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Leucine Metabolism in Skeletal Muscle of the Tumour-bearing Rat

G. A. J. GOODLAD and C. M. CLARK

Department of Biochemistry and Microbiology, Irvine Building, University of St. Andrews, St. Andrews, Fife, Scotland, United Kingdom

Abstract—*Leucine oxidation was increased and its incorporation into protein decreased in incubated hemidiaphragms from rats bearing a Walker 256 carcinoma. Homogenates of diaphragms from these rats exhibited a moderate increase in branched-chain oxo-acid dehydrogenase activity but no change in leucine aminotransferase. Hemidiaphragms of tumour-bearing rats incubated with 0.1 mM leucine showed a decreased release of alanine, no alteration in glutamine release, and an increased output of ammonia. When glucose was added to the medium, leucine oxidation was still increased in hemidiaphragms of tumour-bearing rats, alanine release increased but only to control levels, ammonia release decreased and glutamine release remained at control levels. It was concluded that the increased leucine oxidation accompanying increased net protein breakdown in the tumour-bearing animal is not providing an increase in the output of gluconeogenic precursors.*

INTRODUCTION

SKELETAL muscle has been shown to be the major site of leucine catabolism in the body [1, 2]. In situations such as fasting or diabetes where there is a lack of available glucose, the oxidation of this amino acid by muscle is increased [3–6]. The carbon skeleton of leucine arising from protein degradation can thus serve as a metabolic fuel for muscle and this assumes more importance in the fasting state [7]. The amino group of leucine, along with those of valine and isoleucine, is available for the formation of the gluconeogenic precursors, alanine and glutamine which are released in increased amounts from muscle in fasting [8, 9].

Accompanying the growth of many tumours there is evidence of hypoglycaemia, decreased levels of plasma insulin and an increase in gluconeogenic processes [10–14]. Since depletion of muscle protein, due to both an increase in its rate of degradation and a decrease in its rate of synthesis, is a common systemic effect of a growing tumour [15–19], muscle amino acids might be called upon to play an important role in energy metabolism in the tumour-bearing subject. In the present work we have investigated this possibility with particular reference to the oxidation of leucine and the formation of alanine and glutamine in hemidiaphragms of rats bearing a Walker 256 carcinoma.

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MATERIALS AND METHODS

Materials

L-[1-^{14}C] Leucine (59 mCi/mmole), L-[U-^{14}C] leucine (324 mCi/mmole) were from the Radiochemical Centre Ltd., Amersham, Bucks, U.K.; NE 233 and NE250 liquid scintillators were from Nuclear Enterprises (U.K.) Ltd., Edinburgh; lactate and alanine dehydrogenases, ATP, NAD^+, NADH, pyridoxal phosphate and 4-methyl-2-oxopentanoate were from Sigma (London) Chemical Co., Poole, Dorset, U.K.; glutamine synthetase was prepared from *E. coli* [20]; all other chemicals were of the highest grade commercially available.

Animals

Female virgin Wistar rats of fasting body weight 190–210 g were inoculated with 0.2 ml of a suspension of Walker 256 carcinoma cells in the dorsal region as described previously [16]. Tumour-inoculated rats and groups of weight-paired control rats were housed individually on a 12 hr light–dark cycle (lights on 07.00 hr). Each rat was fed daily at

16.00 hr 12 g ground rat cake (North Eastern Cooperative Ltd., Aberdeen) made into a paste with water. During the experimental period none of the tumour-bearing rats exhibited anorexia and rats in both groups consumed all food offered to them. Tumour-bearing rats with their respective controls were killed 10–12 days after tumour inoculation between 09.00 and 10.30 hr. During this time tumour-bearing rats exhibited a mean loss of carcase weight (tumour weight excluded) of 2.4 g. No allowance was made for the considerable amount of oedematous fluid present in most tumour-bearing animals. Control rats showed a mean loss of 1.0 g in carcase weight over this period.

