Effects of Fasting, Feeding, and Bethanechol Chloride on Pancreatic Microsomal Protein Synthesis *in Vitro*[1,2] (36664)

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Introduction. It has been shown that feeding, bethanechol chloride, and cholecystokinin-pancreozymin administration were associated with augmentation of pancreatic protein synthesis in rats and pigeons (1, 2). Increases in protein synthesis following bethanechol chloride and cholecystokinin-pancreozymin administration were of 1–4 hr duration, were not completely blocked by actinomycin-D pretreatment, and were not associated with parallel increases in incorporation of [^{3}H] uridine into nuclear-RNA (3–5). These characteristics suggested that "short-term" increases in pancreatic protein synthesis might be mediated at the translational level of control. On the other hand, augmentation of protein synthesis following refeeding was associated with increases in both [^{14}C]-L-phenylalanine incorporation into protein and [^{3}H] uridine incorporation into nuclear-RNA (3, 6). These characteristics suggested that "long-term" increases might be mediated by changes occurring at both translational and transcriptional levels of control.

Protein synthesis at the translational level is influenced by amounts and activities of a number of constituents, such as amino acids, activating enzymes, *t*-RNA, initiating and terminating factors, monosomes, and polysomes. These cytoplasmic constituents concerned with protein synthesis at the translational level may be separated by use of high speed centrifugation into soluble and particulate fractions. The soluble or supernatant fraction contains factors such as amino acids, activating enzymes, *t*-RNA, etc.; the particulate or ribosomal fraction contains monosomes, polysomes, and constituents precipitated therewith.

The experiments presented here were designed to determine if increases in pancreatic protein synthesis following feeding or bethanechol chloride administration resulted from changes in factors isolated in the supernatant or particulate fraction of cytoplasm. It is anticipated that these simple experiments employing cell fractionation by high speed centrifugation and ordered *in vitro* incubation of cell sap and particulate fractions will provide preliminary information which will enable additional investigations directed at identification and isolation of selected constituents, *i.e.*, changes in *t*-RNA, changes in activating enzymes or changes in ratios of monosomes–polysomes.

Materials. A mixture of 19 amino acids, ATP, GTP, PEP, pyruvate kinase, polyuridylic acid, and gelatin were purchased from Sigma Chemical Company, St. Louis, MO. Bethanechol chloride (BC) was purchased from Merck, Sharp, and Dohme, West Point, PA. Uniformly labeled [^{14}C]-L-phenylalanine (0.37 mCi/μmole) was obtained from New England Nuclear Corporation, Boston, Massachusetts; [^{14}C]-amino acid mixture (UL) (1 mCi/mg) was obtained from International Chemical and Nuclear Corporation, Irvine, California. Sources of other materials have been described (2).

Methods. White Carneau pigeons (6–8 weeks of age; 500 g body weight) were obtained from Palmetto Pigeon Farms, Sumter,

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South Carolina. Animals referred to as fasted were denied food for 3 days; those referred to as "fed" had food in their cages at all times. All animals had free access to water.

Bethanechol chloride (BC) (2 mg/kg) was dissolved in physiologic saline and injected into a pectoral muscle.

Preparation of Tissue. For all studies the pigeons were decapitated in the morning, between 8:00 and 10:00 AM, the pancreas excised, fat and connective tissue removed, and the tissue placed in ice-cold Krebs–Ringer phosphate (KRP) buffer. All subsequent operations were performed at 4°.

Preparation of Microsomal and Supernatant Fractions. Pancreases were minced with scissors and then homogenized using a teflon Potter homogenizer (0.004 in. clearance) in 0.44 *M* sucrose. The homogenate (10% w/v) was centrifuged in a Sorval RC-2B and a Spinco L2-65B centrifuge to obtain microsomal and supernatant fractions as described by Redman *et al.* (7). The supernatant portion was aspirated, the tube drained, the microsomal pellet washed twice by swirling with 5 ml 0.25 *M* sucrose and the pellet resuspended (0.5 ml per g of tissue wet weight) using a teflon homogenizer in 0.25 M sucrose. The supernatant fraction was used as a source for activating enzymes and *t*-RNA.

