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Hepatic Synthesis of Carnitine from Protein-Bound Trimethyl-lysine

LYSOSOMAL DIGESTION OF METHYL-LYSINE-LABELLED ASIALO-FETUIN

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The biosynthesis of carnitine in the rat was studied by following the metabolism of two radioactive derivatives of asialo-fetuin. The first contained ^{14}C-labelled methyl groups covalently bound to the 6-*N*-amino fraction of its lysine residues as 6-*N*-monomethyl- and dimethyl-lysine. By treating this protein with iodomethane, a second derivative was produced in which the radioactivity was preferentially incorporated as 6-*N*-[*Me*-^{14}C]-trimethyl-lysine. These desialylated glycoproteins, like other asialo-proteins, were immediately cleared from the blood by rat liver. Within hepatocyte lysosomes, the ^{14}C-labelled proteins were rapidly hydrolysed, producing free amino acids containing the various 6-*N*-[*Me*-^{14}C]methylated lysine residues. The radioactive amino acids crossed the lysosomal membrane and were further metabolized in the cytosol. Carnitine was the major radioactive metabolite detected in extracts of rat carcass and liver after intravenous injection of 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-fetuin. Within 3h, at least 34.6% of the trimethyl-lysine in the administered protein was converted into carnitine. Similarly, an isolated perfused rat liver converted 30% of the added peptide-bound trimethyl-lysine into carnitine within 90min. On the other hand, in numerous attempts we failed to detect radioactive carnitine in both rat liver and carcass between 20min and 22h after injection of 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin. These data provide evidence for a pathway of carnitine biosynthesis that involves trimethyl-lysine as a peptide-bound precursor as proposed by R. A. Cox & C. L. Hoppel [(1973) *Biochem. J.* **136**, 1083–1090] and V. Tanphaichitr & H. P. Broquist [(1973) *J. Biol. Chem.* **248**, 2176–2181]. The findings also show that rat liver can synthesize carnitine without the aid of other tissues, but cannot convert free partially methylated lysines into trimethyl-lysine.

Cox & Hoppel (1973*a*) and Tanphaichitr & Broquist (1973) have shown that the free amino acid, 6-*N*-trimethyl-lysine, is a precursor of carnitine (3-hydroxy-4-*N*-trimethylaminobutyrate) in the rat. However, it has been suggested (Cox & Hoppel, 1973*b*; Horne & Broquist, 1973; Cantoni, 1975; Paik & Kim, 1975) that this precursor is not directly synthesized from free lysine, but appears initially bound within polypeptide chains of protein, the degradation of which must be a prerequisite for the biosynthesis of carnitine. This unproven hypothesis is based both on the existence of methylases capable of converting lysine residues on protein into 6-*N*-trimethyl-lysine and the apparent lack of mammalian enzymes that methylate free lysine (Paik & Kim, 1975).

We have obtained evidence strongly supporting this thesis by studying the metabolism of two radioactive derivatives of the glycoprotein, asialo-fetuin. One derivative (6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-fetuin) contains ^{14}C preferentially incorporated into the methyl groups of 6-*N*-trimethyl-lysine. The second protein derivative (6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin) also contains methylated lysine residues, but only as the partially methylated structures, 6-*N*-monomethyl- and dimethyl-lysine.

We have previously shown (LaBadie *et al.*, 1975) that ^{125}I-iodinated asialo-fetuin rapidly accumulates within rat liver lysosomes after intravenous administration and is degraded therein, producing free amino acids. These products are then metabolized in other parts of the cell and the metabolites leave the liver within 1h. It was considered that if the methylated asialo-fetuin derivatives were processed in a similar manner, then the resulting ability to produce what is essentially a pulse-labelling of the liver with either trimethyl-lysine or a mixture of monomethyl- and

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dimethyl-lysine would be of some advantage in studying the biosynthesis of carnitine.

Our findings indicate that the methylated asialofetuins are extensively degraded by rat liver lysosomal enzymes, both *in vivo* and *in vitro*. The radioactivity from the methyl-lysine products was subsequently found in a variety of other metabolites. Only after administration of 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-fetuin was radioactivity detected in extractable carnitine. Further, it was shown that isolated perfused rat liver can also catalyse the synthesis of radioactive carnitine from 6-*N*-[*Me*-^{14}C]-trimethyl-lysine-labelled asialo-fetuin without the assistance of other tissues.

Experimental

Intravenous administration of radioactive proteins, fractionation of liver homogenates, preparation of ^{125}I-labelled asialo-fetuins, digestion of proteins within intact lysosomes and by lysosomal extracts *in vitro*, and chromatography of digests on Sephadex G-25 (fine grade), were done as previously described by LaBadie *et al.* (1975).

Materials

Chromatography standards of 6-*N*-monomethyl-L-lysine and DL-carnitine were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 6-*N*-Dimethyl-L-lysine was purchased from Cyclo Chemical Co., Los Angeles, CA, U.S.A. 6-*N*-Trimethyl-L-lysine was synthesized by the method of DeLange *et al.* (1969).

Methylation of fetuin

Fetuin (Grand Island Biological Co., Grand Island, NY, U.S.A.) was methylated with [^{14}C]-formaldehyde (New England Nuclear Corp., Boston, MA, U.S.A.) by the procedure of Means & Feeny (1968). This method results in the nearly exclusive incorporation of radioactivity into the lysine residues of the peptide as 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine residues.

