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Incorporation of DL-[2-^{14}C]Ornithine and DL-[5-^{14}C]Arginine in Milk Constituents by the Isolated Lactating Sheep Udder

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1. Lactating mammary glands of sheep were perfused for several hours in the presence of DL-[2-^{14}C]ornithine or DL-[5-^{14}C]arginine and received adequate quantities of acetate, glucose and amino acids. 2. In the [^{14}C]ornithine experiment 1·4% of the casein and 1% of the expired carbon dioxide came from added ornithine; 96% of the total radioactivity in casein was recovered in proline; 13% of the proline of casein originated from plasma ornithine. 3. In this experiment the results of chemical degradation of proline of casein as well as relative specific activities in the isolated products are consistent with the view that ornithine is metabolized, by way of glutamic γ-semialdehyde, to proline or glutamic acid. 4. In the [^{14}C]arginine experiments 3% of the casein and 1% of the expired carbon dioxide came from arginine; 84% of the arginine and 9% of the proline of casein originated from plasma arginine. 5. In these experiments the relative specific activities of arginine, ornithine and proline in plasma are in agreement with the view that arginine is metabolized by way of ornithine to proline. The conversion of arginine into ornithine is probably catalysed by arginase, so that arginase in mammary tissue may be involved in the process of milk synthesis.

In a series of measurements of arteriovenous differences across the udder of cows, we observed that the lactating udder absorbs important quantities of free ornithine from the bloodstream (Verbeke & Peeters, 1965). These observations were confirmed by Mepham & Linzell (1966) on the lactating goat. However, ornithine is absent from the milk proteins. On the basis of the biochemical relationship between ornithine, proline and glutamic acid (for review see Greenberg, 1961) we suggested that ornithine was acting as a precursor of proline in the udder of ruminants. Proline is a constituent of casein, but the amount absorbed from the bloodstream is inadequate to provide the corresponding residues in the milk proteins. Our hypothesis was studied by adding DL-[2-^{14}C]ornithine to the perfusion blood of an isolated sheep udder. It was shown that this precursor was incorporated into the proline of casein.

Arginine is another amino acid that is absorbed by the lactating udder of ruminants. In the cow the uptake seems to be of the same order as the amount secreted as casein arginine (Verbeke & Peeters, 1965). In the goat the amount taken up largely exceeds the quantities of arginine in milk proteins (Mepham & Linzell, 1966). On the other hand, it is known that arginase occurs in mammary tissue of ruminants though in much smaller amounts than in mammary tissue of rat and mouse (S. J. Folley & A. L. Greenbaum, unpublished work cited by Folley, 1949). It is obvious that arginase is involved in the synthesis of milk, but its precise role is unknown. When we observed that the isolated sheep udder converts free ornithine of the plasma into proline, we supposed that proline could also to some extent be derived from arginine via ornithine and that arginase could be involved in these reactions. This hypothesis was studied in perfusion experiments carried out on isolated sheep udders in which we added DL-[5-^{14}C]arginine to the perfusion blood. As expected, arginine acted as a precursor for casein proline. Preliminary accounts of part of this work have been published (Verbeke, Peeters, Cocquyt & Lauryssens, 1965; Verbeke, Massart-Leën & Peeters, 1966).

METHODS

Perfusion of mammary glands. Mammary glands were taken from sheep giving 25–38 ml. of milk/hr./gland. Apart from some minor modifications, the technique described by Hardwick & Linzell (1960) and Hardwick, Linzell & Price (1961) for the perfusion of the isolated goat udder was used. Before the experiment the animals were starved for 24 hr., but water was available *ad libitum* and glucose (100 g.) was administered twice by intravenous infusion. On the morning of the experiment the sheep was milked out. The

