Tissue enzyme levels as indices of protein status in sheep

BY E. PAYNE* AND L. LAWS

Animal Research Institute, Department of Primary Industries, Yeerongpilly, Queensland 4105, *Australia*

(*Received* 11 *April* 1977 – *Accepted* 4 *November* 1977)

1. An investigation was made to determine the value of tissue enzymes as indices of protein nutrition.

2. Sheep were given a reduced protein intake for 6 weeks, whereupon some were given a protein supplement for 1 week and then the sheep were refed their initial diet. In the first experiment sheep were taken from pasture and given a reduced protein intake for 6 weeks whereupon some were given a protein supplement for 1 week and then all the sheep were grazed on pasture. In the second experiment sheep were fed on lucerne (*Medicago sativa*) chaff *ad lib.* in place of grazing before and after the period of reduced protein intake during which one group was also fed on molasses. Again, another group was given a protein supplement for 1 week after 6 weeks on the reduced protein intake.

3. As a result of reduced protein intake there were significant ($P < 0{\cdot}01$) changes in the liver in the content of DNA, protein:DNA, arginine synthetase (combined activity of argininosuccinate synthetase (*EC* 6.3.4.5) and argininosuccinate lyase (*EC* 4.3.2.1)), aspartic aminotransferase (*EC* 2.6.1.1), glutamic dehydrogenase (*EC* 1.4.1.2), arginase (*EC* 6.5.3.1) and tyrosine aminotransferase (*EC* 2.6.1.5).

4. There were few consistent changes in muscle in the levels of DNA, protein:DNA, creatine kinase (*EC* 2.7.3.2) and aspartic aminotransferase.

5. The feeding of molasses to one group tended to reduce the change in DNA level but still resulted in significant changes in the enzymes.

6. It is suggested that the levels of arginine synthetase, aspartic aminotransferase and DNA in liver could be useful indices of protein status.

In areas where periods of undernutrition occur in the grazing ruminant due to adverse climate, it is difficult to supplement efficiently with a source of nitrogen because it is difficult to measure the protein intake of a grazing animal. Obtaining a representative sample of the pasture eaten is difficult, even under experimental conditions. An accurate, easily measured criterion in the animal is required.

Various criteria have been investigated including blood urea (Nolan, Cocimano & Leng, 1970) and faecal N (Moir, 1960) but most have had shortcomings as predictors of N intake. Masters (1963) reported that protein:DNA of liver and muscle decreased in sheep when on almost a zero-digestible protein ration. Robinson & Lambourne (1970) also reported a decrease in protein:DNA of muscle in cattle that were emaciated and weak from undernutrition. Though the differences in less severe nutritional conditions would probably not be great they made the suggestion that this index might be of use in predicting N status.

In rats changes in various enzymic activities in the liver, including enzymes of the urea cycle, as a result of varying the levels of protein intake have been reported by Soberon & Sanchez (1961), Muramatsu & Ashida (1962), Schimke (1962), Freedland & Avery (1964), Freedland (1967), Szepesi & Freedland (1968*a*, *b*) and Wirthgen & Bergner (1969). In these experiments the rations contained either very high or very low levels of protein.

In sheep, Payne & Morris (1969) found that various enzymes of the urea cycle decreased when rations containing 7·5 g N/kg were fed after an initial ration containing 57 g N/kg. These results are difficult to relate to conditions of the grazing ruminant.

There have been few reports on changes in muscle enzymes. Wainio, Allison, Kremzner, Bernstein & Aronoff (1959) reported that various oxidative enzymes decreased by 10–20%/

* Present address: Ruakura Animal Research Station, Hamilton, New Zealand.

