plasma concentration bears any relation to the concentration in the cells.

The finding that trimethylamine is released by alkali from the washed proteins of plasma and whole blood is of some interest. In the absence of other known trimethylamine-liberating substances, this must be assumed to originate from 'protein-bound' ergothioneine. The main part of the total blood ergothioneine (about 60%) is present in this bound form. Moreover, the values found are unaffected by different ways of precipitating the proteins or by dialysis, so that the binding cannot be due to a loose association complex or to adsorption of ergothioneine on the proteins during their precipitation. It was suggested by Melville & Lubschez (1953) that the existence of a protein-ergothioneine-disulphide complex would account for their observation that, when rat blood was treated with reducing agents before precipitation of protein with trichloroacetic acid, greatly increased values for blood ergothioneine resulted. With human blood, however, the failure to diminish the level of the 'protein-bound' ergothioneine by treatment with cysteine, glutathione or sulphite is evidence against the existence of such a disulphide linkage.

The slightly greater values found for albumin than for globulin binding are consistent with the greater concentration of the former in human plasma and render it unlikely that a specific protein is associated with the ergothioneine.

SUMMARY

1. A new method of estimating ergothioneine in biological fluids is described which depends on liberating trimethylamine from the thiol in

alkaline solution and its determination as the picrate by spectrophotometry.

2. Values are given and discussed for the concentration ranges of ergothioneine found in human blood, plasma and packed cells.

3. Ergothioneine does not diffuse either out of or into human blood cells.

4. Evidence is given for the existence in blood of 'protein-bound' ergothioneine.

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Toxopyrimidine Phosphate as an Inhibitor of Bacterial Enzyme Systems that Require Pyridoxal Phosphate

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Elucidation of the structure of thiamine was followed by attempts to determine the roles of the two components of the molecule in the nutrition of higher animals and micro-organisms (Abderhalden, 1939). Surprisingly, the pyrimidine unit (4-amino-5-hydroxymethyl-2-methylpyrimidine; toxopyrimidine; pyramin) proved markedly toxic to rats. Makino, Kinoshita, Sasaki & Shioi (1954) showed

that the animals could be protected by simultaneous administration of vitamin B₆. They noted the structural similarity between toxopyrimidine (I) and pyridoxamine (II), and suggested that the former was converted *in vivo* into toxopyrimidine phosphate (4-amino-5-hydroxymethyl-2-methylpyrimidine O-phosphate), which might then competitively displace pyridoxal phosphate from some

(I)

(II)

essential enzyme. Support for this idea came when Makino & Koike (1954*a, b*) synthesized toxopyrimidine phosphate and demonstrated inhibition of the tyrosine decarboxylase of *Streptococcus faecalis* by competition with the pyridoxal phosphate coenzyme. Inhibition of growth of certain microorganisms by toxopyrimidine can also be reversed competitively by pyridoxal, pyridoxamine or pyridoxine (Scheunert, Haenel & Meyer, 1957).

The present work was undertaken to determine whether toxopyrimidine phosphate is a useful competitive inhibitor in studies with vitamin B₆-enzymes. Several inhibitor-analogues are known (Hurwitz, 1952), but so far only deoxypyridoxine phosphate has attracted much attention. It suffers from the disadvantage that optimum inhibitory action is achieved only when the enzyme under examination has been resolved and the inhibitor allowed to react with the apoenzyme in the absence of pyridoxal phosphate. It appears that toxopyrimidine phosphate suffers from this drawback too.

A preliminary account of this work has appeared (Haughton & King, 1957).

MATERIALS AND METHODS

Organisms. *Streptococcus faecalis*, National Collection of Industrial Bacteria, strain nos. 6782 and 6783, was used. *Proteus vulgaris* 10L and *Escherichia coli* 106E were stock laboratory strains. Each organism was maintained by subculture at 2-monthly intervals on nutrient agar slopes. For inoculation purposes subcultures were made in nutrient broth [Lab-Lemco, 1% (w/v); peptone, 1% (w/v); sodium chloride, 0.5% (w/v); obtained in the form of dried granules from Oxo Ltd., London].

