Nuclease Activity in Cell-Free Amino Acid-Incorporating Systems from Chicken Liver

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1. Comparative studies of the polyuridylic acid-directed phenylalanine-incorporating activity of cell-free systems derived from rat and chicken livers demonstrated markedly lower activity in the chicken liver system. 2. The chicken liver cell sap contained the factor(s) responsible for this lower activity. Ribosomes from chicken and rat performed equally well in the presence of rat liver cell sap. Chicken liver cell sap, when mixed with rat liver cell sap, caused an inhibition of incorporation of phenylalanine into acid-insoluble material. 3. Though ribosomal preparations and cell sap from both rat and chicken liver degraded polyuridylic acid to some extent, the chicken liver cell sap contained the largest amount of activity. 4. Rat liver cell sap inhibited the nuclease activities of ribosomal preparations, but no such nuclease inhibition could be demonstrated with chicken liver cell sap.

Experiments to demonstrate amino acid incorporation into protein by microsomal or ribosomal fractions derived from chicken liver have met with little success, though incorporation by such fractions from a variety of other animals and tissues has been observed. Campbell & Kernot (1959) were unable to develop a practical chicken liver microsomal fraction, though similar systems from other domestic fowl were found to be satisfactory. Heald & Pohlman (1966), who also investigated the chicken liver microsomal fraction, observed 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. Such reports are inconsistent with descriptions of successful experiments in which cell-free extracts of foetal chicken liver were used (Carey, 1964; Hultin, 1950). These workers studied preparations of foetal chick liver and noted similarities in requirements and activity between these embryonic extracts and mammalian systems. Thus it would appear that a cell-free system derived from an organism at one stage of its life history is able to mediate protein synthesis, whereas an analogous preparation obtained at another stage in the life cycle may fail to perform. One possible explanation for the differences between species and between preparations obtained from organisms at different stages in the life cycle may be that there is in chicken cell sap a factor that

interferes with protein synthesis. Such a factor might correspond to the nuclease activity described by Roth (1956), by Lawford, Langford & Schachter (1966) and by Hunter (1967).

In related studies Shortman (1961) and Roth (1962) presented evidence that cell sap from rat liver contains an inhibitor of nuclease activity and also observed that chicken liver cell sap contained no substance inhibitory to commercial ribonuclease preparations.

The present paper describes an inhibitory effect of chicken liver cell sap on amino acid incorporation by chicken or rat ribosomes and presents evidence that this inhibition may be ascribed to the presence in the chicken liver cell of an uninhibited nuclease.

MATERIALS AND METHODS

Animals. The animals used were small (150–200 g.) male albino rats obtained from the Holtzman Co. (Madison, Wis., U.S.A.) and young (6–8 weeks) roosters of the brown Leghorn strain obtained from the Poultry Science Department of the University of Florida. The animals were maintained on water and food *ad libitum*, commercial rat chow for the rats and cracked corn for the chickens. Before being killed by decapitation the animals were kept without food overnight.

Chemicals. ATP, GTP, pyruvate kinase and phosphoenolpyruvate were purchased from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). Unlabelled polyuridylic acid, poly[³H]uridylic acid and L-[U-¹⁴C]phenylalanine were obtained from Schwarz BioResearch Inc. (Orangeburg, N.Y., U.S.A.). All other chemicals were analytical or reagent grade. All solutions were prepared with distilled deionized water.

