proteins offers a possible way to the final understanding of them.

SUMMARY

1. The polarization spectrum of insulin, ribonuclease and zein is qualitatively identical with that of tyrosine or cresol, but the lower absolute values of the principal polarization indicate the existence of energy transfer among the tyrosine residues.

2. The polarization spectrum of proteins containing tryptophan is similar to that of *N*-glycyl-tryptophan but differs from it in the lower values of the polarization in the 270 m μ region. The ratio of the polarization on excitation by 305 m μ and by 270 m μ (p_{305}/p_{270} ratio), which varies from 1.4 to 1.7 in the simple indole derivatives, is found to be greater than two in nine out of ten globular proteins studied.

3. The polarization spectrum in 50 % propylene glycol-water at -70° shows an increased p_{305}/p_{270} ratio (2.3–3.0), and the polarization spectrum in 8M-urea shows this ratio decreased to the range 1.4–1.9 in eight out of ten proteins studied.

4. The changes of proteins in urea are only partially reversible. The soluble fractions obtained in three proteins had a distinctly changed polarization spectrum with a lowered p_{305}/p_{270} ratio.

5. Though energy transfer among the tryptophan residues may play a part in the observed effects, it is believed that a change in the relative intensities of the $G-S_1$ and $G-S_2$ transitions in tryptophan can explain these effects equally well.

REFERENCES

- Förster, T. (1951). *Fluoreszenz Organischer Verbindungen*, p. 186. Göttingen: Vandenhoeck und Ruprecht.
 Teale, F. W. J. & Weber, G. (1957). *Biochem. J.* **65**, 467.
 Teale, F. W. J. & Weber, G. (1959). *Biochem. J.* **72**, 15P.
 Warner, R. C. (1954). In *The Proteins*, vol. 2, part A, p. 437. Ed. by Neurath, H. & Bailey, K. New York: Academic Press Inc.
 Weber, G. (1953). *Advanc. Protein Chem.* **8**, 415.
 Weber, G. (1954). *Trans. Faraday Soc.* **50**, 552.
 Weber, G. (1960). *Biochem. J.* **75**, 335.
 Weber, G. & Teale, F. W. J. (1959). *Disc. Faraday Soc.* **27**, 134.

Biochem. J. (1960) **75**, 352

Amino Acid-Activating Systems from Pig Liver

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A reaction between amino acids and adenosine triphosphate catalysed by specific enzymes has been termed amino acid activation by Hoagland (1955). This is considered to consist of the formation of an enzyme-amino acyl adenylate complex with release of pyrophosphate. The amino acid-activating systems further catalyse a reaction between amino acid and 'soluble' ribonucleic acid (Hoagland, Zamecnik & Stephenson 1957) to form an amino acyl-ribonucleic acid compound which is usually assumed to be formed by the transfer of the amino acyl adenylate to 'soluble' ribonucleic acid. Evidence for these two reactions has been summarized by a number of authors (Lipmann, 1958; Zamecnik, Stephenson & Hecht, 1958; Berg & Ofengand, 1958; Novelli, 1958). It is not yet

clear whether these two reactions are catalysed by one or more enzymes, and purification of the system might help to elucidate this point. Moreover, these reactions have special interest as possible early stages in protein synthesis, and further elucidation of their function might be assisted by the availability of the purified enzymes. Pig liver was found by us to be a source of protein catalysing both reactions, and one from which the isolation and purification of these enzymes might be attempted.

In this paper we describe the isolation and simple properties of amino acid-activating preparations, which are essentially free of ribonucleic acid, including 'soluble' ribonucleic acid. This in no way impairs their ability either to catalyse amino acid-dependent exchange of pyrophosphate, or the transfer of [^{14}C]amino acids to added 'soluble' ribonucleic acid. We have compared these preparations with pH 5.0 fractions from the same liver and found them not dissimilar in their general behaviour.

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The removal of ribonucleic acid from these amino acid-activating preparations profoundly alters certain of their properties, in that the enzymes are no longer precipitable at pH 5.0. Addition of ribonucleic acid to the preparations restores this property, the activating enzymes becoming precipitable with some degree of purification. 'Soluble' ribonucleic acid from liver is more effective than yeast ribonucleic acid in these respects.

The observation that the amino acid-activating enzymes may be freed from 'soluble' ribonucleic acid without impairing the activation reaction, as measured by pyrophosphate exchange, was made during attempts to remove microsomal particles from pig-liver extracts without the use of high-speed centrifuging, a technique not practicable when 1–2 kg. of tissue is being processed. We found that the addition of protamine sulphate to the liver extracts caused the microsomes to aggregate, allowing them to be removed at relatively low centrifuge speeds. At the same time the 'soluble' ribonucleic acid was also removed. A preliminary account of this work has already been published (Finch & Hele, 1959).

MATERIALS AND METHODS

Chemicals. L-Isoleucine, L-valine, 2-amino-2-hydroxy-methylpropane-1,3-diol (tris) and adenosine triphosphate (disodium salt; ATP) were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. ATP was neutralized to pH 7.2 (indicator paper) with MgCO_3 , to give disodium magnesium ATP. Tris was titrated to the required pH with HCl. L-Leucine was purified in the laboratory. A sample of L-valine from Roche Biochemicals used in early experiments showed considerable quantities of other amino acids on chromatography. L-Cysteine HCl, glycine and one sample of glutathione were obtained from Hopkin and Williams Ltd. All other amino acids (laboratory grade), iodoacetamide and protamine sulphate (salmon) were obtained from British Drug Houses Ltd. The solutions of iodoacetamide were freshly prepared just before use. The protamine sulphate was dissolved with gentle warming to give a final concentration of 10 mg./ml., and the slightly acidic solution was not neutralized. Another sample of glutathione was obtained from the Schwartz Laboratories Inc. *p*-Chloromercuribenzoic acid (sodium salt) and protamine sulphate (herring) were supplied by L. Light and Co. Ltd. Coenzyme A was obtained from Pabst Laboratories. Yeast ribonucleic acid (RNA) was supplied by the California Corporation for Biochemical Research and was purified by precipitation with ethanol and dialysis against distilled water for 18 hr. Nu-char C activated charcoal, supplied by West Virginia Pulp and Paper Co., N.Y., U.S.A., was treated with 6N-HCl under vacuum for at least 2 hr., then washed with water until the washings were above pH 4.0 (indicator paper).

Radioactive tracers. Uniformly labelled L-[^{14}C]amino acids and $\text{H}_3^{32}\text{PO}_4$ in HCl were obtained from The Radiochemical Centre, Amersham, Bucks. The [^{14}C]amino acids were

mixed with unlabelled amino acid for use at a specific activity of 4–5 $\mu\text{C}/\mu\text{mole}$. The $\text{H}_3^{32}\text{PO}_4$, as the monohydrogen salt, was converted into pyrophosphate by pyrolysis at 400° for 1 hr. (Lee Peng, 1956).

