PROTEIN SYNTHESIS IN PANCREAS OF FASTED PIGEONS

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ABSTRACT

The regulation of protein synthesis in the pigeon has been studied by comparing the capability of cell-free amino acid incorporating systems of membrane-bound and membrane-free polysomes prepared from fasted and fed birds. New methods were developed for isolating polysomes since techniques used for other tissues did not provide quantitative recovery of polysomal RNA. The sucrose gradient profile of polysomes from pigeon pancreas showed a predominance of trisome species. Although initiation factors are present on polysomes, it was found that polysomes in cell-free systems would not initiate protein synthesis without exogenous initiation factors. This suggested the presence of an inhibitor or regulator of protein synthesis. These studies show that fasting resulted in: (*a*) decreased amounts of polysomes; (*b*) disaggregation of polysomes to monosomes; (*c*) decreased capability of polysomes to synthesize nascent peptides and to initiate additional synthesis, apparently not related to concentration of initiation factors.

INTRODUCTION

The exact mechanisms of control of changes in synthetic and secretory phenomena in pancreas under fasting conditions are not well understood. Early evidence (1, 2) has indicated that the pancreas might be synthesizing proteins continually, regardless of the secretory activity. More recent observations (3–5), however, have shown that protein synthesis does not occur at a continuous rate. During fasting, for instance, Veghelyi and Kemeny (6) have reported a decrease in digestive enzyme activity and content. On the other hand, Stenram and Hirschman, by means of autoradiography (7), observed increased protein labeling suggesting enhanced synthesis. Recently we have shown (8, 9) that slices of pancreas from fasting rats undergo not only a loss in total protein and RNA content but a loss in protein synthesizing capability. Further, we have shown (9, 10) that in rats and pigeons fasting results in a lower incorporation of amino acids into protein and that this appeared to involve changes in both the microsomal and supernatant elements. To more fully understand the events that occur during fasting, we have studied this effect in more detail.

Evidence presented in this paper indicates that protein synthesis in the pancreas is highly regulated. Fasting results in a decreased protein synthesis due, in part, to (*a*) reduction in polysome content, (*b*) reduction in capability of the polysome to synthesize protein, and (*c*) disaggregation of the polysomes.

MATERIALS AND METHODS

White Carneau pigeons, 6–8 wk of age, weighing 450–500 g, were used in these experiments. The birds were fed ad lib. or fasted for 72 h before sacrifice. In

all experiments the pigeons were decapitated; the pancreas was removed and placed in cold buffer solution.

Polyribosomes were isolated initially by two different methods: one was a modification (11) of the method of Wettstein et al. (12) and Noll et al. (13), and a second method is described below. With the modified Wettstein method, pancreases were homogenized in 2 vol of cold TMN buffer (0.03 M Tris-HCl, pH 7.5; 0.15 M NH_4Cl; and 3.5 mM $MgCl_2$) made 0.1 M in sucrose (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.; ribonuclease-free) containing 2 mg/ml coarse bentonite (Fisher Scientific Company, Pittsburgh, Pa.; U.S.P.). The homogenate was centrifuged at 12,000 *g* for 10 min. With this procedure, the upper two thirds of the supernatant was discarded and the lower third was made 1.5% final concentration in sodium deoxycholate. This latter solution was layered over 5 ml of a discontinuous gradient made up as follows: 2.3 ml of 0.5 M sucrose and 2.7 ml of 1.5 M sucrose (sucrose solutions were made up in the above TMN buffer). The gradients were centrifuged in a Beckman 50 rotor at 41,500 rpm for 5 h and the resultant pellets were rinsed in TMN buffer.

To determine whether ribonuclease activity might be present and thus have an effect on polyribosomal patterns, experiments using the following ribonuclease inhibitors were performed: bentonite (4 and 6 mg/ml); macaloid, 1% (Baroid Division National Lead Company, Houston, Tex.); 0.5% sodium dodecyl sulfate (SDS); or 0.5% SDS with 2 mg/ml of yeast RNA. All of these experiments showed that ribonuclease activity was not present in sufficient quantity to affect the polysomal sucrose gradient patterns.

