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Inhibitory Effects of pH5 Enzyme from Non-Lactating Bovine Mammary Gland on Various Stages of Protein Synthesis in the Rat Liver Amino Acid-Incorporating System

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1. pH5 enzyme from non-lactating bovine mammary gland was found to contain potent inhibitors of protein synthesis in the rat liver cell-free system. These inhibitors affect (*a*) formation of aminoacyl-tRNA where tRNA represents transfer RNA, (*b*) transfer of labelled amino acids from rat liver amino[^{14}C]acyl-tRNA to protein in rat liver polyribosomes, and (*c*) incorporation of ^{14}C-labelled amino acids into peptide by rat liver polyribosomes supplemented with rat liver pH5 enzyme. 2. Increasing amounts of pH5 enzyme from bovine mammary gland progressively inhibited the incorporation of labelled amino acids into protein by a complete incorporating system from rat liver. Approx. 80% inhibition was observed at a concentration of 2mg. of protein of pH5 enzyme from bovine mammary gland. The inhibitory effect of the bovine pH5 enzyme fraction could not be overcome by the addition of increasing amounts of rat liver pH5 enzyme. 3. Fractionation of bovine pH5 enzyme with ammonium sulphate into four fractions showed that all the fractions inhibited the incorporation of ^{14}C-labelled amino acids in the rat liver system, but to varying extents. The highest inhibition observed (90%) was exhibited by the 60%-saturated-ammonium sulphate fraction. 4. Heat treatment of bovine pH5 enzyme at various temperatures caused only a partial loss of its inhibitory effect on labelled amino acid incorporation by the rat liver system. Treatment at 105° for 5min. resulted in the bovine pH5 enzyme fraction losing 30% of its inhibitory activity. 5. pH5 enzyme from bovine mammary gland strongly inhibited the charging of rat liver tRNA in the presence of its own pH5 enzymes. 6. The transfer of labelled amino acids from rat liver amino[^{14}C]acyl-tRNA to protein in a system containing rat liver polyribosomes and pH5 enzyme was almost completely inhibited by bovine pH5 enzyme at a concentration of 2mg. of protein of the enzyme fraction. 7. One of the inhibitors of various stages of protein synthesis in rat liver present in bovine pH5 enzyme was identified as an active ribonuclease, and the second inhibitor present was shown to be tRNA.

Experiments to demonstrate the incorporation of amino acids into protein by microsomes or by a fraction sedimenting at 14000*g* derived from non-lactating bovine mammary gland have met with little success (Herrington & Hawtrey, 1969). When rat liver pH5 enzyme was substituted into the system for bovine pH5 enzyme, however, amino acid incorporation was observed. Similar observations have been made with the microsomal fraction from chicken liver by Campbell & Kernot (1959) who were unable to develop a practical system. More recent investigators (Heald & Pohlman, 1966) working with the same fraction found that in replicate experiments the addition of chicken liver cell sap to the microsomal fraction sometimes caused a definite stimulation of amino acid incorporation, whereas at other times there was a distinct inhibitory effect.

Hultin (1950) and Carey (1964), on the other hand, were able to obtain similarities in requirements and activity between other mammalian systems and cell-free extracts of foetal chicken liver. It appears that this system, derived from an organism at an early stage of its life history, is able to mediate protein synthesis whereas the same preparation obtained at a later stage in the life cycle cannot. Siler & Fried (1968) have shown that the factor in chicken liver cell sap that interferes with protein synthesis corresponds to the nuclease activity described in rat liver cell sap by Hunter

(1967). The activity is, however, much more pronounced in chicken liver cell sap than in the corresponding rat liver fraction.

It seemed likely that a similar type of inhibitor, that disappears at the onset of lactation, could be present in the supernatant fraction of non-lactating bovine mammary gland. The present paper describes the inhibitory effect of pH 5 enzyme from non-lactating bovine mammary gland on amino acid incorporation by rat liver polyribosomes and presents evidence that this inhibition is due to tRNA* and an uninhibited nuclease.

MATERIALS AND METHODS

Tissue. Mammary tissue from adult non-lactating cows was used for the preparation of pH 5 enzyme. The mammary glands, which were kindly made available by the Cold Storage Commission, Salisbury, Rhodesia, were placed in ice immediately after being removed from the animals. Livers from Wistar albino rats (150–200 g. body wt.) were used for preparation of subcellular fractions. The rats were starved for 24 hr. before being killed by a blow on the head.

