Lack of Effect of Amino Acid Concentration on Protein Synthesis in the Perfused Rat Liver

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The effect of increasing the perfusate concentration of amino acids on the incorporation of labelled valine into protein was followed in perfusions of rat livers lasting for 2 h. A fixed amount of labelled and unlabelled valine was added to the perfusate as the other amino acids were increased in multiples of the concentrations normally found in rat plasma. Under these conditions no increase in valine incorporation was observed, which appeared to be in conflict with results published by other workers. However, a different method of labelling from that used here was used in the earlier studies. An increasing amount of a labelled amino acid was added as the concentrations of the unlabelled amino acids were increased in the perfusate. An experiment directly comparing the two labelling methods produced results that indicated that the apparent increase in liver protein synthesis observed by the other workers could have been due to the method of radioisotope addition. It is therefore concluded that increasing the perfusate concentration of amino acids does not increase amino acid incorporation into liver protein.

Numerous experiments have been performed in which the synthesis of proteins by the perfused rat liver has been studied (Miller & Griffin, 1973; Ross, 1972). For short-term perfusions, one might assume that an amino acid mixture similar to that present in rat plasma would provide adequate substrate for normal rates of protein synthesis. However, for more extended perfusions these concentrations might not be sufficient to support protein synthesis. Jefferson & Korner (1969) observed that only 11 amino acids were required for protein synthesis in livers perfused for 1 h. Optimum rates of incorporation of labelled amino acid into liver protein were obtained when these 11 amino acids were present at ten times their normal concentrations in rat plasma.

In the present study, an attempt was made to repeat the experiments of Jefferson & Korner (1969) in perfusions lasting for 2 h. The use of an alternative method of radioisotope addition produced results in conflict with those of Jefferson & Korner (1969); that is, no increase in amino acid incorporation was observed when the concentration of the 11 amino acids was increased. Further experiments using a double-label system confirmed these results. It is suggested that the apparent increase in incorporation could have been due to the method of radioisotope addition used by Jefferson & Korner (1969).

A preliminary report of the results described here has been presented (Peavy & Hansen, 1975).

Materials and Methods

Chemicals

Bovine serum albumin (fraction V) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; unlabelled amino acids were from Nutritional Biochemicals Corp., Cleveland, OH, U.S.A.; L-[2,3-³H]-valine (16 Ci/mmol) and L-[U-¹⁴C]leucine (312 mCi/mmol) were from Schwarz/Mann, Orangeburg, NY, U.S.A.; stabilized tungstic acid was from Hycel, Houston, TX, U.S.A.; NCS tissue solubilizer was from Amersham/Searle, Arlington Heights, IL, U.S.A.

Animals

Specific-pathogen-free male Sprague–Dawley rats from Hilltop Lab Animals (Scottsdale, PA, U.S.A.) were used. They were allowed free access to standard lab chow and water at all times except for the 24 h period before perfusion, when food was withdrawn. Animals weighed 120–150 g at the time of perfusion.

Liver perfusion

The methods of Hems *et al.* (1966) for liver perfusion *in situ* was used. The basic perfusion medium consisted of 2.6% (w/v) bovine serum albumin and 5% (v/v) aged human erythrocytes in Krebs–Ringer

bicarbonate buffer (Krebs & Henseleit, 1932), pH 7.4, which was continuously gassed during the perfusion with $O_2 + CO_2$ (95:5). The 11 amino acids that Jefferson & Korner (1969) found were required in the perfused liver for optimum protein synthesis were added to the medium in various multiples of the concentrations found in normal rat plasma, except for valine, which was always added at 5 mM. The concentrations (μM) of the other ten amino acids were similar to the values reported by Scharff & Wool (1966) and Schimassek & Gerok (1965) for normal rat plasma and were as follows: arginine (230), asparagine (70), isoleucine (106), leucine (160), lysine (430), methionine (70), phenylalanine (80), proline (290), threonine (400) and tryptophan (80). For Expt. 1, two sterile stock solutions were prepared. The first contained 0.1 M-valine with 200 nCi of [3H]valine/ μmol . The second contained the other ten amino acids as listed above at 200 times the normal concentrations in rat plasma. Appropriate volumes of each solution were added to the perfusion medium to give the desired perfusate concentrations. For Expt. 2, three sterile stock solutions were used. The first was the valine solution as used in Expt. 1. The second was a solution of leucine at 200 times the normal plasma concentration with 625 nCi of [^{14}C]leucine/ μmol . The third was a solution of the other nine amino acids at 200 times their normal concentrations. As in Expt. 1, appropriate volumes of each solution were added to the medium to give the desired perfusate concentration of each. Pyruvate (15 mM) served as the added energy source in all perfusions. The final perfusate volume was 100 ml. Livers were first perfused for 17 min to allow for equilibration of the system. All additions were present in the medium except for the labelled amino acid(s), which was added at 17 min. Zero-time samples were taken after the perfusions were continued for 3 min, to allow for complete distribution of the labelled amino acid throughout the medium. The perfusions were then continued for 120 min.