To prepare the labelled trimethyl-lysine-containing protein, 20mg of the 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled fetuin, in 7ml of 1% (w/v) NaCl, was added to 40ml of methanol in a round-bottom flask. Non-radioactive methyl iodide (10ml) was then added to the slightly cloudy solution, and the pH was adjusted to greater than 10 (as measured with indicator paper) by dropwise addition of NaOH-saturated methanol. Periodic re-adjustment of the pH was made during an 18h gentle reflux of the mixture. The organic solvents were removed under reduced pressure and the resulting acidic solution was neutralized with NaOH. Tris buffer (1M; pH7) was added to a concentration of 0.1M, and the product was left at 4°C overnight. The solution of modified protein, containing some insoluble material, was then applied to a column (2.5cm × 45cm) of Sephadex G-100 equilibrated with 1% NaCl. Nearly all of the radioactive and u.v.-absorbing components in the sample were eluted in a single coincident peak, across which there was an essentially constant specific radioactivity. These fractions were pooled and concentrated by ultrafiltration. Some 67% of the methylated protein was recovered, and the 6-*N*-[*Me*-^{14}C]-trimethyl-lysine-labelled fetuin product had a specific radioactivity (4.68×10^6 d.p.m./mg) identical with that of its precursor, the partially methylated fetuin.

The radioactive components of the modified proteins were analysed after hydrolysis in 6M-HCl. Determinations were performed on a Beckman amino acid analyser by using System I of Kuehl & Anderson (1969) and on a Technicon analyser by using the carnitine program described below. Com-

Table 1. *Analysis of methyl-lysine residues in methylated fetuin derivatives*

Carnitine analysis procedure is described in the text. The analysis performed on a Beckman analyser used System I of Kuehl & Anderson (1969); monomethyl- and dimethyl-lysine were not resolved by this procedure.

	Radioactivity (% of total in protein derivative)					
	6-*N*-[*Me*-^{14}C]-Monomethyl- and -dimethyl-lysine-labelled fetuin			6-*N*-[*Me*-^{14}C]Trimethyl-lysine-labelled fetuin		
Analytical procedure ... Amino acid	Technicon	Beckman	Average value	Technicon	Beckman	Average value
Monomethyl-lysine	25.7	—		0	—	
Dimethyl-lysine	58.8	—		11.0	—	
Monomethyl- plus dimethyl-lysine	84.5	84.7	84.6	11.0	17.8	14.4
Trimethyl-lysine	0	0	0	79.8	73.6	76.7
Unknown aliphatic component	11.9	15.3	13.6	6.5	8.6	7.6

plete methylation of all 6-amino groups of lysines to quaternary amines was not achieved and 14.4% of the radioactivity remained as 6-*N*-[*Me*-^{14}C]dimethyl-lysine (Table 1). Radioactivity in 6-*N*-trimethyl-lysine accounted for an additional 76.6%, and the remainder was found in a single component eluted with the aliphatic amino acids. This latter substance probably arose from the methylation of the amino group of the *N*-terminal amino acid (Means & Feeny, 1968). Desialylation of the modified proteins (removal of 75–80% of the total sialic acid) was performed enzymically with 75μl of insolubilized neuraminidase obtained from Sigma Chemical Co. This reaction was performed for 22h at 37°C in 50mM-sodium acetate buffer, pH4.7, and at a substrate concentration of 1mg/ml.

Carnitine extraction and analysis

At 3h after intravenous administration of radioactive asialo-fetuin, the treated rat was killed by prolonged diethyl ether anaesthesia. The liver was removed, homogenized in ice-cold $HClO_4$ and frozen for separate analysis. Urine and faeces were collected during the experiment and also analysed for radioactivity. The carcass was extracted at 5°C with 1 litre of ice-cold redistilled methanol in a large blender. Three 1min blendings at high speed, separated by 1min intervals, were used to homogenize the carcass. Insoluble material was collected on a Buchner funnel, and the methanol filtrate was concentrated nearly to dryness under reduced pressure at 37°C. This latter residue was diluted to approx. 60ml with 0.2M-NaOH and heated with gentle shaking at 50°C for 1h. The cooled suspension was acidified (below pH2) with 2M-HCl, and its lipids were extracted with an equal volume of chloroform/methanol (5:1, v/v), followed by a second treatment with 30ml of this organic solution. The resulting deep-yellow aqueous phase was concentrated under vacuum at 55°C, and the residue was dissolved in a minimum volume of water (approx. 5ml). This solution was desalted by adding 10vol. of ethanol, chilling the suspension in a solid CO_2/acetone bath and sedimenting the precipitated inorganic salts in a low-speed centrifuge at −15°C (approx. 1500*g* for 10min). The salts were dissolved in water and reprecipitated with ethanol. The combined ethanol solutions were taken to dryness and desalted again by the above procedure. The final desalted solution was dried, dissolved in water and assayed for carnitine as described below.

Liver carnitine was extracted by homogenizing the tissue with 3 × 6ml of 5% (w/v) $HClO_4$ per g of tissue. The combined extracts were adjusted to pH12 with 50% (w/v) KOH and heated at 50°C for 1h. The saponified extract was neutralized with 71% (w/v) $HClO_4$, and the precipitated $KClO_4$ was removed by centrifugation. The supernatant was dried and further desalted with ethanol as described above.

Carnitine was also isolated from freeze-dried perfusion medium to which 2mg of unlabelled carnitine had been added as a carrier. Three repetitive extractions of the dried perfusate were made by refluxing with ethanol. This combined extract was concentrated and desalted by cold-ethanol treatment (see above). The desalted solvent was evaporated and the residue dissolved in 0.5ml of water. A portion (0.25ml) was directly analysed for radioactive carnitine by ion-exchange chromatography (see below) and the remaining 0.25ml was saponified by adding 0.1ml of 1M-NaOH followed by heating at 80°C for 1h. The latter sample was acidified with 2M-HCl and diluted to 0.8ml with water before column analysis.