Chemicals. The disodium salt of ATP, phosphoenolpyruvate (tricyclohexylammonium salt), crystalline GSH and pyruvate kinase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The trisodium salt of GTP was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and yeast RNA (sodium salt) from British Drug Houses Ltd., Poole, Dorset. A ^{14}C-labelled protein hydrolysate from yeast (1500 μc/mg.) was obtained from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A.

Solutions of ATP, GTP, GSH and phosphoenolpyruvate were prepared before use and adjusted to pH 7·6 with 0·3 M-KOH.

Scintillation chemicals. 2,5-Diphenyloxazole (PPO) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) were purchased from Packard Instrument Co. Inc., La Grange, Ill., U.S.A. The scintillation fluid contained 0·5% PPO and 0·03% POPOP in chromatography-grade toluene.

Preparation of subcellular fractions. Rat liver homogenates and bovine mammary-gland homogenates were prepared in medium A [containing (final concn.): 0·25 M-sucrose, 5 mM-$MgCl_2$, 25 mM-KCl and 50 mM-tris–HCl buffer, pH 7·6], as described by Hawtrey, Schirren & Dijkstra (1963) and Herrington & Hawtrey (1969) respectively.

Polyribosomes. Rat liver polyribosomes were prepared by the method of Wettstein, Staehelin & Noll (1963) as modified for centrifuge tubes of the no. 30 rotor (Spinco model L. 50 ultracentrifuge) by Hawtrey & Nourse (1966). Each pellet of polyribosomes was suspended in 2 ml. of medium A and allowed to dissolve overnight at 0°. Denatured material was removed by centrifugation at 1400 *g* for 10 min. The final clear suspension of polyribosomes was kept at 0° and used within 2 days.

pH 5 fractions. These were prepared from the cell sap of rat liver and bovine mammary gland as described by Hawtrey *et al.* (1963). The fractions, suspended in medium A, were assayed for protein concentration and stored at −15° until required. For each experiment a separate batch of pH 5 enzyme fraction was prepared.

Preparation of deacylated tRNA. tRNA was prepared from the pH 5 enzyme fraction by extraction in the presence of 90% (w/v) phenol and 1% (w/v) sodium dodecyl sulphate for 90 min. at room temperature. The emulsion was broken by centrifugation at 2000 *g* for 20 min. The aqueous phase was carefully removed and to this was added 0·1 vol. of 20% (w/v) potassium acetate and 2·5 vol. of 96% (v/v) ethanol. The RNA was allowed to precipitate overnight at −15° before being deacylated by the procedure of Sarin & Zamecnik (1964) by incubation with 1·8 M-tris–HCl buffer, pH 8·0, at 37° for 1 hr. The deacylated tRNA was reprecipitated twice with ethanol and potassium acetate before being dissolved in water and dialysed against water for 6–8 hr. The concentration of RNA was measured spectrophotometrically at 260 nm. ($E^{1\%}_{1cm}$.170). The tRNA fractions had sedimentation coefficient approx. 4 s as determined by sucrose-density-gradient centrifugation.

Rat liver amino[^{14}C]acyl-tRNA preparation. The tRNA of the pH 5 enzyme fraction was labelled with yeast ^{14}C-labelled protein hydrolysate by the method of Hawtrey (1965). The preparation, which was stored at −15°, was found to have a specific activity of 409×10^3 c.p.m./mg. of RNA.

Measurement of incorporation of labelled amino acids into protein. The reaction medium used for all incorporation studies contained (final concn.): 40 mM-tris–HCl buffer, pH 7·6; 18 mM-KCl; 6 mM-$MgCl_2$; 0·2 M-sucrose, plus the components given in the legends to the tables and figures. Incubations were carried out under aerobic conditions at 37° for 15 min. For studies of protein synthesis by rat liver polyribosomes, the reaction was terminated by the addition of an equal volume of cold 10% (w/v) trichloroacetic acid. The samples were kept overnight at 0° before collection of the protein precipitates by centrifugation. The precipitates were washed successively with cold 5% (w/v) trichloroacetic acid, ethanol–ether (1:1, v/v) and ether and then heated at 90° for 15 min. in trichloroacetic acid solution. After thorough cooling in ice, the protein precipitates were filtered on Millipore HA 0·45 μm. filters (Millipore Filter Corp., Bedford, Mass., U.S.A.) and washed at least three times with 5 ml. of cold 5% (w/v) trichloroacetic acid. The filters were dried in air for 1 hr. before being placed in counting vials with 10 ml. of scintillation fluid and their radioactivity was counted in a liquid scintillation spectrometer (Packard Tri-Carb model 2002).

