In vitro energy costs of NA^+, K^+ATPase activity and protein synthesis in muscle from calves differing in age and breed

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1. An in vitro preparation was used to measure rates of oxygen consumption, Na^+, K^+-ATPase-dependent respiration, [^{14}C]phenylalanine incorporation and tyrosine release of skeletal (sternomandibularis) muscle from 10-21-d-old (three) and 7-month dairy (three) calves and control (CDM; four) and extreme double-muscled (EDM; two) calves.

2. Rate of oxygen consumption was greatest ($P < 0.001$) for muscle from 10–21-d-old dairy calves and lowest ($P < 0.05$) for CDM calves.

3. Ouabain (10^{-6} M) caused a 40% inhibition of muscle respiration.

4. Na^+, K^+-ATPase-dependent respiration was similar for muscle from all calf groups except 10–21-d-old dairy calves which had a value 26% greater ($P < 0.001$) than that of older dairy calves.

5. Na^+, K^+-ATPase-independent respiration was 16% greater ($P < 0.001$) for muscle from 10–21-d-old than that of older dairy calves while muscle from EDM calves had a value 11% greater than that of CDM calves.

6. The rate of [^{14}C]phenylalanine incorporation was greater ($P < 0.05$) for muscle from 10–21-d-old dairy than from older dairy calves, similar between older dairy and CDM calves, and decreased ($P < 0.05$) for EDM calves.

7. Rate of tyrosine release was greatest ($P < 0.05$) for muscle from CDM and EDM calves; both dairy groups had similarly low rates of muscle tyrosine release.

8. The energy estimated to be required for peptide bond synthesis accounted for 2·0–3·3% of the O_2 consumption of the muscle preparations.

In order to more fully understand whole animal energy expenditure it is necessary first to identify the causes of metabolic energy expenditure, and then to determine their quantitative importance under a variety of physiological conditions.

Active sodium ion-potassium ion transport, that is, the activity of the plasma membrane Na^+, K^+-ATPase (*EC* 3.6.1.3) in counteracting transmembrane movement of Na^+ and K^+ along their concentration gradients, has been suggested to be a major component of the energy expenditure of animals and has been estimated to account for 20–45% of the oxygen uptake of resting cells (Whittam, 1961). Protein synthesis has also been suggested to be a major energy cost of animals, accounting for up to 30% of the heat production of cattle (Lobley *et al.* 1980). The extent to which energy expended by processes such as active Na^+-K^+ transport and protein synthesis can vary between animals and is influenced by genetic and environmental factors is not clear. Evidence that exposure of animals to a cold environment selectively increased energy expenditure at the level of the Na^+, K^+-ATPase has been presented for muscle preparations from sheep (Gregg & Milligan, 1982).

The objectives of this experiment were to obtain physiologically-realistic estimates of the magnitudes of the energy costs of active Na^+-K^+ transport and protein synthesis in skeletal muscle from calves and to examine the effects of breed and age on the relative costs of these two processes as components of background or maintenance energy expenditure of the tissue.

EXPERIMENTAL

Animals

Muscle samples were obtained from six 7-month-old male calves from a beef crossbred population selected for a high incidence of double-muscling (DM), two calves did not exhibit

heavy muscling (control DM; CDM) and four calves exhibited overt muscular hyperplasia (extreme DM; EDM); three 10–21-d-old male dairy (Holstein) calves; and three 7-month-old male dairy calves. All animals were housed indoors in heated barns (approximately 20°) for at least 4 weeks before surgery. The 10–21-d-old dairy calves were given a milk-substitute diet; 7-month-old calves were given good-quality grass hay *ad lib.* and a ration of barley–oat concentrate mix containing minerals. Water was available *ad lib.* Animals were fasted overnight before surgery.

