.61±0.18	1.33±0.05	0.46±0.07	3.25±0.07	3.04±0.16	4.59±0.21	1.45±0.08
Thiamine Deficient		0.97±0.08	2.72±0.28	1.58±0.08	1.11±0.04	1.16±0.09	1.60±0.10	1.25±0.06	0.74±0.05	3.21±0.14	3.55±0.48	4.11±0.10	1.59±0.07
Control		1.04±0.07	2.78±0.33	1.78±0.06	1.14±0.04	1.21±0.10	1.78±0.23	1.31±0.05	0.47±0.04	3.13±0.08	3.30±0.47	4.12±0.20	1.58±0.09
p	Pyr./Cont.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Th.Def./Cont.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	**	N.S.	N.S.	N.S.	N.S.
	Pyr./Th.Def.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	**	N.S.	N.S.	N.S.	N.S.

TABLE 6

Taurine Concentrations

Groups		Brain Region								Spinal Cord			
		Cerebellum (12)	Medulla Oblongata (12)	Midbrain (12)	Cortex (12)	Caudate Nucleus (12)	Hypo-thalamus (12)	Hippo-campus (12)	Olfactory Bulbs (12)	Cervical (8)	Dorsal (8)	Lumbo-sacral (8)	Retina (12)
Pyrithiamine		3.04±0.21	1.92±0.12	1.78±0.11	3.31±0.20	6.16±0.38	1.57±0.24	4.80±0.32	10.66±0.57	1.60±0.22	0.55±0.08	0.40±0.17	20.51±1.29
Thiamine Deficient		3.36±0.26	2.19±0.09	1.69±0.15	4.13±0.16	5.70±0.46	1.53±0.21	5.41±0.38	9.85±0.48	1.53±0.25	0.74±0.17	0.55±0.12	19.94±1.12
Control		3.33±0.16	2.07±0.15	1.97±0.18	4.56±0.36	6.22±0.40	1.41±0.20	4.63±0.45	9.58±0.50	1.89±0.15	1.23±0.30	0.77±0.18	20.73±0.90
p	Pyr./Cont.	N.S.	N.S.	N.S.	*	N.S.	N.S.	N.S.	N.S.	N.S.	*	N.S.	N.S.
	Th.Def./Cont.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
	Pyr./Th.Def.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.

TABLES 1 to 6: — Amino acid levels are expressed in μmole per g wet weight and are presented in the upper part of the table. Values are the mean of triplicate determinations for each animal. Numbers in parentheses represent the number of animals used in each of the 3 groups. Data was analysed by means of a one way ANOVA for the 3 groups of animals in each of the 12 regions. Post hoc Dunnett and Tukey (a) tests were performed when significant differences were revealed by a given analysis of variance. p values obtained from these tests are shown in the lower part of the table; * $p < 0.005$, ** $p < 0.01$, N.S. Not significant.

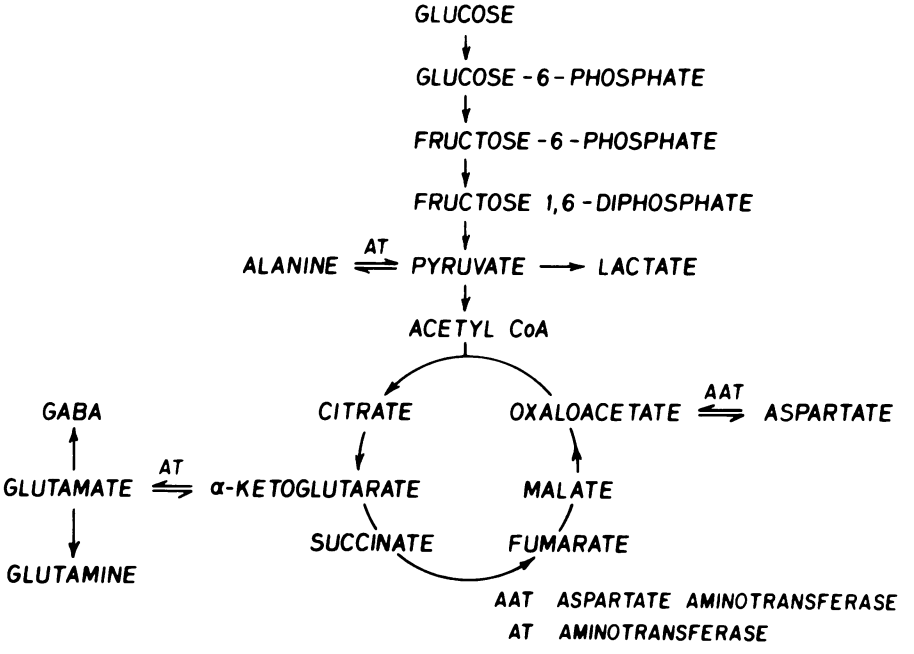
conversion of thiamine to thiamine pyrophosphate (TPP). It has been shown that whole brain activities of the TPP — dependent pyruvate dehydrogenase (PDH) and α-keto-glutarate dehydrogenase (α-KGDH) complexes are decreased by 50% in symptomatic pyrithiamine-treated rats (Gubler, 1961). Similar decreases of activities of these enzymes were reported in the brains of pyrithiamine treated mice (Hollowach, 1968), in addition to increased levels of brain pyruvate and α-ketoglutarate. These findings are consistent with the observed decreased activity of the PDH and αKGDH complexes (see Figure 2).

The modifications of GABA, glutamic acid, and aspartic acid resulting from pyrithiamine treatment may be explained in terms of diminished entry of pyruvate into the tricarboxylic acid cycle, resulting in impaired synthesis of the amino acids. Conversely, it is conceivable that these amino acids may be consumed as an alternative energy source such as has been shown to take place during the neonatal period (Devivo et al., 1975), under certain pathological conditions (Owen et al., 1967), and in *in vitro* experiments in which the supply of glucose to the brain is restricted (Mukherji et al., 1971). A selective depletion of these key amino acids could then lead to severe neuronal dysfunction resulting in the observed neurological symptoms associated with thiamine deficiency.

TABLE 7

Neurological and Biochemical Similarities between Friedreich's Ataxia and Thiamine Deficiency in Rats

	Thiamine Deficiency in Rats	Friedreich's Ataxia
Neurological	— Ataxia (neuropathy) — Curvature of the spine — Nystagmus — Cardiac abnormalities	— Ataxia — Neuropathy — Scoliosis — Nystagmus — Cardiomyopathy — Diabetes
Biochemical	— Glucose intolerance — Abnormal insulin response — Brain PDH and αKGDH activities diminished — Brain glutamate, aspartate and GABA diminished	— Glucose intolerance — Abnormal insulin response — Pyruvate intolerance to oral glucose load — Fibroblast PDH and αKGDH activities diminished (?) — Spinal cord glutamate and aspartate diminished — Cerebellar glutamate, GABA diminished (see this issue)



Metabolic pathways of labelled glucose in adult brain.

Figure 2 — Metabolic pathways of labelled glucose in adult brain.

These results are particularly interesting because thiamine-deficient encephalopathy in the rat shows many neurological and biochemical similarities to Friedreich's ataxia (see Table 7). For example, in addition to the ataxia, characteristic of prolonged thiamine deprivation, both curvature of the spine (scoliosis and cardia abnormalities, two cardinal features of Friedreich's ataxia, are also reportedly

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present (Warnock et al., 1968; Iwata et al., 1968).

Friedreich's ataxia is accompanied by clinical diabetes in 20% of patients, glucose intolerance in a further 20-30%, and abnormal insulin responses in 60% of patients studied (Shapcott et al., 1976). Similar glucose intolerance and insulin abnormalities have been reported in thiamine deficient encephalopathy in the rat (Iwata et al., 1974; 1970). The diminished activities of the PDH and αKGDH complexes, accompanied by increased pyruvate levels have been found in cases of intermittent cerebellar ataxia in children (Lonsdale et al., 1969; Blass et al., 1971) and defective pyruvate utilization has been described in Friedreich's ataxia (Barbeau et al., 1976). Whether or not Friedreich's ataxia patients' fibroblasts and platelets have enzymic defects in the PDH and αKGDH complexes is equivocal (Blass et al., 1976; Barbeau et al., 1976; Melançon et al., 1978; Stumpf et al., 1978).

The present studies suggest that defective pyruvate oxidation is accompanied by significant decreases in GABA, glutamic acid, and aspartic acid concentrations in the central nervous system. Whether such changes in amino acid levels are responsible for the neurological symptoms of Friedreich's ataxia is speculative. It is of interest, however, that diminished levels of glutamic acid and aspartic acid in the spinal cord of Friedreich's ataxia patients have been reported (Robinson et al., 1968) and that levels of GABA and glutamic acid appear to be diminished in certain areas of the cerebellum in Friedreich's ataxia (Huxtable et al., this issue).

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The Syrian Golden Hamster: A Model for the Cardiomyopathy of Friedreich's Ataxia

J. AZARI, T. REISINE, A. BARBEAU, H. I. YAMAMURA AND R. HUXTABLE

SUMMARY: *In light of the available information on the cardiomyopathy of Friedreich's ataxia, the cardiomyopathic Syrian hamster may be an appropriate laboratory model. Cardiomyopathy in these animals is a result of calcium accumulation. We analyzed the atria and right and left ventricles from cardiomyopathic (CM) and random bred (RB) animals for calcium, magnesium, and iron concentrations at 30-40 and 60-70 days of age (age of maximum lesioning). There are no significant differences in the concentration of iron or magnesium among age-matched groups. The concentration of calcium in the left ventricles of the CM animals at 60 days old is 14 fold higher than that of RB animals. Although there is a significant difference in the concentration of calcium in the left ventricles of younger animals, it is not as pronounced as the difference in older animals. Analysis of the taurine concentration in 30-40 day old animals revealed that the CM animals show slightly higher taurine concentrations than RB in the whole heart. In 60 day old CM hamsters the β-adrenergic receptor density of the ventricles is unchanged. This indicates that calcium overload is not due to adrenergic supersensitivity.*

RÉSUMÉ: *Nous croyons que le hamster Syrien pourrait constituer un modèle animal adéquat de la cardiomyopathie retrouvée dans l'ataxie de Friedreich. Chez ces animaux la cardiomyopathie résulte d'une accumulation de calcium. Nous avons analysé le contenu en calcium, magnesium et fer des oreillettes et des ventricules droits et gauches de hamsters cardiomyopathiques (CM) ou contrôles (random bred; RB), et ce à 30-40 et à 60-70 jours. Il n'existe aucune différence dans la concentration de fer ou de magnésium dans les groupes apparillés pour l'âge. La concentration de calcium est augmentée de 14 fois dans les ventricules gauches des animaux CM à 60 jours, par rapport aux contrôles RB. La différence dans le même paramètre n'est pas aussi marquée chez les animaux plus jeunes. La concentration de taurine est légèrement augmentée chez les animaux CM à 30-40 jours par rapport aux animaux RB (coeur entier). A 60 jours les hamsters CM ne montrent aucune modification dans la densité des récepteurs β-adrénergiques. Ceci indique que la surcharge calcique n'est pas due à une supersensibilité adrénergique.*

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INTRODUCTION

A common feature of Friedreich's ataxia is the presence of cardiomyopathy. It appears to be an integral part of Friedreich's ataxia and not a phenomenon secondary to the disease process (Sanchez-Casis et al., 1976). The one heart autopsied by Sanchez-Casis showed severe and diffuse intracellular fibrosis, with iron deposits and areas of intracellular calcification. There are a number of studies indicating that alterations in cell calcium uptake and content are closely related to cardiac necrotic processes (Fleckenstein, 1971; Fleckenstein et al., 1974; Fleckenstein et al., 1975). Calcium overload may be caused by a number of mechanisms. Hartman and Booth (1966) have suggested that sympathetic overactivity secondary to lesion of the vagal nuclei is responsible for myocardial damage. In such a case, the cardiac muscle is abnormally sensitive to adrenergic stimulation, which thus causes an increased flux of calcium across the cell membrane. Another possible biochemical abnormality is an impairment of oxygen transportation or utilization (Malo et al., 1976). An intrinsic impairment of oxygen availability to the cell would have a synergistic effect on calcium overload.

Taurine (2-amino ethane sulfonic acid) is a β-amino acid and is transported in the brain, heart, platelets, and kidney by a system specific for β-amino acids (Chubb and Huxtable, 1978; Hruska et al., 1978; Goldman and Scriver, 1967). As taurine concentrations in the heart are several hundred times higher than in the serum, its transport into the heart is energy dependent. The level of this amino acid in the heart is altered in certain cardiac disorders. Huxtable

and Bressler (1974) have shown heart taurine content is increased in humans dying of chronic congestive heart failure. The same laboratory has shown that taurine has a modifying influence on calcium kinetics (Huxtable and Chubb, 1976). Furthermore, it appears that taurine causes an increase in the affinity of calcium for various intracellular structures (Huxtable and Bressler, 1973; Dolara et al., 1973). These authors have suggested that the pathological effects of high cellular calcium concentrations are due to the free calcium ion, and taurine protects against energy depletion and calcified deposits by holding the extra calcium in a bound form. In a recent review (Huxtable, 1978), it was suggested that taurine may be closely involved in the pathogenesis of the cardiomyopathy associated with Friedreich's ataxia.

Since the direct study of cardiomyopathy in humans has numerous and obvious limitations, we have examined cardiomyopathic states in laboratory animals to find an appropriate model system. Huxtable (1978) suggested the use of the cardiomyopathic Syrian hamster as an experimental model for the cardiomyopathy that occurs in Friedreich's ataxia. Hamsters of the Bio 14.6 strain suffer a genetic cardiomyopathy that is autosomal and recessively transmitted (Lossnitzer and Bajusz, 1973). Cardiomyopathic lesions are detectable at approximately 60 days of age (Bajusz et al., 1969). The lesions are followed progressively by cardiac hypertrophy at about 120 days of age, compensated cardiac failure at about 200 days, and full failure with typical sequellae culminating in death at an age of approximately 300 days (Schwartz et al., 1972). In these animals we have made regional ionic and taurine analyses and have tested the responsiveness of the β-adrenergic system.

MATERIALS AND METHODS

Male cardiomyopathic Syrian golden hamsters (CM) of the Bio 14.6 strain and random bred control hamsters (RB) were purchased from Telaco, Bar Harbor, Maine.

Methods:

Spectral analysis: The CM and RB animals were sacrificed by decapitation. Hearts were removed and washed in 0.9% saline solution containing glucose (1.3 g/l). Atria, right and left ventricles were dissected, washed, and blot-dried before transfer to preweighed calcium-free volumetric flasks. The samples were digested for one hour in 65% ultrapure nitric acid (EM Laboratories, Elmford, New York) on a steam bath. Sufficient ionization buffer was added to make the final solution 1000 ppm cesium. After cooling, the samples were diluted with distilled deionized water. Precipitated proteins were filtered, and the solution analyzed by conventional atomic absorption spectrophotometry with a Varian AA5. An air/acetylene flame was used for iron and a nitrous oxide/acetylene flame for calcium and magnesium. The analytical wave lengths used were 422.7, 285.2, and 248.3 nm respectively for calcium, magnesium, and iron.

Protein determination: Protein determinations for receptor binding studies of the ventricle were performed by the method of Lowry et al. (1951).

Receptor binding assay: Tissue from each heart ventricle was homogenized with a polytron homogenized (Brinkman, setting 5 for 30 sec) to make a 2.5% homogenate in 0.05 M Na^+-K^+ phosphate buffer (pH 7.4). The homogenate was passed through several layers of cheese cloth, and centrifuged at 48,000 g for 20 min in a Sorvall RC 2-B centrifuge. The supernatant was measured and discarded. The pellets were resuspended in Na^+-K^+ phosphate buffer in a volume equal to that of the discarded supernatant. The suspension was rehomogenized for 5 seconds. β-Adrenergic receptors were assayed by the method of Bylund and Snyder (1976). Tissue homogenates containing 80 μg protein were incubated for 30 min at 25°C in 2 ml of 0.05 M Na-K phosphate buffer containing 0.25 μM

TABLE 1

Regional Cation Concentrations in Hearts from 30-40 Day Hamsters

	Location	Wet tissue weight (mg)	μg/g tissue Ca	Mg	Fe
RB	Right and left atria	8.7±0.4	45±5	223±23	not detectable
	Right ventricle	31.5±1.8	68±6	253±1	45±6
	Left ventricle	124.5±4.0	40±3	233±21	41±3
CM	Right and left atria	9.4±0.5	59±11	239±12	not detectable
	Right ventricle	40.0±3.5	68±5	247±1	39±2
	Left ventricle	133.0±5.6	68±8*	246±3	51±2

N=4 All values±SEM *$p<0.025$

TABLE 2

Regional Cation Concentrations in Hearts from 60-70 Day Hamsters

	Location	Wet tissue weight (mg)	μg/g tissue Ca	Mg	Fe
RB	Left and right atria	13.4±1.0	67±6	205±4	54±2
	Right ventricle	54.2±1.0	52±2	236±6	56±1
	Left ventricle	208.0±6.5	36±0	221±3	46±2
CM	Left and right atria	14.0±1.5	81±8	197±1	61±7
	Right ventricle	43.8±1.0	63±4	222±2	56±2
	Left ventricle	190.0±5.8	510±93**	217±5	47±0

N=4 All values±SEM **$p<0.005$

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^{3}H-dihydroalprenolol (DHA; 58 Ci/nmole, NEN) in the presence and absence of 0.1 μM (-)-propranolol. The reaction was terminated by vacuum filtration through GF/B glass fiber filters followed by four 5 ml rinses with buffer kept at 25° C. Bound ^{3}HDHA retained on the filter was extracted in 9 ml of a toluene based scintillation cocktail and radioactivity counted. Specific ^{3}H-DHA binding was defined as the binding displaceable by 0.1 μM (-)-propranolol.

Taurine analysis: Animals were sacrificed by decapitation and the heart removed, washed in saline, and dissected into areas. After being transferred to preweighed centrifuge tubes, the tissues were diluted 1:10 with3.5% 5-sulfosalicylic acid, sonicated to homogenity, centrifuged, and the supernatant analyzed for taurine.

RESULTS

There are no significant alterations in the concentrations of Fe and Mg in the atria, right or left ventricles of CM compared to RB hamsters at either 30 or 70 days of age. However, the calcium concentration of the left ventricle increases with age in CM relative to RB hamsters (Tables 1 and 2). Left ventricular calcium concentration is increased 70% in 30-40 day old animals, and is increased 1300% by 60-70 days of age. There are no corresponding changes in the right ventricle. In RB hamsters, the calcium concentration of the right ventricle is higher than that of the left at both age ranges (Table 1 and 2).

CM hearts show a moderate but significant increase ($p<.05$) in taurine concentration (Table 3). Regional analyses indicate that the right ventricle is the only area not showing an increased concentration relative to heart from RB hamsters (Table 4).

Analysis of a small number of hearts indicates that the development of hypertrophy is not associated with alteration in β-adrenergic receptor density (Table 5, Bmax unchanged). However, the affinity for ^{3}H-dihydroalprenolol may be decreased in CM hamsters. This altered affinity also is indicated by a Scatchard plot (Fig. 1). These data are based on only one RB and two CM hamsters.

DISCUSSION

Our study has shown a substantial increase (14 fold) in the concentration of calcium in the left ventricles of CM animals versus RB ones at 60-70 days. A less pronounced but statistically significant increase in calcium concentrations is also present at 30-40 days. Taurine concentrations are elevated in the atria and left ventricle in 30-40 day old CM hamsters. Our results, based on a limited study, do not indicate that supersensitivity of the β-adrenergic system is responsible for the calcium overload.

A decrease in the concentration of magnesium and an increase in the concentration of iron occurs in CM hamsters between ages of 30 and 60 days. However, when each age group is compared to its appropriate control group, such alterations are not

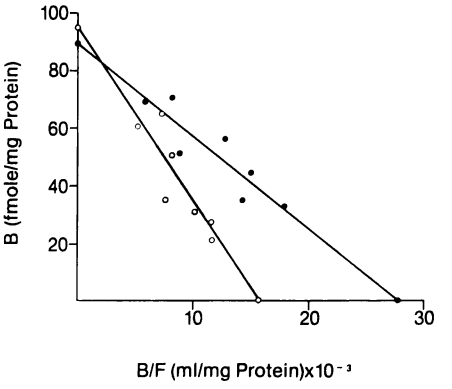

Fig. 1 — Scatchard plot of [^{3}H]-dihydroalprenolol binding to ventricular protein.
B = bound [^{3}H]-dihydroalprenolol (fmole/mg protein)
F = free [^{3}H]-dihydroalprenolol (fmole/ml)
• RB hamster ○CM hamsters (mean of two preparations).

TABLE 3

Taurine Concentration in Hamster Hearts (30-40 Days)

		Animal weight (g)	Heart weight (mg)	Taurine (μmole/g wet tissue)
RB	(N=9)	58.6±2.9	172±5	24.9±1.6
CM	(N=8)	62.4±2.6	183±7	30.4±1.8*

*$p<0.05$ All values±SEM

TABLE 4

Regional Concentration of Taurine in Hamster Hearts (30-40 Days)

	Taurine (μmole/g wet tissue) RB	CM
Right atrium	26.4±3.3	36.8±9.3
Left atrium	26.7±1.7	33.5±1.0**
Right ventricle	36.3±5.9	35.0±2.0
Left ventricle	26.9±3.6	29.4±1.8
Total Taurine	28.5±2.3	30.8±2.7

N=4 **$p<0.025$ All values±SEM

TABLE 5

β-Adrenergic Receptor Binding In Heart Ventricles (60 Days)

	Ventricular weight (mg)	Protein (mg/ml)	Bmax (fmole/mg protein	Bmax (nmole/ventricle)	K_d (nM)
RB	222.0	1.61	89.0	5.55	0.257
CM	260.4	1.58	95.0	5.85	0.477

These data are derived from one RB and two CM hearts.

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significant. Therefore, we believe such alterations to be an age-dependent phenomenon and are not directly involved in the cardiomyopathy.

The cardiomyopathy in these hamsters seems to be calcium dependent. Calcium overload may lead to cardiomyopathy in hamsters by activation of calcium dependant ATPases, leading to energy depletion; by impairment of energy production at the cellular level, or by increased calcium content not accompanied by comparable taurine alterations, leading to increased concentrations of free (energy depleting) calcium ions.

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Quebec Cooperative Study
of Friedreich's Ataxia

Erythrocyte Protoporphyrin Levels in Patients with Friedreich's and other Ataxias

R. O. MORGAN, G. NAGLIE, D. F. HORROBIN AND A. BARBEAU

SUMMARY: *Of 13 patients with Friedreich's ataxia (Type Ia) and 17 with type IIa recessive ataxias, all were found to have levels of "free erythrocyte protoporphyrin" (FEP) above the normal range. The rise in FEP in Friedreich's ataxia correlated well with the age of the individual and thus appears to be related to the course of the disease. Subjects with olivo-ponto-cerebellar atrophy, Charlevoix syndrome, Duchenne muscular dystrophy, and Parkinson's disease were also found to have significantly elevated FEP, although the distribution overlapped with the normal range.*

The finding of elevated FEP may indicate a relative heme deficiency in ataxia due to inhibition of ferrochelatase leading to a state of ineffective, persistent erythropoiesis. The possibility of a prostaglandin abnormality being related to this defect and to the pathogenesis of ataxia is considered.

RÉSUMÉ: *Nous avons trouvé des niveaux élevés de protoporphyrines érythrocytaires libre (FEP) chez chacun de 13 patients avec ataxie de Friedreich (type Ia) et 17 avec une ataxie récessive (type IIa). L'augmentation des FEP dans l'ataxie de Friedreich, et ses variantes, est reliée à l'âge des individus et donc semble liée à l'évolution de la maladie. Une légère augmentation des FEP fut également observée dans l'OPCA, le syndrome de Charlevoix, la dystrophie musculaire de Duchenne et la maladie de Parkinson, même s'il y a chevauchement des données avec les valeurs normales.*

Cette augmentation des FEP indique peut-être une déficience en hème dans l'ataxie par inhibition de la ferrochelatase avec, comme conséquence, une érythroporèse inefficace et persistante. Nous discutons du rôle possible des prostaglandines dans cette anomalie.

From the Clinical Research Institute of Montreal.

Reprint requests for the complete supplement on Friedreich's Ataxia (Phase Two, Part Two) to:

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INTRODUCTION

Despite the many neurological disturbances of inherited disorders of heme metabolism, the "porphyrias" (Goldberg, 1959), there have been, conversely, very few investigations of hematological indices in the hereditary ataxias (Szanto and Gallyas, 1966; Barbeau et al, 1976). A possible role of prostaglandins (PG's) and thromboxanes in ataxia has been suggested (Horrobin, 1978) and is supported by the importance of essential fatty acid metabolism in the CNS myelination process (Merton and Meade, 1977; Trapp and Bernsohn, 1978). Essential fatty acids are precursors of prostaglandins. Heme plays an important role in PG endoperoxide synthesis (Hemler et al, 1976) and, conversely, PG's are important modulators of erythropoiesis and attending heme synthesis (Dukes et al, 1975). If there is abnormal PG metabolism in hereditary ataxias, either the cause or consequence of the disease, then there should be abnormalities of porphyrin-heme biosynthesis as well. In particular, our recent studies have demonstrated a critical role for PG's in regulating protoporphyrin accummulation in chick embryo liver cells in culture (unpublished observations). The ease and simplicity of erythrocyte porphyrin analysis encouraged us to attempt to verify the predicted porphyrin abnormality in blood samples from ataxic individuals.

SUBJECTS AND METHODS

Heparinized blood samples were obtained from 164 patients of both sexes at this and other clinics in the province of Quebec. The ataxia groups included were type Ia (autosomal

recessive) Friedreich's ataxia (n=13), type IIa recessive ataxias endemic to New Brunswick (n=4)* and the Rimouski region of Quebec (n=13), autosomal dominant olivo-ponto-cerebellar atrophy (n=10), and Charlevoix syndrome (n=32), a recessive spastic ataxia. Known obligatory heterozygotes or children at risk for these conditions were also included (n=47). For comparison, several other groups were studied, including Duchenne muscular dystrophy (n=11), Werdnig-Hoffman syndrome (n=3), myasthenia gravis (n=1), Wilson's disease (n=1), Huntington's chorea (n=1), and Parkinson's disease (n=22). Normal control blood samples were obtained from healthy laboratory personnel, 5 male and 1 female in their twenties. Hematocrit or microhematocrit values were obtained for all individuals.

Assays for FEP were performed in quadruplicate on 50 μl aliquots of frozen-thawed whole blood lysates or, in the case of patients with muscular dystrophy, on fresh whole blood following an overnight fast. The method used was the double-extraction method described by Sassa et al (1975) modified only for larger volumes. 3 ml ethylacetate: glacial acetic acid (2:1, v:v) were mixed with 50 μl blood in a large test tube. 3 ml 0.5N HCl were then mixed in and the two phases allowed 15-30 min to separate. The lower aqueous phase (now 4 ml) containing better than 80% of all porphyrins was transferred by Pasteur pipet to a separate test tube. Fluorescence emission spectra were obtained using a Hitachi Perkin-Elmer model 204 spectrofluorometer equipped with an R777 Hammamatsu red-sensitive photomultiplier. The excitation wave length was 410 nm and emission peak heights at 602 and 660 nm were determined for each sample after zeroing with solvent and calibrating with standard solutions of 50 pM Rhodamine B in ethylene glycol and 10 nM protoporphyrin IX in 0.5 N HCl:glacial acetic acid (3:1, v:v) the peak height ratio (602/660 nm) allowed identification of protoporphyrin as the main porphyrin component in the samples analysed, and the second peak height at 660 nm was used for calculation of concentrations. Assays of uroporphyrinogen-I-synthetase were performed as described by Sassa et al (1974) on 10 μl aliquots of frozen-thawed whole blood lysates. Statistical comparisons of experimental data were made using the Student's two-tailed t-test.

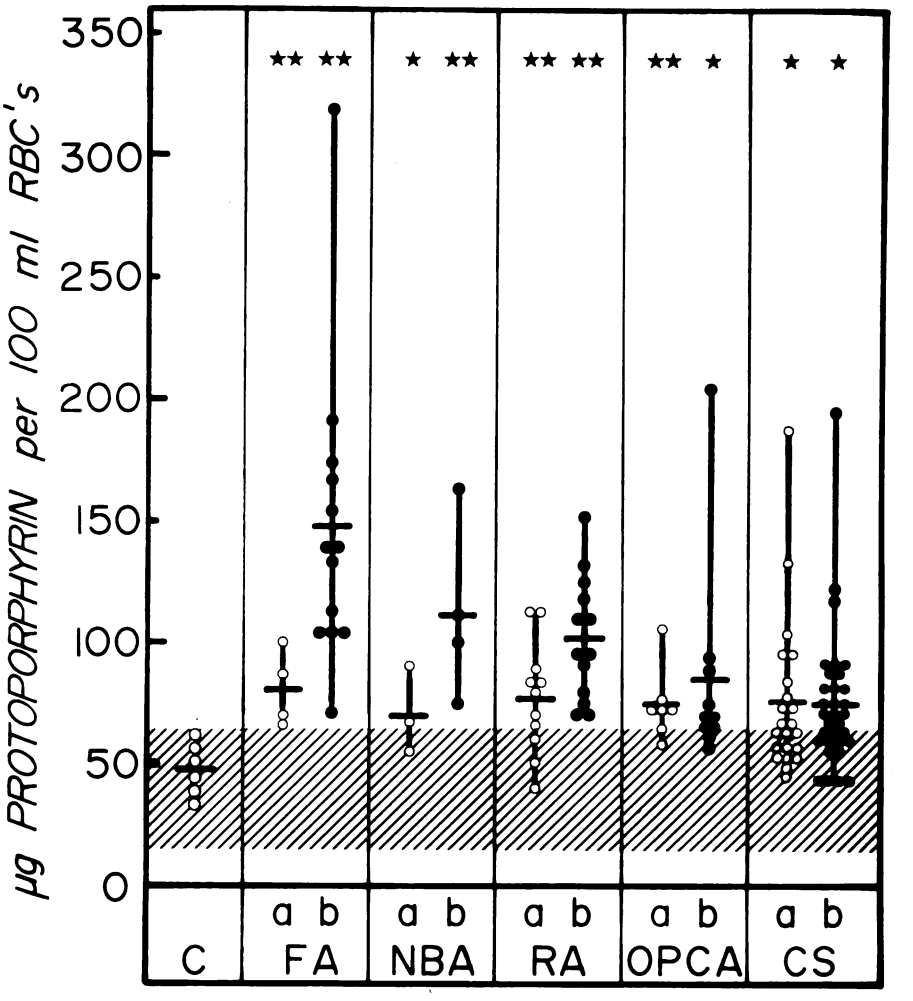

Figure 1—Free erythrocyte protoporphyrin levels in ataxic patients and their relatives. Abbreviations identify the groups as normal controls (C), Friedreich's ataxia (FA), "New Brunswick Ataxia" (NBA), "Rimouski Ataxia" (RA), Olivo-ponto-cerebellar atrophy (OPCA) and Charlevoix Syndrome (CS). Solid circle symbols denote affected individuals (b-columns) while open circles (a-columns) represent obligatory heterozygotes or, in the OPCA group, children at risk. The shaded area covers the expected range of normal values. Vertical lines span the range of experimental values obtained while horizontal lines mark the mean value. Stars appearing above a subgroup indicate a statistically significant difference from the normal control group: 1 star, $p<0.05$ or 2 stars, $p<0.005$.

* "New Brunswick" and "Rimouski" recessive ataxias are slowly progressive forms of ataxia, most likely variants of Friedreich's ataxia. They will be described elsewhere.

RESULTS

The mean FEP for our normal controls was 48±11 (S.D.) μg protoporphyrin/100 ml red blood cells with a range of 34 to 62. These values are similar to ones previously reported: 56±10 (mean±S.D.) μg protoporphyrin/100 ml RBC's (Anderson et al, 1977), range 35 to 65 μg/100 ml RBC's (Orfanos et al, 1977) and range 20 to 50 μg100 ml RBC's

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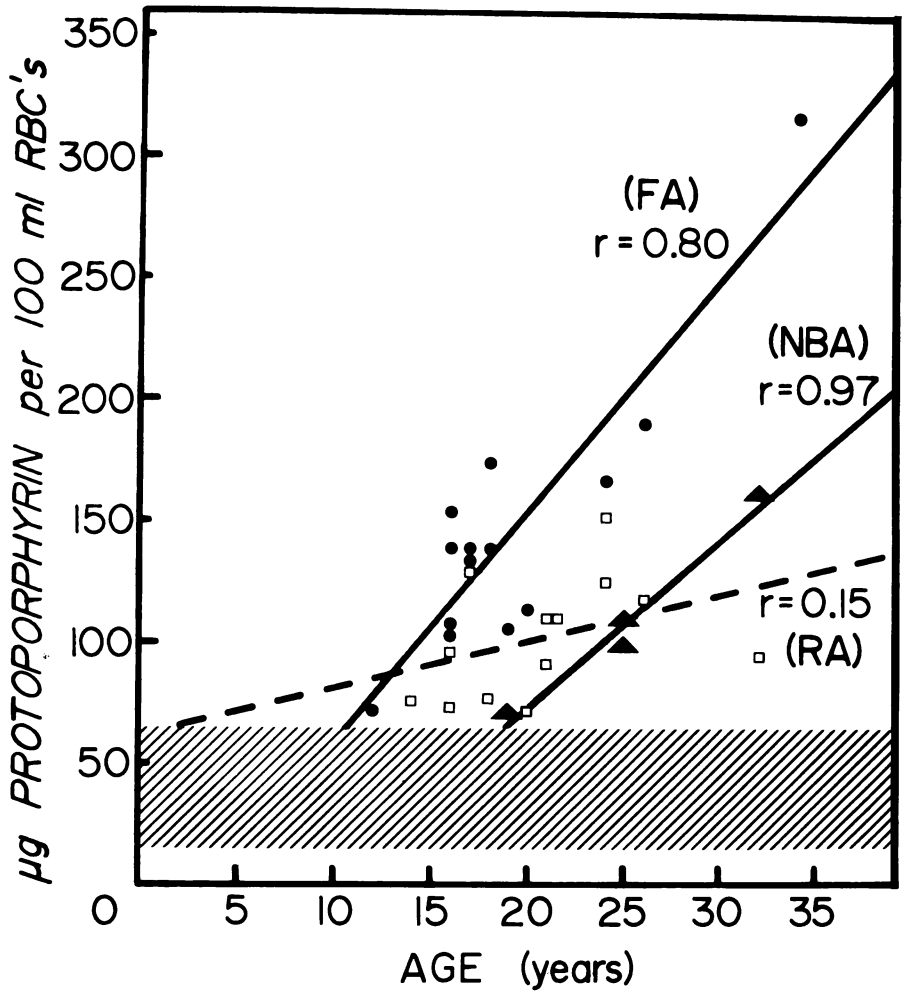

Figure 2— Free erythrocyte protoporphyrin in ataxias as a function of age. Lines represent patients with Friedreich's ataxia (FA, n=13, solid circles), "New Brunswick Ataxia" (NBA, n=4, solid triangle symbols) and "Rimouski Ataxia" (RA, n=13, open squares). Correlation coefficients are given as r-values adjacent to the corresponding line.

(Granick et al, 1972). Values may be slightly higher in young children and females (Orfanos et al, 1977; Krammer et al, 1954). No consistent or marked sex differences were noted in this study. With regard to the above data, the shaded area in Figs. 1, 2 and 3 is intended to represent a normal range of FEP from 15 to 65 μg protoporphyrin/100 ml RBC's.

It is apparent from the distributions of FEP in Fig. 1 that individuals with types Ia and IIa ataxia showed highly significant elevations of FEP; indeed, there was no overlap of FEP values between those 30 patients and the normal range. Groups of OPCA and CS and obligatory heterozygotes for types Ia and IIa hereditary ataxias showed lesser but nonetheless significant elevations of FEP with some overlap in the normal range. Subjects with Rimouski ataxia had FEP levels significantly greater than their corresponding obligatory heterozygotes which were in turn, significantly elevated over control levels. Differences between other ataxic individuals (FA and NBA) and their respective heterozygotes fell just short of significance, due to the small sample size of heterozygotes.

Among the ataxic patients there was excellent correlation of FEP levels with age or duration of the disease in the case of FA and the more slowly progressive NBA (Fig. 2). Within these two groups, FEP appeared to rise progressively above the normal range at about the same rate, but beginning at age 10 in FA and at age 20 in NBA.

In order to assess the "disease specificity" of the elevated FEP in ataxias, it was deemed of interest to investigate subjects with non-ataxic disorders. The results in Fig. 3 indicate that patients with progressive myopathies (DMD, WHS, MG) show relatively minor elevations of FEP and most have levels in the normal range. Those afflicted with Parkinson's disease, aged 23 to 78 years, displayed FEP values ranging from 54 to 127 μg/100 ml RBC's i.e. from the normal range to maximum levels that were significantly elevated but less than the maxima in ataxia groups.

Assays of uroporphyrinogen-I-synthetase, the third enzyme of the heme biosynthetic pathway, revealed a small but significant elevation of enzyme activity of 25 to 75% in ataxic patients and their relatives compared with control levels. Unfortunately, blood samples were subjected to one or more days at room temperature prior to assay due to a refrigerator breakdown. These data are therefore not shown as they likely underestimate the true elevation of uroporphyrinogen synthetase in ataxia.

DISCUSSION

This is the first report of a "paraporphyria" existing in ataxic disorders. Although there has been, hitherto, no recognized nosological association between ataxia and porphyria, the two diseases can nevertheless coexist in a single patient (Goldberg, 1959) and certain subgroups of both of these heterogenous diseases are well known to show a high incidence of diabetes mellitus, enhanced skin photosensitivity, and abnormal pituitary hormone regulation. It is of interest that acute intermittent porphyria was once considered to have a primary neurological basis characterized by defective acetylcholine synthesis, peripheral demyelination, and paralysis, thus illustrating the importance of heme metabolism in nerve function.

The elevations of FEP were most apparent in the patients with

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Friedreich's, New Brunswick, and Rimouski ataxias in which all observed values were above the normal range. Also, only in these three groups was there a clear difference between affected individuals and obligatory heterozygotes. In all three groups the mean value for the heterozygotes was above normal. In the Friedreich's and New Brunswick groups there was a highly significant correlation between age (and therefore severity of the disease) and the FEP values. The correlation was much weaker and not significant for the Rimouski group. This association between FEP and disease progress suggests that both the clinical features of the disease and the FEP abnormalities may be secondary to some underlying primary problem. The elevated FEP is not a cause of the disease but may provide clues as to the cause. It might also provide a readily available and sensitive monitor of new approaches to therapy.

FEP is known to be elevated in a variety of conditions including infection (Krammer et al, 1954), hemorrhage, iron deficiency, nutritional deficiency anaemias of vitamin E (Chou et al, 1978), or vitamin B_{12} and copper (Allen, 1956), following birth, and after irradiation. Elevation of FEP is an indicator of stimulated erythropoiesis and an increased population of mitrochondria-containing erythroblasts and reticulocytes (Johnson and Schwartz, 1972) which are the only red blood cells engaged in active heme synthesis.

Elevations of FEP can result from a number of different abnormalities all pointing to a primary defect at the level of ferrochelatase, the final, regulatory mitochondrial enzyme of the heme pathway that inserts ferrous iron into the protoporphyrin ring (Fig. 4). Possible causes could be 1) a deficiency of the iron substrate 2) a decreased heme requirement or catabolism 3) inhibition of ferrochelatase, as through depletion of cofactors (see below) and/or 4) enhanced flux of intermediates through the porphyrin pathway following induction (derepression) of the rate-limiting enzyme ALA synthetase.

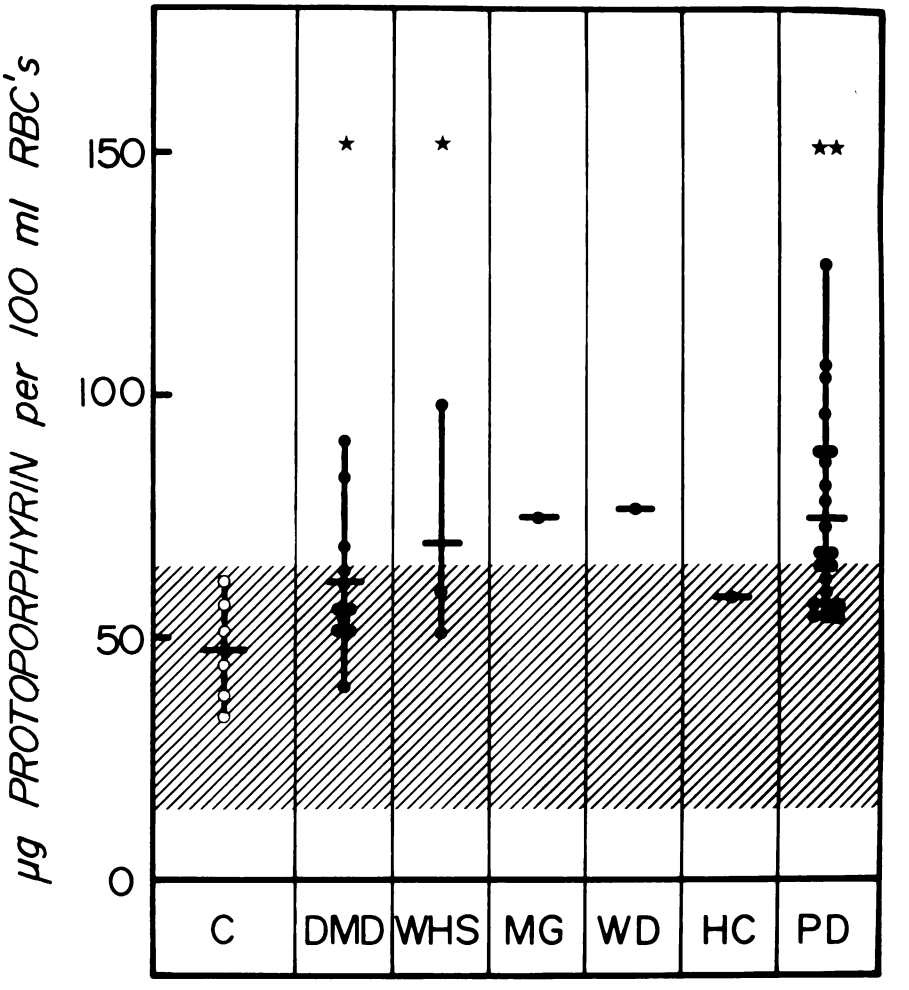

Figure 3—Free erythrocyte protoporphyrin levels in patients with non-ataxic disorders. Abbreviations identify the groups as normal controls (C), Duchenne Muscular Dystrophy (DMD), Werdnig-Hoffman Syndrome (WHS), Myasthenia Gravis (MG), Wilson's Disease (WD), Huntington's Chorea (HC) and Parkinson's Disease (PD). Explanations of data presentation, stars, and shading are given in Figure 1. Note scale change from Figures 1 and 2.