Reagents. Pyridoxal phosphate and toxopyrimidine phosphate were generous gifts from Roche Products Ltd., through the courtesy of Dr A. L. Morrison. Tryptone was

obtained from Oxo Ltd., London. All other reagents were from commercial sources; amino acids were examined by paper chromatography before use.

Buffers. Phosphate and phthalate buffers were prepared by adding the appropriate amount of sodium hydroxide to potassium dihydrogen phosphate or potassium hydrogen phthalate (Cole, 1933).

Cell-free enzyme preparations. Three methods were employed: (1) Acetone-dried powders were prepared by slowly pouring an ice-cold suspension of the organism into 4 vol. of cold acetone with continual stirring; the precipitate was collected on the centrifuge, washed with cold acetone and ether and dried and stored *in vacuo*. (2) Suspensions were shaken for 45 min. at 600 strokes/min. with grade 12 Ballotini beads (Empire Glass Co., Leicester: King & Alexander, 1948); the cell debris was centrifuged down and the supernatant used as source of the enzyme. (3) Suspensions were disintegrated by crushing at -20° , without abrasive, in the Hughes press (Hughes, 1951). For cell-free extracts prepared by methods 2 and 3, the density of the washed suspension before disintegration was measured turbidimetrically; the instrument was calibrated against suspensions whose dry weight had been determined by freeze-drying. The enzyme activity of the cell-free extract was then expressed in terms of the dry weight of cells from which the extract was derived.

Preparation and assay of individual enzymes

Arginine and glutamic acid decarboxylases. *E. coli* 106E was grown on nutrient broth containing 1% (w/v) of glucose for 17 hr. at 28° (Gale, 1940). The washed cells were made into a thick cream with 0.05M-phthalate buffer (pH 4.5), and transferred to the previously chilled Hughes press. After crushing, the suspension of disrupted cells was centrifuged and the clear supernatant was used as the source of both enzymes.

Enzymic activity was determined by adding the buffered enzyme preparation (2.3 ml.) to the main compartment of a Warburg flask and placing 0.1M-L-glutamic acid or -L-arginine (0.7 ml. in phthalate buffer, pH 4.5) in the side arm. Toxopyrimidine phosphate was added, where required, to the main compartment and the flasks were equilibrated at 30° for 30 min., to permit interaction of enzyme and inhibitor before addition of the substrate. The output of CO₂ was linear for at least 15 min., and the Q_{CO_2} values were calculated for this initial period as μ l. of CO₂/hr./mg. dry wt. of uncrushed cells.

Neutral amino acid decarboxylase. A cell-free extract from *P. vulgaris* was prepared by growing the organism on nutrient broth for 16 hr. at 37° and disintegrating the washed cells (suspended in 0.1M-phosphate buffer, pH 6), by shaking with Ballotini beads (Ekladius, King & Sutton, 1957).

A suitable volume of the extract was placed in the main compartment of a Warburg flask, along with pyridoxal phosphate or toxopyrimidine phosphate, or both, where indicated, and phosphate buffer (pH 6) added to bring the volume to 2.3 ml. with a phosphate concentration of 0.1M. One side arm contained 0.7 ml. of L-leucine (0.25M) or DL-valine (0.5M) in buffer. In some cases pyridoxal phosphate was placed in a second side arm. The manometers were filled with N₂ and shaken for 30 min. at 37° to allow equilibration and interaction of the enzyme with the coenzyme or inhibitor. The substrate was then added and,

15 min. later, pyridoxal phosphate tipped in from the second side arm where indicated. $Q_{CO_2}^{N_2} = \mu\text{l. of CO}_2/\text{hr./mg. dry wt. of intact cells.}$

Glutamic acid-alanine transaminase. *E. coli* 106E was grown in Roux bottles on a medium containing, per litre, KH_2PO_4 , 1.5 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 18 g.; NaCl , 0.5 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.8 g.; glucose, 4 g. Each bottle was inoculated with a few drops of a broth culture and harvested after 16 hr. incubation at 28°. Washed suspensions were prepared and cell-free extracts obtained by shaking with Ballotini beads.