Respiration and Na^+, K^+-ATPase-dependent respiration

A section of the sternomandibularis muscle was taken from each animal and preparations made following the method of Gregg & Milligan (1982). On removal from the animal, muscle sections were placed immediately in cooled (15°) HEPES buffer containing 10 mM-glucose and 5 mM-acetate as substrates, and the small tied fibre bundles (approximately 20·0 × 0·5 mm) were prepared at room temperature with the aid of a dissecting microscope. Following measurement of initial respiration rates (Gregg & Milligan, 1982) for five preparations per animal in an O_2 electrode system, these muscle preparations were then incubated in buffers containing 0, 10^{-8}, 10^{-7}, 10^{-6} or 10^{-5} M-ouabain, respectively. Respiration rates were then measured again. Care was taken to observe that the ties on the muscle bundles had not loosened by the end of incubation; in instances in which this did occur, respiration was drastically depressed and the results were discarded. Percentage inhibition of respiration by ouabain was calculated using the ratio, the difference between initial and post-incubation respiration rates:the initial respiration rate. The values for maxim ouabain inhibition, which occurred at concentrations of 10^{-6} M and greater, are reported.

Measurement of [^{14}C]phenylalanine incorporation

Muscle sections were placed in HEPES buffer to which the following additions had been made: essential and non-essential amino acids, except phenylalanine, at the concentrations reported for sheep plasma (Bergman *et al.* 1974); phenylalanine 500 μM; insulin 0·1 unit/ml; chloramphenicol 0·3 mg/l. Four muscle preparations from each animal were incubated in 3 ml complete HEPES buffer containing approximately 0·28 μCi L-[U-^{14}C]phenylalanine/ml (Amersham Corp., Ontario); two preparations were incubated for 1·0 h and two for 2·5 h in a shaking water-bath at 37°. Preliminary studies established the rate of [^{14}C]phenylalanine incorporation to be linear for incubation periods of up to 3·0 h. At the end of the incubation period the muscle preparations were rinsed, blotted and weighed. They were then homogenized in 1 ml cold trichloroacetic acid (500 g/l), centrifuged, and the precipitate washed according to the method of Fulks *et al.* (1975). Acid-precipitated pellets were combusted in a Beckmann biological material oxidizer and $^{14}CO_2$ collected in 10 ml carbon-dioxide-trapping cocktail (500 ml toluene/l, 300 ml methyl cellosolve/l, 200 ml monoethanolamine/l, 5·0 g PPO, 0·2 g POPOP). Radioactivity was measured with a Searle Mark III liquid-scintillation counter and counting efficiency determined with the channels-ratio method.

In a separate experiment, acid-precipitated material from muscle preparations incubated for 2·5 h was dried, hydrolysed and chromatographed according to the method of McBride *et al.* (1979). Sections of the thin-layer chromatography plates were scraped into separate counting vials and counted as described previously. Radioactivity was found to be present only at the position corresponding to the phenylalanine standard.

Measurement of tyrosine release

The rate of tyrosine release from muscle was measured in the HEPES buffer used for the study of [^{14}C]phenylalanine incorporation, in which was included 0·5 mM-cycloheximide and from which tyrosine was omitted, according to the method of Fulks *et al.* (1975). Two muscle

preparations from each animal were pre-incubated in 2 ml of buffer in a shaking water-bath at 37° for 0·5 h then transferred to similar flasks; one preparation was incubated for 0·5 h and the other for 1·5 h. At the end of the incubation period, muscle preparations were blotted and weighed. The amount of tyrosine in the buffer was measured fluorometrically by the method of Waalkes & Udenfriend (1957). Preliminary studies established the rate of tyrosine release from muscle preparations into buffer to be linear for incubation periods of up to 2·5 h after a 0·5 h pre-incubation period.

Analysis of results

Results are expressed as mean values with their standard errors. An unpaired Student's *t* test was used to establish the significance of differences between the means for muscle O_2 consumption, percentage inhibition of respiration by 10^{-6} M-ouabain, and Na^+, K^+-ATPase-dependent and independent respiration. The rates of [^{14}C]phenylalanine incorporation and tyrosine release were determined by least squares analysis. Differences between groups were located by the Student–Newman–Kuel range test (Steel & Torrie, 1960).

RESULTS

O_2 consumption and Na^+, K^+-ATPase activity

Total muscle O_2 consumption and the proportion of respiration inhibited by 10^{-6} M-ouabain are shown in Table 1.

The rate of muscle O_2 consumption was greatest ($P < 0·05$) for 10–21-d-old dairy calves and was reduced by 16% in the muscle from the older dairy calves. Among calves of similar age, dairy calves had a muscle O_2 consumption rate greater ($P < 0·05$) than CDM calves.