Incorporation of [^{14}C]-L-phenylalanine into Microsomal Proteins. For these studies, microsomal and supernatant fractions made up in 0.25 *M* sucrose were incubated *in vitro* with the following: 1 μmole ATP, 0.6 μmoles GTP, 10 μmoles PEP, 200 μg pyruvate kinase, 200 μmoles KCl, 5 μmoles $MgCl_2$, 50 μmoles of Tris (pH 7.4), 8–9 mg microsomal protein, and 0.8–0.9 mg supernatant protein. To this incubation mixture was added 1 μCi of [^{14}C]-L-phenylalanine (UL), final volume, 1.0 ml. *In vitro* incubation was carried out for 10 min at 37°; the reaction was stopped by the addition of 2 ml 10% perchloric acid (PCA).

Preparation and washing of protein. The protein precipitate was washed initially with 10 ml of 10% TCA and heated for 20 min at 90°. The resulting precipitate was washed two times with 10 ml of 10% TCA, one time with 10 ml 95% ethanol and two times with 10 ml of ether–ethanol (3:1) mixture. The precipitate was dissolved in potassium hydroxide, 0.5 *N*, and a sample of the solution placed in a counting vial with a scintillation mixture developed by Patterson and Greene (10). Radioactivity was measured using a Packard Tri-Carb Liquid Scintillation Spectrophotometer.

Protein was assayed by the biuret or Lowry method (11, 12) using bovine serum albumin as standard. DNA was isolated by the method described by Schmidt and Thannhaus-

TABLE I. Incorporation of [^{14}C] L-Phenylalanine into Pigeon Pancreatic Microsomal Protein *In Vitro*.[a]

Composition of system	cpm/mg protein	% Change
Complete	9,113	100%
Boiled microsomes	25	
No ATP, PEP, PK	194	Inhibition
No Mg^{++}	185	
No supernatant	3,575	—61%
No GTP	6,326	—31%
No K^+	8,394	—8%

[a] An incubating flask contained 8.5 mg of microsomal protein, 0.85 mg of postmicrosomal supernatant protein, 1 μmole ATP, 0.6 μmoles GTP, 10 μmoles PEP, 200 μg of pyruvate kinase, 20 μmoles of KCl, 5 μmoles of $MgCl_2$, 50 μmoles Tris (pH 7.4), and 1 μCi of [^{14}C] L-phenylalanine. Incubation was for 10 min at 37°.

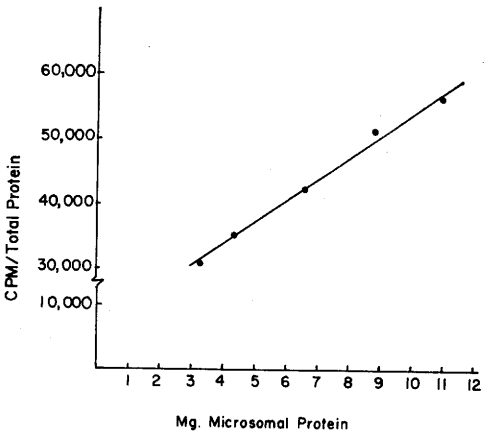

FIG. 1. Time course of incorporation of [^{14}C]-L-phenylalanine into microsomal protein. Incubation conditions described under Table I.

er and assayed by the diphenylamine method using calf thymus DNA as the standard (13, 14). RNA was determined by measuring absorption at 260 nm using a Gilford 240 spectrophotometer. Yeast RNA served as the standard.

Results. Characteristics of in vitro *microsomal incorporation system.* Table I, Figs. 1 and 2, depict characteristics of the *in vitro* incorporation system. Intact microsomes, an ATP generating system, and magnesium ions were required for significant incorporation of [^{14}C]-phenylalanine into protein. Compared with the complete system, deletion of supernatant resulted in a 61% decrease in incorporation; deletion of GTP and KCl reduced incorporation by 31% and 8%.

Figure 1 shows incorporation increased rapidly with increases in incubation time ranging from 1 to 3 min, whereas incorporation changed only slightly for periods of incubation ranging from 5 to 20 min. Linear increases in incorporation of [^{14}C]-phenylalanine were observed with increases in amounts of microsomal protein ranging from 3 to 11 mg (Fig. 2).