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Preparation of liver fractions. Liver fractions were obtained from both rats and chickens by the method of Howell, Loeb & Tomkins (1964), which yields primarily monomeric ribosomes with low endogenous amino acid incorporation. Weighed portions of liver from freshly killed animals were finely minced with scissors and homogenized in 3 vol. of 0.25 M-sucrose in TKM buffer (50 mM-tris-HCl, 25 mM-KCl and 5 mM-MgCl₂ at final pH 7.6) in a Potter-Elvehjem Teflon-glass homogenizer. The homogenate was centrifuged at 600g for 10 min. to remove whole cells, nuclei and debris. The resulting supernatant was centrifuged for 20 min. at 20000g. The sediment from this centrifugation (the FII pellet) was resuspended in approx. 2 vol. (with respect to the original liver weight) of 0.25 M-sucrose in TKM buffer and the solution made 1.3% with respect to DOC.* Then 5 ml. of the resulting solution was layered on to 4 ml. of 0.5 M-sucrose in TKM buffer, which in turn lay over 3 ml. of 1.0 M-sucrose in TKM buffer, in a 12 ml. cellulose nitrate centrifuge tube (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.). The DOC-treated FII preparation was then centrifuged through the two layers of sucrose for 2 hr. at 105000g in the 40 rotor of a Spinco model L centrifuge at 4–5°. The clear light-yellow pellet that resulted was resuspended in TKM buffer without sucrose (2 vol. with respect to original liver weight) with a ground-glass homogenizer. The suspension was clarified by centrifugation at 600g for 5 min., and the ribosomes were used immediately. These ribosomes are referred to as DOC-FII ribosomes.

Cell sap was prepared by centrifuging the supernatant from the 20000g centrifugation described above for 2 hr. at 105000g. The clear lipid-free portion of the resulting supernatant was used as the cell sap.

Amino acid incorporation. The complete amino acid-incorporating mixture contained 1 μmole of ATP, 10 μmoles of phosphoenolpyruvate, 40 μg. of pyruvate kinase, 0.2 μmole of GTP, 15 μmoles of MgCl₂ (a concentration experimentally determined to give maximal incorporation rates), 50 μmoles of tris-HCl, 10 μmoles of KCl, 100 μg. of casein hydrolysate ('casamino acids') plus tryptophan, 0.3 ml. of the DOC-FII ribosomal suspension (33–150 μg. of ribosomal protein) and 0.05–0.1 ml. of cell sap (2–4 mg. of protein) in a final volume of 1.00 ml. The final pH of the mixture was 7.6. Mixtures were preincubated at 37° for 10–15 min. before the reaction was initiated by the addition of 0.25 μC of L-[U-¹⁴C]phenylalanine (360 mc/m-mole) and 100 μg. of polyuridylic acid (an amount that was found to saturate the system). Samples were removed from the mixture immediately after the addition of L-[U-¹⁴C]phenylalanine and placed in 10% (w/v) trichloroacetic acid to determine zero-time values, i.e. radioactivity adsorbed by filter paper or protein (approx. 50 counts/min., including physical background). At the end of a 1 hr. incubation, the reaction was stopped by the addition of 10% trichloroacetic acid and the samples were prepared for counting.

Collection, washing and counting of radioactive samples. Samples (0.1 ml.) of the amino acid-incorporating mixture were allowed to soak (10–15 sec.) into filter-paper circles (Whatman 3MM, 2.3 cm. diam.) and the circles were placed in 10% trichloroacetic acid. The samples were washed according to the procedure of Bollum (1966) and dried with hot air after a final ether wash. The filter-paper circles were placed in scintillation vials, scintillation fluid (Bray, 1960)

was added and the samples were counted for 10 min. in a model 3003 Packard Tri-Carb scintillation counter at approx. 80% efficiency.

Degradation of polyuridylic acid. The non-liver components of the reaction mixture were as described for the amino acid-incorporation studies with the exceptions of the omission of L-[U-¹⁴C]phenylalanine and the substitution of poly[³H]uridylic acid (approx. 90000 counts/min./100 μg.) for the unlabelled polynucleotide. Liver components were present in concentrations approximately equivalent to those used for amino acid-incorporation studies.

At the indicated times 0.1 ml. samples were removed from the polyuridylic acid degradation mixture and pipetted into an equal volume of cold 10% trichloroacetic acid. Zero-time values were obtained by adding 0.09 ml. of the mixture (before the addition of polyuridylic acid) directly to trichloroacetic acid and subsequently adding 0.01 ml. of the poly[³H]uridylic acid solution. After about 30 min., to allow for flocculation, the samples were poured on to Millipore filters (2.5 cm. diam., 0.45 μ pore size) under suction and washed four or five times with approx. 5 ml. portions of cold 5% trichloroacetic acid. The Millipore filters were placed in scintillation vials and dried for 30 min. in a 110° oven. Scintillation fluid was added and the vials were incubated at 37° for several hours to ensure dissolution of the filter and dispersal of the precipitate. Samples were counted for 10 min. at approx. 16% efficiency.