udder was then dissected, and the two halves were separated, removed and perfused independently. The surgical operations were performed on the living animal under general (fluothane and N_2O+O_2, 75:25) and epidural (2% xylocaine) anaesthesia. The glands were perfused at 36° and a constant pressure of 140 cm. H_2O. The perfusion circuit was an arrangement in series of reservoir, pump A, gland, outflow recorder, artificial lung and reservoir. This circuit was connected to an additional one of reservoir, pump B, artificial kidney and reservoir. The total capacity of the circuit was about 1·6 l. and usually contained 1 l. of perfusate. The glands were perfused with washed sheep erythrocytes suspended in a Krebs–Henseleit solution as modified by Hardwick *et al.* (1961) and containing proteins of sheep plasma (70 g./l.). The perfusate was oxygenated by a mixture of air+CO_2 (93:7) and O_2+CO_2 (93:7). The glands were emptied by milking after intra-arterial injection of 0·1 i.u. of oxytocin. Milking was performed at the start of perfusion, immediately before adding the labelled compounds and every hour thereafter. We started adding substrate solutions and labelled precursors to the blood 60–90 min. after the onset, and the perfusions were maintained for 7–8 hr. A substrate solution containing glucose, acetic acid, amino acids, proteins and labelled precursors was infused into the perfusate continously at 10–37 ml./hr. A second solution containing $CaCl_2$ and $MgCl_2$ was added at 2–10 ml./hr.

In Expt. 1 we dissolved 482 mg. of DL-ornithine + 50 μc of DL-[2-^{14}C]ornithine (Calbiochem, Los Angeles, Calif., U.S.A.) in 0·9% NaCl (final specific activity 0·22 μc/mg. of ornithine C). We added a quantity of this mixture containing 25·8 μc to 250 ml. of substrate solution. At the onset of the experiment 1·7 μc was added to the perfusion blood by way of the artificial lung and 22·4 μc to the kidney fluid.

In Expts. 2 and 3, DL-[5-^{14}C]arginine (C.E.A., Saclay, France) was diluted in the system with unlabelled DL-arginine. The L-arginine in the substrate (0·8 g./l.) was replaced by 1·6 g. of DL-arginine/l. A hydrolysate of casein, which had been freed of arginine by means of a column of Dowex 1 (OH^- form), was added to the kidney fluid. Afterwards DL-arginine was added to this fluid (11·75 mg./l.). The final specific activity in the system was 189·3 mμc/mg. of arginine C. At the onset 17·5 and 0·75 μc of [5-^{14}C]-arginine were added respectively to kidney fluid and perfusion blood. A total of 31·75 μc was added to 250 ml. of substrate solution but only part of this was infused during the experiments. In this way in Expts. 2 and 3 the total radioactivity added amounted to 36·8 and 37·7 μc respectively.

Analytical methods. Total N, non-casein N and non-protein N were determined in milk by a micro-Kjeldahl procedure. Colorimetric methods were used for the determination of citric acid (Natelson, Pincus & Lugovoy, 1948), glycerides (Morgan & Kingsbury, 1959) and lactose (reagents of Somogyi, 1952; Nelson, 1944) in milk.

Carbon dioxide. The $^{14}CO_2$ output was calculated from the blood flow and the $^{14}CO_2$ content in arterial and venous blood samples collected every hour. The CO_2 of blood (1 ml.) was released by acidification with 0·5 N-lactic acid (4 ml.) and the CO_2 evolved was trapped under liquid N_2. This trap was transferred to a vacuum manifold, the CO_2 passed through a water trap and after manometric determination swept with methane to a gas-counter tube (Sakami, 1955). The counting efficiency of this was 65%. The blood samples were also analysed for O_2 and CO_2 content (Van Slyke & Neill, 1924). The specific activity of the expired CO_2 was calculated from the quotient: venous−arterial $^{14}CO_2$ difference divided by venous−arterial CO_2 difference.