Measurement of combination of labelled amino acids with tRNA. pH 5 enzyme fractions (1 mg. of protein) from rat liver and bovine mammary gland were incubated with 2 μmoles of ATP, 1·6 μmoles of GSH, ^{14}C-labelled protein hydrolysate from yeast (1 μc), 0·2 M-sucrose, 35 mM-tris–HCl buffer, pH 7·6, 18 mM-KCl and 7 mM-$MgCl_2$ under aerobic conditions at 37°. The total volume was 1·0 ml. At selected times during incubation the reactions were stopped by the addition of an equal volume of cold 10% (w/v) trichloroacetic acid. After standing for 20 min. at 0° the precipitates were washed successively with cold 5% (w/v) trichloroacetic acid, ethanol–ether (1:1, v/v) and ether before being filtered and thoroughly washed on Millipore HA 0·45 μm. filters. The radioactivity-counting procedure was as described above.

* Abbreviations: tRNA, transfer RNA; aminoacyl-tRNA, aminoacyl transfer RNA.

Assay of nuclease activity in pH 5 *enzyme fractions.* Two methods for the determination of nuclease activity in pH 5 enzyme fractions were used. (*a*) With yeast RNA (previously dialysed against water for 24hr. to remove free nucleotides) as a substrate, by measuring the increase in E_{260}. Nuclease activity was determined in duplicate for each pH 5 enzyme under aerobic conditions at 37°. The incubation tubes contained 1ml. of pH 5 enzyme (approx. 5mg. of protein), 0·8ml. of medium A and 0·2ml. of RNA substrate (2mg.). At selected times during the incubation, 0·5ml. samples were removed and placed in tubes kept in ice, containing 5ml. of 0·5M-$HClO_4$. After 20min. at 0° the RNA and protein were precipitated by centrifugation at 1400*g* for 10min. before the supernatant was assayed for free nucleotides. Tubes containing no pH 5 enzyme were included for the selected times during the incubation to ensure that no breakdown of RNA occurred under experimental conditions. Nuclease activity was expressed as E_{260}/mg. of protein of pH 5 enzyme. (*b*) The second procedure for the assay of nuclease activity was a modification of the method of Siler & Fried (1968), involving observing the rate of degradation of ^{3}H-labelled rat liver ribosomal RNA. Incubations were carried out in duplicate at 37° with pH 5 enzyme (approx. 1mg. of protein), 2μmoles of ATP, 0·5μmole of GTP, 5μmoles of phosphoenolpyruvate, 50μg. of pyruvate kinase, 1·6μmoles of GSH and ^{3}H-labelled rat liver ribosomal RNA (7920 c.p.m.) in a total volume of 2·0ml. At the indicated times, 0·5ml. samples were removed from the incubation medium and pipetted into 3ml. of cold 10% (w/v) trichloroacetic acid. After 30min. to allow for precipitation, the samples were poured on to Millipore filters and washed five or six times with portions (approx. 10ml.) of cold 5% (w/v) trichloroacetic acid. The radioactivity-counting procedure was as described above.

Heat treatment. Four equal portions of pH 5 enzyme from bovine mammary gland (5mg. of protein/ml.) were heated at 50°, 70°, 90° and 105° for exactly 5min. No change in the appearance of the solutions was observed at 50° and 70°, whereas at the higher temperature protein coagulation occurred within the first 3min. The coagulated samples were resuspended and 0·2ml. portions used for amino acid-incorporation studies.

Ammonium sulphate fractionation. Successive $(NH_4)_2SO_4$ fractions (15, 20, 30 and 60% saturation) were precipitated from 30ml. of pH 5 enzyme from bovine mammary gland. After each fraction had been removed by centrifugation at 1400*g* for 20min., the remaining supernatant was then brought to the required $(NH_4)_2SO_4$ concentration. The fractions were dissolved in a minimum volume of medium A at 4° and dialysed against medium A until free of sulphate.