Determination of protein. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) as described by Layne (1957). Bovine serum albumin was used as the protein standard.

RESULTS

Preliminary experiments involved repetition of the studies of Howell *et al.* (1964) on amino acid incorporation with rat liver components to ensure reproducibility in our hands. Some of the results are shown in Table 1. When analogous experiments were performed with chicken liver components, isolated by identical procedures, it was found that the amount of polyuridylic acid-directed phenylalanine incorporation was much less in the chicken liver system than in the rat liver system (Table 1). Doubling the concentration of polyuridylic acid led to no increase in incorporation. Since the cell-free system contained only two components from the liver, the cell sap and the ribosomal fraction, it was possible to determine whether one or both of these were responsible for the low incorporation by the chicken liver system. This was done by measuring phenylalanine incorporation in systems containing the four possible combinations of rat and chicken liver components. The results of such an experiment (Table 1) show that ribosomes from both rat and chicken liver incorporated phenylalanine into acid-insoluble material to a greater extent in the presence of rat liver cell sap than they did in the presence of chicken liver cell sap. This would indicate that the low incorporation by the whole chicken liver system may be ascribed to some

* Abbreviation: DOC, sodium deoxycholate.

Table 1. Incorporation of L-[U-¹⁴C]phenylalanine by cell-free systems containing rat and chicken liver components

Components of the reaction mixtures and reaction conditions are described in the Materials and Methods section. Concentrations of rat and chicken cell sap (2 mg. of protein/ml.) and ribosome (300 mg. of protein/ml.) were approximately the same. Samples (0.1 ml.) were counted after 1 hr. incubation and were corrected for acid-insoluble radioactivity present at zero time. Washing and counting procedures were as described in the text.

System		Incorporation* (counts/min./ml.)
Ribosomes	Cell sap	
Rat	Rat	1200
Chicken	Chicken	370
Rat	Chicken	360
Chicken	Rat	1000

* Incorporation in the absence of polyuridylic acid ranged from 20 to 50 counts/min./ml. above background.

Table 2. Inhibition of phenylalanine incorporation by chicken liver cell sap in systems containing rat liver cell sap

Components of reaction mixtures and reaction conditions are described in the Materials and Methods section. DOC-FII ribosomes from chicken liver (300 mg. of protein/ml.) were incubated with rat liver cell sap or chicken liver cell sap or both in the indicated combinations and concentrations. Percentage inhibitions by chicken liver cell sap are relative to the incorporations obtained with the system containing rat liver cell sap alone.

Rat liver cell sap (mg. of protein/ml.)	Chicken liver cell sap (mg. of protein/ml.)	Incorporation (counts/min./ml.)	Inhibition (%)
2.0	—	1190	—
2.0	1.0	720	40
2.0	2.0	760	37
1.0	—	840	—
1.0	2.0	520	38
—	2.0	330	—

Fig. 1. Extent of phenylalanine incorporation by chicken liver ribosomes in the presence of increasing concentrations of rat (○) or chicken (●) liver cell sap. Experimental conditions were as described in the text. Incorporation in the absence of polyuridylic acid ranged from 20 to 50 counts/min./ml. above background.

property of the cell sap and that the DOC-FII ribosomes from chicken liver are capable of mediating protein synthesis.

To ascertain whether the diminished amino acid incorporation in the presence of chicken liver cell sap was caused by deficiency of enzymes required for the biosynthetic process, the following experiment was performed. Different concentrations of the chicken liver cell sap were used in the chicken cell-free system and the amounts of incorporation

were compared with those obtained with corresponding concentrations (in mg. of protein/ml.) of rat liver cell sap. In contrast with the response of the system to rat liver cell sap, increasing the amounts of the chicken liver cell sap had essentially no effect in stimulating incorporation (Fig. 1). It would appear that the extent of amino acid incorporation into protein was more dependent on the source of the cell sap than on its concentration, with chicken liver cell sap exhibiting no ability to stimulate the uptake of L-[U-¹⁴C]phenylalanine.

