PROTEIN SYNTHESIS IN RAT PANCREAS

I. INTRACELLULAR DISTRIBUTION OF AMYLASE*

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Current interest in protein synthesis has focussed attention on the large-scale synthesis of digestive enzymes which takes place in the pancreas[1-5], and has led to isolation of secretory granules and localization of the digestive enzymes within the pancreas cell of the dog[6].

In the present study, the intracellular distribution of amylase was studied in rat pancreas, and because a large proportion of the amylase activity was found in the microsome pellet, as well as in the "soluble" fraction, the microsome material was sub-fractionated in an effort to define the unit with which the amylase is associated.

MATERIALS AND METHODS

Male Sprague-Dawley rats, weighing 300 to 400 g, were used throughout the experiments. They were starved overnight, or fed *ad libitum*, or given pilocarpine by intraperitoneal injection (5 mg of pilocarpine-HCl in 0.5 ml of isotonic sodium chloride per animal) as indicated in the results of the various experiments.

The pancreas was dissected free of fat and extraneous connective tissue, rinsed in isotonic sucrose, and then strained through a plastic tissue mincer. Pancreas from 6 to 8 rats was pooled for each fractionation. The strained tissue was homogenized in seven parts by weight of 0.88 *M* sucrose, in an all-glass homogenizer of the DOUNCE type[7]. The homogenate was strained through a fine stainless steel screen to remove small connective tissue masses, and the homogenate was then fractionated by differential centrifugation as previously described[8]. In the experiments in which the microsome material was to be sub-fractionated, the homogenate was first centrifuged at 18,000 × *g* for 15 minutes. The sediment, which contained the nuclei, secretory granules, and mitochondria, was discarded, and the supernatant fluid, which contained the microsome material and soluble protein, was subjected to differential centrifugation under various conditions. More detailed description of individual experiments is given in the RESULTS section.

The fractions obtained were assayed for amylase activity by the method of MEYER *et al.*[9], slightly modified, and were analyzed for protein-nitrogen[10], nucleic acids[11], and phospholipid-phosphorus. For the determination of phospholipid, aliquots of the various fractions were precipitated with 10% trichloroacetic acid (TCA), and washed twice with 5% TCA. The precipitates were then extracted twice with ethyl alcohol, three times with hot alcohol–ether (3 : 1) and once with ethyl ether, and the combined extracts were evaporated to dryness. The residues were digested overnight at about 140°C with 6 *N* H_2SO_4, and the digests were cleared with hydrogen peroxide. The diluted digests were heated in a boiling water bath for 30 minutes and were analyzed for phosphorus by the method of FISKE AND SUBBAROW[12].

RESULTS

The intracellular distribution of amylase activity in the relatively quiescent pancreas from starving animals was compared with that in the relatively active tissue from animals which had received pilocarpine by injection either two hours (experiments

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TABLE I

INTRACELLULAR DISTRIBUTION* OF AMYLASE ACTIVITY IN RAT PANCREAS

Treatment	Experiment 1**	Experiment 2**	Experiment 3**
Starvation:			
Homogenate	100 (1.47***)	100 (2.40***)	100 (3.19***)
Nuclear fraction	13	3	1
Mitochondria and secretory granules	9	4	12
Microsomes	26	18	30
Supernatant fluid	29	26	29
Pilocarpine:	2 hours	2 hours	15 minutes
Homogenate	100 (0.542***)	100 (1.93***)	100 (4.38***)
Nuclear fraction	9	2	1
Mitochondria and secretory granules	7	6	7
Microsomes	32	25	25
Supernatant fluid	15	18	25

* As % of amylase activity of unfractionated homogenate.

** In experiments 2 and 3, the homogenate was filtered through a fine stainless steel screen before separation of the fractions. The lower % recovery of amylase in the nuclear fraction in these experiments is probably due to a lower proportion of contaminating whole cells.