Influence of fasting, feeding, and bethanechol chloride administration on [^{14}C]-*phenylalanine incorporation* in vitro. For the studies presented in Table II, microsomal and supernatant fractions were prepared from pancreatic tissue of animals either fasted, fed, or pretreated with bethanechol chloride (BC). For facility throughout the paper, association of microsomal and supernatant fractions will be designed as a "set."

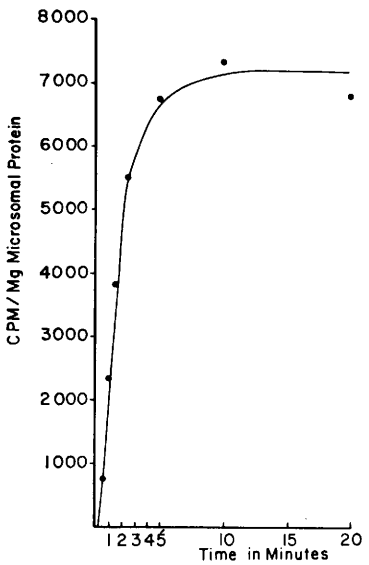

FIG. 2. Effect of increasing amounts of pancreatic microsomes on [^{14}C]-L-phenylalanine incorporation into TCA insoluble material. *In vitro* incubation conditions described in Table I.

For the first trio of experiments, microsomes prepared from fasted birds were incubated with supernatant prepared from fasted birds, fasted birds treated with bethanechol chloride (BC), or fed birds. Microsomes prepared from fasted pigeons incubated with supernatant from fasted pigeons (Set 1) incorporated 1116 ± 395 cpm into protein. Microsomes from fasted animals incubated with supernatant from fasted bethanechol treated animals incorporated 1255 ± 452 cpm/mg protein (Set 2). This 12.5% increase in [^{14}C]-amino acid incorporation into protein was statistically significant ($p < 0.01$) and appeared to result from factors isolated along with the supernatant portion. Similarly, microsomes from fasted pigeons incubated with supernatant from fed pigeons (Set 3) incorporated 1784 ± 730 cpm, a 59.9% increase when compared with Set 1 ($p < 0.02$). A comparison of Sets 2 and 3 with Set 1 suggests that increases were due to factors isolated in the supernatant fraction of fasted bethanechol treated or fed pigeons.

For the second trio of experiments (Sets 4–6), microsomes prepared from fasted pigeons pretreated with bethanechol chloride were incubated with supernatant fractions prepared from fasted, fasted-BC, or fed groups of pigeons. Microsomes from fasted-BC treated animals incubated with supernatant from fasted animals incorporated 951 ± 316 cpm. Microsomes from fasted-BC treated pigeons incubated with supernatant from fasted-BC treated groups incorporated 1256 ± 375 cpm, a 32% increase which was statistically significant ($p < 0.01$). A greater increase in incorporation was observed when microsomes from fasted-BC groups were incubated with supernatant from fed animals, 1784 ± 775 cpm, an 87.6% increase compared with Set 4. These three studies show

TABLE II. Effect of Fasting, Feeding, and Bethanechol Chloride (2 mg/kg IM) on [^{14}C]-L-Phenylalanine Incorporation into Protein by Microsomes and Supernatant from Pancreas.[a]

Set	Microsome		Supernatant	Cpm/mg microsomal prt Mean ± SD	% Diff	p
1	Fasted	+	Fasted	1116 ± 395		
					12.5	<.01
2	Fasted	+	Fasted bethanechol	1255 ± 452		
					59.9	<.02
3	Fasted	+	Fed	1784 ± 730		
4	Fasted bethanechol	+	Fasted	951 ± 316		
					32.0	<.01
5	Fasted bethanechol	+	Fasted bethanechol	1256 ± 375		
					87.6	<.02
6	Fasted bethanechol	+	Fed	1784 ± 775		
7	Fed	+	Fasted	4128 ± 1514		
					2.2	NS
8	Fed	+	Fasted bethanechol	4221 ± 1323		
					65.3	<.05
9	Fed	+	Fed	6824 ± 3111		

[a] Microsomes and supernatant were prepared from pancreata of pigeons fasted, fed, or fasted and given BC. Conditions of incubation are the same as those described in Table I.

that supernatant prepared from fasted bethanechol-treated or fed animals facilitated incorporation by microsomes from fasted-BC treated groups to a greater degree than supernatant from fasted animals.

