possesses intrinsic-factor activity, the other inhibits this activity and that of hog intrinsic factor.

3. It is concluded that a part of the intrinsic-factor activity of gastric mucosal cells is located on the mitochondria in association with a substance which inhibits its action.

4. The existence of this inhibitor may account for the relative inactivity of intrinsic-factor preparations from human material as compared with those from hog pyloric mucosa.

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Amino Acid-Activating Enzymes in Rat-Liver Mitochondria

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Studies on the incorporation of labelled amino acids into mitochondria both in intact animals (McLean, Cohn, Brandt & Simpson, 1958) and in cell-free systems (McLean *et al.* 1958; Reis, Coote & Work, 1959; Greengard, 1959; Greengard & Campbell, 1959; Korner, 1959; Fraser & Gutfreund, 1958; Mager, 1960) provide evidence for a role of these subcellular particles in protein synthesis. It was of interest to determine whether amino acid activation in mitochondria occurs by a mechanism similar to that which has been shown to exist in other parts of the cell (Hoagland, Keller & Zamecnik, 1956) and in many micro-organisms (De Moss & Novelli, 1956). In the series of experiments reported here, rat-liver preparations were used.

If the amino acid-activating enzymes play a role in protein synthesis in mitochondria, they must be associated with the mitochondria themselves because, although the addition of the soluble frac-

tion of the cell enhances amino acid incorporation (McLean *et al.* 1958), this effect is almost fully retained after heat treatment and can be accounted for, at least in part, by the amino acid content of this fraction (Reis *et al.* 1959; J. Lucas & M. V. Simpson, unpublished work). The methods used in testing for the activating enzymes included amino acid-dependent hydroxamic acid formation and amino acid-dependent pyrophosphate-adenosine triphosphate exchange (Hoagland *et al.* 1956). The results obtained with an amino acid mixture suggest the presence of activating enzymes, in confirmation of the results of Reis *et al.* (1959) on rat-liver mitochondria and of Davis & Novelli (1958) on pea-seedling mitochondria. Further work with individual amino acids revealed the presence of an activation mechanism for tryptophan and leucine. However, enzyme activities for most of the other amino acids could not be demonstrated, and the question of the general mode of amino acid activation in mitochondria is left unsettled.

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A preliminary account of some of this work has already appeared (Craddock & Simpson, 1959).

EXPERIMENTAL

Preparation of enzyme fraction. Rat-liver mitochondria were prepared as described by McLean *et al.* (1958), and were washed six times in 0.25 M-sucrose. To obviate permeability problems and for purposes of protein fractionation, the mitochondria were disrupted before testing for amino acid activation. This was accomplished by subjecting the preparation to a 5 min. period of sonic oscillation at maximum intensity in a Raytheon 10 kcyc. sonic oscillator. The resulting suspension was adjusted to pH 5.2, and the precipitate which formed was suspended in 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7.4. Undissolved protein was removed by centrifuging (Hoagland *et al.* 1956). This preparation is called the pH 5 fraction. In preliminary investigations on the unfractionated material after sonic disruption, both high zero-time hydroxamate values and high amino acid-independent formation of hydroxamate were encountered. After the above fractionation was carried out, it became evident that the tris-soluble fraction gave the least amino acid-independent formation of hydroxamic acid and that it was only in this fraction that it was possible to obtain a stimulation of hydroxamate formation by the addition of amino acids. The troublesome high zero-time values could not be eliminated by this fractionation procedure, however. The results reported here were obtained with the tris-soluble material.

Hydroxamate formation. Salt-free hydroxylamine was prepared according to Berg (1956*a*), and the aqueous stock solution obtained was assayed for hydroxylamine by its colour reaction with 8-hydroxyquinoline (Frear & Burrell, 1955). The hydroxylamine was incubated with the enzyme preparation under the conditions (De Moss & Novelli, 1956) shown in Table 1. After incubation, trichloroacetic acid was added and any hydroxamate present was estimated by its colour reaction with ferric chloride (Schweet, 1955). Chemically synthesized glycine hydroxamate (Safr & Williams, 1952) was used as a standard. The addition of pyrophosphatase to the reaction mixture was necessary because, without the addition of this enzyme, the formation of hydroxamate with time could not be demonstrated, either with or without the addition of amino acids. Pyrophosphatase, prepared from yeast (Heppel, 1955), presumably acts by hydrolysing the pyrophosphate liberated, pulling the reaction to the right (Davie, Koningsberger & Lipmann, 1956).