*** mg $\times 10^4$ maltose produced in 30 minutes per mg DNA of unfractionated homogenate.

1 and 2) or 15 minutes (experiment 3) before sacrifice (Table I). In each experiment, the material from the two types of tissue was centrifuged simultaneously in the same rotor at each step in the fractionation. The results of such paired fractionations are given in Table I. In these early experiments, recovery of amylase activity was low, ranging from 50 to 80 %. In part this was due to delay of 24 hours or more in assaying the activity, since in later experiments a decay of activity was demonstrated over this period of time, even though the samples were kept at 0 to 2°C. In such experiments, the rate of decay of amylase activity was greater in isolated secretory granules than in the microsomes and supernatant fluid fractions, but was appreciable in all fractions, amounting to about 30% for the microsomes and 50% for the secretory granules in 24 hours. Even if all the missing activity were assumed to have been derived from the secretory granules, it is evident from Table I that about 50% of the total amylase activity of rat pancreas was recovered in association with the microsomes and supernatant fluid.

In pancreas of starved rats, in which relatively little secretory activity is taking place, the extra-granular amylase is about equally divided between the microsomes and supernatant fluid in 0.88 *M* sucrose. However, when the gland is stimulated to secrete, either by pilocarpine or by feeding, the loss of amylase is greater from the supernatant fluid, with the result that the distribution of this enzyme then favors the microsome fraction. This predominant loss of amylase from the supernatant fluid fraction during secretion has been observed in all the experiments that we have carried out.

In two experiments that are not described in detail in the present report, the secretory granules were isolated by the method of HOKIN[6], modified for use with 0.88 *M* sucrose. Although the secretory granules were not obtained completely free from contamination with other cytoplasmic components, the amylase activity of this

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TABLE II

BIOCHEMICAL PROPERTIES OF MICROSOME PELLETS SEDIMENTED FROM DIFFERENT MEDIA, AT 105,000 × *g* × 60 MINUTES

	Amylase	*RNA*	*Phospholipid-P*	*Protein-N*
A. Quantities sedimented*:				
0.88 *M* sucrose	11.0	4.13	0.207	2.76
0.25 *M* sucrose	15.9	5.49	0.255	3.52
0.25 *M* sucrose, pH 5	19.4	10.0	0.446	8.52
B. Concentrations relative to protein nitrogen:				
0.88 *M* sucrose	3.98	1.50	0.075	
0.25 *M* sucrose	4.52	1.56	0.072	
0.25 *M* sucrose, pH 5	2.27	1.17	0.052	
$\frac{\text{0.25 } M \text{ sucrose, pH 5}}{\text{0.25 } M \text{ sucrose}}$	0.50	0.75	0.73	

* RNA, phospholipid-P, and protein-N: mg per gram equivalent of pancreas. Amylase: mg × 10^4 maltose produced in 30 minutes at 37°C per gram equivalent of pancreas.

fraction with respect to protein-nitrogen was two to three times that of any of the other cell fractions, in agreement with the results of HOKIN with dog pancreas[6].

In order to study in more detail the extra-granular amylase, the microsome material was subjected to two types of sub-fractionation procedure: in one, the centrifugal force and time were kept constant while the concentration and pH of the medium were varied; in the other, the medium was kept constant, and the centrifugal force was varied. The results of the former type of procedure are shown in Table II. As would be expected, higher proportions of all the constituents studied were sedimented when the concentration (and consequently the viscosity and density) of the medium was reduced. When the diluted medium was brought to pH 5 by the addition of a few drops of 0.2 *N* acetic acid, still larger quantities of the constituents were sedimented (Table IIA). The composition of the larger pellets thus obtained was similar to that obtained in the unbuffered 0.88 *M* sucrose medium (Table IIB), although it is evident that reducing the pH resulted in the sedimentation of additional protein which was associated with RNA and phospholipid, but not with amylase activity, since it is evident from the values of the ratios given in the last line of Table IIB that the ratio of amylase activity to protein nitrogen has decreased to a greater extent than has the ratio of phospholipid-phosphorus or RNA to protein-nitrogen.