Using the modified Wettstein method with pancreas, we were able to recover only 25–30% of total RNA as ribosomal RNA. Blobel and Potter (14), however, recovered about 80% of total RNA as ribosomal RNA. Because of extensive rough endoplasmic reticulum found in pancreas, we considered it likely that heavy membrane-bound polyribosomes were being removed in the initial centrifugation steps. Thus, in an effort to increase percent recovery of ribosomal RNA, a second method of isolation was devised. The homogenate was treated with sodium deoxycholate (DOC) in a final concentration of 1.5% to release bound ribosomes before initial centrifugation steps. The homogenate was then centrifuged at 12,000 *g* for 10 min. With this procedure, the entire supernatant was layered over the discontinuous gradient described above. Polyribosomes isolated by this method were similar to those isolated by the other method insofar as sucrose gradient patterns and activities in amino acid incorporating systems were concerned. Since the DOC method resulted in increased recovery of polysomal RNA, it was used for experiments reported in this manuscript. RNA samples were washed as previously described (15) and assayed by the orcinol method (16).

For polysomal sucrose gradients, 25 A_{260} U of polysomes (1 A_{260} U was found to be equivalent to 8 μg RNA in pigeon pancreas polyribosomes) were layered over a 10–40% linear sucrose gradient made with a Beckman density gradient device. These gradients were centrifuged in an SW-27 rotor at 25,000 rpm for 3 h. The gradient 260 nm absorbance profile was determined by puncturing the tube from the bottom and passing the contents through a flow cell in a Gilford 240 spectrophotometer with a chart recorder.

The amino acid incorporation system of Redman et al. (17) was used as reported previously (9). For those experiments utilizing endoplasmic reticulum (ER), the final pellet in Redman's procedure was resuspended in 0.44 M sucrose with light homogenization and recentrifuged to further remove soluble components. The incubation mixture contained 1 μmol of ATP, 0.5 μmol of GTP, 10 μmol of freshly prepared phospho-enol-pyruvate (Sigma Chemical Co., St. Louis, Mo.), 160 μg of pyruvate kinase (Sigma Chemical Co., Type II), 20 μmol of KCl, 5 μmol of $MgCl_2$, 50 μmol of Tris-HCl (pH 7.4), 1 μCi of [L-^{14}C]-phenylalanine (2.4 pmol; New England Nuclear Corp., Boston, Mass.), 0.5 mg of pH 5 fraction (21), and either 5 mg ER, 4 mg polysomes, or 2 mg salt-washed ribosomes. Incubation was carried out at 37°C for 30 min. Protein was precipitated with 10% TCA containing unlabeled phenylalanine (1%). The precipitate was washed with 5% TCA (with unlabeled phenylalanine) and heated at 90°C for 20 min. After centrifuging this mixture, the pellet was washed once with 5% TCA, once with 95% ethanol, and twice with an ethanol-ether mixture (3:1). The final pellet was resuspended in 1 ml of 0.3 *N* KOH and heated at 90°C for 20 min. Samples were assayed for protein by the biuret method (18) using bovine serum albumin as standard and for radioactivity by liquid scintillation techniques reported previously (19).

Polysomes were washed in 0.5 M KCl by the method of Miller and Schweet (20) for the preparation of both the washed ribosomes and the initiation factors (IF). IF were additionally passed through Sephadex G-75 and the excluded protein fraction was used. Preliminary experiments had indicated that the amino acid incorporation capacity of washed ribosomes was dependent on the concentration of IF added.

RESULTS

The effects of DOC treatment on the recovery of ribosomal RNA by two different methods of isolation are shown in Table I. Table I indicates that

TABLE I

*Recovery of Cellular Ribonucleic Acid Isolated by Two Different Methods**

Fraction	Modified Wettstein method		DOC-treatment method	
	mg RNA/g tissue	Percent of homogenate	mg RNA/g tissue	Percent of homogenate
12,000 *g* pellet	1.71	39	0.32	7
Polysomes	1.46‡	33	3.05§	69
Supernatant	0.15	4	0.13	3
Total recovered		76		79

* Pancreases from five fed pigeons were homogenized in TMN buffer containing bentonite as outlined in Materials and Methods. The homogenate, which contained 4.43 mg RNA/g tissue was divided; one half was processed by the modified Wettstein method and the other half was made 1.5% in DOC and processed as described in Materials and Methods. The data above is the mean of three experiments using pooled pancreases from five pigeons each. The 12,000 *g* pellet refers to the pellet resulting after the 12,000 *g* centrifugation step. The supernatant refers to the supernatant fraction remaining after the 105,000 *g* centrifugation step.