Since plasma and tissue iron is normal or elevated in Friedreich's ataxia (Szanto and Gallyas, 1966) the first possibility seems unlikely. The recent findings of hyperbilirubinemia in a large percentage of ataxic individuals (Barbeau et al, 1976; Hamel et al, 1978) and the heme deficiency in brain mitochondrial cytochrome oxidase of lambs with enzootic ataxia allow us to eliminate the second possibility described above. Incidently, studies of experimental protoporphyria caused by pharmacologic inhibition of ferrochelatase have shown that accumulated protoporphyrin can serve as an alternate source of bilirubin (Liem et al, 1977). Thus the third possibility, "inhibition of ferrochelatase" with consequent protoporphyria, could account for the observed hyperbilirubinemia which the aforementioned studies could not attribute to hemolytic anemia. Although erythrocyte turnover rate may be enhanced in Friedreich's ataxia (Szanto and Gallyas, 1966), hemolytic anemia could also be discounted on the basis of protoporphyrin, as opposed to coproporphyrin, accumulation in RBC's, the latter

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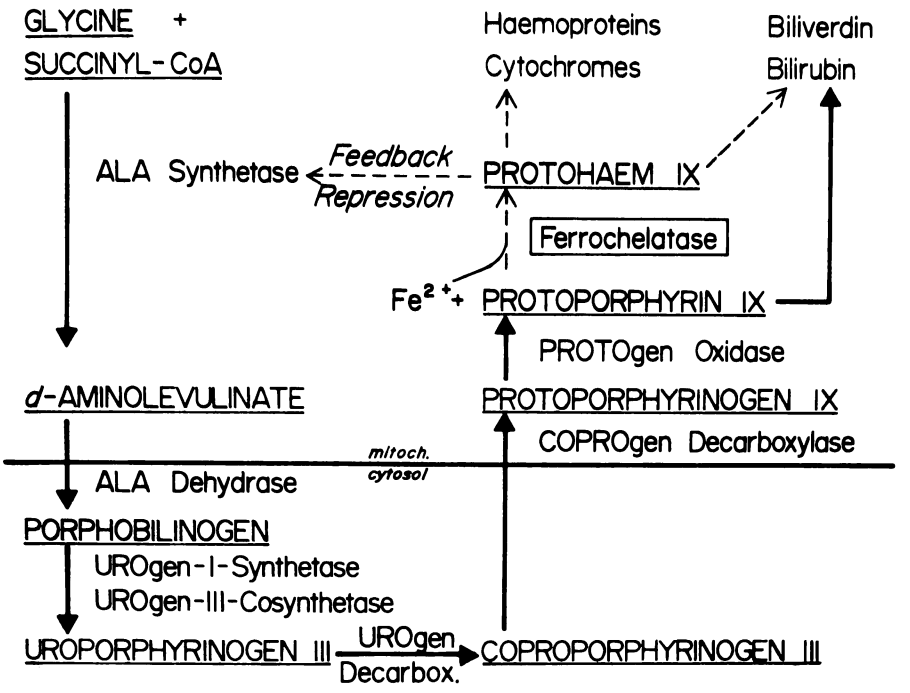

Figure 4 — Synopsis of heme metabolism in ataxia. See text for discussion.

being primarily elevated in hemolytic anemias (Krammer et al, 1954).

The consequences of decreased conversion of protoporphyrin to heme would become manifest as a deficiency of heme proteins and enzymes in cells where heme serves an essential function. Diminished cytochrome activity and respiratory efficiency (Smith et al, 1976) and possible impaired conversion of tryptophan to nicotinic acid via tryptophan pyrrolase in animal ataxias may be examples of this (Fischl and Rabiah, 1964). Diminished cyclooxygenase activity with impairment of prostaglandin synthesis might also be anticipated (Hemler et al, 1976). Derepression of ALA synthetase is an essential prelude to porphyria and would be expected to be elevated in erythropoietic tissues.

To establish the cause of ferrochelatase inhibition we have considered possible depletion of essential enzyme cofactors Cu^{++} (Wagner et al, 1976) and pyridoxal phosphate (Labbe and Nielsen, 1976). In addition, the importance of polyunsaturated fatty acids and calcium as they affect the mitochondrial environment are recognized determinants of ferrochelatase activity (Simpson and Poulson, 1977). Copper deficiency, known to be associated with the enzootic ataxia of lambs (Smith et al, 1976) and with elevated FEP in sheep (Allen, 1956), may or may not be a contributing factor in human ataxias. (Note the elevation of FEP in the one case of Wilson's disease, Fig. 3). Pyridoxal phosphate deficiency does not seem likely and is actually known to impair protoporphyrin biosynthesis since this vitamin is an essential cofactor for ALA synthetase which is rate-limiting for porphyrin biosynthesis.

We next considered a pharmacologic approach to understanding the relation between ataxia and apparent ferrochelatase inhibition. Curiously, substituted pyridine derivatives are used to produce experimental models of cerebellar ataxia viz. 3-acetylpyridine (Perry et al, 1976) and of protoporphyria due to ferrochelatase inhibition viz. 3,5-diethoxycarbonyl-1,4-dihydrocollidine (De Matteis et al, 1973). On the other hand, certain pyridine derivatives such as nicotinic acid and nicotinamide are able to attentuate experimental porphyria (Pinelli and Favalli, 1972; Gibbard and Schoental, 1977). Alteration of pyridine metabolism in hereditary ataxias has not been investigated.

The importance of unsaturated fatty acids and calcium in stimulating ferrochelatase activity prompted us to investigate the actions of prostaglandins on ferrochelatase activity. Preliminary studies in liver cell cultures have established that F-type prostaglandins are powerful modulators of ferrochelatase activity and protoporphyrin accumulation. Using this model system to study heme biosynthesis we have found that certain imidazole and pyridine derivatives known to inhibit thromboxane synthesis and enhance formation of F-type prostaglandins (Moncada et al, 1977; Wennmalm, 1977; Vincent and Zijlstra, 1978; Shimamoto et al, 1976) also produce marked effects on protoporphyrin accumulation. The action of vitamin A acid was also of interest in relation to three, perhaps coincidental observations: apart from its known ability to stimulate lipid peroxidation (prostaglandin synthesis), vitamin A (as beta-carotene) is unique in its ability to abolish symptoms of erythropoietic protoporphyria (Mathews-Roth et al, 1974) and finally, vitamin A deficiency has been used as a model of experimental ataxia (Howell and Thompson, 1967).

In conclusion, it appears that ataxia and elevated FEP may be related in conditions associated with deficiencies of copper, vitamin A, and/or essential fatty acids. Whether any of these nutritional deficiencies contribute to symptoms of hereditary ataxias is at present uncertain. Regretably, such nutritional models have a history of falling short when it comes to applying them to hereditary human diseases e.g. vitamin E's ineffectiveness in hereditary human muscular dystrophies. Elevated FEP may account in part for the hyperbilirubinemia previously observed in hereditary ataxias. Insofar as the mechanisms regulating porphyrin-heme biosynthesis are known or can be investigated pharmacologically, an understanding of the cause(s) of elevated FEP in ataxias may contribute ideas about the etiology of,

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and potential therapy for, human hereditary ataxias.

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Quebec Cooperative Study
of Friedreich's Ataxia

Glucose Tolerance and Erythrocyte Insulin Receptors in Friedreich's Ataxia

P. DRAPER, D. SHAPCOTT, A. LAROSE, J. STANKOVA, F. LEVESQUE AND B. LEMIEUX

SUMMARY: *Detailed in vivo and in vitro studies of glucose and insulin metabolism in Friedreich's ataxia patients and unaffected family members have further defined the extent of the abnormalities in carbohydrate metabolism. The high incidence of glucose intolerance and a hyperinsulinemic response to a glucose challenge in a high percentage of Friedreich's ataxia patients has been confirmed. An increased incidence of glucose intolerance among heterozygotes is suggested, while the siblings show a more normal distribution of diabetes and a nearly normal insulin response to the glucose tolerance test. Human growth hormone patterns are normal for all groups.*

Preliminary studies of insulin binding to erythrocytes suggest a difference in the binding characteristics among diabetic Friedreich's ataxia patients, while the binding in the non-diabetic Friedreich's ataxia group is similar to that of non-diabetic controls. Results from a small group of non-diabetic siblings suggest a normal insulin binding, while a tendency toward increased binding at low insulin concentrations among diabetic family members is noted.

RÉSUMÉ: *Nous avons confirmé l'incidence élevée d'intolérance au glucose et de réponse hyperinsulinique à une surcharge glucosée chez un pourcentage élevé de patients avec l'ataxie de Friedreich. Une incidence augmentée est également suggérée chez les hétérozygotes alors que les taux de diabète sont plus près de la normale dans la fratrie, chez qui la réponse insulinique est à peu près normale. Les valeurs d'hormone de croissance humaine sont normales dans tous les groupes.*

Des études préliminaires concernant la liaison de l'insuline au niveau des érythrocytes indique qu'il semble exister une différence dans les caractéristiques de liaison chez les patients ataxiques diabétiques. La liaison de l'insuline est semblable à celle des sujets contrôles non diabétiques chez les Friedreich non-diabétiques.

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INTRODUCTION

Previous studies in this and other laboratories have shown an elevated incidence of clinical and chemical diabetes in Friedreich's ataxia patients. Shapcott et al, (1976) found an incidence of 18.2% clinical diabetes and 21.2% chemical diabetes for a total of 39.4% in a group of 33 typical Friedreich's ataxia patients (Group Ia). In addition, an abnormally high insulin response to the glucose challenge was noted in 73% of the patients, and it was postulated that this could be the result of a deficiency of membrane receptors for insulin as has been reported for diabetic humans and animals.

Results of earlier studies on diabetes in Friedreich's ataxia have been summarized by Shapcott et al (1976).

The incidence of diabetes in relatives of Friedreich's ataxia patients has been studied less extensively. However, the results of a survey conducted by Andermann et al (1976) suggested an increased frequency among family members.

The possibility of a generalized membrane defect in Friedreich's ataxia has also been suggested. This was based in part on the preliminary observation of a difference in the stability of erythrocyte membranes in Friedreich's ataxia patients, as determined by SDS polyacrylamide electrophoresis (Shapcott et al, 1979).

In light of these findings, we have undertaken a study of the erythrocyte insulin receptors in Friedreich's ataxia patients and in unaffected family members. The survey of glucose and insulin metabolism of Shapcott et al (1976) has also been extended to include the family members in order to further delineate the extent of

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abnormalities in carbohydrate metabolism in obligatory heterozygotes and siblings.

SUBJECTS AND METHODS

Subjects

Typical Friedreich's ataxia (Group Ia) patients and non-affected family members (obligatory heterozygotes and siblings*) were studied. Control subjects were apparently healthy adult volunteers from laboratory and secretarial personnel.

Materials and Methods:

Glucose tolerance test (G.T.T.). The subjects were given a high carbohydrate diet for three days before the test. They were tested on an outpatient basis and they were maintained in a resting position (sitting) for 30 minutes before the test, as well as during the test period. An oral glucose load of 100 g (1.75 g/kg for smaller subjects) was given and blood samples were collected at 0, 30, 60, 90, 120 and 180 minutes. Plasma glucose was determined by the solid phase radioimmunoassay. Glucose tolerance curves were evaluated according to the method of Danowski et al (1973).

Insulin binding studies — erythrocytes

Insulin binding to viable erythrocytes was determined (with fasting blood samples) using the method of Gambhir et al (1977) with the following modifications: the erythrocyte preparations were obtained by washing red cells three times with saline and aspirating the top portion of the cell pellet with the saline after each wash. Suspensions of one volume red cells and one volume pH 7.8 tris-HEPES buffer were incubated in the presence of 0.1 ng/ml ^{125}I labelled insulin and varying concentrations of unlabelled insulin (total incubation volume was 550 ul) as described by Gambhir (1977).

The cell pellet containing bound insulin was separated from free insulin by layering duplicate 200 μl samples of suspension on 0.5 ml dibutylphthalate and 0.5 ml buffer at 4° in 12 x 75 mm plastic tubes. After aspiration of the upper layer, the walls of the tubes were rinsed with 0.4 ml buffer to ensure maximum removal of free insulin.

Specific binding of ^{125}I labelled insulin to erythrocytes was calculated by subtracting the binding of ^{125}I insulin in the presence of 10^5ng/ml unlabelled insulin (non-specific binding) from the total binding. Hemoglobin was determined in the cell suspensions for each subject and the specific binding normalized to a hemoglobin concentration of 15.7 g/100 ml in the suspensions.

Insulin binding to lyophilized ghosts were measured by incubations of about 2-2.5 mg ghosts in the tris-HEPES buffer for 4h at 4°C. Following the incubations, suspensions were centrifuged in μ centrifuge tubes and the ghost pellets washed with buffer to remove unbound insulin.

RESULTS

Distribution of the Danowski scores for the G.T.T. among the various groups (a total of 44 subjects) is shown in Table I. The increased incidence of glucose intolerance in the Friedreich's ataxia group is consistent with the results of previous studies. Similarly, a tendency toward increased glucose intolerance is observed for the obligatory heterozygotes, while the results for the siblings resemble those of the external controls.

Table II shows the insulin and H.G.H. responses to the glucose load in the different groups. These are presented graphically in Fig. 1a - d, with each group of subjects (where numbers are sufficient) being divided into "diabetic" and "non-diabetic" sub-groups. Here, a Danowski score of 600 was chosen arbitrarily as the dividing point between the two sub-groups. It can be seen (Fig. 1a) that the diabetic Friedreich's ataxia patients studied exhibit a delayed hypo-insulinemic response to the glucose challenge, while the non-diabetic Friedreich's ataxia patients show a delayed and eventually a hyperinsulinemic response, relative to control subjects. The curve for the non-diabetic siblings (Fig. 1b) resembles that of the non-diabetic external controls. An increased insulin response appears to result from the glucose load in the obligatory heterozygote group. However, the number of subjects (N=6) available precludes a more precise classification of this group. No significant differences in the H.G.H. results among the groups are apparent (Table II, Fig. 1, c-d).

The specific binding of labelled insulin to erythrocytes as a function of

TABLE I

Distribution of Danowski Scores for Glucose Intolerance Sum of Plasma Glucose Levels at 0, 30, 60 and 120 min

	<501 (Normal)	501-650 (chemical diabetes, mild glucose intolerance)	650-800 (chemical diabetes, moderate glucose intolerance)	>800 (diabetes)	Mean Danowski Scores (±S.D.)
Friedreich's Ataxia * (N=15)	1	11	3		582.7±77.0
Obligatory Heterozygotes N=6)	1	4		1	639.9±201.0
Siblings (N=11)	6	4	1		513.1±92.7
External Controls (N=12)	5	6	1		517.6±105.6

*Siblings: possible heterozygotes and normal.

*Excluded from this group was at least one known diabetic where the glucose tolerance test was not administered.

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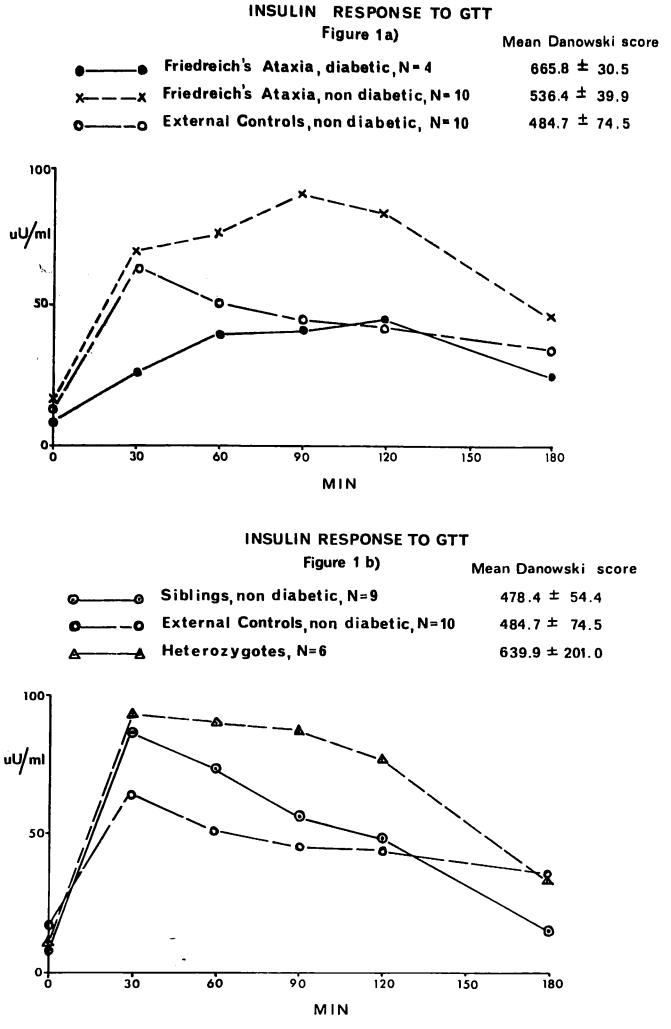

Figure 1, a-b—Insulin response to GTT. a) Friedreich's ataxia, diabetic and non diabetic. b) Siblings, non diabetic. Heterozygotes (diabetic + non diabetic).

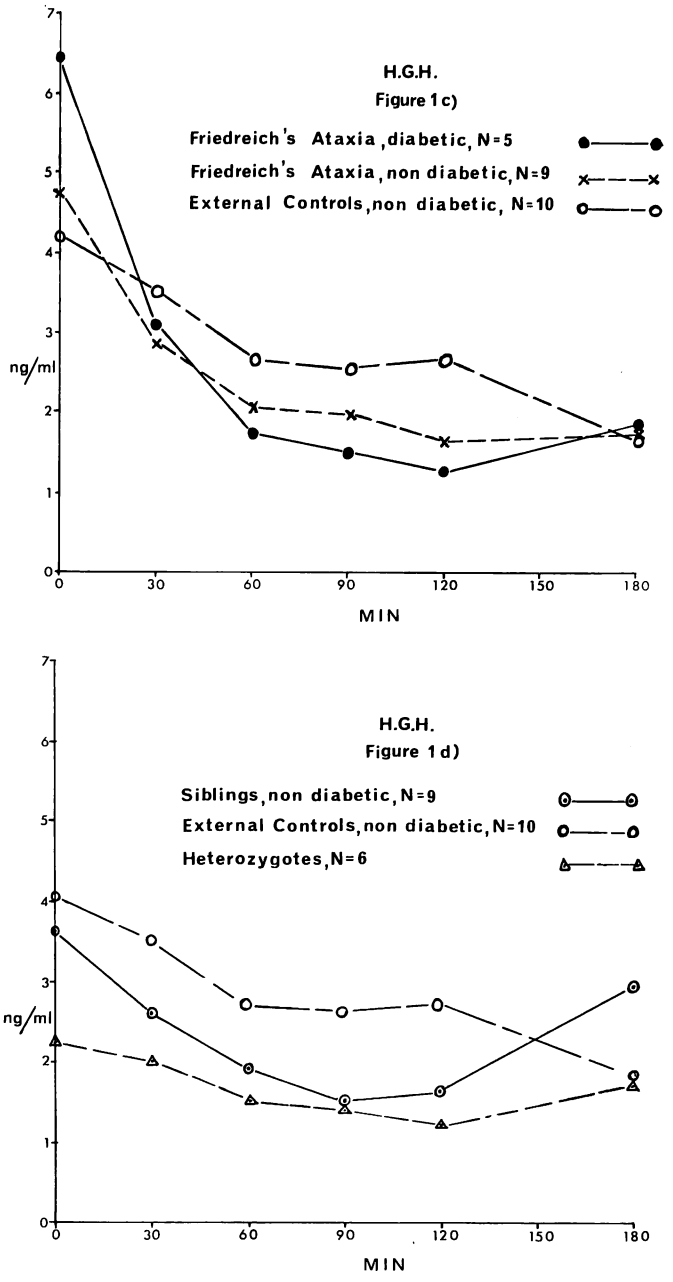

Figure 1, c-d—HGH response to GTT. c) Friedreich's ataxia, diabetic and non diabetic. d) Siblings, non diabetic. Heterozygotes (diabetic + non diabetic).

the concentration of unlabelled insulin is summarized in Table III and is shown graphically in Fig. 2 a, b. Scatchard analyses of the binding are illustrated in Fig. 3 a, b.

The specific binding of insulin to erythrocytes of diabetic Friedreich's ataxia patients is significantly higher than that for non-diabetic controls at low concentrations of unlabelled insulin. The Scatchard plot also suggests a difference in the binding characteristics at high insulin concentrations in the diabetic Friedreich's ataxia group. However, because of the limited amounts of erythrocytes available, these results are based on the binding at just two insulin concentrations and a small uncertainty in the two points may result in a large uncertainty in the intercept. The specific binding for the non-diabetic Friedreich's ataxia group is not significantly higher than the non-diabetic control group. The binding curve for non-diabetic siblings is very similar to that for non-diabetic controls while a trend toward elevated binding at low unlabelled insulin concentrations is noted for diabetic heterozygotes and siblings. Due to the small number of subjects available, it was not considered practical to separate the diabetic heterozygotes and siblings into two groups.

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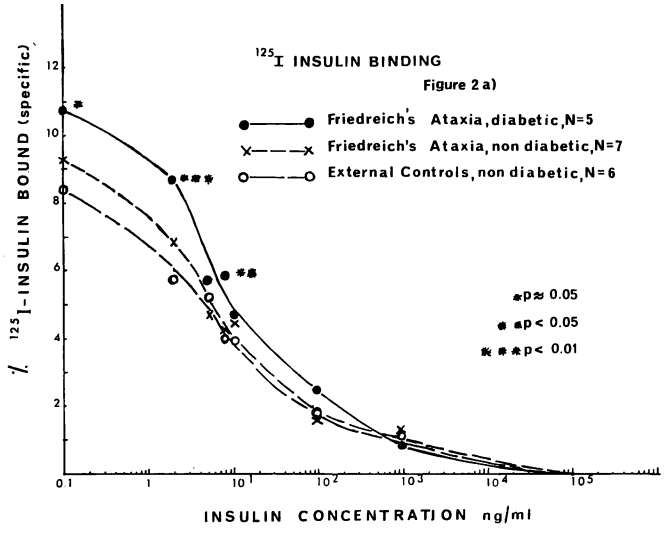
DISCUSSION

The increased incidence of glucose intolerance in genetic disorders such as inherited ataxias and some neuromuscular disorders has been extensively documented (Shapcott et al, 1976, Barbosa et al, 1974). However, the relationship between the glucose intolerance and the primary biochemical defect remains obscure. An hypothesis which we are currently investigating is that the clinical signs in these disorders are the result of a defect in cell membranes. Since insulin receptors are located on the surface of cell membranes, it is conceivable that the membrane defect could affect insulin binding characteristics and hence carbohydrate metabolism. From an alternative point of view, a study of insulin receptors could be used as a probe for the further elucidation of membrane function and possible defects.

The increased incidence of glucose intolerance in Friedreich's ataxia, is shown in Table I. The majority of the patients exhibit a mild degree of glucose intolerance. Thoren (1978), in a follow-up study of a group of Friedreich's ataxia patients first reported in 1964, noted an increase from 17.9 to 30.4% in the incidence of clinical diabetes. Since the age of onset distribution did not match that for non-ataxic diabetics, it is possible that the onset of diabetes reflects the progression of Friedreich's ataxia.

The tendency toward higher Danowski scores in the heterozygotes group may be in part due to the natural trend toward hyperglycemia with increasing age. (Obligatory heterozygotes are the parents of Friedreich's ataxia patients and therefore are older than the other groups). The siblings appear to be less prone to diabetes and the distribution of Danowski scores in our group resembles that of the controls.

Diabetes is a familial disorder (Renold et al, 1978). Thus, in some



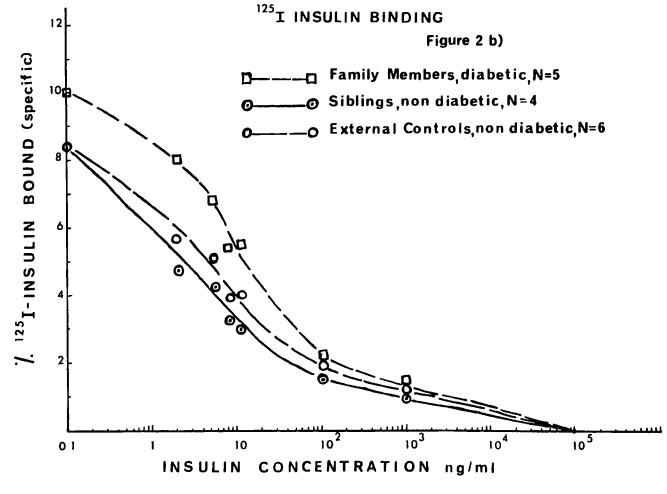
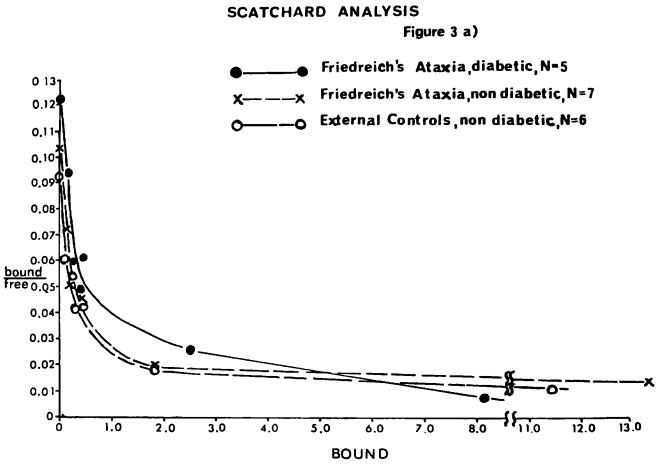


Figure 2, a-b — ^{125}I insulin binding to erythrocytes. a) Friedreich's ataxia, diabetic and non diabetic. b) Family members, diabetic. Siblings, non diabetic.



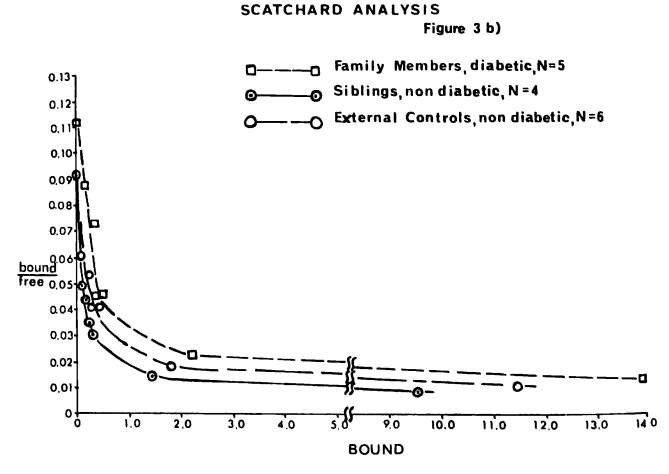


Figure 3, a-b — Scatchard analysis of ^{125}I Insulin binding to erythrocytes. a) Friedreich's ataxia, diabetic and non diabetic. b) Family members, diabetic. Siblings, non diabetic.

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TABLE II

Insulin and HGH Responses to GTT (Mean ± S.D.)

		0 min	30 min	60 min	90 min	120 min	180 min
Friedreich's Ataxia (Diabetic)	HGH (ng/ml N=5)	6.38±7.70	3.12±2.16	1.84±0.69	1.60±0.35	1.34±0.32	1.86±1.14
	INS (uU/ml N=4)	8.4±5.7	25.5±17.5	39.8±22.3	40.8±23.8	44.8±28.1	23.8±17.6
Friedreich's Ataxia (Non-Diabetic)	HGH (N=9)	4.66±4.74	2.92±1.61	2.13±1.39	2.01±1.20	1.73±1.51	1.83±0.96
	INS (N=10)	15.7±18.1	69.6±38.5	77.4±55.0	91.0±51.9	84.1±52.6	46.2±38.2
Siblings	HGH (N=9)	3.63±2.90	2.59±2.36	1.89±1.63	1.47±0.85	1.64±0.78	2.88±1.29
(Non-Diabetic)	(N=9)	7.8±3.3	86.0±52.7	73.0±53.9	55.8±32.8	47.2±20.2	13.4±9.5
External Controls (Non-Diabetic)	HGH (N=10)	4.10±4.27	3.52±4.15	2.69±2.36	2.66±3.00	2.74±2.19	1.76±1.36
	INS (N=10)	13.3±17.0	63.9±31.7	51.0±27.6	42.5±33.2	44.4±30.8	33.4±21.0
Heterozygotes (diabetic + non-diabetic)	HGH (N=6)	2.28±1.02	2.05±0.96	1.52±0.67	1.45±0.61	1.22±0.46	1.72±0.70
	INS (N=10)	10.16±3.75	92.92±87.97	90.0±55.32	87.33±36.11	76.33±37.38	32.92±28.00

families glucose intolerance appears in heterozygotes and in siblings as well, as in the Friedreich's ataxia patients, while other families appear to be relatively free of diabetic complications.

An additional factor is that Friedreich's ataxia patients are poor subjects for a GTT since their low muscle mass would cause them to utilize glucose abnormally, irrespective of their glucose intolerance. While there are a number of obviously diabetic Friedreich's ataxia patients in our group, it is possible that this abnormal glucose utilization may be an important factor in the large number of borderline cases. We are currently investigating the concentration of the minor hemoglobin A_{1C} which gives an indication of the extent of hyperglycemia over the preceding several weeks without resorting to the GTT (Renold et al, 1978; Gonen et al, 1978).

For the purpose of this discussion of insulin metabolism, the subjects have been divided into "diabetic" and "non-diabetic" sub-groups, with a Danowski score of 600 being arbitrarily chosen as the dividing point between the two. Thus, subjects having a Danowski score of less than 600 will have either a normal GTT or they will exhibit a mild degree of glucose intolerance. Subjects with a score of greater than 600 show a more severe degree of intolerance and hence are classified as "diabetic". A more extensive classification as shown in Table I was not considered practical due to the limited number of subjects.

The hyperinsulinemic response to the GTT among the non-diabetic Friedreich's ataxia patients parallels the slight hyperglycemic response of this group and thus a certain degree of insulin resistance is suggested. A similar type of hyperinsulenemia has been reported in patients with myotonic dystrophy (Barbosa et al, 1974). However, the degree of hyperinsulinemia found in our study is much less than that reported by Bar et al (1978) for two siblings with ataxia telangiectasia. In the diabetic Friedreich's ataxia patients where

TABLE III

% ^{125}I Insulin Bound (Specific), ± S.D./15.7 g % Hb

Concentration of non-labelled insulin (ng/ml)	Friedreich's Ataxia, Diabetic (N=5)	Friedreich's Ataxia, Non-Diabetic (N=7)	Family Members Diabetic (N=5)	Siblings Non-Diabetic (N=4)	External Controls, Non diabetic (N=6)
0	10.79±2.18*	9.31±1.64	10.04±2.71	8.38±2.81	8.41±1.14
2	8.60±0.69***	6.75±1.05	8.07±3.28	4.66±1.66	5.73±1.48
5	5.64±1.17	4.78±1.11	6.83±2.28	4.23±1.16	5.11±0.98
8	5.87±1.15**	4.10±0.66	4.39±1.57	3.37±1.24	3.99±0.91
10	4.60±0.46	4.39±1.69	4.49±1.60	2.97±1.68	3.96±1.25
10^2	2.49±0.80	1.87±1.03	2.21±1.77	1.47±1.53	1.84±0.59
10^3	0.81±0.44	1.36±0.60	1.38±1.20	0.95±0.82	1.14±0.57
10^5					

* $p \approx 0.05$
** $p < 0.05$
*** $p < 0.01$
Diabetic = Danowski Score > 600
Non-Diabetic = Danowski Score < 600

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insulin concentration was measured, a delayed hypoinsulinemic response was observed (Fig. 1a).

The first step in the metabolism of insulin is considered to be the interaction of insulin with its target cell receptors. Since defective insulin binding has been associated with abnormalities of insulin metabolism, it was therefore of interest to attempt to make a correlation between the in vivo insulin responses and the data obtained from the insulin binding studies.

Most previous studies on circulating insulin receptors have been conducted on monocytes. However, the amount of blood required to isolate sufficient quantities for detailed studies is large and we felt that the procedure was too invasive. While less is known of the characteristics of erythrocyte insulin receptors, the development of a convenient procedure for the measurement of these receptors by Gambhir et al (1977) prompted us to use erythrocytes. In a more recent paper, Gambhir et al (1978) have shown that binding characteristics of erythrocytes are similar to other human cell types.

Lyophilized erythrocyte membranes (ghosts) represent a very stable and therefore convenient preparation, and since we were already preparing ghosts for other membrane studies, our initial work with erythrocyte receptors was done with the ghost preparation. We were able to demonstrate specific binding of radiolabelled insulin to the erythrocyte ghost-preparation and insulin binding at three different concentrations of non-labelled insulin was determined for Friedreich's ataxia patients, obligatory heterozygotes, siblings, and external controls. No differences among the groups were observed. However, our method resulted in a high percentage of non specific binding (about 60% of the total binding), and the quantity of insulin specifically bound was much less than measured with an equivalent quantity of fresh erythrocytes. Therefore, it appears that the insulin receptors are partially lost or modified during the preparation of the ghosts. We thought it unsound to draw conclusions from the results obtained with the ghosts.

The most important feature of the insulin binding curves (fig. 2a, b) is probably the increased binding of ^{125}I insulin in the presence of low concentrations of unlabelled insulin to erythrocytes of diabetic Friedreich's ataxia patients. Bar and Roth (1977) have reviewed the correlations found between the concentration of a number of circulating hormones and the concentration and affinity of insulin receptors on circulating monocytes. Of particular importance is the concentration of insulin itself; studies of hyperinsulinemia in obesity and adult diabetes, as well as animal studies of hypoinsulinemia show an inverse relationship between plasma insulin levels and receptor concentrations. In view of the low fasting insulin concentrations in many of the diabetic Friedreich's ataxia patients, the higher binding at low insulin concentrations therefore appears reasonable. The apparently lower total receptor concentration for this group, as determined by the intercept of the Scatchard curve (Fig. 3a), seems surprising. However, the intercept of the curves is obtained by extrapolation of just two points in this case and thus they are subject to a considerable degree of uncertainty. Although the non-diabetic Friedreich's ataxia patients give a delayed hyperinsulinemic response to the GTT, the near normal fasting insulin levels are consistent with the near normal binding characteristics of this group.

A number of other factors including the presence of circulating inhibitors of insulin binding (antireceptor antibodies) (Bar et al, 1977), HGH concentrations (Sommen et al, 1978), and ingestion of glucose (Muggeo et al, 1977) have been shown to affect insulin binding. Our results show no significant differences in the HGH responses to the GTT in any of the groups studied. It seems unlikely that the binding differences are related to HGH abnormalities.

There are no data on insulin binding to erythrocytes of diabetic subjects. We are currently carrying out binding studies on diabetic controls not suffering from neurological disorders to confirm whether or not the difference in binding occurs independently of the ataxia. Of probable significance here is a similar trend toward an increase in ^{125}I insulin binding at low concentrations of unlabelled insulin to erythrocytes of diabetic heterozygotes and siblings, compared to non-diabetic controls and siblings.

Defects in insulin binding to monocytes, as are found in insulin resistance or in HGH deficiency are generally acquired defects, since the receptors usually return to normal when the precipitating factor is regulated (Bar et al, 1977). In a relevant example, a large decrease in insulin receptor affinity on monocytes of two siblings with ataxia telangiectasia and severe insulin resistance was reported by Bar et al (1978). However, the defect was not expressed in cultured fibroblasts, suggesting that it was not a basic genetic defect. In this example, evidence for the presence of circulating antireceptor antibodies was obtained.

In contrast, it has been suggested that defects in insulin receptors in thin, insulin resistant diabetics are of genetic origin (Blecher, 1979).

Studies of insulin binding to cultured fibroblasts of Friedreich's ataxia patients are therefore planned to confirm whether or not the difference observed in erythrocytes is expressed.

A number of other possibilities have been suggested to account for insulin resistance. These include secretion of an abnormal insulin or a defect at the second messenger level, but evidence of these is less conclusive. The failure to observe a significant difference in the binding to erythrocytes of non-diabetic Friedreich's ataxia patients where a hyperinsulinemic response to the GTT was observed leaves open the possibility of a defect at a later control stage. Alternatively, it is possible that defects in insulin receptors in the major target cells are not manifested in erythrocytes.

In conclusion, we have confirmed the increased incidence of diabetes in Friedreich's ataxia patients and the hyperinsulinemic response to the GTT in many cases. An increased incidence of glucose intolerance in the obligatory heterozygotes is suggested but the siblings show a more normal distribution of diabetes. Binding of ^{125}I

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insulin to erythrocytes of non-diabetic Friedreich's ataxia patients and siblings does not differ significantly from normal while an increased binding is observed for diabetic Friedreich's ataxia patients. Since the binding appears normal in many cases, it seems unlikely that Friedreich's ataxia is related to a primary defect in insulin receptors; however, the insulin resistance may be secondary to a generalized membrane defect.

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Quebec Cooperative Study
of Friedreich's Ataxia

Pyruvate Dehydrogenase, Lipoamide Dehydrogenase and Citrate Synthase Activity in Fibroblasts from Patients with Friedreich's and Charlevoix-Saguenay Ataxia

S. B. MELANCON, M. POTIER, L. DALLAIRE, P. ROLLIN, G. FONTAINE AND B. GRENIER

SUMMARY: *The activity of lipoamide dehydrogenase and two closely related enzymes was studied simultaneously in early, mid, and late passage fibroblast cultures. Friedreich's ataxia fibroblasts tended to lose pyruvate dehydrogenase and citrate synthase activities, while lipoamide dehydrogenase activity remained constant with aging of the cells. Mean pyruvate dehydrogenase activity was lower over-all in fibroblasts from ataxics. Mean citrate synthase activity was higher in ataxic fibroblasts. Present tissue culture media do not represent the best conditions in which to reproduce cofactor binding defects such as those found in other genetic diseases with structural enzyme mutations.*

RÉSUMÉ: *L'activité de trois enzymes du carrefour "pyruvate-acetyl CoA" ne varie pas de façon significative au cours du vieillissement des cultures de fibroblastes cutanés provenant de patients ataxiques et témoins. L'activité moyenne des déshydrogénases du pyruvate et de la lipoamide, ainsi que de la citrate synthétase ne difère pas entre les trois groupes sur le plan statistique. Cependant, certains éléments essentiels contenus dans le milieu nutritif des cellules peuvent masquer des défauts de régulation dans l'activité de ces enzymes, défauts suggérés par des activités moyennes haussées de la citrate synthétase et réduites de la pyruvate déshydrogénase dans les fibroblastes provenant d'ataxiques.*

From le Centre de Recherche Pédiatrique, Hôpital Sainte-Justine Département de Pédiatrie, Université de Montréal

Reprint requests for the complete supplement on Friedreich's Ataxia (Phase Two, Part Two) to:

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INTRODUCTION

Previous studies by our group (Melançon et al.,1977) and other investigators (Filla et al.,1978) disclosed low levels of lipoamide dehydrogenase (LAD) in serum of patients with Friedreich's ataxia. Repeated attempts to demonstrate a comparable reduction of LAD in platelets (Filla et al.,1978) and cultured skin fibroblasts (Melançon et al., 1978a,1978b) from ataxic patients have, however, not been successful. In order to assess the effect of prolonged culture conditions upon enzyme activity, we have measured the level of LAD and two closely related enzymes, PDH (pyruvate dehydrogenase) and CS (citrate synthase) in skin fibroblasts from Friedreich's and Charlevoix-Saguenay ataxias at different times of passages in culture.

MATERIALS AND METHODS

Skin biopsies obtained from 3 normal controls, 4 patients with Charlevoix-Saguenay ataxia (CSA), and 5 patients with Friedreich's ataxia (FA) were cultured as previously described (Melançon et al., 1972). Confluent monolayers were harvested after 7, 14, and 21 passages and assayed within a week.

LAD was determined as previously described (Melançon et al.,1978b). PDH was assayed according to Blass et al. (1972) and CS by the method of Schulman and Blass (1971). All assays were performed in duplicate, patients and control cell lysates being studied simultaneously each time. Cell protein was determined according to Lowry et al. (1951).

TABLE I

Effect of Time in Culture on Enzyme Activity of Normal and Ataxia Fibroblasts

		Specific Activity* At Passage #7	#14	#21	Mean Activity
Lipoamide Dehydrogenase					
Control	(3)	68.3±13.1	86.8±17.0	88.5±11.5	81.2±15.6
Charlevoix	(4)	65.8±27.0	65.7±14.0	88.7±10.3	72.0±20.4
Friedreich	(5)	79.3±11.5	87.0± 6.4	74.3±13.9	80.6±11.2
Pyruvate Dehydrogenase		20.6± 8.7	40.3± 5.7	25.5± 6.9	29.2±11.2
Control	(3)				
Charlevoix	(4)	39.0±26.7	27.6±11.4	12.3±11.7	27.6±20.0
Friedreich	(5)	23.2±29.4	20.1±19.2	15.3± 6.6	20.2±20.0
Citrate Synthase					
Control	(3)	1.11± .31	0.86± .17	1.01± .18	.99± .23
Charlevoix	(4)	1.14± .47	1.03± .29	1.96±1.34	1.26± .66
Friedreich	(5)	1.44± .24	1.40± .26	1.07± .34	1.32± .37

* Specific activity (M ± SD) in μmol/min/mg protein; LAD × 10^{-3}, PDH × 10^{-4} and CS × 10^{-5}

RESULTS

Our results are summarized in Table I. LAD activity was not affected by aging of either patients or control fibroblast cultures. Although Charlevoix-Saguenay cells showed lower over-all LAD values, no significant difference could be demonstrated. PDH activity was higher in mid and late passage control fibroblasts and lower in ataxias fibroblasts as culture aged. These differences were not statistically different. CS activity followed the opposite trend, with increased levels in early passage FA cells and late passage CSA cells. Mean CS activity was higher over-all in fibroblasts from ataxic patients as compared with control values.