Transaminase activities were compared by setting up (in duplicate) test tubes containing (in a total vol. of 2 ml.) a suitable amount of the cell-free extract with sodium pyruvate (0.05M), L-glutamic acid (0.025M), toxopyrimidine phosphate (0.5 mM), and phosphate buffer (pH 8; 0.1M). The controls omitted inhibitor, enzyme and pyruvate respectively. The tubes were incubated for 1 hr. at 37° and the reaction was stopped by immersing them in boiling water for 5 min. The denatured protein was removed by centrifuging and uniform amounts (approx. 10 $\mu\text{l.}$) of each supernatant were applied to a sheet of Whatman no. 1 filter paper. This was run as a descending chromatogram for 40 hr. with a mobile phase of butanol (480 ml.), acetic acid (95 ml.), butyl acetate (35 ml.) and water (195 ml.). Development was by spraying with 0.1% (w/v) ninhydrin in chloroform containing 0.1% (v/v) of collidine, followed by heating at 40–50° in a current of air. The degree of inhibition (if any) could be assessed by visual comparison of the alanine spots in the 'inhibitor' series and the 'no-inhibitor' controls. The quantity of material chromatographed was sufficient to give an alanine spot of only moderate intensity, and under these conditions a twofold difference would have been detected easily. Although not quantitative, this technique was adequate for our purpose of detecting any gross reduction in transaminase activity in the presence of the inhibitor.

Tryptophanase. *E. coli* 106E was grown on tryptone-agar at 37° for 18 hr. The cells were acetone-dried and extracted with 1.6M-KCl at 37° overnight (Dawes & Happold, 1949). The tryptophanase activity of the extract was measured by a modification of the method of Wood, Gunsalus & Umbreit (1947). The enzyme preparation (1.0 ml.), 0.5M-phosphate buffer (pH 7.8; 0.2 ml.) and water or 1.66 mM-toxopyrimidine phosphate (0.6 ml.) were placed in test tubes and equilibrated at 37° for 10 min. A solution (0.2 ml.) of 0.05M-L-tryptophan in 0.05N-NaOH was then added. After 30 min. the reaction was stopped by the addition of a saturated aqueous solution of trichloroacetic acid (0.2 ml.) and the indole extracted with toluene (2.0 ml.). A portion (1 ml.) of the toluene extract (pure toluene for the reagent blank) was transferred to a colorimeter tube and 2 ml. of absolute ethanol and 5 ml. of Ehrlich reagent (*p*-dimethylaminobenzaldehyde, 5 g.; absolute ethanol, 380 ml.; conc. HCl, 80 ml.) were added. After 10 min. the density was read in an EEL portable colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with a 626 filter (maximum transmission 570 μm); this instrument was adjusted to zero against the reagent blank. Density readings were converted into $\mu\text{g. of indole}$ by a standard curve.

Tyrosine decarboxylase. The appropriate strain of *S. faecalis* was grown on a tryptone-yeast-glucose medium, acetone-dried powders were prepared, and suspended

(20 mg./ml.) in 0.022M-phosphate buffer (pH 5.5) (Epps, 1944). After incubation for 5.5 hr. at 28° the debris was spun down and the extract used as source of the enzyme. In a few experiments, cell-free extracts made by disintegrating the cells with Ballotini beads were used.

The apoenzyme was partially separated from the coenzyme (pyridoxal phosphate) by precipitating the extracts with 3 vol. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution containing 3.3% (v/v) of aq. NH_3 (Epps, 1944). After standing for 10 min. at 4° the mixture was centrifuged and the precipitate resuspended in the original volume of water. The precipitation was repeated and the final solution freeze-dried after removal of the $(\text{NH}_4)_2\text{SO}_4$ by dialysis against distilled water overnight at 4°. This apoenzyme preparation showed about 20% of the maximum activity obtained in the presence of excess of coenzyme (Table 4).

Determination of tyrosine decarboxylase activity. A suitable amount of the apoenzyme preparation was dissolved in 0.2M-acetate buffer (pH 5.5; 2.5 ml.) and placed in the main compartment of a Warburg flask. A 0.033M-suspension of L-tyrosine (0.5 ml.) was placed in the side arm. Pyridoxal phosphate or toxopyrimidine phosphate, or both, were placed either in the main compartment or in a second side arm as required. The manometers were filled with N_2 and placed in the bath at 30°. After allowing time, as indicated, for equilibration and for interaction between apoenzyme and coenzyme or inhibitor, the substrate was added from the side arm and gas output measured over a 15 min. period. (Over longer periods output was often not linear.) Enzyme activity was expressed in terms of $Q_{CO_2}^{N_2}$.

