The Interaction of 'Soluble' Ribonucleic Acid, Magnesium Ions and Sulphydryl Groups in the Control of Amino Acid-Dependent Pyrophosphate-Exchange Reactions

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During an attempt to purify a leucine-activating enzyme from pig-liver-protein fractions low in 'soluble' ribonucleic acid (Hele & Finch, 1960), some fractions became very unstable and lost over half their activity when kept overnight at -15° . In some instances, enzyme activity has been found to be preserved by the presence of 'soluble' ribonucleic acid. In this paper the effect has been further studied with preparations from rat liver. The evidence suggests that the terminal nucleotide sequence of the 'soluble' ribonucleic acid (Hecht, Stephenson & Zamecnik, 1959) is particularly involved in conferring protection upon the enzyme system. Also, removal of 'soluble' ribonucleic acid from preparations of the leucine-activating enzyme greatly enhances the pyrophosphate exchange at high concentrations of Mg^{2+} ion, and the terminal nucleotide sequence (Hecht *et al.* 1959) again seems to be particularly concerned.

The work has also been extended to the activation of isoleucine and lysine. Several investigators (Rendi & Hultin, 1959; Hoagland, Keller & Zamecnik, 1956; Novelli, 1958) have found little or no ability to activate lysine, as determined by the pyrophosphate-exchange reaction, in extracts from rat liver. Rat-liver-protein fractions low in 'soluble' ribonucleic acid (Hele & Finch, 1960) have now been found to catalyse a vigorous lysine-dependent pyrophosphate exchange in the presence of unexpectedly high concentrations (above 20 mM) of Mg^{2+} ions; at 5 mM concentration of Mg^{2+} ions this enzyme cannot be detected.

I have also observed some effects of oxidized and reduced glutathione and of *p*-chloromercuribenzoate on the activity and stability of the leucine- and lysine-activating enzymes.

Preliminary accounts of this work have already been published (Hele, 1960*a, b*).

MATERIALS AND METHODS

Chemicals. ATP (disodium salt), AMP, tris, L-leucine, L-isoleucine and L-valine were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Tris was titrated to the required pH with HCl. ATP was neutralized to pH 7.2

with NaOH or $MgCO_3$ (indicator paper) and AMP likewise neutralized with NaOH or tris. L-Lysine and L-phenylalanine were supplied by the California Corp. for Biochemical Research, Los Angeles 63, Calif., U.S.A. DL-Methionine, DL-threonine, L-tryptophan and protamine sulphate (salmon) were obtained from British Drug Houses Ltd. The preparation of protamine sulphate solutions has already been described (Hele & Finch, 1960). Another sample of protamine sulphate (salmon) and *p*-chloromercuribenzoic acid (sodium salt) were obtained from L. Light and Co. Ltd. *p*-Chloromercuribenzoic acid was prepared as a 2.5 mM solution in 20 mM-tris-HCl, pH 8.4, by suspension in the buffer and treating with ultrasonic vibrations for 30–60 sec. (H. R. Levy, personal communication). Glutathione, predominantly in the reduced form (GSH), was obtained from The Distillers (Biochemicals) Co. Ltd., Liverpool, and was neutralized to pH 7.2 with NaOH or tris (indicator paper). Oxidized glutathione (GSSG) was prepared by passing a stream of oxygen through a 100 mM solution of GSH, pH 7.2, until only about 2% of the nitroprusside-reacting material remained (Grunert & Phillips, 1951). Crystalline ribonuclease was obtained from C. F. Boehringer und Soehne, Mannheim, Germany. EDTA was neutralized to pH 7.4 with KOH. The source and treatment of the yeast ribonucleic acid and the charcoal have been described by Hele & Finch (1960). Ammonium sulphate was either recrystallized from water or, when indicated, from mM-EDTA. One batch of each was prepared, and the same batches were used throughout all the experiments described here. Solutions of $(NH_4)_2SO_4$, saturated at 4° , were neutralized with tris to pH 6.5. The tris was added to the saturated solution in the cold, but the pH was determined at room temperature with a 1 in 5 dilution of the saturated solution. The mixture of recrystallized $(NH_4)_2SO_4$ and potassium acetate, used as an enzyme inhibitor, contained 530 mM- $(NH_4)_2SO_4$ and 100 mM-potassium acetate; the pH was about 7.4. Sucrose, freed from traces of heavy metals by chromatography on Amberlite resin (monobed MB-1), and used in a few experiments, was a generous gift from Mr D. Tyler of this Unit. Calcium triphosphate gel was prepared according to Keilin & Hartree (1938). Stock solutions of sodium phosphate, pH 8.0, from which the 'elution mixture' was prepared, were obtained by titrating $m-Na_2HPO_4$ with the required volume of $m-NaH_2PO_4$. Distilled water, further purified upon a deionizer (Elgastat, Elga Products Ltd., London, S.W. 19), was used in all experiments.

Radioactive tracers. These have been described by Hele & Finch (1960).

Preparations of amino acid-activating fractions from ribonucleic acid-low fractions of rat liver