Table 1. *The rations, sampling protocol and protein intake for Expts 1 and 2*

Expt no.	Experimental period	Group no.*	Ration	Week of Expt when biopsy sample taken: Liver	Week of Expt when biopsy sample taken: Muscle	Protein intake (g/d)
1†	Before start of Expt	1–4	Pasture	0	0	70–100§
	Week 1–6	1–4	Rh chaff	4	4	31
	Week 7	1	Rh chaff	7	—	31
		2	Rh chaff + l. chaff	—	—	103
		3	Rh chaff + safflower meal	—	—	104
		4	Rh chaff + l. chaff + safflower meal	—	—	173
	Week 8–11	1–4	Pasture	11	11	70§
2‡	−2 weeks−0 weeks	1–3	l. chaff *ad lib.*	0	0	140
	Week 1–6	1, 2	Rh chaff	4	4	24
		3	Rh chaff + molasses	4	—	24
	Week 7	1	Rh chaff	7	—	24
		2	Rh chaff + safflower meal	7	—	97
		3	Rh chaff + molasses	7	—	24
	Week 8–11	1–3	l. chaff *ad lib.*	11	11	140

Rh chaff, Rhodes grass (*Chloris gayana*) chaff; l. chaff, lucerne (*Medicago sativa*) chaff.

* In Expt 1 there were four groups of four sheep except group no. 1 which only contained one sheep during weeks 8–11; in Expt 2 there were three groups of six sheep except during weeks 8–11 when groups nos. 1 and 3 only contained three sheep due to losses from a urea tolerance test.

† Each sheep received 500 g Rh chaff/d; supplemented sheep also received 500 g l. chaff/d or 170 g safflower meal/d.

‡ Each sheep received 400 g Rh chaff/d; supplemented sheep also received 150 g molasses/d or 170 g safflower meal/d.

§ Levels are only estimates.

mg protein-N in the muscle of rats on a protein-free ration and Masters & Horgan (1962) reported a slight decrease in aspartic aminotransferase (*EC* 2.6.1.1) in the muscle of sheep on a very-low-N ration.

Most of the published results have been related to wet weight or protein content. The results of Masters (1963) suggested that total DNA was constant for a given organ or tissue of the sheep irrespective of the nutritional state. If this suggestion is valid, specific activities expressed in terms of DNA should show greater differences than any other derivations. Thus for certain enzymes a more sensitive indicator may be the ratio, enzyme activity: DNA, as during a period of N deprivation there may be a decreased synthesis of enzymes of non-essential reactions. The present paper reports the effects of feeding reduced-protein diets on some enzyme activities of liver and muscle of sheep.

EXPERIMENTAL

Design and sampling

The sheep, rations and design of the experiments were described by Payne & Laws (1976) and are described briefly in Table 1.

At the end of supplementation (week 7) all sheep received a drench of 0·4 g urea/kg live weight for the experiment described by Payne & Laws (1976). The biopsy schedule was as frequent as thought desirable if the effects of biopsy on the experiment were to be minimal. In Expt 1 insufficient liver biopsy sample was obtained from many of the sheep in groups nos. 2, 3 and 4 to allow determination of enzymes at 7 weeks except for arginine synthetase (combined activity of argininosuccinate synthetase (*EC* 6.3.4.5) and argininosuccinate

lyase (*EC* 4.3.2.1)), therefore only results for group no. 1 have been included. There were only three sheep surviving in groups nos. 1 and 3 of Expt 2 at the final sampling after the urea tolerance test reported elsewhere (Payne & Laws, 1976).

Diet analyses

On an 'air-dry' basis the Rhodes grass (*Chloris gayana*) chaff, lucerne (*Medicago sativa*) chaff and safflower meal contained 10·2, 22·0 and 65·0 g N/kg respectively. Thus 170 g safflower meal was isonitrogenous with 500 g lucerne chaff.

Sampling methods

Liver samples were obtained by aspiration biopsy using the instruments described by Dick (1952) and the samples were frozen immediately in glass tubes in solid carbon dioxide. Samples of the semitendinosus muscle were obtained by surgical dissection after local anaesthesia was induced by injection of procaine (20 ml/l).

Analytical methods

Homogenization of the liver samples was as described by Payne & Laws (1976). For muscle homogenates a looser-fitting pestle was used and the medium was 0·05 M-Tris buffer, pH 9·1.

Arginine synthetase and arginase (*EC* 6.5.3.1) were determined according to the methods of Payne & Morris (1969). One unit of activity resulted in the production of 1 μmol product/h at 38°. Aspartic aminotransferase was determined, basically, according to the method of Reitman & Frankel (1957). The unit of activity was equivalent to the production of 1 μmol oxaloacetate/min at 25°. Glutamic dehydrogenase (*EC* 1.4.1.2) was determined by the method of Snoke (1956). It was found necessary to add NAD after the homogenate to obtain reaction. The unit of activity was the oxidation of 1 μmol NAD/min at 30°.