Isolation of milk components. Milk fat and casein were obtained from milk as described by Verbeke, Lauryssens, Peeters & James (1959). The fat was repeatedly washed with an acid solution of either unlabelled ornithine or unlabelled arginine and dried. The whey was freeze-dried, the residue was dissolved in 3 ml. of water and the proteins were separated from lactose and citric acid on a column (2 cm. × 25 cm.) of Sephadex G-25, with 0·3 M-pyridine–0·1 N-acetic buffer as eluent. The protein fractions were pooled, freeze-dried, washed with ethanol followed by ether and dried. The peak containing lactose and citric acid was concentrated and passed through a column (0·9 cm. × 15 cm.) of Dowex 1 (formate form). The effluent and washings, containing the lactose, were freeze-dried and crystallized from a minimal volume of ethanol–water. The citric acid was eluted from the column of Dowex 1 by using a linear gradient of 0–6 N-formic acid (Norman & DeLuca, 1964). The citric acid content in the fractions was determined by the method of Natelson *et al.* (1948).

Isolation of amino acids from plasma. Plasma was prepared for analysis as described by Verbeke & Peeters (1965). Ornithine or arginine was isolated on a column (0·9 cm. × 50 cm.) of Dowex 50 (X8) as described by Moore, Spackman & Stein (1958). In Expt. 3, proline was isolated from one sample by chromatography on a column (0·9 cm. × 150 cm.) of Amberlite IR-120. The fractions containing the investigated amino acids were pooled, desalted and concentrated to a small volume. The solutions containing ornithine or proline were analysed for these amino acids by the Chinard (1952) reaction. The arginine concentration was determined by a modified Sakaguchi reaction (Izumi, 1965).

Isolation of amino acids from casein. The incorporation of ^{14}C into casein amino acids was measured after hydrolysis and chromatographic separation (Verbeke *et al.* 1959).

Degradation of proline. A 0·0625 m-mole sample of proline, isolated from casein hydrolysate, was diluted with 5 ml. of 1·00 M-L-proline solution. Then 0·2 m-mole of proline was decarboxylated with 0·5 M-periodate (3 ml.) in the presence of 2 m-moles of $HgCl_2$ at pH 5·8 (4 ml. of phosphate buffer). The reaction was allowed to proceed at room temperature for 24 hr. (Skursky, 1959). The system was swept with N_2 and the carboxyl carbon trapped as CO_2 in NaOH. The reaction mixture was placed in the refrigerator (4°) and after 3 days the crystals were filtered off (pyrollidine–$HgCl_2$). Opening of the ring was performed with 0·1 M-$KMnO_4$ at pH 7 and 70°. Then 30 min. later the mixture was boiled briefly and MnO_2 was filtered off. The filtrate was passed through a column (0·9 cm. × 20 cm.) of Dowex 50 (H^+ form) and γ-aminobutyric acid was eluted with 2 N-NH_3. This amino acid was purified by paper chromatography (Raafat, Verbeke & Peeters, 1963). The yield of γ-aminobutyric acid varied between 5 and 20% of the amount of proline used in the reaction. The pure γ-aminobutyric acid was decarboxylated with NaN_3 in chloroform as described by Hendler & Anfinson (1954) and the CO_2 evolved trapped in NaOH (α-carbon of proline). The resulting propylenediamine solution was made alkaline, extracted in chloroform, acidified and concentrated to dryness. Combustion of this propylenediamine with

$AgNO_3$–persulphate (Sakami, 1955) yielded C-3+C-4+C-5 of proline. In the degradation experiments a portion of the proline was oxidized to CO_2 as a check on the procedure.

Radioactivity measurements. The radioactivity measurements were made with a windowless flow counter. After wet combustion, lactose and casein were counted as $BaCO_3$. Citric acid, total fat and amino acids were plated on plastic disks and counted as described by Verbeke *et al.* (1959). The specific activities are recorded as mμc/mg. of C.

Product quotient. The product quotient, giving the percentage of product formed from precursor, was calculated from the ratio of the specific activity of the isolated product to the specific activity of the precursor in plasma at steady state. This steady-state value was generally reached after 6hr. of perfusion for casein, casein amino acids, citric acid and lactose. If no steady-state value was reached or maintained, the reported product quotient was calculated from the maximal specific activity observed in the isolated product. If no equilibrium values are reached, it is to be expected that the estimated values given are minimal.