Method 1. Young Wistar rats of about 100 g. (two to four animals for each preparation) were employed. The livers were cooled in ice, weighed and minced through a stainless-steel tissue press into 2.3 vol. of medium A (Keller & Zamecnik, 1956). All manipulations were carried out at 0–4°. The mince was homogenized in a glass homogenizer, fitted with a motor-driven Teflon pestle, for three up-and-down strokes, taking about 45 sec. The homogenate was centrifuged for 15 000g for 10 min. in the Spinco model L preparative ultracentrifuge. The supernatant was pipetted off, the heaviest particles being rejected, diluted with 0.66 vol. of medium A and then centrifuged again at 105 000g for 30 min. The supernatant was again pipetted off and the protein concentration estimated (Gornall, Bardawill & David, 1949). A freshly prepared solution of protamine sulphate, 10 mg./ml., was added during 5 min. with mechanical stirring, in the proportion of 1.5 mg. of protamine to each 100 mg. of supernatant protein (about 7.5 mg. of protamine/10 g. of liver). The precipitate formed was removed by centrifuging at top speed (approx. 25 000g) for 3 min. in the superspeed angle head no. 69404 (4 × 25 ml.) of the MSE Major (refrigerated) centrifuge. From the supernatant, a fraction was precipitated with $(\text{NH}_4)_2\text{SO}_4$ -potassium acetate (ASAc fraction, Hele & Finch, 1960) by adding 0.5 ml. of a cold 10M-potassium acetate solution in 20 mM-EDTA to each 10 ml., followed by the addition of $(\text{NH}_4)_2\text{SO}_4$, precooled to –15° (2.8 g./10 ml.). All preparations by method 1 employed $(\text{NH}_4)_2\text{SO}_4$ recrystallized from mM-EDTA. When almost all the salt had dissolved the ASAc fraction was obtained by centrifuging at approx. 25 000g as described above. The precipitate was dissolved in 20 mM-tris-HCl, pH 7.2 (5 ml./10 g. of liver). The yield of protein was about 120 mg./10 g. of liver.

These amino acid-activating enzymes, like the acetate-activating enzyme of ox-heart mitochondria (Hele, 1954), can be further purified by gels in the presence of $(\text{NH}_4)_2\text{SO}_4$. These fractions are designated as 'RNA-low fractions'. The ASAc fraction (undialysed) was diluted with cold water to give a protein concentration of 10 mg./ml. The $(\text{NH}_4)_2\text{SO}_4$ saturation (Dixon & Webb, 1958) was then about 2.5%. Slight variations of this value did not appear to affect the treatment with calcium triphosphate gel, nor did the age of the gel; 1 mg. of gel (dry wt.) was added for every 15 mg. of protein. The gel was stirred in as rapidly as possible, and the mixture centrifuged without further delay in the superspeed head by bringing the head to top speed and allowing it to slow down as soon as top speed was reached. This preliminary treatment with a small amount of gel was found to remove an inhibitor. This precipitate was discarded and a further quantity of gel added in the proportions 1.0 mg. of gel/1.5 mg. of the starting amount of protein, and the mixture centrifuged as before. The precipitate was eluted with 0.75 vol. of the original diluted ASAc fraction, a mixture of 3 parts of 0.1M-sodium phosphate, pH 8.0, and 1 part of saturated $(\text{NH}_4)_2\text{SO}_4$, pH 6.5, being used. The gel was spun down, eluted with 0.5 vol. of the same elution mixture and the eluates were combined. 10M-Potassium acetate was added, equivalent to 0.025 vol. of the combined eluates, followed by saturated $(\text{NH}_4)_2\text{SO}_4$ (0.25 vol. of the combined eluates). The final $(\text{NH}_4)_2\text{SO}_4$

saturation was approx. 40%. After 5 min. the precipitate was collected by centrifuging as before, and dissolved in medium A (Keller & Zamecnik, 1956) to give a protein concentration of 10–15 mg./ml. The $(\text{NH}_4)_2\text{SO}_4$ saturation of this RNA-low fraction was about 5% (approx. 250 mM). About 15–20 mg. of protein was obtained from 10 g. of liver.

Method 2. The cooled, weighed livers were minced into 3 vol. of medium C (Hele & Finch, 1960), stirred briskly for 2 min. and then filtered through two layers of cheesecloth. A freshly prepared solution of protamine sulphate (10 mg./ml.) was added to the filtrate (1.0 ml./10 g. of liver), the addition taking 2 min. with constant stirring. The bulk of the precipitated material was removed by centrifuging at 1880g for 10 min. and the supernatant further centrifuged at 105 000g for 10 min. The ASAc fraction was prepared as for method 1, but required an additional centrifuging at 105 000g for 10 min. to remove a little insoluble material. The yield of ASAc fraction was a little higher than with method 1, being about 140 mg. for 10 g. of liver. This differs from pig liver, where the yield of soluble protein from tissue-mince extracts was 50% of the yield obtained from homogenates (Hele & Finch, 1960). The ASAc fraction was further purified, as under method 1, and the final yield of protein in the RNA-low fraction dissolved in medium C was about 15–20 mg. for 10 g. of liver. All preparations by method 2 employed potassium acetate without EDTA and $(\text{NH}_4)_2\text{SO}_4$ which had been recrystallized from water.

The yield of total enzyme units (substrates: leucine, isoleucine and lysine) in the RNA-low fractions was much the same with either method, and could be in excess of 100% of the total units present in the ASAc fractions. The increase in specific activity was five- to ten-fold, and was probably due to removal of inhibitors and lowering of 'blank' reactions, as well as to concentration of enzyme protein. The yield of enzyme units from 10 g. of liver in the RNA-low fractions was comparable with the yield of enzyme units in pH 5.0 fractions, if the marked effects of MgCl_2 concentration and 'soluble' RNA on some of the reaction rates were taken into account.

The RNA-low fractions contained no RNA detectable by the method of Hele & Finch (1960). The extinction ratio of the fractions, 280 m μ :260 m μ , varied between 1.4 and 1.6.

With pig liver, Hele & Finch (1960) found it necessary to perform pilot runs to determine the correct amount of protamine needed to remove particles from the liver extracts. This was not necessary with rat liver, provided that freshly prepared solutions of protamine were employed. In some of the earlier experiments, with method 2, the protamine solutions used were stored at –15° and thawed just before use. Enzyme preparations made with these protamine solutions gave leucine-activating enzymes of higher specific activity (measured at 6 mM- MgCl_2) than those in which freshly prepared protamine solutions were employed. After about six cycles of freezing and thawing, these protamine solutions suddenly and unpredictably lost much of their ability to precipitate particles.

The use of potassium acetate in conjunction with $(\text{NH}_4)_2\text{SO}_4$ was retained, for, although no longer required as a stabilizer (Hele & Finch, 1960), better yields of enzyme units were obtained in its presence.