Tyrosine aminotransferase (*EC* 2.6.1.5) was determined by the method of Lin, Pitt, Civen & Knox (1958), while the method of Gianetto & de Duve (1955) was used for cathepsin. The unit of tyrosine aminotransferase activity was equivalent to the conversion of 1 μmol/min substrate at 25° whilst the unit of cathepsin activity was the release of 1 μmol tyrosine/min at 38°.

Creatine kinase (*EC* 2.7.3.2) was determined by a modification of the methods of Colombo, Richterich & Rossi (1962) and Bernt & Bergmeyer (1963). The following quantities were pipetted into a 1 ml quartz spectrophotometric cell and mixed: 0·055 ml 0·3 M-potassium chloride, 0·04 ml 0·05 M-Tris buffer (pH 9·0), 0·1 ml M-sodium fluoride, 0·1 ml tissue extract, 0·2 ml buffer coenzyme mixture of Bernt & Bergmeyer (1963), 0·01 ml lactic dehydrogenase (*EC* 1.1.1.27) containing pyruvic kinase (*EC* 2.7.1.40) (4 mg protein/ml). Then 0·1 ml creatine glycine buffer of Bernt & Bergmeyer (1963) or of glycine buffer alone was added and the change in extinction at 340 nm recorded. The unit of activity was the oxidation of 1 μmol NAD/min at 30°. The addition of the NaF effectively decreased the non-specific reactions due to ATPase and phosphatases without affecting the creatine kinase activity.

DNA was estimated in liver by the method of Munro & Fleck (1966) using the method of Giles & Myers (1965) for colour development. The following modifications were made in order to obtain sufficient sensitivity in muscle. All the precipitate of DNA and protein was taken for final colour development instead of a portion of an alkaline mixture of DNA and dissolved protein. To reduce the protein present in the final stages a portion of the homogenate was centrifuged at 1700 ***g*** and the precipitate taken for analysis. Thus the soluble protein was removed.

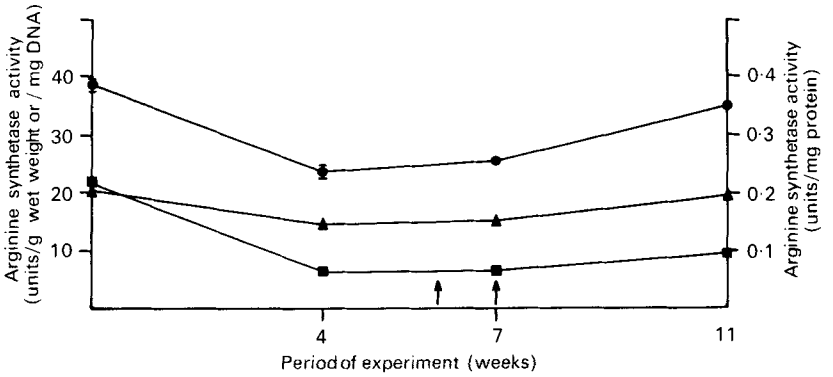

Fig. 1. Expt 1. The changes in liver arginine synthetase activity (combined argininosuccinate synthetase (*EC* 6.3.4.5) and argininosuccinate lyase (*EC* 4.3.2.1) activities) of sheep during protein deprivation and replenishment in groups of sheep during the experimental period when they were given different feeding regimens (for details, see Table 1). (↑), The 7 d supplementation period. Arginine synthetase activity in liver is expressed as: units/g wet wt (●—●); units/mg protein (▲—▲); units/mg DNA (■—■). Each point represents the mean value for all sheep in groups nos. 1, 2, 3 and 4 except for those for week 7 which represent the mean value for group no. 1 only. One unit of enzyme activity was defined as 1 μmol ammonia formed/h at 38°. For each point the standard error is indicated by vertical bars.

Total protein of homogenates was measured by the method of Miller (1959).

Statistical analysis of the differences between means for all sheep at different intervals of sampling was by analysis of variance.