Incubations with hemidiaphragms

Hemidiaphragms were prepared as described previously [21], weighed and placed in flasks with a centre well and side-arm. The medium used was Krebs–Henseleit buffer [22] gassed with O_2/CO_2 (95:5). ^{14}C-labelled substrates were added to the incubation medium. Where the effect of a given metabolite was studied, one of a pair of hemidiaphragms was incubated with the metabolite while the other served as a control. Incubations were carried out for 90 min at 37°C in a metabolic shaker (100 oscillations/min). Since preliminary experiments indicated that more than 95% of the CO_2 produced was present in the medium at the end of the incubation the following procedure was followed. The flasks were placed in an ice bath and the muscle removed. Hyamine hydroxide (0.4 ml–1M in methanol) and a pleated strip of Whatman No. 1 filter paper were placed in the centre well and 1 ml 20% (w/v) H_2SO_4 in the side arm. Flasks were resealed, the acid tipped from the side-arm into the medium and the flask returned to the metabolic bath for a further hour. The filter paper was removed and counted in 17 ml NE233 scintillation fluid (59% efficiency) using an Intertechnique SL-30 scintillation counter. Radioactivity present as [1-^{14}C] 4-methyl-2-oxopentanoate, formed from leucine by transamination, was estimated as the additional $^{14}CO_2$ released from the medium by the addition of a saturated solution of $Ce(SO_4)_2$ in 3.9 M H_2SO_4 after $^{14}CO_2$ had been displaced [23].

To estimate the radioactivity present in the tissue as CO_2 and 4-methyl-2-oxopentanoate, diaphragms were removed from the flask at the end of incubation, blotted and homogenised in distilled water at 0°C with an Ultra-Turrax homogeniser (Janke und Kunkel Kg., Staufen i. Br., W. Germany). Radioactivity was measured in suitable aliquots as described above. Ice-cold 30% (w/v) trichloracetic acid was added to another aliquot to give a final concentration of 5%. The precipitate was centrifuged at 4°C washed three times with 5% (w/v) trichoracetic acid and dissolved by incubating in 0.3 M NaOH at 37°C. The protein content of the solution was estimated by the biuret reaction [24] and suitable aliquots counted on glass-fibre discs (82% efficiency).

Estimation of the specific activity of [1-^{14}C] 4-*methyl-2-oxopentanoate*

Two millilitres medium were sampled after incubation, acidified with 0.1 ml 3M HCl and extracted three times with 2 ml *n*-amyl alcohol by vortexing for 1 min followed by centrifuging for 10 min at 300 rev/min. The *n*-amyl alcohol extract was extracted with 2 ml 10% (w/v) sodium carbonate by vortexing for 1 min and the emulsion clarified by centrifugation. One and a half ml of the aqueous layer was cooled to 0°C and mixed with 1.5 ml 3M-HCl and 1 ml 15 mM 2:4-dinitrophenylhydrazine. The 2:-dinitrophenylhydrazone of 4-methyl-2-oxopentantanoate was extracted into 5 ml cyclohexane as described by Taylor and Jenkins [25]. One ml of this extract was counted in 5 ml NE233 scintillator (73% efficiency) and the amount of the 2:4-dinitrophenylhydrazone present in 3 ml extract was estimated following extraction into 1.5 ml 10% (w/v) sodium carbonate and measurement of the absorbance at 380 nm [23]. The molecular extinction of a purified sample of the dinitrophenylhydrazone in sodium carbonate was found to be 27.45/mmole/1.

Estimation of leucine decarboxylation and amino-transferase activities in muscle homogenates

Leucine decarboxylation by homogenates of diaphragm was assayed essentially as described by Paul and Adibi [6]. Diaphragms were homogenised at 0°C using a Potter–Elvehjem homogeniser in 9 volumes of a medium containing 50 mM Tris–HCl, pH 7.4, 100 mM KCl, 5 mM $MgSO_4$, 1 mM EDTA and 1 mM ATP. Homogenate (0.3 ml) was added to 2.7 ml medium containing 137.5 mM NaCl, 1.8 mM $MgSO_4$, 0.6 mM KH_2PO_4, 24.3 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 0.11 mM pyridoxal phosphate, 1.1 mM 2-

