REFERENCES

- Bertolacini, R. J. & Barney, J. E. (1957). *Analyt. Chem.* **29**, 281.
 Dodgson, K. S. & Lloyd, A. G. (1957). *Biochem. J.* **66**, 532.
 Dodgson, K. S., Lloyd, A. G. & Spencer, B. (1957). *Biochem. J.* **65**, 131.
 Dodgson, K. S., Rose, F. A. & Tudball, N. (1958). *Biochem. J.* **71**, 10.
 Dodgson, K. S. & Spencer, B. (1953). *Biochem. J.* **55**, 436.
 Dodgson, K. S. & Spencer, B. (1957). *Meth. biochem. Anal.* **4**, 211.
 Dodgson, K. S., Spencer, B. & Thomas, J. (1953). *Biochem. J.* **53**, 542.
 Duff, R. B. (1949). *J. chem. Soc.* p. 1597.
 Lloyd, A. G. (1959). *Nature, Lond.*, **183**, 109.

Location of ^{14}C in Protein from Isolated Rat Diaphragm Incubated *in vitro* with ^{14}C Amino Acids and with $^{14}\text{CO}_2$

BY K. L. MANCHESTER AND F. G. YOUNG
Department of Biochemistry, University of Cambridge

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When isolated rat diaphragm is incubated *in vitro* with radioactively labelled amino acids, radioactive material is transferred to the protein (the trichloroacetic acid-insoluble portion) of the diaphragm (Sinex, MacMullen & Hastings, 1952; Krahl, 1953; Manchester & Young, 1958*a*). Manchester & Young (1958*b*) found that when rat diaphragm is incubated with ^{14}C glucose, ^{14}C pyruvic acid, ^{14}C oxoglutaric acid or with $^{14}\text{CO}_2$, ^{14}C is incorporated into protein. By the use of column chromatography we have now determined in which amino acids this incorporated ^{14}C is contained, and to what extent ^{14}C amino acids are converted by diaphragm into other ^{14}C amino acids before incorporation into protein.

MATERIALS AND METHODS

Radioactive materials. Labelled substances were obtained from The Radiochemical Centre, Amersham, Bucks. Glycine and sodium pyruvate were $[1\text{-}^{14}\text{C}]$ labelled; L-alanine, L-serine, L-glutamic acid, L-aspartic acid and D-glucose were all uniformly ^{14}C -labelled ($[\text{U-}^{14}\text{C}]$). Sodium α -oxoglutarate contained ^{14}C in the C-5 position. ^{14}C Glucose had a specific activity of about $67\text{ }\mu\text{C}/\text{mg.}$, ^{14}C sodium pyruvate about $43\text{ }\mu\text{C}/\text{mg.}$, ^{14}C alanine $36\text{ }\mu\text{C}/\text{mg.}$, ^{14}C glycine $37\text{ }\mu\text{C}/\text{mg.}$ and ^{14}C serine $15\text{ }\mu\text{C}/\text{mg.}$ Each of these materials was used at a final concentration of $2\cdot5\text{ }\mu\text{C}/\text{ml.}$ ^{14}C Glutamic acid (specific activity $62\text{ }\mu\text{C}/\text{mg.}$) and ^{14}C aspartic acid (specific activity $32\text{ }\mu\text{C}/\text{mg.}$) were used at a concentration of $10\text{ }\mu\text{C}/\text{ml.}$ The protein from diaphragm incubated with ^{14}C glutamic acid and with ^{14}C oxoglutaric acid had been prepared in a previous experiment (Manchester & Young, 1958*b*).

The bicarbonate medium of Gey & Gey (1936), pH 7.4, gassed with $\text{O}_2 + \text{CO}_2$ (95:5, v/v), was used throughout. The incubation flasks each contained 2 ml. of medium. No unlabelled glucose was added to the medium in any experiment. In the experiment with $^{14}\text{CO}_2$, $16\text{ }\mu\text{C}$ of $\text{NaH}^{14}\text{CO}_3$ was

added to each flask, its final specific activity being $160\text{ }\mu\text{C}/\text{m-mole.}$

Preparation of diaphragm. Diaphragm was removed from female rats, of about 120 g. weight, which had starved for 20–24 hr. The two halves of the diaphragm were washed in freshly gassed buffer, then gently blotted and transferred to small conical flasks containing the incubation medium. Two hemidiaphragms were added to each flask. The flasks were gassed, sealed and incubated at 37° in a Dubnoff shaker.