RESULTS

Live weight

The changes in live weight during Expts 1 and 2 are shown in Tables 2 and 3. It was the small change in live weight in Expt 1 that led to setting up the high level feeding initially and the more severe restriction of intake in Expt 2 which led to greater weight loss. The provision of an energy supplement in the form of molasses had no significant effect on loss in live weight.

Liver enzymes and DNA

Expt 1. In order to determine whether the observed effects of nutrition on enzyme activity might be influenced by the mode of expression of that activity the results for arginine synthetase activity were expressed per g wet weight, per mg protein and per mg DNA (Fig. 1). The enzyme activity:DNA values gave the largest relative changes though the variance was sometimes lower for values of activity expressed per g wet weight. All further results were expressed in terms of DNA.

The effect of protein depletion on various enzyme activities, DNA levels and protein:DNA is shown in Table 2. Both arginine synthetase and aspartic aminotransferase decreased considerably after 4 weeks on the low protein intake, the differences being highly significant ($P < 0{\cdot}01$). On returning the sheep to pasture initial levels were not regained as the pasture had deteriorated. Tyrosine aminotransferase activity was reduced also but to a lesser extent, whereas cathepsin activity was unpredictable.

The DNA level and protein:DNA also showed significant changes ($P < 0{\cdot}01$) due to restricted protein intake though the relative change was not as great as for arginine synthetase activity. Like the enzyme activities these measurements showed only slight recovery to initial values after the sheep were put out to graze. This contrasts with enzyme activities expressed on a wet weight basis which showed greater recovery towards initial values.

Table 2. *Expt* 1. *The effect of protein depletion on live weight, liver DNA concentration, protein : DNA and various liver enzyme activities in sheep removed from grazing and fed on Rhodes grass* (Chloris gayana) *chaff and various protein supplements**

(Mean values with their standard errors, no. of sheep in parentheses)

Time of sampling (week of Expt)	Protein intake (g/d)	Live wt (kg)		DNA (mg/g wet wt)		Protein:DNA		Arginine synthetase (*EC* 6.3.4.5, 4.3.2.1) (units†/mg DNA)		Aspartic aminotransferase (*EC* 6.2.1.1) (units†/mg DNA)		Tyrosine aminotransferase (*EC* 2.6.1.5) (units†/mg DNA ($\times 10^{-3}$))		Cathepsin (units†/mg DNA ($\times 10^{2}$))	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0 (16)	70–100‡	37·1	0·6	2·11	0·19	100·2	10·0	21·8	1·1	7·52	0·53	2·21	0·23	1·23	0·44
4 (16)	31	35·2	0·6	4·19	0·31	42·5	3·8	6·1	0·6	2·78	0·24	1·30	0·15	0·37	0·14
7 (3)§	31	34·9	—	4·05	—	41·3	—	6·3	—	2·59	—	1·32	—	0·66	—
11 (13)‖	70‡	36·9	0·6	3·92	0·19	47·5	2·3	9·1	0·5	3·47	0·24	1·57	0·17	0·17	0·03

* For details of rations, see Table 1 and p. 443.

† For definition of units of activity for all enzymes, see p. 443.

‡ Levels are only estimates.

§ Results only for group no. 1 (control) as analyses for arginine synthetase only done for other groups supplemented with protein to satisfy requirement of another experiment.

‖ Reduced no. of animals due to losses in group no. 1 during urea tolerance test (Payne & Laws, 1976).

Table 3. *Expt 2. The effect of protein depletion on live weight, liver DNA concentration and various liver enzyme activities in sheep previously well fed on lucerne* (Medicago sativa) *and then fed on Rhodes grass* (Chloris gayana) *chaff with or without molasses or a protein supplement**

(Mean values with their standard errors; no. of sheep in parentheses)