oxoglutarate, 2.2 mM NAD^+ and 1 mM-L-[1-^{14}C] leucine (62 μCi/mmole) in flasks adapted for CO_2 collection. Both homogenate and incubation media were kept for 5 min at 37°C before mixing. Incubations were carried out for 20 min in a metabolic shaker at 37°C. The reaction was stopped and the $^{14}CO_2$ and [1-^{14}C] 4-methyl-2-oxopentanoate formed were estimated as described above. The amount of leucine decarboxylated was expressed in nmoles using the specific activity of leucine present initially in the incubation medium. The amount of leucine transaminated was taken as the sum of the leucine decarboxylated and the [1-^{14}C] 4-methyl-2-oxopentanoate formed. Preliminary experiments showed that the enzyme reaction was linear with time up to at least 40 min.

Leucine aminotransferase was also measured in the absence of decarboxylation by carrying out the incubation in an atmosphere of nitrogen under conditions reported to give optimal activity [26]. Homogenates of diaphragm (5%, w/v) were prepared in a medium containing 50 mM Tris–HCl, ph 7.5 and 12 mM mercaptoethanol using an Ultra–Turrax homogeniser. Half a ml homogenate was incubated at 37°C in an atmosphere of nitrogen with 0.5 ml medium containing 20 mM L-[1-^{14}C] leucine (12.5 μCi/mmole), 20 mM 2-oxoglutarate, 0.2 mM pyridoxal phosphate and 20 mM sodium phosphate, pH 7.4 in a metabolic shaking bath (100 oscillations/min). The reaction was terminated by the addition of 0.1 ml 0.7 M HCl and 0.8 ml of the mixture was added to a suspension of 300 mg Amberlite CG-120 (H^+, coarse) in 1 ml water. This mixture was vortexed for 1 min to allow adsorption of unreacted ^{14}C-leucine and then centrifuged. An aliquot of the supernatant was counted in 5 ml NE250 scintillator (72.5% efficiency). Assay blanks were obtained by adding 0.1 ml 0.7 M HCl to the homogenate before adding it to the incubation medium. Activity was expressed as nmole leucine transaminated using the initial specific activity of the leucine in the medium. Leucine aminotransferase activity was found to be linear with time of incubation for periods up to 15 min and was proportional to the amount of tissue added over the range 0–40 mg. Estimation of [^{14}C] 4-methyl-2-oxopentanoate by decarboxylation with acidified ceric sulphate and by use of the cation-exchange resin gave the same result.

Alanine and aspartate aminotransferase activities of these homogenates were also estimated [27].

Estimation of free amino acids and ammonia in medium and tissue

At the end of the incubation period trichloracetic acid was added to the medium at 0°C to a final concentration of 5% (w/v) and any precipitated material was removed by centrifugation. Muscles were extracted at 0°C with 5% (w/v) trichloracetic acid. Trichloracetic acid was removed from the extracts with ether and amino acids estimated automatically on a Joel JLC-5AH autoanalyser. In some cases the specific activity of the leucine fraction was measured.

Alanine, glutamine and ammonia contents of the medium were also measured enzymically [20, 28, 29].

Estimation of pyruvate and lactate in medium and glycogen content of tissue

The amounts of pyruvate and lactate present in the medium at the end of incubation were estimated enzymically [30, 31]. Glycogen in hemidiaphragms was extracted with hot 40% (w/v) potassium hydroxide, precipitated with ethanol and estimated by the anthrone reaction.

RESULTS

Hemidiaphragms from normal and tumour-bearing rats were incubated in the presence of 0.1 mM-L-[1-^{14}C] leucine and the radioactivity appearing in various metabolites estimated (Table 1). The radioactivity present as CO_2 was significantly increased with hemidiaphragms from tumour-bearing rats. Less than 4% of the total $^{14}CO_2$ produced was present in the tissue. More than 98% of the radioactivity present as 4-methyl-2-oxopentanoate was found in the medium. The presence of the major part of this oxoacid in the medium and the fact that it can be metabolised when incubated with diaphragm [32, 33] indicates that it passes readily through cell membranes. Reports of the occurence of 4-methyl-2-oxopentanoate in plasma [34, 35] and urine [36] suggest that this metabolite is also formed and released from the tissue *in vivo*.

