5. The results indicate that free amino acids are utilized by embryonic tissues by routes similar to those found in adult tissues, and at rates that could probably account for the requirements of the organism for protein synthesis.

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The Utilization of *p*-Fluorophenylalanine for Protein Synthesis by the Phenylalanine-Incorporation System from Rabbit Reticulocytes

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The amino acid analogue *p*-fluorophenylalanine is incorporated into the proteins of both bacteria (Richmond, 1962) and animals, including specific proteins such as haemoglobin (Kruh & Rosa, 1959), aldolase and glyceraldehyde 3-phosphate dehydrogenase (Westhead & Boyer, 1961). The replacement of phenylalanine by *p*-fluorophenylalanine is only partial, however, and it therefore seemed possible that different amino acyl-transfer ribonucleic acids could act on the two amino acids and be responsible for the less efficient utilization of the analogue. Alternatively, the existence of degeneracy in the code for phenylalanine, i.e. the participation of more than one transfer RNA in the incorporation of phenylalanine, could result in discrimination against *p*-fluorophenylalanine, if one of the codes happened to favour phenylalanine more than the other. Recent work showing that polyuridylic acid is capable of stimulating the incorporation of phenylalanine by cell-free preparations from *Escherichia coli* (Nirenberg & Matthaei, 1961; Lengyel, Speyer & Ochoa, 1961) or mammalian cells (Arnstein, Cox & Hunt, 1962; Griffin & O'Neal, 1962; Maxwell, 1962; Weinstein & Schechter, 1962) made possible a study of the incorporation of phenylalanine and *p*-fluorophenylalanine into protein in relation to the code for phenylalanine.

The cell-free system used in the present work consisted of ribosomes, amino acid-activating enzymes and transfer RNA from rabbit reticulocytes, and is capable of catalysing the whole process of protein synthesis, i.e. 'activation' of the amino acids followed by attachment to the appro-

priate transfer ribonucleic acids and thence incorporation into peptide linkage. It has also been shown that a considerable proportion of the amino acids utilized for protein synthesis is incorporated into haemoglobin (Schweet, Lamfrom & Allen, 1958; Lipmann & Ehrenstein, 1961).

To measure the relative efficiency of the normal or polyuridylic acid-induced incorporation of phenylalanine and its *p*-fluoro analogue, the relative rates of activation of the two amino acids as well as their incorporation into the product insoluble in hot trichloroacetic acid has to be measured. Without these measurements it is not possible to decide whether any discrimination between the amino acids exists at the step involving the incorporation of the activated amino acid into protein.

In practice, competition between phenylalanine and *p*-fluorophenylalanine at the stage of amino acid activation and/or transfer to transfer RNA was found, but there was no evidence for degeneracy in the code, since the relative rates of activation of phenylalanine and its *p*-fluoro derivative were similar both in the normal and polyuridylic acid-stimulated system. These results are consistent with those showing that alkaline phosphatase synthesized by *E. coli* in the presence of *p*-fluorophenylalanine showed the same degree of replacement of phenylalanine by the *p*-fluoro analogue at each phenylalanine site in the protein (Richmond, 1963).

EXPERIMENTAL

Materials. ATP (dipotassium salt) and GTP (sodium salt) were obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) and Sigma Chemical Co. (St Louis, Mo., U.S.A.) respectively. Pyruvate kinase and phosphoenolpyruvate (silver–barium salt) were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The silver–barium salt was converted into potassium phosphoenolpyruvate by acidifying a suspension in water at 4° with N-HCl and N-H_2SO_4, removing the precipitate by centrifuging and neutralizing the solution with N-KOH. For use in the cell-free system, a solution containing phosphoenolpyruvate (200 μmoles/ml.), ATP (10 μmoles/ml.) and GTP (2·5 μmoles/ml.) was prepared, the pH was adjusted to 7·6 with N-KOH and the mixture was stored frozen at −18°.

Yeast transfer RNA was a gift of Dr G. L. Brown, King's College, London. The polyuridylic acid used in some of the experiments was prepared by Dr J. Fresco, and was obtained from Dr R. A. Cox. Most of the experiments, however, were carried out with material purchased from Miles Chemical Corp. (Elkhart, Ind., U.S.A.).