Preparation of protein from diaphragm. After incubation for 2 hr. the diaphragm was removed, blotted and homogenized in a Potter-Elvehjem homogenizer in 10 ml. of 10% trichloroacetic acid. The precipitate was spun down, resuspended in 10 ml. of 10% trichloroacetic acid and heated for 15 min. at 90° . The insoluble material was extracted twice with 1.5 ml. of 0.4*N*-NaOH, and the insoluble residue rejected (protein content about 5%). The dissolved protein was reprecipitated by the addition of 5 ml. of 10% trichloroacetic acid. It was washed with 2 ml. of ethanol-ether (50:50, v/v), followed by 2 ml. of ether, and was dried in a vacuum desiccator.

Hydrolysis of protein. Protein (10 mg.) in 0.3 ml. of 6*N*-HCl was sealed in a thick-walled glass tube and heated to 105° for 20–24 hr. The hydrolysate was left to dry in a vacuum desiccator over CaCl_2 and redissolved in 1 ml. of 1.5*N*-HCl.

Chromatographic separation of amino acids. The amino acids in the protein hydrolysate were separated on a column (50 cm. $\times$ 1 cm.) of either Dowex-50 or Amberlite CG-120. The resin of both columns had been prepared for use by the method of Moore & Stein (1951), and was prepared for each experiment by washing with 2*N*-NaOH, followed by water and 1.5*N*-HCl. Washing with HCl was continued until the effluent left no deposit of NaCl when evaporated to dryness. The protein hydrolysate was introduced at the top of the column and solvent run through at room temperature at the rate of about 4 ml./hr. As solvents 1.5*N*-, 2.5*N*- and 4*N*-HCl were successively used (Stein & Moore, 1949). The eluate was collected in fractions of about 3.3 ml. by means of an automatic fraction collector.

In the majority of experiments only 450 ml. of eluate (which contained most of the amino acids) was collected; no attempt was usually made to recover arginine and phenylalanine, which were present in the last 150 ml. to emerge from the column. Preliminary experiments revealed no significant radioactivity in this part of the eluate.

Detection of amino acids in the eluate. Amino acids were detected by the ninhydrin method described by Chibnall, Mangan & Rees (1958). A sample (0.2 ml.) of each fraction was neutralized with NaOH and diluted with water to 2 ml. To this was added 1 ml. of a buffer made up as follows. To 23 ml. of 5M-sodium acetate buffer (pH 5.5) was added 2 ml. of 0.1M-KCN and 75 ml. of 2-methoxyethanol (methyl Cellosolve; Hopkin and Williams Ltd.). Ninhydrin (0.2 ml. of 10%, w/v, solution in 2-methoxyethanol) was then added and the mixture heated in a boiling-water bath for 20 min. The mixture was cooled, diluted to 10 ml. with 60% ethanol and the light absorption at 570 m μ measured in a spectrophotometer.

Detection of ^{14}C in the eluate. The remaining portion of each fraction was evaporated to dryness *in vacuo*, redissolved in 0.5 ml. of water, transferred to an aluminium disk (2 cm. diameter) and dried as a thin film by the method of Bourns, Coombs & Rizk (1953). Fractions containing no amino acid, or amino acid expected to have no ^{14}C , were combined and similarly treated. The radioactivity was measured beneath a thin end-window Geiger-Müller tube over a period of 20 min. Counts/min. ranged between 0 and 1000.

RESULTS

The order of elution from the column of those amino acids with which we were principally concerned was aspartic acid, serine + threonine, glutamic acid, glycine and alanine. All the other amino acids emerged after these. It was not possible to distinguish between serine and threonine. The peak for serine + threonine to some extent overlapped that for aspartic acid, but it was possible

to distinguish between the ^{14}C contained in the aspartic acid fraction and that in the serine + threonine fraction, from the shape of the peak of the curve and the position of its maximum point. Likewise, some overlap between the glycine and alanine fractions occurred, but it was possible similarly to determine whether ^{14}C was contained in the alanine or in the glycine fraction. No ^{14}C was detected in any fraction other than those considered below.