From the increase in the sum of the radioactivity present as $^{14}CO_2$ and ^{14}C-4-methyl-2-oxopentanoate it appears that more ^{14}C-leucine had undergone transamination in the hemidiaphragm of the tumour-bearing rat.

The radioactivity incorporated into tissue protein was significantly reduced in hemi-

Table 1. Radioactivity incorporated into $^{14}CO_2$, 4-methyl-2-oxopentanoate and tissue protein by hemidiaphragms from control and tumour-bearing rats following incubation with L-[1-^{14}C] *leucine**

	dis/min × 10^{-2}/100 mg diaphragm		
	Control	Tumour-bearing	*P*
(a) CO_2 in medium	93.7±7.0	129.2±6.0	<0.01
(b) CO_2 in tissue	3.0±0.2	3.0±0.2	N.S.
(c) Total CO_2 (a+b)	96.8±7.1	132.3±6.2	<0.01
(d) 4-methyl-2-oxo-pentanoate in medium	22.9±1.9	26.8±3.8	N.S.
(e) 4-methyl-2-oxo-pentanoate in tissue	0.4±0.04	0.4±0.05	N.S.
(f) Tissue protein	41.4±4.3	30.6±2.6	<0.05
(g) Total transaminase activity (c+d+e)	120.0±6.6	159.5±6.4	<0.01
(h) Total CO_2/tissue protein (c/f)	2.6±0.31	4.04±0.47	<0.02

*Initial specific activity—0.25 mCi/mmole.
Results are expressed as mean±S.E.M. of 7 rats. *P* was calculated using Student's paired *t*-test. Diaphragm weight per 100 g initial body weight: control rats, 197±6 mg; tumour-bearing rats, 152±2 mg. Mean tumour weight, 30±3 g.

Table 2. Production of lactate and pyruvate by hemidiaphragms from control and tumour-bearing rats incubated with 0.1 mM *leucine and* 10 mM *glucose*

	nmole/100 mg/90 min		Lactate/ pyruvate
	Lactate	Pyruvate	
Control	2458±159	107±8	23.2±1.0
Tumour-bearing	3720±318	150±11	24.8±1.3
P	<0.01	<0.01	N.S.

Results are expressed as the mean±S.E.M. of 8 rats. Diaphragm weight per 100 g initial body weight: control rats, 194±5 mg; tumour-bearing rats, 162±8 mg. Mean tumour weight, 28±3 mg. *P* was calculated using Student's *t*-test.

diaphragms from tumour-bearing rats which is in agreement with data obtained previously using ^{3}H-lysine [21]. The value of the ratio of radioactivity in CO_2 to that in protein in hemidiaphragms from tumour-bearing rats was increased suggesting that there is an alteration in the normal pattern of leucine metabolism in these muscles.

Although the initial weights of control and tumour-bearing rats were similar, at the time of sacrifice the mean weights of diaphragms of tumour-bearing rats were 20–24% less than those of control rats (see legends to tables). Experiments were undertaken to establish whether this difference in size of the diaphragms from the two groups of rats might give rise to differences in the degree of oxygenation of the tissues under the incubation conditions employed which could affect both the penetration of substances into the tissues and their subsequent metabolism. Table 2 shows the lactate and pyruvate released into the medium when diaphragms were incubated with 0.1 mM leucine in the presence of 10 mM glucose. While there was an increased output of both metabolites from diaphragms of tumour-bearing rats, the lactate/pyruvate ratio was not significantly different, suggesting that the cytoplasmic NADH/NAD ratio and therefore the extent of oxygenation were similar in diaphragms from both groups of rats.

There is evidence that amino acids which are being incorporated into protein and those which are undergoing catabolism arise from different metabolic pools [37, 38]. Leucine undergoing catabolism is transaminated to 4-methyl-2-oxopentanoate and the occurence of measurable amounts of this metabolite in the incubation medium (Table 1) provides a suitable means of estimating the specific activity of that pool of leucine which is being oxidised. The oxoacid was isolated as the 2:4-dinitrophenylhydrazone following incubation of diaphragms with [1-^{14}C] leucine at an initial concentration of 0.1 mM and a specific activity of 555 dis/min/nmole.