Carnitine analyses were performed with an ion-exchange system similar to that described by Horne *et al.* (1971). Samples were applied to a column (0.6cm × 100cm) of Technicon type A resin maintained at 56°C and equilibrated with sodium citrate buffer, pH3.35. All solutions applied to the column were prepared to be 0.25M-Na^+. A pH gradient, formed in a four-chamber gradient maker, was used to elute the column. The four 80ml chambers contained respectively: the citrate buffer, pH3.35; NaOH; sodium borate buffer, pH8.75; NaOH. The column flow rate was maintained at 0.5ml/min, and 3ml fractions were collected. Carnitine was assayed enzymically (Pearson *et al.*, 1969) in the fractions, and quaternary amines were detected qualitatively by the procedure of Wall *et al.* (1960). Carnitine was eluted sharply in one or two fractions.

Liver perfusion

A perfusion apparatus and medium as described by Gonzalez de Galdeano *et al.* (1973) was used. The animal was anaesthetized with sodium pentabarbital, the abdominal cavity was opened, the bile duct was cannulated with PE 10 Intramedic Tubing (Clay Adams, Parsippany, NJ, U.S.A.), and an 18 gauge cannula was inserted into the hepatic portal vein. After the cannula was tied in place, the inferior vena cava was cut and perfusate flow was begun at approx. 10ml/min. The liver was excised and when the perfusate appeared to be clear of erythrocytes, the liver was mounted above a collecting funnel to return the effluent to a heat and O_2 exchanger. Flow rate was then increased to 45ml/min. The total volume of perfusate was adjusted to 70ml and its temperature was maintained at 37°C.

Analysis of radioactivity

Radioactive samples (1ml) were counted in 10ml of scintillation fluid containing 6g of PPO (2,5-diphenyloxazole) and 75mg of dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] per litre of toluene/Triton X-100 (2:1, v/v) in a Beckman liquid-scintillation spectrometer. Efficiencies were

determined by the external-standard-ratio method. Small aqueous samples and column effluents were made to 1 ml with water before the addition of scintillation fluid. Tissue samples were first digested in 4 vol. of an organic base (Protosol, New England Nuclear Corp.) for 24h at 50°C.

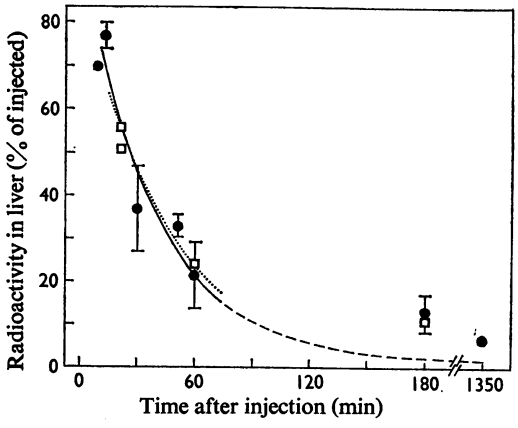

Fig. 1. *Radioactivity in rat liver after injection of 6-N-[Me-^{14}C]methyl-lysine-labelled asialo-fetuins*

The total radioactivity in rat liver was measured at various times after injection of either 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin or 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-fetuin. Data with S.D. bars are the average of two or three experiments. The lines drawn through the data points have been calculated by regression analysis to best fit a first-order decay curve. ●, ——, 6-*N*-[*Me*-^{14}C]-Monomethyl- and -dimethyl-lysine-labelled asialo-fetuin; □, ····, 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-fetuin; ----, extrapolation of the curve that was calculated from the data obtained between 12 and 60min after injection of the radioactive protein.

Results

Plasma and liver clearance of radioactivity

Intravenous administration of either methylated derivative of asialo-fetuin resulted in the rapid and nearly quantitative transfer of that protein from the blood to the liver. The half-time for this first-order process was 2.2 ± 0.9min (15 experiments) for the partially methylated glycoprotein and 3.7 ± 0.8min (four experiments) for 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-fetuin. The specificity of the uptake was shown by determining the amount of radioactivity in various tissues 13min after an injection of 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin. The lungs, spleen, heart and stomach combined contained less than 1% of the injected radioactivity. Blood accounted for another 1.6% of the radioactivity, and 5.3% was found in the kidneys. Nearly all of the remaining radioactivity was found in the excised liver (82.6%).

Although rapid, the blood clearance of 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-fetuin was somewhat slower than that of the partially methylated derivative. To ensure that trimethyl-lysine-labelled asialo-fetuin was cleared from blood by the same mechanism as other asialo-proteins (Ashwell & Morell, 1974), 5.4mg of unlabelled asialo-fetuin was mixed with 0.24mg of 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-fetuin, and the mixture was administered to a rat. The resulting half-time for clearance of ^{14}C from the blood increased more than 10-fold to 41min, indicating that the two proteins were competing for the same uptake system.

After the uptake of the asialo-proteins by the liver, the proteins were degraded in the lysosomes and the radioactive metabolites were released. The amount of radioactivity in the liver, after injection of asialo-fetuin labelled with 6-*N*-[*Me*-^{14}C]-labelled monomethyl- plus dimethyl-lysine or trimethyl-lysine

Table 2. *Distribution of radioactivity in rat liver fractions 20 min after injection of 6-N-[Me-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin, 6-N-[Me-^{14}C]trimethyl-lysine labelled asialo-fetuin, and ^{125}I-labelled asialo-fetuin*

Individual animals were injected with one of the three radioactive derivatives of asialo-fetuin, and after 20min the animals were killed and their livers removed, homogenized and fractionated as described previously (LaBadie *et al.*, 1975). Radioactivity was measured by the methods given in the text. The results are the average values from two experiments.

	Radioactivity (% of injected)		
Labelled protein ...	6-*N*-[*Me*-^{14}C]-Monomethyl- and -dimethyl-lysine	6-*N*-[*Me*-^{14}C]Trimethyl-lysine	^{125}I-labelled tyrosine
Fraction			
Liver homogenate	65	52	32
Nuclear	7.9	2.9	1.2
Heavy mitochondrial	3.6	5.6	1.3
Light mitochondrial	6.4	6.6	6.2
Microsomal	15	8.0	14
Final supernatant	30	24	7.7

reached a maximum at approx. 10min and decreased exponentially for 1h (Fig. 1). The half-time for this latter phase of heterophagy was 28.5min (10 experiments) for the monomethyl- and dimethyl-lysine-labelled derivative, and 31.8min (four experiments) for 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialofetuin.