Dose–response curves constructed for each calf group differed in absolute values for percentage inhibition of respiration observed at each concentration of inhibitor but had a similar sigmoidal shape and a similar value for the lowest concentration of inhibitor yielding maximum inhibition (10^{-6} M-ouabain).

The proportion of respiration inhibited by ouabain ranged from $39·4 \pm 2·9$% for older dairy to $42·7 \pm 1·4$% for CDM calves. Na^+, K^+-ATPase-dependent respiration, the amount of O_2 calculated to have been consumed to support the ouabain-inhibitable portion of Na^+, K^+-ATPase activity, was statistically similar for muscle from all calf groups except 10–21-d-old dairy calves which had a value 26% greater ($P < 0·001$) than that of older dairy calves.

Na^+, K^+-ATPase-independent respiration, the residual portion of muscle O_2 consumption not accounted for by activity of the Na^+, K^+-ATPase, was greatest for muscle from 10–21-d-old dairy calves. The value for muscle from 10–21-d-old dairy calves was 16% greater ($P < 0·001$) than that of older dairy calves. For calves of similar age, Na^+, K^+-ATPase-independent respiration was measured to be 11% greater ($P < 0·05$) for muscle from EDM than that of CDM calves and 19% greater ($P < 0·005$) for muscle from older dairy than that of CDM calves.

Rates of [^{14}C]phenylalanine incorporation and tyrosine release

Rates for [^{14}C]phenylalanine incorporation into muscle protein are shown in Table 2. The rate of [^{14}C]phenylalanine incorporation into muscle differed ($P < 0·05$) between all groups except between older dairy and CDM calves. Values for the percentage of muscle protein synthesized/d (K_s) ranged from 0·7 to 1·5%/d (Table 2); muscle preparations from CDM calves had a K_s value 57% higher than those from EDM calves. The K_s values for muscle preparations from older dairy and CDM calves were similar. The estimated cost of incorporation of amino acids into growing peptide chains ranged from 2·0 to 3·3% of the total in vitro O_2 consumption of the muscle preparations (Table 2).

Table 1. *In vitro oxygen consumption, maximum ouabain inhibition and Na^+, K^+-ATPase-dependent* and independent† respiration of calf muscle preparations*

(Mean values with their standard errors)

Group	O_2 consumption (μl O_2/mg per h)		Percentage inhibition of O_2 consumption by ouabain		Na^+, K^+-ATPase-dependent O_2 consumption (μl O_2/mg per h)		Na^+, K^+-ATPase-independent O_2 consumption (μl O_2/mg per h)	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
10–21-d-old dairy (3 calves)	3·27	$0·27^{a}$	41·3	$4·5^{ab}$	1·36	$0·11^{a}$	1·93	$0·16^{a}$
7-month-old dairy (3 calves)	2·75	$0·27^{b}$	39·4	$2·9^{b}$	1·08	$0·11^{b}$	1·67	$0·16^{b}$
CDM (4 calves)	2·44	$0·24^{c}$	42·7	$1·4^{a}$	1·04	$0·12^{b}$	1·40	$0·16^{c}$
EDM (2 calves)	2·61	$0·27^{bc}$	40·6	$2·2^{ab}$	1·06	$0·10^{b}$	1·55	$0·14^{b}$

CDM, calves not exhibiting heavy muscling; EDM, extreme double-muscled calves.

a, b, c, Means within a column that do not share a common superscript letter differ significantly ($P < 0·05$).

* Na^+, K^+-ATPase-dependent respiration = total O_2 consumption × inhibition by ouabain.

† Na^+, K^+-ATPase-independent respiration = total O_2 consumption − Na^+, K^+-ATPase-dependent respiration.

Table 2. *Rate of [^{14}C]phenylalanine incorporation into protein of calf muscle preparations*

Group	Rate of [^{14}C]phenylalanine incorporation (nmol/mg per h)		K_s* (%/d)	Percentage of O_2 consumption expended by protein synthesis†
	Mean	SE		
10–21-d-old dairy (3 calves)	0·023	$0·002^{a}$	1·5	3·3
7-month-old dairy (3 calves)	0·015	$0·002^{b}$	1·0	2·5
CDM (4 calves)	0·017	$0·002^{b}$	1·1	3·1
EDM (2 calves)	0·011	$0·001^{c}$	0·7	2·0

CDM, calves not exhibiting heavy muscling; EDM, extreme double-muscled calves.

a, b, c, Means within a column that do not share a common superscript letter differ significantly ($P < 0·05$).