These procedures for obtaining RNA-low fractions were not devised with the object of achieving a systematic purification of the enzymes under investigation, but to

provide material that could be compared with pH 5.0 fractions. For this purpose it was necessary that the specific activities of the enzymes in the two types of preparation should be comparable, that the time consumed in isolating the two types of fraction was similar, and that the manipulations used should be of comparable duration and severity. For this reason dialysis procedures were avoided. In the experiments upon enzyme stability, $(\text{NH}_4)_2\text{SO}_4$ and potassium acetate were added to the pH 5.0 fractions for the preincubation tests so that the salt concentrations were similar to those in the RNA-low fractions.

No correlation was found between the RNA content of these preparations as measured by chemical or optical methods, and the behaviour of the enzymes in these preparations. Some preparations, made by method 2 with frozen and thawed protamine solutions that had lost much of their ability to precipitate particles, had extinction ratios, $280 \text{ m}\mu:260 \text{ m}\mu$, of 0.75. Comparable ratios were obtained with pH 5.0 fractions, yet the enzymes in these preparations behaved as would be expected of enzymes in RNA-low fractions. The results obtained with ribonuclease-treated pH 5.0 fractions suggest that fragments of 'soluble' RNA, not detectable by simple chemical methods, can still influence pyrophosphate-exchange rates.

Fractions precipitated at pH 5.0. These were made as described by Hele & Finch (1960), by a procedure based on that of Keller & Zamecnik (1956). Usually, two to four animals were used for each preparation. The medium A employed was freshly prepared immediately before use, by the addition of solid KHCO_3 to a stock solution of all the other components of this medium.

Ribonuclease-treated pH 5.0 fractions. These fractions, dissolved in medium A and of protein concentration about 20.0 mg./ml., were incubated at 37° for 15 min. with 400 μg . of ribonuclease and 2 μmoles of EDTA/ml. These conditions destroyed all the RNA detectable as cold perchloric acid-precipitable ribose (Hele & Finch, 1960). The EDTA stimulated the action of ribonuclease upon the RNA of pH 5.0 fractions. This procedure was used only with the experiments on MgCl_2 concentrations.

'Soluble' ribonucleic acid. This was isolated as described by Hele & Finch (1960).

Assays of enzyme activity

Pyrophosphate exchange. The standard assay was similar to that described by Hele & Finch (1960), except that 6 μmoles of sodium ATP were employed. Magnesium was added as MgCl_2 in the quantities indicated. To prevent the formation of precipitates at the higher concentrations, MgCl_2 was added to the other reagents after they had been diluted to the final volume. In some of the earlier experiments 6 mM-disodium magnesium ATP was used. In experiments with high concentrations of AMP it was necessary to add extra charcoal to adsorb both AMP and ATP. The assay for pyrophosphate exchange in this case consisted of a mixture of 0.5 μmole of amino acid, 3 μmoles of sodium ATP, 1.5 μmoles of $[^{32}\text{P}]$ pyrophosphate, 50 μmoles of tris-HCl, pH 7.2, AMP, NaCl and MgCl_2 added as indicated in individual experiments, and 0.1–0.5 mg. of protein, in a final volume of 0.55 ml. Charcoal equivalent to 400 mg. dry wt. was used. Recovery of 65–75% of orthophosphate from ATP was obtained.

A number of pilot experiments were performed with fractions stored at -15° , with or without added GSH; but

all definitive experiments were carried out with fractions tested upon the day of preparation.

A unit of enzyme activity is defined as that amount of enzyme which catalyses the incorporation of 1.0 μmole of $[^{32}\text{P}]$ pyrophosphate into ATP in 1 min. under standard assay conditions. Since no single standard of MgCl_2 concentration can be defined, the MgCl_2 concentration employed will be stated where necessary for the individual enzymes. Specific activity is defined as enzyme units/mg. of protein. All rates for amino acid-dependent pyrophosphate exchange are given after subtraction of the appropriate amino acid-free blank even when the blank value is also given.

Attachment of $[^{14}\text{C}]$ amino acids to 'soluble' ribonucleic acid. The procedure used has been described by Hele & Finch (1960). Where less than 1 mg. of protein was incubated with 'soluble' RNA an extra 2 mg. of protein, dissolved in a volume of 0.1–0.2 ml., was added immediately before stopping the reaction with cold perchloric acid. The recoveries of RNA in this instance were about 70% of the added RNA, but were as consistent amongst themselves as the recoveries (about 90%) obtained when the protein was present in the incubation from the start. The protein used was obtained from rat-liver or pig-liver fractions which had been treated with calcium triphosphate gel to remove activating enzymes. Such fractions were precipitated with $(\text{NH}_4)_2\text{SO}_4$ and dialysed for 18 hr. against 20 mM-tris-HCl, pH 7.2. This protein could not incorporate $[^{14}\text{C}]$ amino acids into 'soluble' RNA.

Measurement of radioactivity. The procedures were carried out as previously described (Hele & Finch, 1960). All samples containing $[^{14}\text{C}]$ amino acid were counted to give an accuracy of $\pm 5\%$ on the less radioactive samples (i.e. giving an incorporation of less than about 0.5 μmole /mg. of 'soluble' RNA). The accuracy on the more radioactive specimens was $\pm 2-3\%$.

Chemical estimations. These were as described by Hele & Finch (1960).

RESULTS

Influence of magnesium chloride concentration and 'soluble' ribonucleic acid upon amino acid-dependent pyrophosphate exchange

The rates of leucine-, isoleucine- and lysine-dependent pyrophosphate exchange catalysed by pH 5.0 fractions showed a response to changes in MgCl_2 concentration that were highly characteristic for each amino acid (Fig. 1). The main features were observed with a number of pH 5.0 fractions, although some variation occurred in detail.

With isoleucine as substrate the maximum rate of pyrophosphate exchange was achieved between 10 and 20 mM- MgCl_2 , and was between two and three times the rate measured with 5 mM- MgCl_2 . It was highly characteristic of this enzyme that rates of this magnitude (relative to the rate at 5 mM- MgCl_2) were maintained between 10 and 30 mM- MgCl_2 . With leucine as substrate the maximum rate of pyrophosphate exchange occurred at 5 mM- MgCl_2 , and fell off sharply with further

increases in MgCl_2 concentration. With lysine as substrate, little pyrophosphate exchange could be detected with less than 10 mM- MgCl_2 . Above this concentration, the rate rose rapidly until concentrations of 20–30 mM were reached.