Glutathione, L-phenylalanine, DL-phenylalanine and *p*-fluoro-DL-phenylalanine were products of Roche Products Ltd., Welwyn Garden City, Herts.

The amino acid mixture had the composition given by Borsook, Fischer & Keighley (1957), except that half the quantities were used and phenylalanine was replaced by either phenylalanine or *p*-fluorophenylalanine, unlabelled or labelled with ^{14}C, as stated in different experiments.

L-[^{14}C]Phenylalanine (35·7 μc/μmole) was obtained from The Radiochemical Centre, Amersham, Bucks.

p-Fluoro-DL-phenyl[β-^{14}C]alanine (3·5 μc/μmole) was obtained from the Commissariat à l'Energie Atomique, Gif sur Yvette (S.-et-O.), France.

Animals. Rabbits (sandy lop strain, approx. 2·5–3 kg. body wt.) were made anaemic by intraperitoneal injections of 2·5% phenylhydrazine hydrochloride, neutralized to pH 7·0 with N-NaOH, given in daily doses of 0·3 ml./kg. body wt. for 6 or 7 days. On the eighth day the animals were anaesthetized by intravenous injection of Nembutal (0·8 ml.), 1% heparin (1 ml.) being added to the injection as an anticoagulant, and finally with ether. Blood was collected from the heart and cooled in ice. The subsequent operations were carried out at 4° unless otherwise stated. The cells were separated by centrifuging at 1200*g* for 10 min. and washed three times with 0·154M-NaCl–10 mM-sodium phosphate buffer, pH 7. The washed cells (usually 20–30 ml. from one animal) were lysed by adding 100 ml. or 4 vol. (whichever was less) of 5 mM-$MgCl_2$ followed by 1 vol. of 1·5M-sucrose–0·15M-KCl (max. 15 ml.). Leucocyte counts showed that white cells did not lyse under these conditions.

The lysate was centrifuged first at 10000*g* for 10 min. to sediment intact cells and debris, then at 105000*g* for 60 min. to isolate ribosomes. The ribosome pellets were resuspended in medium A (0·25M-sucrose–0·05M-tris, pH 7·6–25 mM-KCl–5 mM-$MgCl_2$) and either used as such after briefly centrifuging (700*g* for 1 min.) or resedimented at 105000*g* for 1 hr. to give a washed preparation after resuspending in medium A.

Amino acid-activating enzymes. After sedimentation of the ribosomes, the supernatant was acidified to pH 5·1 with N-acetic acid and the precipitated protein was collected by centrifuging at 1800*g* for 10 min. The precipitate was redissolved in medium A (10 ml.), the pH being adjusted to 7·6 with N-KOH (approx. 0·1 ml.). Towards the end of this work it was found that this preparation also contained some ribosomes (about 20% of the total), but since this fraction appears to be relatively inactive in protein synthesis (Arnstein & Cox, 1963) the results of these experiments are unlikely to be significantly affected by the presence of these ribosomes.

Estimation of protein and RNA. Protein and RNA were estimated by the u.v. absorption of suitably diluted portions at 260 and 280 mμ, a nomograph based on the extinction coefficients of enolase and nucleic acid (California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A.; cf. Warburg & Christian, 1942) being used. The yield of ribosomes was usually about 3 mg./ml. of washed packed cells, that of the pH 5 enzyme fraction about 3–5 mg. of protein/ml. of cells. Both the ribosomes and the pH 5 enzyme fraction were stored frozen at −18° for up to 3 weeks before use in the cell-free amino acid-incorporation system.

Amino acid incorporation by cell-free preparations. In general the conditions used were similar to those described by Schweet *et al.* (1958). Each tube contained ATP (1 μmole) and GTP (0·25 μmole) with phosphoenolpyruvate (20 μmoles) and pyruvate kinase (0·1 mg.) as the ATP-generating system. Other details are given for each experiment.

