

Liver Amylase

III. SYNTHESIS BY THE PERFUSED LIVER AND SECRETION INTO THE PERFUSION MEDIUM*

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(Received for publication, February 28, 1963)

The presence of α -amylase in liver tissue has been reported previously by several investigators (1-3). In the liver from rats deprived of food, the enzyme is found primarily in the microsomal fraction (4) whereas in fed animals a considerable proportion is bound to glycogen (5). In perfused liver systems, amylase activity is secreted into the perfusion medium under conditions in which other enzymes, normally released on liver damage, remain within the tissue (6). On the basis of these facts and supplementary experimental information, several physiological functions of liver amylase have been postulated (6). Of these, the possible role of the liver as a prominent source of serum amylase under normal physiological conditions is of particular interest because it implies a specific metabolic process directed toward the synthesis and secretion of a serum enzyme.

The experimental observations reported to date provide only circumstantial evidence for the synthesis of amylase by the liver.

1. The immunochemical evidence of McGeachin and Reynolds (7, 8) that liver amylase is different from salivary and pancreatic amylase is based upon the action of antibodies on the amylase activity in crude extracts. Since the liver enzyme is primarily bound to glycogen or microsomal particles, it is possible that its interaction with antibodies is modified.

2. The accumulation of amylase in the perfusion medium during the course of perfusion of rat liver is not unequivocal evidence for synthesis by this organ because of the following alternatives. (a) There may be a release of intracellular bound amylase under the conditions normally employed in the perfusion studies. (b) There may be a conversion of an inactive to an active form of this enzyme. (c) Perfused rat liver systems are usually contaminated with small quantities of pancreas, which is a diffuse organ in this animal. Since the specific activity of amylase is approximately three orders of magnitude higher in the pancreas than in the liver, the accumulation during perfusion may be of pancreatic and not hepatic origin.

The experiments presented in the present paper demonstrate that amylase accumulation during perfusion of liver tissue is not the result of bound amylase or of contaminating pancreatic tissue. Evidence is presented for the synthesis *de novo* of amylase by liver tissue and for its release into the medium under normal

physiological conditions. These experiments therefore provide a sound basis for the roles of liver amylase previously postulated (6), and especially implicate the liver as a source of serum amylase.

EXPERIMENTAL PROCEDURE

Sprague-Dawley albino rats weighing 200 to 400 g and in a few instances 800-g guinea pigs were employed in the perfusion experiments. Unless otherwise stated, animals were fed a stock laboratory diet *ad libitum* before death as either blood or liver donors for perfusion experiments.

Perfusion Methods—The surgical removal of functionally intact livers for perfusion was accomplished by the technique described by Brauer, Pessotti, and Pizzolato (9). The perfusion apparatus has been previously described (6). The total time of the operation was 25 to 35 minutes with an ischemia time of usually 5 and never more than 10 minutes. After blanching of the liver by slow perfusion with 50 ml of nutrient medium, a small lobe was removed for analysis of initial enzyme and glycogen levels. The liver was then placed on the perfusion apparatus. Substrate additions were made to the reservoir as soon as the rate of flow of perfusion medium through the liver (15 to 30 ml per minute) and secretion of bile fluid indicated that the system was functioning normally. The beginning of the perfusion experiment was arbitrarily taken as the time of addition of test substances. Samples of blood were removed at appropriate intervals, and the red cells were separated by centrifugation at 3°. Supernatant fluids were then stored at -15° until analyzed. At the end of the experiment (usually 4 hours), the liver was removed, blotted, and weighed. Both the initial and final lobes were homogenized in 3 volumes of 0.05 M histidine, pH 6.5, at 0° and were stored at -15° until analyses could be made.

Unless otherwise stated, the perfusion medium consisted of 170 ml of Waymouth's nutrient medium (10) with 0.01 M lactate replacing the glucose, 80 ml of the nutrient medium containing rat erythrocytes (at the concentration of whole blood), 6 g of bovine serum albumin, 10,000 units of penicillin, and 10 mg of streptomycin. The erythrocyte suspension was prepared as previously described (6).

Radioactive Tracer Experiments—Uniformly labeled L-leucine- C^{14} , 5 μ c, was added to the medium at the beginning of liver perfusion experiments. At appropriate intervals, incorporation of the labeled leucine into liver or plasma proteins was determined as follows: 1 ml of perfusate or liver homogenate was treated with 2 ml of 10% trichloroacetic acid and then centrifuged, and the supernatant solution was separated from the precipitate.

* This investigation was supported by Grant C-3765 from the National Institutes of Health, United States Public Health Service. Part of the data are taken from a dissertation submitted by Marjorie L. Arnold to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the Doctor of Philosophy degree, February 1963. A preliminary report of this work was presented at the 143rd Meeting of the American Chemical Society in Cincinnati, Ohio, January 1963.