‡ This is 44% of the total RNA recovered in the three fractions.

§ This is 87% of the total RNA recovered in the three fractions.

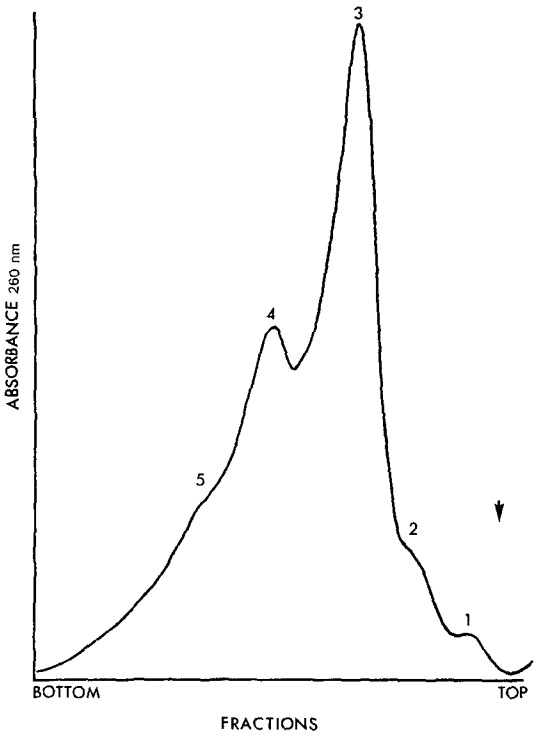

FIGURE 1 Graph showing the polyribosomal centrifugation pattern from fed pigeon pancreases. The graph shows 10–40% linear sucrose gradient running from right to left. Numbers 1–5 represent the number of ribosomes per polysome as determined by comparison to rat liver preparations. The arrow represents the position of a 60 S ribosomal subunit run under identical conditions as a marker. Further details are outlined in the Materials and Methods section.

total recovery of cellular RNA in the three fractions was essentially the same, 76% and 79%. However, release of ribosomes with DOC before the first centrifugation step results in recovery of almost twice the amount of RNA as polysomes, an increase from 33% to 69%. The first pellet shows a decline from 39% to 7% in RNA content. This DOC-treatment method results in a percentage recovery more closely approaching that found by Blobel and Potter (14) than did the modified Wettstein method. Samples of the first and polysomal pellets from both treatments were examined by electron microscopy. Polysomal pellets were free of contaminating organelles in both cases. The first pellet from the modified Wettstein method contained considerably more membrane-bound ribosomes than the corresponding DOC-treated pellet, thus confirming the RNA measurements. Treatment of the homogenate with DOC before the first centrifugation step had no effect on the polysomal sucrose gradient profile (Fig. 1). This figure also shows that the trisome species was the predominant form of polyribosome. Doubling and tripling amounts of bentonite and use of sodium dodecyl sulfate and macaloid did not substantially alter the patterns. This indicates that these patterns were not artifacts of ribonuclease activity. Further, minimal degradation is indicated by the fact that increases in monosomes and disomes are not seen although such increases do occur with fasting (Fig. 2). Breillatt and Dickman (22) have shown that when degradation occurs the monosome and disome peaks increase markedly. The