Isolation of protein and estimation of radioactivity. After the incubation of the cell-free system the tubes were cooled in ice and protein was precipitated by adding 0·5 vol. of 30% trichloroacetic acid. The precipitate was isolated by centrifuging, washed twice with 10% trichloroacetic acid at 90°, redissolved in N-NaOH (1 ml.) and reprecipitated from 10% trichloroacetic acid. After washing with ethanol–ether (3:1, v/v) and ether, the protein was dried in air at room temperature and counted on 1 cm.^{2} polythene planchets with a statistical accuracy of at least ±5%. Since the incorporation has been found to be proportional to the amount of ribosomes, all results have been corrected for differences in the quantity of ribosomes used in the various experiments and are expressed as μm-mole of amino acid incorporated/mg. of ribosomes.

Amino acid activation and transfer to transfer RNA. (*a*) Expt. no. 1. Each tube contained, in 0·8 ml.: glutathione, 5 μmoles; $MgCl_2$, 6 μmoles; KCl, 35 μmoles; tris buffer, pH 7·6, 30 μmoles; pyruvate kinase (0·1 mg.) with the usual ATP-generating system; 0·1 ml. of the amino acid mixture containing ^{14}C-labelled amino acid [L-phenylalanine or *p*-fluoro-DL-phenylalanine (0·1 μmole of the L-enantiomorph)]; 6 mg. of pH 5 enzyme fraction. After incubation at 37° for 15 min., the reaction mixture was cooled, unlabelled L-phenylalanine or *p*-fluoro-DL-phenylalanine (10 μmoles in 0·5 ml.) was added to the tubes containing the corresponding ^{14}C-labelled amino acid and the pH was adjusted to 5 with 2N-acetic acid (0·02 ml.). The precipitated protein containing the amino acyl-RNA was isolated by centrifuging at 1500*g* for 20 min., and redissolved in 0·1M-tris, pH 7·6 (1 ml.). Carrier unlabelled amino acid (10 μmoles) was added as before and the precipitation was repeated. After redissolving, adding carrier and reprecipitating a third time as before, the washed pH 5 fraction in 1 ml. of 0·1M-tris, pH 7·6, containing 10 μmoles of carrier amino acid, was made pH 9·6 by adding 0·01N-NaOH (1·5 ml.). The solution was then incubated for 30 min. at 37° to hydrolyse the amino acyl-RNA to the free amino acid. Protein was precipitated by adding 0·5 vol. of 30% trichloroacetic acid and removed by centrifuging. The ^{14}C-labelled amino acid and carrier was then extracted three times with an equal volume of ether to remove most of the trichloroacetic acid and the aqueous phase loaded on to columns (0·5 cm. diam. × 10 cm. long) of Dowex 50 (H^+ form; X4; 200 mesh). The columns were washed with 25 ml. of distilled water and the phenylalanine (or *p*-fluorophenylalanine) was then eluted with 10 ml. of aq. 2N-NH_3 soln. The ammonia eluates were evaporated to dryness at 35° and the solid residue was dissolved in 1·0 ml. of water. Samples (0·5 ml.) were transferred to planchets containing lens tissue and dried over P_2O_5. A minimum of 10^3 counts above background were recorded.

(*b*) Expt. no. 2. In this experiment, ribosomes were removed from the pH 5 enzyme fraction by centrifuging for 2hr. at 105000*g*.

The complete system contained, in 0·7 ml.: glutathione, 5 μmoles; $MgCl_2$, 4 μmoles; KCl, 39 μmoles; tris buffer, pH 7·6, 19 μmoles; pyruvate kinase (100 μg.) with the usual ATP-generating system; 0·1 ml. of the amino acid mixture with 0·1 μmole of the ^{14}C-labelled L-amino acid (L-phenylalanine or *p*-fluoro-DL-phenylalanine); yeast transfer RNA, 0·4 mg. After incubation at 37° for 15 min., the activated ^{14}C-labelled amino acid was estimated by the procedure in Expt. no. 1.