Pyrophosphate-adenosine triphosphate exchange. Potassium [32 P]pyrophosphate was prepared according to the method of Kornberg & Pricer (1951). The enzyme preparation was incubated with labelled pyrophosphate under the conditions given in the legends to the Tables. Fluoride was used in order to inhibit pyrophosphatase activity. 2,4-Dinitrophenol was added to suppress possible oxidative phosphorylation so as to decrease the incorporation into adenosine triphosphate (ATP) of any labelled orthophosphate which might be formed by the hydrolysis of pyrophosphate. After the incubation period, a 1 ml. sample of the reaction mixture was added to 2 ml. of 5% (w/v) trichloroacetic acid, and the specific activity of the terminal pyrophosphate group of ATP was determined with the

Norit absorption method (Crane & Lipmann, 1953) as modified by De Moss & Novelli (1956), and the percentage exchange was calculated (De Moss & Novelli, 1956). Comparison of the amount of acid-hydrolysable phosphate liberated from the Norit at zero time and after incubation for 10 min. indicated little, and in many cases no, breakdown of ATP during incubation.

Protein. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Amino acid mixture. The amino acid mixture was composed of equimolar amounts of the following amino acids: glycine, L-alanine, L-serine, L-threonine, L-leucine, L-isoleucine, L-valine, L-glutamic acid, L-aspartic acid, L-methionine, L-phenylalanine, L-histidine (free base), L-arginine (free base), L-tryptophan, L-proline and L-lysine, pH 7.0. Tyrosine, cysteine, cystine, glutamine and asparagine were not present in the mixture.

RESULTS

Hydroxamate formation

Substantial evidence exists that amino acid-activating enzymes catalyse the formation of a mixed carboxyl-phosphate anhydride of an amino acid and adenosine 5'-phosphate (Hoagland *et al.* 1956; De Moss & Novelli, 1956; Berg, 1956*b*; Karesek, Castlefranco, Krishnaswamy & Meister, 1958; Kingdon, Webster & Davie, 1958). In the presence of hydroxylamine, the mixed anhydride yields adenosine 5'-phosphate and the amino acid hydroxamate (Hoagland *et al.* 1956; De Moss & Novelli, 1956). The formation of the latter can be detected by a colour reaction with iron. Table 1 shows that the addition of an amino acid mixture stimulates the formation of hydroxamic acid, and that this formation is approximately linear with time. However, the zero-time values are large, decreasing both the precision and the already low

Table 1. *Hydroxamate formation by 'pH 5 fraction' of rat-liver mitochondria*

The reaction mixture consisted of the following additions: ATP, 100 μ moles; $MgCl_2$, 50 μ moles; hydroxylamine, 12.4 m-moles; tris buffer, pH 7.0, 60 μ moles; enzyme preparation, 6.9 mg. of 'pH 5 fraction', protein equivalent to 30 g. of liver; in a final volume of 10 ml. The mixture was incubated at 37°. Where an amino acid mixture* was added, its final concentration was 20 μ moles/ml. of each L-amino acid.

Incubation time (min.)	Hydroxamate formed (μ moles/mg. of protein)		
	Additions		Hydroxamate formation due to amino acid addition
	None	Amino acid mixture	
0	0.87	0.91	0.04
30	0.91	1.07	0.16
60	0.93	1.26	0.33

* See text for the composition of the amino acid mixture.

Table 2. *Pyrophosphate-adenosine triphosphate exchange by 'pH 5 fraction' of rat-liver mitochondria*

The reaction mixture consisted of the following additions: ATP, 15 μ moles; $MgCl_2$, 12 μ moles; potassium pyrophosphate, 15 μ moles, 2×10^6 counts/min./ μ mole; tris buffer, pH 7.5, 450 μ moles; KF, 90 μ moles; 2:4-dinitrophenol, 0.9 μ mole; and the amount of 'pH 5 fraction' protein given in the Table, in a final volume of 3 ml. Where an amino acid mixture* was added, the concentration in the incubation medium was 3 μ moles/ml. of each L-amino acid. The mixture was incubated at 37°.

Incubation time (min.)	Amount of protein added (mg.)	Extent of exchange		Exchange resulting from addition of amino acids (%)
		No additions (%)	L-Amino acid mixture added (%)	
0	0.48	0.03	0.22	—
10	0.48	0.61	1.30	0.69
0	4.80	0.30	0.14	—
10	4.80	5.34	10.08	4.74

* See text for the composition of the amino acid mixture.

sensitivity of the method. It was thus difficult to proceed further with this method of assay, particularly when testing for the activation of individual amino acids. Inasmuch as all attempts to reduce the zero-time values were unsuccessful, this method was abandoned and the much more sensitive pyrophosphate-ATP exchange reaction was employed.