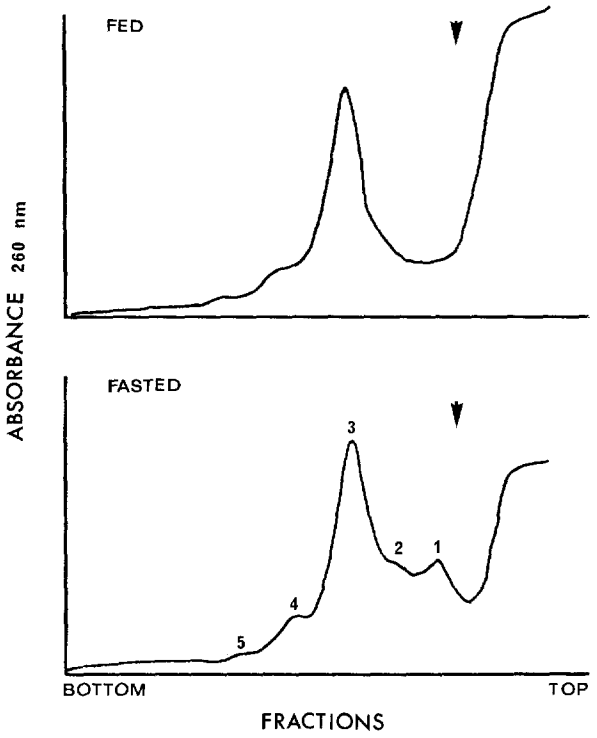

FIGURE 2 Graph showing the polyribosomal centrifugation pattern from fed and fasted pigeon pancreases. The 10–40% linear sucrose gradients were run from right to left. The numbers represent the number of ribosomes per polysome, as in Fig. 1. The arrows represent the position of a 60 S ribosomal subunit run under identical conditions.

TABLE II

*Recovery of Polysomal Ribonucleic Acid in Fasted and Fed Birds**

Fraction	Fed		Fasted		Percent of change
	mg RNA/g tissue	Percent of homogenate RNA	mg RNA/g tissue	Percent of homogenate RNA	
Homogenate	26.30 ± 1.88		14.79 ± 1.11		−44
12,000 *g* pellet	3.55 ± 0.69	14	1.52 ± 0.29	10	
Polysomes	20.46 ± 0.95	78	11.25 ± 1.09	76	−45
Supernatant	1.35 ± 0.08	5	0.96 ± 0.14	6	
Total recovered		97		92	

* Pancreases from nine fasted and six fed pigeons were each pooled and fractions isolated by the DOC method as described in Materials and Methods. The data above is the mean ± SE of three experiments. The 12,000 *g* pellet refers to pellet from 12,000 *g* centrifugation step. Supernatant refers to that supernatant fraction remaining from the 105,000 *g* centrifugation step.

number of ribosomes per polysome was determined by comparison to rat liver polyribosomes isolated concurrently, by comparison to a 60 S ribosome subunit marker run concurrently, as well as from mixing experiments (not shown).

Changes with fasting in polysomal RNA are shown in Table II. This table indicates that with a greater than 90% recovery of all cellular RNA, polysomal RNA from both fasted and fed pigeons amounts to 78 and 76% of the total. The table

also shows that polysomal RNA decreases 45% with fasting. This decrease reflects the 44% decrease in total cellular RNA.

The polyribosomal sucrose gradient patterns from fed and fasted pigeon pancreases are shown in Fig. 2. With fasting, there was an increase in amounts of monosomes and disomes. Although amounts of monosomes and disomes varied widely among individual fasted pigeons, there were consistently more monosomes and disomes observed in tissue from fasted compared with tissue from fed birds.

In Table III, comparison is made of the abilities of ER from fed and fasted birds to respond to IF in an amino acid incorporating system. This table shows: (*a*) amino acid incorporation, using ER from fasted birds, was 33% less than ER from fed birds; (*b*) systems prepared from fasted and fed birds did not incorporate additional amino acids with added poly U (although IF are presumed to be present on the ribosome); (*c*) both systems incorporated additional amino acids with exogenous RNA (poly U) if additional IF were added; amino acid incorporation was approximately 38% less in fasted than in fed systems; and (*d*) the effects seen above were not due to gross differences in amounts of RNA present in the assay.

To determine to what extent the above experimental results represent a function of attachment of polysomes to the ER, we tested the amino acid incorporating activities of membrane-free polysomes prepared from fasted and fed birds. This data, Table IV, shows: (*a*) that the amino acid incorporation system of fasted birds incorporated 23% less amino acid than did the fed system; (*b*) as in Table III, neither the fasted nor the fed systems incorporated additional amino acids with exogenous messenger; (*c*) both systems incorporated additional amino acids with added IF; however, this incorporation was 94% less in fasted compared with fed systems.