When protein from isolated rat diaphragm which had been incubated in a medium containing D-[U- ^{14}C]glucose was hydrolysed and the amino acids were separated, ^{14}C was found to be present in the fractions containing aspartic acid and glutamic acid and alanine (Table 1), the greatest part being in the alanine fraction. With diaphragm incubated with [1- ^{14}C]pyruvic acid, ^{14}C occurred in the fractions of the protein hydrolysate which contained aspartic acid and glutamic acid and alanine, the major portion of the activity again being in the alanine fraction. When diaphragm was incubated with a mixture of L-[U- ^{14}C]glutamic acid and [5- ^{14}C]oxoglutaric acid, under conditions in which Manchester & Young (1958b) found that 75% of the ^{14}C incorporated into protein was derived from the oxoglutaric acid, ^{14}C was found chiefly in glutamic acid and to a less extent in aspartic acid (Table 1). Analysis of the amino acids obtained by hydrolysis of protein from diaphragm incubated with $\text{NaH}^{14}\text{CO}_3$ revealed ^{14}C to be present in the fractions containing aspartic acid and glutamic acid to an approximately equal extent (Table 1).

Because these observations suggested that a significant rate of synthesis of amino acids from non-amino acid sources occurred in diaphragm under the conditions of our experiments, it was of

Table 1. Location of ^{14}C in protein obtained from rat-diaphragm muscle which had been incubated *in vitro* with ^{14}C -labelled substances

Diaphragm incubated with	Total counts/min. eluted from column	Radioactivity in hydrolysate from 10 mg. of protein					
		Percentage of ^{14}C appearing in					Other amino acids
		Aspartate	Serine + threonine	Glutamate	Glycine	Alanine	
D-[U- ^{14}C]Glucose	185	17	0	24	0	59	0
*D-[U- ^{14}C]Glucose	227	15	0	28	0	57	0
[1- ^{14}C]Pyruvate	214	14	0	10	0	76	0
L-[U- ^{14}C]Glutamate and [5- ^{14}C]oxoglutarate	142	28	0	72	0	0	0
$\text{NaH}^{14}\text{CO}_3$	314	48	0	52	0	0	0
L-[U- ^{14}C]Alanine	2168	6	0	12	0	82	0
*L-[U- ^{14}C]Alanine	3232	7	0	16	0	77	0
L-[U- ^{14}C]Glutamate	1535	23	0	74	0	3	0
L-[U- ^{14}C]Aspartate	1425	43	0	42	0	15	0
[1- ^{14}C]Glycine	1436	0	6	0	94	0	0
*[1- ^{14}C]Glycine	2194	0	7	0	93	0	0
L-[U- ^{14}C]Serine	1567	0	86	0	14	0	0

* Insulin (0.5 unit/ml.) was added to the medium.

interest to investigate the extent to which certain amino acids might be broken down and converted into other amino acids under these conditions. When protein from isolated diaphragm which had been incubated in a medium containing L-[U- 14 C]-alanine was hydrolysed, and the amino acids were separated, 14 C was found to be present largely in alanine but also to a less extent in aspartic acid and glutamic acid (Table 1). Incubation of diaphragm with L-[U- 14 C]glutamic acid led to a recovery of 14 C chiefly in glutamic acid, but also to a less extent in

aspartic acid, with a very little in alanine (Table 1). Incubation with L-[U- 14 C]aspartic acid led to an almost equal division of 14 C between aspartic acid and glutamic acid, with a smaller but appreciable amount in alanine (Table 1).

With [1- 14 C]glycine most of the 14 C recovered was found in the glycine fraction of the protein from diaphragm but a small proportion occurred in the serine + threonine fraction (Table 1). Incubation of diaphragm with L-[U- 14 C]serine led to a recovery of 14 C almost entirely in the serine + threonine fraction, but a small proportion of 14 C was also found in the glycine fraction (Table 1).

All these observations were made with a medium containing no added insulin. Addition of insulin (0.5 unit/ml.) to the medium when diaphragm was incubated with [14 C]glycine, [14 C]alanine or [14 C]glucose, raised the amount of 14 C incorporated into protein of the diaphragm by 25–50% but in no case was the distribution of 14 C amongst the amino acids of the protein hydrolysate changed (Table 1). The results are summarized in Fig. 1.