Preparations of amino acid-activating fractions

Fraction precipitated by ammonium sulphate-potassium acetate. Pig liver, obtained from T. Wall and Sons (London, N.W. 10), was taken directly from the animal within 10 min. of death (and not selected from the pooled offal) and packed in ice and conveyed to the Laboratory cold room, where work upon the tissue commenced within 30 min. of the death of the animal. All subsequent operations were carried out between 0° and 4°.

It was first necessary to carry out a pilot preparation upon a sample of the liver to ascertain the optimum amount of protamine sulphate needed to aggregate the microsomes. A portion (100 g.) of tissue was sliced, chopped with a heavy knife to give a thick pulp, stirred mechanically for 1 min. with 2 vol. of medium C (sucrose, 0.35M; KHCO_3 , 0.035M; KCl, 0.025M) and filtered through cheese cloth, yielding a volume of extract about equivalent to the volume of medium used. Four 25 ml. samples of the extract were treated with protamine at levels ranging from 0.5 mg./ml. of extract to 2.0 mg./ml. Salmine sulphate was used, since herring protamine was found not to be effective. The protamine solution was added to the samples of extract over 1–2 min. with constant stirring; the samples were then centrifuged at 1880 g for 10 min., which sedimented the bulk of the particles. The concentration of protamine for treating the main preparation was chosen as slightly below the lowest level giving tight packing of the precipitate. This errs on the side of caution.

The main preparation, involving 1–2 kg. of liver, was carried out upon identical lines, 500 g. batches of tissue being used. If the concentration of protamine used in the main preparation proved insufficient to remove the particles completely, a further centrifuging at 10 000 g for 5–10 min. was performed in the Spinco model L preparative ultracentrifuge. The supernatant was treated with a mixture of solid $(\text{NH}_4)_2\text{SO}_4$ and potassium acetate (28 g. and 5 g./100 ml. of supernatant respectively) and spun at 10 000 g for 5–10 min. The precipitate, which comprised 25% of the protein, was dissolved in 0.01M-tris-HCl buffer, pH 7.2, and spun for 10–30 min. at 105 000 g to remove glycogen and small, variable amounts of particulate materials. The supernatant, referred to as ASAc fraction, was stored in small batches at –15°. Liver gave 2.5–3.5 g. of ASAc-fraction protein/500 g. The ASAc fractions used in the experiments upon the incorporation of [^{14}C]amino acids into 'soluble' RNA were dialysed for 2–4 hr. against 0.01M-tris-HCl buffer, pH 7.5, to remove some of the salts, and centrifuged at 105 000 g for 10–20 min. before use.

The ASAc fraction retained its pyrophosphate-exchange activity for at least 6 weeks if kept at –15°. Similar preparations, precipitated at 60% saturation with $(\text{NH}_4)_2\text{SO}_4$, either un-neutralized or neutralized with KOH or tris during the addition of the salt, gave better yields of threonine- and methionine-activating enzymes, but did not retain their pyrophosphate-exchange activity for more than 2 or 3 days.

The ASAc fraction contains about 0.1–0.3 mg. of RNA/50 mg. of protein/ml. The lower value is just detectable by our methods. This detectable RNA can be completely

removed by centrifuging at 105 000 *g* for 10 min., without affecting either the pyrophosphate-exchange rate or the labelling of added 'soluble' RNA.

Rat-liver ASAc fractions were made from minced tissue along lines identical with those used with pig liver. Their properties were very similar to those of the pig-liver preparations.

We found that the effective precipitation of microsomal particles with protamine sulphate could be achieved only if liver extracts were prepared in the manner described above. Extracts made with phosphate buffers, extracts in which the ratio of tissue to medium C was doubled or homogenates prepared in Waring-type blenders required more protamine sulphate to remove the particles and the ensuing particle-free extracts contained very little activating-enzyme activity.

We suggest that the success of this method for the removal of particles may depend upon a lowered ratio of particulate elements to soluble protein. Compared with homogenates prepared in a blender, the chopping and extracting procedure described above releases about 50 % of the soluble protein, 25 % of the RNA and only 15–20 % of the particulate material, including glycogen. The soluble protein obtained from both chopping and homogenizing gives pH 5.0 fractions with the same specific activity for amino acid-dependent exchange of pyrophosphate, tested with 18 amino acids separately.

We observed that if the cheesecloth filtrate was allowed to stand for more than a few minutes without the addition of protamine, then virtually all amino acid-activating activity was lost. This did not happen if protamine were added immediately after the preparation of the filtrate. In practice this required that the pilot run should be commenced before the main bulk of the material was chopped.

Fraction precipitated at pH 5.0. Preparations were made from the livers of young Wistar rats, weight 80–100 g., by the method of Keller & Zamecnik (1956), but with adjustment of the supernatant from the centrifuging for 2 hr. at 105 000 *g* to pH 5.0 rather than pH 5.2. Similar preparations were made from homogenized pig liver and from the cheesecloth-filtered extract of chopped pig liver. For all of the fractions precipitated at pH 5.0 (pH 5.0 fractions) the tissues were dispersed in medium A (Keller & Zamecnik, 1956), from which our medium C is derived by omission of $MgCl_2$.

Isolation of 'soluble' ribonucleic acid. 'Soluble' RNA was isolated from the pH 5.0 fractions by the phenol method of Kirby (1956) as described by Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958).

Assays of enzyme activity

Pyrophosphate exchange. For the standard assay a mixture of 1 μ mole of amino acid, 6 μ moles of disodium magnesium ATP, 3 μ moles of [^{32}P]pyrophosphate containing 20 000–50 000 counts/min., 100 μ moles of tris-HCl buffer, pH 7.2, and 1–2 mg. of protein in a total volume of 1 ml. was incubated in 15 ml. centrifuge tubes at 38° for 5 min. without shaking, and then treated with 3.5 ml. of ice-cold 6 % trichloroacetic acid. Amino acid was omitted from the control incubation. After centrifuging in the cold to remove the protein precipitate, the supernatant was decanted on to 0.4 ml. of moist charcoal (approx. 160 mg. dry wt.) for the separation of ATP and pyrophosphate by a modification of the method of Crane & Lipmann (1953).