Liver fractionation after injection of labelled asialofetuin

The respective distributions of radioactivity in liver homogenates 20min after administration of 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin, 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-fetuin and ^{125}I-labelled asialo-fetuin were examined. The content of protein and marker-enzyme activity in the fractions was normal and similar for all three glycoproteins (LaBadie *et al.*, 1975). However, great differences were found in the distribution of ^{14}C and ^{125}I (Table 2). The higher amount of ^{14}C in the tissue reflects the very rapid release of ^{125}I from the liver after the uptake of ^{125}I-labelled asialo-fetuin (LaBadie *et al.*, 1975). The major difference in the location of radioactivity from the two methylated proteins as compared with that from the iodinated asialo-fetuin was the high concentration of radioactive isotope in the final supernatant fraction. The concentration of radioactive metabolites in this liver fraction was three- to six-fold greater in animals treated with the proteins containing methyl-lysine. In over 30 separate experiments in which liver fractionation was done between 1.5 and 60min after injection of ^{125}I-labelled asialo-fetuin, the amount of radioactive isotope found in the supernatant was always less than 10% of the total radioactivity injected.

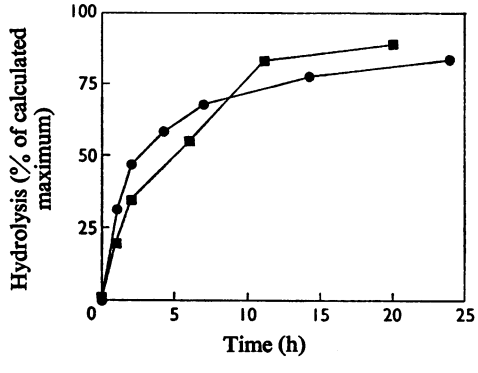

Fig. 2. *Hydrolysis of methylated derivatives of fetuin by lysosomal extracts in vitro*

In separate experiments, iced solutions of rat liver lysosomal extracts and radioactive protein were mixed in a buffered solution at pH4.7 as previously reported for the digestion of ^{125}I-labelled asialo-fetuin *in vitro* (LaBadie *et al.*, 1975). At zero time, the mixtures were incubated at 40°C and 50μl portions were periodically removed for ninhydrin assay. Corrections were made for incubations containing either enzyme or substrate alone, and the extent of fetuin hydrolysis was calculated as described by LaBadie *et al.* (1975). ●, 7.7mg of 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin incubated with 3mg of lysosomal protein in a total volume of 6ml; ■, 0.35mg of 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled fetuin incubated with 0.6mg of lysosomal protein in a total volume of 1ml.

Hydrolysis of methylated derivatives of asialo-fetuin in vitro

Iodinated asialo-fetuin was degraded within liver lysosomes after entering rat hepatocytes and was also degraded *in vitro* by lysosomal extracts (LaBadie *et al.*, 1975). Modification of fetuin by methylation did not affect the susceptibility of the protein to lysosomal hydrolysis. When 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin and 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled fetuin were incubated with extracts of highly purified lysosomes, no significant differences in the rate or the extent of peptide-bond cleavage was found for any of the derivatives. This hydrolysis was greater than 80% in 20h (Fig. 2) and is similar to that observed for the digestion of ^{125}I-labelled asialo-fetuin.

The nature of the radioactive products of the lysosomal hydrolysis of the ^{14}C-methylated fetuins was investigated. Samples of the digests described in Fig. 2 were analysed by gel filtration on a column (1.5cm × 95cm) of Sephadex G-25 (fine grade) as described by LaBadie *et al.* (1975). Nearly all of the radioactivity in a 24h digest of 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin was eluted in a single peak of material at the same volume as standard dimethyl-lysine. The presence of free 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine in the digest was confirmed by rechromatography of the pooled gel-filtration fractions on two ion-exchange systems and by t.l.c. (see Fig. 6) of a portion of the digest.

More than 80% of the radioactivity in a 20h lysosomal digest of 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled fetuin was eluted from a gel-filtration column at the same volume as standard trimethyl-lysine. Approx. 90% of the radioactivity in these fractions co-migrated with trimethyl-lysine on t.l.c. in the system described by Horne & Broquist (1973).

When fraction (L), obtained from the liver of a rat treated 13min before killing with 0.4mg of 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin, was incubated in iso-osmotic buffer, pH7 (LaBadie *et al.*, 1975), radioactivity in the non-sedimentable portion of the reaction mixture increased from 3% to 17% during the first 2h. A significant release of the lysosomal enzyme β-D-hexosaminidase did not occur. This indicated that

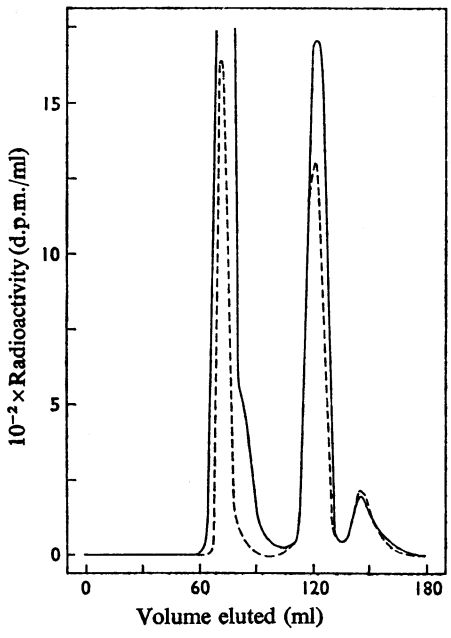

Fig. 3. *Separation of products released from intact lysosomes after digestion of methylated glycoprotein*