Pyrophosphate-adenosine triphosphate exchange

Results with an amino acid mixture. Because of the reversibility of the reaction catalysed by amino acid-activating enzymes, the amino acid-dependent exchange of inorganic pyrophosphate with the terminal pyrophosphate group of ATP can be used as a test for the presence of these enzymes. In general, this method may be expected to possess considerably greater sensitivity than the hydroxamic acid method, particularly as the sensitivity can be increased simply by using pyrophosphate of higher specific activity. However, the precision of the method is limited by, among other things, blank values resulting from exchange which is due to the occurrence, in crude preparations, of reactions which proceed in the absence of added amino acids. This last problem has been a source of constant difficulty in the work with mitochondria.

Table 2 shows that the enzyme preparation catalyses an exchange of pyrophosphate into ATP and that the rate of this exchange is increased two-fold upon the addition of an amino acid mixture. The dependence of the rate of exchange on enzyme concentration can also be seen, a tenfold increase in the latter resulting in a sevenfold increase in the amino acid-dependent rate of exchange. The appreciable extent of exchange in the absence of added amino acids is evident and makes the interpretation of the results difficult. The possibility was considered that this exchange resulted from the presence of amino acids not removed by the pH 5 precipitation or that enzymes responsible for

acetate or fatty acid activation and their substrates were present. However, attempts to lower this exchange by various means, including extensive dialysis of the enzyme preparation against buffer (De Moss & Novelli, 1956) and against ion-exchange resins, did not result in a decrease of the amino acid-independent exchange.

Assay of groups of amino acids. The stimulation of pyrophosphate exchange by an amino acid mixture is in itself not conclusive evidence for the presence of amino acid-activating enzymes, inasmuch as the metabolic pathways of certain amino acids may conceivably involve this type of exchange. In mitochondria, this is particularly true of those amino acids whose degradative pathway involves fatty acid-coenzyme A derivatives. To ascertain which of the amino acids of the mixture were responsible for the stimulation, a preliminary experiment was performed in which some amino acids were added in groups. The results, shown in Table 3, point to a stimulation of pyrophosphate

Table 3. *Effect of groups of amino acids on pyrophosphate-adenosine triphosphate exchange by the 'pH 5 fraction' of rat-liver mitochondria*

The reaction conditions were the same as those given in Table 2. The concentration of each L-amino acid was 3 μ moles/ml. and the amount of pH 5 fraction protein added was 0.48 mg. For abbreviations of amino acids see *Biochem. J.* (1957) **66**, 6.

Amino acids added	Extent of exchange (%/mg. of protein)
None	12.3
Amino acid mixture*	16.0
Glu Asp	10.6
Lys Arg His	9.5
Ser Thr Pro	10.3
Try Phe Met	14.6
Leu Val Ileu Ala	14.1
Tyr	13.0
Gly	9.7

* See text for the composition of the amino acid mixture.

Table 4. *Effect of individual amino acids on the extent of exchange of pyrophosphate with adenosine triphosphate*

The reaction conditions are the same as those given in Table 3.

Amino acid added	Amino acid concn. (μ moles/ml.)	Extent of exchange (%/mg. of protein)
None	—	13.7
Amino acid mixture	0.3*	16.8
Amino acid mixture	3*	19.7
L-Tryptophan	3	19.7
L-Leucine	3	15.8
L-Phenylalanine	3	14.9
L-Methionine	3	14.8
L-Alanine	3	13.4
L-Valine	3	12.7
L-Isoleucine	3	12.5

* Concn. of each amino acid; see text for the composition of the amino acid mixture.

exchange by groups containing tryptophan, phenylalanine, methionine, leucine, valine, isoleucine and alanine. Tyrosine seems to have had virtually no effect whereas the remainder of the amino acids were inhibitory. Asparagine, glutamine, cystine or cysteine were not tested in this series of experiments. Since the combined effect of the two groups which stimulated could account for the total stimulation by the complete amino acid mixture, the amino acids which comprised these groups were tested individually.

Effect of individual amino acids. The results obtained with individual amino acids (Table 4) are consistent with those obtained with groups of amino acids. In the two groups which stimulated pyrophosphate exchange, the effect can be accounted for, at least qualitatively, by the stimulation exerted by phenylalanine, methionine and especially tryptophan in one group and by leucine in the other. Of these four amino acids, only tryptophan and leucine exert an appreciable effect; whether the apparent small differences in the presence of methionine and phenylalanine are significant is open to question. The other amino acids tried (see also Table 3) had either no effect or were inhibitory.

