PARTICIPATION OF FREE AMINO ACIDS IN PROTEIN SYNTHESIS*

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There has long been speculation as to whether protein can be synthesized only from free amino acids or whether large or small peptide fragments can be incorporated directly into a new protein molecule without prior degradation to individual amino acids. In the case of bacterial protein synthesis, the work of Spiegelman and coworkers (3, 4) and Monod *et al.* (5, 6) strongly suggests that all of the new protein in growing bacteria is derived from the free amino acid pool with essentially no contribution from the protein already present. For instance, in several cases bacteria have been cultured on a radioactive substrate which is ultimately incorporated into all the protein of the organism. After transfer to a non-radioactive medium, the synthesis of a new enzyme was induced. The induced enzyme contained no radioactivity and hence could not have been derived from the preexisting labeled protein.

The situation is less clear with higher organisms such as mammals. Simpson and Velick (7) and Heimberg and Velick (8) measured the extent of incorporation of several radioactive amino acids into several proteins of rabbit muscle and concluded that these proteins are synthesized at different rates from a common amino acid pool. Most recently, Askonas and her associates (9, 10) have isolated some thirty peptides from a partial hydrolysate of casein and of β-lactoglobulin obtained from milk secreted after an injection of labeled lysine and valine into a goat. There was no variation in specific activity of the valine or the lysine from one peptide to another. Barring a most unlikely coincidence, the explanation is that these proteins are synthesized rapidly and exclusively from a common amino acid pool.

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On the other hand, Francis and Winnick (11) report that phenylalanine-labeled proteins from embryo extract are incorporated directly into the protein of a tissue culture, because addition of considerable amounts of unlabeled phenylalanine to the medium did not prevent the incorporation. For much the same reason, Babson and Winnick (12) argue that leucine and tyrosine are transferred from rat plasma protein directly to tissue protein in the rat, since there was no inhibition of the transfer on injection of unlabeled amino acids. Finally, as the result of similar experiments, Friedberg and Walter (13) report that S^{35}-containing amino acids in labeled plasma proteins are transferred directly to tissue protein.

In rats, the intravenous injection of saccharated iron oxide stimulates the production of liver ferritin in a way reminiscent of adaptive enzyme formation (14, 1). Because of the problems of protein breakdown and resynthesis, it is not possible to convert abruptly from a fully labeled medium to a completely non-labeled medium as it is with bacteria. However, if labeled L-amino acids are used, it is possible to determine the actual intracellular specific activities of individual amino acids after chromatographic isolation.[1] Thus it is possible to examine a protein being synthesized *de novo* in the presence of amino acids of known specific activity.

Materials and Methods

All animals used in these experiments were adult male Wistar rats weighing between 200 and 265 gm. Four of the six rats used in the 6 hour to 3 day infusion experiment were litter mates.

L-Valine-C^{14}, L-isoleucine-C^{14}, and L-leucine-C^{14} were prepared by variations of the Bucherer hydantoin synthesis from $BaC^{14}O_3$ through $KC^{14}N$ (15). Resolution was effected with the acetyl derivatives with acylase I, which was kindly supplied by Dr. Birnbaum (16, 17). The saccharated iron oxide used was the commercial preparation Feojectin (courtesy of Smith, Kline and French).

Rats, in apparently good health as measured by daily weight gain, were anesthetized, and a femoral vein was catheterized with polythene tubing. The catheter was brought out through the rat's back, where it was secured by a loose-fitting plaster collar. After recovery from anesthesia, injections were given or continuous infusion was begun. The rats were given water *ad libitum* and had good appetites. After three days, they still seemed healthy, the only death during these experiments being due to pneumonia.

At the end of the experiment, the rat was decapitated, the liver was excised, and part of the liver was homogenized in a Waring blendor with

[1] The use of DL-amino acids is precluded because only the specific activity of the L form is of consequence. This would be impossible to determine when the C^{14} DL-amino acid is mixed with an uncertain amount of C^{12} L-amino acid in the cell if, as is likely, the rates of destruction of the L and D forms are different.

30 ml. of 1 per cent picric acid (18), all within 1¼ minutes after the infusion was stopped. In order to minimize errors due to proteolysis and continued metabolism, no time was used in an attempt to perfuse the livers.