* Calculated assuming an average muscle amino acid molecular weight of 130 g/mol and a phenylalanine content of 3·0 mol% (Chang & Goldberg, 1978).

† The calculation of the percentage of muscle O_2 consumption expended for protein synthesis assumed 5 mol ATP to be required for incorporation of 1 mol amino acid into a peptide chain and 1 mol O_2 to be required for synthesis of 5 mol ATP.

Rates of release of tyrosine from muscle preparations are shown in Table 3. The rate of tyrosine release from muscle differed ($P < 0·05$) between all calf groups except between 10–21-d-old and older dairy calves. Both dairy groups had tyrosine release rates which were too low to allow accurate measurement. Muscle preparations from EDM calves had a greater ($P < 0·05$) rate of tyrosine release (Table 3) than those from CDM calves.

The percentage of muscle protein degraded/d (K) was calculated assuming a muscle tyrosine content of 2·8 mol% (Chang & Goldberg, 1978). K_s values thus calculated were 0·1%/d for 10–21-d-old and older dairy calves, 0·8%/d for CDM calves, and 5·4%/d for EDM calves. It was assumed that tyrosine released from muscle originated from muscle protein since Fulks *et al.* (1975) have shown that under similar experimental conditions muscle free tyrosine content did not change during 3 h of incubation.

Table 3. *Rate of tyrosine release from calf muscle preparations*

Group	Rate of release (nmol/mg per h)	
	Mean	SE
10–21-d-old dairy (3 calves)	0·002	0·009[a]
7-month-old dairy (3 calves)	0·002	0·011[a]
CDM (4 calves)	0·016	0·008[b]
EDM (2 calves)	0·078	0·008[c]

a, b, c, Means within a column that do not share a common superscript letter differ significantly ($P < 0·05$).

DISCUSSION

Exposure of muscle to 10^{-6} M-ouabain resulted in an average of 40% for inhibition of respiration. This value is in agreement with values for inhibition of respiration by ouabain reported for skeletal muscle from sheep (Gregg & Milligan, 1982), mice (Gregg & Milligan, 1980*a*), and rats (Asano *et al.* 1976; Ismail-Beigi & Edelman, 1970).

The rates of O_2 consumption measured for calf muscle preparations were similar to those previously measured with identical preparations of the same muscle for adult sheep (Gregg & Milligan, 1982). A decrease of metabolic rate with age has been previously observed for cattle (Webster *et al.* 1974). The greater rate of muscle O_2 consumption measured for 10–21-d-old than for older dairy calves resulted from increases in both the Na^+, K^+-ATPase-dependent and independent components of respiration. For calves of similar age, the differences measured for total O_2 consumption between EDM and CDM calves and between dairy and CDM calves were due to an increased amount of O_2 consumed in the Na^+, K^+-ATPase-independent component of respiration.

The use of ouabain to determine Na^+, K^+-ATPase-dependent respiration was criticized (Himms-Hagen, 1976) primarily on the basis that the decreased O_2 consumption observed in the presence of ouabain may be due to altered intracellular Na^+ and K^+ concentrations and may, therefore, be secondary to the inhibition of the Na^+, K^+-ATPase. However, Asano *et al.* (1976) found measurements of the ouabain-inhibitable O_2 consumption of rat skeletal muscle to be independent of a wide range of experimentally-induced changes in intracellular Na^+ and K^+ concentrations. Also, inhibition by ouabain of the rate of glycolytic substrate-level phosphorylation in ascites tumor cells was shown to have resulted from direct inhibition of Na^+, K^+-ATPase rather than from a disturbance of glycolytic enzyme function through alterations of intracellular K^+ concentrations (Scholnick *et al.* 1973). A second objection (Chinet *et al.* 1977) concerned the use of sliced tissue preparations for measurement of Na^+, K^+-ATPase-independent respiration; rapid leakage of Na^+ into the cell at sites of membrane damage may have occurred and caused a non-physiological stimulation of the Na^+, K^+-ATPase leading to the large values measured for Na^+, K^+-ATPase-dependent respiration. Investigations conducted with intact organ perfusions have measured Na^+, K^+-ATPase-dependent respiration or heat production to account for less than 6% of total tissue O_2 consumption or heat production (Chinet *et al.* 1977; Folke & Sestoft, 1977). In contradiction to this criticism, Gregg & Milligan (1980*b*) have presented evidence that at least part of the low response to ouabain obtained in the micro-calorimetric studies of perfused organs was due to previous inhibition of the enzyme by the experimental conditions.