RESULTS

Composition of milk. The composition of the milk (fat, lactose, protein fractions) obtained during the perfusions was normal as compared with that observed on the living animal in the week preceding the experiments. However, the citric acid content was somewhat higher in the milk collected during perfusion in every experiment.

Specific activities of plasma amino acids. In Expt. 1, ornithine was isolated from six different samples of plasma. The specific activity of the precursor varied less than 6% during the last 4hr. of the experiment (Fig. 1).

In Expt. 3, the specific radioactivity of plasma arginine was determined in four blood samples (Fig. 2). The specific activity of the precursor remained practically constant between 4 and 8hr. after the onset of perfusion. In blood, collected 8hr. after the beginning of the experiment, ornithine and proline were isolated: the specific activity of plasma ornithine amounted to 54mμc/mg. of C, whereas that of plasma proline was 10·8mμc/mg. of C. In Expt. 2 no plasma arginine was isolated; the specific activity of arginine in the perfusate was used as a basis for the calculation of the product quotients.

Carbon dioxide. In Expt. 1, 2·2μc was recovered as $^{14}CO_2$, indicating that ornithine is metabolized to a considerable extent by the isolated mammary gland. The product quotient (Fig. 1 and Table 1) suggests that only 1% of the expired carbon dioxide was derived from ornithine.

In total, 2·3μc was recovered as $^{14}CO_2$ during Expt. 3. The product quotient (Fig. 2 and Table 1) shows that 1% of the expired carbon dioxide was derived from arginine carbon.

Synthesis of milk components. Preliminary perfusion experiments (Verbeke *et al.* 1965) showed

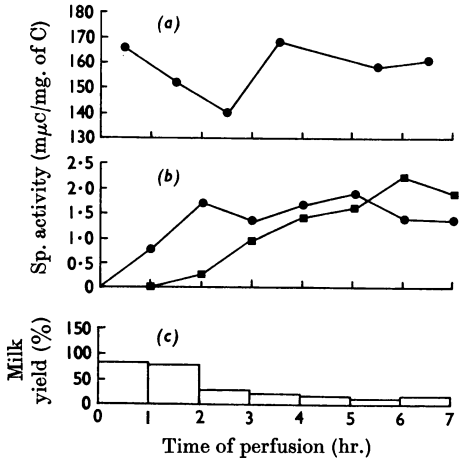

Fig. 1. Perfusion of one mammary gland with DL-[2-^{14}C]-ornithine (Expt. 1). The gland, weighing 855g., yielded 38ml. of milk/hr. before the experiment (average of 8 days, shown as 100%). The infusion of substrates with [2-^{14}C]-ornithine was started at 0hr. The mean blood flow during perfusion was 31ml./100g./min.; the O_2 consumption was about 1ml./100g./min. at maximum. (*a*) Specific activity of plasma ornithine (●); (*b*) specific activities of casein (■) and expired CO_2 (●); (*c*) hourly milk yield (% of previous hourly yield).

that practically no ^{14}C from [2-^{14}C]ornithine was transferred to lactose, citric acid or milk fat, but an efficient ^{14}C incorporation was found in the milk protein fractions, mainly in casein.

In Expt. 1, we recovered in total 0·9μc of the administered ^{14}C in casein. The specific activity of casein increased linearly during the first 4hr. of perfusion, reaching a constant value in the last 2hr. (Fig. 1). It was calculated that 1·4% of the casein carbon is derived from ornithine (Table 1).

The specific activities of the different milk components, during Expt. 3, were followed as a function of the perfusion period (Fig. 2). A very small but definite radioactivity was detected in lactose and milk fat: 0·007μc and 0·001μc were recovered in these fractions. After separation of the fatty acids of the triglycerides, the radioactivities of milk fat appeared to be localized entirely in C_4, C_6 and C_8 fatty acids. A higher specific activity was detected in citric acid: 0·007μc was recovered in this fraction. The highest specific activities in milk were found in casein. The radioactivity of this protein rose during the first hours of the experiment, reaching a constant value during the last 3hr. of perfusion.