In RNA-low fractions (Fig. 2a), the responses of leucine- and isoleucine-dependent pyrophosphate-exchange rates to changes in MgCl_2 concentration were remarkably similar. With isoleucine as substrate the maximum rate of pyrophosphate exchange occurred at 10 mM- MgCl_2 , and fell off sharply with further increases in MgCl_2 concentration. With leucine as substrate the maximum rate of pyrophosphate exchange occurred at 15 mM- MgCl_2 . With lysine as substrate the MgCl_2 concentration curve was similar to that observed with pH 5.0 fractions.

Addition of phenol-isolated 'soluble' RNA, made from fresh pH 5.0 fractions, resulted in MgCl_2 concentration curves for leucine and isoleucine very similar to those obtained with pH 5.0 fractions, but these curves (Fig. 2b) were not identical with those observed with pH 5.0 fractions.

Fig. 1. Magnesium chloride concentration and amino acid-dependent pyrophosphate exchange catalysed by pH 5.0 fractions. For details see Materials and Methods section. Open symbols and filled-in symbols represent different samples. Some of the latter, for isoleucine and lysine, which were almost identical with the open symbols at low MgCl_2 concentrations, have been omitted for the sake of clarity. □, ■, Isoleucine; ○, ●, leucine; △, ▲, lysine.

The curve for lysine-dependent pyrophosphate exchange did not alter significantly in this experiment, which did not include MgCl_2 concentrations above 30 mM.

The pH 5.0 fractions, preincubated at 37° to remove the terminal nucleotide sequence of 'soluble' RNA (Hecht *et al.* 1959), showed significant alterations in the response of these enzymes to changes in MgCl_2 concentration at and above 10 mM (Fig. 3b). The ability of the leucine-activating enzyme to catalyse pyrophosphate exchange at high MgCl_2 concentrations increased, whereas that of the isoleucine-activating enzyme declined markedly, the rates with isoleucine at the higher concentrations being no longer two to three times the rate observed at 5 mM- MgCl_2 , as was the case with fresh pH 5.0 fractions. With lysine as substrate the rate of increase of pyrophosphate exchange fell off at

Fig. 2. Magnesium chloride concentration and amino acid-dependent pyrophosphate exchange catalysed by: (a) RNA-low fractions; (b) by such fractions with added 'soluble' RNA. □, Isoleucine; ○, leucine; △, lysine. For (a), the assay was as described under Materials and Methods, but for (b) it was scaled down to a total volume of 0.5 ml. The concentration of RNA-low fraction protein in the assay for (a) was 0.44 mg./ml. and for (b) was 0.76 mg./ml., that of 'soluble' RNA being 90 μg./ml. The RNA-low fraction was prepared by method A, and the 'soluble' RNA was isolated from a freshly prepared pH 5.0 fraction. Different enzyme preparations were used in the two experiments.

MgCl_2 concentrations above 15 mM, instead of continuing to rise fairly sharply, as found both with pH 5.0 fractions and RNA-low fractions. Similar modifications to the MgCl_2 concentration curves were observed (Fig. 3a) with ribonuclease-treated pH 5.0 fractions. These preparations contained no 'soluble' RNA detectable by simple chemical methods, and could not incorporate $[^{14}\text{C}]$ amino acids into 'soluble' RNA.

The effects of MgCl_2 concentration upon the rates of pyrophosphate exchange were also investigated in the presence of other amino acids (Figs. 4a and b). For threonine and valine they were similar to that observed with lysine.

Hele & Finch (1960) reported that, relative to leucine, less $[^{14}\text{C}]$ threonine and $[^{14}\text{C}]$ valine were

transferred to 'soluble' RNA in pH 5.0 fractions than in 'recombined' systems. The transfer of $[^{14}\text{C}]$ lysine shows the same phenomenon (Tables 1 and 2). The best incorporations of $[^{14}\text{C}]$ lysine into recombined systems were sometimes obtained with the smallest amounts of protein and short incubation periods.

Depression of amino acid-dependent pyrophosphate exchange by adenosine 5'-phosphate

The effect of adding AMP upon amino acid-dependent pyrophosphate exchange was studied at four different MgCl_2 concentrations (Table 3). At the two higher MgCl_2 concentrations sodium AMP depressed the lysine-dependent pyrophosphate exchange to about half of that in the presence of a control addition of NaCl. This effect was observed with both pH 5.0 fractions and RNA-low fractions. Similar results were obtained with 36 mM- MgCl_2 . Inorganic orthophosphate (13.5 mM, sodium salts, pH 7.2) was without effect. With leucine as substrate, similar results were obtained, but only when RNA-low fractions were used. The degree of depression produced by AMP at 27 mM- MgCl_2 is dependent upon the amount of AMP added (Fig. 5). Tris-AMP was somewhat more effective than

Fig. 3. (a) Effect of ribonuclease treatment on amino acid-dependent pyrophosphate exchange catalysed by pH 5.0 fractions at varying MgCl_2 concentrations. Details of the ribonuclease treatment are given under Materials and Methods. □, Isoleucine; ○, leucine; Δ, lysine. (b) Effect of preincubation on amino acid-dependent pyrophosphate exchange catalysed by pH 5.0 fractions at varying MgCl_2 concentrations. The pH 5.0 fraction (20 mg. of protein/ml.) was incubated at 37° for 30 min. and cooled in ice, before testing as described under Materials and Methods. The preincubated fraction contained 2.0 mg. of 'soluble' RNA/100 mg. of protein, but could only incorporate the following amounts of $[^{14}\text{C}]$ amino acid into this 'soluble' RNA: isoleucine, 0.27; leucine, 0.23; lysine, 0.12 (μm-mole/mg. of 'soluble' RNA/4 min.). The assay for $[^{14}\text{C}]$ amino acid incorporation is described under Materials and Methods. □, Isoleucine; ○, leucine; Δ, lysine.