RESULTS

Kinetics of the normal and polyuridylic acid-induced incorporation of phenylalanine in the reticulocyte system. The kinetics of the incorporation of phenylalanine into material insoluble in hot trichloroacetic acid by the cell-free system from rabbit reticulocytes are shown in Fig. 1. It will be seen that normal amino acid incorporation was rapid for about 10 min., then levelled off and was essentially complete after 60 min. incubation.

Addition of polyuridylic acid increased the incorporation of phenylalanine from about 0·08 to almost 2 μm-moles/mg. of ribosome. There appeared to be only a short lag before the maximum effect of polyuridylic acid became established, and, like the normal incorporation, the polyuridylic acid-induced system showed little activity after 60 min.

In subsequent experiments therefore incubation was always terminated after 1 hr. or less.

Comparison of the incorporation of phenylalanine and p-fluorophenylalanine. A comparison of the incorporation of phenylalanine and its *p*-fluoro derivative into the product insoluble in trichloro-

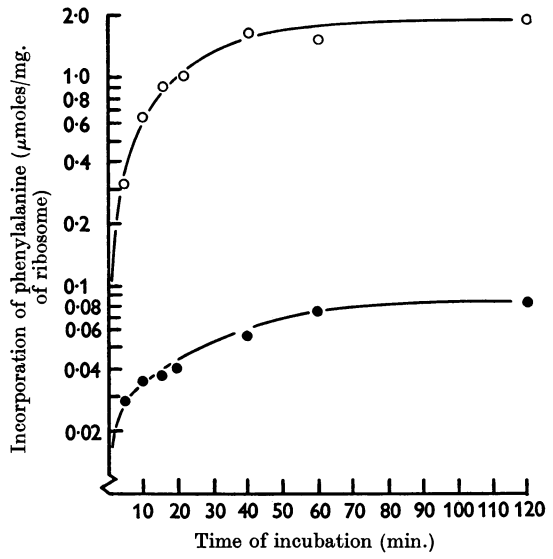

Fig. 1. Kinetics of the polyuridylic acid-induced incorporation of phenylalanine into polypeptide. Each tube contained, in 0·7 ml.: 5 μmoles of glutathione; 6·5 μmoles of $MgCl_2$; 32 μmoles of KCl; 12·5 μmoles of tris buffer, pH 7·6; 0·1 ml. of the amino acid mixture with L-[^{14}C]-phenylalanine (0·25 μc, sp. radioactivity 46 μc/μmole); ribosomes (1·9 mg.); pH 5 enzyme fraction (3·1 mg. of protein) and the standard ATP-generating system. Polyuridylic acid (50 μg.) was added where necessary. ○, Tube containing polyuridylic acid; ●, control tube.

Table 1. *Incorporation of phenylalanine and* p-*fluorophenylalanine by cell-free preparations from rabbit-reticulocyte ribosomes*

In Expt. no. 1, each tube contained the following components in 1·5 ml., the composition of the reaction mixtures for Expts. nos. 2 and 3 being given in parentheses (final volumes 1·4 and 0·8 ml. respectively): glutathione, 5 μmoles (5, 5); $MgCl_2$, 7·5 μmoles (6·7, 6·4); KCl, 50 μmoles (34, 40); tris buffer, pH 7·6, 42 μmoles (47, 70); phosphoenolpyruvate, 20 μmoles (20, 10); ATP, 1 μmole (1, 0·5); GTP, 0·25 μmole (0·25, 0·125); pyruvate kinase, 100 μg. (100, 100); unlabelled amino acid mixture, 0·1 ml. (0·1, 0·125); L-[^{14}C]phenylalanine, 0·1 μmole, sp. radioactivity 9·9 μc/μmole [or *p*-fluoro-DL-[β-^{14}C]phenylalanine, 0·2 μmole, sp. radioactivity 3·5 μc/μmole (same labelled amino acids in Expts. nos. 2 and 3)]; ribosomes, 9·6 mg. (4·5, 2·3); pH 5 enzyme fraction, 10 mg. of protein (10, 2·3). Incubation was at 37° for 1 hr.