DISCUSSION

The presence of amino acid-activating enzymes in rat-liver mitochondria is indicated both by hydroxamate formation and by pyrophosphate-ATP exchange, both of which reactions were stimulated by a mixture of L-amino acids. The stimulation observed upon addition of tryptophan is of particular significance since its catabolic products are not known to be involved in pyrophosphate-liberation reactions.

Because of the failure to find most of the activating enzymes, the question of whether they play a role in protein synthesis in mitochondria remains open. It is premature to conclude, however, that because the enzymes have not been found, they are not present. It is possible that even the brief sonic treatment used here has destroyed many of the enzyme activities; such an effect has been reported both in pea seedlings and in microorganisms (Davis & Novelli, 1958; Nisman, 1959). However, sonic treatment of calf-heart mitochondria does not result in the loss of ability of the disrupted preparation to incorporate amino acids into protein (Kalf & Simpson, 1959). It is also possible that some activities have been lost during the pH 5 fractionation, although this is unlikely since all fractions were tested with an amino acid mixture before being discarded. Another difficulty in the interpretation of the negative findings is the high base-line pyrophosphate-ATP exchange, i.e. the amino acid-independent exchange, which could well mask small stimulatory effects of added amino acids (Davis & Novelli, 1958). It is not clear at present what type of reaction is causing this exchange. The presence in the enzyme preparation of acetate-activating enzyme or of a higher fatty acid-activating enzyme plus the appropriate substrate tightly bound (inasmuch as dialysis was without effect) is one possibility. On the other hand, exchange due to the presence of the amino acid-activating enzymes themselves is not excluded if it is assumed that the amino acids are so tightly bound that they would not be removed by dialysis. Were this the case, the exchange reaction may already be proceeding at a nearly maximum rate so that addition of exogenous amino acid has little or no stimulatory effect. Other possibilities involve the existence in mitochondria of different types of activation mechanisms. Such mechanisms might not involve the cleavage from ATP of pyrophosphate (Cormier, Stulberg & Novelli, 1959; Beljanski, 1960; Wachsmann, Fukuhara & Nisman, 1960) or might not be dependent upon the presence of a substrate for pyrophosphate exchange to occur.

SUMMARY

1. The presence of amino acid-activating enzymes in rat-liver mitochondria was investigated.
2. Evidence was obtained that mitochondrial preparations can catalyze the formation of amino acid hydroxamates, and can bring about an amino acid-dependent exchange between pyrophosphate and adenosine triphosphate.
3. Tryptophan and leucine are the amino acids most active in the latter respect, whereas most of the other amino acids tried failed to promote the exchange reaction.

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Ethionine Administration in the Rat

1. EFFECTS ON THE LIVER AND PLASMA LIPIDS AND ON THE DISPOSAL OF DIETARY FAT

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Dyer (1938) showed that the administration of ethionine (α -amino- γ -ethylmercaptobutyric acid) to the rat decreased the growth rate of immature animals and caused a loss of body weight in the adult. Since then several other effects of the administration of ethionine to experimental animals have been established (see Farber, 1959). Two of these, a marked and rapid accumulation of triglyceride in the liver (Farber, Simpson & Tarver, 1950; Koch-Weser, Farber & Popper, 1951) and a fall in the concentration of the plasma lipids to very low values (Feinberg, Rubin, Hill, Entenman & Chaikoff, 1954; Furman, Norcia, Robinson & Gonzalez, 1957), suggest that ethionine may interfere with fat transport in the body. The present study was undertaken to obtain evidence on this point.

MATERIALS AND METHODS

Wistar-strain albino rats weighing 160–200 g. were fed on the laboratory stock diet, containing 4% of fat, throughout the experimental period. DL-Ethionine (25 mg./ml.) was given subcutaneously in 0.9% NaCl soln. Control animals were given corresponding injections of 0.9% NaCl soln. Chyle was collected by establishing fistulae in the thoracic ducts of normal rats which had been previously fed with 1 ml. of olive oil by stomach tube (French, Robinson & Florey, 1953). Labelled chyle was obtained by feeding rats, in which thoracic-duct fistulae had been established 24 hr. previously, with 20 μ C of glyceryl tri-[1-¹⁴C]palmitate dissolved in 1 ml. of olive oil. Chyle samples were collected in 3.8% (w/v) trisodium citrate soln., approx. 1 vol. of citrate being used for 9 vol. of chyle. The chyle was stored at 0° until needed. No samples were kept for more than a week before use.