Fig. 4. (a) Concentration of MgCl_2 and amino acid-dependent pyrophosphate exchange catalysed by 'RNA-low' fractions. The standard assay system, described under Materials and Methods, was employed. □, Methionine; ■, valine. (b) Concentration of MgCl_2 and amino acid-dependent pyrophosphate exchange catalysed by pH 5.0 fractions. □, Methionine; ○, tryptophan; Δ, threonine.

sodium AMP, suggesting that Na^+ ions may counter-act the AMP.

At the two lowest MgCl_2 concentrations used (Table 3), the effect of adding AMP was not reproducible. For example, in two experiments

Table 1. Incorporation of $[^{14}\text{C}]$ leucine and $[^{14}\text{C}]$ lysine into 'soluble' ribonucleic acid catalysed by pH 5.0 fractions

The assay system described under Materials and Methods was used with samples (0.25 ml.) of pH 5.0 fractions. In Expt. 1 the MgCl_2 concentration was 30 mM; in Expts. 2 and 3 it was 10 mM. In all experiments the protein concentration was about 10 mg./ml.

Expt. no.	Incubation time (min.)	$[^{14}\text{C}]$ Amino acid incorporated into 'soluble' RNA ($\mu\text{m-moles/mg.}$)	
		Leucine	Lysine
1	4	3.2	1.0
2	2	4.5	1.8
	4	4.5	2.4
	10	5.0	2.5
3	4	3.3	1.9
	10	3.3	4.0

with RNA-low fractions, similar to that shown in the Table, the pyrophosphate-exchange rate, observed at 0.9 mM- MgCl_2 with and without leucine, was unaffected by the addition of 13.5 mM-sodium AMP. Leucine-dependent pyrophosphate exchange, catalysed by pH 5.0 fractions in the presence of 13.5 mM- NaCl , was greater at 13.5 mM- MgCl_2 than at 4.5 mM- MgCl_2 . This change was not due to changes in the amino acid-free blank. This blank was more variable when pH 5.0 fractions were used than when RNA-low fractions were employed.

Stability of the enzymes

RNA-low fractions containing recrystallized $(\text{NH}_4)_2\text{SO}_4$ and preincubated before assay by keeping at 37° lost much of their capacity to catalyse leucine-dependent pyrophosphate exchange (Table 4). This loss was reduced by the addition of GSH or EDTA. The addition of yeast RNA, or of 'soluble' RNA from liver, in amounts equivalent to those present in pH 5.0 fractions, did not give protection.

The pH 5.0 fractions were very stable when kept at 37° under similar conditions (Table 5). The

Table 2. Incorporation of $[^{14}\text{C}]$ leucine and $[^{14}\text{C}]$ lysine into 'soluble' ribonucleic acid catalysed by 'recombined' systems

The assay system described under Materials and Methods was used. The final MgCl_2 concentration was 10 mM. Times in parentheses refer to the period of incubation. The RNA-low fraction was prepared by method B with a frozen and thawed solution of protamine. 'Soluble' RNA (113 $\mu\text{g.}$) was added to the assay in Expt. 1; 100 $\mu\text{g.}$ was used in Expt. 2.

Ribonucleic acid-low protein added (mg.) to 0.4 ml. of reaction mixture	Amino acid incorporated into 'soluble' RNA ($\mu\text{m-moles/mg.}$)			
	Without GSH		With 10 mM-GSH	
	Leucine	Lysine	Leucine	Lysine
Expt. 1. 3.8 (10 min.)	6.3	4.9	6.2	5.0
0.05 (2 min.)	0	2.1	4.8	4.6
0.10 (0.5 min.)	0.1	2.1	3.7	4.2
Expt. 2. 1.1 (10 min.)	3.3	2.8	5.7	3.5
0.05 (2 min.)	0	2.9	4.0	4.5

Table 3. Effect of magnesium concentration and adenosine 5'-phosphate on amino acid-dependent pyrophosphate exchange catalysed by ribonucleic acid-low and pH 5.0 fractions

The assay system described under Materials and Methods was used. The RNA-low fraction was prepared by method B. Sodium AMP was employed, 7.5 μmoles being added to the appropriate assays, and 7.5 μmoles of NaCl were added to the controls. Figures in parentheses refer to the amino acid-free controls and apply to experiments with lysine as well as leucine. Final vol. 0.55 ml.

MgCl_2 (μmoles)	Pyrophosphate exchange ($\mu\text{m-moles/mg. of protein/min.}$)							
	RNA-low fraction				pH 5.0 fraction			
	Leucine		Lysine		Leucine		Lysine	
	- AMP	+ AMP	- AMP	+ AMP	- AMP	+ AMP	- AMP	+ AMP
0.5	23 (26)	5 (28)	0	0	14 (13)	7 (11)	0	0
2.5	77 (25)	66 (19)	15	7	19 (18)	25 (9)	3	1
7.5	147 (23)	79 (31)	131	64	37 (17)	9 (11)	51	24
15.0	82 (26)	43 (20)	246	116	10 (19)	8 (13)	65	26

(NH₄)₂SO₄ and potassium acetate present in these experiments prevented the loss of the terminal nucleotide sequence of the 'soluble' RNA, as is indicated by the ability of the 'soluble' RNA to undergo labelling with [¹⁴C]amino acids (Table 6). The pH 5.0 fractions did not lose ability to catalyse leucine-dependent pyrophosphate exchange, when kept at 37° either with or without the addition of the salts. However, after incubation for 10–15 min. in the absence of salts they became unstable on addition of the salts (Table 5). This suggested that the stability of the enzyme was connected with the completeness of the terminal nucleotide sequence of the 'soluble' RNA. Inclusion of 10 mM-GSH, along with the salts, conferred a considerable degree of protection upon the enzymes. Ribonuclease-treated fractions were also unstable when tested in this system.