RESULTS

Arginine and glutamic acid decarboxylases

0.5 mM-Toxopyrimidine phosphate did not inhibit the decarboxylation of either arginine or glutamic acid (Table 1). The enzyme preparations were crude extracts and no attempt had been made to resolve them, so it was necessary to consider whether this negative result was due to an excess of endogenous pyridoxal phosphate. Samples were boiled and the codecarboxylase activity estimated by means of the leucine apodecarboxylase of *Proteus vulgaris* (Ekladius *et al.* 1957). Final concentrations of pyridoxal phosphate not greater than μM and $5 \mu\text{M}$ were found in the arginine and

Table 1. Effect of toxopyrimidine phosphate on the decarboxylation of arginine and glutamic acid

Cell-free extracts of *E. coli* were incubated, with or without toxopyrimidine phosphate, for 30 min. at 30° in the main compartment of a Warburg flask. Arginine or glutamic acid was then added from a side arm. The initial rate of CO_2 output is given.

Concn. of toxopyrimidine phosphate (mM)	Decarboxylase activity (Q_{CO_2})	
	Arginine	Glutamic acid
0	424	71
0.5	461	63

glutamic acid experiments respectively. The inhibitor:coenzyme ratio must therefore have been at least 100:1.

Neutral amino acid decarboxylase

This enzyme is of interest in coenzyme studies (Ekladius *et al.* 1957). It catalyses the decarboxylation of leucine, valine and some other amino acids. The coenzyme (pyridoxal phosphate) is less

Fig. 1. Effect of toxopyrimidine phosphate on the decarboxylation of leucine. Warburg flasks contained enzyme and 0.06 M-L-leucine (added at $t=0$). Curve I, no additions; curve IV, excess of pyridoxal phosphate (27 μ M); curve III, pyridoxal phosphate (2.5 μ M) at $t=15$ min.; curve II, toxopyrimidine phosphate (0.5 mM) 30 min. before substrate, pyridoxal phosphate (2.5 μ M) at $t=15$ min. The points on curves II and III represent the mean of duplicates.

Table 2. Effect of toxopyrimidine phosphate on valine decarboxylation

Flasks containing cell-free enzyme preparation buffered at pH 6 with or without toxopyrimidine phosphate were incubated at 37°. 0.13 M-DL-Valine was added after 30 min. and pyridoxal phosphate after a further 15 min. Final volume, 3.0 ml. Enzyme activity is expressed as $Q_{CO_2}^{N_2}$ (= μ l. of CO_2 evolved/hr./mg. dry wt. of uncrushed cells).

Concn. of toxopyrimidine phosphate (mM)	Concn. of added pyridoxal phosphate (μ M)	Rate of decarboxylation ($Q_{CO_2}^{N_2}$)
0	0	18
0	1.3	26
0.5	0	21
0.5	1.3	27

firmly bound to the apoenzyme when leucine is the substrate than when valine is being decarboxylated; this was seen clearly in the *Proteus* extracts used as an enzyme source in these experiments. On adding leucine, the coenzyme dissociated from the apoenzyme spontaneously to such an extent that only a slight and transient reaction was observed (Fig. 1, curve I; final $Q_{CO_2}^{N_2}=1$) unless extra coenzyme was added (curve IV; $Q_{CO_2}^{N_2}=58$). When valine was offered to the enzyme, however, the coenzyme was more firmly bound and, even without the addition of extra coenzyme, decarboxylation proceeded at about two-thirds of the optimum rate (Table 2). One would expect the leucine decarboxylation to be an exceptionally favourable system for demonstrating inhibition by toxopyrimidine phosphate. In Fig. 1, curve III, the system was supplied with sufficient exogenous coenzyme to permit about half-maximal rate of decarboxylation ($Q_{CO_2}^{N_2}=34$), whereas in curve II the same experimental conditions were employed with addition of the inhibitor in 200-fold excess over the coenzyme. Even so, only slight inhibition occurs ($Q_{CO_2}^{N_2}=27$), though the inhibitor had been in contact with the apoenzyme for 30 min. before addition of the substrate, and a further 15 min. was allowed to elapse before the coenzyme was added. With valine, we should expect less inhibition than with leucine, and Table 2 shows that toxopyrimidine phosphate does not appreciably affect the decarboxylation of valine either in the absence of added coenzyme or with the addition of sufficient exogenous pyridoxal to give nearly maximal rate of action; the ratio inhibitor:added coenzyme was 400:1.