The mean values obtained from five experiments for the dinitrophenylhydrazone at the end of incubation were 116±7 (S.E.M.) and 127±9 dis/min/nmole for the control and tumour-bearing rats respectively. The difference between these two values was not statistically significant. It would appear therefore that the increase in $^{14}CO_2$ production by diaphragms of tumour-bearing rats is not a consequence of an increase in the specific activity of the intracellular leucine pool but represents an increase in the amount of the amino acid being decarboxylated. Table 3 shows the result of a series of experiments carried out under similar conditions to those reported in Table 1 in which the amounts and radioactivity of leucine in the tissue and in the medium at the end of incubation were measured. The release of some other amino acids into the medium was also measured in these experiments (Table 7). An increase in the content and a decrease in the specific activity of leucine in the medium was observed with hemidiaphragms from control and tumour-bearing rats. This would suggest that a significant amount of unlabelled leucine has entered the leucine pool. This would also account for the lower level of the specific activity of the tissue leucine compared to that of the leucine in the medium. Buse *et al.* [3] have reported a similar difference between the specific activities of tissue and medium leucine with incubated diaphragms. An estimate of the amount of unlabelled leucine arising from protein breakdown can be obtained from the amount of tyrosine released into the medium and the leucine content of diaphragm protein [8]. From the tyrosine in the medium at the end of the incubation (Table 7) it can be calculated that per 100 mg diaphragm, 138 and 166 nmole respectively of leucine could be supplied from this source with muscles from control and tumour-bearing rats.

To investigate whether the tumour affects the ability of the diaphragm to metabolise the carbon skeleton of leucine after the decarboxylation of carbon-1, one hemidiaphragm from each rat was incubated with L-[1-^{14}C] leucine and the other with L-[U-^{14}C] leucine, both substrates having the same specific activity. As seen in Table 4 the amount of $^{14}CO_2$ produced from L-[U-^{14}C] leucine by muscles of control and tumour-bearing rats was 61 and 72% respectively of that found with L-[1-^{14}C] leucine. If no catabolism of the carbon chain occurred beyond the initial decarboxylation step, the radioactivity in the $^{14}CO_2$ would not exceed one sixth of that obtained with the L-[1-^{14}C] leucine. The result obtained indicates therefore that in both cases metabolism of the carbon skeleton had proceeded beyond carbon-1, a finding in agreement with observations made by other workers using normal animals [37, 39]. While the amount of $^{14}CO_2$ produced from [U-^{14}C] leucine by hemidiaphragms of tumour-bearing rats exceeded that obtained with hemidiaphragms of control animals, the values of the

Table 3. Content and radioactivity of leucine in medium and tissue at end of incubation

	Control	Tumour-bearing
Amount of leucine in medium (nmole/3 ml)	350±15	341±11
Radioactivity of leucine in medium (dis/min/nmole)	382±20	399±3
Concentration of leucine in hemidiaphragm (nmole/100 mg)	26±4	25±5
Radioactivity of leucine in hemidiaphragm (dis/min/nmole)	142±3	154±12
Estimated amount of leucine arising from protein degradation per 100 mg diaphragm*	138±10	166±13
Mean weight hemi-diaphragm incubated (mg)	205±11	156±17

Results are the mean±S.E.M. of 4 experiments. Mean tumour weight, 31±3 g. Medium contained initially 300 nmole [1-^{14}C]leucine, specific activity, 555 dis/min/nmole.

*Calculated from the amount of tyrosine released into medium (Table 8).

Table 4. Comparison of $^{14}CO_2$ production from L-[1-^{14}C] leucine and L-[U-^{14}C] leucine* by hemidiaphragms from control and tumour-bearing rats*

	$^{14}CO_2$ produced dis/min × 10^{-2}/100 mg diaphragm)		
	Control	Tumour-bearing	*P*
L-1-^{14}C leucine	78±10	101±10	<0.01
L-U-^{14}C leucine	48±9	73±13	<0.01
$\frac{\text{L-U-}^{14}\text{C leucine}}{\text{L-1-}^{14}\text{C leucine}}$	0.61±0.04	0.72±0.08	N.S.