A 150g rat was treated with 0.4mg of 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin (3.22×10^6 d.p.m.) and killed 12min later. The liver was fractionated and the light mitochondrial fraction (L) was suspended in 10ml of iso-osmotic sucrose, pH7, (LaBadie *et al.*, 1975) and incubated at 30°C. At various times (120min in this Figure), 1ml portions of this reaction mixture were analysed on Sephadex G-25 (fine grade). ——, Total radioactivity obtained by treating the sample with 0.1ml of 20% (w/v) Triton X-100; – – – –, non-sedimentable radioactivity obtained by sedimenting the particulate material in the reaction at 31000*g* for 15min and analysing the supernatant fraction.

the protein was hydrolysed within intact lysosomes and that the radioactive products were released across the lysosomal membrane. Protein degradation during this reaction was also followed chromatographically (Fig. 3). A build-up of radioactive products was found which were eluted at the same position as standard dimethyl-lysine (component eluted at 130ml) and a small amount of radioactive material was detected in the region of the chromatogram where most free amino acids were eluted (elution volume 145ml). The radioactive hydrolysis products passed freely through the lysosomal membrane, since nearly all of the low-molecular-weight radioactive material (over 80% of the 6-*N*-[*Me*-^{14}C]methyl-lysine and 95% of the free amino acid component) was found in the supernatant after high-speed centrifugation of the reaction mixture (Fig. 3). On the other hand, only 10% of the corresponding high-molecular-weight radioactive component was in this supernatant fraction, presumably released from damaged lysosomes or phagosomes during incubation.

Degradation of 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin also occurred *in vivo*. The size of the labelled products produced in rat liver decreased during the first hour after injection of the radioactive protein as shown by the fraction of total liver radioactive material that was able to be dialysed. At 9, 30 and 60min after injection, 8, 73 and 95% of the radioactivity was recovered in a water diffusate of the homogenized liver. These diffusates were concentrated by freeze-drying and chromatographed on Sephadex G-25. Each gave a single radioactive component which was detected at the elution volume of dimethyl-lysine.

Attempts to detect the synthesis of carnitine from 6-N-[Me-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin

The incorporation of ^{14}C into carnitine from 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin was not seen in several different experiments. Ion-exchange chromatographic analysis of organic extracts of liver and muscle (Tanphaichitr *et al.*, 1971) at 3 and 22h after injection of this radioactive protein revealed the presence of numerous labelled components (at least 15), but no radioactivity was detected in those fractions containing carnitine.

Our failure to detect hepatic radioactive carnitine in the above experiments could be a consequence of the rapid transport of the newly synthesized carnitine to other tissues (Brooks & McIntosh, 1975). To test this possibility, a rat was injected with 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin and killed 3h later. The liver was removed for separate analysis and the remaining carcass was extracted with methanol. A carnitine analysis was then performed on the extract as described in the Experimental section. Three major and a number of minor radioactive components were detected (Fig. 4). The extracted carnitine in the sample was eluted entirely in two fractions. These fractions contained less than 200d.p.m. of ^{14}C, accounting for only 0.2% of the total radioactivity in the analysed sample. Thus the protein-bound partially methylated lysine residues that were released by the lysosomal hydrolysis of 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin did not serve as direct precursors of carnitine in the rat.

Detection of radioactive carnitine in rat liver and carcass after injection of 6-N-[Me-^{14}C]trimethyl-lysine-labelled asialo-fetuin

The livers of two rats were individually processed for carnitine analysis at 1 and 3h after the injection of

Table 3. *Analysis of rat liver for carnitine after injection of 6-N-[Me-^{14}C]trimethyl-lysine-labelled asialo-fetuin*

Two animals were given separate injections of 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-fetuin (9.2×10^6 and 2.4×10^6 d.p.m. respectively). After 1 and 3h the respective animals were killed and their livers removed and extracted to isolate carnitine. The final extracts were analysed by ion-exchange chromatography (see the text), and these extracts contained 70–71% of the total liver radioactivity. The recovery of the radioactivity from this column was 84–94%. At 1 and 3h, 24 and 12% respectively of the injected radioactive isotope was present in the liver.

Separated components			Radioactivity (% of total in liver)	
Order of elution	Identity	Time after injection ...	1h	3h
1	Unknown		7.8	18.9
2	Carnitine		24.2	28.8
3	Unknown		11.4	11.2
4	Trimethyl-lysine		12.8	2.3

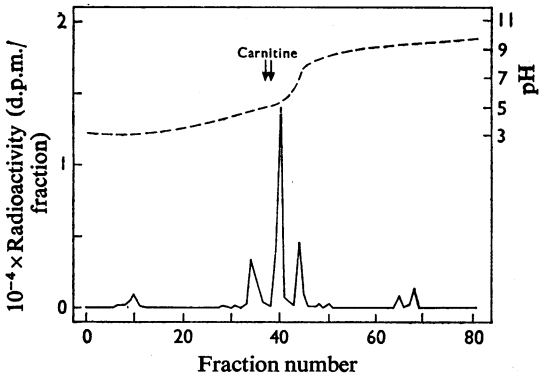

Fig. 4. *Ion-exchange analysis of the radioactive components of a saponified methanol extract of a rat carcass 3h after injection of 6-N-[Me-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin*

A 173g rat was treated with 0.41mg of 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin and killed 3h later. The carcass was then extracted and analysed as described in the Experimental section. Enzyme-assayable carnitine was eluted at the arrows. ----, pH of the column fractions.

6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-fetuin. Four major regions of radioactivity were detected by ion-exchange chromatography which accounted for over 98% of the radioactive isotope recovered from the column (Table 3). The radioactive component that was eluted in the second position coincided with enzyme-measurable carnitine. The fourth and final substance was eluted in the fractions that corresponded to 6-*N*-trimethyl-L-lysine.