The modification consisted of using filtration instead of centrifuging for the washing of the charcoal, in a micro-filter tube (A. Gallenkamp Ltd., no. AG 12 A $\times$ 4; capacity 8 ml.) with the outlet stoppered with a polyvinyl chloride cap. The filter tube was fitted into a receiving vessel with a side arm. (Filter tube with side arm, 150 mm. $\times$ 25 mm. diam., capacity 40 ml.; Griffin and George Ltd., no. S25-720.) Layering with ethanol was unnecessary. With a manifold connexion to the vacuum pump up to forty incubations could be treated at one time. The arrangement of the filter tube and receiving vessel is shown in Fig. 1. Charcoal was measured into the filter tube with a sawn-off pipette fitted with a glass tube as a plunger. The radioactive samples were added to the charcoal and mixed with glass rods. The filter tubes were capped to prevent loss of liquid before adsorption of ATP had taken place. After a brief interval, when all the samples had been well mixed with the charcoal, the caps were removed and suction was applied through a manifold. The charcoal was washed with water up to the 15 ml. graduation on the receiving vessel. This solution was on occasion retained for estimation of liberated orthophosphate. Suction was stopped, the filter tube and top stopper were removed together and the cap was replaced on the bottom of the filter tube. The charcoal was stirred with 2 ml. of 2*N*-HCl and the filter tube, top stopper and cap were transferred to a boiling-water bath for 15 min. to hydrolyse the labile phosphates of ATP. At the end of this period the caps were removed from the

Fig. 1. Filtration equipment used for washing charcoal precipitates. A, Filter tube, top stopper and cap; B, receiving vessel, graduated at 15 ml.

bottom of the filter tubes and the filter tubes and top stoppers were quickly returned to the receiving vessels, which had meantime been washed out six times with water and dried in an oven. Suction and washing were applied as before, resulting in a 15 ml. sample suitable for estimation of radioactivity and orthophosphate (P_i). After correction for the recovery of P_i from ATP (usually 80–90%) the rates of exchange were calculated according to Davie, Koningsberger & Lipmann (1956).

The amino acid-dependent exchange of pyrophosphate was taken as the difference between test and control incubations. All our data on pyrophosphate exchange are expressed after the subtraction of the control, except where specifically stated otherwise.

The rate of amino acid activation is influenced by the amount of pyrophosphate in the system (Davie *et al.* 1956). P_i found (about 0.8 μ mole) in the first washings from the charcoal indicates a maximum loss of pyrophosphate of 10%.

Attachment of [14 C]amino acids to 'soluble' ribonucleic acid. A mixture of 0.04 μ mole of [14 C]amino acid, 4 μ moles of disodium magnesium ATP, about 100 μ g. of 'soluble' RNA and about 4 mg. of protein in a total volume of 0.4 ml. was incubated in 15 ml. centrifuge tubes at 38° for 4 min. without shaking and then treated with 1 ml. of ice-cold 0.6N-perchloric acid, followed by centrifuging at 1880 g in the cold. Controls were performed with the omission of either ATP or 'soluble' RNA as appropriate. The supernatant was discarded and the precipitate washed with cold 0.2N-perchloric acid and lipid solvents according to the procedure of Hoagland *et al.* (1958). In some experiments the RNA was extracted from the washed precipitate with hot NaCl by the method of these authors, but more generally the total precipitate was dissolved by warming in 0.1 ml. of N-NaOH followed by 0.2 ml. of water. Volumes of 0.1 ml. were taken for plating and counting and for estimation of RNA (ribose) content. In certain control experiments the labelled RNA was extracted from the total precipitate with hot 0.8N-perchloric acid before counting (Rendi & Campbell, 1959). 'Soluble' RNA recoveries in the samples used for counting rarely differed by more than 25% within any one experiment, and agreement between samples was usually of the order of 10–15%.

Measurement of radioactivity

32 P was counted in a M. 6 liquid counter of 9 ml. capacity (20th Century Electronics Ltd.) and corrected for the decay of the isotope.

Materials containing 14 C were plated as thin samples on lens paper in 2 cm.² polythene planchets and counted with an end-window counter. The counts were corrected to negligible thickness by the use of an experimentally determined self-absorption curve. The maximum corrections thus introduced were less than 10%.

Owing to the large numbers of samples that had to be counted the time of counting of any one specimen was restricted to 5 min. The standard error of counting of weakly radioactive specimens was as high as $\pm 15\%$, but for more active specimens this ranged between ± 3 and $\pm 8\%$. The background counting rate was always determined to an accuracy of $\pm 5\%$, generally being 10 ± 0.5 counts/min.

In the studies with both pH 5.0 fraction and our 'recombined system' the control experiments, from which either ATP or added 'soluble' RNA was omitted, seldom

gave counting rates higher than the background count. The highest control count observed on one occasion only was 3 counts/min. above background. Experiments with complete systems gave counting rates of between 10 and 250 counts/min. above the background count. Counting rates of 5 counts/min. above background or less were of little significance.

We have expressed our experimental findings on the labelling of 'soluble' RNA in terms of μ m-moles of amino acid incorporated/mg. of 'soluble' RNA. This allows us to correct for the varying specific activities of our [14 C]amino acid solutions, and for the amount of RNA used in the assay of radioactivity. This latter correction is important, for although the recoveries of RNA were very consistent within any one experiment, wide variations occurred between experiments. Standard errors for these corrected values were calculated from the standard errors of counting the samples.

Chemical estimations

RNA was estimated as ribose by the orcinol method of Albaum & Umbreit (1947), the colour being developed by heating for 40 min. in stoppered tubes in a boiling-water bath. ATP was used as a ribose standard, since ribose itself or adenosine monophosphate gave poorly reproducible results. The results were expressed as RNA on the basis of a conversion factor of 670 mg. of RNA $\equiv$ 1 μ mole of ATP ribose, determined for a standard preparation of RNA. This standard preparation was isolated from mouse-ascites-tumour cells by the method of Davidson & Weymouth (1944) and contained 8.1% of P.

Determinations of the RNA content of enzyme preparations or tissue fractions were carried out on the precipitate of denatured protein obtained with 0.8N-perchloric acid and washed with 0.2N-perchloric acid and lipid solvents as in the assay for attachment of [14 C]amino acids to 'soluble' RNA. The estimation was carried out on either the extract obtained by overnight incubation at 38° with 0.8N-perchloric acid or on the solution in NaOH as used for plating and counting. The estimations by these two procedures showed good agreement. When the precipitates from perchloric acid contained large amounts of protein relative to the amount of RNA present (less than 200 μ g. of RNA/100 mg. of protein) a brown or yellow colour often developed during the boiling of the orcinol reagents, which interfered with measurements of E at 660 μ . This made it very difficult to detect small quantities of RNA with any certainty. For this reason we were unable to detect with confidence small amounts of 'soluble' RNA added to ASAc-fraction protein (Fig. 6), and for this reason we considered all values below 5 μ g. of RNA found in the portions taken for plating and counting to be of little significance.

Like Simpkin & Work (1957) we have found that microsomes often lose a considerable quantity of their RNA in the presence of cold dilute perchloric acid. This loss can be avoided by precipitating the microsomes with protamine, resuspending and then reprecipitating them with cold perchloric acid and washing in the usual manner.

Protein was determined usually by the biuret procedure of Gornall, Bardawill & David (1949) and on occasion by the turbidimetric method used by Hoagland, Keller & Zamecnik (1956) and by Stadtman, Novelli & Lipmann (1951). These three methods agreed to within 10%.

Sulphydryl groups were determined by the procedure of Grunert & Phillips (1951) and P_i by the method of Allen (1940).