With differences in enzyme concentration ruled out as the sole basis for the difference between the cell saps, an alternative possibility, i.e. that chicken liver cell sap contains a factor that inhibits amino acid incorporation, was investigated. The phenylalanine-incorporating properties of chicken ribosome systems supplemented with mixtures of rat and chicken liver cell saps were compared with those of systems containing only the rat liver cell sap. The results appear in Table 2. The decrease in incorporation observed with systems containing chicken liver cell sap indicates that there is an inhibition of phenylalanine incorporation by some factor (or factors) present in the chicken liver cell sap. It is important to note that doubling the amount of chicken liver cell sap in the mixed cell sap system did not further increase the inhibition. This precludes the possibility that the inhibition of amino acid incorporation is merely an artifact due to dilution of the L-[U-¹⁴C]phenylalanine by unlabelled phenylalanine that might be present in the chicken cell sap.

One of the several points in the protein biosynthetic process at which inhibition could occur is

Fig. 2. Incorporation of radioactivity from poly[³H]-uridylic acid into acid-soluble form by the cell-free phenylalanine-incorporating systems derived from rat (○) or chicken (●) liver. Poly[³H]uridylic acid (100 μg., 90 000 counts/min.) in 0.1 ml. of buffer was added to the cell-free system (minus labelled phenylalanine) described in the Materials and Methods section. The final volume was 1.0 ml. Samples (0.1 ml.) were removed at the times indicated and counted as described in the Materials and Methods section.

Table 3. *Degradation of polyuridylic acid by components of liver homogenates*

Reaction conditions and non-liver components were those of the amino acid-incorporating system described in the Materials and Methods section, with L-[U-¹⁴C]phenylalanine omitted and unlabelled polyuridylic acid replaced by poly[³H]uridylic acid. Liver components were added as indicated. Samples (0.1 ml.) were counted at the times indicated as described in the Materials and Methods section.

Liver component added	% of radioactivity rendered acid-soluble	
	5 min.	30 min.
None	0	0
Rat liver cell sap	20	60
Rat liver DOC-FII ribosomes	40	95
Chicken liver cell sap	70	98
Chicken liver cell sap (diluted 1:10)	20	67
Chicken liver DOC-FII ribosomes	50	85

the formation of the ribosome-messenger-RNA-aminoacyl-transfer-RNA complex. Degradation of polyuridylic acid, the synthetic messenger RNA, in the presence of chicken liver cell sap might explain the decreased incorporation in systems containing this cell sap. This possibility was evaluated by measuring the extent of the incorporation of radio-

Table 4. *Degradation of polyuridylic acid by homologous liver systems*

Experimental details were as described in Table 3 and the legend to Fig. 2.

Liver components added		% of radioactivity rendered acid-soluble		
		5 min.	15 min.	30 min.
Ribosomes	Cell sap			
—	Rat	35	49	67
Rat	—	42	53	81
Rat	Rat	35	53	69
—	Chicken	70	—	98
Chicken	—	76	92	96
Chicken	Chicken	87	93	98

Table 5. *Degradation of polyuridylic acid by heterologous liver systems*

Experimental details were as described for Table 4.

Liver components added		% of radioactivity rendered acid-soluble		
		5 min.	15 min.	30 min.
Ribosomes	Cell sap			
Chicken	Rat	58	81	90
Chicken	Rat+chicken	81	96	99
Chicken	Rat+chicken (diluted)*	75	90	98

* Chicken liver cell sap diluted 1:1 with TKM-sucrose buffer.

activity from labelled polyuridylic acid into acid-soluble form, a measure of the hydrolytic degradation of the polyuridylic acid, in cell-free systems identical with those used for studies of phenylalanine incorporation.