The last trio of experiments (Sets 7–9) incubated microsomes prepared from fed pigeons with supernatant prepared from fasted, fasted-BC, or fed birds. Interchange of supernatant from fasted to fasted bethanechol animals did not influence amounts of ^{14}C-label incorporated. On the other hand, incubation of fed microsomes with fed supernatant yielded values of 6824 ± 3111 cpm. The 65% increase compared to Set 7 suggests increases resulted from factors isolated in the supernatant portion.

An appreciation of the role of factors isolated with microsomes may be obtained by comparing data obtained when similar supernatant fractions were incubated with different microsomal fractions. Matching Sets 1, 4 and 7, Sets 2, 5 and 8, or Sets 3, 6 and 9 (Table II) provide such comparision. Microsomes in Sets 1 and 4 incorporated [^{14}C]-amino acids to about the same degree (1116 vs 951). Comparison of fasted microsomes (Sets 1 and 4) with fed microsomes (Set 7) shows a 255% increase in incorporation (1116 vs 951 vs 4128). Such an increase suggests that microsomes isolated from fed animals have greater capabilities for incorporation of [^{14}C]-amino acids than microsomes from fasted animals. Microsomes from fasted (Set 2) and fasted-BC treated pigeons (Set 5) incorporated [^{14}C]-amino acids about the same level. Microsomes from fasted and fasted-BC groups (Sets 3 and 6) incorporated [^{14}C]-amino acids about the same degree; however, microsomes from fed animals (Set 9) compared with microsomes from fasted and fasted bethanechol animals (Sets 3 and 6) showed significant increases in incorporation (1784 vs 6824).

The data presented in Table II show that augmentation of [^{14}C]-amino acid incorporation obtained with fed birds or bethanechol chloride administration to fasted birds resulted from changes in factors which were isolated in both the supernatant and microsomal fractions. Enhancement of incorporation following bethanechol chloride adminis-

E III. Effect of Bethanechol Chloride (2 mg/kg IM) on *In Vitro* Incorporation of [^{14}C]-L-Phenylalanine by Microsomes and Super
Fed Pigeons.[a]

Microsome		Supernatant	Killing time				
			15 min		30 min		60 min
			Cpm/mg microsomal protein				
			Mean ± SD	*p*	Mean ± SD	*p*	Mean ± S
Fed	+	Fed	8254 ± 1588		9594 ± 960		11886 ± 11
				<.05		<.01	
Fed	+	Fed bethanechol	9281 ± 1716		11451 ± 1129		12342 ± 17
Fed bethanechol	+	Fed bethanechol	9208 ± 2348		10842 ± 1213		9558 ± 11
				<.05		<.01	
Fed bethanechol	+	Fed	7649 ± 1541		9218 ± 1075		9564 ± 7

ups of pigeons were given either bethanechol chloride (2 mg/kg IM) or saline and killed after 15, 30, or 60 min. Pancreatic tissue
enized, and microsomal and supernatant fractions prepared by centrifugation. Conditions of incubation are the same as described in

tration to fasted pigeons resulted from changes in factors which were isolated in supernatant fractions; increases in [^{14}C]-amino acid incorporation associated with feeding resulted from changes in factors which were isolated in both supernant and microsomal fractions. Microsomes isolated from fed pigeons incorporated greater amounts of [^{14}C]-amino acids than microsomes isolated from fasted animals. Thus, increases in [^{14}C]-amino acid incorporation resulted from changes in both microsomal and supernatant components. Increases resulting from factors isolated with microsomes appeared to augment incorporation to a greater degree than factors isolated in the supernatant.