Comparing Table III with Table IV, it is apparent that: (*a*) on a per milligram RNA basis, membrane-bound polysomes were about four to five times more effective in incorporating amino acids than membrane-free polysomes; (*b*) the membrane-free fed systems responded about two

TABLE III

*Fasted and Fed Endoplasmic Reticulum Amino Acid Incorporation Systems**

Additions	Incorporation	
	dpm/mg protein ± SE	dpm/mg RNA ± SE
Fed		
(*a*) None	1,339 ± 172	15,216 ± 1,955
(*b*) Poly U	1,343 ± 195	15,261 ± 2,216
(*c*) Poly U + IF	1,874 ± 216	21,295 ± 2,455
$\frac{c-b}{a}$	0.40	0.40
Fasted		
(*a*) None	898 ± 172	10,565 ± 2,024
(*b*) Poly U	943 ± 175	11,094 ± 2,059
(*c*) Poly U + IF	1,167 ± 222	13,729 ± 2,612
$\frac{c-b}{a}$	0.25	0.25
Differences (fasted and fed [%])		
a	−33	−30
$\frac{c-b}{a}$	−38	−25

* Endoplasmic reticulum was isolated from pancreases of fasted and fed pigeons as outlined in Materials and Methods. Incubation mixture contained 5 mg endoplasmic reticulum protein; 0.8 mg poly U; IF factors 175 μg protein; and 0.5 mg pH 5 fraction. Values given are the means ± SE of three fed and four fasted experiments.

TABLE IV

*Fasted and Fed Polyribosomal Amino Acid Incorporation System**

Additions	Incorporation	
	dpm/mg protein ± SE	dpm/mg RNA ± SE
Fed		
(*a*) None	979 ± 74	3,059 ± 232
(*b*) Poly U	963 ± 54	3,009 ± 169
(*c*) Poly U + IF	1,824 ± 144	5,700 ± 450
$\frac{c-b}{a}$	0.88	0.86
Fasted		
(*a*) None	750 ± 4	2,500 ± 13
(*b*) Poly U	756 ± 24	2,520 ± 80
(*c*) Poly U + IF	795 ± 79	2,650 ± 263
$\frac{c-b}{a}$	0.05	0.06
Differences (fasted and fed [%])		
a	−23	−18
$\frac{c-b}{a}$	−94	−93

* Polyribosomes isolated from fed pigeons and pigeons fasted 72 h as described in Materials and Methods. Incubation conditions same as Table III with mg polyribosomal protein per assay. Values given are the means of three experiments ± SE.

times greater to IF than did the membrane-bound systems; (*c*) membrane-free fasted systems were five times less effective in initiating new protein synthesis with addition of IF; and (*d*) changes brought about by fasting did not appear to be due to lack of IF since the addition of IF to fasted systems failed to produce higher rates of incorporation than was observed in fed systems.

DISCUSSION

Polysomal sucrose gradient profiles of many tissues and especially canine pancreas (22) show a predominance of polysomes containing five and six ribosomes per polysome. Means et al. (23) have shown that polyribosomes from rat testicular tissue exhibit a preponderance of trisome species. Thus, it is of interest to note that pigeon pancreas also has a predominance of three ribosomes per polysome suggesting that the predominant protein(s) being synthesized are coded for by the messenger RNA of these trisomes. Work of Staehelin et al. (24) suggests that the molecular weight of messenger RNA on a polysome is directly related to the number of ribosomes in the aggregate. Further, these authors state that there are approximately 90 nucleotides per ribosome or 30 amino acid codons per ribosome. Thus, a trisome would be expected to contain approximately 270 nucleotides or 90 amino acid codons. After the above author's analogy with the hemoglobin chain synthesized on pentosomes, one can compute the molecular weight of the protein coded for by trisome messenger RNA to be around 9,000. Danielsson (25) has shown the molecular weight for pig pancreas α-amylase to be 45,000 while molecular weights of other pancreatic digestive enzymes have been shown (26) to be in the range of 20,000–25,000. Thus, theoretical considerations would suggest the possibility that trisome messenger RNA codes for the synthesis of subunits of one or more of the pancreatic enzymes. Alternatively, there may be greater molecular weight of mRNA per ribosome in pigeon pancreas than shown in the work of Staehelin et al. (24). That is, rather than having an mRNA molecular weight of around 80,000 for the trisome species, as suggested by Staehelin et al., pigeon pancreas trisome may have mRNA molecular weight around 420,000. Effort is underway in this laboratory to further investigate this problem.