In Expt. 2, constant specific activities were noted for casein 5hr. after the addition of labelled substrate. In both experiments 2·8μc of ^{14}C was

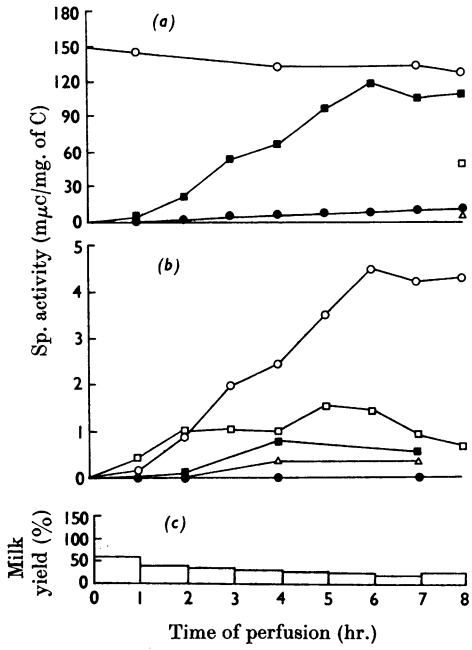

Fig. 2. Perfusion of one sheep mammary gland with DL-[5-^{14}C]arginine (Expt. 3). The gland, weighing 724g., yielded 25ml. of milk/hr. before the experiment (average of 8 days, shown as 100%). The infusion of substrates with DL-[5-^{14}C]arginine was started at 0hr. The mean blood flow during perfusion was 52ml./100g./min.; the O_2 consumption was about 1·4ml./100g./min. at maximum. (*a*) Specific activities of plasma arginine (○), plasma ornithine (□), plasma proline (△), casein arginine (■) and casein proline (●); (*b*) specific activities of casein (○), expired CO_2 (□), whey proteins (■), citric acid (△) and lactose (●); (*c*) hourly milk yield (% of previous hourly yield).

recovered in the casein fraction. As shown by the product quotients, about 3% of the casein carbon originated from arginine (Table 1).

Synthesis of casein amino acids. The amino acids were isolated from hydrolysates of four samples of casein obtained in two preliminary perfusion experiments with [2-^{14}C]ornithine (Verbeke *et al.* 1965). The results of these analyses are shown in Table 2. In each of the samples analysed, proline showed a very high ^{14}C incorporation: 96% of the total casein radioactivity was recovered in the proline residues of this protein. If compared with proline, only low levels of radioactivity were found in glutamic acid, aspartic acid and glycine. No appreciable radioactivity was found in the other non-essential amino acids of casein. No ^{14}C incorporation could be detected in arginine any more than in the other essential casein amino acids.

These results demonstrate that ornithine functioned as an important precursor for proline. Assuming that 96% of the total casein radioactivity was due to proline, it may be calculated from the specific activities of casein obtained in Expt. 1 that 13% of the proline residues of casein originated from plasma ornithine.

Proline isolated from casein (Table 2, Expt. B, perfusion time 5hr.) was degraded in order to localize the ^{14}C within the molecule. The specific radioactivities of total proline, carboxyl carbon, α-carbon and C-3+C-4+C-5 were respectively 0·42, 0·00, 2·00 and 0·03mμc/mg. of carbon. Almost all of the ^{14}C of proline was recovered from the α-carbon, only 4·3% being measured in C-3+C-4+C-5. No radioactivity was detected in the carboxyl group of this amino acid. The recovery of ^{14}C in the degradation experiment amounted to 99·5%.

Table 1. *Transfer of* ^{14}C *from* [2-^{14}C]*ornithine or* [5-^{14}C]*arginine to carbon dioxide and milk constituents*

Experimental details are given in the text. —, Not determined.