DISCUSSION

Insulin and incorporation of amino acids. Manchester & Young (1958a) found that with isolated rat diaphragm incubated *in vitro* incorporation into protein of radioactive material from all the 11 labelled amino acids tested was stimulated by addition of insulin to the medium. Except with alanine, this stimulating action of insulin was little affected by the addition of glucose or of pyruvic acid to the medium. The action of insulin in stimulating the incorporation of 14 C from [14 C]alanine into diaphragm protein was abolished by the addition of glucose to the medium. This atypical behaviour of [14 C]alanine, and the observation, again peculiar to alanine, that addition of glucose or of pyruvic acid to the medium in the absence of insulin depressed incorporation of 14 C from the amino acid into diaphragm protein, was explicable by a dilution of labelled alanine with unlabelled alanine formed from the glucose or pyruvic acid added (Manchester & Young, 1958b). It was therefore not necessary to postulate an action of insulin on the incorporation of alanine qualitatively different from that of insulin on the incorporation of other amino acids.

In the absence of evidence to the contrary it is reasonable to assume that insulin stimulates incorporation of radioactive material from labelled amino acids into protein of diaphragm by a mechanism that is similar for all amino acids. This assumption is supported by the observation reported here, that addition of insulin to the medium when diaphragm was incubated with [14 C]glycine, [14 C]alanine or [14 C]glucose raised the total in-

Fig. 1. Location of [14 C]amino acids in hydrolysates from protein obtained from isolated rat diaphragm incubated *in vitro* with [14 C]amino acids. The amino acids were separated by column chromatography (see text).

corporation but did not affect the distribution of ^{14}C among the amino acids of the protein. Since the rise in incorporation of ^{14}C from glucose induced by insulin was 23 %, whereas the rise in ^{14}C from alanine and glycine with insulin was 49 and 53 % respectively, the effect of the hormone on the incorporation of amino acids by diaphragm *in vitro* is of a magnitude at least similar to that of its effect on glucose utilization. These observations provide evidence against the idea that, in influencing protein biosynthesis, insulin acts by stimulating reactions specific for the metabolism of only a limited number of amino acids, or for that of substances involved in glycolysis. They agree with the view that insulin stimulates protein biosynthesis by promoting the transfer of amino acids across the cell membrane, but provide no direct evidence for it.

Fixation of carbon dioxide. Manchester & Young (1958b) found that addition of [^{14}C]glucose or of [^{14}C]pyruvic acid to the medium in the presence of [^{14}C]alanine results in no diminution of incorporation of ^{14}C into protein of isolated rat diaphragm such as normally occurs when unlabelled glucose or pyruvate is added to the medium. This observation suggested that both glucose and pyruvic acid can rapidly provide the carbon skeleton of alanine in isolated rat diaphragm *in vitro*. We have now confirmed this suggestion by finding that the principal [^{14}C]amino acid formed from [U- ^{14}C]glucose or [1- ^{14}C]pyruvic acid is alanine, although indeed some labelling of the aspartic acid and glutamic acid occurred. The appearance of [^{14}C] in aspartic acid and glutamic acid under these conditions is of particular interest, because on the basis of present views it would be expected that if the carbon skeletons of these amino acids are formed from [1- ^{14}C]pyruvic acid by the action of pyruvic dehydrogenase together with the operation of the tricarboxylic acid cycle, the amino acids thus made would be unlabelled (see Krebs, 1954). It is possible, however, that the [^{14}C]-aspartic acid and -glutamic acid are formed by processes which involve fixation of $^{14}\text{CO}_2$ into protein of diaphragm (Manchester & Young, 1958b), the $^{14}\text{CO}_2$ being formed by the decarboxylation of [1- ^{14}C]pyruvic acid by pyruvic dehydrogenase. If this is so, $^{14}\text{CO}_2$ produced by the action of pyruvic dehydrogenase on [1- ^{14}C]pyruvic acid must be trapped in some way in our experiments before it has time to equilibrate with the unlabelled carbon dioxide and HCO_3^- ions in the diaphragm and buffer. Bachhawat & Coon (1957) have suggested that carbon dioxide used in carboxylation first forms a complex with adenylic acid; in this way the carbon dioxide liberated from pyruvic acid by pyruvic dehydrogenase may be trapped. On the other hand, if pyruvic dehydrogenase is located in the mito-

chondrion (Vennesland, 1951), and the mitochondrion is not very permeable to carbon dioxide, the trapping of a significant proportion of the carbon dioxide might also be expected.