Expt. no.	^{14}C-labelled amino acid	Incorporation (μm-mole/mg. of ribosome)	Ratio of incorporation (phenylalanine/*p*-fluoro-phenylalanine)
1	Phenylalanine	0·079	1·53
	p-Fluorophenylalanine	0·051	
2	Phenylalanine	0·435	2·13
	p-Fluorophenylalanine	0·204	
3	Phenylalanine	0·105	1·30
	p-Fluorophenylalanine	0·081	

acetic acid in four different experiments (Table 1 and Fig. 2) shows that, whereas the amounts of amino acid converted into polypeptide were somewhat variable, the incorporation of phenylalanine was always slightly greater (25–100 %) than that of *p*-fluorophenylalanine. Fig. 2 also shows that there is no apparent difference between the two amino acids in the kinetics of their incorporation into protein, and that *p*-fluorophenylalanine is used without any lag. The relative incorporation of phenylalanine and *p*-fluorophenylalanine into protein after incubation for 1 hr. was almost identical with the relative rates of activation of the two amino acids by the pH 5 enzyme fraction in the absence of ribosomes (Table 2).

Effect of polyuridylic acid on the incorporation of p-*fluorophenylalanine.* The incorporation of *p*-fluorophenylalanine into protein by the cell-free system was stimulated by polyuridylic acid to about the same extent as that of phenylalanine, and the kinetics of the polyuridylic acid-induced increase were similar in both cases (Fig. 3). In this experiment the utilization of *p*-fluorophenylalanine for polypeptide synthesis was as good as or even better than that of phenylalanine.

Competition between phenylalanine and p-*fluorophenylalanine.* The incorporation of *p*-fluorophenylalanine, present in a concentration of 0·1 μmole/ml., into protein insoluble in trichloroacetic acid is completely inhibited by phenylalanine added in equimolar amounts (Table 3). On the other hand, even a fivefold excess of *p*-fluorophenylalanine (10 μmoles of *p*-fluoro-DL-phenylalanine/ml.) reduced the incorporation of phenylalanine by less than 50 %. A similar relationship was found in the competition between phenyl-

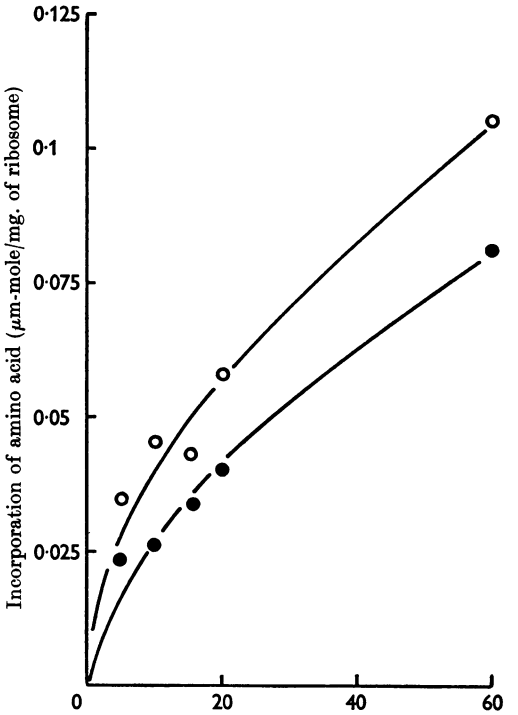

Fig. 2. Incorporation of phenylalanine and *p*-fluorophenylalanine into polypeptide. Each tube contained, in 0·8 ml.: 5 μmoles of glutathione; 6 μmoles of $MgCl_2$; 30 μmoles of KCl; 50 μmoles of tris, pH 7·6; the standard ATP-generating system; 0·13 ml. of the unlabelled amino acid mixture with either ○, L-[^{14}C]phenylalanine (4·6 μc/μmole, 0·1 μmole), or ●, *p*-fluoro-DL-[β-^{14}C]phenylalanine (3·5 μc/μmole; 0·2 μmole).

alanine and the *p*-fluoro analogue for amino acid activation (Table 4), where a tenfold excess of the *p*-fluoro derivative reduced activation of phenylalanine by less than 50 %, although activation of fluorophenylalanine was almost completely inhibited by the presence of about 20 % of phenylalanine.