Determination of protein. Protein was determined by the biuret method of Gornall, Bardawill & David (1949) with bovine serum albumin as standard.

RESULTS

Effect of pH 5 *enzyme from bovine mammary gland on protein synthesis in the rat liver system.* The effect of the enzyme fraction on protein synthesis in the rat liver system was investigated in three different ways: (*a*) on total protein synthesis by rat liver polyribosomes; (*b*) on the formation of aminoacyl-tRNA; (*c*) on the transfer of labelled amino acids from rat liver amino[^{14}C]acyl-tRNA into protein by rat liver polyribosomes.

pH 5 enzyme from non-lactating bovine mammary gland appears to contain a potent inhibitor of protein synthesis in the rat liver system. This is evident from the results shown in Fig. 1, where an almost linear inhibition of protein synthesis by rat liver polyribosomes is obtained by the addition of increasing quantities of bovine pH 5 enzyme to the incorporating system. At a concentration of 2mg. of protein, the bovine fraction almost completely masks the effect of rat liver pH 5 enzyme, giving an inhibition of up to 80%. In all probability the inhibitory effect would be greater if more of the bovine fraction was added to the system.

To ascertain whether the bovine pH 5 enzyme had a detrimental effect on the different steps of protein synthesis, the formation of amino[^{14}C]acyl-tRNA in both rat liver and bovine mammary gland fractions was investigated. As expected the activating enzymes of rat liver were capable of rapidly catalysing the formation of enzyme-bound aminoacyl adenylates and of transferring the aminoacyl moeity to a specific type of RNA (Fig. 2). The formation of bovine amino[^{14}C]acyl-tRNA was extremely slow, being about one-fifth the rate of the rat liver system. However, when both pH 5

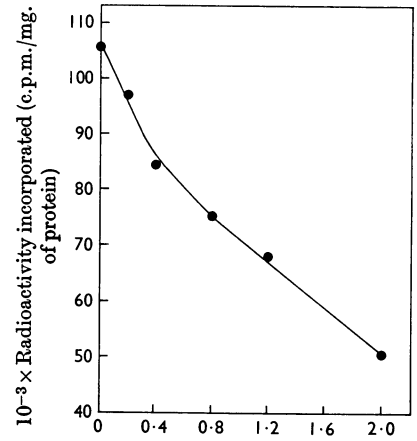

Fig. 1. Effect of pH 5 enzyme from bovine mammary gland on the incorporation of labelled amino acids into rat liver polyribosomes. The incubation mixture contained: 2μmoles of ATP, 0·5μmole of GTP, 5μmoles of phosphoenolpyruvate (tricyclohexylammonium salt), 50μg. of pyruvate kinase, 1·6μmoles of GSH, yeast ^{14}C-labelled protein hydrolysate (1μc), rat liver pH 5 enzyme (0·5mg. of protein), rat liver polyribosomes (0·5mg. of protein) and increasing concentrations of bovine pH 5 enzyme. All incubations were carried out in duplicate at 37° for 15min.

enzyme fractions were combined, the bovine fraction appeared to prevent the charging of the rat liver tRNA, since the formation of the ^{14}C-labelled activated complex was also slow.

Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958) showed that during protein synthesis the amino acid is transferred from tRNA to uncharacterized protein chains being formed on the ribosome. In the rat liver system the transfer of the amino acids was found to be grossly inhibited (up to nearly 100%) by the presence of bovine pH5 enzyme, as shown in Fig. 3. These results provide clear evidence of the inhibitory effect of pH5 enzyme from bovine mammary gland on protein synthesis in the rat liver system. That this inhibition of total protein synthesis cannot be overcome by increasing the amount of pH5 enzyme from rat liver in the system is evident from the results presented in Table 1. Reasons for this are unknown.

Stability of rat liver amino[^{14}C]*acyl-tRNA.* In an attempt to verify that bovine pH5 enzyme did not contain a hydrolytic enzyme capable of splitting the aminoacyl-tRNA complex, rat liver amino-[^{14}C]acyl-tRNA was incubated with both bovine and rat liver pH5 enzyme. No deacylation was observed in either case.