Manchester & Young (1958b) suggested that ^{14}C from $^{14}\text{CO}_2$ which enters the protein in diaphragm does so through a fixation of carbon dioxide by pyruvic acid to form oxaloacetic acid (Krebs & Eggleston, 1940), which is then aminated to form aspartic acid. If this were so, ^{14}C should be recoverable from the aspartic acid fraction of the protein hydrolysate, and this has now been done. Although Stern (1948) failed to find direct evidence for the presence in muscle of a reversible oxaloacetic decarboxylase, our results can be easily and simply explained on the basis of the presence of such an enzyme system in rat-diaphragm muscle. On the other hand, as much ^{14}C is found in glutamic acid as in aspartic acid in our experiments, and although this might be explained by interconversion of aspartic acid and glutamic acid through the tricarboxylic acid cycle, a possibility exists that a second way of fixing carbon dioxide can function in muscle. Such a way might be by fixation of carbon dioxide by succinyl-coenzyme A or bound succinic semialdehyde to form oxoglutaric acid, which would then be aminated to form glutamic acid (Kaufman, 1951). It has not been shown that the decarboxylation of oxoglutaric acid to succinyl-coenzyme A and carbon dioxide can be reversed, but since Ochoa (1945) has found that the enzymic decarboxylation of oxalosuccinic acid to oxoglutaric acid and carbon dioxide is reversible, there is no reason to suppose that the former reaction cannot also be reversed.

When $\text{NaH}^{14}\text{CO}_3$ is incubated with diaphragm similar amounts of $^{14}\text{CO}_2$ enter aspartic acid and glutamic acid. Furthermore, the incubation of diaphragm with [U- ^{14}C]aspartic acid leads to as much incorporation of ^{14}C into glutamic acid as into aspartic acid, with a small incorporation into alanine also. We believe that since this transfer of ^{14}C from aspartic acid to glutamic acid is quite large, it is more probably a result of operation of the tricarboxylic acid cycle than a fixation by succinyl-coenzyme A into oxoglutaric acid of $^{14}\text{CO}_2$ released from oxaloacetic acid (formed from aspartic acid) during the operation of the tricarboxylic acid cycle. Nevertheless, our experiments do not at present provide decisive evidence by which a distinction between the various possible interpretations we have considered may be made.

Hiatt, Goldstein, Lareau & Horecker (1958) have recently found that when isolated diaphragm is incubated with $\text{NaH}^{14}\text{CO}_3$, ^{14}C is incorporated into glycogen. They considered the possibility that ^{14}C may become incorporated into phosphopyruvic acid formed from pyruvic acid by operation of the

'dicarboxylic acid shuttle' (Ochoa, Mehler & Kornberg, 1948; Utter & Kurahashi, 1953), but concluded that these reactions, though occurring rapidly in liver, take place to only a small extent if at all in muscle. They therefore considered the possibility of an exchange of the pyruvic acid carboxyl group with $^{14}\text{CO}_2$ in the pyruvic acid oxidation reaction demonstrated with a mammalian enzyme system by Goldberg & Sanadi (1952). We have detected no ^{14}C in alanine, and our results therefore provide no support for this view.

On the other hand, Goldberg & Sanadi (1952) found that ^{14}C could become incorporated into oxoglutaric acid when the latter reacts with $\text{NaH}^{14}\text{CO}_3$ in the presence of α -oxoglutaric oxidase prepared from pig heart. The occurrence of such a reaction in diaphragm would explain the entry of ^{14}C into glutamic acid in diaphragm protein which we have observed when $\text{NaH}^{14}\text{CO}_3$ is incubated with isolated rat diaphragm.