Lysine-activating enzymes present in pH 5.0 fractions were less stable than leucine-activating enzymes when kept at 37° with (NH₄)₂SO₄ and potassium acetate. Both enzymes became more unstable upon storage at –15° for a few days, as tested by keeping at 37° in the presence of these

salts. The loss of stability of the leucine-activating enzyme was accompanied by a rise in specific activity, measured with 10 mM-MgCl₂, which could not be accounted for by changes in protein concentration. The lysine-activating enzyme was the more unstable enzyme of the two and there was no indication that 'soluble' RNA conferred protection upon this enzyme in these tests. The two enzymes were about equally unstable when RNA-low fractions were kept at 37° with (NH₄)₂SO₄ and potassium acetate. The lysine-activating enzyme in

Table 4. *Effect of certain substances on the stability of the leucine-activating enzyme present in ribonucleic acid-low fractions*

The assay system described under Materials and Methods was used with 6 mM-disodium magnesium ATP. An additional 10 μ moles of MgCl₂ were added to the assays for the sample containing EDTA. The RNA-low fraction was prepared by method 2, with a frozen and thawed solution of protamine. This fraction was kept at 37°, at a protein concentration of approx. 5 mg./ml. for 30 min., and the recrystallized (NH₄)₂SO₄ concentration was about 100 mM. The 0 min. samples were assayed after the additions had been mixed with the enzyme preparation but before keeping at 37°. The [¹⁴C]leucine transfer to 'soluble' RNA amounted to 2.5 μ m-moles/mg. of 'soluble' RNA/4 min. This was tested the same day, 1 mg. of RNA-low fraction protein and 115 μ g. of 'soluble' RNA in a final volume of 0.4 ml. being used. No GSH was added to the assay.

Time of preincubation ... Addition during preincubations	Pyrophosphate exchange (μ m-moles/mg. of protein/min.)	
	0	30
None	156	42
100 mM-Tris-GSH	174	144
'Soluble' RNA (188 μ g./ml.)	134	12
Yeast RNA (135 μ g./ml.)	168	34
45 mM-EDTA	140	120

Table 5. *Effect of preincubation on the stability of leucine-activating enzyme in pH 5.0 fractions*

The assay system described under Materials and Methods was used, with 6 mM-disodium magnesium ATP. Samples of pH 5.0 fraction were preincubated at 37° before assay at a protein concentration of about 5 mg./ml. No significant changes in any of the amino acid-free blanks were observed.

Time of preincubation (min.)	Leucine-dependent pyrophosphate exchange (μ m-moles/mg. of protein/min.)		
	0	15	40
No addition	42	—	44
Salts added at 0 min.	38	—	36
Salts added at 15 min.	—	41	26
Salts plus GSH added at 15 min.	—	45	40

Fig. 5. Effect of tris-AMP on amino acid-dependent pyrophosphate exchange, catalysed by 'RNA-low' fractions. The assay system is described under Materials and Methods. Concentration of MgCl₂: 27 mM. The 'RNA-low' fraction was prepared by method B. O, Leucine; Δ, lysine; □, no amino acids.

Table 6. *Effect of certain substances on the ability of preincubated pH 5.0 fractions to incorporate [14 C]amino acids into 'soluble' ribonucleic acid*

Samples (0.2 ml.) of pH 5.0 fractions dissolved in medium A were preincubated in 15 ml. centrifuge tubes at 37° for 30 min. with the additions as indicated. The tubes were cooled in ice, and 0.02 ml. of N -KHCO₃ was added to those containing EDTA to prevent the formation of a precipitate when the other reagents were added. The final pH was not significantly changed (indicator paper). Magnesium chloride (6 μ moles) and 4 μ moles of ATP were added to all tubes, bringing the final volume to 0.4 ml., and the reaction was started by the addition of 0.04 μ mole of [14 C]amino acid. Incubation was for 4 min. at 37°. Figures shown in parentheses in Expt. 2 were obtained from samples tested in the presence of the additions, but without preincubation.

Additions to 0.2 ml. samples of pH 5.0 fraction	Amino acid incorporated into 'soluble' RNA (μ m-moles/mg.)	
	Leucine	Lysine
Expt. 1		
No additions	0.5	0
Salts*	3.7	1.6
EDTA (2.5 μ moles)	3.8	0.9
EDTA (0.25 μ mole)	1.1	0.3
Not preincubated	2.9	1.1
Expt. 2		
No additions	0.1 (3.2)	
Salts*	3.6 (3.2)	
EDTA (5 μ moles)	2.6 (3.0)	
EDTA (0.5 μ mole)	0.6 (3.5)	

* 26 μ moles of recrystallized (NH₄)₂SO₄ and 5 μ moles of potassium acetate.

pH 5.0 fractions and in RNA-low fractions was stimulated by keeping these preparations at 37° in the presence of 10 mM-GSH, whereas the leucine-activating enzyme was not significantly stimulated.

In other experiments in which pH 5.0 fractions and RNA-low fractions were treated with *p*-chloromercuribenzoate or kept at 37° in the presence of GSSG plus EDTA, the leucine-activating enzyme was more readily depressed than the lysine-activating enzyme (Fig. 6*a, b*). Omission of EDTA from experiments with pH 5.0 fractions enhanced the effect of GSSG upon the lysine-activating enzyme, but not that on the leucine-activating enzyme. Since EDTA prevents the loss of the terminal nucleotide sequence from 'soluble' RNA (Table 6) this suggests that the sequence protects the lysine-activating enzyme, but not the leucine-activating enzyme, from attack by GSSG. Alternatively, the lysine-activating enzyme is more sensitive than the leucine-activating enzyme to combined attack by traces of metals and GSSG.

The transfer of [14 C]leucine to 'soluble' RNA catalysed by 'recombined systems' (Hele & Finch, 1960) was considerably stimulated by the addition

of 10 mM-GSH at low concentrations of protein. The transfer of [14 C]lysine was stimulated by GSH to a lesser extent under similar experimental conditions (Table 2). Addition of mM-EDTA to the reaction mixture, or treatment of the 'soluble' RNA preparation with mM-EDTA before addition to the reaction mixture, also protected the enzyme, suggesting that small amounts of heavy metals were bound to the 'soluble' RNA and acted as inhibitors (Goldstein & Holley, 1960) at low concentrations of protein.