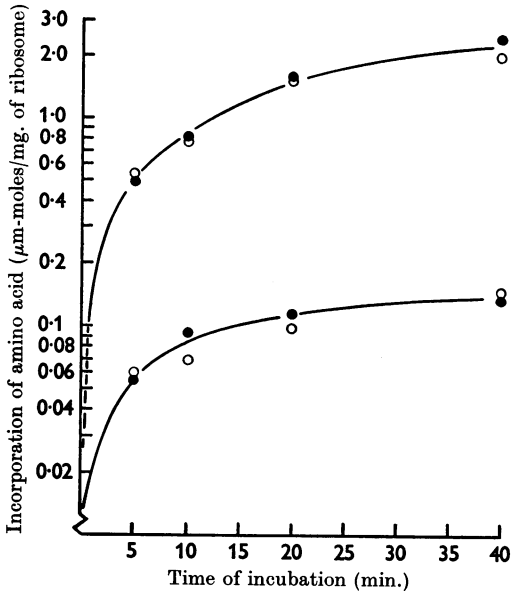

Fig. 3. Comparison of the polyuridylic acid-induced incorporation of phenylalanine and *p*-fluorophenylalanine into polypeptide. Each tube contained, in 0·8 ml.: 5 μmoles of glutathione; 6 μmoles of $MgCl_2$; 40 μmoles of KCl; 40 μmoles of tris, pH 7·6; the standard ATP-generating system but with 65 μg. of pyruvate kinase; 0·1 ml. of the mixture of unlabelled amino acids (except phenylalanine) and either ○, L-[^{14}C]phenylalanine (10·5 μc/μmole; 0·1 μmole), or ●, *p*-fluoro-DL-[β-^{14}C]phenylalanine (3·5 μc/μmole; 0·2 μmole); ribosomes, 2·7 mg.; pH 5 enzyme fraction (3·5 mg. of protein). Polyuridylic acid (50 μg.) was added in experiments shown in the upper curve.

DISCUSSION

The incorporation of *p*-fluorophenylalanine into protein by the cell-free system from rabbit reticulocytes was quantitatively similar to that of phenylalanine. This result was unexpected since *in vivo* only 16–25 % replacement of phenylalanine by the *p*-fluoro analogue had been found for two rabbit enzymes (Westhead & Boyer, 1961) and in earlier experiments on haemoglobin biosynthesis by cell-free preparations from rabbit reticulocytes *p*-fluorophenylalanine replaced about 33 % of the phenylalanine (Kruh & Rosa, 1959). The low replacement of phenylalanine by the *p*-fluoro analogue in the intact animal is probably explained by the inhibitory effect of phenylalanine in rela-

Table 2. *Activation of phenylalanine and* p-*fluorophenylalanine and conversion into protein*

Details of the amino acid-activation assay are given in the Experimental section (Expt. no. 1). For the incorporation into protein, ribosomes (2·5 mg.) were also added to each tube and the incubation was at 37° for 1 hr.

Amino acid activated (μm-mole/15 min.)		Ratio (phenylalanine/*p*-fluorophenylalanine)
Phenylalanine	*p*-Fluorophenylalanine	
0·158	0·120	1·3
0·191	0·155	
Amino acid incorporated into protein (μm-mole/mg. of ribosome/hr.)		
Phenylalanine	*p*-Fluorophenylalanine	
0·279	0·177	1·4
0·279	0·208	

Table 3. *Effect of phenylalanine on the incorporation of* p-*fluorophenylalanine*

In addition to the energy-generating system (see the Experimental section), each tube contained, in 0·75 ml.: 5 μmoles of glutathione; 8·9 μmoles of $MgCl_2$; 44 μmoles of KCl; 77 μmoles of tris, pH 7·5; unlabelled amino acid mixture with either L-[^{14}C]phenylalanine (0·1 μmole) or *p*-fluoro-DL-[β-^{14}C]phenylalanine (0·2 μmole). Unlabelled phenylalanine or *p*-fluorophenylalanine was added as shown. Incubation was at 37° for 1 hr.