Metabolism of amino acids. In the studies with ^{14}C amino acids, ^{14}C from ^{14}C alanine enters the glutamic acid and aspartic acid fractions to a surprising extent, and the transfer of ^{14}C from ^{14}C glutamic acid to ^{14}C aspartic acid is also large. A substantial transfer of ^{14}C from ^{14}C aspartic acid to ^{14}C alanine has also been observed, presumably through the decarboxylation of oxaloacetic acid to pyruvic acid, the oxaloacetic acid being derived from the added aspartic acid. A smaller transfer of ^{14}C to alanine from ^{14}C glutamic acid could be a consequence either of the presence at the site of action of a smaller concentration of endogenous aspartic acid than is the case when this substance is added to the medium, or of the location of oxaloacetic decarboxylase outside of the mitochondrion associated with a formation of aspartic from glutamic acid within the mitochondrion.

Though all the observations reported here cannot easily be explained in terms of known processes, it is clear that in isolated diaphragm rapid operation of the tricarboxylic acid cycle takes place, together with interconversion of aspartic acid, glutamic acid and alanine and their corresponding keto acids.

Though the major portion of ^{14}C incorporated into diaphragm protein from ^{14}C glycine is contained in the glycine fraction, some conversion of the labelled glycine into serine (or into constituents of the serine + threonine fraction) appears to occur. Similarly, though most of the ^{14}C from ^{14}C serine can be recovered in the serine + threonine fraction, some formation of labelled glycine from the serine appears to take place. The interconversion of serine and glycine has been demonstrated previously in liver (Siekevitz, Winnick & Greenberg, 1949; Vilenkina, 1952), but not in muscle.

SUMMARY

1. Isolated rat diaphragm has been incubated with various ^{14}C amino acids and metabolites, and protein from the diaphragm has been hydrolysed and separated by column chromatography into its component amino acids.

2. When diaphragm is incubated with $[\text{U-}^{14}\text{C}]$ -glucose or with $[\text{1-}^{14}\text{C}]$ pyruvic acid, ^{14}C is incorporated mainly into the alanine fraction and to a less extent into aspartic acid and glutamic acid.

3. Incubation of diaphragm in a medium containing $^{14}\text{CO}_2$ leads to a recovery of similar quantities of ^{14}C in aspartic acid and glutamic acid.

4. Diaphragm incubated with $[\text{U-}^{14}\text{C}]$ alanine incorporates ^{14}C mainly into the alanine fraction of the protein, though some ^{14}C is also found in aspartic acid and glutamic acid. Incubation with $[\text{U-}^{14}\text{C}]$ aspartic acid gives rise to ^{14}C in the glutamic acid and alanine fractions as well as in aspartic acid, whereas incubation with $[\text{U-}^{14}\text{C}]$ glutamic acid leads to an appreciable recovery of ^{14}C in aspartic acid and glutamic acid alone.

5. ^{14}C incorporated into protein of diaphragm from $[\text{1-}^{14}\text{C}]$ glycine is recovered almost entirely as ^{14}C glycine, the remainder being found in the serine + threonine fraction. Conversely, protein of diaphragm incubated with $[\text{U-}^{14}\text{C}]$ serine contains ^{14}C mainly in the serine + threonine fraction but also to a small extent in the glycine fraction.

6. Addition of insulin to the medium, although increasing the amount of ^{14}C incorporated by 25–50%, did not change the distribution of ^{14}C incorporated from ^{14}C glucose, alanine and glycine.

7. The results are largely explicable in terms of rapid transaminations and operation of the tricarboxylic acid cycle in isolated rat diaphragm, together with incorporation of carbon dioxide by pyruvate, succinyl-coenzyme A or related substances.

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REFERENCES

- Bachhawat, B. K. & Coon, M. J. (1957). *J. Amer. chem. Soc.* **79**, 1505.
- Boursnell, J. C., Coombs, R. R. A. & Rizk, V. (1953). *Biochem. J.* **55**, 745.
- Chibnall, A. C., Mangan, J. L. & Rees, M. W. (1958). *Biochem. J.* **68**, 111.