	Product quotient (% of product formed from precursor)		
Substrate	DL-[2-^{14}C]Ornithine	DL-[5-^{14}C]Arginine	
Expt. no.	1	2	3
CO_2	0·99	—	1·09
Casein	1·42	3·0	3·29
Whey proteins	—	—	0·57
Lactose	—	—	0·003
Citric acid	—	—	0·26
Casein proline	13·2	9·1	9·5
Casein arginine	0·0	84	83·9
Casein glutamic acid	0·21	0·285	0·31
Casein aspartic acid	0·10	0·07	0·14

Table 2. *Specific activities of amino acids isolated from casein obtained in preliminary perfusion experiments with* DL-[2-^{14}C]*ornithine*

Experimental details are given in the text.

Expt.	Sp. activity (mμc/mg. of C) A	B			% of ^{14}C of casein recovered in amino acids
Time of perfusion (hr.)	3	1	3	5	
Isolated substance					
Casein	1·24	0·157	1·93	3·66	100
Proline	11·50	1·48	16·00	34·20	96
Glutamic acid	0·27	0·03	0·36	0·55	3
Aspartic acid	0·09	0·03	0·15	0·27	0·6
Glycine	0·00	0·01	0·12	0·26	0·1
Serine	0·00	0·01	0·03	0·04	0·02
Alanine	0·01	0·00	0·02	0·02	0·05

Table 3. *Specific activities of amino acids isolated from casein after perfusion with* DL-[5-^{14}C]*arginine*

Experimental details are given in the text. —, Not determined.

Expt. no.	Sp. activity (mμc/mg. of C) 2	3			% of ^{14}C of casein recovered in amino acids
Time of perfusion (hr.)	3	2	5	8	
Casein	4·79	0·89	3·53	4·34	100
Arginine	137	22·6	99·8	115	68
Proline	14·8	2·53	9·82	13·1	31
Glutamic acid	0·24	0·02	0·24	0·42	0·9
Aspartic acid	0·11	0·01	0·13	0·19	0·15
Alanine	0·01	—	—	0·01	0·002
Glycine	0·04	—	—	—	0·01
Serine	0·01	—	—	—	0·04

One sample of casein from Expt. 2 and three samples from Expt. 3 were hydrolysed, followed by chromatographic isolation of the amino acids. The results of these analyses are summarized in Table 3. Practically all the radioactivity found in casein (99%) was recovered in the amino acids arginine and proline. From Table 3 it is also apparent that the specific activities of arginine were ten times those found in casein proline. The product quotients show that 84% of the arginine residues of casein and 9% of the casein proline were derived from plasma arginine (Table 1). As in experiments with [2-^{14}C]-ornithine, only traces of radioactivity were detected in the other non-essential amino acids of casein. No measurable ^{14}C incorporation was found in the essential amino acids.

DISCUSSION

It is clear that DL-[2-^{14}C]ornithine and DL-[5-^{14}C]-arginine are metabolized by the isolated sheep mammary gland. The product quotients show that about 13% and 9% of the casein proline are derived respectively from free ornithine and free arginine of the plasma. This means that nearly one-quarter of the total amount of proline residues of the casein originates from these sources. The high product quotients obtained for casein arginine in the [5-^{14}C]arginine experiments suggest that this amino acid originates mainly from plasma arginine. All these observations are in accordance with the finding that free ornithine and free arginine of the blood plasma are absorbed to an important extent by the lactating udder of ruminants *in vivo* (Verbeke & Peeters, 1965; Mepham & Linzell, 1966).

The first step in the metabolism of ornithine would be a transfer of the δ-amino group of ornithine to a keto acid leading to the formation of glutamic γ-semialdehyde (for review see Greenberg, 1961). The nitrogen of this transamination thus becomes available for synthesis of non-essential amino acids within the mammary gland. From the glutamic γ-semialdehyde (or its cyclization product) proline or glutamic acid may be formed. If the conversion

of [2-^{14}C]ornithine proceeds by the indicated pathway, the proline formed must acquire its radioactivity in the α-carbon of the molecule. Chemical degradation of proline isolated from casein shows that practically all of the ^{14}C in this molecule is localized in the α-carbon, in accordance with the suggested metabolic route.