RESULTS

Removal of particles with protamine

The contents of some constituents of the supernatant obtained after treatment with varying amounts of protamine are given in Fig. 2. The ribonucleic acid content of the supernatant drops rapidly, consistent with the observed loss of microsomal particles. The turbidity in the turbidimetric estimation (the value measured deriving both from soluble protein and from particulate material) drops initially in a similar manner, reaching a constant low level which represents largely a mixture of soluble protein and glycogen. With concentrations of protamine above that necessary to bring down the bulk of the ribonucleic acid and the particles, a loss of activating enzymes as obtained in the ASAc fraction is observed. We do not know the extent to which the upward trend in activity

Fig. 2. Removal of particulate material and amino acid-activating enzymes from a pig-liver extract treated with protamine sulphate. Increasing amounts of a protamine sulphate solution (10 mg./ml.) were added, with stirring, over 1 min. to 50 ml. samples of a pig-liver extract prepared as described under Materials and Methods. The samples were centrifuged for 10 min. at 1880 g and determinations made upon the supernatants so obtained of RNA content (●), turbidity by the method of Hoagland, Keller & Zamecnik (1956), the results being given here in arbitrary turbidity units/ml. (○) and protein by the biuret procedure (■). Amino acid-dependent exchange of pyrophosphate was measured upon samples of ASAc fractions prepared from the 1880 g supernatants by the assay described under Materials and Methods, with leucine (Δ) and an impure sample of valine ($\blacktriangle$) as substrates, the latter containing isoleucine and methionine. Very small amounts of RNA could not be detected accurately (see Materials and Methods). The broken line indicates the amount assumed to be present.

shown in this curve is maintained at concentrations of protamine above 2.0 mg./ml. of extract, although early experiments with higher concentrations of protamine than those employed here did not give very encouraging results in terms of enzyme activity. No significant decrease in $-SH$ occurs over this range of protamine concentrations.

Fig. 3. Effect of protein concentration upon the rate of isoleucine-dependent exchange of pyrophosphate catalysed by a pig-liver ASAc fraction. The standard assay, as described under Materials and Methods, was used with varying amounts of protein as indicated. The ASAc fraction was dialysed for 2 hr. before use. Δ , Total pyrophosphate exchange; $\circ$, amino acid-free pyrophosphate exchange; $\bullet$, amino acid-dependent pyrophosphate exchange, shown as the difference between the first two determinations.

Fig. 4. Effect of amino acid concentration upon the rate of amino acid-dependent exchange of pyrophosphate catalysed by a pig-liver ASAc fraction. The standard assay, as described under Materials and Methods, was used. The ASAc fraction was dialysed for 4 hr. before use and 2.1 mg. of protein was used in the incubation mixture. $\square$, L-leucine; $\circ$, L-isoleucine; Δ , L-valine.

Amino acid-dependent exchange of pyrophosphate catalysed by pig-liver preparations

Without dialysis the ASAc fraction gave high control values in the pyrophosphate-exchange assay in the absence of added amino acids. Dialysis against 0.01 M-tris-HCl buffer, pH 7.2, for 2-3 hr. reduced the control values to the order of 10-20 μ m-moles/mg. of protein/min. The rate of amino acid-dependent exchange of pyrophosphate remained linear with time for at least 5 min. With the dialysed preparations the amino acid-dependent exchange of pyrophosphate was linear with protein concentration to at least 5 mg. of protein (Fig. 3). For L-isoleucine, L-leucine and L-valine the rate increases with concentration of amino acid as shown in Fig. 4, with half-maximal rates being reached in the range 0.5-0.1 mM. The rate at the standard-assay concentration of mM was about three-quarters of the maximum for these amino acids. No inhibition was observed at high concentrations.

The ASAc fraction and the pH 5.0 fraction predominantly activate leucine and isoleucine (Table 1). The activities of the pH 5.0 fractions from the same liver are usually 20-50 % higher than those of the ASAc fraction. The rat-liver fractions are two to three times as active as the comparable pig-liver ones. With the majority of preparations the values of the amino acid-dependent exchange of pyrophosphate were higher than those in Table 2, but the relative order of the rates remained much the same.

The addition of 'soluble' RNA to incubations of ASAc fractions did not influence the amino acid-dependent exchange of pyrophosphate (Table 2), even if pre-incubated with the protein. In some cases a considerable reduction of the exchange in the amino acid-free control has been observed. Yeast RNA at a concentration of 0.5 mg./ml. has no effect on the pyrophosphate exchange.

The ASAc fraction contains about 0.3 μ mole of SH/ml. (20-50 mg. of protein) as determined upon boiled extracts, or upon cold perchloric acid extracts. Periods of dialysis up to 18 hr. had no effect on the amino acid-dependent exchange of pyrophosphate, although decreasing the blank. The presence of 0.05 M-glutathione, mM-ethylene-diaminetetra-acetate or 0.01 M-MgCl₂ in the dialysis buffer did not affect the amino acid-dependent rate. The addition of glutathione, coenzyme A and acetate was without effect on the level of pyrophosphate exchange. The amino acid-dependent rate in the standard assay was reduced to 20 % for both ASAc and pH 5.0 fractions in the presence of 0.1 mM-p-chloromercuribenzoic acid and was unchanged and reduced to 50 % respectively in the presence of 10 mM-iodoacetamide.

Effect of ribonucleic acid on the precipitation of amino acid-activating enzymes at pH 5.0

Adjustment of pH to 5.0 does not cause precipitation from the dialysed ASAc fraction, provided that it was centrifuged after dialysis. If 'soluble'

Table 1. *Typical rates of amino acid-dependent exchange of pyrophosphate catalysed by pig-liver preparations*

The standard assay, as described under Materials and Methods, was used.

Amino acid	Pyrophosphate exchange (μ m-moles/min./mg. of protein)	
	ASAc fraction	pH 5.0 fraction
Alanine	0	0
Arginine	0	1
Asparagine	0	1
Aspartic acid	0	5
Glutamic acid	0	5
Glycine	4	1
Histidine	0	7
Isoleucine	21	22
Leucine	17	18
Lysine	0	2
DL-Methionine	6	10
Phenylalanine	2	0
Proline	4	7
Serine	4	4
DL-Threonine	2	5
Tryptophan	6	5
Tyrosine	0	4
Valine	7	6

Table 2. *Effect of added 'soluble' ribonucleic acid upon the rate of amino acid-dependent exchange of pyrophosphate catalysed by the 'ASAc fraction'*

The standard assay as described under Materials and Methods was used.

In Expt. 1 the ASAc fraction was dialysed for 2 hr. against 0.01 M-tris buffer, pH 7.2, and then centrifuged for 10 min. at 105 000 g; 0.75 mg. of protein was used and 0.75 mg. of 'soluble' RNA was added as indicated.

In Expt. 2 the ASAc fraction was dialysed for 2.5 hr. against the same buffer and then pre-incubated for 3.5 min. at 37° with added 'soluble' RNA (2.3 mg./100 mg. of protein). The pre-incubated mixture was cooled in ice for 2.5 min. and then tested in the standard assay.

All data obtained with added amino acids are expressed as the excess over the amino acid-free blank.