Analytical methods

For the determination of radioactivity incorporated into liver trichloroacetic acid-precipitable proteins, the carcass was removed from the perfusion apparatus and the liver rapidly excised, washed with 0.9% NaCl, blotted and weighed. A 100 mg piece of the right lobe was removed and homogenized in a Teflon/glass homogenizer in 2 ml of cold 10% (w/v) trichloroacetic acid (which for Expt. 1 also contained 10 mM-valine and for Expt. 2 contained 10 mM-valine and 10 mM-leucine). The sample was centrifuged at 1000g for 10 min and the resulting pellet was washed with 2 $\times$ 3 ml of cold 10% trichloroacetic acid. The pellet was solubilized in 1.0 ml of 0.3 M-NaOH by heating at 80°C for 20 min with occasional agitation.

The protein in a 0.2 ml sample of the solubilized protein solution was re-precipitated by the addition of 2 ml of 10% trichloroacetic acid. The precipitate was collected on a Millipore filter (0.45 μm pore size) and washed with 3 $\times$ 2 ml of 10% trichloroacetic acid. The filter was then placed in a scintillation vial and the protein dissolved by the addition of 0.7 ml of conc. formic acid. After 20 min, 15 ml of BBOT scintillation fluid was added and the filter dissolved by vigorous shaking. BBOT scintillation fluid consisted of 4 g of 2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen/litre of toluene/methyl Cellosolve (1:1, v/v). The radioactivity was determined in a Packard model 3320 scintillation counter, with the radioactivity (d.p.m.) being calculated by a channels-ratio technique. Preliminary tests with several liver samples showed this method to be reproducible within 5%.

For the determination of radioactivity incorporated into perfusate proteins, a 0.5 ml sample of the perfusion medium was taken and the erythrocytes were removed by centrifugation at 1000g for 5 min. A 0.1 ml sample of the resulting plasma was precipitated by addition of 2 ml of buffered 0.78% (w/v) Na_2WO_4 in 0.042 M- H_2SO_4 (Hycel, Houston, TX, U.S.A.) which had been diluted 1:1 (v/v) with water. This reagent also contained 10 mM-valine or 10 mM-valine plus 10 mM-leucine in Expts. 1 and 2 respectively. After centrifugation at 1000g for 10 min, the resulting pellet was washed with 2 $\times$ 2 ml of the Hycel reagent. The pellets were then dissolved in 2.2 ml of NCS tissue solubilizer by heating at 45°C for 10 min with occasional agitation. The samples were left to cool to 23°C and a 2.0 ml sample was withdrawn and placed in a scintillation vial, to which 15 ml of PPO/POPOP scintillation fluid was added. PPO/POPOP scintillation fluid consisted of 4 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre of toluene. The radioactivity was then determined as for the liver protein samples. The radioactivity values obtained at zero time (3 min after radioisotope addition) were used to indicate any radioactivity trapped in the protein precipitates and were subtracted from samples taken later in the perfusion. The values were 2 and 10% of the radioactivity present in perfusate proteins after 2 h of perfusion with [^{14}C]leucine and [3H]valine respectively.

Except where indicated, all extraction steps for the liver and perfusate proteins were carried out at 0–4°C.