Nuclease activity of the pH5 *enzyme fractions.* The fact that pH5 enzyme from bovine mammary gland is unable to support protein synthesis in the bovine cell-free system and inhibits total protein synthesis in the rat liver system suggests that it contains a factor or factors that inhibit amino acid incorporation. The points in the protein-biosynthetic process at which inhibition occurs have been located (Figs. 1, 2 and 3). A partial explanation may be the degradation of messenger RNA, thereby decreasing the incorporation of amino acids in systems containing this pH5 enzyme from bovine mammary gland. This possibility was evaluated by measuring the extent to which RNA was degraded in cell-free systems identical with those used for amino acid incorporation. When yeast RNA or ^{3}H-labelled rat liver ribosomal RNA was incubated with pH5 enzyme fractions from either rat liver or bovine mammary gland, the results shown in Figs. 4 and 5 were obtained respectively. After 40min. of incubation, over 70% of the acid-insoluble radioactivity had been rendered acid-soluble in the bovine system, whereas only about 25% had been solubilized in the rat liver system (Fig. 5).

These findings indicate that the bovine pH5 enzyme contains an active ribonuclease. Experiments have shown that rat liver cell sap (2mg. of protein), which contains a ribonuclease inhibitor (Roth, 1956), markedly depresses the ribonuclease activity of bovine pH5 enzyme.

Heat treatment of pH5 *enzyme from bovine mam-*

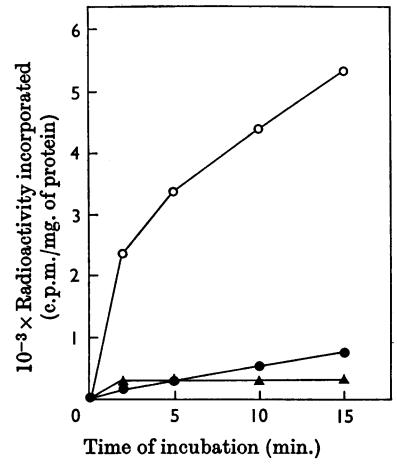

Fig. 2. Time-course of formation of amino[^{14}C]acyl-tRNA with protein hydrolysate from yeast in the presence of pH5 enzyme from bovine mammary gland (●), from rat liver (○) and an equal concentration of both enzymes (▲). See the Materials and Methods section for details.

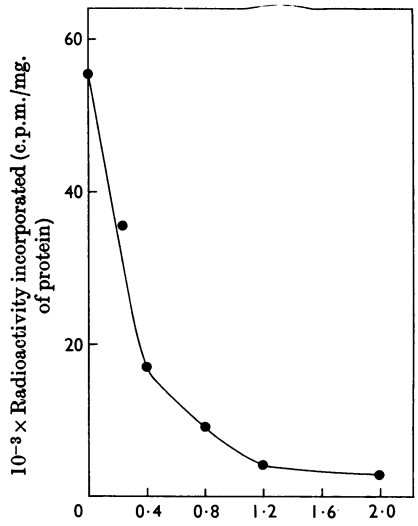

Fig. 3. Influence of pH5 enzyme from bovine mammary gland on the transfer of labelled amino acids from rat liver amino[^{14}C]acyl-tRNA into protein by rat liver polyribosomes. The complete system contained: 0·5 μmole of GTP, 1·6 μmoles of GSH, 5 μmoles of phosphoenolpyruvate (tricyclohexylammonium salt), 50 μg. of pyruvate kinase, amino[^{14}C]acyl-tRNA (60600 c.p.m.), rat liver pH5 enzyme (1mg. of protein), rat liver polyribosomes (0·4mg. protein) and increasing concentrations of bovine pH5 enzyme. All incubations were carried out in duplicate at 37° for 15min.

Table 1. *Effect of increasing concentrations of* pH 5 *enzyme from rat liver on the inhibitory properties of* pH 5 *enzyme from bovine mammary gland during amino acid incorporation by rat liver polyribosomes*

The incubation mixture contained: 2 μmoles of ATP, 0·5 μmole of GTP, 5 μmoles of phosphoenolpyruvate (tricyclohexylammonium salt), 50 μg. of pyruvate kinase, 1·6 μmoles of GSH, ^{14}C-labelled protein hydrolysate from yeast (1 μc), bovine pH 5 enzyme (2 mg. of protein), rat liver polyribosomes (0·6 mg. of protein) and increasing amounts of rat liver pH 5 enzyme, in a total volume of 1·70 ml. All incubations were carried out in duplicate at 37° for 15 min. The control contained the complete incubation mixture with the exception of pH 5 enzyme from bovine mammary gland.