In the 3h experiment, methanol extraction of the remaining carcass (minus the liver) was performed to determine the extent of overall incorporation of the ^{14}C radioactivity into carnitine. A 70% recovery of the injected radioactivity was found in the combined

Table 4. *Distribution of radioactivity recovered from rat carcass 3h after injection of 6-N-[Me-^{14}C]trimethyl-lysine-labelled asialo-fetuin*

A rat was given an injection of 0.4mg of 6-*N*-[*Me*-^{14}C]-trimethyl-lysine-labelled asialo-fetuin, and 3h later the animal was killed and its liver removed (see Table 3). The carcass was processed for carnitine extraction as described in the text (see Fig. 5). The amount of radioactive protein injected was 19.2×10^5 d.p.m. (100%).

Sample	Radioactivity (10^{-5} × d.p.m.)	(% of injected dose)
Methanol extract	8.2	43
(Saponified methanol extract)	(5.5)	(29)
Liver	2.2	12
Residue from carcass	1.6	8.6
Excrement	1.4	7.2
Total	13.4	71

methanol extract, liver, extracted carcass residue, and excrement, with the bulk of the labelled material being in the methanol extract (Table 4). On ion-exchange chromatography of the saponified methanol extract, it was found that a number of fractions, including those eluted with trimethyl-lysine, contained only low amounts of radioactivity. This total accounted for less than 11% of the applied radioactive label, and fraction 44 contained an additional 13% (Fig. 5). The remainder (76%) of the radioactivity recovered from the column was located in the single fraction that contained all of the enzyme-measurable carnitine. Standard carnitine and a large portion of the radioactivity present in the methanol extract also co-migrated on t.l.c. (Fig. 6). On the basis of column separation, and the amount of radioactivity in the saponified methanol extract, the total

carnitine-associated radioactivity found in the carcass extract was 21.8% of that injected into the animal.

Synthesis of carnitine from 6-N-[Me-^{14}C]trimethyl-lysine-labelled asialo-fetuin by perfused rat liver

When the trimethyl-lysine-labelled asialo-fetuin was added to the perfusate medium flowing in a closed system through an isolated rat liver, the circulating radioactivity decreased exponentially for approx. 15 min with a half-life of 6.0min (Fig. 7). A minimum concentration (8% of the initial perfusate concentration) was reached at 25min, followed by a continual increase in radioactivity in the perfusate for the duration of the experiment. Carnitine analysis of the liver 90min after addition of the modified protein revealed the presence of the same four radioactive compounds found in rat liver *in vivo* (Table 5). Trimethyl-lysine and carnitine accounted respectively for 22.5 and 25.6% of the liver radioactivity. The liver contained 61% of the added ^{14}C.

The perfusate was also processed for carnitine analysis, but differently from the liver or carcass extracts from the experiments done *in vivo*. One portion of the perfusate extract was not saponified and, on column analysis, this sample had a radioactive component not detected in the other analyses (Fig. 8). The new component was eluted between the first

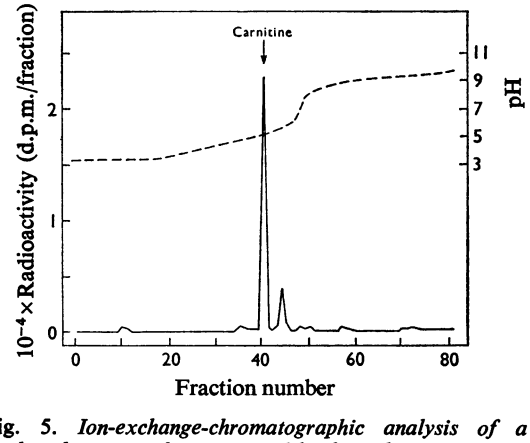

Fig. 5. *Ion-exchange-chromatographic analysis of a methanol extract of rat carcass 3h after administration of 6-N-[Me-^{14}C]trimethyl-lysine-labelled asialo-fetuin*

A 165g rat was given an intravenous injection of 0.4mg of 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-fetuin (1.9×10^6d.p.m.) and killed 3h later. The liver was removed and analysed separately (see Table 3), and the remaining carcass was extracted with methanol and analysed by ion-exchange chromatography as described in the text. Carnitine was eluted in a single fraction as indicated by the arrow. ----, pH of the column fractions.

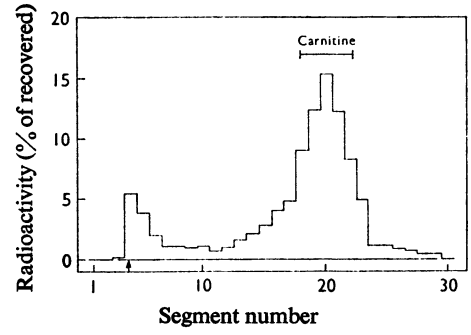

Fig. 6. *T.l.c. of a methanol extract obtained from the carcass of a rat given an injection of 6-N-[Me-^{14}C]trimethyl-lysine-labelled asialo-fetuin*

The saponified and desalted methanol extract (8μl; 1×10^4d.p.m.) described in the legend to Fig. 5 was applied to silica gel coated on a glass plate. The chromatogram was developed in methanol/aq. NH_3 (3:1, v/v) for a distance of 16cm. Segments (0.6cm) of the gel were scraped into scintillation vials for measurement of ^{14}C radioactivity. The bar indicates the migration of standard carnitine detected in an adjacent channel with I_2 vapour. The origin is indicated by the arrow.