*Initial specific activity, 0.25 mCi/mmole.

Results are expressed as mean±S.E.M. of 5 observations. Statistical analysis was carried out using analysis of variance. Diaphragm weight per 100 g initial body weight: control rats, 197±11 mg; tumour-bearing rats, 152±7 mg. Mean tumour weight, 38±3 g.

ratio:

$$\frac{^{14}CO_2 \text{ from U-}^{14}\text{C leucine}}{^{14}CO_2 \text{ from 1-}^{14}\text{C leucine}},$$

obtained with diaphragms from both groups of rats did not differ significantly, indicating that metabolism of carbon atoms 2–6 of the amino acid was proceeding normally in the muscle of the tumour-bearing animal.

Leucine metabolism was also studied using homogenates prepared from diaphragms from control and tumour-bearing rats. Under conditions optimal for transamination and also under conditions optimal for decarboxylation [6, 26], leucine aminotransferase activity of preparations from tumour-bearing rats did not differ significantly from that observed with preparations from control rats (Table 5). The levels of alanine and aspartate aminotransferase activities were also unaltered in diaphragms of tumour-bearing rats. In contrast to the lack of effect of the tumour on aminotransferase activities, the ability of muscle homogenates from tumour-bearing rats to decarboxylate leucine showed a moderate (20%) but significant increase over that observed with homogenates from control rats.

The catabolism of leucine by incubated hemidiaphragms of normal and diabetic rats has been reported to be affected by the addition of glucose, pyruvate or octanoate to the medium [3, 6, 32]. The effect of these three metabolites on $^{14}CO_2$ production from ^{14}C-leucine by hemidiaphragms of tumour-bearing rats is shown in Table 6. Analysis of variance of the data obtained showed that 5 mM sodium pyruvate caused a significant ($P<0.01$)

Table 5. Transamination and decarboxylation reactions in cell-free extracts of diaphragms from control and tumour-bearing rats

	nmole/min/100 mg wet wt.		
	Control	Tumour bearing	P
Leucine decarboxylated (6)	1.7±0.2	2.0±0.2	<0.05
Leucine aminotransferase (4)*	21.5±0.5	22.1±1.4	N.S.
Leucine aminotransferase (8)†	84.6±5.2	79.0±5.1	N.S.
Alanine aminotransferase (3)	740±74	719±70	N.S.
Aspartate aminotransferase (3)	6520±170	6400±290	N.S.

*Leucine aminotransferase assayed under conditions optimal for assay of leucine decarboxylation (see text).
†Leucine aminotransferase assayed under nitrogen (see text). Results are expressed as mean±S.E.M. The number of observations is shown in parenthesis. P was calculated using Student's t-test. Diaphragm weight per 100 g initial body weight: control, 208±4 mg; tumour-bearing, 161±5 mg. Mean tumour weight, 33±9 g.

Table 6. The effect of pyruvate, octanoate and glucose on $^{14}CO_2$ production from ^{14}C leucine by hemidiaphragms from control and tumour-bearing rats*

	$^{14}CO_2$ produced (dis/min × 10^{-2}/100 mg diaphragm)	
	Control	Tumour-bearing
0.1 mM L-[1-^{14}C] leucine (4)	61.3±3.2	79.4±10.5
0.1 mM L-[1-^{14}C] leucine + 5 mM sodium pyruvate (4)	33.4±0.5	64.8±4.1
0.1 mM L-[1-^{14}C] leucine (4)	66.4±5.8	103.4±3.4
0.1 mM L-[1-^{14}C] leucine + 1 mM sodium octanoate (4)	82.5±4.5	128.9±10.8
0.1 mM L-[U-^{14}C] leucine (5)	44.4±2.8	73.6±5.1
0.1 mM L-[U-^{14}C] leucine + 10 mM glucose (5)	47.1±4.1	63.6±2.2