^{14}C-Labelled amino acid present	Unlabelled amino acid added (μmoles)	Incorporation of labelled amino acid (μm-mole/mg. of ribosome)
p-Fluorophenylalanine	None	0·052
	L-Phenylalanine (0·1)	0·001
	L-Phenylalanine (1)	0·002
	L-Phenylalanine (10)	0·003
Phenylalanine	None	0·048
	p-Fluoro-DL-phenylalanine (0·1)	0·047
	p-Fluoro-DL-phenylalanine (1)	0·026
	p-Fluoro-DL-phenylalanine 10)	0·012

Table 4. *Competition between phenylalanine and* p*-fluorophenylalanine for amino acid-activating enzyme*

Details of the assay system are given in the Experimental section. The labelled amino acid was present at 0·1 μmole of L-enantiomorph in a total volume of 0·7 ml. The amount of pH 5 enzyme fraction was 3·2 mg. of protein.

Labelled amino acid	Unlabelled amino acid added (μmoles)	^{14}C-Labelled amino acid activated (μm-mole)
Phenylalanine	None	0·45, 0·35
	p-Fluoro-DL-phenylalanine (0·2)	0·41, 0·33
	p-Fluoro-DL-phenylalanine (2·0)	0·28, 0·29
	p-Fluoro-DL-phenylalanine (10·0)	0·09, 0·04
	None	0·02*
p-Fluorophenylalanine	None	0·18, 0·22
	L-Phenylalanine (0·002)	0·14, 0·15
	L-Phenylalanine (0·02)	0·08, 0·06
	L-Phenylalanine (0·2)	0·04, 0·03
	None	0·05*

* ATP and ATP-generating system omitted.

tively low concentration on the activation of *p*-fluorophenylalanine, which would ensure the preferential utilization of phenylalanine for protein synthesis under these conditions. This is probably a reflexion of the lower affinity of *p*-fluorophenylalanine than phenylalanine for the activating enzyme, as suggested by our competition experiments (see Table 4).

In the experiments of Kruh & Rosa (1959) on haemoglobin biosynthesis in a cell-free system very low substrate concentrations (1 μM) were used, and in this case also the presence of a trace of phenylalanine in the incubation mixture would result in a low incorporation of the *p*-fluoro compound. At these low amino acid concentrations, equimolar amounts of either *p*-fluorophenylalanine or phenylalanine inhibited the incorporation of the other amino acid by 50%. In our experiments, however, with much higher concentrations (100 μM) of substrate, equimolar *p*-fluorophenylalanine had little or no effect on the activation of phenylalanine or its incorporation into protein.

The activation and attachment to transfer RNA of *p*-fluorophenylalanine is inhibited by phenylalanine by about the same extent as is its incorporation into protein (Tables 3 and 4), suggesting that the transfer of the analogue from transfer RNA to protein is not discriminated against by the coding and enzyme systems involved. In agreement with this interpretation, almost identical ratios were found for the activation and incorporation into protein of *p*-fluorophenylalanine relative to phenylalanine (Table 2). It is concluded that competition between phenylalanine and the *p*-fluoro derivative occurs at the stage of amino acid activation and/or transfer to transfer RNA, but not subsequently.

The similarity in the magnitude and kinetics of the polyuridylic acid-induced incorporation of phenylalanine and *p*-fluorophenylalanine also indicates that at least one code, the UUU triplet, for phenylalanine does not discriminate against the *p*-fluoro analogue. Although there is at present no evidence for degeneracy in the code for phenylalanine, it may also be concluded that, if such degeneracy should exist in the reticulocyte system, the various transfer ribonucleic acids must have similar specificities for phenylalanine and *p*-fluorophenylalanine.

SUMMARY

1. A cell-free preparation from rabbit reticulocytes incorporates both phenylalanine and *p*-fluorophenylalanine into material insoluble in hot trichloroacetic acid (protein) at approximately the same rate and in similar amounts.