When poly[³H]uridylic acid was incubated with a complete cell-free system from either rat or chicken liver, the results shown in Fig. 2 were obtained. After 5 min. of incubation over 80% of the acid-insoluble radioactivity had been rendered acid-soluble in the chicken system, whereas only about 30% had been solubilized in the rat system. The results of attempts to assign this polyuridylic acid-degrading activity to separate components of the cell-free preparations are shown in Table 3. Ribosomes from both rat and chicken liver exhibit nuclease activities of about the same order of magnitude. The nuclease activity of chicken liver cell sap is significantly greater than that of rat liver cell sap, and indeed this seems to be the most active cell fraction. Even when diluted 1:10 it was still quite active. Table 4 shows the results of experiments in which the nuclease activities of homologous cell sap and ribosome combinations were measured. Here,

too, it is apparent that the chicken liver systems were much more active than those from rat liver. It is noteworthy that in systems derived from the rat the reconstituted ribosome plus cell sap system was less active in polyuridylic acid degradation than were the ribosomes alone. This was not true of the chicken system. When heterologous combinations of cell sap and ribosomes were studied, the results shown in Table 5 were obtained. The presence of chicken liver cell sap increases the degradative activity significantly, particularly at shorter reaction times. It also appears that the nuclease activities of ribosomes and rat liver cell sap are not additive. In fact, rat liver cell sap inhibited the nuclease activity of both rat and chicken liver ribosomes.

These findings indicate that the degradation of polyuridylic acid in the complete amino acid-incorporating system is much more pronounced when soluble components from chicken rather than those of rat liver are used. It is apparent that chicken liver cell sap contains a potent ribonuclease (or at least a 'polyuridylic acid-ase') and that this degradative activity is dependent on the concentration of the chicken liver cell sap. Other liver cell components exhibit nuclease activity, but with concentrations of these components equivalent to concentrations used in the amino acid-incorporation studies the amount of nuclease activity was less than that of the chicken liver cell sap.

DISCUSSION

The results obtained from the polyuridylic acid degradation experiments show an interesting correlation with the inhibition of phenylalanine incorporation by chicken liver cell sap. One possible explanation for the parallel results is that the polynucleotide, which serves as messenger, is degraded to such a large extent by chicken liver cell sap that the concentration remaining is insufficient to direct amino acid incorporation to the degree that obtains in experiments with rat liver cell sap. However, since the degradation of polyuridylic acid by chicken liver cell sap appears to depend on concentration of cell sap, it becomes necessary to explain why the phenylalanine incorporation remains the same with increasing amounts of chicken liver cell sap. If it is assumed that incorporation of the amino acid is dependent on the amount of polynucleotide present, and the amount of polynucleotide present is inversely dependent on the amount of chicken liver cell-sap 'nuclease' present, then larger amounts of cell sap should decrease the amount of incorporation. Indeed, if the rate at which polyuridylic acid is degraded in the presence of chicken liver cell sap is compared with the rate in the rat liver system, it is surprising that there is as much phenylalanine

incorporation in the chicken liver system as the results indicate.

The same paradox appears in a comparison of the phenylalanine incorporation and polyuridylic acid degradation results obtained with systems containing rat liver cell sap and various amounts of chicken liver cell sap. Addition of increasing amounts of chicken liver cell sap to rat liver cell sap caused an increase in the rate and extent of degradation of polyuridylic acid, a result that is reasonable if one equates chicken liver cell sap with nuclease activity. However, though the addition of chicken liver cell sap to the incorporating system containing rat liver cell sap inhibited incorporation, addition of twice the amount of chicken liver cell sap did not increase the inhibition of incorporation to a corresponding extent. Instead this doubling had no significant effect. Again, if incorporation is directly related to the amount of undegraded polynucleotide remaining, one would expect a doubling of the amount of chicken liver cell sap to produce a further decrease in phenylalanine incorporation.