*Initial specific activity 0.25 mCi/mmole. The number of observations is shown in parenthesis. Results are expressed as the mean ±S.E.M. Diaphragm weight per 100 g initial body weight: control rats, 210±4.9 mg; tumour-bearing rats, 141±5.0 mg. Mean tumour weight, 33±2 g.

inhibition while 1 mM sodium octanoate brought about a significant ($P<0.025$) increase in $^{14}CO_2$ production by hemidiaphragms from both control and tumour-bearing rats. Glucose at a concentration of 10 mM had no effect on $^{14}CO_2$ production. The leucine-oxidizing system in the diaphragm of the tumour-bearing rat responded to all three metabolites in a manner similar to that observed with muscles from control animals. In the presence of all three substances the increased oxidation of leucine by hemidiaphragms of tumour-bearing rats persisted ($P<0.01$).

The release of alanine and some other amino acids by hemidiaphragms of control and tumour-bearing rats was was also studied when 0.1 mM leucine was present initially in the medium. Table 7 shows that hemidiaphragms from tumour-bearing rats released less alanine than did muscles from control rats. The release of tyrosine, phenlalanine and methionine on the other hand was significantly increased with muscles of tumour-bearing rats. This may only reflect a diminished demand for amino acids for protein synthesis but the possibility exists that an increase in protein degradation may also be a factor. In experiments with isolated muscle fibres from tumour-bearing subjects a diminution in protein synthesis was observed which was not accompanied by any alteration in the degree of reutilisation of leucine but was associated with an increase in the rate of protein degradation [17, 40]. The release of alanine, glutamine and ammonia was also studied when hemidiaphragms were incubated with 0.1 mM leucine in the presence of 10 mM glucose (Table 8). The addition of glucose to the medium increased the amount of alanine released by muscles of tumour-bearing rats to the level observed with muscles of control animals. The release of alanine by hemidiaphragms of control rats was not affected by glucose. There was no difference between the amount of glutamine released by muscles of control and tumour-bearing rats. In the presence of 10 mM glucose there was a tendency for the output of the amide to be increased in both cases. In the absence of exogenous glucose, hemidiaphragms of tumour-bearing rats

Table 7. Amino acids released by hemidiaphragms from normal and tumout-bearing rats incubated with 0.1 mM *leucine*

Amino Acid	nmoles/100 mg diaphragm/90 min		P
	Control	Tumour-bearing	
Alanine	228±10	164±12	<0.02
Methionine	36±1	47±2	<0.02
Phenylalanine	47±3	62±2	<0.02
Tyrosine	44±2	54±2	<0.02

Amino acids analysed by automatic ion-exchange chromatography. Results are expressed as the mean ±S.E.M. of 4 experiments. Diaphragm weight per 100 g initial body weight; control rats, 211±6 mg; tumour-bearing rats, 161±7 mg. Mean tumour weight, 31±3 g. P was calculated using Student's paired t-test.

Table 8. Release of alanine, glutamine and ammonia by hemidiaphragms of control and tumour-bearing rats in presence of 0.1 mM leucine and 10 mM *glucose*

	mM Glucose	nmole/100 mg diaphragm/90 min		P
		Control	Tumour-Bearing	
Alanine	0	210±7	150±5	<0.01
(4)	10	208±11	199±5	N.S.
		N.S.	P<0.02	
Glutamine	0	645±22	653±43	N.S.
(7)	10	729±17	709±34	N.S.
		P<0.05	N.S.	
Ammonia	0	175±36	234±30	<0.05
(6)	10	141±39	158±21	N.S.
		N.S.	N.S.	

Metabolites assayed enzymically (see Methods). Results are expressed as the mean±S.E.M. The number of animals in each group is shown in parenthesis. P was calculated using Student's paired t-test. Mean diaphragm weight per 100 g initial body weight: control rats, 192 ±7 mg; tumour-bearing rats, 151±4 mg. Mean tumour weight, 24 ±2 g.

released significantly more ammonia than did diaphragms from control rats. The addition of glucose to the medium had no effect on ammonia release by control diaphragms but decreased that from tumour-bearing rat diaphragms to a mean value similar to that of control diaphragms. This decrease, which just failed to attain statistical significance, was equivalent in magnitude to the increased release of alanine from hemidiaphragms of tumour-bearing rats due to the presence of glucose.