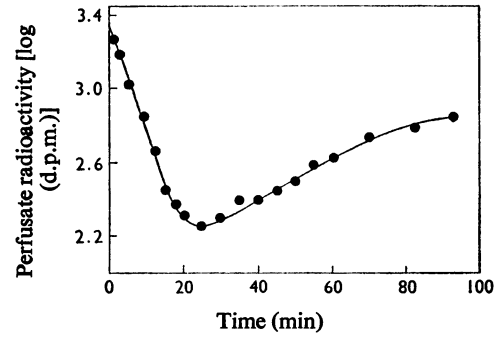

Fig. 7. *Radioactivity in perfusate medium after adding 6-N-[Me-^{14}C]trimethyl-lysine-labelled asialo-fetuin to a perfused rat liver*

A liver (7g) of a rat was perfused as described in the text, and 0.2mg of 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-fetuin (1.06×10^6d.p.m.) was added to the perfusate medium. During the next 90min, portions (0.2ml) of the perfusate were removed and their radioactivity was determined. A plot of the logarithm of radioactivity versus time was linear with time over the initial 16min. The slope of this line corresponds to a half-life of 6.0min.

labelled compound found *in vivo* and carnitine. Saponification of the perfusate before analysis caused the disappearance of this radioactive substance and a concomitant increase of a similar magnitude in the radioactivity that was associated with

Table 5. *Carnitine analysis in rat liver after perfusion with 6-N-[Me-^{14}C]trimethyl-lysine-labelled asialo-fetuin*

The liver and perfusate from the experiment described in the legend of Fig. 8 were analysed for carnitine by ion-exchange chromatography. The ethanol extracts of the liver and freeze-dried perfusate contained 79 and 55% respectively of the total radioactivity in each of those two samples. There was a 90–97% recovery of the added radioactivity from the ion-exchange column. The liver contained 61% of the added radioactivity and the perfusate contained 27%. One portion of perfusate sample was not saponified before ion-exchange analysis (see the text).

Separated component		Radioactivity (% of total in liver or perfusate)		
Order of elution	Identity	Liver	Perfusate (see Fig. 8)	Perfusate (saponified)
1	Unknown	11.5	1.6	1.7
2a	Acyl carnitine?	—	7.4	0
2	Carnitine	25.6	20.8	26.6
3	Unknown	10.0	11.1	10.5
4	Trimethyl-lysine	22.5	6.1	5.3

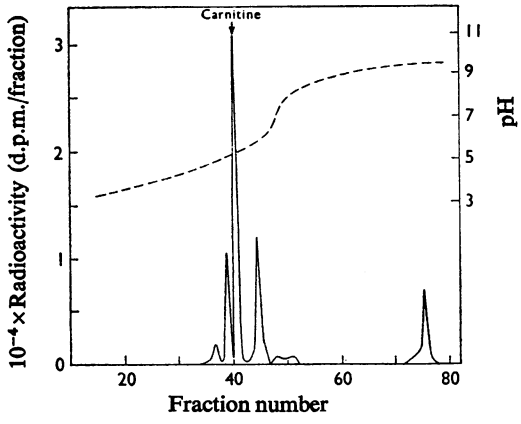

Fig. 8. *Ion-exchange-chromatographic analysis of perfusate medium from a perfused rat liver 90 min after the addition of 6-N-[Me-^{14}C]trimethyl-lysine-labelled asialo-fetuin*

The perfusion medium from the experiment described in the legend to Fig. 7 was collected 90min after the addition of the labelled asialo-fetuin and then freeze-dried. The resulting material was extracted with ethanol (see the text) and a portion (0.25ml) of this extract was analysed for radioactive carnitine. The arrow indicates the only fraction in which carnitine was detected enzymically. ----, pH of the column fractions.

carnitine. The distribution of all other radioactivity was not changed by saponification. Since this new radioactive component gave a positive reaction in the analytical test for quaternary amines, it has tentatively been considered to be acylated carnitine.

Discussion

Lysine and methionine have been shown to be precursors of carnitine in the rat. The 6-positioned nitrogen and four carbons of the lysine side chain form the carnitine backbone, and the methyl group of methionine is utilized in methylating the amino group. Low incorporation of these atoms of lysine can be demonstrated by administering the appropriately labelled molecule and analysing the newly synthesized carnitine (Tanphaichitr & Broquist, 1973). A much greater incorporation of radioactivity into carnitine has been demonstrated by using labelled 6-*N*-trimethyl-lysine as the precursor (Cox & Hoppel, 1973*a*; Tanphaichitr & Broquist, 1973). Other metabolites, intermediate in the pathway between trimethyl-lysine and carnitine, e.g. 4-*N*-trimethylaminobutyrate, are also actively incorporated into carnitine in the rat (Cox & Hoppel, 1973*b*). The metabolic conversion of free or partially methylated lysine directly into 6-*N*-trimethyl-lysine has not been demonstrated in mammalian systems, although this pathway appears to be present in *Neurospora* (Rebouche & Broquist, 1974). On this basis, 6-*N*-monomethyl- and -dimethyl-lysine should not serve as carnitine precursors, if mammalian systems are indeed unable to further methylate these free amino acids.

Our results show that the acid proteinases of rat liver lysosomes were able to hydrolyse extensively 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin. This hydrolysis, which released labelled monomethyl-lysine and dimethyl-lysine, occurred both *in vivo* and *in vitro*. In a pseudo system *in situ*, in which the radioactive protein was contained within intact lysosomes, hydrolysis was also observed and the radioactive products crossed the lysosomal membrane. On the basis of our experiments *in vitro*, lysosomal enzymes do not contribute to further metabolism of methylated lysine residues. The appearance of other labelled metabolites in rat liver extracts after injection of 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin was probably due to ε-alkyl-lysinase (Paik & Kim, 1974). The action of this demethylating enzyme

would have produced formaldehyde which then could have been redistributed as a member of the C_1 pool.

The high flux rate of carnitine between liver and blood (Brooks & McIntosh, 1975) could make it difficult to detect newly synthesized carnitine in rat liver. This was not the situation after injections of 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine-labelled asialo-fetuin as no labelled carnitine could be detected in methanol extracts of the carcass. The absence of labelled carnitine from this extract, despite the proven appearance of 6-*N*-[*Me*-^{14}C]-monomethyl- and -dimethyl-lysine in the hepatocyte cytosol, strongly suggests that rat liver does not have the enzymic capability to convert the free partially methylated lysine residues into trimethyl-lysine. The latter amino acid otherwise would have been actively transformed into carnitine.