DISCUSSION

These data and our previous observations using skin fibroblasts cultured from patients with typical Friedreich's ataxia (Melançon et al.,1978a) do not favor a genetic defect in the lipoamide dehydrogenase apoenzyme as the basic mechanism for pyruvate accumulation in Friedreich's ataxia. We have investigated twenty-two fibroblast cultures from such patients over a two-year period and found no significant reduction of LAD in any of the cultures tested. Other investigators (Strump, D., personal communication) have also experienced results similar to ours.

The artificial nutrient mixture used in skin fibroblast cultures does not, however, reproduce *in vivo* conditions. Eagle MEM nutrient mixture, for instance, contains pharmacological levels of a number of vitamins not normally encountered in living tissues (thiamine 1mg/1, riboflavin 0.1mg/1, pyridoxine 1mg/1, and nicotinamide 1mg/1). A point mutation in coenzyme binding affinity would therefore be masked under such conditions. Blass and Gibson (1977) have recently uncovered a higher than normal apparent Km value of transketolase for thiamine in extracts of fibroblasts from patients with Wernicke-Korsakoff syndrome. In their study, the activity of transketolase in the presence of excess thiamine pyrophosphate was slightly higher in patients than in control fibroblasts. However, the Km for binding of thiamine pyrophosphate was 10 to 20 times higher in fibroblasts from the patients than the controls. If a comparable structural mutation existed in one of the three components of the PDH complex in Friedreich's ataxia fibroblasts, normal enzyme activities would remain meaningless under present tissue culture conditions.

ACKNOWLEGMENTS

These studies were supported by grants from l'Association Canadienne de l'Ataxie de Friedreich, the Quebec Network of Medical Genetics (Ministère des Affaires Sociales) and the Medical Research Council of Canada. We thank Jean-Pierre Bouchard, Richard Gagné, Bernard Lemieux, Guy Geoffroy and André Barbeau who provided us with the skin biopsies.

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Quebec Cooperative Study
of Friedreich's Ataxia

Pre- And Postsynaptic Effects of Taurine and Gaba in the Cockroach Central Nervous System

B. HUE, M. PELHATE AND J. CHANELET

SUMMARY: *Taurine resembles GABA in its synaptic effects in the cockroach cercal nerve giant fiber synapse where it exerts a depressant action upon synaptic transmission. Both taurine and GABA produce an increased conductance of pre- and postsynaptic membranes through changes in the permeability of chloride ions.*

RÉSUMÉ: *La Taurine ressemble de près au GABA dans son action sur le synapse géant de la blatte, où les deux substances produisent une dépression de la transmission synaptique. La Taurine et le GABA produisent une augmentation de la conductance des membranes pré- et postsynaptique par leur effet sur la perméabilité des ions de chlore.*

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INTRODUCTION

Taurine and γ-aminobutyric acid have been shown to be involved in inhibitory mechanisms in vertebrate and invertebrate nervous system (Curtis and Watkins, 1965). Analysis of synaptic effects of amino-acids are well known at peripheral synapses (Dudel, 1965; Takeuchi and Takeuchi, 1975; Constanti, 1977). Other studies at the unitary level in the sixth abdominal ganglion of the cockroach (Callec, 1974), in bullfrog spinal ganglion (Nishi et al., 1974), in rat spinal ganglion (Deschesnes et al., 1976) and cat spinal ganglion (Gallagher et al., 1978) have generated much data concerning the site and/or the ionic basis of the action of γ-aminobutyric acid.

Previous results have respectively demonstrated the lack of effect of taurine on the cockroach giant axon (Pelhate et al., 1978) and its depressant action on the cockroach cercal-nerve giant fiber synapse (Hue et al., 1978). These findings have led us to investigate in further detail the effects of taurine and γ-aminobutyric acid on the afferent and efferent pathways located in the sixth abdominal ganglion of the cockroach (Periplaneta americana).

METHODS

Experiments were performed on adult male cockroaches. The methods (oil-gap and mannitol-gap techniques) used to investigate the amino-acid sensitivity of the cockroach cercal nerve giant fiber synapse have been described in detail previously (Pichon and Callec, 1970; Callec and Satelle, 1973; Callec, 1974; Hue et al., 1976, 1978). In addition the record of the presynaptic terminal polarization was made with the mannitol-gap apparatus (we have adapted that technique to the presynaptic cercal nerve XI which contains many cholinergic fibers).

Polarization of pre- and postsynaptic membranes was continuously monitored on a rectilinear ink writing paper recorder. Postsynaptic events were observed on storage and conventional oscilloscopes, and results were stored on a magnetic tape recorder or filmed immediately with a camera.

In some experiments postsynaptic events were computed using a programmable signal analyser (Histomat S) and presented as a mean of 3 to 50 signals (see fig. 1 and 5).

All experiments were carried out at constant room temperature (18° C). A Ringer solution (Ri) containing 210 mM NaCl, 3.1 mM KCl, 5.4 mM $CaCl_2$ was applied continuously to the desheathed sixth abdominal (A6) ganglion. The pH value was adjusted to 7.2 with a phosphate-bicarbonate buffer.

Taurine (Merck,) γ-aminobutyric acid (Sigma) were used in addition to the Ringer. When testing the interaction of picrotoxin and/or strychnine with taurine and γ-aminobutyric acid, the amino acids were added to the picrotoxin and/or strychnine solution. In some experiments, in order to study the eventual chloride dependency of taurine and/or γ-aminobutyric acid responses, the chloride content of the standard Ringer solution was reduced by replacement of a portion of the sodium chloride with equimolar sodium acetate.

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GABA and Taurine log-dose response curves were obtained using non-cumulative external applications (Feltz, 1971; Constanti, 1977).

*Drug abbreviation: Tau. = Taurine; GABA = γ-aminobutyric acid; PTX = Picrotoxin; STRY = Strychnine.

RESULTS

1- Comparative effects of Tau. and GABA on evoked EPSP amplitude.

Tau. is known to mimic the action of GABA in nervous tissues. At the cercal-nerve giant-fiber synapse, no quantitative studies have been made. In order to study the potency of Tau. and GABA to depress the synaptic transmission, preliminary experiments were performed using the mannitol-gap technique at the postsynaptic level. Evoked excitatory postsynaptic potentials (EPSP) and continuous postsynaptic polarization were recorded on a magnetic tape. The analysis of Tau. and GABA-mediated potential changes was done later on a programmable Histomat S signal analyser. Fig. 1 gives an example indicating the stronger synaptic depressant effect on Tau. compared to GABA's at the same concentration : 10 mM. On the other hand Tau. and GABA induced a postsynaptic hyperpolarization (see fig. 1 and Table 1).

Are Tau. and GABA acting on the same or different membrane receptors? In order to answer this question, experiments were performed using the single-fiber oil-gap technique. This unitary technique allows the control of the postsynaptic membrane resistance by passing pulses of hyperpolarizing current using a Wheatstone bridge circuit. Control of the evoked EPSP amplitude can be achieved at the same time.

Fig. 2 shows semi-logarithmic dose-responses curves in which the postsynaptic resistance decrease was plotted versus the logarithm of the amino-acid concentration. Results were obtained using non cumulative application of amino-acid. GABA often produced responses which faded during the drug application; for this reason we have taken the maximal effect of the drug. Comparable studies were made using the evoked EPSP amplitude as a test of amino-acid effect. Results appear in fig. 3.

The results of the experiments illustrated in fig 2 and 3 and data enclosed in Table I suggest that Tau. is a more potent compound than GABA to depress the evoked EPSP, whereas the effects of GABA on the postsynaptic membrane resistance are more important than those obtained with Tau.

In order to clarify the nature of the receptor mechanism involved in Tau. we have studied the combined application of GABA and Tau. on a single postsynaptic fiber. The control GABA curve was compared with curves obtained by combining varying concentrations of GABA with 2.5 and 10mM of Tau. GABA "combination" curve obtained was shifted upwards in a non parallel manner. According to the "occupation theory" (Ariens and Simonis, 1964) Tau. appears to be an agonist of GABA at this level. (Fig. 4 and 5)

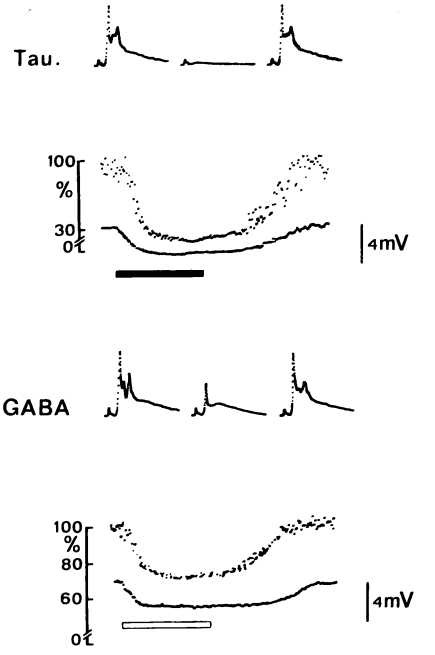

Figure 1. — Effects of Tau. (10mM) and GABA (10 mM) on the evoked EPSP amplitude and postsynaptic polarization. (mannitol-gap technique). Graphs are issued from the Histomat signal analyser and drawn with an XY writing table.

For each amino-acid, the mean of 30 postsynaptic responses before, during and after the effect is represented (upper trace).

Below, the time course of the evoked EPSP amplitude is shown (scale on the left indicates the percentage of the remaining EPSP). The third line depicts the evolution of the postsynaptic polarization (scale on the right). Each point of the second and third line represents the mean issued from 3 postsynaptic events.

Black and white bars denote period (5 mn) of application of amino-acids.

TABLE I

N° Experiment	%tage of variation after 15 minutes of experiment -(increase) +(decrease)									
Oil-gap technique	Control		Tau 20 mM		GABA 20 mM		Tau+GABA		GABA+Tau	
	Rmb	EPSP ampl.	Rmb	EPSP ampl.	Rmb	EPSP ampl.	Rmb	EPSP ampl.	Rmb	EPSP ampl.
1	+2%	0	-26,8%	-74%	-34,8%	-23,5%	-60,2%	-75,3%	62,4%	-78,6%
2	0	0	-20%	-76%	-30%	-25,2%	-50,7%	-78,1%	52%	-78,8%
3	-2%	-3%	-23%	-81%	-32,2%	-26,4%	-57,3%	-89,8%	-56,2%	-90,2%
4	+1%	+2%	-17%	-72%	-29,2%	-25,8%	-50,2%	-75,1%	-49,8%	74,3%
5	0	0	-28%	-80%	-35%	-27,2%	-62,4%	-87%	-63,1%	-88,2%
Average	+0,2%	+0,2%	-22,96%	-76,6%	-32,24%	-25,62%	-56,16%	-81,06%	-56,7%	-82,02%

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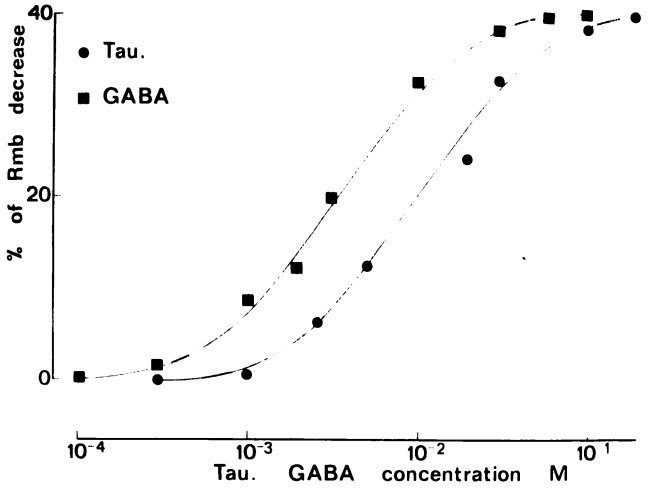

Figure 2. — Effects of Tau. and GABA on the postsynaptic membrane resistance (Rmb). No — normalized curves.

The same maximal decrease of Rmb (40%) is obtained under Tau. or GABA treatment.

Dose-response curves are issued from a representative experiment. Single-fiber oil-gap technique.

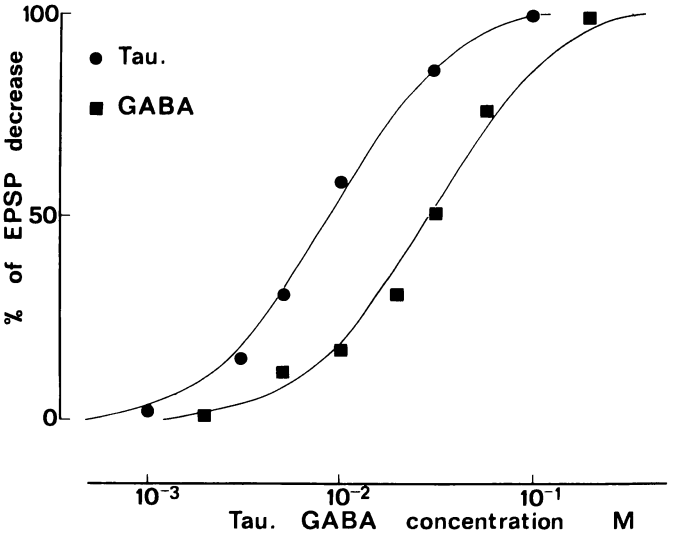

Figure 3. — Effects of Tau. and GABA on the evoked EPSP amplitude. The normalized decrease of the EPSP is plotted versus the logarithm of Tau. and GABA concentration. Singlefiber oil-gap technique.

2- Presynaptic effects of Tau. and GABA

Pharmacological investigations at the presynaptic level can be done using the preparation including the cercal nerve XI and the postsynaptic giant axon. For this, we have used the mannitol-gap technique previously described. Recording of the polarization of the presynaptic fiber was made between the end of the cercal nerve XI and the A6 ganglion, which is connected to the ground.

In normal conditions Tau. 20 mM and GABA 20 mM produced a depolarization of about 1.5 mV on the afferent pathways included in the cercal nerve XI. On the other hand, effects of amino-acids have been tested on degenerated cercal nerve XI. Degeneration was obtained by cutting the nerve at the basis of the cercus, 15 days before the test. Under these conditions, the presynaptic effects of Tau. and GABA are abolished. These last data enhance the suggestion of a presynaptic depolarizing effect of Tau. and GABA.

As seen in fig. 6 these experiments have been completed by a test of combined solutions (GABA + Tau.) which are active on the presynaptic polarization. Nevertheless, at this level, it is not possible to be precise about the direct effect of Tau. and GABA upon the membrane resistance. Presynaptic depolarization was taken as a test of increased membrane conductance. By analogy with the postsynaptic effect of combined solutions we could expect the same synergistic effect of Tau. at the presynaptic level.

In an earlier report (Hue et al., 1978) we had suggested a presynaptic action of Tau. i.e. a weak decrease of the presynaptic action potential. In order to delineate and compare this putative effect, we have recorded and stored on a magnetic tape the electrotonically transmitted presynaptic action potentials, triggered by strong electrical presynaptic stimulations, in standard Ri and under Tau. and GABA treatment. Fifty signals were computed and averaged in each case. The results presented in fig. 7 are in accordance with the wealth of evidence for the generally accepted view that the presynaptic spike decrease is the result of presynaptic Tau. or GABA-induced depolarization. Nevertheless, the presynaptic spike being recorded by electrotonic transmission and the decrease of the postsynpatic membrance resistance could partially explain the presynaptic spike decrease.

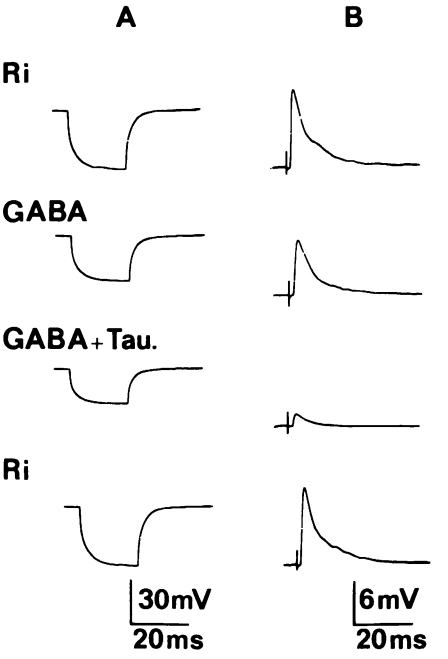

Figure 4. — Effects of Tau. 10 mM and combined solutions Tau. 10 mM +GABA 20 mM on the postsynaptic membrane resistance (A) and evoked EPSP (B).

Resistance was tested as an hyperpolarizing current pulse applied through the postsynaptic membrane. EPSP was evoked by a presynaptic electrical stimulation applied on the homolateral cercal nerve XI.

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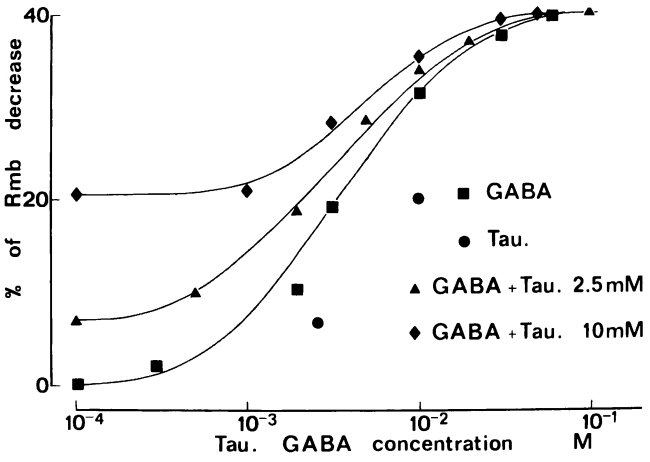

Figure 5. — Interaction between GABA and Tau. on the postsynaptic membrane resistance (Rmb). Varying concentrations of GABA are applied with a fixed dose of Tau. 2.5 and 10 mM. Results obtained were compared with the normal dose-response curve. Note the upward non-parallel shift of the combined curves. No-normalized curves.

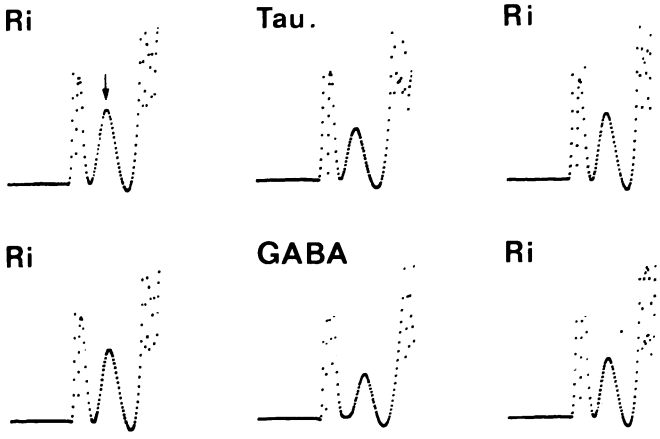

Figure 7. — Effects of Tau. 20 mM and GABA 20 mM on the electrotonically transmitted presynaptic action potential (arrow).

Synaptic events are issued from mannitol-gap technique, stored on a magnetic tape, computed and presented in form of dot-display recordings as a mean of 50 signals.

Note that the induced Tau. and/or GABA decrease of the presynaptic spike is reversible by washing with normal Ri.

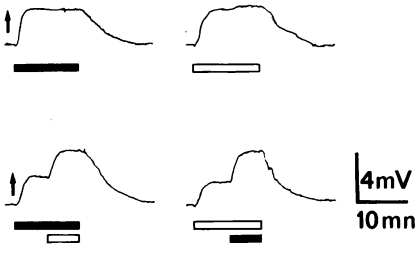

Figure 6. — Effects of Tau. 20 mM (black bars) and GABA 20 mM (white bars) and combined solutions on the presynaptic fiber polarization. Arrow indicates the depolarizing direction from a stable resting polarization.

Presynaptic fiber polarization was recorded using the mannitol-gap technique.

3-Chloride and potassium dependency of pre- and postsynaptic effects of Tau. and GABA.

Experiments were performed with standard Ri and low chloride solutions as indicated in fig. 8 et 9. Results are consistent with the hypothesis of an increase in pre- and postsynaptic membrane permeability to chloride ions for both, Tau. and GABA. It appears therefore in both cases, that the presynaptic chloride dependency corresponds via a shift to the left of the postsynaptic chloride dependency linear relationship.

For the potassium dependency of Tau. and GABA effects, the results of some experiments (not illustrated here) in which external potassium was increased, lead us to conclude that Tau. and GABA allowed a weak increase in potassium permeability of pre- and postsynaptic membranes.

4- Action of antagonistic substances: Picrotoxin and Strychnine

According to numerous findings, PTX which is known as an antagonist of GABA (Takeuchi and Takeuchi, 1969; De Groat, 1972; Levy and Anderson, 1972; Barker et al., 1975 a) has also an antagonistic action to Tau. (Barker et al., 1975; Koidl and Florey, 1975; Nistri and Constanti, 1976). At the cercal-nerve giant-fiber synapse of the cockroach, Callec (1974) has shown that PTX was able to suppress spontaneous and evoked IPSP and to enhance the subthreshold evoked EPSP (see also fig. 10).

On the other hand, STRY which is known to block spinal inhibition postsynaptically by interacting with glycine receptors (Curtis et al., 1971), has been reported to antagonize Tau. effect (Barker et al., 1975 a, b; Nistri and Constanti, 1976). At the crayfish neuromuscular junction (Parnas and Atwood, 1966) and at the cockroach neuromuscular junction (Atwood and Jahromi, 1967) STRY has been found to block the synaptic transmission by acting on the presynaptic endings. We have shown in fig. 10 that an irreversible block of the cercal-nerve giant-fiber synapse occurs during application of STRY 10^{-4} M.

In the experiments described here, PTX was employed at various concentrations and applied 20 minutes before the amino-acid test. Results summarized in fig. 11 and 12 show that Tau. responses were more antagonized by PTX than GABA responses and were completely suppressed at 10^{-5} M PTX. In other respects, in some experiments, we have noted that PTX 10^{-7} M to 5 x 10^{-7} M does not strongly antagonize the Tau.-induced postsynaptic resistance change but partially antagonizes the important depressant effect of Tau. on the evoked EPSP amplitude. Perhaps there is a particular antagonistic action of PTX which explains the different potencies of Tau. and GABA to depress the evoked EPSP amplitude.

In many cases, especially high concentrations of PTX increased the postsynaptic membrane resistance (see Callec, 1974) by 10 to 20% and inhibited all the effects of Tau. and GABA.

In some experiments the interaction between STRY and GABA and/or

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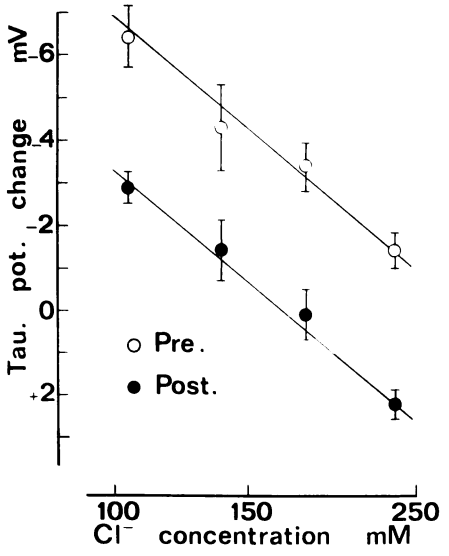

Figure 8. — Pre- and postsynaptic potential changes during addition of Tau. 20 mM to Ringer solution containing a various amount of chloride. Mannitol-gap technique.

Each point represents the average of six to fifteen values (±S.E.).

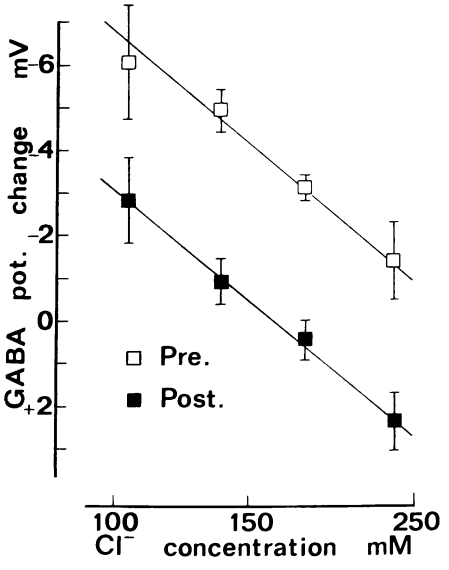

Figure 9. — Pre- and postsynaptic potential changes during addition of GABA 20 mM to Ringer solution containing a various amount of chloride. Mannitol-gap technique.

Each point represents the average of five to eight values (±S.E.).

Tau. was tested on the Tau. and GABA postsynaptic resistance changes. Increasing concentrations of STRY were applied on the A6 ganglion and Tau. or GABA tests were done after 30 minutes of pre-treatment with the drug. Tau. responses apear reduced by strychnine while GABA's are not affected. Nevertheless, high doses of strychnine are required to antagonize Tau. responses.

DISCUSSION

The results of the experiments presented here demonstrate that synaptic transmission in the insect central nervous system is affected by Tau. and GABA (Callec, 1974).

Earlier results of the Tau. effects have been published (Hue et al., 1978) but no quantitative study and comparison with the GABA effects have been made. Callec (1974) has shown that GABA was able to block the cercal-nerve giant-fiber synapse and was antagonized by PTX. In a previous report (Hue et al., 1978), we presented the evidence that Tau. may depress the synaptic transmission in the same preparation.

In the cockroach A6 ganglion Tau. was a more potent compound than GABA in blocking synaptic transmission. If under GABA treatment the slight decrease of EPSP amplitude can be interpreted as due to change in postsynaptic membrane resistance (Callec, 1974), our data for Tau. show an identical or a lower decrease in postsynaptic membrane resistance involve a major reduction of EPSP amplitude (fig. 1, 2 and 3). These results suggest that Tau. acts on EPSP amplitude by a passive effect on the postsynaptic membrane resistance like GABA, but, in addition another mechanism must be required to explain the strong effect of Tau. on EPSP amplitude.

The observed depolarization of presynaptic terminals induced by Tau. and GABA is in agreement with the literature (Schmidt, 1963; Dudel, 1965; Tebecis and Phillis, 1969; Davidson and Southwick, 1971; Davidoff, 1972; Nishi et al., 1974; Barker et al., 1975 a; Gallagher et al., 1978). According to these authors, it is possible to explain the weak decrease of the presynaptic action potential by the Tau. and/or GABA induced presynaptic terminal depolarization. This depolarization and especially the associated increase of presynaptic membrane conductance to chloride ions would shunt the presynaptic action potential amplitude. When one knows the importance of the amplitude of presynaptic spike on the influx of calcium governing the process of transmitter release (Katz et Miledi, 1967) we should expect a reduction in transmitter release by Tau. or GABA action at the presynaptic level.

The dose-conductance relationships obtained with GABA and Tau. suggest for both the same intrinsic activity, but a lower affinity for Tau. However the receptor mechanism of Tau. effect remains unclear. On the one hand GABA/Tau. "combination" curves obtained are similar to those obtained with GABA/5-aminovalerianic acid at the lobster inhibitory neuromuscular junction by Constanti (1977). In this case we should expect an agonist effect for Tau. On the other hand 5-aminovalerianic acid is part of a group which has in common with Tau. and β-alanine antagonism to STRY and PTX (Barker et al., 1975 a). In addition, at the cercal-nerve giant fiber synapse we have noted that STRY antagonizes only Tau. and that PTX is able to antagonize in varying degrees both Tau. and GABA responses. These last results are similar to those obtained at the crayfish neuromuscular junction (Dudel, 1965), at the rat superior cervical ganglion (Bowery and Brown, 1974) and in the frog spinal cord (Barker et al., 1975 a).

The ionic mechanism whereby Tau. and GABA produce an increased conductance of pre- and postsynaptic membranes involves primarily chloride ions. This is supported by the direct dependency of the responses to Tau. and/or GABA application in low chloride solutions. A linear relationship was obtained both at the pre- and postsynaptic levels. The behavior of presynaptic terminal varying in a parallel manner in various chloride solutions suggests that Tau. and/or GABA cause a comparable increase in presynaptic membrane permeability to chloride ions. The parallel shift of the Tau. and GABA potential changes in the presynaptic fiber raises a question relating to the different

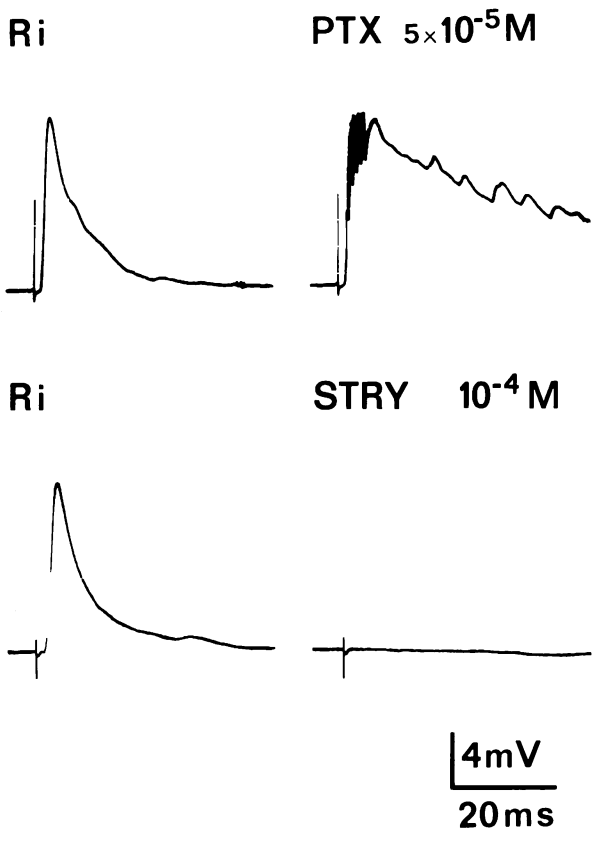

Figure 10. — Effects of PTX and STRY on the evoked EPSP in the A6 ganglion. Single-fiber oil-gap technique.

After 30 minutes of PTX treatment, the evoked EPSP is increased both in amplitude and duration and is able to trigger repetitive action potentials (which appears truncated) in the giant axon.

STRY 10^{-4} M blocks irreversibly the synaptic transmission in less than 20 minutes.

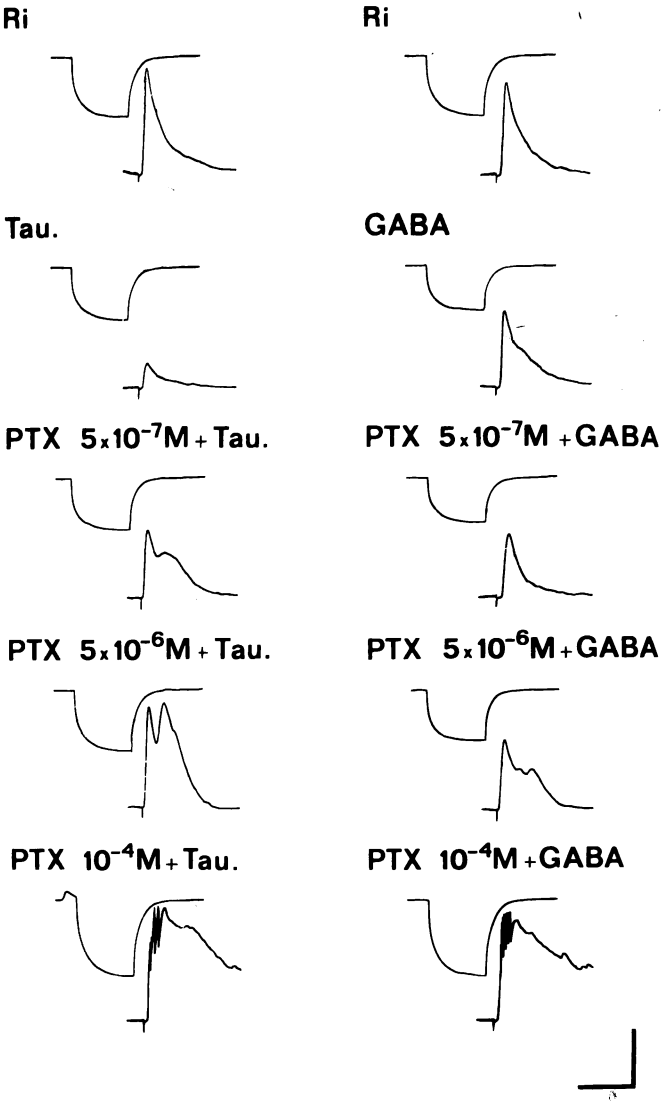

Figure 11. — Effect of Tau. and GABA on postsynaptic resistance and evoked EPSP amplitude before and after a 20 minutes PTX pretreatment of the A6 ganglion.

— Note the beginning of the antagonistic effect of PTX 5 x 10^{-7} M to Tau.-reduced EPSP, and the increase of postsynaptic membrane resistance under PTX 10^{-4} M treatment.

Horizontal scale: 20 ms

Vertical scale: 40 mV for hyperpolarizing pulses
8 mV for EPSP

responses of the pre- and postsynaptic membranes to Tau. and GABA in various chloride solutions. Why are such responses opposite at the pre- and postsynaptic level, if one obtains in both cases an increase in membrane chloride permeability? A simple explanation may be that the intracellular chloride concentration is relatively higher in the presynaptic terminal. One would suppose the presence in these presynaptic fibres of a metabolically dependent inwardly directed chloride pump. Such a mechanism has already been suspected in various preparations (Keynes, 1962; De Groat, 1972; Nishi et al., 1974).

However, the possibility that movements of other ions are involved in Tau. and GABA responses cannot be excluded. Following a 2 fold increase of external potassium concentration (from 3.1 to 6.2 mM), the Tau. and GABA induced postsynaptic hyperpolarizations decreased slightly in amplitude.

In conclusion, a similarity has been observed between Tau. and GABA in their effects on ion conductance changes. For Tau. there is a greater potency to block the cercal-nerve giant-fiber synapse. According to several authors who have studied. the ganglionic action of homotaurine and Tau. (De Groat, 1970; Horii et al., 1971; Hilton, 1977), the effects of Tau. on cholinergic receptors cannot be eliminated.

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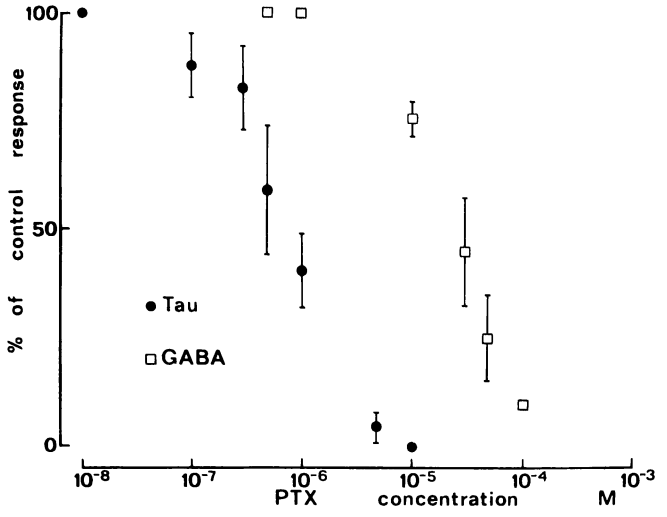

Figure 12. — Effect of increasing concentrations of PTX on Tau. and GABA responses.

The Tau. and GABA induced postsynaptic resistance changes, expressed as a percent of control, are plotted against the logarithm of the PTX concentration.

Each point represents the mean±S.E. of two to six values.

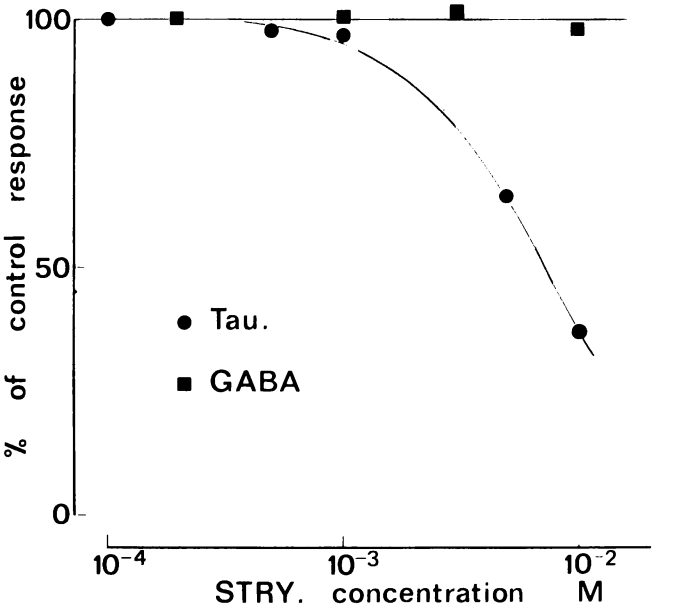

Figure 13. — Effects of increasing concentrations of STRY on Tau. and GABA responses.

The Tau. and GABA induced postsynaptic resistance changes, expressed as a percent of control, are plotted against the logarithm of the STRY concentration.

Results are issued from a representative experiment.

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Quebec Cooperative Study
of Friedreich's Ataxia

Taurine and Beta-Alanine Uptake in Cultured Human Skin Fibroblasts from Patients with Friedreich's Ataxia

S. B. MELANCON, B. GRIGNON, M. POTIER AND L. DALLAIRE

SUMMARY: *Taurine and β-alanine uptake kinetics were studied in cultured skin fibroblasts from 9 patients with Friedreich's Ataxia and 8 controls. No significant difference was observed. The data support the presence of normal β-amino acid carrier protein in Friedreich's Ataxia cell membrane.*

RÉSUMÉ: *L'incorporation de taurine et de β-alanine procède selon des paramètres de cinétique comparables dans les fibroblastes cutanés normaux et de patients souffrant d'Ataxie de Friedreich. Ces résultats militent en faveur de la normalité des protéines membranaires responsables du transport des acides β-aminés dans l'Ataxie de Friedreich.*

From le Centre de Recherche Pédiatrique de l'Hôpital Ste-Justine, Département de Pédiatrie, Université de Montréal

Reprint requests for the complete supplement on Friedreich's Ataxia (Phase Two, Part Two) to:

Dr. André Barbeau, M.D., Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec, Canada H2W 1R7.

INTRODUCTION

Amino acid transport studies in cultured human skin fibroblasts have been relatively few and were generally intended to demonstrate defects comparable to those found in kidney and intestinal cells of patients with diverse metabolic errors such as; cystinuria (Groth and Rosenberg, 1972), cystinosis (Kaye and Nadler, 1976), Hartnup disease (Booth and Nadler, 1975; Groth and Rosenberg, 1972), hyperglycinemia (Revsin and Morrow, 1976), γ-glutamyl transpeptidase deficiency (Pellefigue et al., 1976), and α-methyl-acetoacetyl-CoA β-ketothiolase deficiency (Hillman and Otto, 1974). Recent work by Ullrich and Fromter (1978) have suggested that amino acid transport in the contraluminal plasma membrane of kidney tubular epithelium differs from basal-lateral membranes and behaves more like the fibroblast transport systems. Fibroblasts then would appear suitable for investigation of amino acid conservation defects not limited to kidney and intestinal cells. Previous studies by Lemieux et al. (1976) and Butterworth et al. (1978) in Friedreich's and experimental ataxias have shown reduced levels of taurine in cerebrospinal fluid and cerebellum, respectively. These observations led us to investigate taurine transport in cultured fibroblasts from patients with Friedreich's Ataxia.

The finding of reduce uptake rates of taurine in fibroblasts could have been extended to other body systems and might provide an explanation for some of the neurologic, cardiac, hepatic, and renal defects found in Friedreich's Ataxia (Barbeau, 1978).

METHOD

Skin biopsies were obtained with informed consent from nine patients with Friedreich's Ataxia and eight control subjects. Fibroblast cultures and amino acid uptake experiments were conducted as previously described (Melançon et al., 1979) using taurine-1-^{14}C and β-alanine-1-^{14}C (New England Nuclear).

RESULTS

Taurine and β-alanine uptake was linear with time up to 90 minutes at extracellular concentrations varying from 5 μM to 0.25 mM (fig. 1). The rate of β-amino acids uptake was also linear, with increasing concentrations of the amino acids in the incubation medium up to 0.25 mM. At higher concentrations of taurine and β-alanine, the rate of uptake decreased without saturation. The apparent Km and Vmax of taurine and β-alanine uptake are illustrated in table I. Friedreich's Ataxia fibroblasts showed lower Km and higher Vmax than control fibroblasts, but these differences were not statistically significant.