	Pyrophosphate exchange (μ m-moles/min./mg. of protein)	
	Without added 'soluble' RNA	With added 'soluble' RNA
Expt. 1		
No amino acid	20	18
Leucine	14.7	14.7
Isoleucine	21.4	24.2
Expt. 2		
No amino acid	21	12.8
Leucine	7	7.4
Isoleucine	8.7	6

RNA or yeast RNA be added before adjusting the pH to 5.0, then the lowering of pH causes precipitation, the amount of protein precipitated being proportional to the amount of RNA added.

This precipitation results in a purification of some of the amino acid-activating enzymes. The optimum ratio of protein to RNA giving the best increase in

specific activity of the enzyme appears to be about 50:1; the specific activity increases with the ratio up to this point, and thereafter decreases. The same value for the optimum ratio is found with both yeast RNA and 'soluble' RNA but 'soluble' RNA gives a sixfold purification, as compared with the threefold purification obtained with yeast RNA (Table 3). About twice as much 'soluble' RNA as yeast RNA is precipitated at and below the ratio (50:1) of RNA to protein, although the amount of protein precipitated is the same (Table 4). This relatively lower yield of yeast RNA to protein in pH 5.0 precipitates has been observed in a number of experiments.

Table 3. *Purification of amino acid-activating enzymes in the 'ASAc fraction' by precipitation at pH 5.0 in the presence of added ribonucleic acid*

ASAc fraction was diluted to a concentration of 1 mg. of protein/ml. with 0.01M-tris-HCl buffer, pH 7.5, and yeast RNA or 'soluble' RNA was added in the proportion of 2 mg. of RNA to 100 mg. of protein. pH 5.0 fractions were prepared from these mixtures as described under Materials and Methods and assayed in our standard system for pyrophosphate exchange.

	Pyrophosphate exchange (μ m-moles/min./ mg. of protein)	
	ASAc fraction	pH 5.0 fraction
Expt. 1 (with 'soluble' RNA)		
Isoleucine	16.0	122
Leucine	20.4	124
Expt. 2 (with yeast RNA)		
Isoleucine	15.0	48

'pH 5.0 Fraction' prepared from
pig liver and rat liver

Pig-liver pH 5.0 fraction does not differ greatly from rat-liver pH 5.0 fraction in its ability to incorporate [14 C]amino acids into 'soluble' RNA (Table 5). A far greater difference exists between the rates of amino acid-dependent exchange of pyrophosphate catalysed by the pH 5.0 fractions from the two different sources. Two pig-liver pH 5.0 fractions tested with [14 C]phenylalanine and [14 C]lysine gave values for incorporation of the same order as valine and threonine. [14 C]Tryptophan was not incorporated. The value for [14 C]-

Table 4. *Precipitation of protein and ribonucleic acid at pH 5.0*

ASAc fraction was dialysed for 3 hr. against 0.01M-tris-HCl buffer, pH 7.5, and then centrifuged at 105 000 g for 15 min. Samples of this protein (50 mg.) were diluted with tris buffer to a final concentration of 2 mg./ml., and RNA was added in the quantities indicated in the Table. pH 5.0 fractions were prepared as described in the Materials and Methods section. No precipitation occurred in the absence of added RNA.

Total quantity found in pH 5.0 fraction	'Soluble' RNA				Yeast RNA			
	RNA added (mg./100 mg. of protein)							
	1	2	3	4	1	2	3	4
	Protein (mg.)	8.1	14.2	20.2	24.2	7.8	14.8	22.4
RNA (mg.)	0.15	0.26	0.26	0.26	0.07	0.13	0.28	0.29

Table 5. *Incorporation of [14 C]amino acids into 'soluble' ribonucleic acid and the amino acid-dependent exchange of pyrophosphate catalysed by pig- and rat-liver pH 5.0 fraction*

The assay systems described under Materials and Methods were used.

	Amino acid incorporated into 'soluble' RNA (μ m-moles/mg./4 min.)			
	Leucine	Isoleucine	Valine	Threonine
Pig pH 5.0 fraction	2.2 $\pm$ 0.17*	6.7 $\pm$ 0.69	0.74 $\pm$ 0.12	1.1 $\pm$ 0.15
Rat pH 5.0 fraction	4.3 $\pm$ 0.18	7.2 $\pm$ 0.21	0.60 $\pm$ 0.15	1.0 $\pm$ 0.08
	Pyrophosphate exchange (μ m-moles/mg. of protein/min.)			
	Leucine	Isoleucine	Valine	Threonine
Pig pH 5.0 fraction	24.3	28.7	6.1	7.4
Rat pH 5.0 fraction	90.0	152.0	—	17.5

* S.E. derived from standard error of 14 C counting.

valine obtained with the rat-liver preparation is abnormally high. In most cases essentially zero incorporation was obtained with rat-liver pH 5.0 fractions with [^{14}C]valine.

Incorporation of [^{14}C]amino acids into added 'soluble' ribonucleic acid catalysed by 'recombined' systems

ASAc fractions of low RNA content easily transfer [^{14}C]amino acids to added 'soluble' RNA, previously isolated by phenol extraction from pH 5.0 fractions (see Materials and Methods section). We term such a system a 'recombined' system. ASAc fractions over 6 weeks old are as effective as freshly prepared material, but solutions of isolated 'soluble' RNA deteriorate after about 3 days, and the best results with 'recombined' systems are obtained with freshly isolated 'soluble' RNA. The extent of incorporation of [^{14}C]amino acid into the added 'soluble' RNA in the 'recombined' system is usually comparable, although not identical, with the incorporation obtained with a sample of the pH 5.0 fraction from which the

'soluble' RNA was originally isolated (Table 6). The rate of pyrophosphate exchange, and therefore of the potential quantity of acyl amino acid formed, is far greater than the rate of incorporation of amino acid into 'soluble' RNA, or of the total quantity of labelled 'soluble' RNA formed. As we noted with the pH 5.0 fractions, there was no clear relationship between the abilities of our various systems to catalyse the two reactions. Thus in Expt. 1 (Table 6) the total amount of valine adenylate potentially generated by the rat-liver pH 5.0 fraction amounts to 680 $\mu\text{m-moles}$, by the rat-liver ASAc fraction 1200 $\mu\text{m-moles}$ and by the pig-liver ASAc fraction 280 $\mu\text{m-moles}$. In Expt. 2 the rat-liver pH 5.0 fraction would have produced 20 000 $\mu\text{m-moles}$ of leucine adenylate during the period of incubation and the pig-liver ASAc fraction 2900 $\mu\text{m-moles}$.