- Gey, G. O. & Gey, M. K. (1936). *Amer. J. Cancer*, **27**, 45.
- Goldberg, M. & Sanadi, D. R. (1952). *J. Amer. chem. Soc.* **74**, 4972.
- Hiatt, H. H., Goldstein, M., Lareau, J. & Horecker, B. L. (1958). *J. biol. Chem.* **231**, 303.
- Kaufman, S. (1951). *Phosphorus Metabolism*. Ed. by McElroy, W. D. & Glass, B. 1, 370. Baltimore: Johns Hopkins Press.
- Krahl, M. E. (1953). *J. biol. Chem.* **200**, 99.
- Krebs, H. A. (1954). In *Chemical Pathways of Metabolism*, vol. 1, p. 109. Ed. by Greenberg, D. M. New York: Academic Press Inc.
- Krebs, H. A. & Eggleston, L. V. (1940). *Biochem. J.* **34**, 1383.
- Manchester, K. L. & Young, F. G. (1958*a*). *Biochem. J.* **70**, 353.
- Manchester, K. L. & Young, F. G. (1958*b*). *Biochem. J.* **70**, 297.
- Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
- Ochoa, S. (1945). *J. biol. Chem.* **159**, 243.
- Ochoa, S., Mehler, A. H. & Kornberg, A. (1948). *J. biol. Chem.* **174**, 979.
- Siekevitz, P., Winnick, T. & Greenberg, D. M. (1949). *Fed. Proc.* **8**, 250.
- Sinex, F. M., MacMullen, J. & Hastings, A. B. (1952). *J. biol. Chem.* **198**, 615.
- Stein, W. H. & Moore, S. (1949). *Symp. quant. Biol.* **14**, 179.
- Stern, J. R. (1948). *Biochem. J.* **43**, 616.
- Utter, M. F. & Kurahashi, K. (1953). *J. Amer. chem. Soc.* **75**, 758.
- Vennesland, B. (1951). *Keto acid decarboxylases*. In J. B. Sumner & K. Myrbäck, *The Enzymes*, vol. 2, p. 199. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press Inc.
- Vilenkina, G. Y. (1952). *C.R. Acad. Sci. U.R.S.S.* **84**, 559.

The Reactions of Amino Acids with Soluble Ribonucleic Acid from Guinea-Pig Mammary Cells

By M. J. FRASER, H. SHIMIZU AND H. GUTFREUND
National Institute for Research in Dairying, Shinfield, near Reading

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During the last 5 years many cell-free systems of animal, plant and bacterial origin have been prepared which were able to perform some of the intermediate reactions which are likely to be on the pathway to protein synthesis. The mammary gland in full lactation is a most suitable system for the study of every aspect of protein synthesis; not only are large quantities of protein continuously formed but they are also excreted from the cells and thus the complications introduced by catabolism are avoided. Barry (1958) has described various studies of the formation of milk proteins from injected labelled amino acids *in vivo*. He concluded that milk proteins are formed from free amino acids of the blood stream. The development of cell-free preparations from liver, which could incorporate [¹⁴C]amino acids into the peptide linkage of microsomal protein (Zamecnik & Keller, 1954; Keller & Zamecnik, 1956), led to the discovery of three distinct steps in this process (Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958). The three steps are: first, the activation of amino acids by the enzymic formation of amino acid adenylates; secondly, the combination of activated amino acids with cell-sap ribonucleic acid; and thirdly the transfer of amino acids from cell-sap ribonucleic acid to microsomal nucleoprotein.

In this Laboratory we have studied many

aspects of amino acid incorporation with a number of different systems prepared from lactating mammary glands. The pattern of incorporation of amino acids into subcellular particles and soluble protein of intact mammary cells and tissue homogenates *in vitro* has been described recently (Fraser & Gutfreund, 1958), and we have shown that the three steps studied by Hoagland *et al.* (1958) can be usefully investigated in mammary-tissue homogenates. We have now studied the formation of cell-sap ribonucleic acid-amino acid compounds in greater detail. Our results do not enable us to draw any conclusions about the chemical mechanism of this reaction, but they give a quantitative picture of the proportions of different amino acids involved, of their competition with each other and of the rates and equilibria of the compound formation. It will be seen that certain conclusions can be drawn from such data about the role of cell-sap ribonucleic acid-amino acid coupling in the overall process of protein synthesis.

EXPERIMENTAL

Materials. All [¹⁴C]amino acids were obtained from The Radiochemical Centre, Amersham, Bucks. Their specific activities are quoted in relevant places in the text. The L-amino acids are generally labelled, and the DL-amino acids are [1-¹⁴C]. The adenosine triphosphate (ATP) used