DISCUSSION

Our results suggest that fibroblasts from patients with Friedreich's Ataxia incorporate taurine and β-alanine at rates comparable to normal fibroblasts. The Km for maximum velocity of taurine uptake in fibroblasts is approximately half the mean plasma taurine concentration in man (Lemieux et al., 1976). This comparison

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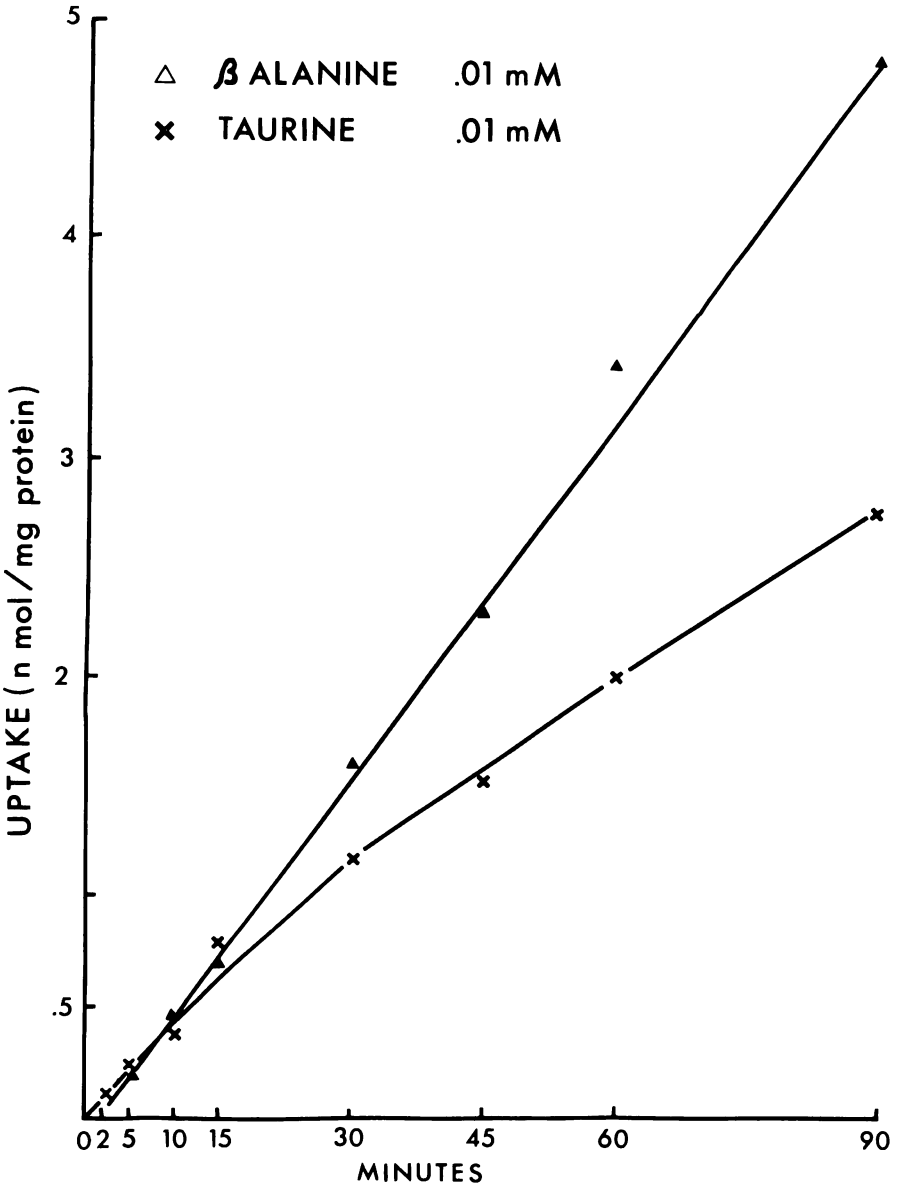

Figure 1—Time course of β-amino acid uptake by cultured human skin fibroblasts.

TABLE I

Kinetics of taurine and β-alanine uptake by cultured skin fibroblasts at low substrate concentration (.005 - .250 mM)

	Taurine		β-Alanine	
	Km*	Vmax*	Km*	Vmax*
Controls (8)	.023±.012	.152±.110	.081±.059	.226±.230
Friedreich (9)	.014±.006	.222±.102	.051±.023	.240±.103

*Km in mM; Vmax in nmol/min/mg protein; mean ±SD.

would support the view that intracellular taurine levels are easily maintained through this high affinity low Km uptake system even at half reduced plasma taurine concentration. The uptake of β-alanine follows a slightly different pattern. Plasma levels of β-alanine are normally too low for accurate estimation by standard automated amino acid analyzer methods. It is thus expected that fibroblast uptake would proceed below maximum velocity *in vivo*.

Taurine uptake by platelets from patients with Friedreich's Ataxia was recently studied (Filla et al, 1978) and found to be normal. These results and our data with cultured fibroblasts do not support the presence of a genetic defect in β-amino acid membrane protein carriers in Friedreich's Ataxia. On the contrary, an apparently reduced Km and increased Vmax as compared to control cells would tend to protect Friedreich's Ataxia cells against β-amino acid depletion. More work is presently under way in our laboratory in order to elucidate the mechanisms of regulation of β-amino acid transport in fibroblasts.

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Quebec Cooperative Study
of Friedreich's Ataxia

Regional Distribution of Amino Acids in Friedreich's Ataxia Brains

R. HUXTABLE, J. AZARI, T. REISINE, P. JOHNSON, H. YAMAMURA, AND A. BARBEAU

SUMMARY: *The distribution of amino acids in two Friedreich's ataxia brains is compared to 4 control brains. Glutamate and GABA were decreased in the cerebellar hemispheres and/or in the vermis. Taurine concentrations were uniformly elevated throughout the areas studied. Taurine/glutamate and taurine/GABA ratios were consistently elevated in Friedreich's ataxia brains, whereas glutamate/glutamine ratios were consistently decreased (with normal glutamine concentrations).*

RÉSUMÉ: *Nous avons étudié la distribution des acides aminés dans le cerveau de deux cas d'ataxie de Friedreich et de 4 cerveaux témoins. La concentration du glutamate et du GABA est diminuée dans les hémisphères cérébelleux et/ou le vermis. La concentration en taurine est uniformément élevée dans les régions étudiées. Les rapports taurine/glutamate et taurine/GABA sont constamment élevés dans les cerveaux d'ataxie de Friedreich, alors que le rapport glutamate/glutamine est diminué en présence de concentrations normales de glutamine.*

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Reprint requests for the complete supplement on Friedreich's Ataxia (Phase Two, Part Two) to:

Dr. André Barbeau, M.D., Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec, Canada, H2W 1R7.

INTRODUCTION

There are no reports of the concentrations of free amino acids in the brains of Friedreich's ataxia patients. The possibility that alterations in amino acid concentrations are related to the pathogenesis of the disease has assumed greater importance as a result of the findings of Lemieux et al. (1976) on the disturbances of amino acide is plasma and urine. Among the many disturbances found by these authors, was an apparent defect in the tubular resorption of taurine in the kidney. The taurine content of urine was doubled in Friedreich's ataxia patients compared to control subjects.

Disturbances in taurine regulation are of potential significance in that Friedreich's ataxia is a spinocerebellar degeneration, and there is strong evidence implicating taurine as a neurotransmitter in the cerebellum (McBride and Frederickson, 1978). The possible involvement of taurine in the cardiomyopathy of Friedreich's ataxia has been discussed by Huxtable (1978).

In this paper, we report the result of amino acid analyses of the brains of two Friedreich's ataxia patients, and four control brains from subjects dying of non-neurological disorders.

METHODS

The subjects are described in an accompanying paper (Reisine et al., 1979). The regions of the brain examined were; dentate nucleus, anterior cerebellar vermis, posterior cerebellar vermis, cerebellar hemisphere, inferior olive nucleus, red nucleus and dorsal root ganglion. In addition, on one of the Friedreich's ataxia patients, we obtained analyses of the lumbar spinal cord and the thoracic spinal cord.

Amino Acid Analyses:

Brain tissues were thawed and homogenized 10:1 in 3.5% sulfosalicylic acid. Homogenates were centrifuged and the supernatants added to a half volume of 0.3N lithium hydroxide. Aliquots of this solution were analyzed on a Beckham Model 118 amino acid analyzer. α-Amino-n-butyric acid was used as internal standard.

Statistical analyses were performed by the Student unpaired 't' test. Data are reported as means $\pm$ one standard deviation.

RESULTS

Analytical results are shown in Table 1. Numerous differences can be observed, although the small number of samples militates against statistical significance. For areas in which six brains were analyzed, six statistically significant differences occur. Glutamate concentration is significantly lowered in the cerebellar hemisphere and the posterior cerebellar vermis. GABA concentrations are significantly decreased in the same two areas, and also in the anterior cerebellar vermis. Aspartate, glutamate, phenylalanine, and GABA are uniformly decreased in concentration in all brain areas examined. Taurine is the only amino acid to be increased in all the brain areas examined. Figure 1 illustrates the individual values of the neurotransmitter amino acids in the various brain areas. In the Friedreich's ataxia samples, taurine shows a marked regional variability, ranging from 0.6 μmole/g in the dorsal root ganglion to 3.8 μmole/g in the cerebellar hemisphere. However, a different

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TABLE 1

Amino acid content (μmole/g wet weight) in selected brain areas

Control	CH (4)	PCV (4)	ACV (4)	ION (3)	DN (3)	RN (1)	DRG (1)
Tau	2.21±1.03	1.96±0.92	1.61±0.73	0.95±0.49	1.35±0.71	1.58	0.47
Thr	0.94±0.32	0.97±0.25	0.82±0.20	0.62±0.33	0.78±0.15	0.35	0.31
Asp	1.57±1.01	1.75±1.14	1.44±1.03	0.66±0.41	1.22±0.22	1.53	0.38
Ser	1.16±0.77	1.24±0.56	1.00±0.48	0.75±0.18	1.10±0.50	0.75	0.38
Gln	5.38±1.62	6.36±3.38	3.59±1.89	5.33±4.52	6.19±3.83	8.62	0.21
Glu	10.14±2.63	12.51±3.36	7.71±3.93	4.98±3.30	6.75±2.70	7.49	1.09
Gly	1.96±0.93	2.20±0.86	1.71±0.82	1.68±0.87	1.85±0.56	1.58	0.54
Ala	2.11±0.61	2.39±0.56	1.95±0.59	1.83±0.84	1.76±0.31	1.89	0.90
Val	0.75±0.38	0.77±0.26	0.58±0.30	0.54±0.16	0.66±0.29	trace	0.37
Cysta	0.45±0.12	0.49±0.11	0.37±0.05	0.96±0.78	1.52±0.79	1.46	0.00
Met	0.39±0.18	0.41±0.10	0.36±0.13	0.36±0.04	0.33±0.09	0.52	0.13
Isoleu	0.47±0.23	0.47±0.14	0.41±0.14	0.38±0.02	0.42±0.13	0.35	0.22
Leu	1.00±0.43	1.05±0.30	0.92±0.28	0.69±0.13	0.81±0.23	0.74	0.31
Tyr	0.49±0.13	0.52±0.09	0.45±0.14	0.43±0.21	0.46±0.09	0.21	0.20
Phe	0.59±0.15	0.62±0.08	0.53±0.11	0.49±0.18	0.52±0.03	0.26	0.21
GABA	1.41±0.45	1.99±0.52	1.55±0.20	1.02±0.32	3.18±1.15	1.75	—

Friedreich's ataxia	CH (2)	PCV (2)	ACV (2)	ION (1)	DN (2)	RN (2)	DRG (1)	LSC (1)	TSC (1)
Tau	3.78±0.61	2.38±0.84	2.56±0.07	3.05	3.49±3.26	1.13±0.06	0.63	0.96	1.37
Thr	0.48±0.01	0.51±0.08	0.59±0.03	1.13	0.86±0.94	1.33±1.43	0.43	0.42	0.49
Asp	0.75±0.36	0.92±0.08	1.00±0.13	1.20	0.93±0.11	0.90±0.21	0.31	0.90	1.29
Ser	0.64±0.14	0.71±0.23	0.83±0.21	2.30	1.47±1.56	0.89±0.45	0.36	0.51	0.60
Gln	4.98±2.22	5.13±1.35	5.60±2.80	4.08	9.27±5.71	5.36±3.54	1.27	0.45	4.95
Glu	4.42±0.83[a]	4.55±0.73[a]	4.12±0.88	4.02	4.12±1.90	4.98±0.39	0.97	2.84	7.05
Gly	1.17±0.33	1.28±0.34	1.33±0.37	3.35	2.33±1.99	1.98±1.14	0.88	2.06	1.48
Ala	1.25±0.30	1.26±0.42	1.43±0.22	2.96	2.52±1.73	1.78±0.39	1.33	1.39	1.36
Val	0.38±0.13	0.58±0.43	0.60±0.06	1.54	1.04±1.29	0.48±0.01	0.37	1.06	0.36
Cysta	0.23±0.18	0.58±0.54	0.21±0.15	0.27	0.51±0.40	0.66±0.49	0.05	0.19	0.49
Met	0.24±0.01	0.29±0.10	0.38±0.01	1.03	0.61±0.66	0.43±0.16	0.19	0.28	0.26
Isoleu	0.21±0.06	0.31±0.11	0.15±0.00	0.72	0.47±0.47	0.43±0.13	0.26	0.13	0.22
Leu	0.43±0.12	0.59±0.16	0.49±0.19	1.63	1.09±1.10	0.78±0.28	0.41	0.36	0.39
Tyr	0.37±0.00	0.35±0.10	0.45±0.01	0.84	0.58±0.47	0.46±0.01	0.36	0.21	0.33
Phe	0.32±0.04	0.32±0.08[c]	0.42±0.03	0.64	0.48±0.39	0.48±0.01	0.33	0.16	0.28
GABA	0.73±0.08[a]	0.95±0.17[a]	0.97±0.11[b]	0.83	1.52±1.10	2.13±0.78	0.07	0.55	0.37

The numbers of brains analyzed are indicated at the column heads. Statistical significances are indicated only for brain areas for which a total of six brains were analyzed. [a]$p<.05$, [b]$p<.025$, [c]$p<.01$

The abbreviations are: CH cerebellar hemisphere; PCV posterior cerebellar vermis; ACV anterior cerebellar vermis; ION inferior olive nucleus; DN dentate nucleus; RN red nucleus; DRG dorsal root ganglion; LSC lumbar spinal cord; TSC thoracic spinal cord.

picture is seen with glutamic acid (Fig. 1b). Control brains show marked regional variations in concentration and the standard deviation about the mean in a given brain area is high. In the Friedreich's ataxia brains, however, glutamic acid concentrations are invariant in all the areas examined. Furthermore, little difference is seen between the two brains. Qualitatively, the same phenomenon occurs with the other neurotransmitter amino acids GABA (Fig. 1c) and aspartic acid (Fig. 1d).

The ratios of concentrations of neurotransmitter amino acids may be of greater relevance than the absolute concentrations (Van Gelder 1978). Selected ratios are illustrated in Table 2. In the four brain areas reported, taurine/glutamate and taurine/GABA ratios are consistently elevated in Friedreich's ataxia brains, whereas glutamate/glutamine ratios are consistently decreased. No alteration occurs in GABA/glutamate ratio. The changes in glutamate/glutamine ratios are due to altered glutamate concentration, glutamine levels being normal in Friedreich's ataxia brains.

DISCUSSION

Although our data are based on only two Friedreich's ataxia brains and four control brains, they are of interest in that brain amino acid levels have rarely been reported for any ataxia, and are unreported for Friedreich's ataxia.

Despite the small number of brains reported on here, a number of differences are clearly established. In

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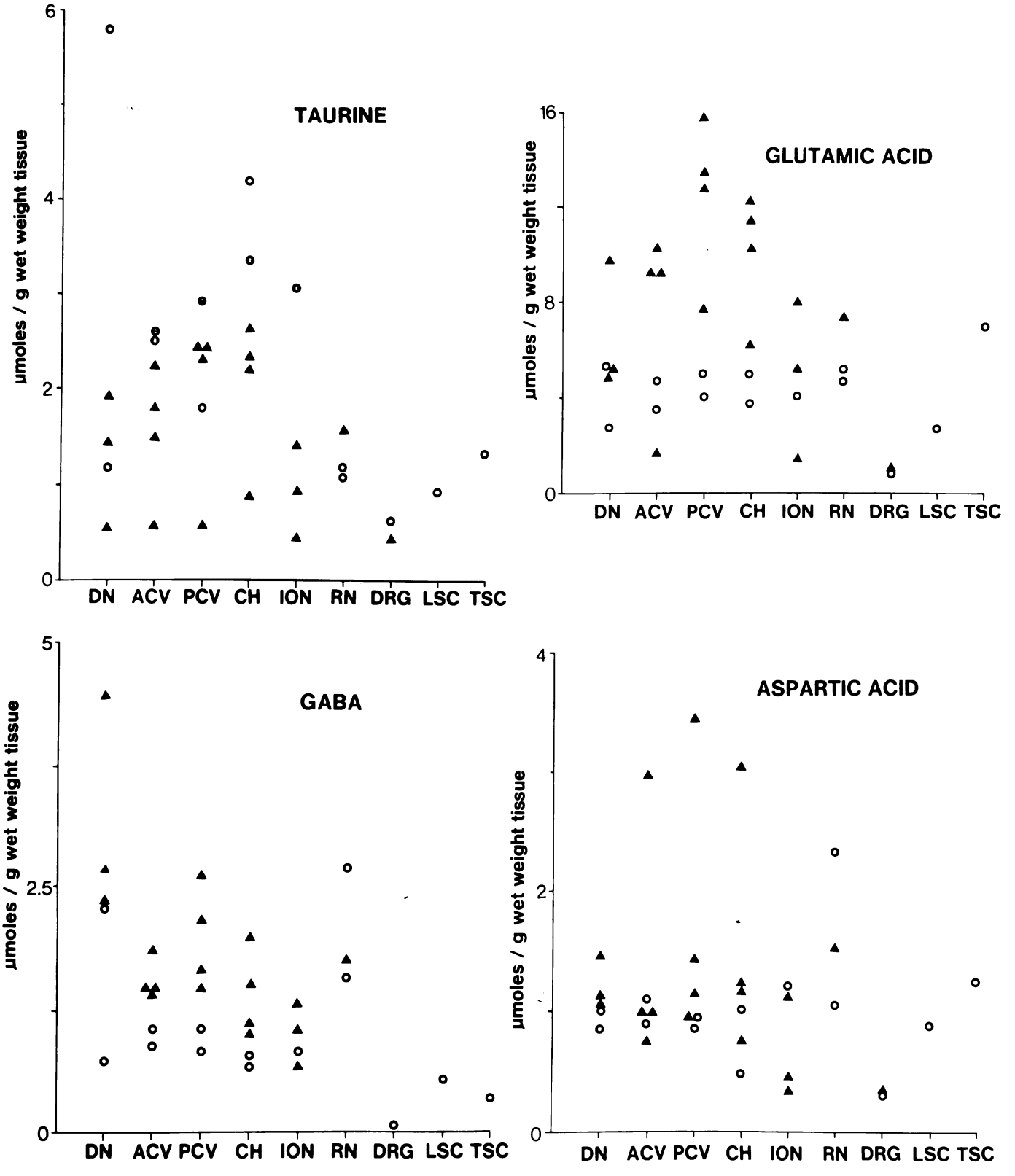

Figure 1 — Neurotransmitter amino acid concentrations. Abbreviations for brain areas are given in Table 1.
▲ : control brains 0 : Friedreich's ataxia brains

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TABLE 2

Selected Amino Acid Ratios

Control	CH (4)	PCV (4)	ACV (4)	DN (3)
Tau/glu	0.24±0.14	0.18±0.11	0.37±0.42	0.23±0.15
Tau/GABA	1.57±0.63	1.00±0.52	1.05±0.50	0.51±0.36
GABA/glu	0.14±0.04	0.16±0.04	0.32±0.30	0.47±0.04
Glu/gln	1.94±0.55	2.32±1.23	2.20±1.05	1.22±0.38
Friedreich's ataxia	(2)	(2)	(2)	(2)
Tau/glu	0.89±0.30[a]	0.55±0.28	0.64±0.12	0.74±0.45
Tau/GABA	5.17±0.23[c]	2.62±1.38	2.66±0.23[b]	4.17±5.17
GABA/glu	0.17±0.06	0.21±0.00	0.24±0.02	0.49±0.49
Glu/gln	0.94±0.25	0.90±0.10	0.80±0.24	0.63±0.59

[a]$p<.025$; [b]$p<.01$; [c]$p<.001$

general, changes in amino acid content are limited to neurotransmitter substances. Glutamic acid, glutamine, and GABA are metabolically closely linked. Glutamic acid, an excitatory amino acid, is converted to GABA, an inhibitory amino acid, by the enzyme glutamic acid decarboxylase. Both of these amino acids are significantly decreased in concentration in the cerebellar hemisphere and the posterior cerebellar vermis. Additionally, GABA concentration is significantly decreased in the anterior cerebellar vermis. Glutamine concentration, on the other hand, is normal in all the areas examined. Glutamine is present largely in glia (Martinez-Hernandez et al., 1977), whereas glutamate is present largely in neuronal elements (Berl et al., 1970). One significant normal function of glutamine is to buffer the glutamate concentration by means of a ready transamination reaction (Van Gelder, 1978). It seems that in Friedreich's ataxia brains this buffering capacity has been lost. The most remarkable observation made in this study is the uniform concentration of glutamic acid found in the different areas of the Friedreich's ataxia brains. The significance of this must await further work.

ACKNOWLEDGMENTS

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Quebec Cooperative Study
of Friedreich's Ataxia

Brain Neurotransmitter Receptors in Friedreich's Ataxia

I. D. REISINE, J. AZARI, P. C. JOHNSON, A. BARBEAU, R. HUXTABLE AND H. I. YAMAMURA

SUMMARY: *The binding of ^{3}H-quinuclidinyl benzilate, a muscarinic cholinergic antagonist, of ^{3}H-dihydroalprenolol, a beta adrenergic antagonist, and of ^{3}H-flunitrazepam, a ligand which labels benzodiazepine receptors, was examined in several regions of control and Friedreich's ataxia (FA) brains. ^{3}H-Quinuclidinyl benzilate binding appeared to increase in the inferior olivory nucleus, anterior and posterior cerebellar vermi but was unaltered in the dentate nucleus and cerebellar hemisphere of FA brain. The binding of ^{3}H-dihydroalprenolol seemed to increase in the inferior olivary nucleus yet was not different from controls in the dentate nucleus, cerebellar hemisphere, anterior and posterior cerebellar vermi of FA brains. ^{3}H-Flunitrazepam binding was slightly lowered in the inferior olivary and dentate nuclei but was unchanged in the other FA brain regions examined. The present study suggests possible trends in neurotransmitter receptor alterations in post-mortem brain tissue of FA patients.*

RÉSUMÉ: *Nous avons examiné dans plusieurs régions de cerveaux contrôles et provenant d'ataxie de Friedreich (AF) la liaison de plusieurs ligands: ^{3}H-quinuclidinyl benzilate (QNB), un antagoniste chlorinergique muscarinique; ^{3}H-dihydroalprenolol (DHA), un antagoniste β-adrénergique; ^{3}H-flunitrazepam (FLU), un ligand qui marque les récepteurs à la benzodiazepine. La liaison de QNB semble augmentée dans le noyau olivaire inférieur, le vermis cérébelleux antérieur et postérieur, mais est inchangée dans le noyau dentelé et les hémisphères cérébelleuses de cerveaux AF. La liaison du DHA semble également augmenté dans le noyau olivaire inférieur, mais ne diffère pas des contrôles dans le noyau dentelé, les hémisphères cérébelleuses et le vermis antérieur et postérieur. Par contre la liaison FLU était légèrement diminuée dans les noyaux olivaires inférieurs et dentelés mais était intacte dans les autres régions examinées de cerveaux AF.*

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INTRODUCTION

Friedreich's ataxia (FA) is an inherited neurological disorder that was first described in 1861 (Friedreich, 1861). Neuropathological studies have revealed that there is severe neuronal degeneration in several regions of the central nervous system. In particular, the sensory fibers of the posterior columns, the spinocerebellar and corticospinal tracts, the brainstem, and cerebellum are most affected in the disease (Wintrobe et al., 1974).

At present, little information is available concerning the possible neurochemical alterations that might be present in the FA central nervous system. Previous studies on such neurological disorders as Parkinson's disease and Huntington's chorea have revealed much information concerning the neurochemical abnormalities of these degenerative diseases (Reisine et al., 1977; Yamamura, 1978). Thus, in a recent study by Huxtable et al., (1978), marked changes in the levels of several amino acids were observed in various FA brain regions which upon pathological examination exhibited extensive neuronal destruction. In the present study, using a limited number of brain samples, we report on the density of muscarinic cholinergic, β-adrenergic, and benzodiazepine receptors in brains obtained post-mortem from two subjects diagnosed as having FA as compared to four patients devoid of any neurological disorders.

SUBJECTS AND METHODS

Post-mortem brain tissue from two patients (average age 19 years) diagnosed as having Friedreich's ataxia were used in this study (Table 1). Four brains were obtained post-mortem from individuals (average

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age 40.5 years) devoid of any neurological or psychiatric disorders. The onset (5 years old) of ataxia and death (19 years old) in both FA patients was early in life. Pathological examination of the brain and spinal cord of both FA patients revealed degeneration of the posterior columns, pyramidal and spinocerebellar tracts. Each FA patient had distal peripheral neuropathy with kyphoscoliosis and pes cavus. Both patients had cardiomyopathy.

Attempts were made to obtain age matched controls for this study. Two of the controls patients were between 20-30 years old at the time of death (Table 1). Two older patients were included in the control group since no apparent differences were noted in neurotransmitter receptor binding in the older patient's brain tissue as compared to binding in brain tissue from the younger control patients. Thus, although age matched controls were not used in this study, no significant differences in receptor binding characteristics were observed with age. Therefore, any differences in receptor binding between the FA and control groups is not the result of differences in the ages of the patients at the time of death.

On the average, five hours elapsed between the time of death and freezing (–80° C) of the brain tissue. Before freezing the tissue, the inferior olivary and dentate nuclei, anterior and posterior cerebellar vermi, and cerebellar hemisphere were dissected from each brain. On the day of the experiment, the tissue from each brain region (about 50 mg) was thawed and then homogenized with a Polytron homogenizer (Brinkman, setting 5 for 30 sec.) to make a 5% homogenate in 50 mM sodium-potassium phosphate buffer (pH 7.4). The tissue was washed once by diluting it with 15 ml of buffer and centrifuging it at 48,000 x g for 15 minutes in a Sorvall RC2-B centrifuge. The pellets were resuspended in buffer to make a 5% homogenate. Protein determinations were performed by the method of Lowry et al., (1951).

The ligands employed to measure the various receptor levels were as follows: cholinergic muscarinic receptor, ^{3}H-quinuclidinyl benzilate (^{3}H-QNB); β-adrenergic receptor, ^{3}H-dihydroalprenolol (^{3}H-DHA); and the benzodiazepine receptor, ^{3}H-flunitrazepam (^{3}H-Flu). The interaction of these ligands with their respective receptors has been described elsewhere (Yamamura and Snyder, 1974; Bylund and Snyder; 1976; Speth et al., 1978).

Briefly, the muscarinic cholinergic receptor was assayed in tissue homogenates (50-100 μg protein) which were incubated in 2 ml of 50 mM sodium-potassium phosphate buffer (pH 7.4) for 60 minutes with 100 pM ^{3}H-QNB (29.4 Ci/mmole) in the presence and absence of 1 μM atropine. The reaction was terminated by vacuum filtration through GF/B glass fiber filters, followed by four 5 ml rinses of ice cold buffer. Bound ^{3}H-QNB retained on the filter was extracted in 9 ml of a toluene based scintillation cocktail and radioactivity was monitored in a Searle Mark III liquid scintillation counter. The amount of isotope displaced by atropine is termed specifically bound QNB and is a measure of the number of receptor sites present.

The benzodiazepine receptor was assayed in tissue homogenates (50-100 μg protein) which were incubated for 90 minutes at 0° C in 2 ml of buffer containing 0.5 nM ^{3}H-Flu 87.5 Ci/mmole) in the presence and absence of 1 μM clonazepam. Termination of the reaction was similar to that

TABLE 2

Regional ^{3}H-QNB Binding in FA and Normal Brains

Brain Number	Brain Region				
	Inferior Olivary Nucleus	Dentate Nucleus	Ant. Cere. Vermis	Post. Cere. Vermis	Cere. Hemisphere
FA					
A-77-178	23.8	37.0	48.2	52.2	47.9
A-77-218	40.3	18.9	57.2	59.6	48.3
Average	32.1	28.0	52.7	55.4	48.1
Normal					
A-77-101	—	28.8	31.1	34.2	45.2
A-77-71	15.6	31.8	40.7	33.7	50.1
A-77-51	23.2	—	26.1	31.0	61.1
A-75-144	—	19.2	24.6	38.5	52.3
Average	19.2	26.6	30.6	34.3	52.2

The values are expressed as fmole of ^{3}H-QNB bound per mg protein.
The concentration of ^{3}H-QNB used was 100 pM

TABLE 1

Description of patients from which post-mortem brain tissue was obtained

Brain Number	Interval between Death and freezing (hrs.)	Patient Age (yrs.)	Cause of Death
FA			
A77-178	3.5	19	Heart Failure due to Cardiomyopathy
A77-218	5.5	19	Pneumonia
Average	4.5	19	
Normal			
A77-101	3.0	21	Respiratory Failure
A77-71	9.0	27	Lymphoma
A77-51	2.0	63	Liver cirrhosis
A75-149	5.0	51	Peritonitis
Average	5.0	41	

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TABLE 3

Regional ^{3}H-Flunitrazepam Binding in FA and Normal Brains

Brain Number	Brain Region				
	Inferior Olivary Nucleus	Dentate Nucleus	Ant. Cere. Vermis	Post. Cere. Vermis	Cere. Hemisphere
FA					
A-77-178	1.9	1.9	58.8	53.2	55.4
A-77-218	5.4	5.6	42.8	41.1	53.7
Average	3.6	3.7	50.8	47.2	54.6
Normal					
A-77-101	—	4.2	31.4	28.6	28.1
A-77-71	9.0	7.3	56.5	35.2	51.4
A-77-51	5.8	—	52.4	54.2	54.8
A-75-149	—	8.1	60.4	50.6	78.3
Average	7.4	6.5	50.2	42.2	53.2

The values are expressed as fmole of ^{3}H-Flu bound per mg protein.
The concentration of ^{3}H-Flunitrazepam used was 500 pM.

described above. Specific ^{3}H-Flu binding was defined as that binding displaceable by 1 μM clonazepam.

The β-adrenergic receptor was assayed in tissue homogenates (300 μg protein) which were incubated for 30 minutes at 25°C in 2 ml of sodium-potassium phosphate buffer containing 0.25 nM ^{3}H-DHA (58 Ci/mmole) in the presence and absence of 0.1 μM (-)-propranolol. Termination of the reaction was similar to the previously described assays except that the filters were rinsed with buffer maintained at 25°C. Specific ^{3}H-DHA binding was defined as that binding displaceable by 0.1 μM (-)-propranolol.

RESULTS

The results of this study reveal that there may be an increase in ^{3}H-QNB binding in the inferior olivary nucleus and in the anterior and posterior cerebellar vermi of FA brains (Table 2). ^{3}H-QNB binding was unaltered in the FA dentate nucleus and cerebellar hemisphere. ^{3}H-Flu binding appeared to be slightly lowered in the inferior olivary and dentate nuclei yet was unchanged in the anterior and posterior cerebellar vermi and cerebellar hemisphere (Table 3). The binding of ^{3}H-DHA was unaltered in the FA dentate nucleus, anterior and posterior cerebellar vermi, and cerebellar hemisphere while increased in the inferior olivary nucleus (Table 4).

DISCUSSION

The inferior olivary nucleus receives neuronal inputs from the spinal cord, brainstem, and cerebral cortex and is the major source of the climbing fibers which innervate the cerebellum (Noback and Demarest, 1972). Pathological studies revealed that this region is often severely atrophied in FA. In this study, both ^{3}H-QNB and ^{3}H-DHA binding were found to increase while ^{3}H-Flu binding decreased in the FA inferior olive. The increased level of muscarinic cholinergic and β-adrenergic receptors suggests that these receptors are located on nondegenerated cell-types and that decreases in cholinergic and/or noradrenergic neuronal activity might have occurred in the FA inferior olive. The lowered levels of ^{3}H-Flu binding suggests that either benzodiazepine receptors are located on degenerated cells in the inferior olive or that these receptors have become desensitized.

The dentate nucleus is a deep cerebellar nucleus that is severely degenerated in FA. A major inhibitory pathway from the cerebellar cortex (via Purkinje fibers) innervated the dentate nucleus which sends output fibers to the ventral lateral and intralaminar thalamic nuclei as well as the red nucleus (Noback and Demarest, 1972). In the present study, benzodiazepine receptors were slightly depleted in FA dentate nucleus whereas muscarinic cholinergic and β-adrenergic receptors were unaltered. The results suggest that benzodiazepine receptors might be on degenerated cell-types in the FA dentate nucleus, while muscarinic cholinergic and β-adrenergic receptors are on non-degenerated cells.

Interestingly, ^{3}H-QNB binding increased in both the FA anterior and posterior cerebellar vermi. Neither ^{3}H-Flu nor ^{3}H-DHA binding were altered in these same regions. The increased level of muscarinic cholinergic receptors in the FA cerebellar vermi suggests that there may be a loss of

TABLE 4

Regional ^{3}H-DHA Binding in FA and Normal Brains

Brain Number	Brain Region				
	Inferior Olivary Nucleus	Dentate Nucleus	Ant. Cere. Vermis	Post. Cere. Vermis	Cere. Hemisphere
FA					
A-77-178	3.9	10.5	5.8	7.4	6.9
A-77-218	20.2	16.8	11.3	10.0	17.0
Average	12.1	13.6	8.5	8.7	11.9
Normal					
A-77-101	—	9.0	6.8	8.5	5.9
A-77-71	—	14.4	7.5	9.2	9.2
A-77-51	6.6	—	5.5	2.8	4.4
A-75-149	—	10.4	6.7	5.8	14.7
Average	6.6	11.3	6.6	6.6	8.6

The values are expressed as fmole of ^{3}H-DHA bound per mg protein.
The concentration of ^{3}H-DHA used was 250 pM.

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cholinergic innervation in the cerebellar vermi of FA brains. Studies measuring the activity of choline acetyltransferase (a marker for cholinergic neurons) are currently in progress in order to determine if such a loss of cholinergic input occurs in the FA cerebellar vermis.

Neuropathological examinations revealed no gross pathological abnormalities in the FA cerebellar hemisphere. In accordance with these findings, no alterations in any of the neurotransmitter receptor levels were detected in this region of FA brains.

In the present study, only a limited number of FA brains were available for analysis. This small sample number prevented statistical comparisons and kinetic analysis of the FA and control groups. Therefore, the results of this study should be viewed with caution. However, this study does reveal trends in neurotransmitter receptor alterations in several FA brain regions. It is hoped that this initial information might promote further examination of the brain neurochemistry of FA.

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Dicarboxylic Amino Acid Uptake in Normal, Friedreich's Ataxia, and Dicarboxylic Aminoaciduria Fibroblasts

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SUMMARY: *Glutamic and aspartic acid uptake was measured in skin fibroblasts from patients with Friedreich's Ataxia, dicarboxylic aminoaciduria, and normal individuals. The results showed no difference in uptake kinetics of either dicarboxylic amino acids between Friedreich's Ataxia and normal cells, but reduced uptake velocities in dicarboxylic aminoaciduria fibroblasts. Friedreich's Ataxia fibroblasts were, however, less calcium-dependant and more magnesium and phosphate-dependent than controls in glucose-free incubation mixture. This difference might be related to some degree of glucose intolerance by Friedreich's Ataxia fibroblasts in culture.*

RÉSUMÉ: *L'incorporation de l'acide glutamique et aspartique a été mesurée dans les fibroblastes cutanés de patients souffrant d'Ataxie de Friedreich, d'aminoaciduurie dicarboxylique et de témoins normaux. Les résultats démontrèrent une cinétique normale de l'incorporation des deux acides aminés étudiés dans les fibroblastes d'Ataxiques et une vélocité d'incorporation réduite dans l'aminoaciduurie dicarboxylique. Les fibroblastes d'ataxiques se montrèrent différents des contrôles normaux et pathologiques dans leur régulation ionique et glycolytique. Ces différences peuvent être reliées à une utilisation réduite du glucose par les cellules ataxiques en culture.*

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INTRODUCTION

Recent studies of amino acid levels in Friedreich's Ataxia have demonstrated a possible dicarboxylic and β-amino acids conservation defect (Lemieux et al., 1976). Although reduced CSF taurine and aspartic acid levels could have been explained on the basis of technical interference during automatic analysis of small concentrated samples (Lemieux et al., 1978), there remained an apparent renal conservation defect for taurine, aspartic acid, and β-alanine. Further information was provided by the finding of reduced aspartic acid levels in the retina, and reduced glutamic acid and taurine levels in the cerebellum of 3-acetyl pyridine injected ataxic rats (Butterworth et al., 1978 a). Decreased levels of aspartic acid were also found in cerebellum of rats rendered diabetic by Alloxan monohydrate injections (Butterworth et al., 1978 b). These findings suggested that the alterations in amino acid concentrations observed in tissues and biological fluids of patients with Friedreich's and other ataxias might be secondary to changes in membrane composition or permeability (Barbeau, 1978).

The present investigation was designed to assess amino acid transport in cultured skin fibroblasts from patients with Friedreich's Ataxia. Although the transport properties of cultured human diploid fibroblasts have not been well characterized, there have been a few investigations showing the presence in fibroblasts of group-specific amino acid transport systems corresponding to the major systems found in other mammalian tissues (Groth and Rosenberg, 1972; Hillman and Otto, 1974; Booth and Nadler, 1975; Kaye and Nadler, 1976; Pellefigue et al., 1976; Revsin and Morrow, 1976). Since it was not ethically acceptable to proceed to transport experiments using kidney needle aspirates from patients with Friedreich's Ataxia, in whom a renal defect is only suspected, we have initiated a study of dicarboxylic amino acid transport using cultured skin fibroblasts. The present investigation was conducted using two sets of controls for the Friedreich's Ataxia fibroblasts; (a) fibroblasts from age-matched normal individuals and (b) from younger subjects with a known familial dicarboxylic amino acids renal conservation defect (Melançon et al., 1977).

The finding of altered dicarboxylic amino acids transport parameters in both Friedreich Ataxia and dicarboxylic aminoaciduria subjects would lead to the recognition of a genetic membrane defect involving the conservation of amino acids rather than changes in membrane permeability secondary to an unknown disease process.

METHOD

Skin biopsies were obtained with informed consent from 5 patients with Friedreich's Ataxia (FA), 5 individuals with dicarboxylic aminoaciduria (DCA), and 13 control subjects. Fibroblasts were cultured in minimum essential medium (MEM Eagle) with Earle's salts and L-glutamine (GIBCO) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250μg/ml Fungizone at 37° in 5% $C0_2$, 95% air. The fibroblasts were harvested at confluency using 0.25% trypsin in Hank's balanced salt solution. All amino acid uptake experiments were performed

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on cells between their 7th and 20th passage. For uptake experiments, confluent fibroblasts from one 75cm² tissue culture flask (Corning) were trypsinized and suspended in 20 ml nutrient mixture. Half of the suspended cells were seeded evenly over 14 size 9x50mm glass coverslips (Belko Glass inc) in a 100cm² square tissue culture dish while the remaining cells were grown back to confluency in the original flask. Twenty-four hours after plating, the micro-coverslips containing flask was fed with MEM containing 10% fetal calf serum without antibiotics. Forty-eight hours later, the coverslips were mounted on a stainless steel rack which holds ten coverslips side by side in a vertical position and permits simultaneous and identical manipulation procedures. The coverslips were immersed 3x10 sec. in Dulbecco's phosphate buffered saline (PBS) and preincubated for 15 min in PBS containing 0.1% glucose (PBSG). Amino acid uptake was measured using uniformly labeled L-aspartic-^{14}C and L-glutamic-^{14}C acids (New England Nuclear) and unlabeled substrates at the indicated concentrations in PBSG at 37° (unless stated otherwise). The radiochemicals were subjected to two-dimensional ascending paper chromatography (Perry et al., 1975) and autoradiography and found to contain less than 1% contamination by α-alanine, threonine, serine and glutamic acid (in L-aspartic-^{14}C acid) and less than 0.5% contamination by pyrolidone-5-carboxylic acid, γ-amino-butyric acid and aspartic acid (in L-glutamic-^{14}Cacid). This low order of contamination, being rendered negligible by further dilution in non-radioactive amino acid solutions, was not taken into consideration.

The incubation medium consisted of PBSG to which the labeled amino acids were added with or without the test compounds. Incubation proceeded at 37° in 15 ml stainless steel waterbaths. Individual coverslips or the whole rack were taken out at the appropriate time and immersed successively in 3 beakers containing ice cold PBSG, touched to an absorbent paper towel, and placed in a test tube containing 1 ml of 0.1N NaOH. The test tubes were mixed at high speed on a vortex mixer and shaken overnight at 37° in a water-bath. Aliquots of 0.5 ml were then taken for protein determination by the method of Lowry (1951) and liquid scintillation counting in 10 ml of Aquasol (New England Nuclear) in a Packard Tricarb model 3375 liquid scintillation counter. Radioactivity of the cells was corrected for protein value of each individual coverslip and a blank value corresponding to the radioactivity absorbed during a one second immersion of duplicate coverslips from each cell line into the incubation medium, as above. Radioactivity of the substrate solution was measured prior to incubation and was used to calculate the amino acid incorporation into fibroblasts. Uptake of L-aspartic and L-glutamic acid was expressed in nmol/min/mg cell protein.

The kinetics of aspartic and glutamic acid uptake were determined in PBSG at substrate concentrations of 0.005, 0.0075, 0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 1.0, 2.5, and 5 mM using a constant amount of radiolabeled marker (0.25μCi) of each amino acid. Uptake kinetics of glutamic acid were also studied at substrate concentrations of 1.0, 2.5, 5, 10, 15, and 20mM, using 1μCi of radiolabeled marker (L-glutamic-^{14}C acid). Vmax and Km were determined by the double reciprocal method of Lineweaver and Burk.

Inhibition of dicarboxylic amino acid uptake was evaluated with respect to ions and glucose, utilizing ion and glucose depleted PBSG and Tris (hydroxymethyl) aminomethane buffers, pH 7.5. The chemicals used for buffer preparation were obtained from Fisher Scientific Co. and their final concentration was determined by current clinical laboratory methods before use (Table I).