Transaminase

Though the method employed for estimation of transaminase yielded little more than qualitative results, it was certain that no gross inhibition of the glutamic acid-alanine transaminase of *E. coli* occurred in the presence of 0.5 mM-toxopyrimidine phosphate. No assay of the pyridoxal phosphate present in the enzyme preparation was performed, but from the results obtained in the arginine decarboxylase experiments, which used the same organism, the ratio inhibitor:coenzyme must have been at least 200:1.

Tryptophanase

There was no significant inhibition of tryptophanase activity in the presence of 0.5 mM-toxopyrimidine phosphate (Table 3).

Tyrosine decarboxylase

Inhibition of unresolved enzyme. Acetone-dried powders of *S. faecalis* 6782 showed $Q_{CO_2}^{N_2}$ (tyrosine) = 65; this figure was reduced to 49, an inhibition of 25 %, by 0.5 mM-toxopyrimidine phosphate.

Partially resolved enzyme (*S. faecalis* 6783). Table 4 shows that about 80% resolution was obtained, and that the concentration of pyridoxal phosphate needed for half-maximal activity agreed well with the value of $0.016 \mu\text{M}$ obtained by Umbreit & Gunsalus (1949). With three times this level of coenzyme and varying amounts of toxopyrimidine phosphate, inhibition was obtained as shown in Table 5. This brings out the following

Table 3. *Effect of toxopyrimidine phosphate on tryptophanase activity*

Tubes containing buffered enzyme extract (1.2 ml.), $0.05 \text{ M-L-tryptophan}$ (0.2 ml.), toxopyrimidine phosphate (0.5 ml.) and water to 2 ml. were incubated at 37° ; the reaction was stopped after 30 min. and the indole estimated.

	Indole formed ($\mu\text{g.}$)
Control (no substrate)	1.8
Control (no inhibitor)	30.8, 30.1
Toxopyrimidine phosphate (0.5 mM)	30.2, 26.4

Table 4. *Activation of tyrosine apodecarboxylase by pyridoxal phosphate*

The enzyme was incubated with $0.2 \text{ M-acetate buffer}$ (pH 5.5) and pyridoxal phosphate at the final concentration given. After equilibration at 30° tyrosine was added from a side arm and the initial rate of CO_2 output measured.

Concn. of pyridoxal phosphate (μM)	$Q_{\text{CO}_2}^{\text{N}}$
1.6	18.5
0.16	18.4
0.016	10.6
0	3.7

Table 5. *Inhibition of tyrosine decarboxylase by toxopyrimidine phosphate*

The apoenzyme preparation was placed in the main compartment of a Warburg flask and coenzyme (pyridoxal phosphate), inhibitor (toxopyrimidine phosphate) and substrate (L-tyrosine) were added at the times indicated.

Compound added		Time of addition (min.)		
Pyridoxal phosphate		0	15	0
Toxopyrimidine phosphate		15	0	0
Tyrosine		30	30	30
Concn. (μM)		Inhibition observed (%)		
Pyridoxal phosphate	Toxopyrimidine phosphate			
0.048	0.48	5	12	4
0.048	4.8	16	18	—
0.048	48	36	47	41
0.048	480	37	79	62
0.016	480	55	92	66

observations: (1) Appreciable inhibition is obtained only when a large excess of inhibitor is added. (2) The order of adding inhibitor and coenzyme is of great importance. If the inhibitor is added first, a ratio inhibitor:coenzyme of 1000:1 causes 50% inhibition; but a ratio of 30 000:1 is needed to produce the same effect if the coenzyme is added first. Intermediate results are obtained if coenzyme and inhibitor are added simultaneously. Makino & Koike (1954b) reported inhibition in this system with much lower inhibitor:enzyme ratios than those reported here; but their results are difficult to interpret as they employed two apoenzyme preparations with very different properties, and did not appear to appreciate the importance of the order of addition of inhibitor and coenzyme.