Time of sampling (week of Expt)	Group no.	Protein intake (g/d)	Live weight (kg)		DNA (mg/g wet wt)		Arginine synthetase (*EC* 6.3.4.5, 4.3.2.1) (units†/mg DNA)		Aspartic aminotransferase (*EC* 2.6.1.1) (units†/mg DNA)		Glutamic dehydrogenase (*EC* 1.4.1.2) (units†/mg DNA)		Arginase (*EC* 6.5.3.1) (units†/mg DNA ($\times 10^{-3}$))	
			Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
0	1–3 (18)	140	32·8	1·1	2·32	0·10	22·5	1·0	16·2	1·3	15·8	0·7	1·97	0·18
4	1–3 (18)	24	26·8	0·8	4·43	0·15	8·1	0·4	4·3	0·2	9·1	1·8	0·57	0·06
7	1 (6)	24	24·6	2·1	4·09	0·23	9·2	0·6	4·0	0·3	7·4	1·3	1·27	0·16
	2 (6)	97	24·5	0·9	3·20	0·11	15·7	1·0	9·6	0·6	11·2	1·9	2·21	0·38
	3 (6)	24	26·5	1·1	3·29	0·17	11·5	0·7	4·5	0·5	9·7	1·5	1·78	0·09
11	1–3‡ (12)	140	31·1	1·0	2·22	0·09	32·5	2·0	21·9	1·3	32·2	2·0	3·77	0·51

* For details of rations, see Table 1 and p. 443.
† For definition of units of activity for all enzymes, see p. 443.
‡ Only three sheep in groups nos. 1 and 3 due to losses from urea tolerance test (Payne & Laws, 1976).

Table 4. *Expts* 1 *and* 2.* *The effect of protein depletion on muscle DNA, protein: DNA, and the activities of creatine kinase and aspartic aminotransferase*

(Mean values with their standard errors; no. of sheep in parentheses)

Expt no.	Time of sampling (week of Expt)	Protein intake (g)	DNA (mg/g wet wt) Mean	DNA (mg/g wet wt) SE	Protein:DNA Mean	Protein:DNA SE	Aspartic aminotransferase (*EC* 2.6.1.1) (units†/mg DNA) Mean	Aspartic aminotransferase (*EC* 2.6.1.1) (units†/mg DNA) SE	Creatine kinase (*EC* 2.7.3.2) (units†/mg DNA) Mean	Creatine kinase (*EC* 2.7.3.2) (units†/mg DNA) SE
1	0 (16)	70–100‡	0·170	0·01			254	14	487	29
	4 (16)	31	0·220	0·02			251	16	363	25
	11 (13)§	70‡	0·181	0·01			212	18	502	57
2	0 (18)	140	0·271	0·01	628	37	131	8	341	19
	4 (18)	24	0·270	0·01	617	30	159	13	321	17
	11 (12)	140	0·242	0·01	744	55	181	13	292	28

* For details for experimental design and rations, see Table 1 and p. 443.
† For definition of units of activity for all enzymes, see p. 443.
‡ Levels are only estimates.
§ For explanations of reduced nos. of animals at week 11, see Tables 2 and 3.

Overall in this experiment there were marked changes in certain enzymes, DNA and protein:DNA though very small changes in live weight.

Expt 2. Whereas in Expt 1 protein and energy intakes before the experiment were probably only slightly above maintenance, in this experiment the initial feeding of lucerne was considerably above maintenance whilst the intake during depletion was less than for Expt 1.

The results for various enzymes and DNA levels are presented in Table 3. Again, arginine synthetase and aspartic aminotransferase showed a similar reduction in activity to that observed in Expt 1. Activity expressed on a per mg DNA basis changed to a greater extent than activity expressed on a per g wet weight basis. Protein supplementation for 1 week partially restored the enzyme levels. Aspartic aminotransferase activity was lower in Expt 2 than in Expt 1 though it is difficult to find an explanation for this. Upon refeeding with lucerne for 4 weeks enzyme activities increased to levels higher than initial levels.

The measurement of tyrosine aminotransferase activity, cathepsin and protein were discontinued in Expt 2 as it was desired to study the activity of glutamic dehydrogenase and arginase under conditions of protein depletion. Both enzymes showed a significant reduction in activity ($P < 0{\cdot}01$) though the change was not as great as for the other enzymes measured. The large variation in the arginase values reduced their predictive value.

The change in DNA concentration with protein depletion was again highly significant ($P < 0{\cdot}01$) but in this experiment initial levels were regained upon return to lucerne feeding.