TABLE I

Composition of Buffers for Amino Acid Uptake Experiments in Cultured Human Skin Fibroblasts

Buffer	Ions (mM)					
	[Na]	[K]	[Mg]	[Ca]	[Cl]	[PO_4]
PBS*	164	4.0	.49	.85	149	13
Na free	1†	3.8	.47	.85	142	13
Na 10mM	10	3.7	.65	ND	149	20
Na 50mM	46	3.7	.65	ND	150	21
Na 100mM	94	3.9	.66	ND	139	21
Na 50,K100mM	50	100	.69	ND	147	21
Mg free	155	4.1	.05†	.9	143	13
Ca free	153	4.2	.49	.05†	142	13
K free	157	0.1†	.48	.9	142	14
Tris-PO_4 free*	155	4.0	.48	.5	164	0
	[Na]	[K]	[Mg]	[Ca]	[Cl]	[PO_4]
PBSG*	160	4.3	.58	.86	151	13
Na free	2	3.7	.54	.68	142	19
Na 10mM	11	3.7	.55	.68	149	19
Na 50mM	48	3.8	.56	.52	149	19
Na 100mM	96	4.0	.55	.62	142	21
Na 50,K100mM	52	95	.57	.42	151	21
Mg free	156	4.2	.07	.70	146	13
Ca free	153	4.4	.54	.04	144	13
K free	155	0.1	.55	.73	143	13
Tris-G-PO_4 free*	153	4.4	.61	.89	168	0.1

* PBS: phosphate buffered saline pH 7.5 without glucose
Tris-PO_4 free: phosphate replaced by equimolar tris (hydroxymethyl) aminomethane PBSG and Tris-G contained glucose 100 mg/l
† Cations replaced by equimolar amounts of lithium. ND:not determined

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Inhibition studies by membrane active chemicals and competitor amino acids and drugs were performed at the indicated concentrations of test compounds after a 15 min preincubation. The test compounds were obtained from Sigma Chemical Co. and Calbiochem. Oxidation of glutamic acid was studied by measuring the $^{14}C0_2$ trapped in suspended plastic wells (Kontes Glass co.) filled with 0.1 ml of 0.1N K0H in glass wool, using fibroblast layered coverslips placed cell up, in a glass culture tube (Belko) containing 0.025 mM L-glutamic acid (0.25μCi) in 1 ml PBS at 37° for 15 min. At the end of the incubation period, the plastic wells were dropped in liquid scintillation vials containing 10 ml of Aquasol and counted for radioactivity. For calculation of oxidation rate, the amount of radioactivity in the wells was compared to the radioactivity in the incubation medium, after correction for the amount of protein on the coverslips and a blank value obtained by incubating cell-free coverslips as above. These results were expressed in pmol. glutamic acid oxidized per min. per mg cell protein. The statistical analyses were carried out using Student's t-test and values of ½.05 were retained.

RESULTS

Time course (fig 1)

The uptake of aspartic (Asp) and glutamic (Glu) was linear with increasing protein content from 5 to 100μg per coverslip and linear with time up to 90 min. at 0.01 mM substrate concentrations. Previous experiments using dicarboxylic amino acids disappearance rates from incubation medium (Melançon et al., 1978) had showed a linear time course with absence of saturation at substrate concentrations of 0.001 to 1 mM. Dicarboxylic amino acid uptake by FA and DCA fibroblasts was also found to be linear with time. An incubation time of 15 min was chosen for further experiments.

Oxidation of glutamic acid (table II)

The rate of Glu oxidation was lower in FA and DCA fibroblasts but without statistically significant differences. Less than 1% of the radioactivity incorporated into the cells was accounted for into the $^{14}C0_2$ trapped.

Kinetic Analysis (table III and IV)

The rate of dicarboxylic amino acid uptake by fibroblasts increased with increasing concentration of dicarboxylic amino acids in the incubation medium. At concentrations above 0.5 mM, the rate of both Asp and Glu decreased without saturation. Analysis of the data obtained for Asp uptake disclosed a significant decrease in velocity at substrate concentrations below 0.5 mM for DCA fibroblasts alone. At high substrate concentrations (above 0.5 mM) the Km and Vmax of FA and DCA fibroblasts were higher, but without statistically significant difference from control cells. The apparent Km for Glu uptake was comparable if not identical to the Km for Asp at low substrate concentrations. However, the uptake velocity was 2.5× greater for Glu than Asp. At high substrate concentration, both Km and Vmax values for Glu were higher than Asp. Although FA and DCA fibroblasts showed lower than control uptake kinetics for Glu at high concentrations, the difference observed was not significant.

Metabolic Inhibition (table V)

Potassium cyanide (KCN) and iodoacetate were the only two amongst respiration blockers and oxidative phosphorylation uncouplers which diminished Glu and Asp uptake in fibroblasts. The reduction of Glu uptake was definitely marked in DCA fibroblasts. The sulfhydryl reagents, N-ethyl-maleimide (NEM), cupric ions ($CuS0_4$) and N, N-dimethyl amide (Diamide) were in decreasing order, effective inhibitors of Glu and Asp uptake. Chloramphenicol, an antibiotic which inhibits protein synthesis by interfering with peptide-forming steps, reduced Glu and Asp acid uptake but to a lesser degree than phosphorylation uncouplers. Chlorpromazine (CPZ) and Ouabain were the most potent of all inhibitors for the low Km Glu uptake system, suggesting that 95% of Glu uptake was dependent on

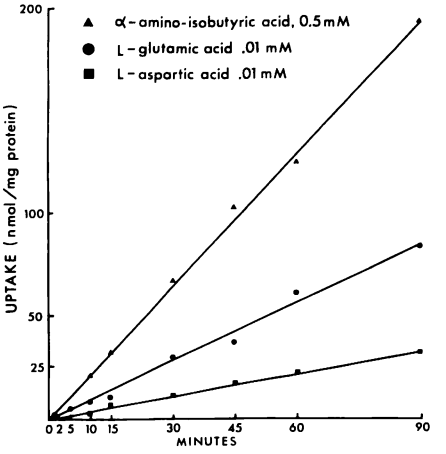

Figure 1—Time course of dicarboxylic amino acids and Aib uptake in cultured skin fibroblasts

TABLE II

Oxidation of Glutamic Acid by Intact Cultured Skin Fibroblasts from Controls and Patients with Friedreich's Ataxia and Dicarboxylic Aminoaciduria

Cell lines	Glutamic Acid Oxidized *
(number)	(pmol/min/mg protein)
	mean±SD
controls (5)	3.0±1.3
Friedreich's (4)	2.1±1.2
dicarboxylic (2)	1.2±0.2

* Calculated from $^{14}C0_2$ trapped in 0.1 N KOH, after incubation of fibroblasts (50μg protein equivalent) with L-glutamic acid 0.025mM (0.25μCi of L-U-14-C-glutamic acid) for 15 min. at 37° in 1 ml of phosphate buffered saline.

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TABLE III

Km (mM) and Vmax (nmol/min/mg protein) of Aspartic Acid Uptake by Cultured Skin Fibroblasts from Normal Controls and Patients with Friedreich Ataxia and Dicarboxylic Aminoaciduria

Cell lines	Aspartic Acid Concentration					
	5μM — 5mM		< .5mM		> .5mM	
	Km	Vmax	Km	Vmax	Km	Vmax
Controls (5)	.024	.813	.015	.569	2.1	4.3
Friedreich's (2)	.027	.855	.017	.622	4.1	6.9
Dicarboxylic (2)	.022	.373*	.012	.255†	3.1	4.9

*p<.05 for differences from controls
†p<.02 for differences from controls and <.05 from Friedreich's

the activity of Na+, K+-ATPase. The inhibitory effect of membrane active compounds such as lidocaine, quinidine and D, L-propanolol was not different in diseased and control lines. Isoproterenol, dibutyryl cyclic AMP, and theophylline enhanced Glu uptake in all cell lines. Insulin, alloxan monohydrate, and 3-acetyl pyridine, which cause significant changes in the intracellular concentration of dicarboxylic amino acids in other mammalian transport systems, did not permit differentiation between FA cells and control fibroblasts. Finally, m-chlorocarbonylcyanide phenylhydrazone (CCCP), an uncoupler which permits the rapid exchange of H^+ across membranes and thus discharges both pH and electrical gradients, was a good inhibitor of Glu uptake only when preincubated with the fibroblasts before uptake experiments.

Buffer composition and temperature (table VI, fig 2 and 3)

Potassium, calcium, and sodium ions were, in increasing order, the most required cations for efficient dicarboxylic amino acids uptake, Magnesium appeared slightly inhibitory when glucose containing PBSG was used in Glu uptake experiments. Similarly, calcium dependent uptake of both Glu and Asp was more evident in glucose free PBS. Sodium dependent dicarboxylic amino acid uptake was progressively restored by increasing concentrations of sodium, from 10 to 100 mM. However, fibroblasts incubated in 100 mM K^+ and 50 mM Na^+ showed a significant decrease in dicarboxylic amino acid uptake corresponding to a level observed with 10 mM sodium alone. Phosphate ions had an inhibitory effect in the presence of glucose and the opposite effect in the absence of glucose. Both Glu and Asp uptake decreased at 4° C, down to approximately 10% of the initial 37° C uptake levels. Significant differences between FA and control fibroblasts were found with respect to calcium dependent Asp uptake in glucose free PBS only. DCA fibroblasts were likewise less magnesium and calcium dependent for Glu uptake in glucose containing PBSG. The same DCA fibroblasts differed from control fibroblasts in their lesser dependance on sodium (in Na^+ free, and Na^+ 50, K^+ 100 mM) and temperature (at 22° only) for Glu uptake in PBSG. Glucose related uptake of Glu was significantly decreased in $P0^4$ depleted FA and DCA fibroblasts, in magnesium depleted FA fibroblasts, and finally in all three calcium depleted cell lines.

Inhibition by other amino acids (table VII)

Previous experiments suggested that the uptake of 0.01 mM dicarboxylic amino acids was not inhibited significantly by 1 mM glycine, α-alanine, leucine, or taurine (fig 4 and 5). The present data (table VII) revealed that Glu uptake is markedly inhibited by L and D-aspartic acid, L-glutamic acid, and L-glutamine. Glu uptake was also inhibited to varying degrees in order of decreasing inhibition by: serine, threonine, isoleucine, asparagine, methionine, cysteine, valine, and the aromatic amino acids tyrosine and phenylalanine. D-glutamic acid was not inhibitory at a concentration of 1mM, and further experiments revealed that the D-isomer of aspartic acid was a significantly more potent inhibitor of both L-glu and L-asp uptake (table VIII and fig. 6 and 7), for the same inhibitor concentrations. We found no significant difference in the degree or stereospecificity of inhibition by other amino acids between control and patients fibroblasts.

Effect of time in culture (fig 8)

Aging slowly but clearly reduced the uptake of Asp by control fibroblasts. This reduction was less apparent in FA fibroblasts and not at all present in DCA fibroblasts where low Asp uptake values were observed at the early passages. Glu uptake increased with time up to the 12th passage, and decreased thereafter, showing very good parallelism between all three groups of fibroblasts studied. The overall uptake values of Asp were

TABLE IV

Km (mM) and Vmax (nmol/min/mg protein) of glutamic acid uptake by cultured skin fibroblasts from normal controls and patients with Friedreich Ataxia and dicarboxylic aminoaciduria

Cell lines	Glutamic Acid Concentration			
	5μM — 1mM		1 — 20mM	
	Km	Vmax	Km	Vmax
Controls	.025	2.0 (13)*	24.7	148.3 (6)
Friedreich's	.031	2.3 (5)	11.3	64.6 (2)
Dicarboxylic	.035	1.6 (5)	15.3	103.3 (2)

*Number of cell lines studied

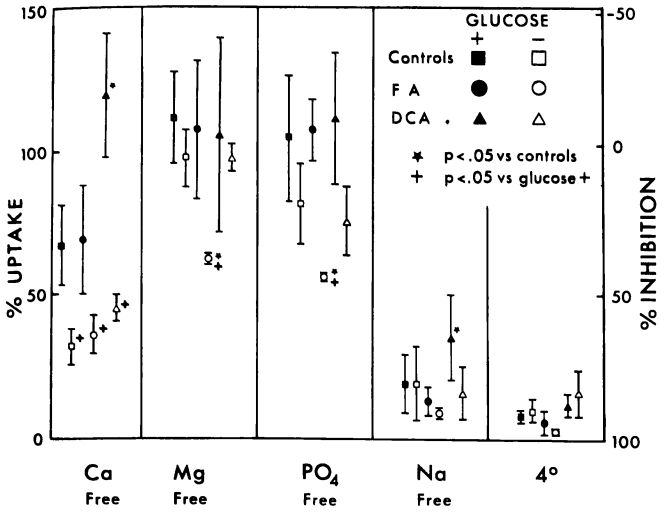

Figure 2—Influence of glucose depletion on dicarboxylic (Glu) amino acids uptake by cultured human skin fibroblasts

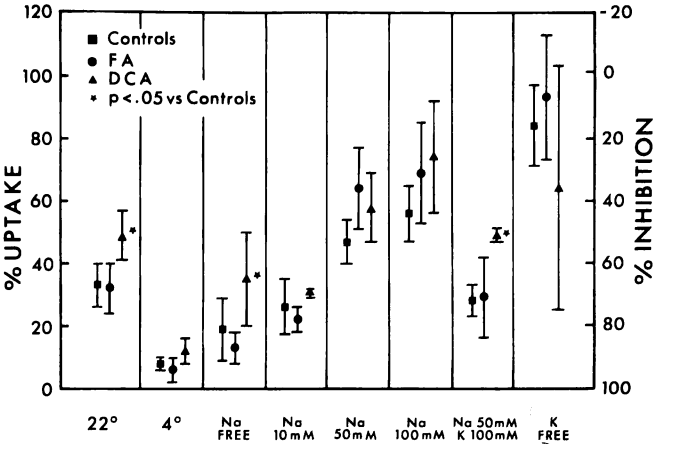

Figure 3—Influence of sodium and potassium on dicarboxylic (Glu) amino acid uptake by cultured skin fibroblasts

TABLE V

Effect of Metabolic Inhibitors on Dicarboxylic Amino Acids Uptake by Cultured Human Skin Fibroblasts

Inhibitor	Concentration	% uptake (mean±SD)					
		Glutamic Acid			Aspartic Acid		
		controls (n:6)	F.A. (n:2)	D.C.A. (n:2)	controls (n:6)	F.A. (n:2)	D.C.A. (n:2)
None		100%	100%	100%	100%	100%	100%
NaF	.025mM	105±13	110±8	103±10	105±15	103±21	94±6
$NaN0_3$	.025mM	95±15	95±28	106±18	102±9	92±13	96±6
KCN	.025mM	75±16	82±1	*44±33	109±19	106±40	117±5
Iodoacetate	.025mM	55±7	64±3	85±34	63±7	68±16	51±23
NEM	.025mM	73±12	59±9	66±4	65±10	73±8	—
$CuS0_4$†	.025mM	87±11	97±25	98±25	—	—	—
Diamide	.025mM	97±13	98±10	84±1	107±14	81±39	94±28
Chloramphenicol	.025mM	85±13	84±5	107±13	89±24	107±36	73±5
CPZ†	.2mM	5±1	7±1	7±5	—	—	—
Ouabain	.025mM	47±7	70±2	44±11	44±9	61±1	*28±4
Lidocaine†	.025mM	144±13	141±17	171±61	—	—	—
Quinidine	.1mM	44±4	44±4	38±4	87±7	85±23	86±10
Propanolol	.2mM	62±14	62±9	75±6	49±9	52±23	*68±1
Isoproterenol†	.2mM	116±20	119±25	141±11	—	—	—
DBcAMP†	.025mM	129±19	144±50	144±5	—	—	—
Theophylline	.025mM	140±31	118±12	166±59			
Insulin†	100μu/ml	135±24	147±54	163±25	—	—	—
Alloxane	.01mM	86±7	96±17	99±18	80±8	77±22	93±16
3-APyr†	.01mM	87±8	92±29	119±14	—	—	—
CCCP	.01mM	50±7	67±1	40±15	67±6	74±8	*48±3
CCCP†	.01mM	84±15	95±32	99±16	—	—	—

*p<.05 for differences from controls
†preincubation did not contain the inhibitor

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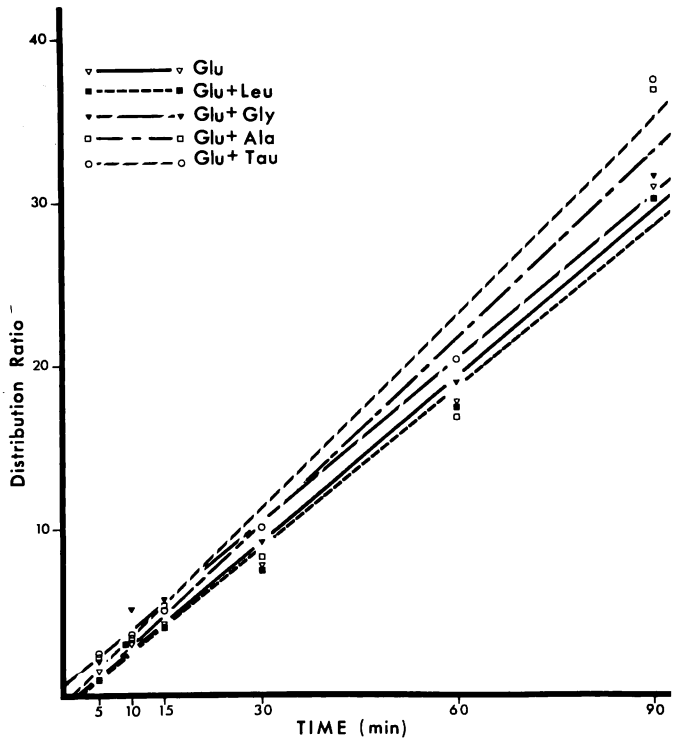

Figure 4—Effect of other acids (1mM) on glutamic acid (10μM) uptake by cultured skin fibroblasts

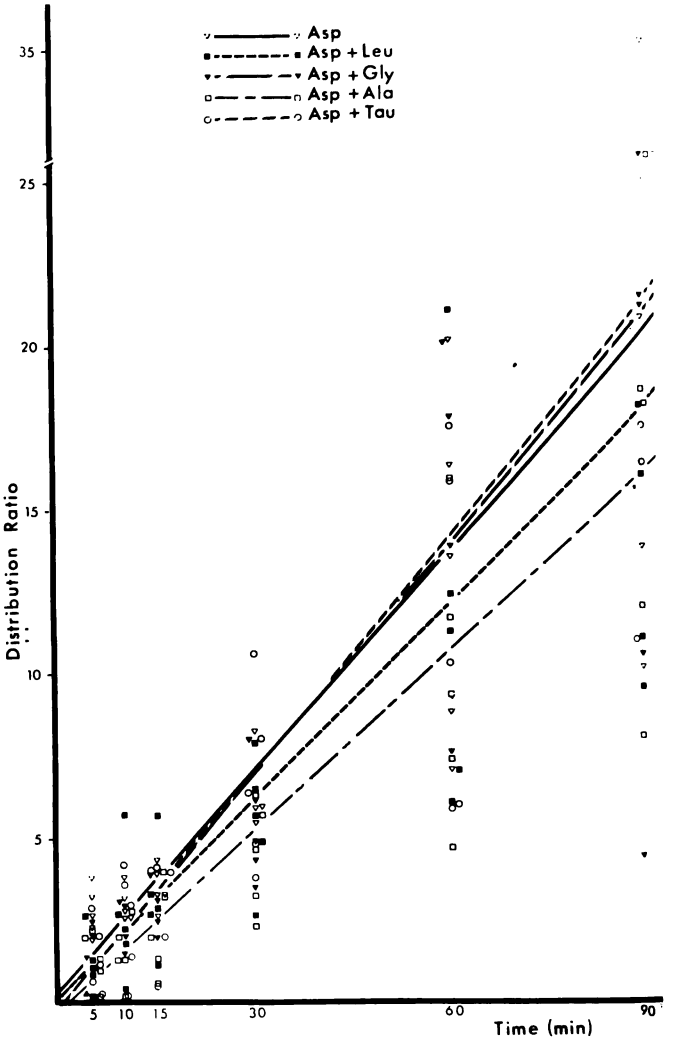

Figure 5—Effect of other amino acids (1 mM) on aspartic acid (10μM) uptake by cultured skin fibroblasts

significantly reduced in DCA fibroblasts ($p<.01$) as compared with control and FA fibroblasts (table IX). Although Glu uptake was reduced in FA and more specifically in DCA fibroblasts, the difference was not statistically significant.

DISCUSSION

The present studies sought to define the uptake processes for glutamic and aspartic acid in cultured fibroblasts from normal individuals and patients with Friedreich's Ataxia, using dicarboxylic aminoaciduria fibroblasts as diseased controls. Results of the oxidation studies revealed that FA fibroblasts do not rely more than control or DCA fibroblasts on external glutamate for operation of the tricarboxylic acid cycle. A very small proportion (1%) of the glutamic acid taken up by the cells was oxidized, suggesting that the greater part of transported Glu remained free or incorporated into cell proteins (Novogrodsky et al., 1977). Although the kinetics of dicarboxylic amino acid uptake must be interpreted cautiously, a number of characteristic observations were valid.

The apparent Km values obtained for all three cell lines were similar, but the Vmax values for the DCA fibroblasts were lower with respect to aspartic acid uptake at low substrate concentration (.025 mM). These data suggest a comparable affinity of the dicarboxylic amino acid carrier for Asp and Glu in all cell lines, but a depressed movement or a reduced rate of dissociation of dicarboxylic amino acid from the carrier in DCA fibroblasts at low substrate concentrations.

Since this low Km system would be the major one operative *in vivo* at physiological concentrations of Asp and Glu in biological fluids (Lemieux et al., 1976), the apparent high Km uptake system of dicarboxylic amino acids was not further investigated.

The effect of metabolic inhibitors revealed that uncouplers of oxidative phosphorylation were inhibitory to Glu and Asp uptake. Sulfhydryl reagents also showed some inhibition, suggesting the relative importance of free sulfhydryl groups in transferring dicarboxylic amino acids across the fibroblast membrane. Chlorpromazine inhibited Glu uptake down to levels seen only with sodium

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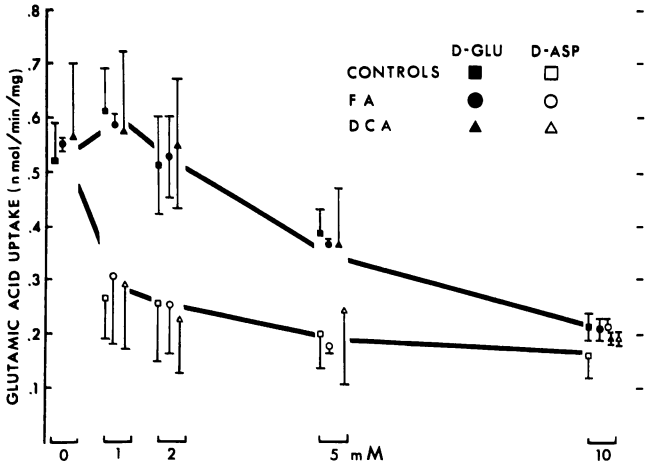

Figure 6 — Inhibitory effect of D-glutamic and D-aspartic acids on L-glutamic uptake by cultured skin fibroblasts

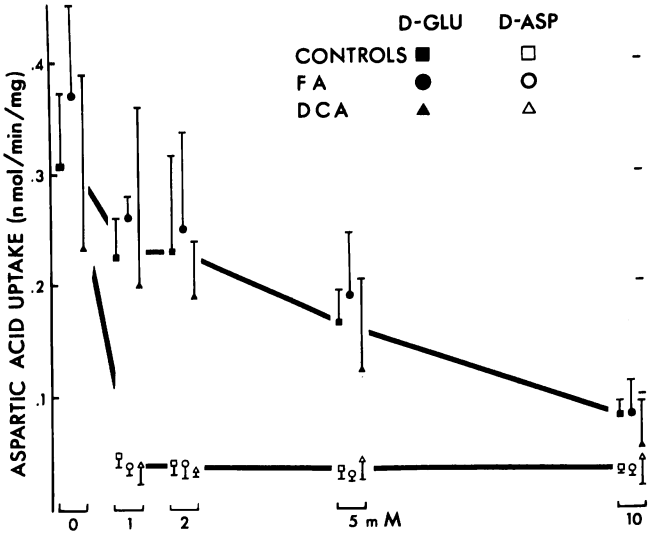

Figure 7 — Inhibitory effect of D-glutamic and D-aspartic acids on L-aspartic acid uptake by cultured skin fibroblasts

deprivation and very low ambient temperature. Chlorpromazine is known to inhibit Na^+, K^+-ATPase activity of brain microsomes, human leukocytes, and isolated rat liver slices (Akeda and Brody, 1969).

It is assumed that the chlorpromazine free radical interacts with the enzyme's free sulfhydryl groups thus creating an irreversible inhibition (Samuel and Carey, 1978). Further, it has been suggested that chlorpromazine can localize at cell membranes phospholipid protein interfaces, causing an expansion of the cell membrane (Seeman et al., 1969). Our results were in agreement with both modes of chlorpromazine action since ouabain or sodium depletion (table VI), which mimics Na^+, K^+ATPase inhibition, could only abolish Glu uptake by approximately 80% with comparison to 95% inhibition by chlorpromazine alone.

Propanolol, lidocaine, and quinidine which inhibit the incorporation of leucine into protein as a consequence of a reduction in uptake at the cell membrane (Schoenfeld et al., 1977) also disorganize lipid molecules and induce changes in the proteins located in the outer layers of the membranes (Poste et al., 1975). These membrane active compounds were less effective against dicarboxylic amino acid than against β-aminoisobutyric acid uptake as previously reported for chick embryo liver cells in culture (Schoenfeld et al., 1977), particularly

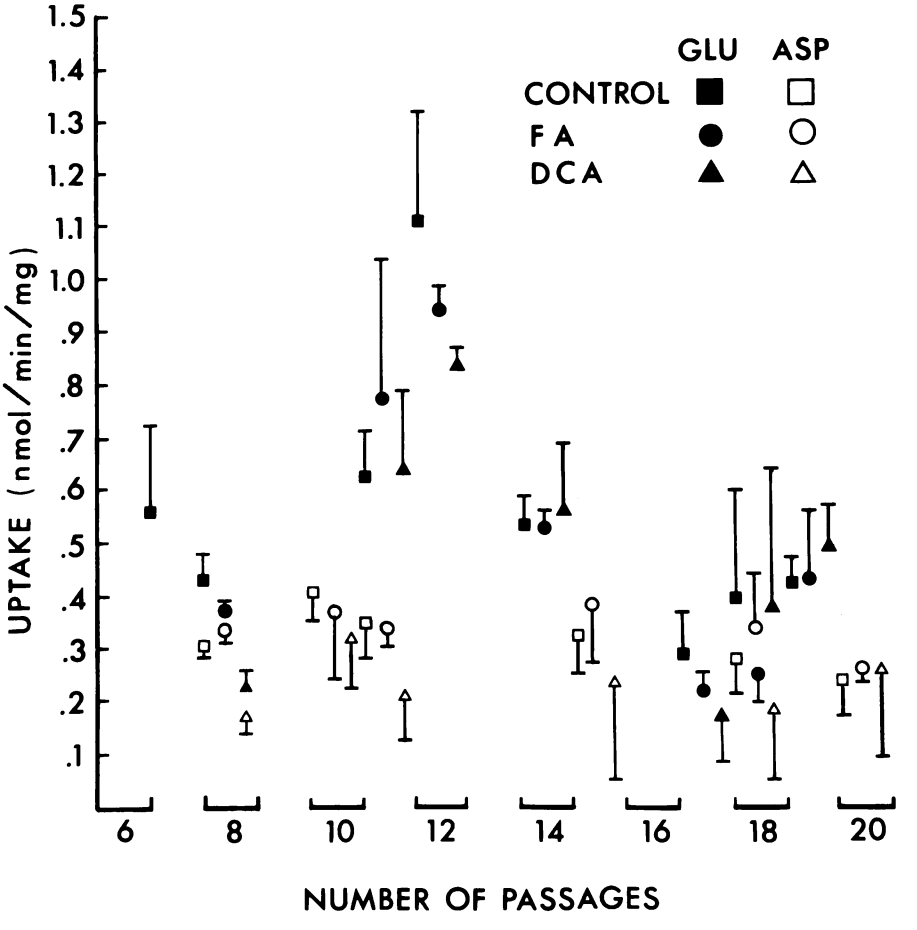

Figure 8 — Influence of time in culture on dicarboxylic amino acids uptake by skin fibroblasts

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TABLE VI

Influence of Buffer Composition and Temperature on Dicarboxylic Amino Acid Uptake in Control, Friedreich's Ataxia and Dicarboxylic Aminoaciduria Cultured Skin Fibroblasts

Buffer	Ion Deleted	% Glutamic Uptake			% Aspartic Uptake		
		Control	FA	DCA	Control	FA	DCA
PBS±G	None*	100±SD(n)	100±SD(n)	100±SD(n)	100±SD(n)	100±SD(n)	100±SD(n)
PBSG	Ca free	67±14(12)	69±19(4)	119±22(4)§c	35±14(12)	39±14(4)	38±10(4)
PBS	Ca free	32± 6(6)§g	36± 7(2)‡g	45± 5(2)†g	29± 2(5)	51± 4(2)§c	32± 2(2)
PBSG	Mg free	111±16(12)	107±24(3)	105±34(3)	89±18(2)	88± 7(4)	100±20(4)
PBS	Mg free	97±10(6)	62± 1(2)‡cg	97± 5(2)	93±14(6)	94±18(2)	99± 1(2)
PBSG	K free	84±13(6)	93±20(2)	64±39(2)	57±15(6)	96±30(2)	80± 1(2)
PBSG	Na free	19±10(12)	13± 5(4)	35±15(4)†c	8±7(12)	8± 5(4)	10± 6(4)
PBS	Na free	19±13(6)	9± 2(2)	16± 9(2)	7± 1(6)	14± 6(2)	10± 1(2)
PBSG	Na 10mM	26± 9(6)	22±44(2)	31± 1(2)	17±10(6)	39±37(2)	24± 5(2)
PBSG	Na 50mM	47± 7(6)	64±13(2)	58±11(2)	33±21(6)	35±22(2)	27± 1(2)
PBSG	Na 100mM	56± 9(6)	69±16(2)	74±18(2)	51±32(6)	33±28(2)	69± 4(2)
PBSG	Na50,K100mM	28± 5(6)	29±13(2)	49± 2(2)‡c	22±12(6)	25±14(2)	25± 8(2)
Tris-G	P04 free	104±22(12)	107±11(4)	111±23(4)	112±37(12)	105±32(4)	124±48(4)
Tris	P04 free	81±14(6)	56± 1(2)†cg	75±12(2)§g	82±13(6)	80± 4	90± 4(2)
PBSG	None 22°	33± 7(6)	32± 8(2)	49± 8(2)†c	25± 4(6)	19± 4(2)	22± 3(2)
PBSG	None 4°	8± 2(12)	6± 4(3)	12± 4(3)	9± 6(11)	12± 6(4)	14± 5(4)
PBS	None 4°	10± 4(6)	4± 1(2)	16± 8(2)	3± 2(6)	14± 1(2)	14±10(2)

*Incubations were performed at 37° (except when stated otherwise) for 15 min at Glu and Asp concentrations of .025 mM; (n) refers to the number of cell lines studied.
†$p<.05$ for differences from control (†c) or from glucose containing bufer (†g)
‡ $p<.01$ for differences from control (‡c) or from glucose containing buffer (‡g)
§ $p<.001$ for differences from control (§c) or from glucose containing buffer (§g)

TABLE VII

Effect of Amino Acids (1mM) on Glutamic Acid (25μ) Uptake by Cultured Human Skin Fibroblasts

Amino Acids	% Glutamic Acid Uptake (mean ± SD)		
	controls (4)	FA (2)	DCA (2)
None100%	100%	100%	100%
Ornithine*	124 ± 34	99 ± 4	114 ± 20
β-alanine	103 17	129 18	115 4
Lysine	104 31	92 1	100 9
Histidine	115 43	91 34	80 4
D-glutamic	97 16	85 20	108 15
Proline	95 30	78 10	83 6
Arginine	85 12	76 15	104 16
Phenylalanine	83 3	97 38	92 1
Tyrosine	78 26	61 5	76 20
Valine	73 26	71 14	72 6
Cysteine	60 10	54 10	97 40
Methionine	69 17	67 10	69 4
Asparagine	67 17	69 11	63 4
Isoleucine	68 15	66 1	60 1
Thréonine	69 23	60 8	59 6
Serine	58 6	59 12	64 3
Glutamine	44 4	44 10	44 4
Glutamic	23 7	21 4	19 1
Aspartic	23 5	40 2	54 17
D-aspartic	22 5	36 7	58 18

*Except for D-aspartic and D-glutamic, all amino acids were L-isomers

lidocaine, which in liver cells inhibited α-AIB uptake by 22 to 38% as compared to a 41 to 71% stimulation in cultured skin fibroblasts.

Propanolol-mediated inhibition of dicarboxylic amino acid uptake may also be through its β-adrenergic antagonism as reported for β-amino acid transport in mammalian kidney slices (Chesney et al., 1978). Isoproterenol, a β-adrenergic agonism enhanced Glu uptake by 16-41% over basal conditions. A possible mechanism might be through activation of adenylate cyclase, with subsequent formation of cyclic AMP.

Involvement of cyclic AMP was further supported by a 29-44% increase in Glu uptake by fibroblasts incubated with dibutyryl cyclic AMP, and a 18-66% increase during incubation with the phosphodiesterase inhibitor theophylline. Cyclic AMP has been reported to stimulate amino acid uptake into a variety of tissues, including bone (Phang et al., 1970), kidney (Weiss et al, 1972), uterus (Griffin and Szego, 1968), liver (Tews et al., 1970), heart (Huxtable and

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TABLE VIII

Effect of D-Glutamic and D-Aspartic Acids on Dicarboxylic Amino Acids Uptake in Cultured Human Skin Fibroblasts

[L-substrate] [D-isomer]	% Uptake *		
	Controls (6)	FA (2)	DCA (2)
L-Glutamic 25μM	100	100	100
+			
D-Glutamic 1mM	117	107	103
2mM	98	96	98
5mM	74	66	66
10mM	42	38	34
+			
D-Aspartic 1mM	51	55	52
2mM	49	45	40
5mM	38	31	43
10mM	31	39	33
L-Aspartic 25μM	100	100	100
+			
D-Glutamic 1mM	73	71	86
2mM	74	68	81
5mM	54	52	54
10mM	27	23	24
+			
D-Aspartic 1mM	16	10	17
2mM	13	11	15
5mM	12	8	19
10mM	11	9	20

*mean percentage of uptake as compared to uninhabited fibroblasts

Chubb, 1977), and intestine (Kinzie et al., 1976). The changes in uptake were apparently due to an altered electrochemical gradient across the cell with an increased external sodium concentration. High sodium concentrations in the incubation medium were also responsible for stimulation of dicarboxylic amino acids. FA and DCA fibroblasts appeared slightly more stimulated than controls in their response to DBc AMP and isoproterenol, but no statistically significant differences were found. Propanolol significantly inhibited Asp uptake in DCA fibroblasts only.

The insulin stimulated Glu uptake by cultured skin fibroblasts bears some similarity to system B described by Kilberg and Neuhaus (1977) for AIB (α-aminoisobutyric acid) transport in liver. These authors have shown that insulin stimulated AIB transport through a neutral Na^+ dependent amino acid transport system other than systems A (alanine preferring) or L (Leucine preferring). The dicarboxylic amino acid transport system of fibroblasts was also found to be Na^+ dependent and insulin sensitive. Alloxane, which produces a significant decrease in the cerebellar concentration of aspartate (Butterworth et al., 1978 a) and aspartate amino transferase (Jayashree and Nayeemunnis, A., 1975), reduced minimally both Glu and Asp uptake in cultured skin fibroblasts. Intraperitoneal injection of 3-acetyl-pyridine in rats produces a cerebellar ataxia and decreased levels of glutamic acid and taurine in the cerebellum and decreased levels of aspartic acid in the retina (Butterworth et al., 1978 b). Our results of a slightly reduced uptake of Glu by skin fibroblasts incubated with 3-APyr were not suggestive of a minor interference by that chemical on dicarboxylic amino acid uptake. We recognize that many of the metabolic inhibitors should have been tested at higher concentrations, but in many cases their low level of solubility in PBSG prevented such a possibility. CCCP which completely abolishes D-lactate-energized uptake of glutamate in membrane vesicles of E. coli, like sodium azide, a potent inhibitor of D-lactate oxidation (Macdonald et al., 1977) had very little effect on Glu uptake in fibroblasts not preincubated with the inhibitor. However, preincubation with CCCP resulted in a 50% inhibition of uptake suggesting the importance of an electochemical gradient not related to oxidation of lactate.

These results and the data from glucose and ion free incubation medium suggested that Glu and Asp uptake into skin fibroblasts involves a sodium-substrate symport. Calcium and phosphate ions were required for optimum dicarboxylic amino acid uptake in glucose-free more than in glucose-containing medium. In addition to these ions, membrane integrity as to -SH groups and protein-lipid interfaces was found of major importance for dicarboxylic amino acid uptake. Magnesium was inhibitory in glucose containing medium, but had very little effect in glucose free medium, with the exception of FA fibroblasts. The effect of inhibitor amino acids revealed that the Glu uptake system is not stereospecific and that competition

TABLE IX

Uptake of Dicarboxylic Amino Acids by Cultured Human Skin Fibroblasts (nmol/min/mg protein)

Cell lines	Aspartic	Glutamic
	(mean ± SD)	(mean ± SD)
Control (6)*	.317 ± .06	.523 ± .26
Friedreich (2)	.337 ± .04	.503 ± .27
Dicarboxylic (2)	.229 ± .06†	.473 ± .25

*number of cell lines in parenthesis
†$p<.01$ for difference from control and Friedreich

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occurs between D and L-Asp and possibly L-glutamine for the L-Glu carrier. In order to compete for the uptake sites, D-Glu concentration had to be raised to 10 mM. The competition by L-glutamine and to a lesser degree L-asparagine has to be reexamined. Either glutamine and asparagine share the dicarboxylic amino acid system as such or they are rapidly transformed into Glu and Asp during the 37° C incubation and act as group specific competitors (Perry et al., 1968).

Another possible explanation comes from the work of Novogrodsky et al., (1977) in human lymphoid cell lines with variable y-glutamyl transpeptidase activity. In their study, transported glutamine was extensively (80-98%) converted to glutamate. In addition, the rate of glutamine uptake at 0.025 mM extracellular concentration was 0.75 - 1.9 nmol/min/mg protein as compared to our data of 0.5 nmol/min/mg protein for Glu uptake at 0.025 mM extracellular concentration. This suggests that 1 mM glutamine would be more rapidly taken up by the cell and transformed into glutamate, thus creating a gradient against Glu uptake.

Inhibition of Glu uptake by neutral aliphatic, branched-chain, and aromatic amino acids ranged between 40% and 0%. Recent studies with cultured skin fibroblasts (Melançon et al., 1978), had shown no competition by alanine (Na^+ dependent A system) or leucine (Na^+independent L system) for the dicarboxylic amino acid uptake system and no significant competition from glutamic acid for both A and L neutral amino acid transport systems (Booth and Nadler, 1975). An ASC (alanine, serine, cysteine) Na^+dependent transport system has also been found operative in human cultured skin fibroblasts (Revsin and Morrow, 1976). However, this ASC system would not accept glutamic acid either since its preference would most certainly go to alanine, serine and cystine.

Taurine and β-alanine which have been shown to utilize a different uptake system in fibroblasts (see Melançon et al., this issue) were not competitors of Glu uptake in FA fibroblasts or in normal fibroblasts. These data would suggest the following possibilities. First, dicarboxylic amino acids are mostly transported into fibroblasts through a Na^+dependent, non stereospecific "uphill" transport system which requires energy from oxidative phosphorylation and is activated by cyclic AMP and insulin, and inactivated by membrane active compounds and Na^+, K^+ ATPase inhibitors. Second, glutamine and certain neutral amino acids compete for dicarboxylic amino acid transport carrier (or carriers) sites, when at 40 times the concentration of dicarboxylic amino acid. Third, less than 10% of dicarboxylic amino acid uptake occurs either passively or through other amino acid transport systems.

Skin fibroblasts from patients with Friedreich's Ataxia seem to have a normal dicarboxylic amino acid uptake system which is less dependent on calcium for aspartic acid uptake and more dependent on phosphate and magnesium for Glu uptake in glucose-free medium. These figures could not be further explained without further investigation of glucose requirements of FA fibroblasts. Dicarboxylic aminoaciduria fibroblasts showed a reduced Vmax for aspartic acid and a number of other differences, including more sensitivity to metabolic inhibition and less dependence on calcium, sodium and temperature.

Passages in culture slowly reduced the effectiveness of aspartic acid uptake by control and FA fibroblasts but not by DCA fibroblasts. This observation suggests that the observed reduction in Asp uptake by DCA fibroblasts was not the result of cell aging or selection in culture. Hillman and Otto (1974) reported a reduced isoleucine uptake rate in a fibroblast line from a patient with α-methyl acetoacetyl-Co A β-ketothiolase deficiency. Isoleucine uptake by the patient's cells was found to decrease with passage in culture until a stable culture was found in which sodiumdependent low concentration isoleucine uptake was lacking. Revsin and Morrow (1976) studied non-ketotic hyperglycinemia fibroblasts and reported lower Vmax for glycine uptake but not secondary to passages in culture.

In conclusion, we have shown that cultured skin fibroblasts from control, Friedreich's Ataxia, and dicarboxylic aminoaciduria patients have a group specific dicarboxylic amino acid uptake system with two unsaturable components. Kinetics, inhibition, and competition studies in all three cell lines revealed major differences from control and DCA fibroblasts with respect to Vmax, and ionic dependency in glucose-free medium only. Since the kinetic characteristics of the fibroblast's Glu uptake system are very similar to those reported for cultured astrocytes (Hertz et al., 1978) we assume that normal dicarboxylic amino acid uptake occurs in Friedreich's Ataxia glial cells also.