In several cases we observed that more [^{14}C]valine, and possibly more [^{14}C]threonine, was incorporated into 'soluble' RNA acid in the 'recombined' system consisting of pig-liver ASAc fraction + rat-liver 'soluble' RNA than occurred with the

Table 6. *Incorporation of [^{14}C]amino acids into added 'soluble' ribonucleic acid in a 'recombined' system*

	Amino acid incorporated into 'soluble' RNA ($\mu\text{m-moles/mg./4 min.}$)			
	Leucine	Isoleucine	Valine	Threonine
Expt. 1				
Rat pH 5.0 fraction (120 $\mu\text{g.}$ of 'soluble' RNA + 4.1 mg. of protein)	1.5 $\pm$ 0.22*	9.8 $\pm$ 0.43	0	0.9 $\pm$ 0.11
Rat 'soluble' RNA (155 $\mu\text{g.}$; 1-day-old) + rat ASAc (3.9 mg. of protein)	2.0 $\pm$ 0.21	4.2 $\pm$ 0.34	1.0 $\pm$ 0.16	1.4 $\pm$ 0.21
Rat 'soluble' RNA (155 $\mu\text{g.}$; 1-day-old) + pig ASAc (a) (3.9 mg. of protein)	2.2 $\pm$ 0.21	2.7 $\pm$ 0.23	1.7 $\pm$ 0.22	1.1 $\pm$ 0.21
Expt. 2				
Rat pH 5.0 fraction (45 $\mu\text{g.}$ of 'soluble' RNA + 2.25 mg. of protein)	59. $\pm$ 0.51	12.3 $\pm$ 0.89	0	1.4 $\pm$ 0.26
Rat 'soluble' RNA (63 $\mu\text{g.}$; 1-day-old) + pig ASAc (b) (4.0 mg. of protein)	6.3 $\pm$ 0.67	10.5 $\pm$ 0.76	1.4 $\pm$ 0.35	2.3 $\pm$ 0.21
Expt. 3				
Pig pH 5.0 fraction (89 $\mu\text{g.}$ of 'soluble' RNA + 5.5 mg. of protein)	4.4 $\pm$ 0.26	5.8 $\pm$ 0.26	1.8 $\pm$ 0.12	1.1 $\pm$ 0.09
Pig 'soluble' RNA (190 $\mu\text{g.}$; 4-day-old) + pig ASAc (b) (4.2 mg. of protein)	1.8 $\pm$ 0.09	—	1.3 $\pm$ 0.10	0.8 $\pm$ 0.09
Expt. 4 (incubation for 5 min.)				
Rat pH 5.0 fraction (240 $\mu\text{g.}$ of 'soluble' RNA + 4.8 mg. of protein)	2.8 $\pm$ 0.10	—	0.3 $\pm$ 0.05	—
Rat pH 5.0 fraction, as above + 100 $\mu\text{g.}$ of rat 'soluble' RNA	2.6 $\pm$ 0.08	—	0.5 $\pm$ 0.05	—
Rat 'soluble' RNA (100 $\mu\text{g.}$) + pig ASAc (b) (9.8 mg. of protein)	2.1 $\pm$ 0.18	—	1.6 $\pm$ 0.20	—

* S.E. derived from standard error of ^{14}C counting.

Table 7. *Incorporation of [^{14}C]amino acid into 'soluble' ribonucleic acid and protein in experiments with 'recombined' systems*

Duplicate samples of ^{14}C -labelled precipitates from perchloric acid were prepared as described in the Materials and Methods section, and one set of precipitates were dissolved in NaOH and counted. Where indicated, the duplicate precipitates were either extracted with hot 10% NaCl and the isolated 'soluble' RNA in this extract was counted, or they were extracted with hot 0.8N-perchloric acid, and the residual precipitate (i.e. minus 'soluble' RNA) was dissolved in NaOH and counted.

		Amino acid incorporated into 'soluble' RNA ($\mu\text{m-moles/mg./4 min.}$)	
		'Total' perchloric acid precipitate	'Soluble' RNA from NaCl extract
Expt. 1	Pig pH 5.0 fraction { Leucine Isoleucine	2.9 $\pm$ 0.18*	2.7 $\pm$ 0.27
		4.0 $\pm$ 0.20	3.7 $\pm$ 0.29
	Pig ASAc + pig 'soluble' RNA { Leucine Valine	1.8 $\pm$ 0.11	2.2 $\pm$ 0.21
		1.3 $\pm$ 0.10	1.5 $\pm$ 0.15
			'Total' perchloric acid precipitate
Expt. 2	Pig ASAc + rat 'soluble' RNA { Isoleucine Valine	'Total' perchloric acid precipitate	after extraction with 0.8N-perchloric acid
		6.7 $\pm$ 0.31	0.0
	1.4 $\pm$ 0.17	0.0	

* S.E. derived from standard error of ^{14}C counting.

appropriate rat-liver pH 5.0 fraction. These effects, although small, represented a significant amount of labelling; indeed, no labelling at all occurred with some of the rat-liver pH 5.0 fractions.

This increased incorporation of [^{14}C]valine might possibly be attributed to the presence of microsomes in the ASAc fraction, which would lead to the incorporation of label into microsome protein, as well as into 'soluble' RNA. These effects would not be differentiated when 'total' perchloric acid precipitates were used for the estimation of radioactivity. Control experiments (Table 7), designed to test this point, showed that 'soluble' RNA could be extracted with hot 10% NaCl from the 'total' perchloric acid precipitate, and that this extracted RNA was labelled to the same extent as that in the 'total' precipitate. Moreover, RNA could be completely extracted from the 'total' precipitate (under conditions that would have left labelled protein behind in the 'total' precipitate) and with it all the radioactivity of the 'total' precipitate. It is unlikely that the increase in [^{14}C]valine incorporated described above was stimulated by the small quantity of -SH in the ASAc fraction (cf. Rendi & Campbell, 1959).

Some simple studies of the kinetics of the 'recombined' system have been made. The rate of incorporation of [^{14}C]isoleucine is proportional to the concentration of ASAc-fraction protein (Fig. 5*b*), when measured on the linear portion of the time curve (Fig. 5*a*). The amount of [^{14}C]leucine and [^{14}C]valine incorporated into added 'soluble' RNA is proportional to the amount of 'soluble' RNA added, for an incubation period of 4 min. (Fig. 6).

DISCUSSION

Exchange of pyrophosphate

Pig-liver preparations catalyse the activation of much the same spectrum of amino acids as do other mammalian-liver preparations, and, broadly speaking, at much the same rates (cf. Novelli, 1958). The results of the amino acid-concentration studies indicate the affinities of L-isoleucine, L-leucine and L-valine for their activating systems to be similar to those found for L-tryptophan (Davie *et al.* 1956), L-tyrosine (Schweet & Allen, 1958) and L-methionine (Berg, 1956).

The relative rates of activation of the various amino acids by both the ASAc fraction and pH 5.0 fraction are similar to those of Webster (1959) observed with pig-liver preparations. It is possible that the small amino acid-dependent exchange of pyrophosphate observed for many of the amino acids might be greater if tested at the optimum pH as proposed by this author.