System			
Polyribosomes (mg. of protein)	Rat liver pH 5 enzyme (mg. of protein)	Bovine pH 5 enzyme (mg. of protein)	Radioactivity incorporated (c.p.m./mg. of protein)
0·6	1·0	0	111117
0·6	0·2	2·0	10403
0·6	0·4	2·0	10458
0·6	0·8	2·0	10852
0·6	1·2	2·0	14562
0·6	2·0	2·0	17530

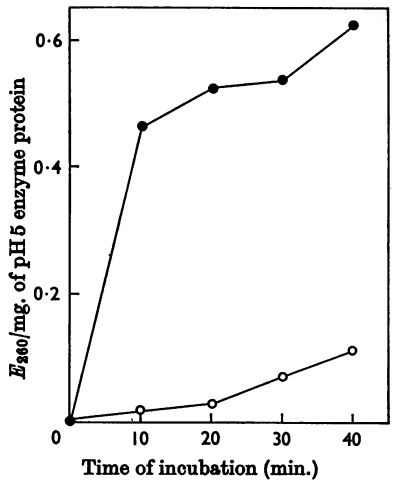

Fig. 4. Nuclease activity on yeast RNA in pH 5 enzyme from bovine mammary gland (●) and from rat liver (○). See the Materials and Methods section for experimental details.

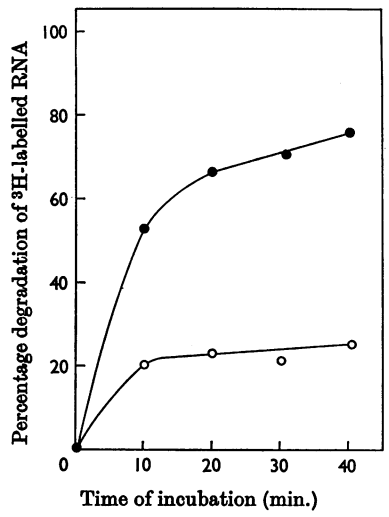

Fig. 5. Degradation of ^{3}H-labelled rat liver ribosomal RNA by pH 5 enzyme from bovine mammary gland (●) and from rat liver (○). The reaction conditions are described in the Materials and Methods section.

mary gland. From the results already presented on the effects of bovine pH 5 enzyme on total protein synthesis, attempts were made to isolate the factor or factors responsible for this inhibition. In conjunction with the conclusions of Siler & Fried (1968) it is possible that ribonuclease activity could be a contributory factor. The higher enzyme activity in bovine pH 5 enzyme, however, does not account for the marked inhibition, as the results presented in Table 2 show.

When the bovine pH 5 enzyme fraction underwent differential heat treatment very little effect was observed except at the elevated temperatures of 70° and 90° (Table 2). Ribonuclease is stable up to temperatures of 70°. These results suggest that ribonuclease contributes only partially towards the inhibitory effect caused by pH 5 enzyme from bovine mammary gland. Some other factor, which is heat-stable, must be mainly responsible.

Isolation of the inhibitor present in the pH 5 *enzyme fraction from bovine mammary gland.* The results in Table 3 show that all the ammonium sulphate

Table 2. *Effect of heat treatment of* pH 5 *enzyme from bovine mammary gland on its inhibitory properties*

The incubation mixture contained: 2 μmoles of ATP, 0·5 μmole of GTP, 5 μmoles of phosphoenolpyruvate (tricyclohexylammonium salt), 50 μg. of pyruvate kinase, 1·6 μmoles of GSH, ^{14}C-labelled protein hydrolysate from yeast (1 μc), rat liver pH 5 enzyme (1 mg. of protein), rat liver polyribosomes (0·4 mg. of protein) and bovine pH 5 enzyme (1 mg. of protein), in a total volume of 1·70 ml. All incubations were carried out in duplicate at 37° for 15 min. For details of heat treatment see the Materials and Methods section.