Since the reactions of arginine leading to proline were supposed to proceed via ornithine, these amino acids were isolated from a plasma sample 8hr. after the onset of perfusion. The specific activity of plasma ornithine was intermediate between those of arginine and proline, indicating that arginine is metabolized by way of ornithine. Enzymic hydrolysis of [5-^{14}C]arginine to [5-^{14}C]-ornithine is probably catalysed by arginase of the mammary tissue, suggesting that this enzyme takes part in the nitrogen metabolism of the udder.

Mepham & Linzell (1967) have reported the results of a perfusion experiment performed on an isolated goat udder in the presence of L-[U-^{14}C]-arginine. In accord with our findings a high radioactivity was found in arginine and proline of the milk and in proline and ornithine of the blood plasma. Moreover, there was a net increase in the concentration of urea in the blood. From their results it is also evident that [^{14}C]arginine is split into [^{14}C]ornithine and [^{14}C]urea, supporting the role of arginase in the nitrogen metabolism of the udder.

The conversion of ornithine, by way of glutamic acid, into α-oxoglutaric acid and catabolism of the latter via the tricarboxylic acid cycle would explain the incorporation of ^{14}C into glutamic acid, aspartic acid, citric acid and carbon dioxide in the [^{14}C]-ornithine and [^{14}C]arginine experiments. However, the low product quotients suggest that neither ornithine nor arginine is acting as an important precursor for these amino acids.

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REFERENCES

Chinard, F. P. (1952). *J. biol. Chem.* **199**, 91.
Folley, S. J. (1949). *Biol. Rev.* **24**, 316.
Greenberg, D. M. (1961). In *Metabolic Pathways*, vol. 2, pp. 173–253. Ed. by Greenberg, D. M. New York: Academic Press Inc.
Hardwick, D. C. & Linzell, J. L. (1960). *J. Physiol.* **154**, 547.
Hardwick, D. C., Linzell, J. L. & Price, S. M. (1961). *Biochem. J.* **80**, 37.
Hendler, R. W. & Anfinson, C. B. (1954). *J. biol. Chem.* **209**, 55.
Izumi, Y. (1965). *Analyt. Biochem.* **10**, 218.
Mepham, T. B. & Linzell, J. L. (1966). *Biochem. J.* **101**, 76.
Mepham, T. B. & Linzell, J. L. (1967). *Nature, Lond.*, **214**, 507.
Moore, S., Spackman, D. H. & Stein, W. H. (1958). *Analyt. Chem.* **30**, 1185.
Morgan, D. M. & Kingsbury, K. J. (1959). *Analyst*, **84**, 409.
Natelson, S., Pincus, J. & Lugovoy, J. (1948). *J. biol. Chem.* **175**, 745.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Norman, A. W. & DeLuca, H. F. (1964). *Biochem. J.* **91**, 124.
Raafat, M. A., Verbeke, R. & Peeters, G. (1963). *Biochem. J.* **88**, 155.
Sakami, W. (1955). In *Handbook of Isotope Tracer Methods*, pp. 3–5 and 135–140. Ed. by Sakami, W. Cleveland, Ohio: Western Reserve University.
Skursky, L. (1959). *Z. Naturf.* **14b**, 473.
Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.
Van Slyke, D. D. & Neill, J. M. (1924). *J. biol. Chem.* **61**, 523.
Verbeke, R., Lauryssens, M., Peeters, G. & James, A. T. (1959). *Biochem. J.* **73**, 24.
Verbeke, R., Massart-Leën, A. M. & Peeters, G. (1966). *Arch. int. Physiol. Biochem.* **74**, 943.
Verbeke, R. & Peeters, G. (1965). *Biochem. J.* **94**, 183.
Verbeke, R., Peeters, G., Cocquyt, G. & Lauryssens, M. (1965). *Meded. LandbHoogesch. Gent*, **30**, 743.