The importance of tissue preparation and condition in achieving measurements indicative of physiological O_2 consumption and Na^+, K^+-ATPase activity cannot be ignored. The

muscle preparation used in this experiment was developed specifically to study the metabolism of muscle from large mammals under conditions which minimize cellular damage and maintain physiological characteristics such as membrane potential and oxygenation (Gregg & Milligan, 1982). The inhibition of respiration induced by ouabain is a function of both enzyme availability to ouabain and the equilibrium reached between formation and dissociation of the inhibitor-enzyme complex (Tobin & Brody, 1972). The proportion of Na^+, K^+-ATPase activity actually inhibited in the muscle preparation under our experimental conditions is not known. Therefore, our measurements of the proportion of muscle O_2 consumption required for support of Na^+, K^+-ATPase activity must be considered as minimum estimates.

Accurate calculation of rates of protein synthesis from incorporation of [^{14}C]phenylalanine into muscle protein requires that the specific activity of phenylalanine-tRNA be known. McKee *et al.* (1978) have shown for perfused rat heart that the specific activities of extracellular, intracellular and tRNA-bound phenylalanine are the same when the perfusate phenylalanine concentration was 0·4 mM or greater. It was assumed that protein degradation that may have occurred during the period of incubation did not significantly influence the estimate of synthesis by causing cleavage of newly-added phenylalanine from peptide chains.

The calculated values for K_s (percentage of muscle protein synthesized/d) (Table 2) are in good agreement with K_s values reported for heifer and cow muscle (0·8–2·0%/d) by Lobley *et al.* (1978) and Lobley *et al.* (1980) as determined by in vivo constant infusion of [^{3}H]tyrosine. The greater rate of muscle protein synthesis measured for 10–21-d-old dairy calves than from older dairy calves is consistent with the higher K_s values for protein synthesis measured for immature in contrast to adult animals (see Garlick, 1980).

Although the rate of protein synthesis was 50% greater in muscle of 10–21-d-old dairy calves than in that of older dairy calves, the increased required rate of peptide bond synthesis would, assuming five molecules ATP per peptide bond synthesized, account for only 7·5% of the difference in total muscle O_2 consumption measured between the two dairy groups. It is not known how much of the greater Na^+, K^+-ATPase-dependent respiration might have been in support of, or association with, the increased rate of protein synthesis as suggested by Reeds *et al.* (1980). Older dairy and CDM calf muscle did yield similar values for both the amount of O_2 consumed in direct support of protein synthesis and Na^+, K^+-ATPase-dependent respiration. However, among 7-month-old calves, the rate of protein synthesis in the muscle of CDM calves was 55% greater than in muscle of EDM calves while no difference was measured for Na^+, K^+-ATPase-dependent respiration between the two calf groups. These results do not appear to support a close association of increased rate of protein synthesis and of Na^+, K^+-ATPase activity. However, the extent to which Na^+, K^+-ATPase-dependent respiration is underestimated and the extent to which differences in protein degradation may have affected muscle O_2 consumption is not known. Therefore, it is not possible to reach a conclusion regarding a relationship between rate of protein synthesis and Na^+, K^+-ATPase activity.

Rates of protein turnover should not be calculated from our results since the rates of [^{14}C]phenylalanine incorporation and of tyrosine release were measured under different experimental conditions, the latter in a medium with tyrosine omitted and cycloheximide added. Nonetheless, estimated rates of protein degradation were less than or similar to rates of protein synthesis for muscle preparations from all calf groups except EDM calves. The CDM and EDM muscle preparations exhibited an enhanced rate of tyrosine release which is certainly suggestive of an increased capacity for protein degradation.

Double-muscled cattle appear to partition energy in a way that is different from non-double-muscled cattle including altered potentials for protein and fat deposition (Holmes & Ashmore, 1972). The metabolic cause of these changes is not known. One might

speculate, on the basis of the possibility of increased protein degradation, that muscles of EDM calves may expend more energy for protein turnover than those of CDM calves and this might account for the tendency for increased total muscle O_2 consumption. In the animal, increased energy expenditure for such a maintenance function could result in a reduction of energy available for fat deposition.