Results

Expt. 1

The results of Expt. 1 are shown in Table 1. In this experiment, valine was held at 5 mM and 200 nCi/ μmol in each perfusion as the other ten amino acids were increased from 0 to 20 times the concentrations normally found in rat plasma. Although the number

of perfusions precluded statistical analysis, it was evident that a trend in amino acid incorporation of the magnitude observed previously (Jefferson & Korner, 1969) was not present. Jefferson & Korner (1969) had observed about a threefold increase in incorporation as the amino acids were increased from normal to 10 times normal concentrations. One difference between the present experiment and those reported previously was that in our experiment the labelled amino acid was held constant in both specific radioactivity and molar amount as the other amino acids were varied, whereas in the earlier work (Jefferson & Korner, 1969) the labelled amino acid was held constant in specific radioactivity but increased in total molar amount as the other amino acids were also increased. To test if this difference might explain the disparity of results, Expt. 2 was performed.

Expt. 2

In this experiment, livers were perfused with both [^3H]valine and [^{14}C]leucine. Valine was given as in Expt. 1, i.e. constant specific radioactivity and molar amount. [^{14}C]Leucine was maintained at a constant specific radioactivity but was increased in molar amount as a multiple of its normal plasma concentration as the other amino acids were increased. The incorporation rates of [^3H]valine and [^{14}C]leucine into total liver proteins at multiples of 1 and 5 times the normal plasma concentrations of amino acids are shown in Table 2. As in Expt. 1, no increase in incorporation of [^3H]valine was observed, whereas in the same livers incorporation of [^{14}C]leucine was increased nearly twofold. Further verification of this point is presented in Table 3 where incorporation of valine and leucine into perfusate proteins in these same experiments is presented. Noteworthy, the effect observed on [^{14}C]leucine incorporation was greater in the perfusate protein than in liver protein. The time-course of radioisotope incorporation into perfusate proteins is presented in Fig. 1. After the characteristic 20–30 min lag period (Mortimore & Mondon, 1970), incorporation in each case was linear with time for the duration of the experiment, indicating that the results in Tables 2 and 3 were not simply due to fortuitous timing of sample collection.

Table 1. *Incorporation of [^3H]valine into total liver protein with various multiples of rat plasma amino acid concentrations present*

The mixture of amino acids consisted of those listed in the Materials and Methods section. Valine was held at 5 mM and 200 nCi/ μmol as the concentrations of the other amino acids were varied. Pyruvate (15 mM) was present in all cases. Values in parentheses represent numbers of livers perfused.

Multiple of plasma amino acid concentration	[^3H]Valine incorporation (nCi/h per g of tissue)
0 (2)	292
1 (2)	300
2.5 (2)	311
5 (4)	308
10 (4)	302
20 (2)	209

Discussion

In the experiments presented in this paper 5 mM-valine was chosen as the labelling source for several reasons. This concentration of valine is approximately 25 times the normal rat plasma concentration (Scharff & Wool, 1966; Schimassek & Gerok, 1965).

Table 2. *Incorporation of [^3H]valine and [^{14}C]leucine into total liver protein*

Valine was given as in Table 1. Leucine was given at 625 nCi/ μmol but increased in total molar amount from 1 to 5 times plasma concentration as the other amino acids were increased. All other conditions were as in Table 1. Results are means $\pm$ S.E.M. for four livers; statistical comparisons were made by Student's *t* test.

Multiple of plasma amino acid concn.	[^3H]Valine incorporation (nCi/h per g of tissue)	[^{14}C]Leucine incorporation (nCi/h per g of tissue)
1	300 $\pm$ 32	344 $\pm$ 19
	N.S.	$P < 0.01$
5	320 $\pm$ 16	586 $\pm$ 35

Table 3. *Incorporation of [^3H]valine and [^{14}C]leucine into perfusate protein*

Results (means $\pm$ S.E.M. of four experiments) are expressed as total nCi incorporated into perfusate proteins at 120 min of perfusion. All other conditions were as in Tables 1 and 2.