DISCUSSION

Interaction of magnesium ions and 'soluble' ribonucleic acid in control of reactions catalysed by amino acid-activating enzymes

The previous failure to detect an effect of added 'soluble' RNA upon the leucine- and isoleucine-pyrophosphate-exchange reactions (Hele & Finch, 1960) probably arose from the use of too little magnesium (6 mM) (Fig. 2*b*). The effects of 'soluble' RNA and higher concentrations of Mg²⁺ ions are in opposite directions for the two reactions. At high magnesium concentrations the terminal nucleotide sequence of the 'soluble' RNA appears to be particularly important, as is shown by the changes in pyrophosphate-exchange rates after incubation of pH 5.0 fractions at 37° (Fig. 3*b*). Increased modification of the 'soluble' RNA, brought about by treatment with ribonuclease, results in further changes, particularly at magnesium concentrations of 10 mM and above. These modified pH 5.0 fractions give magnesium-concentration curves that do not fully resemble the curves observed with RNA-low fractions. In particular, the sharp maximum rate observed with leucine at 15 mM-magnesium chloride is not observed with the modified pH 5.0 fractions. This suggests that after removal of the terminal nucleotide sequence sufficient 'soluble' RNA remains behind to influence pyrophosphate-exchange rates, and that this is also true of the fragments of 'soluble' RNA resulting from ribonuclease treatment. These fragments are sufficiently small to escape detection as ribose, which is insoluble in cold perchloric acid.

These effects of different portions of the 'soluble' RNA molecules suggest that the pyrophosphate exchange may occur by two mechanisms. Perhaps one involves the formation of the amino acyl adenylate, and is controlled by the 'amino acid-specific' part of the 'soluble' RNA molecule (Schweet, Bovard, Allen & Glassman, 1958; Preiss, Berg, Ofengand, Bergmann & Dieckmann, 1959; Lipmann, Hülsmann, Hartmann, Boman & Acs, 1959); the other mechanism could involve amino acyl thioester intermediates and the terminal nucleotide sequence of the 'soluble' RNA (Hecht

Fig. 6. (a) Effect of *p*-chloromercuribenzoate (PCMB) on amino acid-dependent pyrophosphate exchange. Samples of enzyme preparations, containing a final protein concentration of 10 mg./ml., were kept for 5 min. at 4° in the presence of the indicated concentrations of PCMB, before assay for pyrophosphate exchange. With lysine as substrate, the $MgCl_2$ concentration was 30 mM. With leucine, the $MgCl_2$ concentration was 5 mM with pH 5.0 fractions, and 15 mM with RNA-low fractions. The RNA-low fraction was prepared by method A. The 100% rates (symbols for which are omitted from the diagram) were obtained from samples, without PCMB, but otherwise treated in an identical manner. The values of these 100% rates (μ -moles/mg. of protein/min.) were as follows: pH 5.0 fraction with leucine (●) 46, with lysine (▲) 95, RNA-low fraction with leucine (○) 108, with lysine (Δ) 158. Addition of GSH (final concentration 20 mM) for a further 5 min. to samples of both fractions treated with 0.25 mM-PCMB gave complete restoration of all enzyme activities. (b) Effect of GSSG on amino acid-dependent pyrophosphate exchange. Samples of enzyme preparations, containing a final protein concentration of 10 mg./ml., were kept at 37° for 20 min. in the presence of 10 mM-EDTA and the indicated amounts of GSSG. After cooling in ice the samples were assayed as for Fig. 6 (a), except that 6 mM- $MgCl_2$ was used with the pH 5.0 fraction when leucine was used as substrate. EDTA (1 μ mole) was added to each assay along with the samples of enzyme. The RNA-low fraction was prepared by method A. The 100% rates were given by samples that were preincubated at 37° for 20 min. in the presence of EDTA alone, and with the RNA-low fraction were identical with the rates obtained with a fresh sample. The values for the 100% rates (μ -moles/mg. of protein/min.) were as follows: pH 5.0 fraction with leucine (●) 38, with lysine (▲) 110, RNA-low with leucine (○) 141, with lysine (●) 266. In another experiment the EDTA was omitted and purified sucrose used in the composition of the medium A in which the pH 5.0 fraction was dissolved. In this experiment the pH 5.0 fraction gave these values for the 100% rate: with leucine (□) 41 and with lysine (■) 92.

et al. 1959; Canellakis, 1959). Alternatively, reactions priming the 'soluble' RNA molecule with ATP might be involved (Ogata, Nohara & Miyazaki, 1959).

Ogata, Nohara, Ishikawa, Morita & Asaoka (1961), using guinea-pig-liver preparations purified by chromatography on diethylaminoethylcellulose, have shown that the stimulating effect of 'soluble' RNA upon isoleucine-dependent pyrophosphate exchange does not require the terminal

nucleotide sequence (Hecht *et al.* 1959); this is virtually the reverse of the observations made here. This discrepancy may do no more than reflect differences in experimental conditions, but other explanations should not be overlooked. The relatively unpurified pH 5.0 fractions may contain an additional factor which is required if the terminal nucleotide sequence is to exert its maximum effect upon pyrophosphate-exchange rates, and which might itself undergo partial destruction

during the incubation which removes the terminal nucleotide sequence of the 'soluble' RNA. The addition of 'soluble' RNA to RNA-low fractions does not restore completely the type of magnesium-concentration curve observed with pH 5.0 fractions (Figs. 1, 2*b*), especially at higher magnesium concentrations. Some of the factor may have been removed during the protamine treatment.

Under the experimental conditions used lysine-dependent pyrophosphate exchange is not markedly influenced by the presence or absence of 'soluble' RNA. The response to changes in magnesium concentrations, particularly at low concentrations, is the reverse of the response obtained with leucine and isoleucine.