Bovine pH 5 enzyme components added	Radioactivity (c.p.m./mg. of protein)	Inhibition (%)
None	187945	—
Bovine pH 5 enzyme	60723	67·7
Enzyme treated at 50°	55338	70·6
Enzyme treated at 70°	93380	50·3
Enzyme treated at 90°	111118	40·9
Enzyme treated at 105°	97115	48·3

Table 3. *Effect of different ammonium sulphate fractions of* pH 5 *enzyme from bovine mammary gland on amino acid incorporation by rat liver polyribosomes*

The incubation mixture contained: 2 μmoles of ATP, 0·5 μmole of GTP, 5 μmoles of phosphoenolpyruvate, 50 μg. of pyruvate kinase, 1·6 μmoles of GSH, ^{14}C-labelled protein hydrolysate from yeast (1 μc), rat liver pH 5 enzyme (1 mg. of protein), rat liver polyribosomes (0·55 mg. of protein) and pH 5 enzyme fractions from bovine mammary gland (1 mg. of protein) in a total volume of 1·70 ml. All incubations were carried out in duplicate at 37° for 15 min. For the $(NH_4)_2SO_4$ fractionation procedure, see the Materials and Methods section.

Bovine pH 5 enzyme component added	Radioactivity (c.p.m./mg. of protein)	Inhibition (%)
None	225355	—
Bovine pH 5 enzyme	53971	76·1
15%-satd.-$(NH_4)_2SO_4$ fraction	144442	35·9
20%-satd.-$(NH_4)_2SO_4$ fraction	105011	53·4
30%-satd.-$(NH_4)_2SO_4$ fraction	87589	61·1
60%-satd.-$(NH_4)_2SO_4$ fraction	17482	92·2
15%-+20%-satd.-$(NH_4)_2SO_4$ fraction	117769	47·7
15%-+30%-satd.-$(NH_4)_2SO_4$ fraction	102816	54·4
20%-+30%-satd.-$(NH_4)_2SO_4$ fraction	83687	62·9

fractions of bovine pH 5 enzyme possessed inhibitory activity, which increased in fractions obtained at increasing degrees of saturation of ammonium sulphate. This fact, coupled with other investigations (M. D. Herrington & A. O. Hawtrey, unpublished work), suggested that the inhibitory activity was derived from the tRNA component present in the pH 5 enzyme.

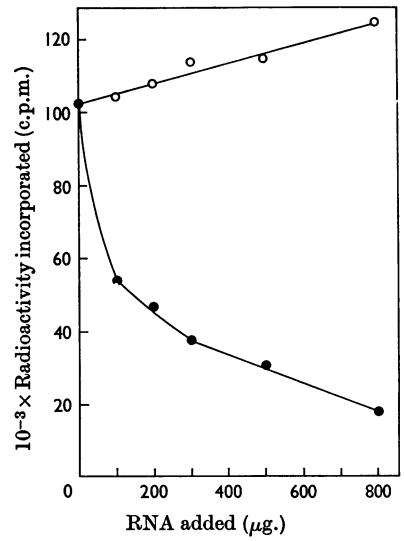

Fig. 6. Effect of deacylated tRNA from rat liver (○) and bovine mammary gland (●) on the incorporation of labelled amino acids into rat liver polyribosomes. The incubation mixture contained: 2 μmoles of ATP, 0·5 μmole of GTP, 5 μmoles of phosphoenolpyruvate (tricyclohexylammonium salt), 50 μg. of pyruvate kinase, 1·6 μmoles of GSH, yeast ^{14}C-labelled protein hydrolysate (1 μc), rat liver pH 5 enzyme (1 mg. of protein), rat liver polyribosomes (0·5 mg. of protein) and increasing amounts of the indicated deacylated tRNA. All incubations were carried out in duplicate at 37° for 15 min.

Verification of this hypothesis can be seen from the results presented in Fig. 6. tRNA from bovine mammary gland was found to inhibit strongly the incorporation of labelled amino acids into protein in rat liver polyribosomes, whereas tRNA isolated from rat liver had a slight stimulatory effect.

DISCUSSION

The above results show that the pH 5 enzyme fraction prepared from non-lactating bovine mammary gland inhibits the protein-biosynthetic processes occurring in the rat liver cell-free system.