Multiple of plasma amino acid concn.	[^3H]Valine incorporation	[^{14}C]Leucine incorporation
1	502 $\pm$ 84	950 $\pm$ 78
	N.S.	$P < 0.01$
5	584 $\pm$ 40	2472 $\pm$ 146

Fig. 1. Time-course of radioisotope incorporation into perfusate proteins

Results shown are from the livers perfused in Expt. 2. (a) [^{14}C]Leucine incorporation at 1 (●) and 5 (○) times plasma amino acid concentrations. (b) [^3H]Valine incorporation at 1 (●) and 5 (○) times plasma amino acid concentrations. Vertical bars represent 1 S.E.M.

Therefore valine should not become limiting to protein synthesis in our experiments if synthesis were to increase in proportion to the concentrations of the other amino acids present. In addition, this concentration of valine will result in a large intracellular pool of valine whose specific radioactivity will approximate the extracellular specific radioactivity (Mortimore *et al.*, 1972). Because of the large intracellular pool, the specific radioactivity should remain relatively constant despite the possibility of changes in the rate of entry of unlabelled valine into the pool owing to alterations in proteolysis rates as a result of exogenous amino acid addition (Woodside & Mortimore, 1972).

Using 15 mM-valine as the labelling source, Woodside & Mortimore (1972) have reported that when a mixture of 20 amino acids was added at 10 times the normal concentration a stimulation of valine incorporation of approx. 20% was observed compared with the case where no amino acids were added. However, in contrast with the experiments reported here, no energy source was added in their perfusions.

It is conceivable that, when no energy source and no amino acids are added, the concentrations of endogenous amino acids could be limiting for protein synthesis owing to the additional drain to meet energy requirements. The addition of large quantities of amino acids could alleviate the limitation by providing sufficient substrate to meet the demands of both processes and would therefore result in a greater incorporation of label into liver protein. Tables 1 and 2 show that with similar labelling conditions, but with an energy source (pyruvate) present in all cases, the addition of 11 amino acids did not increase the incorporation of valine into liver protein.

Mortimore *et al.* (1972), using the perfused liver, have shown that, when the extracellular concentration of valine is increased while the valine specific radioactivity is held constant, the intracellular specific radioactivity increases and the intracellular/extracellular specific radioactivity ratio approaches a value of 1. For a given rate of protein synthesis, the effect of increasing the extracellular valine concentration would be to increase the intracellular valine specific radioactivity and thereby increase the rate of label incorporation into protein. Jefferson & Korner (1969) used phenylalanine as the labelled amino acid and kept the phenylalanine specific radioactivity constant but increased the phenylalanine concentration as a multiple of its plasma concentration as the other 11 amino acids were increased. If one assumes that phenylalanine will behave similarly to valine as described above, then the effect of amino acid addition that Jefferson & Korner (1969) observed on phenylalanine incorporation into total protein would appear to reflect the method of radioisotope administration and not an effect on rates of liver protein synthesis. In the present study, increasing the perfusate concentrations of the 11 amino acids did not increase the rate of valine incorporation into protein.

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References

- Hems, R., Ross, B. D., Berry, M. N. & Krebs, H. A. (1966) *Biochem. J.* **101**, 284–292
- Jefferson, L. S. & Korner, A. (1969) *Biochem. J.* **111**, 703–712
- Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
- Miller, L. L. & Griffin, J. (1973) in *Isolated Liver Perfusion and its Applications*, (Bartosek, I., Guaitini, A. & Miller, L. L., eds.) pp. 139–178, Raven Press, New York
- Mortimore, G. E. & Mondon, C. E. (1970) *J. Biol. Chem.* **245**, 2375–2383

- Mortimore, G. E., Woodside, K. H. & Henry, J. E. (1972) *J. Biol. Chem.* **247**, 2776-2784
- Peavy, D. E. & Hansen, R. J. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 481
- Ross, B. D. (1972) *Perfusion Techniques in Biochemistry*, pp. 133-220, Clarendon Press, Oxford
- Scharff, R. & Wool, I. G. (1966) *Biochem. J.* **99**, 173-178
- Schimassek, H. & Gerok, W. (1965) *Biochem. Z.* **343**, 407-415
- Woodside, K. H. & Mortimore, G. E. (1972) *J. Biol. Chem.* **247**, 6474-6481