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Quebec Cooperative Study
of Friedreich's Ataxia

Fatty Acid Profile of Major Lipid Classes in Plasma Lipoproteins of Patients with Friedreich's Ataxia — Demonstration of a Low Linoleic Acid Content most evident in the Cholesterol-Ester Fraction

JEAN DAVIGNON, Y.S. HUANG, J.P. WOLF AND A. BARBEAU

SUMMARY: *Studies were undertaken to further characterize plasma lipids and lipoprotein abnormalities in Friedreich's ataxia. The high density lipoprotein (HDL) apo AI/AII ratio was quantitated by densitometry and found to be normal. The free to esterified cholesterol ratio in HDL was lower in Friedreich's ataxia because of a reduction in the amount of free cholesterol in this lipoprotein class. The fatty acid profile of the cholesteryl-ester (CE) fraction was markedly deficient in linoleic acid (18:2) in both total plasma and HDL. There was a compensatory increase in saturated acids. The HDL phospholipid (PL) fraction also showed a reduction in the proportion of 18:2 with a concomitant increase in stearic (18:0) and oleic acid (18:1) while the HDL triglyceride (TG) fraction showed only an increase in palmitoleic (16:1) and oleic acids. Feeding of soya lecithin rich in 18:2 failed to increase significantly the 18:2 content of HDL-CE and HDL-PL but lowered the percentage of 16:1 and 18:1 in all 3 lipid classes of HDL. Although the total plasma CE fatty acid profile was perturbed in Friedreich's Ataxia, total plasma PL and TG fatty acid patterns were unaffected. Among the plasma lipoprotein fatty acid profiles, that of the low density lipoprotein (LDL) was most affected, then that of the HDL. The very low density lipoprotein (VLDL) fatty acid composition showed an increase in 16:1 and a decrease in 18:2 which were entirely corrected by lecithin feeding. These results suggest the existence of a metabolic defect in the incorporation of 18:2 into chylomicron phospholipids within the intestinal mucosa.*

RÉSUMÉ: *Nous avons poursuivi notre étude de l'anomalie des lipides et des lipoprotéines plasmatiques dans l'Ataxie de Friedreich. Nous avons mesuré le rapport apo AI/AII des lipoprotéines de haute densité (HDL) par densitométrie et l'avons trouvé normal. Le rapport cholestérol libre/cholestérol estérifié des HDL était plus bas dans l'Ataxie de Friedreich et cela à cause surtout d'une réduction de la quantité de cholestérol libre. L'étude du profil lipidique des esters de cholestérol (CE) nous a révélé une diminution importante de la proportion d'acide linoléique (18:2) tant dans le plasma total que dans les HDL. Il y avait en échange une augmentation de la proportion d'acide gras saturés. Le 18:2 des phospholipides (PL) des HDL était aussi plus bas et compensé par une augmentation concomitante de l'acide stéarique (18:0) et de l'acide oléique (18:1) tandis que les triglycérides (TG) des HDL étaient enrichis en acide palmitoléique (16:1) et en acide oléique. Nous avons constaté que l'addition au régime de lécithine de soya riche en 18:2 ne pouvait corriger ce déficit en 18:2 des fractions HDL-CE et HDL-PL alors qu'elle entrainait une diminution du pourcentage de 16:1 et de 18:1 dans les 3 classes de lipides des HDL. Bien que le profil d'acide gras des CE du plasma total ait été anormal dans l'Ataxie de Friedreich, ce n'était pas le cas pour la composition en acide gras des PL et des TG du plasma total. Pour ce qui est du profil en acide gras des diverses fractions lipoprotéiques, il s'est avéré anormal surtout au niveau des lipoprotéines de faible densité (LDL) mais aussi au niveau des HDL. Nous avons constaté une augmentation du 16:1 et diminution du 18:2 dans les lipoprotéines de très faible densité (VLDL) que l'administration de lécithine de soya a complètement corrigées. Ces résultats nous suggèrent qu'il existe un bloc métabolique dans l'introduction du 18:2 dans les phospholipides des chylomicrons au niveau de la muqueuse intestinale.*

INTRODUCTION

Because of known associations of neurological disorders and plasma lipid abnormalities (Steinberg, 1972; Herbert P.N., et al. 1978; Sidbury et al. 1967; Sandbank U., et al. 1971) we have carried out a systematic study of plasma lipids and lipoproteins in patients with Friedreich's ataxia (Huang Y.S., et al. 1978). This survey disclosed a low level of plasma high density lipoproteins (HDL) which had an abnormal composition, with an increased cholesterol to protein ratio. To further investigate this anomaly we measured the proportion of esterified cholesterol in the HDL fraction, used densitometry to obtain a semi-quantitative appraisal of the apolipoprotein AI/AII ratio, and examined the fatty acid pattern of HDL cholesteryl-esters. We found a higher proportion of cholesteryl-esters which

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were markedly deficient in linoleic acid (18:2) in the HDL of the Friedreich patients. This led us to study the fatty acid profile of the major plasma lipid classes as well as that of the various plasma lipoprotein fractions. We also examined the effect of feeding large amounts of linoleic acid-rich soybean lecithin on the compositional defect of cholesteryl-esters. It is the results of these studies that we wish to report here.

MATERIAL AND METHODS

Subjects:

These studies were carried out in two groups of subjects. Measurement of the proportion of free and esterified cholesterol in HDL, of the Apo AI/AII ratio, and preliminary studies on the fatty acid pattern of HDL cholesteryl-esters (CE) were carried out on frozen aliquots obtained in our first study of 11 Friedreich's ataxia patients and 10 normolipidemic volunteers. Clinical data and details of the procedure followed to obtain the HDL fraction are given in the original study (Huang Y.S. et al., 1978).

The detailed analysis of the fatty acid patterns was carried out on fresh samples obtained in another group of 11 patients affected with Friedreich's ataxia as defined by the criteria of the Quebec Cooperative Study of Friedreich's Ataxia (QCSFA; Barbeau, 1976). There were 7 males and 4 females between the ages of 18 and 33 years. They were compared with 11 normolipidemic healthy volunteers, 6 males and 5 females, between the ages of 19 and 23 years, who were free of any neurological impairment. The characteristics of the subjects are given in table 1. The ponderal index, the plasma cholesterol levels, and the plasma phospholipid concentrations did not differ significantly between the two groups. The Friedreich patients were slightly older. Their plasma triglycerides, though well within the normal range, were significantly higher, as observed previously (Huang et al., 1978). Two women in the ataxia group had overt diabetes requiring insulin treatment, another 2 patients (1 male, 1 female) had an abnormal 2-hour glucose tolerance test.

TABLE 1

Characteristics of Subjects Studied

	Controls	Friedreich	p
Number (Males)	11 (7)	11 (5)	
Age	20.5±1.2*	26.5±3.6	<0.001
Weight (kg)	64.4±10.2	56.6±5.6	<0.05
Height (cm)	174.8±7.0	164.9±10.2	<0.02
Ponderal index †	13.2±0.5	13.0±0.7	NS
Cholesterol (mg/dl)	155±23	147±32	NS
Triglycerides (mg/dl)	59±16	116±32	<0.001
Phospholipids (mg/dl)	186±21	201±43	NS

*mean±SD; unpaired Student *t* test used for comparisons between groups.
† height (inches) over cube roots of weight (pounds)

TABLE 2

Composition of a Commercial Soybean Lecithin Preparation

PL Fraction:		LPC†	PC	PS	PE	Total
Phospholipids (mg/g):		42.3	198.0	106.0	185.0	531.3
Fatty acid*(%)	15:0	3.17	0.25	0.75	0.50	0.13
	16:0	30.69	12.51	30.51	22.00	22.06
	16:1	—	0.26	0.59	0.48	0.46
	18:0	11.93	2.62	6.99	6.36	6.30
	18:1	22.49	6.96	5.09	8.23	8.15
	18:2	23.85	68.23	47.23	54.47	61.88
	18:3	2.23	8.71	7.33	7.19	8.50
	20:2	0.66	—	0.24	0.09	0.34
	22:0	4.17	0.27	0.68	0.35	0.18
	20:4	0.82	0.20	0.59	0.32	0.19

† Abbreviations: LPC: Lysophosphatidyl-choline, PC: phosphatidyl-choline (lecithin), PS: phosphatidyl serine, PE: phosphatidyl ethanolamine.

*Fatty acids having the same retention time as standard samples of 15:0, 16:0, 16:1 etc... run on the same GLC column. See text for the names of the acids.

Five of the 11 Friedreich's ataxia patients had received large doses of soybean lecithin as part of another experimental protocol at the time of sampling. Those of us who carried out the fatty acid studies were not made aware of the actual distribution until the measurements were completed. Looking at the data of the two groups separately (i.e. with and without lecithin) allowed us to make some interesting observations. The soybean "lecithin" was obtained in a granular form from Sigma Chemical Co, St-Louis, MO. It consisted of a mixture of phospholipids containing approximately 20% phosphatidyl-choline by weight. We performed a fatty acid analysis of the various phospholipid fractions (table 2). The most abundant fatty acid was linoleic acid (18:2) which was highest in the phosphatidyl-choline fraction (68.2%) and lowest in the lysophosphatidyl-choline fraction (23.9%). The total linoleic acid content was estimated to be 230 mg per gram of powder. The powder was also rich in palmitic acid (16:0). At the time of sampling, 3 patients had received 7.2 g per day over a period of 21 to 27 weeks, one had received 10.8 g per day over 21 weeks, and another had been given 60 g per day during 13 weeks. The lecithin was administered in orange juice in three divided doses.

Blood sampling and separation of lipoproteins:

Venous blood for plasma lipid determination, fatty acid analysis, and lipoprotein separation was obtained in the morning after a 12-hour fast. The

sample was withdrawn into tubes which contained disodium EDTA (1 mg per milliliter of blood), immediately cooled and centrifuged at 4° C and processed within 24 hours. Small aliquots were used for determination of plasma cholesterol, triglyceride, and phospholipid concentrations as well as for total lipid extraction and measurement of the fatty acid profile of each lipid class. A 5 ml aliquot was used for plasma lipoprotein separation.

The major lipoprotein fractions, very low density lipoproteins (VLDL). low density lipoproteins (LDL), and high density lipoproteins (HDL), were separated by sequential ultracentrifugation at densities 1.006, 1.063, and 1.21 g/ml according to the technique of Havel et al. (1955). After dialysis during 24 hours against several changes of a large volume of normal saline containing EDTA (10^{-4}M), the LDL and HDL fractions were adjusted to the original plasma volume for total lipid extraction.

The apolipoproteins of plasma HDL already separated by polyacrylamide gel electrophoresis in the first group of patients (Huang et al. 1978) using the method of Kane et al. (1973, 1975), were quantitated by densitometry of the Coomassie blue stained bands in a Vitatron Scanner, model TLD 100 (Dieren, Holland).

Lipid measurements:

The plasma cholesterol was determined on a Technicon autoanalyzer by the method of Block et al. (1966). Triglycerides were measured by the method of Laurell (1966) adapted to the autoanalyzer by Kraml and Cosyns (1969). Phospholipids were measured by the method of Bartlett (1959).

For the measurement of the fatty acid profile, total lipids were extracted from plasma and from the lipoprotein fractions with a chloroform/methanol mixture according to the technique of Folch et al. (1957). Aliquots of the total lipid extract were hydrolyzed at 70° C for 90 minutes with 1 N KOH in a 70% methanol solution. After removing the non-saponifiable material by hexane extraction (equal volumes, 3 times), the remaining aqueous phase was acidified with 10 N HCl. The fatty acids were then extracted into hexane.

Separate aliquots of the total lipid extract were fractionated by thin layer chromatography (TLC) on Silica Gel H-coated (0.5 mm thick) 20 x 20 cm glass plates. The plates were developed with hexane/diethyl ether/acetic acid 40:10:1 volume. Cholesteryl-esters (CE), triglycerides (TG), and phospholipids (PL) were visualized by spraying with a 0.1% solution of 2,7-dichlorofluorescein (Brinkmann Instruments), Rexdale, Ontario). The bands corresponding to TG and PL were scraped into a test tube and 2.0 ml of boron trifluoride-methanol reagent (Matheson Coleman & Bell Manufacturing Chemists, Norwood, OH) was added and heated in boiling water for 30 and 90 minutes respectively (Morrison and Smith, 1964). The CE fraction scraped off the plate was first saponified as for total lipids and the free cholesterol extracted before methylation of the fatty acids with BF_3-methanol. The methyl esters in all cases were extracted into hexane and concentrated under a stream of nitrogen for gas liquid chromatography (GLC).

GLC of the methylated fatty acids was performed on a 6-foot U-shaped glass column (3 mm i.d.) packed with 10% Silar 10 C on 100-120 mesh Gas Chrom Q (Applied Science Laboratories Inc. State College, PA) at 185° C using a Hewlett-Packard F & M model 402 apparatus equipped with an hydrogen flame ionization detector. The hydrogen flow rate was adjusted at 35 ml per min. An electronic integrator (Hewlett-Packard 3373B) was used to measure the relative proportion of the various fatty acids. Retention times were compared with those of methylated standards purchased from Applied Science (L-205, L-206, L-207, L-209 and KD mixtures).

To measure the relative proportion of free (FC) and esterified cholesterol in the first group of patients, an aliquot of the HDL total lipid extract was separated by TLC as above. The

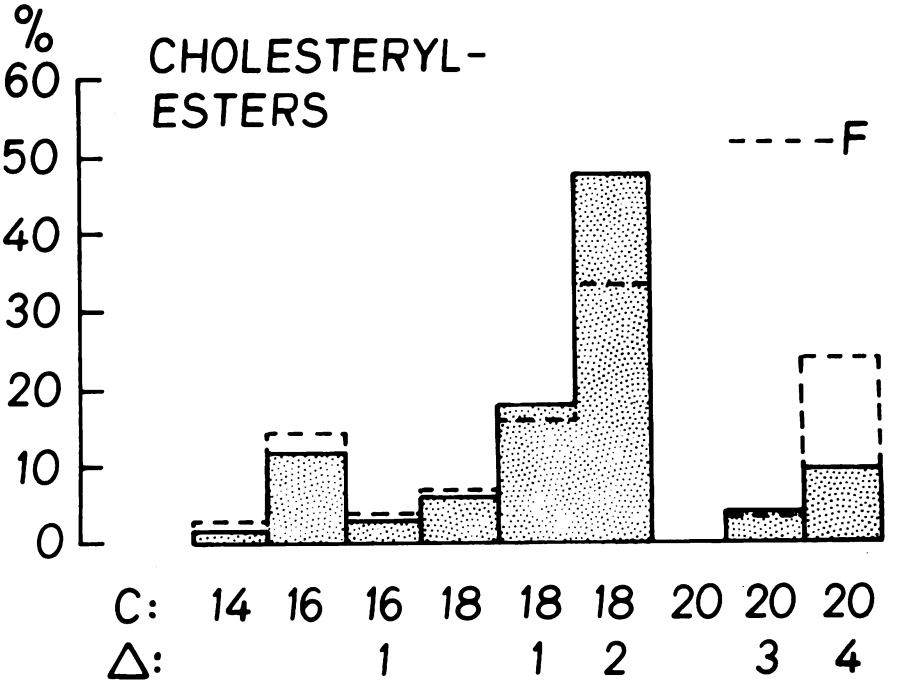

Figure 1 — Fatty acid profile of cholesteryl-esters in high density lipoproteins of normal subjects (shaded bars) and of Friedreich's ataxia patients (dotted lines, F), a preliminary experiment on frozen aliquots. C indicates the number of carbon atoms in the fatty acids, Δ gives the number of double bonds; 20:4 represents here the pooled long chain fatty acids of retention times equal to or greater than arachidonic acid.

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FC and CE bands were recovered and placed in a test tube containing 0.1 mg 5α-cholestane as internal standard. They were saponified with 1 N KOH in a 70% methanol solution at 70° C for 90 minutes, the cholesterol was extracted into hexane which was evaporated to dryness. The trimethyl-silyl-derivatives (TMS) were prepared (Marcel and Vezina, 1973) and measured by GLC on a 4-foot column packed with 3% OV-1 on 80-100 mesh Chromosorb W (Applied Science) at 245° C with an hydrogen flow rate of 30 ml per minute.

RESULTS

The proportion of esterified cholesterol was greater in the Friedreich's ataxia patients (84.14± 2.20%, mean±SD) than in the control subjects (79.69±3.58%) and the difference was statistically significant ($p<0.01$). The percentages of free cholesterol were thus respectively 15.86 and 20.31. Expressed as percentage of the total HDL molecule there was 3.2% of FC and 17.1% of CE in the ataxic patients as compared to 2.8% of FC and 11.0% of CE in the controls. In absolute values, however, the difference is mainly related to a reduction the total amount of HDL free cholesterol. The HDL-FC concentration was 4.85± 1.22 g/dl in the patients and 7.00± 1.80 in the controls, a significant difference ($p<0.01$). In contrast, the HDL-CE levels were 25.69±4.01 in Friedreich's ataxia and 27.30±5.45 mg/dl in the controls (NS).

The densitometric measurement of the AI/AII ratio did not disclose any difference between the two groups. It averaged 4.18±1.24 in the control subjects and 4.05±0.95 in the patients.

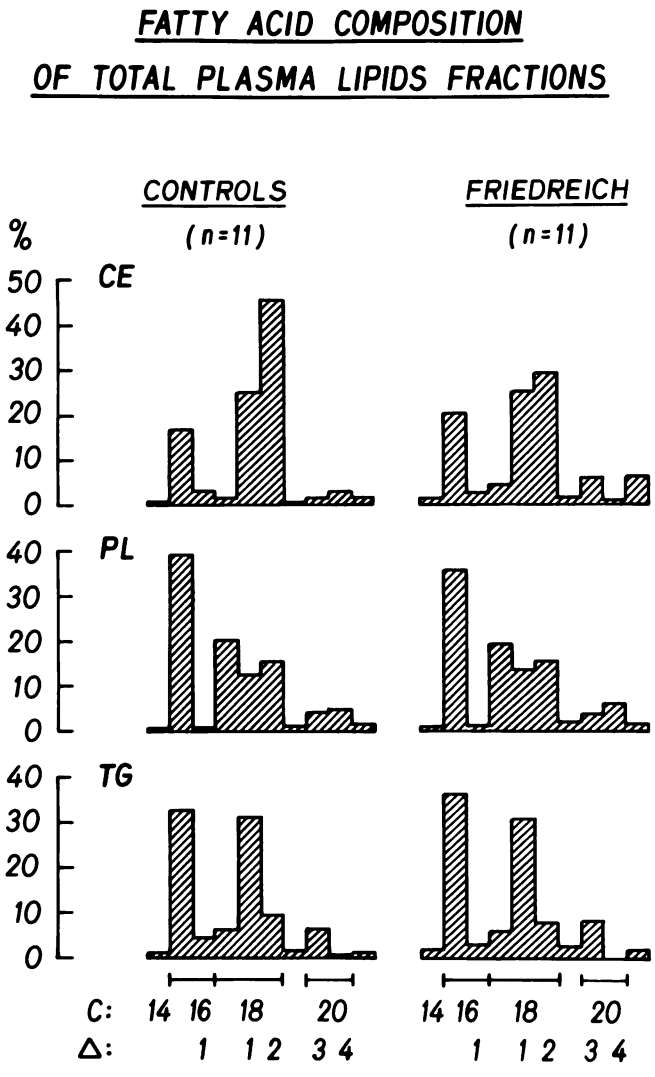

Figure 2—Fatty acid profiles of total plasma cholesterol esters (CE) phospholipids (PL) and triglycerides. C indicates chain length (number of carbon atoms), Δ gives the number of double bonds.

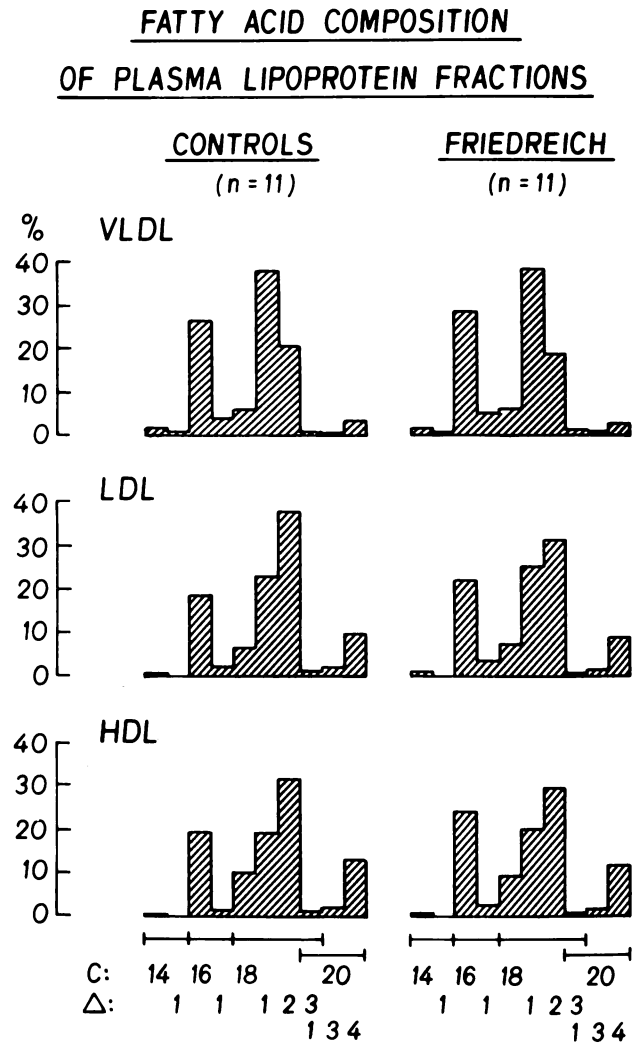

Figure 3—Fatty acid profiles of the total lipid extract of plasma very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). C indicates chain length (number of carbon atoms) and Δ the number of double bonds.

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The abnormalities found in the CE/FC ratio coupled with the lack of obvious differences in the protein moiety of the HDL fraction led us to examine the fatty acid composition of the CE fraction on frozen aliquots of the HDL obtained in the course of our previous study (Huang et al., 1978). The results of this preliminary experiment are depicted in figure 1. The fatty acid composition of the HDL-CE in the normolipidemic volunteers are represented by the shaded bars and contrasted with the measurements made in the Friedreich's cases (dotted lines). The most obvious difference was in the linoleic acid content which was significantly lower in Friedreich's ataxia (28.07 ± 4.29 vs $47.87 \pm 6.67\%$, $p < 0.001$). This difference was compensated by a relative increase in the other fatty acids especially of longer chain length, although the individual variations were quite pronounced at this end of the scale.

We then turned to a new group of patients to explore these abnormalities in depth with fresh plasma. First, we looked separately at the major plasma lipid classes to determine whether HDL-CE abnormality was also present in the total CE fraction of plasma and whether it could be detected also in total plasma triglycerides and phospholipids. The results are presented in figure 2. In Friedreich's ataxia the fatty acid profile of the cholesteryl-ester fraction was markedly modified whereas that of the other two lipid classes were unchanged. There again the proportion of 18:2 was strikingly lower than normal ($29.11 \pm 6.09\%$ vs $45.50 \pm 9.67\%$, $p < 0.001$). In contrast, the percentage of stearic acid (18:0) was higher (4.60 ± 2.19 vs 2.13 ± 0.70, $p < 0.01$) as well as the percentage of eicosa-trienoic acid (20:3) ($5{:}49 \pm 3.94$ vs 1.75 ± 0.88, $p < 0.01$) and of fatty acids with a retention time longer than that of arachidonic acid (20:4) (1.75 ± 2.65 vs 6.11 ± 4.78, $p < 0.02$). Arachidonic acid itself was lower in Friedreich's ataxia (1.11 ± 0.66 vs 3.36 ± 2.13). These differences in the fatty acid profile of plasma cholesteryl-esters were not modified by lecithin supplementation and values for the treated group were virtually identical to those of the untreated groups. For the other plasma lipid classes, there were no significant differences between control subjects and Friedreich's ataxia patients. There was a non significant increase in the 18:2 content of the triglyceride fraction with lecithin treatment in the Friedreich group (6.48 ± 2.55 vs 9.07 ± 2.24 for a control value of $9.48 \pm 3.66\%$). Such a trend was not seen for the 18:2 content of the phospholipid fraction.

Next, we looked at the total fatty acid profile of the various lipoprotein fractions in search of an abnormality which would be specific for a given lipoprotein class. The results of these measurements are given in table 3. There were no differences between control subjects and Friedreich's ataxia patients in the VLDL fatty acid pattern. In the LDL fraction, the proportion of 18:2 was significantly reduced in Friedreich's ataxia whereas that of 14:0 (myristic acid) and 16:0 (palmitic acid) were significantly increased. The HDL fatty acid profile was also perturbed: 14:0, 16:0 (palmitic acid) and 16:1 (palmitoleic acid) percentages were significantly higher whereas the percentage of 18:0 was significantly lower in the Friedreich cases. The proportion of 18:2 was also reduced but the differences did not reach statistical significance because of one outlier. In all lipoprotein classes, there was a non-statistically significant trend for 20:4 to be lower in the patients than in the controls. When the treated and

TABLE 3

Percent Fatty Acid Composition of Major Plasma Lipoprotein Classes

Fatty Acid*	VLDL		LDL		HDL	
	Control (11)	Friedreich (11)	Control (11)	Friedreich (11)	Control (11)	Friedreich (11)
14:0	1.43 ± 0.46†	1.61 ± 0.95	0.29 ± 0.13	0.52 ± 0.30^{c}	0.11 ± 0.06	0.46 ± 0.27^{d}
14:1	0.33 ± 0.14	0.28 ± 0.14	0.16 ± 0.08	0.18 ± 0.12	0.09 ± 0.03	0.29 ± 0.51
16:0	25.92 ± 2.02	27.83 ± 3.43	17.98 ± 1.17	21.91 ± 2.30^{d}	19.53 ± 1.50	23.75 ± 2.66^{d}
16:1	4.00 ± 1.08	4.99 ± 1.38	2.42 ± 0.75	3.11 ± 1.15	1.71 ± 0.42	2.43 ± 0.86^{c}
18:0	5.56 ± 1.31	5.73 ± 1.03	6.20 ± 0.50	6.93 ± 1.12	10.03 ± 0.71	9.27 ± 0.59^{d}
18:1	37.88 ± 2.77	37.32 ± 3.44	22.98 ± 1.98	24.89 ± 3.32	19.20 ± 1.27	19.79 ± 2.44
18:2	20.35 ± 3.64	18.33 ± 6.57	37.58 ± 3.75	31.33 ± 6.25	32.32 ± 4.07	28.98 ± 5.01
18:3-20:2	0.67 ± 0.61	1.10 ± 0.46	0.80 ± 0.29	0.85 ± 0.20	1.11 ± 1.09	0.64 ± 0.14
20:3	0.45 ± 0.33	0.54 ± 0.22	1.45 ± 0.68	1.36 ± 0.46	2.11 ± 0.47	2.12 ± 1.11
20:4	3.38 ± 1.63	2.91 ± 0.80	9.68 ± 1.69	8.46 ± 2.00	13.27 ± 1.84	11.76 ± 2.04
>20:4	—	—	0.46 ± 0.24	0.38 ± 0.17	0.54 ± 0.14	0.51 ± 0.27

*see table 2.
†mean ± SD, statistical comparisons using unpaired Student *t* test
[a] $p < 0.01$
[b] $p < 0.02$
[c] $p < 0.05$
[d] $p < 0.001$

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untreated subgroups were compared, it was found that none of these changes could be accounted for by lecithin feedings. Indeed, exclusion from the calculations of the treated patients made the differences with the control group even more evident. In contrast, the lecithin feeding had corrected a higher level of 16:1 (5.92 ± 1.10 vs 3.88 ± 0.67, $p<0.01$) and a lower proportion of 18:2 (15.72 ± 2.84 vs. 21.46 ± 4.42, $p<0.05$) in the VLDL fraction of the untreated patients.

We then focused our attention upon the HDL fraction and measured the fatty acid pattern in each major esterified lipid class. The results for the cholesteryl-esters are presented in table 4 for the control subjects, the entire group of Friedreich patients, as well as for the lecithin-treated and untreated subgroups. The most striking difference is again in the 18:2 component which is 40% lower and in the 18:0 fraction which is more than doubled in the Friedreich's ataxia patients. These differences, also noted with the untreated subgroup, are not altered by lecithin feeding. In contrast, the 25% higher 16:0 is entirely accounted for by lecithin feeding. There is finally a higher, though not statistically significant, percentage of 14:0 in all Friedreich groups. The fatty acid composition of the HDL phospholipids is given in table 5. It shows a markedly lower 18:2 content and a higher 18:0 percentage which are not appreciably modified by lecithin feeding. In contrast, the higher values of 18:1 in the Friedreich group are in part accounted for by lecithin supplementation. Lecithin feeding also tended to lower 20:40. Table 6 finally summarizes the findings for HDL-TG fatty acid composition. There is a higher percentage of 16:1 which seems to be part of the Friedreich's ataxia pattern and is completely corrected by lecithin administration. The proportion of oleic acid (18:1) is also higher but only partly corrected by lecithin feedings. The 18:2 content tends to be higher in all Friedreich groups but the difference is small (15%) and not statistically significant. The fatty acid of the 20:3-20:4 region also tends to be lower in the Friedreich's ataxia patients.

DISCUSSION

The abnormal cholesterol to protein ratio in the plasma HDL fraction of the Friedreich's ataxia patients previously reported (Huang et al., 1978) could have reflected a disturbance in the composition of either the protein or the lipid portion of the molecule. Visual comparison of the HDL apolipoprotein patterns on polyacrylamide gel had failed to disclose any major difference between the control samples and those of the patients (Huang et al., 1978). Since the apo AI/AII ratio may change the affinity of the molecules for certain lipids (Cheung and Albers, 1977) we quantitated this ratio by densitometry. These measurements failed to demonstrate any difference. Although the proportion of AI was slightly overestimated because of the close proximity of the slower migrating and much smaller apo E band, the results obtained by our procedure compare well with those obtained by more

TABLE 4

Percent Fatty Acid Composition of HDL Cholesterol Esters

		Friedreich		
Fatty Acid*	Controls (11)	All (11)	Untreated (6)	Treated (5)†
14:0	1.23 ± 0.54	2.58 ± 0.86^{a}	2.35 ± 0.82^{b}	2.87 ± 0.90^{a}
16:0	20.63 ± 4.16	25.71 ± 4.16	23.47 ± 4.17	28.41 ± 2.23^{bc}
16:1	2.29 ± 0.89	2.47 ± 1.11	3.23 ± 0.78	1.56 ± 0.65^{d}
18:0	3.33 ± 1.78	7.21 ± 1.35^{a}	6.36 ± 1.04^{b}	8.23 ± 0.92^{ae}
18:1	19.93 ± 3.91	21.98 ± 3.53	23.43 ± 2.79	20.25 ± 3.82
18:2	45.18 ± 6.12	26.78 ± 4.68^{a}	27.43 ± 4.46^{a}	26.02 ± 5.34^{a}
n.i.§	2.54 ± 1.20	3.04 ± 1.17	3.19 ± 1.60	2.87 ± 0.44
20:3-20:4	4.63 ± 2.30	5.54 ± 2.36	6.17 ± 2.62	4.77 ± 2.00
>20:4	trace	1.60 ± 1.34	1.09 ± 0.81	2.15 ± 1.71

*see table 2.
† dietary supplement of soybean lecithin
[a] $p<0.001$ as compared with control group
[b] $p<0.01$ as compared with control group
[c] $p<0.05$ as compared with untreated group
[d] $p<0.01$ as compared with untreated group
[e] $p<0.02$ as compared with untreated group
§ n.i.: not identified

TABLE 5

Percent Fatty Acid Composition of HDL Phospholipids

		Friedreich		
Fatty Acid*	Controls (11)	All (11)	Untreated (6)	Treated (5)†
16:0	31.26 ± 4.31	33.32 ± 3.93	32.01 ± 4.30	34.89 ± 3.13
16:1	n.d.§	0.57 ± 0.50	0.87 ± 0.48	0.21 ± 0.19
18:0	11.82 ± 3.60	17.03 ± 2.32^{a}	15.87 ± 2.38^{c}	18.42 ± 1.38^{b}
18:1	13.15 ± 1.58	15.40 ± 2.52^{c}	16.15 ± 1.48^{b}	14.52 ± 3.36
18:2	28.38 ± 4.04	19.28 ± 3.02^{a}	18.47 ± 3.17^{a}	20.25 ± 2.84^{a}
n.i.§	0.76 ± 0.29	0.58 ± 0.23	0.59 ± 0.20	0.56 ± 0.28
20:3	2.61 ± 1.19	3.39 ± 1.04	3.95 ± 1.10	2.72 ± 0.41
20:4	11.86 ± 4.21	9.68 ± 2.42	11.02 ± 2.49	8.08 ± 1.00
>20:4	n.d.	0.49 ± 0.40	0.68 ± 0.44	0.26 ± 0.19

*see table 2.
† dietary supplement of soybean lecithin
§ n.i.: not identified; n.d.: not detectable
[a] $p<0.001$ as compared with control group
[b] $p<0.01$ as compared with control group
[c] $p<0.05$ as compared with control group

TABLE 6

Percent Fatty Acid Composition of HDL Triglycerides

		Friedreich		
Fatty Acid*	Controls (9)	All (11)	Untreated (6)	Treated (5)†
<16:0	2.75±0.93	3.02±1.22	2.95±1.18	3.09±1.40
16:0	45.45±5.78	47.57±4.80	46.07±5.49	49.37±3.53
16:1	1.31±0.48	2.46±1.22[b]	3.13±1.29	1.65±0.42[c]
18:0	13.48±5.05	10.22±2.41	9.77±2.90	10.77±1.82
18:1	13.82±2.83	20.54±5.04[a]	23.55±4.87	16.92±1.97[b]
n.i.§	1.87±1.02	1.95±1.11	1.18±0.71	2.87±0.69[d]
18:2	4.86±4.82	5.99±1.49	5.98±1.76	6.00±1.31
n.i.	2.08±0.94	1.82±1.03	1.32±0.89	2.43±0.91
20:3-20:4	13.45±7.40	5.97±3.09	5.58±3.05	6.44±3.42
>20:4	0.92±0.87	0.46±0.28	0.48±0.35	0.44±0.21

*see table 2.
†dietary supplement of soybean lecithin
§n.i.: not identified
[a] $p<0.001$, comparison with control group
[b] $p<0.02$, comparison with control group
[c] $p<0.05$, comparison with untreated group
[d] $p<0.01$, comparison with untreated group

accurate techniques. Calculations from the data of Cheung and Albers (1977), who used a specific and sensitive immunoassay, indicate a mean value of 3.76±0.21 for males (n=6) and 4.07±0.34 for females (n=6). Our own values were virtually identical: 3.79±0.84 for males (n=4) and 4.24±1.02 for females (n=6) in the normal group and respectively 4.02±1.48 (n=5) and 4.35±1.09 (n=5) in the Friedreich group. The slightly higher figures for the males in the Friedreich's ataxia patients are not statistically different from control values. Thus, there were no gross abnormality in the protein moiety of the HDL.

We had shown that the plasma HDL-protein concentration was considerably lower in Friedreich's ataxia while the HDL cholesterol concentration was closer to normal (Huang et al., 1978). The present study reveals that the proportion of free cholesterol is markedly reduced relative to the cholesteryl-esters in HDL, another finding which directs our attention towards the HDL-CE fraction in a search for potential anomalies.

Since the degree of saturation of lipids transported by a lipoprotein has been shown to affect both its metabolism and its lipid carrying capacity (Shepherd et al., 1978; Spritz and Mishkel, 1969), it was of interest to examine the fatty acid composition of the various plasma lipids and lipoprotein classes. A preliminary study carried out on the HDL-CE, a fraction most likely to be affected in view of our previous findings, revealed a striking reduction in the proportion of linoleic acid.

Each major plasma lipid class has its own distinctive fatty acid profile which is known to be maintained across the various lipoprotein fractions (Goodman and Shiratori, 1964). The fatty acid profiles of CE, PL, and TG of our control subjects are in close agreements with those reported in the literature (Goodman and Shiratori, 1964) for both the total plasma (figure 2) and the HDL fraction (table 4). Of the 3 lipid classes, only cholesteryl-ester fatty acid pattern was grossly altered in Friedreich's ataxia.

Table 2 reveals that a distinctive pattern also exists for each lipoprotein fraction taken separately. This is most obvious when displayed in a graphic form as seen in figure 3. It can be seen readily that the LDL pattern is the most markedly affected in Friedreich's ataxia while the fatty acid profile of the HDL is less perturbed and that of the VLDL is unchanged. The lower 18:2, 18:0, and 20:4 fractions and the higher 14:0, 16:0, and 16:1 fatty acids in HDL, as well as the lower LDL 18:2 and 20:4 and higher LDL 14:0 and 16:0 fractions in Friedreich's ataxia were not related to lecithin feeding of a subgroup of the patients. In contrast, a closer look at the VLDL changes revealed that dietary lecithin supplementation had corrected an increase in 16:1 and a decrease in 18:2 in this lipoprotein class.

A detailed analysis of the various lipid classes of the HDL fraction revealed the most striking changes in fatty acid composition. Whereas the major changes in total plasma lipid classes had been exclusively in the CE component, all 3 fractions, CE, PL and TG, were markedly altered in HDL. The reduction in 18:2 CE was of the same order of magnitude in HDL (39.4%) as that in plasma CE (37.1%). This was accompanied by a compensatory increase in 14:0 and 18:0 (table 4). The HDL phospholipids had a 32% lower content of 18:2, a 44% higher content of 18:0, and a 12.5% higher proportion of 18:1 (table 5). In the HDL-TG fraction, 16:1 and 18:1 were higher in the Friedreich patients by 88% and 48.6% respectively. Lecithin feeding failed to increase significantly the 18:2 content of HDL-CE and HDL-PL, although it could bring down the percentage of 16:1 and 18:1 in all 3 lipid classes of HDL.

One way to reconcile these findings is by postulating a block in the incorporation of 18:2 into chylomicron phospholipids at the intestinal wall level. According to Tall and Small (1978), the HDL phospholipids would originate from the surface components of chylomicrons. A metabolic defect in phosphatidyl-choline (PC) esterification in the enterocyte would bring into plasma HDL 18:2-deficient PC molecules. The circulating lecithin: cholesterol-acyl-transferase (LCAT) enzyme would then be presented with a poor substrate and be unable to transfer normal amounts of 18:2 from the 2 position of PC to the HDL cholesteryl-esters which would in turn be deficient in 18:2. Exchange with other lipoproteins would allow their CE fraction to reflect this abnormal composition. Since 60% of erythrocyte membrane PC is exchangeable (Reed, 1968) and since the transfer of

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phospholipids from plasma lipoproteins to red blood cell membrane constitutes a major pathway for the removal of erythrocyte PL and is independent of the degree of saturation of their fatty acids (Renooij and Van Golde, 1977) we would expect the membrane phospholipids to reflect the abnormal fatty acid profile described above.

If the 18:2 molecules provided by the intestinal hydrolysis of lecithin behave in the same manner as 18:2 molecules derived from the hydrolysis of polyunsaturated fatty acids (Shepherd et al., 1978) we would expect an enrichment of all HDL lipoprotein fractions in 18:2 upon feeding lecithin. There is evidence for that in a recent publication of Rosseneu et al. (1979). The feeding of polyunsaturated lecithin to chimpanzees was found to raise the 18:2 content of HDL_3. This increase was especially striking in the HDL_3-PL fraction (from 18.7 to 32.2%). The fact that dietary lecithin supplementation failed to enrich either HDL-PL or HDL-CE in 18:2 is in favor of our hypothesis. Indeed, the patient who received the largest amount (60 g per day) of the lecithin preparation had higher than control 18:2 values in all the lipid fractions studied except in the total plasma CE (20.8 vs 45.5%), in HDL-CE (33.8 vs 45.2%), and in HDL-PL (24.8 vs 28.4%). This patient was the outlier mentioned above in relation with the HDL total fatty acid pattern. That lecithin feeding could enrich VLDL fatty acids in 18:2 and increase to some extent the 18:2 content of HDL-TG, would suggest conversely that their 18:2 molecules did not follow the same metabolic pathway.

Whether the defect responsible for the 18:2 deficiency in plasma CE and HDL-PL fractions is related to the pathogenesis of Friedreich's ataxia remains to be determined. That a defect in HDL composition might be related to neurological manifestations has been discussed by Scanu (1978). There are neurological manifestations in Tangier disease where HDL are deficient, of abnormal composition, and their metabolism perturbed (Schaefer et al., 1978). The HDL of abetalipoproteinemia, where ataxia is a prominent feature, are also abnormal. Indeed, as in Friedreich's ataxia, their total amount is reduced, their content of 18:2 is decreased and their CE/FC ratio is abnormal (Scanu, 1978). It is possible that the defect reported here is not specific for Friedreich's ataxia. Indeed, a reduction in 18:2 has been reported in the fatty acid composition of total plasma lipids in multiple sclerosis and other neurological diseases (Baker et al., 1963; Kalofoutis and Jullien, 1974). Yao et al. (1976) have conducted an analysis of serum non polar lipids in a series of hereditary neuropathies. In most of them, they found a significant decrease in the percentage of linoleate to total fatty acids in both CE and TG. These changes were observed in hereditary motor neuropathy, neuronal Charcot-Marie-Tooth disease, Dejerine-Sottas disease, and in 6 cases of Friedreich's ataxia. They did not study the individual plasma lipoproteins and their lipid components. On the other hand, they examined the fatty acid profile of plasma non-esterified fatty acids, which we have not done, and reported a significant increase in 16:1 in Friedreich's ataxia. Though not necessarily specific for Friedreich's ataxia, it remains that the postulated defect might contribute to the phenotypic expression in several of these neurological diseases.

If the hypothesis of a defect in PC esterification at the enterocyte level is true, we would expect LCAT activity to be somewhat reduced because of the abnormal substrate. The slightly lower but not significantly different values reported for LCAT activity in Friedreich's ataxia as compared to healthy subjects (83 ± 7 vs 97 ± 19 mmol/ml/hr) (Yao et al. 1976) would be in accordance with this view. It would be important, however, to determine whether this lowered activity could be enhanced in the presence of a normal substrate.