2. The incorporation of both amino acids is stimulated to approximately the same extent by polyuridylic acid.

3. Phenylalanine markedly inhibits the incorporation of the *p*-fluoro analogue into protein by competing at the stage of amino acid activation and attachment to transfer RNA.

4. At stages after attachment of the amino acid to transfer RNA no discrimination against *p*-fluorophenylalanine occurs in the reticulocyte system.

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The Estimation of Renin in Plasma by an Enzyme Kinetic Technique

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For many years it has seemed possible that the enzyme renin, which reacts with a plasma substrate to form the pressor peptide angiotensin, might be of considerable importance both in the economy of the normal animal and in the pathogenesis of various diseases. Hitherto, assay techniques for the measurement of renin in body fluids have been insufficiently sensitive or specific for a proper evaluation of its function. Lever & Peart (1962) demonstrated renin-like activity in the renal lymph of the dog, but renin was not detectable in the renal-vein plasma of normal or hypertensive rabbits, unless infused in immediately pressor doses (Peart, Robertson & Grahame-Smith, 1961). It was clear at this stage that further development of these aspects required much greater sensitivity and precision of assay. The present paper describes the development of such a method, based on the initial velocity of angiotensin formation under standard conditions. The present description deals with the measurement of renin in rabbit plasma. With modifications, however (Brown, Davies, Lever, Robertson & Tree, 1963*a*, *b*; and unpublished work), the technique may be used for the estimation of renin in plasma and other body fluids of man and the dog. Preliminary reports of the present work have been published (Lever, Robertson & Tree, 1963; Lever & Tree, 1963; Robertson, 1963).

METHODS

To minimize the risk of bacterial contamination, all buffers were made up in recently boiled water. As a further precaution, neomycin sulphate (0·2%) was added where stated. This concentration of neomycin was without demonstrable effect on the renin–substrate reaction.

Preparation of substrate. The properties required of the substrate were that it should: (*a*) form angiotensin when incubated with rabbit renin; (*b*) be devoid of pressor activity; (*c*) be stable when incubated alone; (*d*) be free from both angiotensinase and renin. A convenient source of large quantities of substrate was ox blood. The method of Dexter, Haynes & Bridges (1945) failed to eliminate angiotensinase sufficiently for present purposes and was therefore modified as follows to include two precipitation stages. Serum was separated from two 80 l. batches of ox blood by the technique of Peart (1955). Then NaCl (90 g./l.) was added to 10 l. batches of serum, which were acidified to pH 3·1–3·2 over 10 min., by using 6N-HCl initially and N-HCl for the final adjustment, with stirring throughout. A heavy precipitate formed during this process. The material was kept at room temperature for 1 hr. and then, provided that the pH had remained in the range 3·1–3·2, was mixed with Celite 545 (Johns–Manville Co.) and filtered through a Celite bed previously washed with 1·7M-NaCl adjusted to pH 3·1 with N-HCl. The precipitate, containing angiotensinase, was discarded. Provided that the pH had remained in the range 3·1–3·2, 200 g. of NaCl was added/l. A second, finer, precipitate formed, and was separated by filtration at room temperature on Whatman no. 541 paper. The filtrate from this step was discarded. The total precipitate, which contained the renin substrate, was dissolved in 15 l. of 0·15M-phosphate–saline buffer, pH 5·7 (0·555% NaH_2PO_4; 0·179% Na_2HPO_4,-$12H_2O$; 0·585% NaCl), and further dialysed in Visking cellophan sacs (18/32 in.) against ten 20 l. changes of the same buffer over 5 days at 8°. By this technique 19 l. of substrate was prepared from 70 l. of ox serum.

Preparation of standard rabbit renin. The standard renin was required to be stable on incubation, and free from both substrate and angiotensinase. It was prepared as follows. A sample (150 g.) of ethanol-dried rabbit-kidney powder (Peart, 1955) was stirred with 1·5 l. of 0·15M-NaCl at 8° for 48 hr., and filtered first through muslin and then through a Celite bed. Then $(NH_4)_2SO_4$ (40 g./100 ml.) was added to