DISCUSSION

These investigations indicate that toxopyrimidine phosphate is unlikely to have more than limited applications as a competitive inhibitor of pyridoxal phosphate enzymes. Even in relatively large excess, it did not appreciably inhibit a number of systems which had not been resolved into apo- and co-enzyme. When resolution was achieved—with tyrosine decarboxylase—marked inhibition was observed only at a very high molar inhibitor:coenzyme ratio. Inhibition was also much more difficult to achieve when the apoenzyme was first allowed access to the coenzyme; this goes far to explain the lack of success with unresolved systems, and severely restricts the possible use of this reagent for detecting a pyridoxal phosphate requirement in systems where this cannot be demonstrated directly. It would seem, too, that the union of pyridoxal phosphate with the apoenzyme may not be a strictly reversible reaction. This is in accordance with previous work with other enzymes and with other pyridoxal phosphate analogues. Thus pyridoxine phosphate failed to inhibit the unresolved glutamate-aspartate transaminase of pig heart; nor was the system inhibited if the apoenzyme had been preincubated with pyridoxal phosphate. Inhibition was observed only if pyridoxine phosphate or deoxypyridoxine phosphate was incubated with the apoenzyme before addition of the coenzyme (Meister, Sober & Peterson, 1954). Unresolved bacterial tyrosine decarboxylase and the serine-glycine interconversion of liver were partially inhibited by deoxypyridoxine phosphate, but a much greater degree of inhibition was obtained when these enzymes were resolved and the apoenzymes preincubated with the inhibitor (Umbreit & Waddell, 1949; Blakley, 1955).

The decarboxylation of leucine by the enzyme found in *Proteus vulgaris* by Ekladius *et al.* (1957)

would seem to be a specially favourable case for demonstrating inhibition by toxopyrimidine phosphate, since the coenzyme spontaneously dissociated from the apoenzyme as decarboxylation proceeded (Fig. 1). But, even here, only slight inhibition was observed, whereas in the valine decarboxylation (when the coenzyme is more firmly bound) no inhibition was detected under the experimental conditions. Nevertheless, there are probably cases where toxopyrimidine or its phosphate can severely inhibit unresolved enzymes: otherwise, the toxicity to animals at relatively low toxopyrimidine:pyridoxal ratios is difficult to explain.

SUMMARY

1. Toxopyrimidine phosphate failed to inhibit unresolved bacterial arginine and glutamic decarboxylases, tryptophanase and glutamate-alanine transaminase.

2. The leucine decarboxylase system of *Proteus vulgaris*, in which the coenzyme dissociates spontaneously during enzyme action, was inhibited by 20 %.

3. The degree of inhibition of the resolved tyrosine decarboxylase system of *Streptococcus faecalis* depended on the order of addition of pyridoxal phosphate and toxopyrimidine phosphate to the apoenzyme. Even when the inhibitor was added first, an inhibitor:coenzyme ratio of 1000:1 was needed to produce 50 % inhibition.

4. Toxopyrimidine phosphate seems to be of only limited usefulness in the study of bacterial vitamin B₆ enzymes.

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Intermediates in the Biosynthesis of Bacterial Penicillinase

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The nature of the intermediates in the biosynthesis of proteins from amino acids has for several years been a subject of much concern to biochemists interested in the mechanism of protein formation. Experimental attack on the problem has taken several different forms, which can be roughly divided into (a) direct attempts to isolate stable intermediary precursors and to investigate the behaviour of likely chemical models with *in vitro* reconstructions based on simplified reaction systems, and (b) the 'indirect' approach, designed

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to obtain relevant evidence by following the kinetics of induced enzyme formation in micro-organisms, the extent to which a particular protein may be derived from pre-existing cell material other than free amino acids and the rate of incorporation of amino acids from the medium into the protein. If it can be reasonably assumed, or directly demonstrated, that amino acids in the medium equilibrate with the free amino acids present in the cell without measurable delay, any lag found between addition of a labelled amino acid and the appearance of the label in a specific protein may reasonably be regarded as evidence of the