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Quebec Cooperative Study
of Friedreich's Ataxia

Pilot Studies on Membranes and some Transport Mechanisms in Friedreich's Ataxia

A. FILLA, R. F. BUTTERWORTH AND A. BARBEAU

SUMMARY: *The observed anomalies in high density lipoproteins in Friedreich's ataxia led us to investigate the state of cellular membranes in this illness. As a preliminary screening program, we studied the shape of erythrocytes; the phospholipid content of platelets and the transport properties of these membranes as indirectly reflected in the absorption of Vit E and the renal handling of orally injected taurine. All these investigations were normal, except for a tendency towards more echinocytes in Friedreich's ataxia and the significant increase in taurine urinary excretion after an oral load. We concluded that the possible membrane abnormalities are not major and will have to be searched for with more subtle and specific tests.*

RÉSUMÉ: *Les anomalies observées dans les lipoprotéines à haute densité de l'ataxie de Friedreich nous ont conduit à étudier l'état des membranes cellulaires dans cette maladie. Comme étape préliminaire nous avons investigué la forme des érythrocytes, le contenu en phospholipides des plaquettes et les propriétés de transport indirectement reflétées dans l'absorption de la vitamine E et la disposition rénale d'une dose orale de taurine. Toutes ces études ont donné des résultats normaux, sauf pour une tendance à une plus grande formation d'échinocytes par les ataxiques et une augmentation significative de l'excrétion urinaire de la taurine après une surcharge orale. Il faut donc conclure que les anomalies membranaires postulées ne sont certainement pas majeures, mais il est possible qu'elles soient mises en évidence par des tests plus subtils et plus spécifiques.*

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INTRODUCTION

One of the important findings of our Phase Two studies has been the high density lipoprotein (HDL) fraction (Huang et al., 1978). Because the cholesterol and phospholipid content of membranes is dependent on the surrounding milieu, and can be exchanged with the lipoproteins of the milieu, the eventual significance of the HDL defect could be a modification of membrane structure or fluidity in Friedreich's ataxia.

Such alterations could be evident through modifications in the shape or composition of the membranes of easily accessible cells, such as the erythrocyte or the platelet. Similarly, if membranes are abnormal, impaired intestinal absorption or specific renal handling could be expected in some cases. Since such indications can be obtained through fairly straightforward tests, these were undertaken as a preliminary exploration of possible membrane involvement in Friedreich's ataxia before more specific investigations, such as electron spin resonance, nuclear magnetic resonance, and lectin agglutination could be carried out. The present paper reports the results of such preliminary studies.

SUBJECTS AND METHODS

a) Erythrocyte shape modifications

Venous blood (5 ml with heparin and 10 ml without anti-coagulant) was collected in the fasting state from 10 ataxic patients and 6 age-matched healthy control subjects. The heparinized blood was immediately centrifuged at 2,000 rpm to separate red blood cells (RBC) from plasma. Erythrocytes were suspended in tissue culture medium 199 (Gibco-Grand Island, N.Y.) with 10 percent autologous serum (Mars et al., 1969), and diluted 1/200. 10 ml of the suspension in 25 ml Erlenmeyer Flasks were incubated in an atmosphere of 95 percent 0_2/5 percent $C0_2$ at 37° C, for four hours in a metabolic water bath shaker (New Brunswick Scientific Co. Inc., New Brunswick, New Jersey). Every hour specimens were examined on disposable vinyl plastic microslides (Thomas Scientific Apparatus, Cat. No. 6686-V15) by light microscopy at magnification 400X. One thousand cells were counted for each slide and the percentage of deformed cells calculated. In each experiment at least one control was run together with one or more patients.

b) Platelet phospholipid content

Platelet phospholipid content was determined by thin layer chromatography according to the detailed method of Marcus et al. (1972) in 65 subjects: 5 young controls; 6 controls 40-70 years of age; 10 parkinsonian patients without levodopa, and 10 with; 5 Huntington's chorea patients; 8 with chronic multiple sclerosis, and 10 with typical Friedreich's ataxia.

c) Vitamin E in serum

Serum Vitamin E levels were determined in 10 normal control subjects, 30 ataxic patients (15 with typical Friedreich's ataxia and 15 with other forms of hereditary ataxia) and 16 neurological disease controls, age-matched. Vitamin E assay was performed spectrophometrically in triplicate, as described by Martinek (1964) with modifications according to Tsen (1961).

d) Renal handling of a taurine load

The renal handling of taurine was estimated in 5 patients affected with typical Friedreich's ataxia (from

different sibships) and 5 age-matched normal controls. Specimens of urine were collected for two hours before, and 24 hours after the oral absorption of a 250 mg taurine load (ICN Company). Two hour samples were collected until 17:00 hrs and the last sample was made up of the overnight (17:00-07:00) hrs) collection. Patients and controls received a standard diet during the 3 days preceding the experiments and during the collection period. Taurine was measured by the method of Anfano et al. (1978).

RESULTS

a) Erythrocytes

As can be seen in Table 1, the percentage of distorted erythrocytes (echinocytes) shows no statistical difference between the control subjects and the ataxic patients. However, it is noteworthy that at each time period the mean level for Friedreich's ataxia is always approximately 20% higher than the mean for control subjects, even if the difference is not significant with the relatively small number of individuals tested.

b) Platelet phospholipids

The study of the various phospholipids in platelets, as seen in Table 2, did not reveal any significant difference between ataxic subjects and their age-matched controls. However, the combined levels phosphatidyl-serine and phosphatidyl-inositol are lower than in age-matched controls (approaching significance). It is also interesting to note that in parkinsonian patients treated with levodopa most phospholipid absolute values are lower than without this treatment, and even lower than in age-matched controls.

c) Vitamin E

Similar negative results are observed in the study of vitamin E in the serum. As seen in Table 3 there was no significant difference between levels in ataxic patients and normal or disease controls, although the levels in Friedreich's ataxia tended to be lower than the other groups. A single Friedreich patient had a value of 0.28, which is pathologically low, but not within the range of Bassen-Kornzweig's disease.

e) Oral taurine load

The overall pattern of urinary excretion of taurine after an oral load of 250 mg of taurine (a level just above the normal dietary ingestion) is essentially similar to that seen in age and sex-matched controls (Figure 1). However, the ataxic subjects uniformly excreted twice as much of the load as the controls. This was true in each matched pair and is highly significant statistically.

DISCUSSION

Many diseases are manifested by abnormalities in membranes which are sometimes reflected in the shape of erythrocytes. Thus, increased cholesterol content in erythrocyte membranes associated with β-lipoprotein abnormalities have been detected in "spur cell" anemia and in acquired hypo-β-lipoproteinemia in undernourished infants (McBride et al.,

TABLE 1

% Erythrocyte Distortion

	Hours				
Controls (n=6)	**1**	**2**	**3**	**4**	**5**
Mean	3.8	4.59	4.93	4.27	4.87
S.D.	0.91	1.88	6.0	2.16	4.65
S.E.	0.41	0.84	2.68	0.97	2.08
Patients (n=10)					
Mean	5.24	6.5	6.3	6.22	6.02
S.D.	4.23	4.15	4.42	4.20	3.92
S.E.	1.89	1.38	1.47	1.40	1.31
t=	0.74	1.18	0.45	1.14	0.47

TABLE 2

Phospholipids in Human Blood Platelets

Group	N	Age	Platelet Count ($X10^3$)	μg Phospholipid per 10^8 Platelets					Per Cent Total Phospholipid			
				Total	PE	PC	SPH	PI + Ps	PE	PC	SPH	PI + Ps
Controls 20-30 yrs	5	25.4±1.4	409.4±30.9	21.52±3.07	4.92±0.74	6.92±0.81	6.75±1.02	2.94±0.73	24.9±1.1	44.4±3.1	19.9±1.3	10.8±1.6
Controls 40-70 yrs	6	54.3±5.6	393.0±34.2	30.52±3.16	7.48±0.61	8.95±0.96	9.57±1.00	4.51±0.71	27.5±1.0	40.0±0.9	20.2±0.9	12.2±0.8
Parkinsonians Dopa	10	60.5±4.1	362.3±28.4	24.12±2.72	6.24±0.72	7.67±0.78	7.26±1.05	2.96±0.39	27.5±0.6	42.4±1.3	18.5±0.7	10.6±1.0
Parkinsonians No Dopa	10	60.0±3.2	394.2±32.6	29.18±3.26	7.24±0.82	8.89±0.92	8.66±1.11	4.40±0.67	27.3±0.9	41.8±1.4	18.7±0.7	12.2±1.3
Huntington's Chorea	5	30.6±6.1	337.2±46.0	21.32±4.37	5.15±0.92	5.96±0.88	7.90±2.08	2.89±0.48	27.6±0.9	41.0±2.6	23.3±1.8	10.2±1.0
Multiple Sclerosis	8	42.3±2.0	346.2±38.3	23.67±4.13	5.90±0.94	7.40±1.08	7.37±1.33	3.00±0.81	27.7±1.0	43.9±1.7	19.4±0.6	9.1±1.6
Friedreich's Ataxia	10	26.2±1.5	288.3±24.8	18.79±1.73	4.58±0.47	6.36±0.45	5.90±0.63	1.94±0.28	26.3±0.8	46.2±1.6	19.4±0.5	8.2±0.7

Values reported represent mean values ± S.E.

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1970). Absence of LDL and changes in HDL have been described in plasma of patients with the Bassen-Kornzweig syndrome. In this disease, one finds low levels of cholesterol, phospholipids, and triglycerides in plasma with decreased concentrations of linoleic acid in all fractions and a reduced ratio of phosphatidyl-choline/sphingomyelin. These abnormalities are reflected in red cell membranes which contain less phosphatidylcholine and more sphingomyelin, but almost the same total amounts of phospholipids as normal cells (Fredrickson et al., 1972). Similar, but less evident lipid changes have been found in familial hypobetalipoproteinemia.

Red cell membrane changes from patients with muscular dystrophy have also been shown to differ from normal with respect to fatty acids and phospholipid composition (Kunze et al., 1973). More recently, decreased palmitoleic acid and triglyceride levels have been detected in erythrocytes from Duchenne muscular dystrophy patients (Howland et al., 1977). Many forms of muscular dystrophy (Matheson et al., 1974) and myotonic dystrophy (Roses et al., 1974) can be accompanied by deformations in the shape of erythrocytes. However, the mechanism of "spurring" still remains unclear. Some distortion is attributed to changes in cation distribution consequent to modifications in membrane lipids (Matheson et al., 1974). Abnormal kinetics in the exchange of cholesterol has been implicated in the mechanism of "spurring" in abetalipoproteinemia.

Modifications in HDL lipoproteins in Friedreich's ataxia could lead to disturbed cholesterol exchange with membranes. However, the degree of echinocyte formation observed in this disease, although always more important than in controls, was not significant in our studies. Similarly, we could not demonstrate (Table 2) significant changes in the phospholipid pattern in platelets in patients with Friedreich's ataxia, Parkinson's disease, Huntington's chorea, and multiple sclerosis, all diseases with postulated fatty acid lipid changes in the plasma and occasionally in membranes (Karlsson et al., 1971).

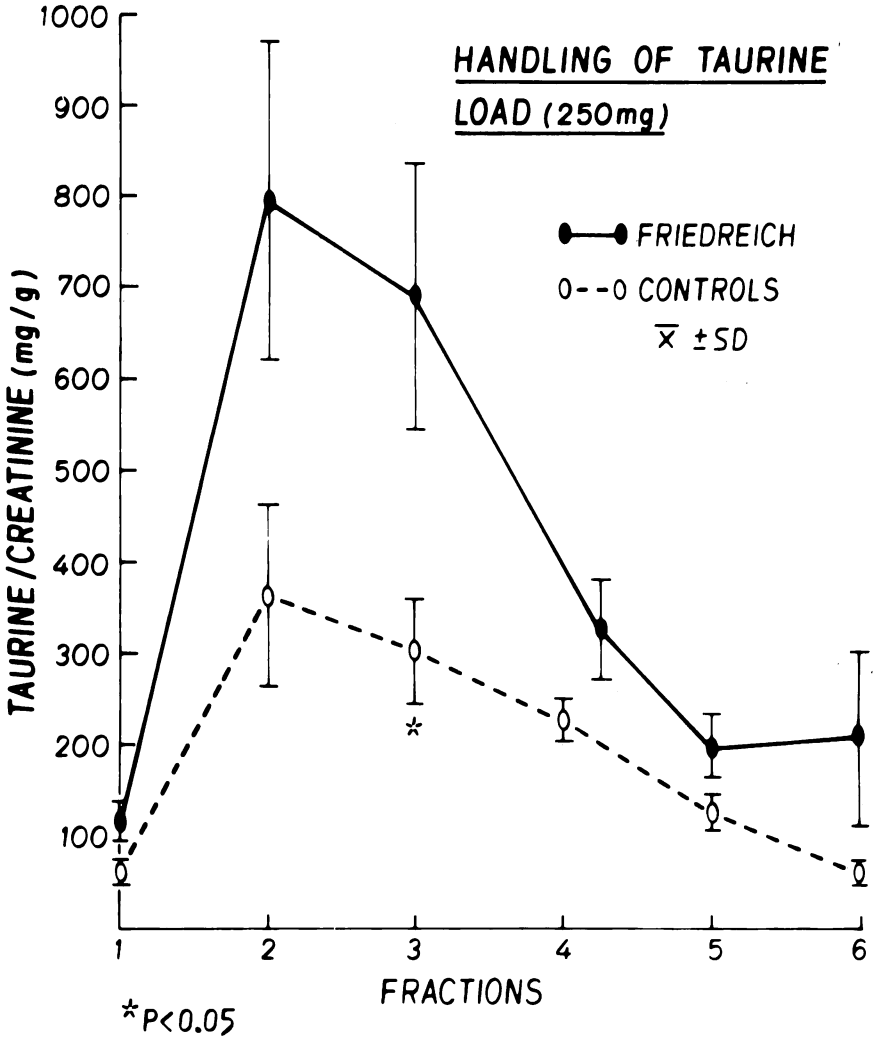

Figure 1 — Urinary excretion of taurine after an oral load of 250 mg of taurine as determined by timed urinary collections in Friedreich's ataxia and age-matched controls.

Vitamin E has been extensively investigated in recent years, but its physiological function and mechanisms of action are still unclear. However, there exists a fat soluble vitamin deficiency syndrome resembling Friedreich's ataxia manifested clinically by progressive ataxia, areflexia, impaired position sense, and absent vibration sense after small bowel resection (Bertoni et al., 1978). Vitamin E deficiency has been recently associated with progressive ataxia, impaired position and vibration sense and areflexia in a patient with cystic

TABLE 3

Serum Vitamin E

	N	Mean (mg%)	S.D.	S.E.
1. Controls	10	0.69	0.11	0.04
2. Ataxic Patients	30	0.65	0.20	0.04
2a. Friedreich	15	0.61	0.18	0.04
3. Disease Controls	16	0.72	0.17	0.04

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fibrosis (Jackson, 1977, quoted by Bertoni et al., 1978). Moreover, the lowest plasma concentrations of vitamin E in humans have been reported in abetalipoproteinemia (Fredrickson et al., 1972). It was thus of interest in Friedreich's ataxia when Takahashi and collaborators (1977) demonstrated that vitamin E is mostly transported by the HDL fraction of serum. Our own results (Table 3) fail to demonstrate a significant change in ataxics taken as a group, although the levels of vitamin E in Friedreich's ataxia were clearly, and almost significantly, lower.

Finally, abnormally high urinary excretion of taurine has been reported in camptodactyly with mental deficiency (Nevin et al., 1969), in familial cerebellar dyssynergia (Hall et al., 1974), in myotonic dystrophy associated with generalized amino-aciduria (Blahd et al., 1955), and in Friedreich's ataxia (Lemieux et al., 1976). It is known that, in the rat, 37% of administered radioactive taurine is eliminated in the urine and 8.6% in the feces while the rest is usually retained and distributed in the organism, mainly muscles (Bouquet and Fromageot, 1965; Portman and Mann, 1955).

Our results (Fig. 1) show that twice as much of a load dose of taurine is excreted in the urine in Friedreich's ataxia as in control patients. Ataxics can thus not retain taurine as well as normal subjects. In the presence of a normal intestinal absorption, serum concentration, and uptake mechanisms of taurine previously demonstrated (Lemieux et al., 1976; Barbeau et al., 1976; Filla et al., 1978), such a result must be due either to an abnormal distribution in the organism or to an abnormality in renal excretion or reabsorption of the amino acid. Because of the important role of muscle for taurine reserves, a decreased size of the muscle pool would explain a decreased ability to retain taurine. The other explanation, of course, would implicate a defect in tubular reabsorption of taurine and β-alanine. A localized renal transport defect for these amino acids, which utilize the same transport system (Chesney et al., 1978), is still possible despite the lack of evidence for a generalized uptake defect for taurine (Filla et al., 1978). At the present time we favor the first hypothesis, a decreased muscle mass or reserve pool. Thus, in the presence of normal dietary intake of taurine, the Friedreich's ataxia patient would be in relative body deficiency of taurine, especially when metabolic stressful situations (such as tissue accumulation of calcium) require the presence of more taurine to immobilize or neutralize by binding this potentially cytotoxic action. The taurine loss thus appears to be an indirect consequence of subtle membrane anomalies in that disease, and possibly an important contributor to the cardiomyopathy (see elsewhere in this issue).

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of Friedreich's Ataxia

Erythrocyte Membrane Lipids in Friedreich's Ataxia

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SUMMARY: *In a study of the lipid composition of erythrocyte membranes in Friedreich's ataxia, the concentration of the major membrane components (phospholipids, cholesterol and protein) in ataxic patients, family members, and control subjects were found to be the same. The total fatty acid distribution was also normal. However, an altered distribution of phospholipid classes in erythrocytes was noted (an increase of PI + PS and a decrease of PE in Friedreich's ataxia patients).*

RÉSUMÉ: *Nous avons étudié la composition lipidique des membranes érythrocytaires, ainsi que la concentration des principales composantes de la membrane (phospholipides, cholesterol et protéines) chez des sujets atteints d'ataxie de Friedreich, des membres de leurs familles et des sujets témoins. Aucune différence majeure ne fut trouvée. La distribution des acides gras totaux était également normale, cependant nous avons noté une altération dans la distribution des classes de phospholipides dans les érythrocytes (une augmentation de PI + PS et une diminution de PE dans l'ataxie).*

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INTRODUCTION

The possibility of a generalized membrane defect in Friedreich's ataxia has been suggested. This was based in part on the preliminary observation of a difference in the stability of erythrocyte membranes in Friedreich's ataxia patients, as determined by SDS polyacrylamide gel electrophoresis (Shapcott et al., unpublished).

Lipids are also of fundamental importance in membrane structure, and the possibility of an abnormality in lipid composition was suggested by the greatly increased cholesterol/protein ratio in the plasma high density lipoprotein (HDL) fraction in Friedreich's ataxia (Huang et al., 1978). As part of the study of membrane function in Friedreich's ataxia, we measured the major lipid components of erythrocyte membranes in Friedreich's ataxia patients, in unaffected family members, and in control subjects.

SUBJECTS AND METHODS

A. Subjects

Typical Friedreich's ataxia (Group Ia) patients and non-affected family members (obligatory heterozygotes and siblings*) were studied. Control subjects were apparently healthy adult volunteers from laboratory and secretarial personnel.

B. Preparation of Ghosts

Fasting blood samples were collected in heparinized tubes and the plasma removed. The red cells were washed once with cold 5mM sodium phosphate (pH 8) — 0.15 M NaCl and the ghosts prepared according to the method of Fairbanks et al. (1971). The ghosts were divided into aliquots for further analysis and were then lyophilized and the weight of each fraction determined.

*Siblings: are either heterozygotes or normal.

Inorganic phosphorus was determined by direct addition of the ammonium molydate — p — semidine reagents to an acidified solution of ghosts (Wybenga, 1974). From this, the weight of phosphate buffer remaining in the ghosts was calculated, and the net weight of lyophilized ghosts was determined by subtraction of the amount of remaining buffer from the lyophilized ghost weight.

Total proteins

Lyophilized ghosts were dissolved by incubation in 0.1 M NaOH overnight at 37°. The method of Lowry (1951) was then used to measure protein in the solution.

Lipids

About 6-8 mg lyophilized ghosts (equivalent to 1 ml ghost preparation before lyophilization) was shaken gently with 1.0 ml chloroform-methanol (2:1) for 5 min. The mixture was centrifuged, the supernatant divided into fractions for the various lipid determinations, and the solvent was evaporated with a stream of nitrogen at room temperature.

For determination of the phospholipid classes, erythrocytes were extracted directly according to the method of Dodge and Phillips (1967).

Total phospholipid

An aliquot (0.1) of the total lipid extract was digested with sulfuric acid and the phosphorus determined according to the method of Wybenga (1974). Quantities of each reagent were reduced proportionally to accommodate the relatively small amount of phosphorus.

Phospholipids

The major phospholipid classes were separated by thin layer

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chromatography (Noel et al., 1972) and lipid phosphorus in each class measured by the method of Bartlett (1959).

Cholesterol

Total and free cholesterol were determined in aliquots of the total lipid extract as described by Dryer (1970).

Fatty acids

Phytanic acid (100μg), methanol (0.4 ml), and 10 M NaOH (60μl) were added to 0.4 of the total lipid extract, and the mixture saponified by heating 15 min at 100°. Methyl esters were prepared by heating the fatty acids in 20 μl of a methylating reagent (made from mixing 0.4 ml methanol and 0.1 ml acetyl chloride) at 120° for 10 min. Excess reagent was evaporated and the produce repeated.

The dried esters were dissolved in methanol (20μl) and 2μl injected onto a 6 ft glass column packed with 10% SP 2330 on 100/120 Chromosorb WAW (Supelco) in a Hewlett-Packard model 5750 gas chromatograph equipped with a flame ionization detector. The oven temperature was programmed from 150 to 200°C at 2°/min. Peak identifications were confirmed for one Friedreich's ataxia patient with a gas chromatograph-mass spectrometer (GC-MS) combination consisting of a Hewlett Packard model 7130-A gas chromatograph connected to an AE1-MS30 mass spectrometer. The fatty acid compositions reported here are calculated directly from relative peak areas and are not corrected for relative recoveries or responses. However, the reproducibility of the procedure was monitored by carrying out the extraction, methylation, and chromatographic steps on mixtures of known quantities of the acids with each lot of samples.

RESULTS

Table 1 summarizes the distribution of the major components (phospholipids, cholesterol, protein) of erythrocyte membranes from Friedreich's ataxia patients, unaffected family members, and external controls. No significant differences among the groups is seen for these components except for a somewhat lower total cholesterol/total protein ratio for the obligatory heterozygotes ($p<002$ compared with external controls and $p<0.05$ compared with Friedreich's ataxia). However, it must be noted that these results are based on a small number (N=4) of heterozygotes. Determination of free cholesterol (by precipitation with digitonin) and gas chromatographic analysis of cholesterol esters showed that most of the cholesterol is in the free form with very low concentrations of cholesterol esters.

In Table 2, the distribution of the major phospholipid classes in erythrocytes of Friedreich's ataxia patients and control subjects is given. There is a significant decrease in phosphatidylethanolamine (PE, $p<0.001$) and an increase in phosphatidylinositol + phosphatidylserine (PI + PS, $p<0.01$) in Friedreich's ataxia patients is noted.

Table 3 shows the distribution of membrane fatty acids in Friedreich's ataxia patients and unaffected family members while in Table 4 the sum of the fatty acid concentrations are expressed as a percentage of the ghost weight and also relative to total ghost protein. The only difference in the distribution and total quantity of fatty acids among the groups is a tendency toward a lower fatty acid/protein ratio for the obligatory heterozygotes.

DISCUSSION

The concept of a generalized membrane defect in several inherited disorders, particularly the neuromuscular diseases, is becoming widely

TABLE 1

Composition of Erythrocyte Membranes (Mean± S.D.)

	Friedreich's Ataxia (N=16)	Obligatory Heterozygotes (N=4)	Siblings (N=10)	External Controls (N=15)
Total Phospholipid (% Dry Ghost Weight)	26.0±2.8	26.8±1.7	26.1±3.3	25.2±4.2
Total Cholesterol (% Dry Ghost Weight)	10.23±1.99	8.93±0.82	9.79±1.32	10.46±2.48
Total Protein (% Dry Ghost Weight)	40.63±2.61	42.63±2.00	40.81±2.14	39.69±6.22
Total Phospholipid/ Total Protein	0.65±0.09	0.63±0.04	0.65±0.07	0.66±0.19
Total Cholesterol/ Total Protein	0.249±0.048	0.210±0.025	0.240±0.031	0.269±0.074

TABLE 2

Percentage Composition of Erythrocyte Phospholipids (Mean ± S.D.)

	Friedreich's Ataxia (N=12)	Controls (N=9)
Total Phospholipid (mg/ml)	3.21±0.35	2.97±0.72
Lysophosphatidylcholine	Trace	Trace
Spingomyelin (S)	28.49±4.63	26.86±2.90
Phosphatidylcholine (PC)	29.22±3.96	32.00±1.87
Phosphatidylinositol (PI) +Phosphatidylserine (PS)	19.84±2.68	14.03±2.99*
Phosphatidylethanolamine (PE)	22.45±3.47	27.10±3.24**

* $p < 0.001$
** $p < 0.01$

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TABLE 3

Percentage Composition of Membrane Fatty Acids (Mean ± S.D.)

	Friedreich's Ataxia (N=13)	Obligatory Heterozygotes (N=4)	Siblings (N=10)	External Controls (N=14)
C14:0	1.57±0.63	1.31±0.99	1.98±1.06	2.27±1.35
C16:0(+C17:0)	27.27±3.68	25.29±3.41	27.09±3.40	26.85±4.11
C16:1	1.17±0.34	1.42±0.48	1.10±0.48	0.93±0.35
C18:0	18.71±2.49	17.54±2.92	17.25±2.70	17.48±2.60
C18:1	21.49±2.74	23.51±2.17	20.22±2.39	21.61±2.20
C18:2	13.16±2.00	13.57±1.00	14.43±2.15	13.70±3.31
C20:3	1.59±1.80	0.61±0.59	0.97±0.55	0.71±0.55
C20:4 +C22:0	15.16±5.44	16.73±2.70	17.13±2.37	16.62±3.58

TABLE 4

Total Fatty Acids ($C_{14:0}$ - $C_{20:4}$) in Erythrocyte Ghosts (Mean ± S.D.)

	Total Fatty Acid (% Dry Ghost Weight)	Total Fatty Acid/ Total Protein
Friedreich's Ataxia (N=13)	13.9±3.6	0.34±0.09
Oblitatory Heterozygotes (N=4)	12.8±2.1	0.30±0.06
Siblings	16.1±4.0	0.39±0.09
External Controls (N=14)	13.4±2.6	0.34±0.07

accepted. Alterations in the composition of the two major membrane components, lipids and proteins, would affect the physical state of the membrane with far reaching consequences in membrane function being likely. Based on the hypothesis that the defect is manifested in membranes of many different tissue cells, and because of its availability and relative ease of isolation, the erythrocyte membrane is a widely used model. The possibility of a difference in erythrocyte and fibroblast membrane protein composition in Friedreich's ataxia will be discussed in detail in other papers in this series.

Defects in lipid metabolism have been associated with a number of inherited diseases, a relevant example being the large increase in plasma phytanic acid in Refsum's disease, a hereditary form of ataxia (Steinberg, 1978).

Erythrocyte lipids have also been measured in a number of disease states and an abnormal composition has been found in patients with familial lecithin: cholesterol acyltransferase (LCAT) deficiency (Gjone et al., 1968). Much work has been described on erythrocyte lipids in Duchenne Muscular Dystrophy with abnormal (Kunze et al., 1973; Kalofoutis et al., 1977) and normal (Kobayashi et al., 1978; Roses and Appel, 1978) phospholipid composition being reported. It has been suggested that oxidation of polyunsaturated fatty acid components during their extraction and analysis may contribute to anomalous results, and differences in lipid content and fatty acid composition of erythrocyte phospholipids between adult and child controls has been reported (Kobayashi et al., 1978). These examples show that comparison of membrane lipid determinations must be based on identical experimental methods, and that variables such as age or the effects of unrelated disease states must also be considered.

In an alternative approach to the elucidation of membrane structure, Butterfield et al. (1974, and this issue) have described the results of ESR experiments which demonstrate different lipid environments in erythrocyte membranes of myotonic muscular dystrophy and Friedreich's ataxia patients. It has been suggested that very subtle differences in chemical composition or altered protein-lipid organization could account for the greater membrane fluidity in myotonic muscular dystrophy, and that these differences would probably not be measurable by the usual chromatographic techniques.

Interpretation of the differences in erythrocyte phospholipids in Friedreich's ataxia is difficult at this stage of our investigation, especially in view of the normal distribution in plasma (Huang et al., 1978) and in platelets (Filla et al., this issue), and also in view of the conflicting results described for the muscular dystrophies. Although our results are consistent with our hypothesis of a generalized membrane defect, they may simply reflect two extremes of the normal range, resulting from differences in diet or other environmental factors. Further studies of the fatty acid composition of the erythrocyte phospholipid classes along with lipid determinations in fibroblasts and biopsy material are planned in order to determine the extent of these differences. The normal fatty acid distribution in erythrocyte membranes of Friedreich's ataxia patients and unaffected family members is in contrast with the anomalies of fatty acid distribution in plasma lipoproteins, where a decrease of linoleic acid ($C_{18.2}$) has been observed in Friedreich's ataxia patients (Davignon et al., this issue). In this respect, dietary fatty acids are incorporated into circulating red cells by exchange as well as by synthesis of new cells. However, it has been suggested that the erythrocyte can maintain its lipid balance even when the plasma levels are altered as long as the enzyme system controlling exchange between the cell and its environment is functioning normally (Nelson, 1972).

The possibility of an error in the identification of a fatty acid or of missing an abnormal acid which elutes with a normal acid peak in a gas chromatographic analysis should

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always be considered. Therefore, in this study, the membrane fatty acids listed in Table 3 from one Friedreich's ataxia patient were determined by mass spectrometry. Branched chain fatty acids, such as phytanic acid, give characteristic fragmentation patterns in their spectra and, thus, it should be possible to identify such compounds even in the presence of normal straight-chain acids. However, no abnormal acids were found in the patient examined and therefore the presence of large amounts of abnormal acids in erythrocyte membranes of Friedreich's ataxia patients seems unlikely. There remains the slight possibility that a subtle difference in acid structure (for example, a difference in the ratio of cis-trans isomers) not readily detectable by mass spectrometry may occur in Friedreich's ataxia.

In our fatty acid determinations, we used an internal standard so that the total fatty acid concentration as well as their distribution could be measured. Phytanic acid was chosen as the standard since it elutes in a peak free area of the chromatogram, and it was found not to be present in significant amounts in erythrocyte ghosts of Friedreich's ataxia or control subjects.

The results of our study of erythrocyte membrane in Friedreich's ataxia suggest a normal concentration of the major components, proteins, phospholipids and cholesterol. Similarly, the total fatty acid concentration and distribution is normal; however, an increased percentage of PI + PS and a decrease of PE in erythrocytes of Friedreich's ataxia patients was found. Although no gross abnormalities in lipid composition were uncovered, the results along with the abnormalities observed by E.S.R. suggest that further work on membrane lipids be done, particularly the study of the fatty acid composition of individual phospholipids.

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Quebec Cooperative Study of Friedreich's Ataxia

Evidence for an Altered Physical State of Membrane Proteins in Erythrocytes in Friedreich's Ataxia

D. A. BUTTERFIELD, P. K. LEUNG, W. R. MARKESBERY AND A. BARBEAU

SUMMARY: *Electron spin resonance, scanning electron microscopic, and SDS-polyacrylamide gel electrophoretic studies of erythrocytes in Friedreich's ataxia have been performed. No alteration in the physical state of membrane lipids, in morphology, or in the staining profile of erythrocytes in Friedreich's ataxia could be demonstrated. An altered conformation and/or organization of proteins in erythrocyte membranes in this disorder was suggested by spin labeling studies ($P<0.025$), favoring the possibility of a generalized membrane abnormality in Friedreich's ataxia. These findings are discussed in relation to other inherited neurological diseases where similar studies have been performed.*

RÉSUMÉ: *Nous avons étudié des érythrocytes provenant de patients avec ataxie de Friedreich grâce à des techniques de résonance paramagnétique électronique, de microscopie à balayage électronique et d'électrophorèse sur gel de polyacrylamide SDS. Nous n'avons pas pu démontrer des modifications dans la morphologie, dans le profil de coloration ou dans l'état physique des lipides membranaires des ataxiques. Cependant une possibilité ($p<0.025$) de conformation ou d'organisation altérée des protéines de ces mêmes membranes est suggérée par les études de résonance paramagnétique électronique. Ceci soulève la possibilité d'une anomalie membranaire généralisée dans l'ataxie de Friedreich. Ces résultats sont comparés à ceux obtenus avec des méthodes semblables dans plusieurs autres maladies neurologiques héréditaires.*

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INTRODUCTION

Friedreich's ataxia (FA) inherited in an autosomal recessive manner is characterized clinically by ataxia of gait, dysarthria, muscle weakness, impaired proprioception and/or vibratory sense, and areflexia. In addition, scoliosis, pes cavus, cardiomyopathy, and an extensor plantar response are observed as the disease progresses. Other accessory symptoms and signs are observed in some FA patients (Barbeau, 1976). The pathological changes in FA include degeneration of posterior nerve roots and ganglia, posterior columns, Clarke's columns, and the spinocerebellar and pyramidal tracts (Barbeau, 1976).

Huang and co-workers (1978) found major differences in the high density lipoprotein (HDL) fraction of plasma lipoprotein in FA. In FA patients the relative proportion of cholesterol and triglycerides was increased, while the relative protein content was decreased. The cholesterol content of membranes can be exchanged with the plasma lipoproteins. Consequently, an abnormality in HDL in FA could result in a modified structure or fluidity of erythrocyte membranes. Moreover, taurine was found to have increased renal clearance rates in FA, suggesting a specific membrane transport defect (Lemieux et al, 1976). Taurine in the heart has been shown to increase calcium ion retention, the extra Ca^{+2} is known to have rigidizing effects on membrane lipids (Ohnishi and Ito, 1974). These possibilities of altered conformation and/or organization of proteins or fluidity of lipids of red cell membranes led to our investigation of erythrocyte membranes in FA by electron spin resonance, scanning

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electron microscopy, and SDS × polyacrylamide gel electrophoresis.

MATERIAL AND METHODS

The spin labels employed in the current studies, 2,2,6,6-tetramethyl-piperidin-1-oxyl-4 maleimide (MAL-6), and 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolindinylolyl (5-nitroxide stearate of 5-NS) were obtained from Syva. All other chemicals and reagents were of the highest purity commercially obtainable.

The studies were carried out on twelve patients with Friedreich's ataxia as defined by the criteria of the Quebec Co-operative Study of Freidreich's Ataxia (Barbeau, 1976) and on an equivalent number of age — and sex matched normal controls. All but one patient were ambulatory and only three were on medication. One subject was given 1200 mg lecithin, six times per day, another was given Beminal® (500 mg), 3 times per day, and the third was given diazepam, 5 mg per day. Blood was obtained in heparinized tubes by venipuncture and erythrocyte membranes (ghosts) were prepared as previously described (Butterfield, 1977b).

Electron spin resonance (ESR) spectra were recorded on a Varian E-109 electron spin resonance spectrometer employing an E-238 quartz aqueous sample cell. Modulation and power broadening of the ESR spectral lines were avoided by use of low microwave powers (16 milliwatts) incident on the E-238 rectangular cavity and by employing a modulation amplitude of 0.2 G. Membrane ghosts were spin labeled with MAL-6 or 5-NS as previously described (Butterfield, 1977a).

Control and FA unheparinized blood were drawn from seven FA patients and six controls by venipuncture through a short catheter infusion set and were processed for scanning electron microscopy (SEM) after the method of Miller et al (1976). Two or three drops of blood were dripped directly from the infusion set into 1% glutaraldehyde in phosphate buffer, pH 7.4. The unmanipulated erythrocytes were gently inverted and allowed to settle for 2 hours. One drop of cells was then pipetted to collagen-coated coverslips in a moist petri dish. Cells were then dehydrated through ascending ethanol solutions, processed through critical point drying in liquid $C0_2$, and coated with gold palladium. Samples were studied in a Cambridge Steroscan MKII-A scanning electron microscope at 20kV. Between 200 and 600 cells were counted for each subject.

SDS — polyacrylamide gel electrophoresis (Fairbanks et al, 1971) and estimation of membrane protein content (Lowry et al, 1951) were performed as previously described.

RESULTS

No alteration in the resulting protein profile of FA erythrocyte membranes, which had been subjected to SDS — polyacrylamide gel electrophoresis, compared to that of corresponding control volunteers could be demonstrated. Also, no altered morphology of FA cells from the seven subjects studied compared to control samples was observed by SEM.

The two spin labels used in these studies report on different environments within the erythrocyte membrane (Butterfield, 1977a,b). 5-NS is thought to orient in the lipid bilayer part of the membrane with its long alkyl chain on the average parallel to the alkyl chains of the membrane lipids, and its polar head group near the polar head groups of the lipid molecules (Hubbell and McConnell, 1971). A typical spectrum of 5-NS in erythrocyte membranes is shown in Figure 1. Several authors have discussed the interpretation of spectra like that in Figure 1 and the use of labels like 5-NS to deduce information about membrane fluidity via the order parameter S (Berliner, 1976; McConnell and McFarland, 1970). The larger the value of S, the more rigid is the local microenvironment reported by the paramagnetic center of the spin label (Berliner, 1976; McConnell and McFarland, 1970) which, in the case of 5-NS, is about 5 carbon atoms into the lipid bilayer from the membrane surface.

In contrast to noncovalently bound lipid-specific spin labels like 5-NS, MAL-6 is covalently bound to membrane protein sulfhydryl (SH) groups, although a small amount of amino group binding may also occur (Chapman et al, 1969). The low field

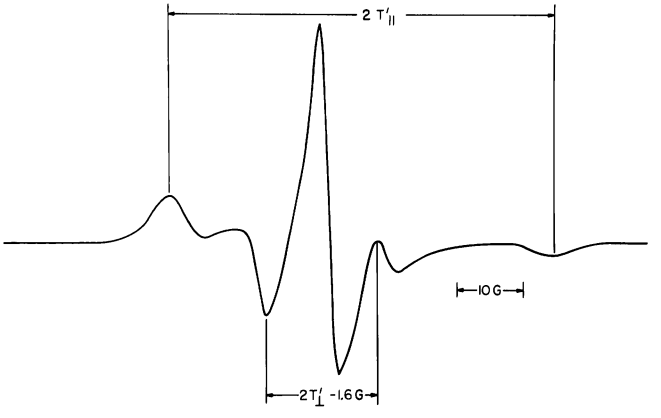

Figure 1 — Typical ESR spectrum of 5-NS in normal erythrocytes. The T-tensor parameters are indicated.

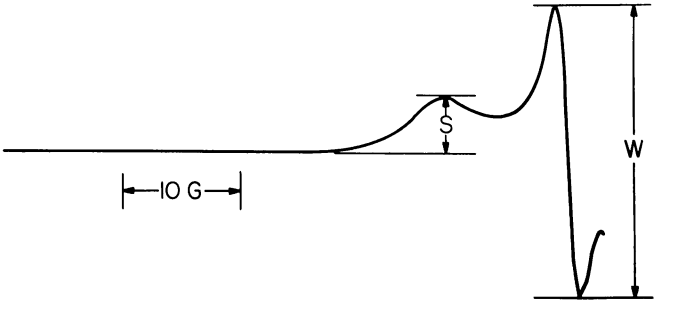

Figure 2 — Typical ESR spectrum of human erythrocyte membranes labeled with MAL-6. Only the $M_I = +1$ weakly and strongly immobilized lines (of amplitudes W and S, respectively) are presented.

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($M_I = +1$) lines of a typical spectrum of erythrocyte ghosts labeled with MAL-6 are shown in Figure 2. Considerable discussion of the interpretation and use of MAL-6 in the study of erythrocyte membrane proteins in several disease states has been presented in previous papers from our laboratory (Butterfield, 1977a,b; Butterfield et al, 1977). The ratio of the ESR spectral amplitude of MAL-6 attached to weakly-immobilized SH groups (W) to that of MAL-6 attached to strongly immobilized SH group (S) is a sensitive and convenient monitor of conformational and/or organizational differences of membrane proteins in erythrocytes (Butterfield 1977a,b; Butterfield et al, 1977).

The W/S ratio of MAL-6 attached to membrane proteins is increased in FA erythrocytes compared to normal controls ($P<0.025$, Table 1), suggesting that there is an alteration in the physical state of membrane proteins in FA. The order parameter of 5-NS is unchanged in FA erythrocytes compared to control ($P<0.25$, Table 1). The half-width at half maximum amplitude of the low-field ESR line of 5-NS has been suggested as being a more sensitive monitor of membrane fluidity than the order parameter (Mason et al, 1977). Use of this approach resulted in a trend indicative of a more rigid lipid environment in FA erythrocytes but the level of significance was borderline ($0.05<P<0.1$) (data not shown)

DISCUSSION

No definitive change in lipid fluidity as reported by spin labeling methods or in the SDS — polyacrylamide gel electrophoretic staining profile of erythrocyte membranes from patients with Friedreich's ataxia was observed in these studies. In addition, no alterations in surface morphology were found in the FA samples examined by SEM. However, altered ESR parameters of a protein specific spin probe, suggesting changes in the physical state of membrane proteins in FA were observed ($P<0.025$, Table 1). The absolute difference of the mean values of the W/S ratio of MAL-6 in FA and control ghost membranes is not large. There could be a small change in a protein present in large amounts in the erythrocyte membrane in FA, or there could be a large change in a protein which constitutes only a small fraction of the total ghost protein. Other interpretations are possible.

It is known that at least two kinds of phospholipids exist in membranes: those that are free to move in the bilayer and those that are tightly attached to integral membrane proteins (so called boundary-lipids) (Jost et al, 1977). A small change in boundary-lipids (perhaps as a result of alterations in HDL) may lead to an altered conformation and/or organization of proteins in the red cell membrane as suggested in this study. The absence of fluidity differences in FA erythrocytes is not inconsistent with such a possible biochemical defect. The resulting spectrum is a time average of all spin label molecules in the membrane. Many more 5-NS molecules will likely be located in the free lipid portion of the bilayer since it constitutes the larger fraction of lipids in the membrane; hence, a small change in boundary lipid might not be detected in these experiments. Also, a slight increase in the amount of bound Ca^{+2} might lead to the results of the current study. This is a possibility since even a small change in Ca^{+2} concentration in the buffer causes a large change in the W/S ratio of MAL-6 attached to proteins in erythrocyte ghosts (D.A.B. unpublished results).