Entirely different results were obtained when successive sub-fractions of the microsome material were sedimented from an isotonic sucrose medium by means of graded increases in the gravitational force applied (Table III). These three experiments gave consistent results in spite of differences in the condition of the animals and in the preparation of the homogenate. In the first experiment the animals were starved overnight, while in the last two experiments the animals were allowed to feed *ad libitum*, with the result that both the total quantity of amylase and the proportion of the amylase in the supernatant fluid were lower in the latter. In both the first and second experiments, the pancreas was homogenized in 0.88 *M* sucrose, and subsequently the mitochondrial supernatant fluid was diluted to bring the sucrose concentration to 0.25 *M* before the microsome sub-fractions were sedimented; in the third experiment, the pancreas was homogenized in 0.25 *M* sucrose. In contrast to the experiments

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TABLE III

BIOCHEMICAL PROPERTIES* OF MICROSOME PELLETS PREPARED BY DIFFERENTIAL CENTRIFUGATION IN 0.25 *M* SUCROSE

	Amylase		*RNA*		*Phospholipid-P*		*Protein-N*
	% *of total***	*Rel. activity****	% *of total***	*Rel. concn.*	% *of total***	*Rel. concn.*	% *of total*
Sediments:§			Experiment 1				
25,000 × *g*	31	2.5	—	—	67	0.10	19
75,000 × *g*	14	2.1	—	—	17	0.04	11
105,000 × *g*	13	1.0	—	—	11	0.01	23
Supernatant fluid	28	3.7	—	—	< 4	< 0.01	25
Sediments:§			Experiment 2				
25,000 × *g*	37	0.91	35	1.4	61	0.11	30
75,000 × *g*	8	0.76	13	2.1	8	0.05	8
105,000 × *g*	5	0.66	11	2.5	1	0.01	6
Supernatant fluid	20	0.54	18	0.80	6	0.01	28
Sediments:§			Experiment 3				
25,000 × *g*	43	0.95	35	1.5	78	0.11	30
75,000 × *g*	6	0.70	10	2.0	8	0.05	6
105,000 × *g*	9	0.60	16	2.2	6	0.02	10
Supernatant fluid	19	0.45	15	0.69	3	0.01	27

* Relative activities and concentrations per mg protein-nitrogen in each fraction.

** Total = total activity or total mg measured in the unfractionated supernatant fluid obtained after sedimentation of nuclei, secretory granules, and mitochondria at 18,000 × *g* × 15 minutes.

*** mg maltose produced in 30 minutes at 37°C, per mg protein-nitrogen.

§ In all cases the 25,000 × *g* and 75,000 × *g* sediments were obtained by centrifuging for 30 min, and the 105,000 × *g* sediments by centrifuging for 90 min.

described above, the microsome sub-fractions obtained in these experiments were distinctly different from one another. The sediment obtained at 105,000 × *g* differed from that obtained at 25,000 × *g* by a factor of 5 to 10 in the ratio of phospholipid-phosphorus to protein-nitrogen, and by a factor of 1.5 to 2 in the ratio of RNA or of amylase to protein-nitrogen.

In a third pair of experiments, microsome sub-fractions were sedimented in 0.88 *M* sucrose; the results are given in Table IV. In this case, the microsome material was sedimented in two sub-fractions, the first at 30,000 × *g* and the second at 105,000 × *g*. Since the total protein in the two sub-fractions from 0.88 *M* sucrose is similar to the total protein sedimented from 0.25 *M* sucrose at 25,000 × *g* and 75,000 × *g* (Table III), a direct comparison of the results of the two procedures is possible. It is evident from the data given in Table IV that for either pilocarpine-treated or starved pancreas, the microsome sediment obtained in 0.88 *M* sucrose at 30,000 × *g* is essentially identical in its biochemical properties to that obtained at 105,000 × *g* on the basis of the ratio of amylase or of RNA or of phospholipid-P to protein-nitrogen. On the other hand, the sediment obtained in 0.25 *M* sucrose at 25,000 × *g* differed from that obtained at 75,000 × *g* by a factor of 1.5 to 2 in the ratio of RNA and of phospholipid-P to protein-nitrogen. The results given in Table IV are typical of those obtained to date, in that the ratio of amylase to protein-nitrogen in the microsome and supernatant fluid fractions of the starved pancreas is consistently much higher than that of the actively secreting pancreas.