The transfer of [^{14}C]lysine to 'soluble' RNA proceeds more rapidly with the 'recombined' systems than with pH 5.0 fractions. The transfer of [^{14}C]valine and [^{14}C]threonine was found to have this peculiarity by Hele & Finch (1960). The block in the transfer of these amino acids to the 'soluble' RNA of pH 5.0 fractions may be closely related to the fact that the effects of magnesium concentration upon the pyrophosphate-exchange reactions are similar for these three amino acids. Clearly, systems reconstructed from RNA-low protein and phenol-isolated 'soluble' RNA may not be identical with the original amino acid-activating system found in the pH 5.0 fraction.

AMP depresses leucine-dependent pyrophosphate exchange when this reaction is catalysed by RNA-low fractions at magnesium concentrations above 10 mM. At these concentrations the terminal nucleotide sequence of 'soluble' RNA exerts a predominant influence over the pyrophosphate-exchange rate. This suggests that the AMP may act by combining with a site upon the enzyme to which the 'acceptor' adenosyl unit of 'soluble' RNA is bound. Sodium ions counteract the effect of both AMP and 'soluble' RNA, probably by competing with Mg^{2+} ions (Hamilton & Petermann, 1959). With the lysine-activating enzyme, AMP influences the pyrophosphate-exchange rate, but 'soluble' RNA does not. This may be connected with the block in the transfer of activated lysine to 'soluble' RNA.

Labile groups of amino acid-activating enzymes

The stability of the leucine-activating enzyme is related both to the manner in which 'soluble' RNA is associated with the enzyme in pH 5.0 fractions, and to the ability of the 'soluble' RNA to accept activated amino acid. The data suggest that the terminal nucleotide sequence of the 'soluble' RNA can protect a labile site associated with the enzyme. The experiments with AMP suggest that it may be the 'acceptor' adenosyl unit which confers this

protection. The lysine-activating enzyme was not protected by 'soluble' RNA in the preincubation tests with ammonium sulphate and potassium acetate.

The relative degrees of deterioration, during storage, shown by the two enzymes are very similar to those reported by Allen, Glassman & Schweet (1960*b*) in pH 5.0 fractions prepared from guinea-pig liver. Stored pH 5.0 fractions retain their ability to transfer [^{14}C]amino acid to 'soluble' RNA for several weeks; therefore the change in the manner of association between 'soluble' RNA and enzyme that gives rise to the changes in specific activity and stability must be rather small and not involve any major destruction of the 'soluble' RNA. The protection conferred upon stored pH 5.0 fractions by GSH (Allen *et al.* 1960*b*) suggests that this change in the association between enzyme and 'soluble' RNA involves sulphhydryl groups. The increase in specific activity of the leucine-activating enzyme, which occurs upon removal of 'soluble' RNA, and accompanies its loss of stability, resembles a similar association of changes observed with the alanine-activating enzyme present in ribonuclease-treated pH 5.0 fractions (Holley & Goldstein, 1959; Goldstein & Holley, 1960).

The effect of *p*-chloromercuribenzoate upon leucine-dependent pyrophosphate exchange is virtually the same with the rat-liver pH 5.0 fractions studied in this investigation and the guinea-pig-liver pH 5.0 fractions studied by Allen *et al.* (1960*b*). The high degree of resistance of the lysine-activating enzyme, as measured by pyrophosphate exchange, to treatment with *p*-chloromercuribenzoate is in striking contrast with its instability and its ability to be stimulated by GSH. Allen, Glassman, Cordes & Schweet (1960*a*) showed that the transfer of [^{14}C]lysine to added 'soluble' RNA catalysed by purified fractions obtained from guinea-pig liver was more resistant to treatment with *p*-chloromercuribenzoate than was the transfer of [^{14}C]leucine. This is consistent with my observations on the stimulatory effect of GSH upon these reactions.

The responses of the leucine- and lysine-activating enzymes to these activators and inhibitors recalls the individual type of response to inhibition with *p*-chloromercuribenzoate reported for the leucine- and threonine-activating enzymes of guinea-pig liver pH 5.0 fractions by Allen *et al.* (1960*b*). Conceivably, the effects may be correlated with their response to Mg^{2+} ions and to 'soluble' RNA.

Allen *et al.* (1960*b*) suggested that sulphhydryl groups might participate in the reaction mechanisms of amino acid activation. Perhaps the transfer of the activated amino acid to the adenosyl

unit involves a chelate mechanism and a thio ester intermediate (Hele, 1960*a*), along the lines suggested by Ingraham & Green (1958) for the activation of acetate.

SUMMARY

1. The pyrophosphate-exchange reactions which are catalysed by rat-liver preparations and depend upon leucine or isoleucine are profoundly modified by 'soluble' ribonucleic acid and by changes in magnesium concentration. The preponderant influence is exerted by the terminal nucleotide sequence of the 'soluble' ribonucleic acid. Lysine-dependent pyrophosphate exchange occurs only at relatively high magnesium concentrations and is not greatly influenced by 'soluble' ribonucleic acid. The transfer of [¹⁴C]lysine to 'soluble' ribonucleic acid is catalysed more rapidly by 'recombined' systems than by pH 5.0 fractions and may be related to the inability of the enzyme to react with low concentrations of magnesium, and to the presence of an inhibitor.

2. The terminal nucleotide sequence of 'soluble' ribonucleic acid may be concerned in the protection of a labile, essential site associated with the activating enzyme. The leucine- and lysine-activating enzymes show highly individual responses to activators and inhibitors of sulphydryl groups. The possible role of the labile site in the reaction mechanism is discussed.

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A Phosphoprotein Phosphatase from Ox Brain

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It has been shown [see Heald (1959) for references] that the phosphorus of a phosphoprotein fraction of brain is exchanged rapidly when the tissue is stimulated *in vitro* by electrical pulses.

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During attempts to isolate this material (Heald, 1961*a*) it became apparent that brain contains an enzyme capable of splitting phosphate from phosphoprotein. Knowledge of such enzymes is limited and, apart from brief reports by Feinstein & Volk (1949) and Norberg (1950) that dispersions of rat