We have previously shown in pancreas of rats fasted for up to 96 h a decrease in total RNA, protein, and capacity to synthesize proteins (8). In addition, rates of incorporation of [^{3}H]uridine into RNA were lower in pancreatic tissue slices prepared from fasted compared with fed birds (15). Preliminary experiments (9) suggested that particulate factors concerned with protein synthesis (in vitro) were more affected by fasting than were soluble factors. In the experiments reported here, there were decreases in amounts of polysomal RNA as well as a shift to monosomes and disomes. Sox and Hoagland (27) have shown that monosomes and disomes are less active than polysomes in protein synthesis. Other laboratories have reported similar results derived from studies of hepatic tissue of fasted animals (27–32). It is apparent from the data in this report that fasting resulted in both quantitative and qualitative changes in the protein synthesizing apparatus.

Initiation factors are present on polysomes and can be removed with a high salt wash (20, 23, 33). Addition of exogenous messenger (poly U), therefore, should result in increased amino acid uptake. The data in Tables III and IV show that poly U did not cause an increase. There are at least two possible reasons: (*a*) some rate limiting factor(s) are not present, or (*b*) initiation is controlled or regulated by factor(s), such as glutathione, suggested by others (33–35). The increase noted with the addition of IF tends to support the first possibility; however, IF are already present on polysomes which support the second possibility. The existence of a regulator is suggested by preliminary experiments, by reports from this laboratory, and by reports in the literature (10, 33). We have shown that amino acid incorporation in a cell-free system prepared from pancreas was complete within 5 min (10), whereas incorporation in cell-free systems prepared from other tissue (23) was nearly linear for up to 60 min. Comstock et al. (33) have shown that oviduct polyribosomes do not accept poly U while KCl-washed ribosomes do accept poly U. In preliminary experiments, we have found that washed ribosomes incorporate amino acids for at least 40 min, whereas unwashed ribosomes incorporate amino acids for only 5 min. Whether or not the substance removed is the inhibitor suggested by others (33–35) is not known at this time. Further work is underway to determine the nature of this substance and its mechanism of action in the pancreas.

A qualitative difference in polysomes prepared from fed and fasted animals is suggested since they did not initiate protein synthesis to the same degree when soluble enzymes, amino acids, initiation factors, mRNA and cofactors were added in excess. Henshaw et al. (32) have shown that in fasted rat liver, ribosomes are altered both in activity and in proportion of polysomes to monosomes. The greater decrease of incorporation with membrane-free polysomes prepared from fasting animals suggests greater instability of these polysomes. The presence of inactive monosomes in fasted systems could explain some of the decrease in amino acid synthesizing capability observed in fasting systems. Coupled with the above possibilities is the probability of decreased concentration of mRNA in fasted systems.

We see that protein synthesis in the pigeon pancreas appears to be a well regulated system. Addition of exogenous messenger does not increase amino acid incorporation unless excess IF are added, although IF are presumed to be present. Membrane-free systems, compared to membrane-bound systems, show greater loss of ability to initiate protein synthesis with fasting. Removal of the membrane does not alter the inhibitory or regulatory phenomenon. Preliminary evidence indicates this regulatory phenomenon might be due to the presence of factors removed in high salt washes. Fasting was associated with decreases in amounts of polysomal RNA available for protein synthesis as well as decreases in synthesizing capability of remaining polysomes. This does not appear to be due to a decrease in concentration of IF but could be partially due to disaggregation of polysomes with resulting increase in inactive monosomes. Decreases in concentrations of mRNA, not measured in this study, could contribute to the above phenomenon.

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