That amino acid activation occurs in the absence of 'soluble' ribonucleic acid and is not stimulated by its addition is in line with the findings of Schweet & Allen (1958) for the tyrosine-activating enzyme from hog pancreas, and by the findings of Schweet, Bovard, Allen & Glassman (1958) for activating enzymes from guinea-pig liver. Our findings are also in line with those of Hoagland *et al.* (1958) and of Decken & Hultin (1959), who showed that treatment of pH 5.0 fractions with ribonuclease did not affect amino acid activation as measured by pyrophosphate exchange, thus differing from the findings of Ogata & Nohara (1957*a, b*).

Unlike Rendi, Di Milia & Fronticelli (1958) we can find no evidence that ribonucleic acid is inhibitory to amino acid activation, at least when this reaction is measured by following pyrophosphate exchange.

Precipitation at pH 5.0 in the presence of ribonucleic acid

Evidently the amino acid-activating enzymes require the presence of a polyanion before they become insoluble at acid pH, and any type of ribonucleic acid will serve this function. The greater degree of purification obtained with 'soluble' ribonucleic acid as compared with yeast ribonucleic acid suggests that the 'soluble' ribonucleic acid is

more selective for the enzyme proteins. It is tempting to associate the twofold efficiency of 'soluble' ribonucleic acid (as compared with yeast ribonucleic acid) in the purification of these enzymes with the fact that twice as much 'soluble' ribonucleic acid is precipitated in the pH 5.0 fraction as is yeast ribonucleic acid. We do not know if it is simply the size of the ribonucleic acid molecule or its specific structure which decides this selectivity, for we have no information about the molecular size of the ribonucleic acids used in these experiments. Rendi & Campbell (1959) have shown that the protamine-treated 'S-protein' of Sachs (1957), of low ribonucleic acid content, is non-precipitable at pH 5.0.

Fig. 5 (a). Effect of incubation time upon the incorporation of [¹⁴C]isoleucine into 'soluble' ribonucleic acid in a 'recombined' system. The assay system described under Materials and Methods was used and contained 3.85 mg. of pig ASAc fraction and 155 μg. of rat-liver 'soluble' RNA (5-day-old). A zero-time sample of the complete system gave the same radioactivity as the background count of 9 counts/min. Isoleucine gave a rate of amino acid-dependent exchange of pyrophosphate of 16 μm-moles/min./mg. of ASAc protein. The standard errors for these four points, calculated from the standard error of the ¹⁴C counting, and reading from left to right, are ±0.12, ±0.13, ±0.09, ±0.12 μm-mole.

(b) Effect of ASAc-fraction protein concentration upon the rate of incorporation of [¹⁴C]isoleucine into 'soluble' ribonucleic acid in a 'recombined' system. The assay system described under Materials and Methods was used and contained 210 μg. of rat-liver 'soluble' RNA (1-day-old) and pig-liver ASAc fraction in the amounts indicated. The incubation time was 1.5 min. The ASAc fraction gave a rate of amino acid-dependent exchange of pyrophosphate of 12 μm-moles/min./mg. of protein with isoleucine. The standard errors for these four points, calculated from the standard error of the ¹⁴C counting, and reading from left to right, are ±0.09, ±0.09, ±0.11, ±0.19 μm-mole.

Attachment of amino acids to 'soluble' ribonucleic acid and its relation to pyrophosphate-exchange activity

Although isoleucine and leucine are, of the amino acids tested, the most effective in both incorporation and pyrophosphate exchange, no very clear correlation exists between pyrophosphate-exchange

Fig. 6. Relationship between [¹⁴C]amino acid incorporation into 'soluble' ribonucleic acid in a 'recombined' system and the amount of 'soluble' ribonucleic acid added. The assay system used is described under Materials and Methods. Each incubation mixture contained 4 mg. of pig-liver ASAc fraction and pig-liver 'soluble' RNA was added in the amounts indicated. Radioactivity is expressed as observed counts/min. (after correcting for self-absorption and the background counts of 10 counts/min.) in experiments with [¹⁴C]leucine (●) and [¹⁴C]valine (■). 'Soluble' RNA determinations were carried out upon portions identical with those used for the estimation of radioactivity, and the amounts of 'soluble' RNA found in these portions in the experiment with [¹⁴C]leucine (○) and with [¹⁴C]valine (□) are also shown. With 180 μg. of 'soluble' RNA added to the system the incorporation of leucine was equivalent to 1.8 μm-moles and of valine to 1.3 μm-moles/mg. of 'soluble' RNA. The standard errors for these points, calculated from the standard error of the ¹⁴C counting, and reading from left to right, are: (i) leucine ±4.1, ±2.0, ±2.5, ±3.2, ±4.3; (ii) valine 0, ±1.9, ±2.3, ±2.5, ±3.8.

activity and the ability of the preparations to incorporate [^{14}C]amino acids into 'soluble' ribonucleic acid. Incorporation of [^{14}C]amino acid into 'soluble' ribonucleic acid can occur in the absence of significant pyrophosphate-exchange activity. This may simply mean that the incorporation of [^{14}C]amino acids into 'soluble' ribonucleic acid is a more sensitive test for the presence of amino acid-activating enzymes than is pyrophosphate exchange, and that all the amino acid-activating enzymes of the liver are of the type described by Acs, Hartmann, Bowman & Lipmann (1959).

If we assume that the attachment of the amino acid to 'soluble' ribonucleic acid proceeds via an amino acyl adenylate, under our experimental conditions the rate and extent of the adenylate formation greatly exceeds that of the attachment of the amino acids to 'soluble' ribonucleic acid. This may in part reflect a lack of saturation with 'soluble' ribonucleic acid at the ribonucleic acid concentrations of the order used here, whereas the reactants in the pyrophosphate exchange are at near-saturation levels; but it may also reflect a difference in the maximum rates for the two reactions.

In our experiments with [^{14}C]valine we observed that pig-liver preparations are better able than rat-liver preparations to catalyse the transfer of this amino acid to 'soluble' ribonucleic acid. Pig-liver ASAc fraction is more effective in promoting the attachment of [^{14}C]valine to 'soluble' ribonucleic acid than is the rat-liver pH 5.0 fraction from which the ribonucleic acid was isolated. This effect is not related to the rates of valine-dependent exchange of pyrophosphate observed with the different preparations used, and is not observed with the more actively incorporated amino acids leucine and isoleucine. Perhaps the effect can be observed only when the conditions for the transfer of an amino acid to 'soluble' ribonucleic acid are in some way suboptimum.

Rendi & Campbell (1959) have shown that the protamine-treated 'S-protein' of Sachs (1957) will attach amino acids to added 'soluble' ribonucleic acid. We also observed that this preparation of Sachs can incorporate [^{14}C]amino acids into 'soluble' ribonucleic acid, but did not investigate this system as extensively as did Rendi & Campbell. Recently Rendi & Hultin (1959) have described a protamine-treated preparation similar to 'S-protein', which, like our ASAc fraction, is able to incorporate [^{14}C]leucine into added 'soluble' ribonucleic acid, but, unlike the ASAc fraction, cannot catalyse pyrophosphate exchange.