The alteration in muscle glycogen levels during incubation was also studied in hemidiaphragms incubated in the absence of exogenous glucose. No significant difference was observed in the amount of glycogen degraded in the two groups of muscles (Table 9).

Table 9. Glycogen breakdown in hemidiaphragms of control and tumour-bearing rats incubated with 0.1 mM *leucine*

incubation Time (min)	μg glycogen/100 mg diaphragm	
	Control	Tumour-bearing
0	266±20	211±40
90	65±7	42±9

Results are expressed as mean±S.E.M. of 4 rats. Diaphragm weight per 100 g initial body weight: control rats, 184±8 mg; tumour-bearing rats, 148±8 mg. Mean tumour weight, 31±3 g.

DISCUSSION

The results obtained in the present work suggest that hemidiaphragms from rats with a Walker carcinoma have an increased ability to catabolise leucine associated with an increased level of branched-chain oxo-acid dehydrogenase, the rate-limiting enzyme in the oxidation of branched-chain amino acids in skeletal muscle [33, 35]. It would also appear that the diaphragm of the tumour-bearing rat can oxidise the decarboxylated carbon skeleton of leucine as extensively to CO_2 as the diaphragm of the control rat (Table 4). This suggests that although decreased levels of coenzyme A have been reported in cancer patients and tumour-bearing animals [41], this factor is not limiting in the oxidation of branched-chain amino acids. The importance of leucine as a metabolic fuel and its involvement in the alanine-glucose cycle in muscle has been stressed by several workers [7, 26]. In other situations where there is an increase in leucine oxidation there is a corresponding increase in the release of alanine by muscle [8, 42]. In the present work the amount of alanine released during incubation in a glucose-free medium in the presence of leucine was significantly less in the case of hemidiaphragms from tumour-bearing rats (Table 7) despite the fact that, in addition to any increased formation from pyruvate related to an increased leucine oxidation, the amount of alanine which would arise from the net protein loss, as estimated from the tyrosine content of the medium [8], is greater in muscles from tumour-bearing rats, viz. 177 ±14 nmole 100 mg diaphragm compared with 142±9 nmole/100 mg diaphragm for control animals. It has been suggested that glucose is the source of the carbon skeleton of alanine synthesised in muscle [8]. When 10 mM glucose was present in the incubation medium the output of alanine by hemidiaphragms of tumour-bearing rats showed an increase but only to levels in control animals (Table 7) while the oxidation of leucine was still significantly elevated. Glucose had no effect on alanine release from hemidiaphragms of control rats. One explanation of the low output of alanine from muscles of tumour-bearing rats in the absence of exogenous glucose might be that pyruvate was limiting for transamination reactions despite the fact that glycogen breakdown was not significantly altered in these tissues. This lack of correlation between alanine release and leucine oxidation in muscles of the tumour-bearing rat would appear to distinguish the action of the tumour from that of starvation or diabetes on muscle amino acid metabolism [8, 42].

The release of the other main gluconeogenic amino acid, glutamine, was similar with incubated diaphragms from control and tumour-bearing rats in the presence and absence of glucose (Table 8). It has been realised for some time that skeletal muscle can produce ammonia from amino acids by a process which involves a transamination with oxaloacetate to form aspartate which subsequently participates in a purine nucleotide cycle [43]. In the absence of exogenous glucose incubated diaphragms from rats with a Walker tumour released significantly more ammonia than did hemidiaphragms from control animals. When glucose was added to the medium there was a decrease in the ammonia release by muscles from the tumour-bearing rat which corresponded to the observed increase in alanine output (Table 8).

While we have obtained evidence of an increase in the catabolism of leucine in skele-

tal muscle of the tumour-bearing rat, the fate of the amino groups arising from this is obscure and a more detailed study of the nitrogenous components of the system is required. It would appear, however, that the net increase in muscle protein breakdown associated with the growth of a tumour [15, 18, 40] and which is occuring in the present instance, is not providing increased amounts of gluconeogenic substrates for the enhanced gluconeogenisis reported to accompany tumour growth [13, 14].

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