The available evidence supports the importance of activity of Na^+, K^+-ATPase as a cause of cellular energy expenditure in muscle. While it would be invalid to conclude that the role of Na^+, K^+-ATPase is of the identical magnitude in vivo as measured in vitro, the comparison of in vitro and in vivo estimates for rate of protein synthesis does provide some confidence that what is measured for these muscle preparations is at least indicative of what occurs in the animal.

In conclusion, activity of the Na^+, K^+-ATPase accounted for a minimum of 40% of muscle O_2 consumption. The energy required for peptide bond synthesis was estimated to account for less than 4% of muscle O_2 consumption. The amount of muscle O_2 consumption due to activity of the Na^+, K^+-ATPase was similar for 7-month-old calves of different breed backgrounds, and was increased for 10–21-d-old dairy calves. The amount of O_2 calculated to have been expended on peptide bond synthesis was increased 50% or greater for muscle from 10–21-d-old dairy over that of older dairy calves, and from CDM over that of EDM calves. The increased amount of O_2 consumption expended in direct support of protein synthesis would not account for the difference between the dairy groups.

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REFERENCES

Asano, Y., Liberman, U. A. & Edelman, I. S. (1976). *J. clin. Invest.* **57**, 368.
Bergman, E. N., Kaufman, C. F., Wolff, J. E. & Williams, H. H. (1974). *Am. J. Physiol.* **226**, 833.
Chang, T. W. & Goldberg, A. L. (1978). *J. biol. Chem.* **253**, 3685.
Chinet, A., Clausen, T. & Girardier, L. (1977). *J. Physiol., Lond.* **265**, 43.
Folke, M. & Sestoft, L. (1977). *J. Physiol., Lond.* **269**, 407.
Fulks, R. M., Li, J. B. & Goldberg, A. L. (1975). *J. biol. Chem.* **250**, 290.
Garlick, P. J. (1980). In *Protein Deposition in Animals* p. 51 [P. J. Buttery and D. B. Lindsay, editors]. London: Butterworths.
Gregg, V. A. & Milligan, L. P. (1980*a*). *Gen. Pharmac.* **11**, 323.
Gregg, V. A. & Milligan, L. P. (1980*b*). *Biochem. Biophys. Res. Comm.* **95**, 608.
Gregg, V. A. & Milligan, L. P. (1982). *Can. J. Anim. Sci.* (In the Press).
Himms-Hagen, J. (1976). *A. Rev. Physiol.* **38**, 315.
Holmes, J. H. G. & Ashmore, C. R. (1972). *Growth* **36**, 351.
Ismail-Beigi, F. & Edelman, I. S. (1970). *Proc. Natl Acad. Sci. USA* **67**, 1071.
Lobley, G. E., Milne, V., Lovie, J. M., Reeds, P. J. & Pennie, K. (1980). *Br. J. Nutr.* **43**, 491.
Lobley, G. E., Reeds, P. J. & Pennie, K. (1978). *Br. J. Nutr.* **37**, 96A.
McBride, R. W., Jolly, D. W., Kadis, B. M. & Nelson, T. E. (1979). *J. Chromat.* **168**, 290.
McKee, E. E., Cheung, J. Y., Rannels, E. & Morgan, H. E. (1978). *J. biol. Chem.* **253**, 1030.
Reeds, P. J., Cadenhead, A., Fuller, M. F., Lobley, G. E. & McDonald, J. D. (1980). *Br. J. Nutr.* **43**, 445.
Scholnick, P., Lang, D. & Racker, E. (1973). *J. biol. Chem.* **248**, 5175.
Steel, R. G. D. & Torrie, J. H. (1960). *Principles and Procedures of Statistics.* New York: McGraw-Hill, Inc.
Tobin, T. & Brody, T. M. (1972). *Biochem. Pharmac.* **21**, 1553.
Waalkes, T. P. & Udenfriend, S. (1957). *J. Lab. clin. Med.* **50**, 733.
Webster, A. J. F., Brockway, J. M. & Smith, J. S. (1974). *Anim. Prod.* **19**, 127.
Whittam, R. (1961). *Nature, Lond.* (1974). **191**, 603.

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