In contrast with the metabolic fate of the partially methylated lysine residues in asialo-fetuin, our data show that the major radioactive product from 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-protein in rat liver is ^{14}C-labelled carnitine. Within 3h of intravenous injection of 6-*N*-[*Me*-^{14}C]trimethyl-lysine-labelled asialo-fetuin, carnitine isolated by methanol extraction of the rat carcass contained 21.8% of the injected radioactivity. Carnitine present in the liver accounted for an additional 4.8% of the radioactive isotope. However, this measured 26.6% incorporation of ^{14}C into carnitine should be considered a minimum value, since the efficiency of carnitine extraction was not known, and no corrections were made for losses incurred during the concentration, saponification, lipid extraction and desalting of the methanol extract. Additional carnitine may also have been contained in the radioactive excrements (Brooks & McIntosh, 1975). However, the percentage conversion of protein-bound labelled trimethyl-lysine into labelled carnitine can be adjusted for that portion of the radioactivity of modified asialo-fetuin which was present in residues other than trimethyl-lysine (Table 1), since these would not serve as carnitine precursors. On this basis, in 3h at least 34.7% of the protein-bound 6-*N*-trimethyl-lysine was converted into carnitine, and similarly the perfused liver converted 30% of this residue into carnitine in 90min.

Haigler & Broquist (1974) have determined that liver is the major site, perhaps the only site, of carnitine biosynthesis in the rat. Slices of liver, kidney, muscle, heart and testis were examined for their capacity to synthesize carnitine from either trimethyl-lysine or trimethylaminobutyrate. Approx. 7% of the added trimethyl-lysine was converted into carnitine. Except for the testis, none of the other tissues was able to catalyse the entire sequence of reactions between trimethyl-lysine and carnitine, although all were able to convert trimethyl-lysine into trimethylaminobutyrate. Our perfused liver experiment extends these observations by showing that isolated rat liver can synthesize carnitine from peptide-bound trimethyl-lysine.

These results, combined with the contrasting absence of carnitine synthesis from monomethyl- and dimethyl-lysine-labelled asialo-fetuin and yet equivalent lysosomal digestion of and product release from the latter glycoprotein are strong support for a pathway of carnitine biosynthesis in the rat that utilizes trimethyl-lysine attached to proteins. The unusual requirement of a peptide-bound amino acid for synthesis of carnitine, a metabolite that ultimately functions in the energy-yielding process of fatty acid oxidation, may involve an intricate physiological regulation of basic energy metabolism. Possibly there is a specific class of proteins whose function is to act as a depot of trimethyl-lysine residues, designated for carnitine biosynthesis after their release by controlled proteolytic digestion. However, we suggest (*a*) that there are large amounts of proteins that normally contain 6-*N*-trimethyl-lysine (such as histones and myosin) and (*b*) that this protein pool is normally turning over at reasonably rapid rates as shown by the high content of monomeric 6-*N*-trimethyl-lysine present in human urine (Kakimoto & Akazawa, 1970); and therefore, at the normal metabolic steady-state, hydrolysis of such proteins plus the intestinal digestion of dietary proteins releases sufficient free 6-*N*-trimethyl-lysine to meet effectively the metabolic demand for carnitine.

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References

Ashwell, G. & Morell, A. G. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol.* **41**, 99–128

Brooks, D. E. & McIntosh, S. E. A. (1975) *Biochem. J.* **148**, 439–445

Cantoni, G. L. (1975) *Annu. Rev. Biochem.* **44**, 435–451

Cox, R. A. & Hoppel, C. L. (1973*a*) *Biochem. J.* **136**, 1075–1082

Cox, R. A. & Hoppel, C. L. (1973*b*) *Biochem. J.* **136**, 1083–1090

DeLange, R. J., Glazer, A. N. & Smith, E. L. (1969) *J. Biol. Chem.* **244**, 1385–1388

Gonzalez de Galdeano, L., Bressler, R. & Brendel, K. (1973) *J. Biol. Chem.* **248**, 2514–2520

Haigler, H. T. & Broquist, H. P. (1974) *Biochem. Biophys. Res. Commun.* **56**, 676–681

Horne, D. W. & Broquist, H. P. (1973) *J. Biol. Chem.* **248**, 2170–2175

Horne, D. W., Tanphaichitr, V. & Broquist, H. P. (1971) *J. Biol. Chem.* **246**, 4373–4375

Kakimoto, Y. & Akazawa, S. (1970) *J. Biol. Chem.* **245**, 5751–5758

Kuehl, W. M. & Anderson, R. S. (1969) *Biochem. Biophys. Res. Commun.* **37**, 59–65
LaBadie, J. H., Peterson, K. & Aronson, N. N., Jr. (1975) *Biochem. J.* **152**, 271–279
Means, G. E. & Feeny, R. E. (1968) *Biochemistry* **7**, 2192–2201
Paik, W. K. & Kim, S. (1974) *Arch. Biochem. Biophys.* **165**, 369–378
Paik, W. K. & Kim, S. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol.* **42**, 227–286
Pearson, D. J., Chase, J. F. A. & Tubbs, P. K. (1969) *Methods Enzymol.* **14**, 615–616
Rebouche, C. J. & Broquist, H. P. (1974) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **33**, abstr. 1815
Tanphaichitr, V. & Broquist, H. P. (1973) *J. Biol. Chem.* **248**, 2176–2181
Tanphaichitr, V., Horne, D. W. & Broquist, H. P. (1971) *J. Biol. Chem.* **246**, 6364–6366
Wall, J. S., Christianson, D. D., Dimler, R. J. & Senti, F. R. (1960) *Anal. Chem.* **32**, 870–874