The precipitated protein was centrifuged, the picric acid removed on Dowex 1 (chloride form), and the amino acids were concentrated by adsorption on a 4 × 1 cm. column of Dowex 50 (hydrogen form) and by elution with 5 ml. of 5 N ammonium hydroxide. The eluate was evaporated to dryness in a stream of nitrogen and, in some cases, was treated with performic acid to oxidize cysteine and glutathione which interfered in varying degrees with the chromatography. The amino acids were chromatographed on Amberlite XE-69 (strong acid resin, hydrogen form; obtained by courtesy of Rohm and Haas) with the use of 100 × 0.4 cm. columns and 2.0 N hydrochloric acid. 1 ml. fractions were collected, concentrated and transferred to clean 22 mm. No. 1 circle cover-slips (Corning), and evaporated to dryness. The total radioactivity of each fraction was determined with a Geiger-Müller end window counter. Each glass planchet was then broken in a colorimeter tube and the total amino acid content determined by the ninhydrin method (19). With as little as 0.2 μmole of amino acid and a 200 c.p.m. total, it was generally found that the three or four peak tubes had specific activities within 10 per cent of each other. An occasional grossly aberrant value was rejected.

The part of the liver not homogenized with picric acid was homogenized with water. After 1 hour the homogenate was centrifuged, and the material in the supernatant fluid which was precipitated between the limits of 16 and 25 gm. of ammonium sulfate per 100 ml. was collected, taken up in water, and heated at 75° for 5 minutes. 1 gm. of ammonium sulfate was added to each 4 ml. of the supernatant solution, and the resulting precipitate, which contained all of the ferritin, was taken up in 3 ml. of water. 4 ml. of ethanol were added and, after 15 minutes at room temperature, the suspension was centrifuged. The sediment, which contained ferritin and denatured protein, was taken up in water, dialyzed, and centrifuged. The insoluble residue at this point is later referred to as the "alcohol denatured discard." The supernatant fluid was a solution of ferritin containing between 17 and 22 per cent of iron. The ferritin was assayed for radioactivity and then hydrolyzed by being heated for 12 hours in 6 N hydrochloric acid at 115°. The hydrolysate was concentrated nearly to dryness, fractionated on an Amberlite XE-69 column, and assayed as described above. In Fig. 1 a typical chromatogram appears.

Results

Young rats growing well on our stock diet accumulate no iron reserves in the form of ferritin. After the intravenous injection of 5 mg. per 100 gm. of saccharated iron oxide, there is a nearly linear increase in liver

ferritin in the interval from 4 hours to 3 days. The amount subsequently remains rather constant at about 8 mg. per liver for several weeks. If the ferritin is synthesized directly from free amino acids, and if the free intracellular amino acids have been maintained at a constant specific radioactivity during this period, the individual amino acids in the ferritin should have exactly the same specific activity as the free amino acids.

In Table I, under conditions of continuous infusion, the intracellular specific activity of each amino acid did not vary from the average by more than about 20 per cent during 3 days. Since the saccharated iron oxide was injected after the infusion was commenced, all of the ferritin isolated

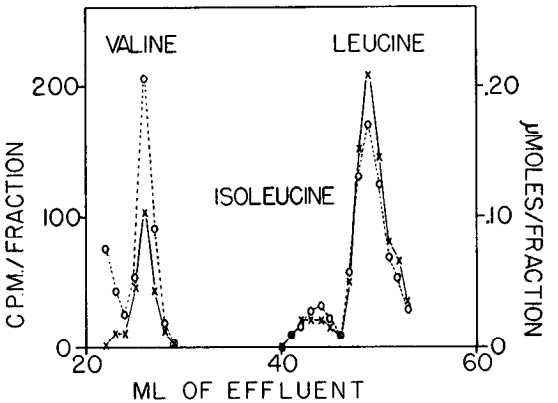

FIG. 1. A typical chromatogram of a ferritin hydrolysate for the calculation of the specific activities of constituent amino acids. 0.4 × 105 cm. Amberlite XE-69 column; elution with 2.0 N HCl. O, micromoles of amino acid per fraction; ×, counts per minute per fraction.

in five experiments was synthesized during this period when the intracellular valine, leucine, and isoleucine possessed constant specific activity. It is noteworthy that the valine, leucine, and isoleucine found in each of five such ferritin samples are almost all within 20 per cent of the average specific activity of the corresponding intracellular amino acid. To the extent that endogenous (hence at the outset non-radioactive) proteins contribute to ferritin synthesis directly, the radioactivity of the amino acids of the ferritin should be lower than that of the corresponding intracellular free amino acids. The figures in Table I are not consistent with such a direct conversion of protein to ferritin. Somewhat lower specific activities were observed for the amino acids in ferritin isolated after 6 hours. These values have been rejected because the ferritin was grossly impure.