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TABLE IV

BIOCHEMICAL PROPERTIES* OF MICROSOME PELLETS PREPARED BY DIFFERENTIAL CENTRIFUGATION IN 0.88 *M* SUCROSE

	Amylase		*RNA*		*Phospholipid-P*		*Protein-N*
	*% of total***	*Rel. activity****	*% of total***	*Rel. concn.*	*% of total***	*Rel. concn.*	*% of total***
Pilocarpine-treated:							
Sediments:§							
30,000 × *g*	41	2.2	36	1.1	50	0.10	28
105,000 × *g*	21	2.5	17	1.1	24	0.10	13
Supernatant fluid	28	1.0	25	0.50	21	0.028	41
Starved:							
Sediments:§							
30,000 × *g*	25	5.0	31	0.84	34	0.079	27
105,000 × *g*	20	6.0	22	0.88	24	0.085	17
Supernatant fluid	43	5.0	23	0.35	18	0.025	44

* Relative activities and concentrations per mg protein-nitrogen in each fraction.
** Total = total activity or total mg measured in the unfractionated supernatant fluid obtained after sedimentation of nuclei, secretory granules, and mitochondria at 18,000 × *g* × 15 minutes.
*** mg maltose produced in 30 min at 37°C, per mg protein-nitrogen.
§ The 30,000 × *g* sediments were obtained by centrifuging for 30 min, and the 105,000 × *g* sediments were obtained by centrifuging for 60 min.

The considerable differences between the microsome sub-fractions obtained by varying the centrifugal force in 0.25 *M* sucrose are in clear contrast to the close similarities in the biochemical properties of the sub-fractions obtained by varying the concentration of the medium or by varying the centrifugal force in 0.88 *M* sucrose.

DISCUSSION

HOKIN[6] has shown that the secretory granules of dog pancreas contain amylase activity in higher concentration than any of the other cell fractions, and this was found to be the case in the present study. However, from the results in Table I, it is evident that a large part of the amylase activity of rat pancreas was also recovered in the microsome and supernatant fluid fractions. The finding of a large proportion of the amylase activity in association with the microsome fraction is quite different from the results of HOKIN[6], who found that the microsome fraction of dog pancreas was the only one essentially free of amylase activity. This discrepancy may be due to species differences, or it may be that the microsome fractions obtained by the two different procedures are not the same. In mouse pancreas, it has been reported that 4% of the amylase remains associated with the microsomes in spite of careful washing and treatment with ribonuclease[3]. The presence of a large amount of amylase in the supernatant fluid has been observed in other studies[2,6].

It is possible that the amylase activity is localized to the secretory granules in the intact tissue, but is found in the microsomes and supernatant fluid after cell fractionation because of disruption of the secretory granules and consequent solubilization of the enzyme protein, as suggested by HOKIN[6]. In rat pancreas, however, where so large a proportion of the activity is recovered in the microsome fraction, it becomes necessary to assume in addition an extensive adsorption of the soluble enzyme protein to