SUMMARY

1. Extracts of pig liver contain 50% of the soluble protein and 15–20% of the particulate

material released by homogenizing the tissue by orthodox methods. The particulate material can be removed by treatment of the extract with protamine sulphate, followed by centrifuging at relatively low speeds.

2. Precipitation of the protamine-treated extract with ammonium sulphate and potassium acetate yields a protein fraction which will catalyse the activation of several amino acids, as measured by exchange of [^{32}P]pyrophosphate, to an extent very similar to that observed with pH 5.0 fractions prepared from the extract.

3. This protein fraction is very low in ribonucleic acid and is non-precipitable at pH 5.0 unless ribonucleic acid be added. The material with little ribonucleic acid readily catalyses the incorporation of [^{14}C]amino acids into added isolated 'soluble' ribonucleic acid obtained from either pig or rat liver.

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REFERENCES

- Acs, G., Hartmann, G., Bowman, H. G. & Lipmann, F. (1959). *Fed. Proc.* **18**, 178.
Albaum, H. G. & Umbreit, W. W. (1947). *J. biol. Chem.* **167**, 369.
Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Berg, P. (1956). *J. biol. Chem.* **222**, 1025.
Berg, P. & Ofengand, E. J. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 78.
Crane, R. K. & Lipmann, F. (1953). *J. biol. Chem.* **201**, 235.
Davidson, J. N. & Waymouth, C. (1944). *Biochem. J.* **38**, 375.
Davie, E. W., Koningsberger, V. V. & Lipmann, F. (1956). *Arch. Biochem. Biophys.* **65**, 21.
Decken, von der, A. & Hultin, T. (1959). *Exp. Cell Res.* **17**, 188.
Finch, L. R. & Hele, P. (1959). *Biochem. J.* **73**, 11P.
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
Grunert, R. R. & Phillips, P. H. (1951). *Arch. Biochem.* **30**, 217.
Hoagland, M. B. (1955). *Biochim. biophys. Acta*, **16**, 288.
Hoagland, M. B., Keller, E. B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **218**, 345.
Hoagland, M. B., Stephenson, M. L., Scott, F. J., Hecht, L. I. & Zamecnik, P. C. (1958). *J. biol. Chem.* **231**, 241.
Hoagland, M. B., Zamecnik, P. C. & Stephenson, M. L. (1957). *Biochim. biophys. Acta*, **24**, 215.
Keller, E. B. & Zamecnik, P. C. (1956). *J. biol. Chem.* **221**, 45.
Kirby, K. S. (1956). *Biochem. J.* **64**, 405.
Lee Peng, C. H. (1956). *Biochim. biophys. Acta*, **22**, 42.
Lipmann, F. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 67.
Novelli, G. D. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 86.

- Ogata, K. & Nohara, H. (1957a). *Biochim. biophys. Acta*, **25**, 659.
- Ogata, K. & Nohara, H. (1957b). *Biochim. biophys. Acta*, **26**, 656.
- Rendi, R. & Campbell, P. N. (1959). *Biochem. J.* **72**, 435.
- Rendi, R., Di Milia, A. & Fronticelli, C. (1958). *Biochem. J.* **70**, 62.
- Rendi, R. & Hultin, T. (1959). *Exp. Cell Res.* **17**, 540.
- Sachs, H. (1957). *J. biol. Chem.* **228**, 23.
- Schweet, R. S. & Allen, E. H. (1958). *J. biol. Chem.* **233**, 1104.
- Schweet, R. S., Bovard, F. C., Allen, E. & Glassman, E. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 173.
- Simpkin, J. L. & Work, T. S. (1957). *Biochem. J.* **65**, 307.
- Stadtman, E. R., Novelli, G. D. & Lipmann, F. (1951). *J. biol. Chem.* **191**, 365.
- Webster, G. C. (1959). *Arch. Biochem. Biophys.* **82**, 125.
- Zamecnik, P. C., Stephenson, M. L. & Hecht, L. I. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 73.

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Reactivation of Phosphorylated Acetocholinesterases by Pyridinium Aldoximes and Related Compounds

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Certain organophosphates, e.g. tetraethyl pyrophosphate, diisopropyl phosphorofluoridate and insecticides of the organophosphate type, inhibit acetocholinesterase by a phosphorylation process. The phosphorylated enzyme is devoid of esterase activity, but this can be restored by substances which displace the phosphoryl group from the enzyme. The best known reactivators are oximes and the most potent oxime is 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (pyridine 2-aldoxime methiodide).

Because of the need for an antidote in organophosphate poisoning which is more effective and of greater water-solubility than 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (Hobbiger & Sadler, 1959) we have investigated a series of pyridinium aldoximes and related compounds. In each case the reaction with tetraethyl pyrophosphate, the reactivation of diethyl phosphorylacetocholinesterase and the affinity for acetocholinesterase were investigated. In some instances the reaction with diisopropyl phosphorofluoridate and the reactivation of diisopropyl phosphorylacetocholinesterase were also studied.

It was found that some of the pyridinium aldoximes investigated are much more potent than 2-hydroxyiminomethyl-*N*-methylpyridinium iodide as reactivators of both diethyl and diisopropyl phosphorylacetocholinesterase and that their potency as reactivators cannot be predicted solely from their reactivity with organophosphates or from their affinity for acetocholinesterase, as measured by the anticholinesterase action.

EXPERIMENTAL

Methods

All experiments were carried out in the Warburg apparatus at 37° in a medium of 0.025M-NaHCO₃ and an atmosphere of N₂+CO₂ (95:5), pH 7.45. All vessels were gassed while being shaken with the mixture of N₂+CO₂ for 5 min. after their transfer to the water bath. All incubation periods mentioned in the text include this period.

Reactivity of oximes with organophosphates. The reaction between oximes and organophosphates leads to the release of acids; this reaction was followed by measuring the displacement of CO₂ from the NaHCO₃ medium and its rate constant *k*, expressed as l.mole⁻¹min.⁻¹, was calculated from the formula

$$k = \frac{2.303}{[\text{Reactivator}] (t_2 - t_1)} \log \frac{a}{a - b}.$$

2 mm-Tetraethyl pyrophosphate or diisopropyl phosphorofluoridate was used in these experiments; *a* represents μl. of CO₂ obtained when 2 moles of acid/mole of organophosphate are released. Ten minutes were allowed for equilibration in the water bath and *t*₁ is the time of addition of the organophosphate from the side arm to the oxime in the main compartment. Only measurements of initial rates were taken and *t*₂ - *t*₁ are 10 and 20 min. in experiments with tetraethyl pyrophosphate and diisopropyl phosphorofluoridate respectively; *b* is μl. of CO₂ produced during this period. Whenever possible concentrations of each oxime were used which on interaction with the organophosphate produced a CO₂ output differing by not more than 10% from that obtained with one of two standard concentrations of 2-hydroxyiminomethyl-*N*-methylpyridinium iodide. In all experiments spontaneous hydrolysis of the organophosphate contributed at the most 20% to the observed hydrolysis and no correction was made for this when calculating rate constants.