The incorporation of labelled amino acids into microsomal proteins requires the presence of the soluble cytoplasm (Zamecnik & Keller, 1954) or the pH 5 enzyme fraction (Keller & Zamecnik, 1956).

The three basic steps in the formation of a peptide may be summarized as follows:

$$\text{Amino acid} + \text{AMP–PP} \underset{\text{enzyme}}{\overset{Mg^{2+}}{\rightleftharpoons}}$$

$$\text{enzyme–AMP}\cdot\overset{\overset{O}{\|}}{C}\cdot CH(NH_2)R + PP_i \qquad (1)$$

$$\text{Enzyme–AMP}\cdot\overset{\overset{O}{\|}}{C}\cdot CH(NH_2)R + \text{tRNA} \rightleftharpoons$$

$$R(NH_2)CH\cdot\overset{\overset{O}{\|}}{C}\sim\text{tRNA} + \text{AMP} \qquad (2)$$

$$R(NH_2)CH\cdot\overset{\overset{O}{\|}}{C}\sim\text{tRNA} \xrightarrow[\substack{GTP\\ \text{transfer factors}}]{\text{Ribosomes}}$$

$$\text{peptide}\sim\text{ribosomes} + \text{tRNA} \qquad (3)$$

The enzymes responsible for the initial steps of protein synthesis are the aminoacyl-tRNA synthetases (Hoagland, Keller & Zamecnik, 1956). These enzymes, along with tRNA, are found in the pH 5 enzyme fraction (Hoagland, Zamecnik & Stephenson, 1957).

However, when pH 5 enzyme from bovine mammary gland is added to such a system, it almost completely prevents the formation of protein. Not only does it block the transfer of labelled amino acids from the aminoacyl-tRNA complex into protein, but it also prevents rat liver tRNA from being activated by its own activating enzymes. From the results reported in Fig. 2 one can conclude that either the pH 5 enzyme from bovine mammary gland lacks the necessary activating enzymes required to activate the tRNA components or that the tRNA is completely incapable of being acylated.

The fact that the inhibitory component was only partially removed by vigorous heat treatment suggested that the main inhibitory factor was not protein in nature. There are several reports indicating the role of lipids in protein synthesis (Hendler, 1959; Gaby & Silberman, 1960; Hunter & Goodsall, 1961). This aspect was investigated but it was found that lipids isolated from the pH 5 enzyme fraction of bovine mammary gland had no effect on rat liver protein synthesis (M. D. Herrington & A. O. Hawtrey, unpublished work).

The results suggested that the tRNA fraction was responsible for the inhibition. Inhibition of protein synthesis by tRNA has been shown by Aaronson, Korner & Munro (1966), Decken & Campbell (1962) and So & Davis (1965). All the inhibitions described by these authors were at the level of incorporation of labelled amino acids into protein.

It is clear from the results that tRNA from bovine mammary gland affects protein synthesis in both the initial and the later stages. The RNA may bind to the aminoacyl-tRNA synthetases from rat liver, thereby preventing the activation of rat liver tRNA. On the other hand, pH 5 enzyme from bovine mammary gland also prevents the transfer of labelled amino acids from rat liver aminoacyl-tRNA to the growing polypeptide chain on the ribosome. Reasons for this are unknown.

Siler & Fried (1968) have demonstrated the presence of high nuclease activity in chicken liver cell sap, which is thought to inhibit amino acid incorporation. An analogous inhibitor appears to be present in the pH 5 enzyme fraction of bovine mammary gland. This activity could be responsible for the degradation of the messenger RNA in the rat liver cell-free system, thus inhibiting protein synthesis.

From the results given in Table 2 and Fig. 6 one may calculate the relative contributions of tRNA and the ribonuclease to the total inhibition. The pH 5 fraction has an RNA/protein ratio of 0·11 (M. D. Herrington & A. O. Hawtrey, unpublished work) so that a sample containing 1 mg. of protein would contain 110 μg. of tRNA. Heat treatment, which is assumed to denature ribonuclease, causes a decrease in inhibition of 19% and, from Fig. 6, 110 μg. of tRNA would produce an inhibition of 48%.

In conclusion it appears that the inhibition of protein synthesis caused by bovine pH 5 enzyme is derived mainly from the tRNA fraction (70%), whereas the ribonuclease activity contributes to a small extent (30%).

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