A simple extension of the explanation given above for inhibition of incorporation by chicken liver cell sap might resolve the above paradoxes. It can be proposed that the amount of polyuridylic acid conventionally used in incorporation studies represents a large molar excess over the concentration of ribosomes and that only that fraction of the polynucleotide that is bound to the ribosomes is responsible for directing phenylalanine incorporation. Some polynucleotide would be bound in the presence of chicken liver cell sap; this bound messenger might then be relatively insensitive to the nuclease (Takanami & Okamoto, 1963) and would be able to direct phenylalanine incorporation. The difference in the extent of degradation observed with the rat liver cell sap system and the combined rat liver and chicken liver cell sap system is relatively large. The difference in the extent of degradation between systems with rat liver cell sap combined with two different concentrations of chicken liver cell sap is not very large, however, and there might be no detectable difference between the amounts of phenylalanine incorporated by these systems.

To recapitulate the evidence for and against a correlation between ribonuclease activity of chicken liver cell sap and the inhibitory effect by the cell sap on incorporation, it is shown that: (1) chicken liver cell sap contains a potent polyuridylic acid-degrading activity; (2) cell-free systems containing chicken liver cell sap exhibit both inhibition of polyuridylic acid-directed phenylalanine incorporation and a marked ability to degrade polyuridylic acid; (3) rat liver cell sap inhibits the nuclease activity associated with both rat and chicken liver ribosomes and promotes phenylalanine incorporation; (4) though the degradation of polyuridylic acid

shows an expected dependence on chicken liver cell sap concentration, addition of increasing amounts of this cell sap to amino acid-incorporating systems results in no corresponding increase in the inhibition of polypeptide synthesis.

No anatomical studies have been reported indicating that compartmentalization of liver ribonuclease into organelles such as lysosomes exists to a greater extent in chicken liver than in rat liver. However, there is a report that, in contrast with livers from many species, chicken liver contains no ribonuclease inhibitor (Roth, 1962). Such an inhibitor has been recognized and partially purified from rat liver (Roth, 1958; Shortman, 1961), and the present work demonstrates the presence of a substance in rat liver cell sap that inhibits nuclease activity in both rat and chicken liver ribosomes. Chicken liver cell sap appears to contain much less of this inhibitor.

The present results serve as evidence for a correlation between the relatively poor incorporation of phenylalanine in the chicken liver cell-free system and the nuclease activity associated with the components of the system.

It is interesting to speculate about the role of such nucleases and nuclease inhibitors in the overall mechanism of protein biosynthesis. It would appear that the nucleases function to break down messenger RNA at a rate that would limit its persistence and hence would limit the repetitive synthesis of protein molecules. The elaboration of nuclease inhibitors would prevent this and would act to extend the time during which individual messenger RNA molecules are able to direct protein synthesis. Such a balanced system could provide for extremely fine control of the rate and extent of protein synthesis. The observation by Carey (1964) that extracts of

embryo chicken liver are significantly active in amino acid incorporation would indicate that such a control system could play a significant role during development.

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REFERENCES

- Bollum, F. J. (1966). In *Procedures in Nucleic Acid Research*, p. 296. Ed. by Cantoni, G. L. & Davies, D. R. New York: Harper and Row.
- Bray, G. A. (1960). *Analyt. Biochem.* **1**, 279.
- Campbell, P. N. & Kernot, B. A. (1959). *Biochem. J.* **72**, 6 p.
- Carey, N. H. (1964). *Biochem. J.* **91**, 335.
- Heald, P. J. & Pohlman, D. (1966). *Biochim. biophys. Acta*, **123**, 390.
- Howell, R. R., Loeb, J. N. & Tomkins, G. M. (1964). *Proc. nat. Acad. Sci., Wash.*, **52**, 1241.
- Hultin, T. (1950). *Exp. Cell Res.* **1**, 3.
- Hunter, A. R. (1967). *Biochem. J.* **105**, 44 p.
- Lawford, G. R., Langford, P. & Schachter, H. (1966). *J. biol. Chem.* **241**, 1835.
- Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 448. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Roth, J. S. (1956). *Biochim. biophys. Acta*, **21**, 34.
- Roth, J. S. (1958). *J. biol. Chem.* **231**, 1085.
- Roth, J. S. (1962). *Biochim. biophys. Acta*, **61**, 903.
- Shortman, K. (1961). *Biochim. biophys. Acta*, **51**, 37.
- Takanami, M. & Okamoto, T. (1963). *J. molec. Biol.* **7**, 323.