Effects of in vivo *bethanechol chloride on [^{14}C]-L-phenylalanine incorporation by microsomes and supernatant* in vitro. Publications from this laboratory have shown that *in vivo* administration of cholinergic drugs to pigeons was associated with increased [^{14}C]-L-phenylalanine incorporation within 5 min, maximal enhanced incorporation within 30 min, and return to basal incorporation within 60–120 min (1). The experiments shown in Table III were designed to determine whether increases in [^{14}C] phenylalanine incorporation following *in vivo* bethanechol chloride resulted from changes in factors isolated in particulate or soluble fractions. Because of the day to day variability in pancreatic protein synthesis in pigeons, the data in Table III must be compared vertically rather than horizontally. Experiments utilizing birds killed after 15 min were done on similar days, whereas experiments utilizing birds killed after 30 and 60 min were done on other days. Differences obtained between 15, 30, and 60 min (*i.e.,* 8254, 9594, and 11,886), represent day to day variability. The comparison is made vertically; that is, between 8254 and 9281.

Groups of fed pigeons were given saline or bethanechol chloride (2 mg/kg im) and killed 15, 30, and 60 min later. Sets 1 and 2 show results with microsomes prepared from fed birds incubated with supernatant preprepared from fed or fed-BC treated birds which had been killed 15, 30, or 60 min after injection. The 12% and 19% increases at 15 and 30 min were statistically significant and were associated with the supernatant of the fed-bethanechol chloride treated birds. When animals were killed 60 min after bethanechol chloride, there were no differences in incorporation (11,886 vs 12,342) between groups.

Microsomes from fed birds incubated with supernatant from fed-BC treated birds incorporated about the same amounts of label as did microsomes from fed bethanechol chloride treated groups incubated with supernatant from fed bethanechol treated animals (Sets 2 and 3). Microsomes from fed-BC animals incorporated greater amounts of label when they were incubated with supernatant from fed-bethanechol treated (Sets 3 and 4) animals. Increases in incorporation were present after 15 and 30 min, but not after 60 min (Sets 3 and 4). The data in Table III indicates that bethanechol treatment, within the time periods studied, initiated changes in factors isolated in the supernatant fraction which increased [^{14}C]-L-phenylalanine incorporation. These factors were present only in animals killed 15 and 30 min after bethanechol chloride administration.

Discussion. Protein synthesis in the pigeon pancreas has been demonstrated to be greater in fed birds compared to fasted or refed compared to fasted birds. In addition, increases in protein synthesis have been shown to occur following administration of the gastrointestinal hormone, cholecystokinin-pancreozymin, and cholinergic agents such as methacholine and bethanechol chloride (1). These studies were designed to determine if increases in pancreatic protein synthesis observed after feeding or bethanechol chloride administration were mediated by changes in components of soluble or precipitable fractions of a cell homogenate. The studies show that increases in protein synthesis associated with feeding were mediated by factors which were isolated in both soluble and particulate fractions. Bethanechol chloride administration was associated with increases in protein synthesis which seemed to result from changes which were observed only in cytoplasmic fractions and these increases were only apparent at 15 and 30 min after bethanechol chloride administration.

These studies suggest that "long-term" increases in protein synthesis, *i.e.*, those associated with feeding, result from changes which are mediated by both soluble and particulate components at the translational level of control. "Short-term" changes, on the other hand, seem to be mediated by changes occurring only in cytoplasmic or soluble components.

The microsomal cell-free incorporating system used in these experiments behaved in a manner similar to that described by Redman *et al.* (7). An ATP generating system consisting of ATP, phosphoenol pyruvate, and pyruvate kinase was shown to be necessary. Microsomal supernatant, GTP, and KCl were necessary for maximal rates of incorporation.

Microsomes from fed pigeons were found to be about four times more active than those from fasted or fasted-urecholine treated pigeons when tested with soluble components from fasted birds and approximately six times more active when tested with soluble components from fed birds. The greater capacity of microsomes from fed compared with fasted birds for protein synthesis suggested a difference in polyribosomes in pancreas from the former group. Preliminary studies have shown that pancreas from fed animals has not only greater amounts of polyribosomes but also greater ratios of polysomes to monosomes and disomes.

These studies then serve as preliminary indicators for other studies to better evaluate hormonal control of pancreatic protein synthesis at translational and transcriptive levels of control.

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