The absence of changes in both lipid fluidity and surface morphology in erythrocytes in FA is in contrast to such alterations observed in other inherited neurological diseases. Electron spin resonance studies of erythrocytes have shown an increased membrane fluidity in myotonic muscular dystrophy (Butterfield, 1977a), an increased membrane rigidity in Duchenne muscular dystrophy (Wilkerson et al 1978), and no change in Huntington's disease (Butterfield et al, 1979). A nonspecific increased number of stomatocytes has been observed in SEM studies of erythrocytes in myotonic and Duchenne muscular dystrophy (Miller et al, 1976) and Huntington's disease (Markesbery and Butterfield, 1977).

Differences in the ESR parameters of MAL-6 have been observed in all these diseases (Butterfield, 1977a,b; Butterfield et al, 1977). However, different alterations in different proteins in each of these diseases could give rise to similarly altered spectra. Biochemical evidence supports the concept of different primary defects in these diseases. No alteration in the activity of membrane-bound protein kinase was observed in Huntington's disease erythrocytes (Butterfield et al, 1978a) while that of myotonic dystrophy was reduced (Roses and Appel, 1975) and that of Duchenne muscular dystrophy was increased (Roses et al, 1975). $Na^+ + K^+$-ATPase activity is increased in Huntington's disease erythrocytes (Butterfield et al,

TABLE 1

Electron Spin Resonance Parameters of MAL-6 and 5-NS in Erythrocyte Membranes from Friedreich's Ataxia Patients and Normal Controls+

MAL-6		5-NS	
$^{(W/S)}$ Control	$^{(W/S)}$ FA	$^{**(S)}$ Control	$^{(S)}$ FA
4.89±0.07	5.14±0.07	0.682±0.004	0.690±0.003
P***<0.025		P<0.25	
N 12	12	7	7

* Means±S.E.M. are presented.

** S- value is calculated by: $S = \frac{T'_{11} - T'_{\perp}}{(T_{11} - T_{\perp})_{XL}} \cdot \frac{TrT}{TrT'}$

where primed values are obtained experimentally (Figure 1) and unprimed crystal values are obtained from Jost et al (1971). TrT is the trace of the nitrogen nuclear hyperfine tensor and equal to $T_{11} + 2T_{\perp}$.

*** P - value calculated by a two-way analysis of variance (Brownlee, 1960).

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1978), while in myotonic muscular dystrophy it is normal (Roses and Appel, 1975), and in Duchenne muscular dystrophy it is abnormally stimulated by oubain (Brown et al, 1967). The biophysical and biochemical studies and the current results suggest that the basic molecular defects in each of these disorders and Friedreich's ataxia are different.

In summary, our findings in the present experiments suggest an alteration in the conformation and/or organization of membrane proteins in erythrocytes in Friedreich's ataxia and support the concept that this disorder may be associated with a generalized membrane abnormality.

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Quebec Cooperative Study
of Friedreich's Ataxia

Hemagglutination by Lectins in Friedreich's Ataxia

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SUMMARY: *Coded erythrocyte samples from ten individuals with Friedreich's ataxia, from parents of five of these individuals, and from five unrelated control individuals were subjected to lectin agglutination tests at three temperatures; before and after trypsinization; and before and after treatment with echinocyte-producing sodium salicylate and stomatocyte-producing tetracaine followed by shape-fixation with glutaraldehyde. The agglutinins tested were the polycationic poly-L-lysine (PLL) and four lectins with different saccharide specificities: soybean agglutinin, wheat germ agglutinin, Ulex europeus agglutinin (UEA) and concanavalin A. Altogether, over 45,000 individual test wells were scored, the status of each blood donor with respect to diagnosis being disclosed to the experimenters only after all results were tabulated.*

The majority of these tests revealed no significant difference among the three groups of blood samples. A few tests did reveal statistically valid ($p<0.01$) differences between groups, the most significant of which were the following: Trypsinized control RBC were more sensitive, on average, to agglutination by UEA (fucose-inhibited) than were RBC of ataxics or their parents. Non-trypsinized control "stomatocytes" were less sensitive, on average, to agglutination by PLL than were those of ataxics or their parents. Trypsinization appeared, on average, to sensitive control but not ataxia or parent RBC to PLL-agglutination. Other differences of borderline ($p=0.01$-0.025) or near borderline ($p=0.025$-0.05) significance were also noted. None of the statistically significant, Friedreich's ataxia-related differences in median agglutination titers were large, the greatest being about three-fold, and in every case the ranges of individual titers within the differing groups overlapped. Thus, none of these tests at present offers a method of preclinical diagnosis or carrier detection, and only further tests can establish whether even the differences observed in the present series of tests are reproducible.

RÉSUMÉ: *Des échantillons codés provenant de 10 individus souffrant d'ataxie de Friedreich, de 5 parents hétérozygotes obligatoires et de 5 sujets contrôles non apparentés ont subi des tests d'agglutination des lectines à trois températures; avant et après trypsinisation; avant et après des traitements avec le salicylate de sodium, qui produit des echinocytes, et la tetracaine, qui produit des stomatocytes, le tout suivi d'une fixation au glutaraldehyde. Les agglutinines employées furent l'agent polycationique poly-L-lysine (PLL) et quatre lectines spécifiques pour différents saccharides: l'agglutinine de fève Soya, l'agglutinine de germe de blé, l'agglutinine Ulex europeus (UEA) et la concanavalin A. Au total plus de 45,000 tests individuels furent évalués, le tout avant de connaître le diagnostic.*

La majorité de ces tests ne révèla aucune différence significative entre les trois groupes d'échantillons sanguins. Quelques tests s'avérèrent significatifs ($p<0.01$) dont: 1) Les globules rouges (GR) trypsinisés contrôles étaient plus sensibles, en moyenne, à l'agglutination par UEA (fucose inhibé) que ne l'étaient les GR des ataxiques ou de leurs parents. 2) Les "stomatocytes" contrôles étaient plus sensibles, en moyenne, à l'agglutination par UEA (fucose inhibé) que ne l'étaient les GR des ataxiques ou de leurs parents. 2) Les "stomatocytes" contrôles non-trypsinisés étaient moins sensibles, en moyenne, à l'agglutination par PLL que les ataxiques et leurs parents. La trypsinisation semble sensibiliser les contrôles mais non les ataxiques ou leurs parents à l'agglutination des GR au PLL. D'autres différences s'avéraient limitropes ($p=0.01$-0.025) on quasi-limitropes ($p=0.025$-0.05) du point de vue signification. Aucune des différences significatives reliées à l'ataxie de Friedreich en ce qui concerne les taux d'agglutination ne s'avère de grande amplitude, la plus grande étant un facteur de trois; dans tous les cas les taux individuels des groupes chevauchaient. Aucun des tests exécutés ne peut servir pour le diagnostic préclinique ou à la détection des porteurs.

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INTRODUCTION

The goal of this research has been to answer the question: Can Lectins discriminate between erythrocytes of affected individuals and carriers from those of non-carriers of the ataxia gene? The rationale is as follows: Lectins are naturally occurring agglutinins that act by bridging between glycoproteins and glycolipids on cell surfaces. Lectins from different plant species "recognize" different sugar linkages. Therefore differences in the spectrum of responses of two cell populations to a panel of lectins can be used to reveal differences in the carbohydrate moieties of their surfaces. But, lectin agglutination is a more versatile tool than this. It is also sensitive to mutual charge repulsion between cells (Schnebli et al., 1976) and possibly to cell surface topogra-

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phy (e.g. the smoothness or irregularity of the plasma membrane) (Willingham and Pastan, 1975; Ukena and Karnovsky, 1977). Being sensitive to the distribution (dispersed vs. clumped) of binding sites as well as to their number and specificities (Nicolson, 1974), it can also reflect differences in the lipid components of cell membranes in which the glycoproteins and lipoproteins "float". For example, changes in the saturation or chain length of the membrane's fatty acids or in its cholesterol content can alter its viscosity-temperature relationship and thereby the temperature at which lectin binding sites are no longer free to be clumped by the addition of lectin (Nicolson, 1974), sensitizing the cells to agglutination by the lectin. Similarly, lectin binding molecules that are anchored in place should respond to lectins differently than those that are free to diffuse in the membrane.

In general, antigens of human erythrocyte membranes have not been observed to form patches or caps in response to antibodies or lectins. Presumably, this is because all of the major integral proteins are linked, directly or indirectly, to a spectrin substructure on the cytoplasmic side of the membrane (Shotton et al., 1978). Peters et al. (1974) have presented evidence that even some lipid components of the membrane diffuse slowly if at all. Yet prostaglandin can induce changes in the fluidity of RBC membrane lipids (Kury et al., 1974; Kury and McConnell, 1975; Meyers and Swislocki, 1974), suggesting that properties detectable by lectin agglutination tests may be subject to regulation. Moreover, Gordon and Marquardt (1975) have demonstrated that RBC crenation (echinocyte-formation) in the presence of anti-A antibody causes clustering of the antigen at the tips of the spicules and suggest that "crenation ... either frees membrane components from ... restriction of movement or ... mobilizes the cytoskeletal elements so that they direct the movement of the anti-A receptors to the apices." Marikovsky et al. (1976) have reported that both fresh and glutaraldehyde-fixed echinocytes are more sensitive to agglutination by both soybean agglutinin and poly-L-lysine, and that reversion of unfixed cells to disc form is accompanied by a return of agglutinability to the previous, lower level. In addition, echinocytes showed clustering of cationized ferritin binding sites, interpreted as due to a rearrangement of negative charge (probably sialic acid) bearing molecules on the cell surface. Finally, Fowler and Branton (1977) have shown that human RBC integral proteins are laterally mobile, and that this mobility is strongly temperature dependent, in the membranes of erythrocytes that have been fused (and hemolysed) by Sendai virus or polyethylene glycol.

Is there any empirical basis for supposing that erythrocytes in Friedreich's ataxia might differ in a property that could be reflected in lectin agglutination tests? Huang et al. (1978) have reported an abnormal composition of high density lipoproteins (HDL) in Friedreich's ataxia. The lipoproteins are involved in the transport of lipids to and from cell membranes (Goldstein and Brown, 1977). Hui and Harmony (1978) have reported that low density lipoprotein (LDL) induces RBC to form spherocytes *in vitro* while HDL inhibits this shape change. Abnormalities of plasma membrane lipid composition in Friedreich's ataxia are thus one possibility.

The sensitivity of human RBC to agglutination by one or another lectin can be altered (1) by protease treatment (Nicolson, 1974); (2) by exposure to amphipathic drugs (Weltzien, 1975) believed to intercalate preferentially into the external (echinocytogens) or internal (stomatocytogens) half of the plasma membrane bilayer (Sheetz and Singer, 1974); and (3) by conducting the agglutination at higher or lower temperatures (Vlodavsky et al, 1972; Shnebli and Bächi, 1975; Gordon and Marquardt, 1974). We have therefore tested combinations of the above variables in lectin agglutination tests on the RBC of individuals diagnosed as having Friedreich's ataxia, their parents (obligatory heterozygotes), and unrelated control individuals.

MATERIALS AND METHODS

a) Case material, blood collection, shipment and storage

Blood samples were collected at the Clinical Research Institute of Montreal from ten patients (3 male, 7 female) with Friedreich's ataxia, from five parents (2 male, 3 female) of these patients, and from five (1 male, 4 female) unrelated control individuals. The blood was drawn on two successive days into code-designated EDTA-Vacutainer tubes and refrigerated, then immediately shipped by overnight express, under refrigeration, to Princeton University, where the tests reported here were conducted. Each sample was immediately typed using Ortho blood grouping serum and a 1 ml aliquot was removed and counted for the proportions of discocytes, echinocytes, stomatocytes and spherocytes (see below). The samples were then washed by centrifugation 3X into sterile human Ringer's acid citrate-dextrose-adenine solution (ACD-A), in which the cells wee resuspended in capped, sterile Corning polystyrene centrifuge tubes. The cell counts were repeated on the 5th day after collection (Fig. 1). Cell suspensions were stored between 0° and 4° C and were handled in plasticware only, at no time being exposed to glass.

b) Trypsinization

Between one and five days after collection, a 1 ml sample of each erythrocyte suspension was washed 3X in chilled phosphate-buffered saline (PBS) and divided into two portions. The cells from one portion ("unfixed; non-trypsinized") were used for agglutination tests immediately after determinatin of the proportions of discocytes, echinocytes, stomatocytes, and spherocytes. Those from the other portion were resuspended to a final concentration of 4% RBC in 0.1% crude trypsin (Difco, 1:250) in PBS and incubated at 37° C for 45 minutes. The cells were then pelleted, resuspended in two volumes of chilled 0.1% ovalbumin trypsin inhibitor (Sigma type II-0) in PBS, and washed 3X in PBS. After determination of the proportions of RBC with the various shapes listed above, the

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cells were used in agglutination tests ("unfixed; trypsinized"). The average distribution of RBC shapes at 1-2 days and at 5 days (data for all samples pooled) is shown in figure 1.

c) RBC shape-altering treatments and fixation

Between two and seven days after collection, trypsinized and non-trypsinized portions of each cell suspension were prepared and counted as described above. Echinocyte formation, monitored by phase contrast microscopy, was induced by the addition of 50-300 μl of 1.5 M sodium salicylate (Deuticke, 1968) to 9 ml of a 1% suspension of RBC in PBS contained in the hydrophilic side (itself echinocytogenic) of a #3024 Falcon plastic tissue culture flask. Stomatocyte formation, similarly monitored, was induced by the addition of 50-200 μl of 0.1 M tetracaine (Deuticke, 1968) to 9 ml of a 1% suspension of RBC in PBS contained in the hydrophobic side (non-echinocytogenic) of a similar flask. These salicylate and tetracaine concentrations were found in preliminary tests with fresh RBC to be adequate to induce these shape changes. The proportions of stomatocytes, discocytes, and echinocytes in these and in untreated RBC suspensions (the latter examined in the hydrophobic side of a plastic culture flask) were monitored and the cells were fixed (to stabilize their shapes) by addition of 3 ml of 18% glutaraldehyde solution (Ladd Research Industries; originally 70%) in PBS and counted (Figure 2). After 15 min, the contents (9 ml) of each flask were transferred to a screw-capped, 15 ml centrifuge tube to which 3 ml of 0.15 M glycine (pH7.4) was added. Ten minutes later, each RBC suspension was pelleted and resuspended in 12 ml of the same glycine buffer (to combine with unreacted aldehyde) for 15 minutes.

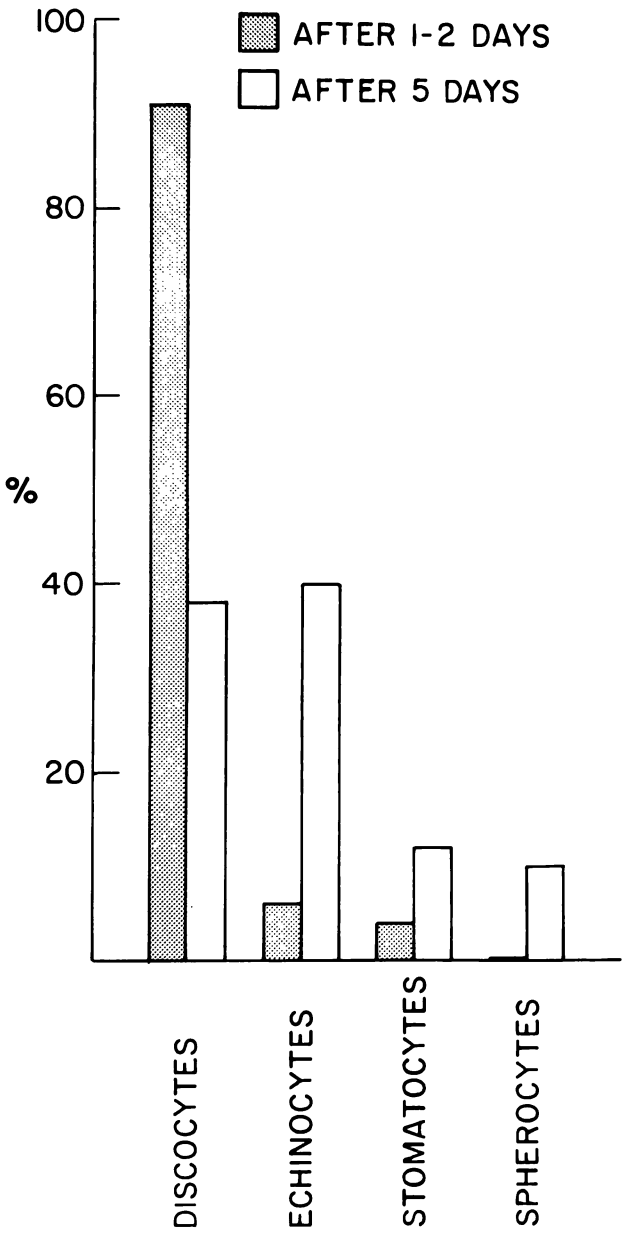

Figure 1 — Distribution of observed cell shapes in unfixed blood samples. Counts of trypsinized and non-trypsinized samples, being similar, were pooled.

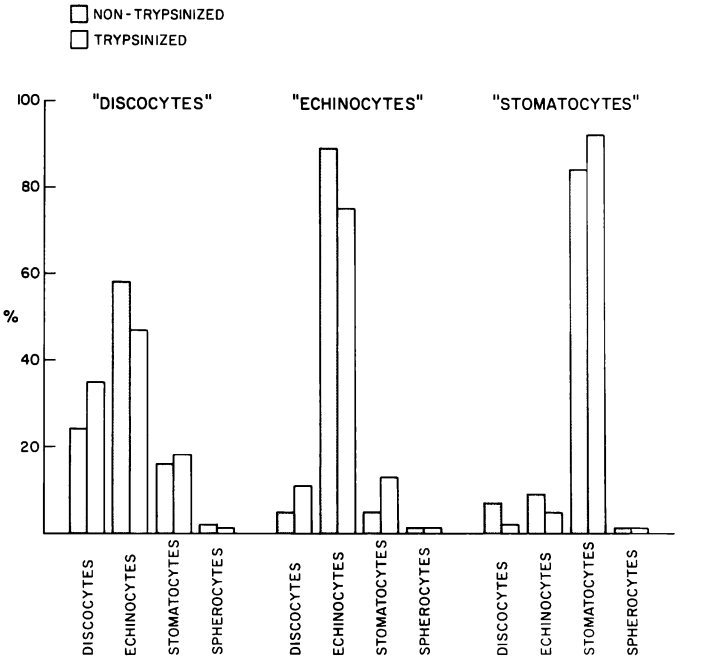

Figure 2 — Distribution of observed cell shapes in glutaraldehyde-fixed preparations of normal discocytes, echinocytes and stomatocytes.

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The fixed erythrocytes were then washed 3X in PBS and stored at 4° C. After several days, all RBC preparations were washed 2X in 10 ml of 0.1% sodium azide in PBS, in which they were kept at 4° C until the agglutination tests were to be performed, when they were again washed and resuspended in PBS.

d) Lectins

Lectins reactive with a variety of glycoside linkages common to the surfaces of RBC of all human ABO blood types were selected. They are characterized below in accordance with their major sugar specificities (Lis and Sharon, 1973).

Soybean agglutinin (SBA) — N-acetyl-D-galactosamine, D-galactose.

Wheat germ agglutinin (WGA) — N-acetyl-D-glucosamine.

Ulex europeus lectin (UEA) — L-fucose.

Concanavalin A (Con A) — D-mannose.

Poly-L-lysine (PLL) — Not a true lectin, this polycationic substance presumably agglutinates cells by electrostatic interaction and bridging with surface anionic groups (Katchalsky et al., 1959), principally sialic acid carboxyls.

WGA was kept as a stock solution of 120 μg/ml in PBS. All other agglutinins were kept as 500 μ/ml stock solutions.

e) Solutions

Acid-citrate-dextrose (USP-A) plus adenine ("ACD-A") (Spielmann and Seidl, 1973)

0.10% citric acid
0.29% sodium citrate (hydr.)
0.32% dextrose (hydr.)
0.5 mM adenine

f) Agglutination tests

0.05 ml of PBS was delivered with a Cooke Microtiter Micropipette (Cooke Laboratory Products, Alexandria, VA 22314) to each well of a Cooke Microtiter V-bottom plate which had earlier been dipped in 0.02% human serum albumin (HSA), rinsed in distilled water, and air dried. This treatment diminished the tendency of certain RBC preparations to stick to the walls of the depressions and thereby to generate false positive agglutination tests (absence of a "button" at the bottom of the V-well). 0.05 ml of a stock lectin solution was added to the first well in each row and a series of 12 two-fold dilutions (table 1) was produced with a Cooke 0.05 ml Micro-diluter. After addition of another 0.05 ml of PBS, each well received 0.05 ml of a 1% suspension of erythrocytes in PBS and the plates were lightly tapped by hand to facilitate mixing. In the case of unfixed cells, the plates were then incubated, immobile, at 4°, 24° or 37° C for two hours, and scored by recording the wells showing agglutination. Agglutination was defined as lack of a distinct "button" of red cells at the base of the well. The distribution of echinocytes, discocytes, and spherocytes among both the trypsinized and the non-trypsinized erythrocytes at the time of plating was determined by phase contrast microscopic examination in hydrophobic Falcon plastic Petri dishes with or without previous treatment of the plastic with HSA followed by rinsing. (Hydrophilic Falcon "tissue culture" plastic, like glass, induced immediate echinocyte formation.)

The above procedure was unsatisfactory for use with fixed cells because they (especially echinocytes) adhered to the plastic, giving false positive scores for agglutination. HSA-pretreatment of the plastic reduced but did not eliminate this tendency. It was largely overcome by conducting these assays on a gyrating device which repeatedly tapped each dish on all four sides successively, dislodging weakly attached cells. The intensity of tapping was regulated so that cells slid down to form a "button" in the absence of any agglutinin but not in the presence of a high concentration of agglutinin. Absolute titers recorded by this procedure are therefore not comparable with the titers obtained by the stationary assay, but relative titers have the same validity. Using this procedure, there were occasional plates in which every well scored positive and others in which every well scored negative. There being no way to determine whether or not this was due to differences in the plates themselves, data from such plates were eliminated from the analyses. Each test was run in duplicate. Altogether, over 45,000 idividual test wells were scored. The diagnostic status of the coded samples was not disclosed to the investigators until after all results had been tabulated.

g) Statistical evaluation

Because of the characteristics of Microtiter test data (a series of discrete values with an upper limit), the data were analyzed by the median test (Siegel, 1956). The null hypothesis in this analysis states that the two groups are from populations having the same

TABLE 1

Lectin Agglutination Titers

titer	Agglutinin concentration (μg/ml)	
	SBA UEA Con A PLL	WGA
1	167	33.3
2	83.5	16.7
3	41.8	8.35
4	20.9	4.18
5	10.4	2.09
6	5.2	1.04
7	2.6	0.520
8	1.3	0.260
9	0.65	0.130
10	0.32	0.065
11	0.16	0.032
12	0.08	0.016

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median. When the total number of cases, N, exceeded 40, the chi square test corrected for continuity was applied. When $20<N<40$ the Fisher test (Fisher, 1934) was used if the smallest "expected frequency" was less than 5. Otherwise, these data were also analyzed by the chi square test. Finally, in all cases where $N<20$, the Fisher test was used. We have regarded the difference between two groups as significant only when the probability (p) that their medians are the same or less than 0.01. The range $p=0.01$-0.025 has been regarded as representing differences of borderline significance.

RESULTS AND DISCUSSION

a) Effects of trypsinization

1) *Unfixed cells:* Trypsinization lowered the minimal concentration ("titer") at which four of the five tested agents agglutinated erythrocytes (table 2). The degree of this sensitization was different from lectin to lectin: about 30-40 fold for SBA, 8-16 fold for UEA, 8-fold for WGA; and from 2- to 8-fold for Con A, depending upon the temperature. The polycationic PLL, the only non-lectin among these agglutinins, showed no significant sensitization at any temperature in this comparison between data for all blood samples pooled (but see table 10).

2) *Glutaraldehyde-fixed cells:* In general, results with fixed cells were similar to those with unfixed cells, although the degree of trypsin sensitization was less: an average of about 8-fold for SBA, 4-5 fold for UEA, 2-3 fold for WGA, and 2-fold for PLL.

b) Effects of temperature

In these tests with unfixed cells, there was no temperature-dependence of agglutination titer of either trypsinized or non-trypsinized cells with SBA, WGA, UEA or PLL. This is in agreement with observations of Schnebli and Bächi (1975) with WGA and of Vlodovsky et al. (1972) and Gordon and Marquardt (1974) with SBA. Agglutination of non-trypsinized erythrocytes by Con A was temperature dependent, agglutination occuring at lower titers at 4° than at 24° or 37° (table 2). This has been observed earlier by Schnebli and Bächi (1975) for Con A agglutination of non-trypsinized (but not of trypsinized) human RBC in stationary test plates.

c) Effects of glutaraldehyde fixation

Because of differences necessitated in the procedures for testing agglutination of fixed versus unfixed cells (see Materials and Methods), valid comparisons of absolute agglutination titers cannot be made between the two groups. In the great majority of tests, fixed cell suspensions were not agglutinated by Con A at any concentration (see tables 3 and 6). Reduction or elimination of Con A-agglutinability by aldehyde fixation has been described by Noonan and Burger (1973) and Inbar et al. (1973) for Py3T3 and lymphoma cells, respectively. This has been suggested previously to result from the immobilization of lectin receptors, since Con A binding was similar after fixation (Noonan and Burger, 1973; Inbar et al., 1973), but the clustering of binding sites was abolished (Inbar et. al., 1973). Inbar et al. also reported

TABLE 2

Effect of Temperature on Agglutination Titers

Unfixed RBC: data for all genotypes pooled

lectin		non-trypsinized 4°	24°	37°	signif diff.	trypsinized 4°	24°	37°	signif diff.
SBA	titer	6	7	7		11-12	11	12	–
	(n)	(46)	(46)	(45)		(46)	(43)	(45)	
WGA	titer	3	3	3		6	6	6	–
	(n)	(46)	(46)	(46)		(46)	(46)	(45)	
UEA	titer	5	5	4		9	8	8	–
	(n)	(46)	(45)	(46)		(45)	(45)	(46)	
Con A	titer	4	2-3	2	+	5	5	5	
	(n)	(46)	(46)	(46)		(45)	(46)	(46)	
PLL	titer	7	6	7		7	7	7	–
	(n)	(46)	(46)	(46)		(46)	(45)	(46)	

	non-trypsinized 4°	24°	37°	p		trypsinized		p	
	4	2-3		$\ll 0.005$	+	5	5	0.1-0.5	–
	(46)	(46)				(46)	(46)		
Con A		2-3	2	0.05-0.1			5	5	<0.9
		(46)	(46)				(46)	(46)	
	4		2	$\ll 0.005$	+	5		5	0.5-0.9 –
	(46)		(46)			(46)		(46)	

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TABLE 3

Effect of Shape-Altering Treatments on Agglutination Titers

Glutaraldehyde-fixed RBC: data for all genotypes pooled

lectin		non-trypsinized				signif diff.	trypsinized				signif diff.
		stomatocytes	discocytes	echinocytes			stomatocytes	discocytes	echinocytes		
SBA	titer	3	4	3		–	5	6	8		+
	(n)	(39)	(18)	(27)			(40)	(36)	(32)		
WGA	titer	4-5	4	2		+	6	6	0*		–
	(n)	(32)	(10)	(22)			(17)	(21)	(9)		
UEA	titer	1	2	0		–	3	3	4		–
	(n)	(44)	(18)	(20)			(34)	(37)	(23)		
Con A	titer	0	0	0		–	0	0	0		–
	(n)	(46)	(18)	(34)			(36)	(30)	(38)		
PLL	titer	9	6	6		–	8	8-9	8-9		–
	(n)	(46)	(15)	(26)			(30)	(36)	(24)		
					p					p	
SBA		3 (39)	4 (18)		0.95-0.98	–	5 (40)	6 (36)		0.05-0.1	–
			4 (18)	3 (27)	0.1-0.2	–		6 (36)	8 (32)	⩽0.005	+
		3 (39)		3 (27)	0.8-0.9	–	5 (40)		8 (32)	⩽0.005	+
		4 (57)		3 (27)	0.02-0.05		6 (76)		8 (32)	⩽0.005	+
WGA		4-5 (32)	4 (10)		0.3-0.5		6 (17)	6 (21)		0.5-0.9	–
			4 (10)	2 (22)	0.0036	+		6 (21)	0* (9)	>0.9	–
		4-5 (32)		2 (22)	0.001-0.01	+	6 (17)		0* (9)	>0.29	–
		4 (42)		2 (22)	0.005-0.01	–	6 (38)		0* (9)	0.5-0.9	–
PLL		9 (46)	6 (15)		0.05-0.10						
			6 (15)	6 (26)	>0.95						
		9 (46)		6 (26)	>0.28	–					
		9 (46)	6 (41)		0.05-0.10						

*Five samples had a titer of 0, two samples had a titer of 8, and two samples had a titer of 10.

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that the duration of fixation required to abolish agglutination differed greatly from lectin to lectin, ranging from 45 minutes for WGA to 6 hours for SBA. This may explain the restriction of this effect to Con A-agglutination in the present experiments, in which fixation was relatively brief (15 minutes).

d) Effects of RBC shape-altering treatments

In preliminary experiments, we observed that RBC shapes often changed dramatically (echinocyte formation) as soon as the cells were deposited in the test plates. Therefore, only fixed cells were used for these tests. Because all of the blood samples,in EDTA solution, were received in a single shipment and tests with fresh cells had to be conducted first, fixation was carried out from two to seven days after the blood was drawn. Even though the cells had been washed into ACD-adenine upon receipt and stored at 0-4°C, significant crenation took place between two and five days after collection (Fig. 1), and the treatments designed to transform them into echinocytes or stomatocytes were in general only partially effective. This suggests that the capacity to phosphorylate spectrin on the inner surface of the RBC plasma membrane might have diminished significantly during this time (Birchmeier and Singer, 1977).

The effects of shape-altering treatments on agglutination titers are shown in table 3. These treatments did not affect the agglutination of fixed RBC by either UEA or PLL. Trypsinized (but not non-trypsinized) echinocytes were agglutinated at significantly lower titers by SBA. Non-trypsinized (but not trypsinized) echinocytes were agglutinated at significantly lower titers by WGA. No significant differences in these respects were found among erythrocytes of ataxics, their parents and unrelated control individuals (table 4). That the differences in agglutination titers were not brought about exclusively by shape-changes *per se* is illustrated by the comparisons in table 5. In the case of RBC from control sample U, it happened that the same proportions of stomatocytes, discocytes, and echino-

TABLE 4

Agglutination Titers after Shape-Altering Treatments, By Friedreich's Ataxia Status

Glutaraldehyde-fixed, non-trypsinized RBC									
		stomatocytes + discocytes				echinocytes			
lectin		control	parent	ataxia	signif diff.	control	parent	ataxia	signif diff.
WGA	titer	4	4	4	–	2	0	2	–
	(n)	(8)	(12)	(22)		(7)	(5)	(10)	
Glutaraldehyde-fixed, trypsinized RBC									
		stomatocytes +discocytes				echinocytes			
lectin		control	parent	ataxia	signif diff.	control	parent	ataxia	signif diff.
SBA	titer	6	6	6	–	9	8	8	
	(n)	(22)	(20)	(34)		(11)	(8)	(13)	–

TABLE 5

Agglutination Titers and Distribution of Actual RBC Shapes after Shape-Altering Treatments: One Example

Glutaraldehyde-fixed, non-trypsinized RBC					
				minimal agglutinating titers (duplicates)	
Control sample U	% stomatocytes	% discocytes	% echinocytes	soybean agglutinin	poly-L-lysine
stomatocytes	75	17	8	4;4	6;6
discocytes	1	19	80	5;6	1;1
echinocytes	1	18	80	2;2	⩾12;⩾12

TABLE 6

Agglutination Titers by Friedreich's Ataxia Status

Glutaraldehyde-fixed RBC; data for all shapes pooled									
		non-trypsinized			signif	trypsinized			signif
		control	parent	ataxia	diff.	control	parent	ataxia	diff.
SBA	titer	3	4	3		6	6	7	–
	(n)	(27)	(20)	(37)		(33)	(28)	(47)	
WGA	titer	4	4	4		7	6	6	–
	(n)	(15)	(17)	(32)		(14)	(9)	(24)	
UEA	titer	1	0	1		4*	1*	3*	–
	(n)	(24)	(20)	(38)		(27)	(25)	(42)	
Con A	titer	0	0	0		0	0	0	–
	(n)	(28)	(26)	(44)		(36)	(23)	(45)	
PLL	titer	8	9	8	–	8	9	8	–
	(n)	(28)	(23)	(36)		(27)	(20)	(43)	

*These differences proved to be non-significant ($p>0.1$) because of large ranges in all groups.

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TABLE 7

Agglutination Titers by Friedreich's Ataxia Status

Unfixed RBC; data for all temperatures pooled

		non-trypsinized control	non-trypsinized parent	non-trypsinized ataxia		signif diff.	trypsinized control	trypsinized parent	trypsinized ataxia		signif diff.
SBA	titer	7*	4*	3*	–	–	>12	11	10		–
	(n)	(48)	(29)	(59)			(45)	(30)	(59)		
WGA	titer	3	3	3			6	6	6		–
	(n)	(48)	(30)	(60)			(48)	(30)	(59)		
UEA	titer	5	4	4			9	7-8	8		+
	(n)	(48)	(29)	(60)			(46)	(30)	(60)		
Con A	titer	2	3	3		+/–	5	5	5		–
	(n)	(48)	(30)	(60)			(47)	(30)	(60)		
PLL	titer	6-7	6	7		=	8	7	7		+/–
	(n)	(48)	(30)	(60)			(48)	(30)	(59)		
					p					p	
	titer	5		4	≃0.5	–	9		8	≪0.005	+
	(n)	(48)		(60)			(46)		(60)		
	titer	5	4		0.1-0.5	–	9	7-8		≪0.005	+
	(n)	(48)	(29)				(46)	(30)			
UEA	titer		4	4	0.5-0.9	–		7-8	8	0.05-0.1	–
	(n)		(29)	(60)				(30)	(60)		
	titer	5	4		0.2-0.3	–	9	8		≪0.005	+
	(n)	(48)	(89)				(46)	(90)			
	titer	2		3	0.01-0.025	+/–	5		5	0.1-0.5	
	(n)	(48)		(60)			(47)		(60)		
	titer	2	3		0.1-0.5	–	5	5		0.1-0.5	–
	(n)	(48)	(30)				(47)	(30)			
Con A	titer		3	3	0.1-0.5			5	5	0.025-0.05	–
	(n)		(30)	(60)				(30)	(60)		
	titer	2	3		0.025-0.05						
	(n)	(48)	(90)								
	titer	6-7		7	>0.9	–	8		7	0.025-0.05	–
	(n)	(48)		(60)			(48)		(59)		
	titer	6-7	6		0.5-0.9	–	8	7		0.1-0.5	–
	(n)	(48)	(30)				(48)	(30)			
PLL	titer		6	7	>0.9	–		7	7	>0.9	–
	(n)		(30)	(60)				(30)	(59)		
	titer						8	7		0.01-0.025	+/–
	(n)						(48)	(89)			

*These differences proved to be non-significant (p>0.1) because of large ranges in all groups.

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cytes, respectively. Notwithstanding, these two preparations differed greatly in their sensitivity to agglutination by both SBA and PLL.

e) Friedreich's ataxia-related differences

In tests with fixed cells, no significant ataxia-related differences in agglutination titers were detected in statistical comparisons between groups when the data for nominal stomatocytes, discocytes, and echinocytes were pooled (table 6). Tests with fresh cells, however, did reveal certain differences. In almost every case, these differences distinguished the group of ataxia-unrelated blood samples (control) from the two groups of ataxia-related samples: from ataxics and their parents. In only one case was a possible difference found between the latter two groups, and this was of borderline significance.

1) *UEA:* The median lowest agglutinating titer for control samples of trypsinized RBC was 9 (0.6 μg/ml), while for samples from ataxics and their parents it was 8 (1.3 μg/ml). This difference, although small, was highly significant, the probability that the two groups were actually identical (p) being much less than 0.005 (table 7). Both the *ataxia* and the *parent* groups individually differed from the *control* group with equal significance, but they did not differ significantly from each other (p=0.05-0.1). None of these differences were apparent in the

TABLE 8

Agglutination Titers at Higher and Lower Temperatures, by Friedreich's Ataxia Status

		Unfixed, non-trypsinized RBC									
		4°					24° + 37°				
		control	parent	ataxia	p	signif diff.	control	parent	ataxia	p	signif diff.
•	titer	3		4	0.7-0.8	–	1		2	0.02-0.05	–
	(n)	(16)		(20)			(32)		(40)		
	titer	3	4		0.8-0.9	–	1	3		0.02-0.05	–
	(n)	(16)	(10)				(32)	(20)			
Con A	titer		4	4	>0.27	–		3	2	0.7-0.8	–
	(n)		(10)	(20)				(20)	(40)		
	titer						1	2-3		0.01-0.02	+/–
	(n)						(32)	(60)			

TABLE 9

Agglutination Titers after Shape-Altering Treatments, by Friedreich's Ataxia Status

		Glutaraldehyde-fixed RBC; poly-L-lysine-agglutination									
		non-trypsinized					trypsinized				
		control	parent	ataxia	p	signif diff.	control	parent	ataxia	p	signif diff.
	titer	8-9		5	≃0.5	–	8		9	0.1-0.5	–
	(n)	(12)		(16)			(17)		(29)		
	titer	8-9	6		0.5-0.9	–	8	9		0.1-0.5	–
	(n)	(12)	(13)				(17)	(14)			
	titer		6	5	>0.5	–		9	9	≃0.9	–
	(n)		(13)	(16)				(14)	(29)		
	titer	8-9	6		>0.3	–	8	9		>0.9	–
	(n)	(12)	(29)				(17)	(43)			
	titer	8		9	0.01-0.05	+/–	8-9		6-7	>0.3	–
	(n)	(16)		(20)			(10)		(14)		
	titer	8	9-10		<0.005	+	8-9	9-10		>0.1	–
	(n)	(16)	(10)				(10)	(6)			
	titer		9-10	9	>0.2	–		9-10	6-7	>0.2	–
	(n)		(10)	(20)				(6)	(14)		
	titer	8	9		0.005-0.01	+	8-9	7-8		≃0.9	–
	(n)	(16)	(30)				(10)	(20)			

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corresponding non-trypsinized samples.

2) *Con A:* As shown in table 7, *control,* non-trypsinized RBC gave a median titer of 2 (83.4μg/ml), while the *ataxia* and *parent* groups both gave a median titer of 3 (41.7μg/ml). None of these differences met the stringent criterion of statistical significance ($p<0.01$) adopted here. Because of greater variance in the *parent* group, only the difference between the *ataxia* and *control* groups met our criterion of borderline significance ($p=0.01$-0.025). The difference between the *ataxia* and *parent* groups (combined) and the *control* group approached borderline significance ($p=0.025$-0.05).

The above comparisons utilize data from tests at 4°, 24° and 37°, these comparisons were repeated for these groups separately. A difference of borderline significance ($p=0.01$-0.02), distinguishing the control group from the parent and ataxia groups combined, was found at the higher temperatures but not at 4° C (table 8). In these comparisons, the differences between the *parent* and *ataxia* groups individually and the *control* group approached borderline significance ($p=0.02$-0.05). No significant differences were observed in the corresponding trypsinized samples.

3) *PLL: Control* trypsinized erythrocytes agglutinated with a median titer of 8 (1.3 μg/ml) compared with 7 (2.6μ/ml) for both *ataxia* and *parent* erythrocytes. Only the comparison between the *control* group and the *ataxia* and *parent* groups combined (table 7) met our criterion for borderline significance ($p=0.01$-0.025).

Comparisons of two other kinds suggested themselves. As shown in table 3, when data for all genotypes were pooled, the median titers for non-trypsinized, fixed discocytes and echinocytes differed from that for stomatocytes, but (because of great variance) not to the level of statistical significance. These data were re-grouped according to Friedreich's ataxia status. Because median titers for the discocyte and echinocyte subgroups tested out as being the same with high probability, these subgroups were combined. A significant difference now emerged (table 9). The stomatocyte *control* group, with a median titer of 8 (1.3μg/ml), differed from the *parent* group, with a median titer of 9-10 (0.6-0.3μg/ml), with a probability $p<0.005$. The *ataxia* group, with a median titer of 9 (0.6μg/ml) differed from the *control* group with borderline significance ($p=0.01$-0.005). *Ataxia* and *parent* groups did not differ significantly, and the two groups combined differed significantly from the control group ($p=0.005$-0.01). The differences found between the non-trypsinized group combining nominal discocyte and echinocyte groups and their controls were not significant, nor were those found among any of the trypsinized groups.

TABLE 10

Effect of Trypsinization upon Poly L Lysine-Agglutination, by Friedreich's Ataxia Status

Unfixed RBC; data for all temperatures pooled				
	non-trypsinized	trypsinized	p	signif diff.
control	6-7 (48)	8 (48)	0.001-0.01	+
parent	6 (30)	7 (30)	0.7-0.8	–
ataxia	7 (60)	7 (59)	0.90-0.95	–

All of the differences described above entail comparisons between ataxia-related and control groups. The PLL data at the bottom of table 7 suggested a set of comparisons each of which utilizes erythrocytes from only a single group. Inspection of these data reveals that trypsinization appears to sensitize *control* but not *ataxia* erythrocytes to PLL agglutination. The *parent* (heterozygote) group appears intermediate. These comparisons were therefore evaluated statistically. As shown in table 10, the difference between trypsinized and non-trypsinized *control* groups is significant at the $p=0.001$-0.01 level. Trypsinized and non-trypsinized *ataxia* groups are identical with a probability (p) exceeding 0.90. The difference between the corresponding *parent* groups is not significant ($p=0.7$-0.8). If these differences prove to be reproducible, the presence or absence of trypsin sensitization to PLL agglutination might serve as an indicator of an individual's status with respect to Friedreich's ataxia.

None of the statistically significant, ataxia-related differences between median minimal agglutination titers found in these tests are large, and the ranges of individual titers within the differing groups overlapped in all cases. Thus, none of these tests, at the present time, offers a method of preclinical diagnosis or carrier detection, and only further tests can establish whether even the differences between groups are reproducible. If they are, they will indicate alterations in erythrocyte membranes in Friedreich's ataxia and may serve as a starting point for investigating these alterations.

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