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the microsome particles (*cf.*[13]). Recently ROSENTHAL *et al.*[14] have demonstrated that when liver homogenates are prepared in sucrose, arginase activity is adsorbed to those particulate fractions that are characterized by a high concentration of nucleic acids, especially the nuclear and microsome fractions, but that such adsorbed activity can be eluted from these fractions to the extent of about 90% by a single extraction in a medium containing ions. In the present study, the nuclear fraction was essentially free from amylase activity when contamination by whole cells was reduced by previous filtration of the homogenate (experiments 2 and 3, Table I). Furthermore, in experiments to be published elsewhere in greater detail, we have found that not more than 50 to 70% of the amylase activity can be eluted from the microsome material by extraction with sodium chloride in concentrations up to 4%; the residual activity can be solubilized only when the microsome material is brought into solution with sodium desoxycholate. Furthermore, although it is not ruled out completely, interpretation of the present data in terms of simple adsorption is unlikely for three reasons: Firstly, there are consistent physiological changes in the distribution of activity between the microsomes and the other amylase-containing fractions when the relatively quiescent pancreas of the starving animal is stimulated to secrete, either by feeding or by pilocarpine. Secondly, the amylase activity in 0.88 *M* sucrose is apparently associated with a complex, containing phospholipid, RNA, and protein, that is broken during preparation into fragments resembling one another in composition, but differing in size[15], as indicated by the consistent ratios of amylase, RNA, and phospholipid-phosphorus to protein-nitrogen in the various microsome sub-fractions obtained by reducing the concentration of the medium and by differential centrifugation in 0.88 *M* sucrose. The third argument against adsorption is that in spite of this consistency in the relation of amylase to the other microsome constituents within one set of experimental conditions, the ratio of amylase to the other constituents varies considerably with the physiological state of the pancreas, but is not directly a function of the total pancreatic amylase activity nor is it directly related to the proportion of the amylase activity found in the secretory granules and in the supernatant fluid. It is possible, however, that the adsorptive capacity of the microsome particles varies with the physiological state of the pancreas, and if so, this might be an important factor in protein synthesis.

If these physiological changes in the association of amylase with the microsome fraction are representative of the conditions inside the living pancreas cell, then, in keeping with the demonstrated importance of the microsome fraction, and especially the ribonucleoprotein components thereof, in the uptake of amino acids into protein[16], it is quite possible that the mature enzyme protein is manufactured there, and liberated into the supernatant fluid as a soluble protein, to be organized, possibly by the Golgi substance[5], for storage in the secretory granules. It is noteworthy that in the experiments in which a dissociation of the microsome complex was demonstrated, *i.e.*, after differential centrifugation of suspensions in 0.25 *M* sucrose, no consistent relation could be shown between the amylase activity and any of the other microsome constituents. It is probable therefore that if the amylase protein is synthesized in elements of the microsome fraction, the new amylase is not associated with the minute ribonucleoprotein granules alone, but with the whole ergastoplasmic complex, which consists of a vesiculated lipoprotein reticulum, to which the ribonucleoprotein granules are attached[15].

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SUMMARY

The intracellular distribution of amylase in rat pancreas has been studied by differential centrifugation of pancreas homogenates. Although the secretory granules showed high activity, at least half the amylase activity was recovered in the microsome and supernatant fluid fractions. In the latter fractions, the proportion of the total enzyme activity and the concentration of amylase activity relative to protein-nitrogen showed consistent variations with changes in the secretory state of the gland. Apparently some of the amylase is associated with the endoplasmic reticulum in the living pancreas cell, and during homogenization in 0.88 *M* sucrose the endoplasmic reticulum is broken into pieces resembling one another in composition but differing in size. During exposure to 0.25 *M* sucrose some dissociation of the components of this material takes place, but when such dissociation occurs the amylase protein is not preferentially associated with either the ribonucleoprotein granules or the lipoprotein reticulum of the microsome fraction.

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SYNTHESIS OF NUCLEIC ACIDS IN ULTRAVIOLET-TREATED *ESCHERICHIA COLI**

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Starvation increases the sensitivity of protozoa[1,2] and of yeast (unpublished observation) to ultraviolet (UV) radiations, in the latter case the absence of nitrogen compounds being especially effective in this respect. On the other hand DEMEREC AND LATARJET[3] have shown that *Escherichia coli* B/r is 2–3 times more resistant to UV in the resting than in the growing state, and WITKIN[4] using the same strain showed that the bacteria are more resistant to UV in the lag phase of a culture than in any other phase of the growth cycle.

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