The proteins in the various subcellular fractions and the protein saved from various steps in the isolation of ferritin were all assayed for radio-

activity. All of the protein samples from a given point of time were approximately equally radioactive, except that the protein remaining soluble in 25 per cent ammonium sulfate solution was consistently one-fourth less radioactive than the average. The average radioactivity increased from about 300 c.p.m. per mg. of protein at 6 hours to about 650 c.p.m. per mg. of protein at 3 days. The most radioactive of such proteins, the alcohol-denatured discard from a 3 day infusion, had 240 c.p.m. per μmole of

TABLE I

Continuous Infusion of C^{14} Amino Acids into Adult Rats

Weight of rat	Time	Ferritin	Counts per minute per micromole of amino acid isolated					
			L-Valine-C^{14}		L-Isoleucine-C^{14}		L-Leucine-C^{14}	
			Intracellular	Ferritin	Intracellular	Ferritin	Intracellular	Ferritin
gm.		*c.p.m. per mg.*						
255	4 hrs.	*	450	*	700	*	1040	*
240	1 day	750	525	520		800	805	1180
244	2 days	850	†	800	†	500	†	990
264	3 "	1000	608	663	660	650	900	755
250	3 "	1050	780	720	575	500	1180	1020
Average.....			590	675	645	610	981	986

Each of six male Wistar rats was given 10 mg. of saccharated iron oxide intravenously, and simultaneously continuous infusion (0.74 ml. per hour) was commenced with a solution containing L-valine-1-C^{14} (30.6 mM; 3230 c.p.m. per μmole), L-isoleucine-1-C^{14} (12.8 mM; 3800 c.p.m. per μmole), L-leucine-1-C^{14} (22.9 mM; 7160 c.p.m. per μmole), 0.04 M sodium chloride, and 0.31 M glucose. The rats received no protein in the diet. The specific activities of the free intracellular amino acids and of the amino acids of the ferritin hydrolysate were determined as described in the text.

* The entire liver was used for determination of intracellular specific activities.

† The entire liver was used for isolation of ferritin.

valine, 140 c.p.m. per μmole of isoleucine, and 480 c.p.m. per μmole of leucine.

In a second series of experiments, the animals were first given a large dose of leucine-C^{14} by tail-vein. 24 hours later the femoral vein was catheterized. Immediately after 10 mg. of saccharated iron oxide were injected through the catheter, an intravenous infusion of unlabeled L-leucine (22 mg. per hour) was begun. We anticipated that the high influx of non-radioactive leucine would maintain the intracellular pool of leucine at a very low specific activity and that the ferritin formed would be non-radioactive, although the other proteins would all be labeled. Table II

indicates that the specific activity of the intracellular leucine remained higher than the average specific activity for total liver protein for at least 1 day, despite the continuous infusion of unlabeled leucine. No conclusions regarding the source of ferritin leucine can be drawn. However, the data suggested a possible explanation of the experiments of Francis and Winnick (11), Babson and Winnick (12), and Friedberg and Walter (13), in which considerable amounts of unlabeled amino acid failed to inhibit the incorporation of radioactive amino acid derived from labeled protein into the protein of a tissue culture or of several organs in a rat. Large amounts of intravenously administered leucine mix only partially with leu-

TABLE II

Specific Activity of Leucine in Total Liver Protein, Ferritin, and Free Intracellular Leucine after Infusion with Unlabeled Leucine

Time	Total protein	Free liver leucine	Ferritin
	c.p.m. per μmole	*c.p.m. per μmole*	*c.p.m. per μmole*
5 hrs.	266	344	
21 "	230	350	373
3 days	174	173	205

Each of three rats weighing 200 gm. was injected with DL-leucine-1-C^{14} (5 μmoles, 1.5×10^7 c.p.m.). 20 hours later saccharated iron oxide (10 mg. of Fe) was injected, and the animal was continuously infused with 1.1 ml. per hour of a solution containing 2.0 gm. of L-leucine, 4.0 gm. of glucose, and 0.2 gm. of sodium chloride per 100 ml. Throughout the experiment the rats ate a normal diet. The time is measured from the beginning of the infusion; specific activities were determined by chromatographic isolation of the leucine.

cine produced intracellularly by proteolysis and hence do not "wash out" the intracellular radioactive amino acids.