In this experiment there were considerable changes in live weight but enzyme activity only changed by a similar amount to that in Expt 1. The feeding of energy in the form of molasses did not exert any significant effect during protein deprivation though there appeared to be a trend towards higher levels of certain enzyme activities and a lower level of DNA. After refeeding with lucerne both the group supplemented with protein and the group fed on molasses appeared to have higher levels of many enzymes and a lower level of DNA than the control group though the differences were not significant under the conditions of the experiment.

Muscle enzymes

In muscle the level of DNA was much lower than it was in liver and was more difficult to estimate with small samples.

It can be seen from Table 4 that in both Expts 1 and 2 there was little change in DNA or in protein:DNA as a result of low protein intake. Similarly there was little change in creatine kinase and aspartic aminotransferase activities as a result of low protein intake in both Expts 1 and 2 (Table 4).

DISCUSSION

The present results suggest that measurements of liver arginine synthetase or aspartic aminotransferase could be useful as indices of protein status. Preferably, the activity expressed on a per mg DNA basis is desirable but activity expressed per g wet weight would be adequate for most situations. In particular, the results in Expt 1, when there were small changes in live weight, suggest that under a mild extent of protein deprivation changes in these enzymes occur before any visible change in body condition.

The ready response of certain enzymes to protein intake as shown by the increased enzyme activity after giving a protein supplement in Expt 2 suggests that the enzyme level will be affected by the protein intake for the previous week. The initial protein intake of the sheep in Expt 1 could not be assessed particularly since pasture selected by sheep can differ markedly in N content from total pasture. However, it can be assessed that the intake was considerably less than the approximately 140 g/d received by sheep on lucerne and probably in the range 70–100 g. The protein intakes during depletion were selected to be much lower than the daily maintenance requirement of approximately 70 g protein. During the refeeding on pasture, which had deteriorated in quality as it was the dry winter months, the pasture intake was probably approximately 70 g protein/d.

In general aspartic aminotransferase is the better enzyme for use as an indicator, as it is fairly stable, present in high concentration and many laboratories are currently performing this assay in plasma. Tissue for assay could be obtained from either freshly slaughtered animals or by liver biopsy. In the latter instance only 0·2 g would be required. The difference in values between the two experiments suggests that further work is required to establish basal levels which can be used to interpret future results on depleted sheep.

Arginase activity may also be a good indicator of protein intake provided that an assay method with a lower coefficient of variation is developed. In the present method the high extinction of the 'blank' led to considerable variation in the results.

Liver DNA level would be a satisfactory criterion also, but the assay is tedious and the change in level is not quite as great as for the enzymes. The proposals made by Masters (1963) and Robinson & Lambourne (1970) that muscle protein:DNA could be a suitable index were not confirmed in the present series of experiments. The difference in results may well be due to the extent of undernutrition involved. In their work the sheep and cattle involved had been in an extreme state of N and energy deprivation. At least in one instance the animals were suffering from starvation. In the present experiments the extent of protein deprivation was less severe and was much closer to that observed in the field. Possibly in the more severe stages of protein deprivation muscle protein is reduced and marked changes occur in protein:DNA and possibly in certain enzyme concentrations. However, our results show that muscle criteria are unlikely to be of any value in assessing protein status of sheep under normal grazing conditions.

The lack of change in liver aspartic aminotransferase activity on an extremely-low-protein diet reported by Masters & Horgan (1962) accentuates the fact observed in other experiments with rats (Awapara, 1953; Muramatsu & Ashida, 1962) that starvation and protein-free diets can produce different effects from moderate protein deprivation. Under these conditions, increases in certain enzymes can develop to cope with the increased protein catabolism that occurs.

Before levels of liver arginine synthetase, aspartic aminotransferase and DNA can be

used as indices, further work will have to be done to delineate what enzyme levels are maintained under various N intakes. It would be desirable to compare liver biopsy samples from animals maintained on a ration of standard protein content with those from animals on unknown protein intakes in order to check the reliability of the determination in relation to new substrates etc. From the results of Expt 2 it appears that energy in the diet has only a small influence and so variations in this component will not radically affect the levels obtained.

The authors wish to thank staff of the Animal Husbandry Branch, Animal Research Institute, for feeding the animals, Miss L. Ballinger and Messrs J. O. Twist, A. May and H. Mawhinney for technical assistance and Mr P. O'Rourke for the statistical analyses.

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Printed in Great Britain