To check this point, L-leucine-C^{14} was injected intravenously into 150 gm. rats in doses of 25 μmoles per 100 gm. every 10 minutes. This is 30 mg. of L-leucine per hour or approximately 3 times the normal rate of dietary intake. After 5 minutes, the first animal was killed, and the specific activity of the intracellular leucine was found to be only 35 per cent of that of the injected leucine. The specific activity of the intracellular leucine did not exceed 40 per cent of the injected specific activity even after 80 minutes.

DISCUSSION

The present evidence complements that of Askonas *et al.* (9, 10) and of Simpson and Velick (7). Entirely different experimental designs applied to three different protein-synthesizing systems have led to one conclusion

that all, or very nearly all, of the amino acid residues in several proteins are derived from the free amino acid pool. If this conclusion is justified for the rapid production and secretion of large quantities of milk protein, for the very slow "turnover" of intracellular muscle enzymes, and for the synthesis *de novo* of ferritin, it is reasonable to suggest that all protein is synthesized from the free amino acid pool. Arguments to the contrary must be closely examined.

As indicated above, experiments in which an effort has been made to limit reincorporation of residues from labeled protein by flooding the tissue with unlabeled amino acid are not conclusive. Another serious criticism of this type of experiment is that no proof is available that the tissue protein finally assayed does not include some of the original completely unmodified protein. Thus Babson and Winnick ((12) Table IV) report that, after 2 hours, the protein specific activities in the liver and kidney protein of the rat were about 5 per cent of the activity of the tagged plasma protein. This much activity could be accounted for by no more than 10 per cent of plasma in the organs. Alternatively, there is evidence from several sources that intact protein molecules can be absorbed into cells, and it seems quite possible that there may be a specific imbibition of certain proteins by certain organs (20). Ebert (21) has shown that, when transplants of radioactive chick embryo kidney, liver, or spleen are made into chick embryos, the radioactivity appears almost exclusively in the corresponding organ of the embryo. Although the possibility of partial breakdown and resynthesis is not excluded, it seems quite likely that entire unmodified protein molecules have been selectively absorbed by the organ for which they are most specific.

The unequal labeling of amino acid residues in proteins by Anfinsen and his coworkers (22–24) suggests a stepwise synthesis of the protein through peptide intermediates. The present evidence requires that, if there are peptide intermediates, these must be derived only from free amino acids and not from preformed protein.

In calculations of turnover rates, it has been tacitly assumed that free amino acids were the precursors of protein. This assumption is justified. However, the specific activity of the intracellular amino acid is not likely to be the same as that of the injected amino acid. Thus, turnover rates based on a single massive injection may be in error by a factor of 2. In the same way, so much reincorporation of amino acids from the breakdown of protein takes place that turnover rates based on apparent rate of decay are quite inaccurate. This is confirmed by the experiments of Penn, Mandeles, and Anker (25), who have recently found that the rate of loss of tagged plasma protein depends on whether other proteins in the animal are labeled.

Finally, although proteins appear to be derived only from free amino acids, it is of interest to note that, in turn, more than half of the free amino acid pool is derived from protein. Thus, in our first experiments in which no amino acids were received in the diet, for every amino acid molecule entering ferritin from the circulation approximately seven came from pre-existing non-labeled liver (and plasma?) protein. In the last experiment, despite the infusion of 225 μmoles of leucine per hour, at least half of the intracellular leucine, and hence half of the protein synthesis, is derived from unlabeled endogenous protein.

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SUMMARY

L-Valine-C^{14}, L-isoleucine-C^{14}, and L-leucine-C^{14} have been infused continuously into rats to maintain a relatively constant level of radioactivity in these amino acids over a 3 day period. Saccharated iron oxide was injected to stimulate ferritin synthesis. The ferritin isolated after various intervals was found to have the same radioactivity in the three amino acids as was present in the liver tissue. It is concluded that ferritin, like several other proteins, is synthesized only from free amino acids and not directly from other proteins.

It has also been noted that the bulk of the free amino acids within the liver tissue is derived from endogenous protein, even when relatively large amounts of amino acid are introduced into the circulation. This observation affects many determinations of "turnover rates" and is important in considering experiments which seem to show a direct conversion of one protein into another.

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