The precipitate was washed three times with 3 ml of 10% trichloroacetic acid, the first wash containing 10 mg of nonradioactive L-leucine. Finally, the precipitate was washed three times with 5 ml of acetone, suspended in water, and plated on stainless steel planchets; radioactivity was determined in an internal gas flow Geiger-Müller counter. Results were expressed as counts per minute after correction for background and self-absorption.

Production of Antibodies to Amylase—Antiserum to crystalline hog pancreatic amylase was prepared in an adult albino rabbit by four separate subcutaneous injections at 2-week intervals of 25 mg of amylase, prepared in Freund's adjuvant (11). The presence of precipitating antibodies was indicated by both the precipitin reaction of Heidelberger and Kendall (12) and by Ouchterlony double diffusion analysis (13). The equivalence point was approximately 300 μ g per ml of antiserum.

Analyses—The determination of amylase activity and the definition of units of activity have been previously described (6). Although the iodine assay was employed in these studies, the units are expressed in terms of the amount of enzyme required to produce an increment in reducing power equivalent to 1 μ mole of glucose per minute (6).

Trypsin and trypsinogen were determined by the method of Schwert and Takenaka (14). Glucose was determined by the glucose oxidase reaction coupled with peroxidase in the presence of the dye, *o*-dianisidine (15), except that 0.1 M phosphate buffer, pH 7.0, replaced acetate buffer, pH 4.5.

Reagents—Crystalline hog pancreatic amylase was obtained from Worthington Biochemical Corporation. Bovine serum albumin (Fraction V) was procured from Pentex, Inc. A sample of puromycin dihydrochloride was kindly supplied by Dr. B. L. Hutchings of Lederle Laboratories. Uniformly labeled leucine having a specific activity of 4.9 mc per mmole was purchased from Volk Radiochemical Company. Triton X-100 was kindly supplied by Rohm and Haas Company.

RESULTS

Amylase Production during Perfusion Experiments—The amylase, bile, and glucose production from six typical perfusion experiments has been plotted in Fig. 1. The amylase activity and bile production increase steadily during the first 3 hours of perfusion. On the other hand, the glucose accumulation in the perfusion medium is initially very rapid and then levels off as the glycogen of the liver becomes depleted. The satisfactory reproducibility in both amylase and bile production is in contrast to the marked variations reported by others in similar perfusion experiments (6, 16); this is possibly related to the defined nature of the perfusion medium as well as to the inclusion of antibiotics.

Elimination of Pancreas as Source of Amylase during Liver Perfusion—It is difficult to remove rat liver without contamination by pieces of pancreatic tissue attached to the mesenteries near the bile duct and portal vein. Several types of experiments, however, have convincingly demonstrated that such pancreatic tissue does not contribute significantly to the accumulation of amylase during the perfusion experiments.

Deliberate variation in the size of the pancreatic contaminant did not alter the rate of accumulation of amylase. Thus, livers contaminated with as much as 200 mg of pancreatic tissue produced approximately the same amount of amylase as those in which essentially all the pancreas was removed by a surgical cauterization needle. Furthermore, there was no demonstrable

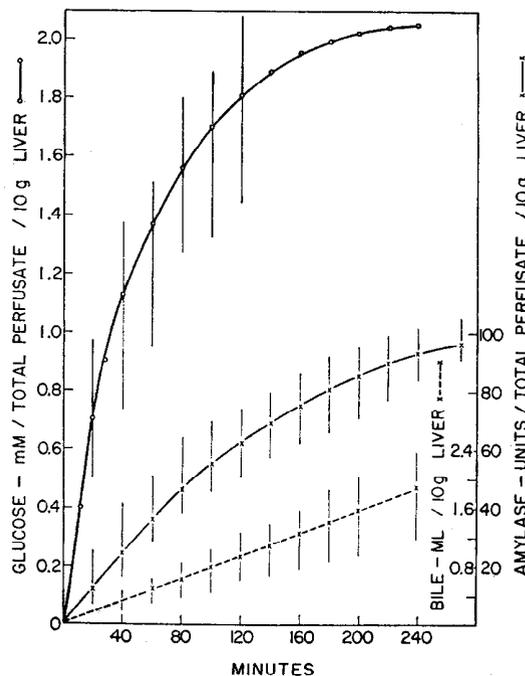


FIG. 1. Glucose, bile, and amylase production by perfused rat livers. The vertical lines represent the range of values obtained in six experiments performed under standard conditions ("Experimental Procedure"). The values are normalized on the basis of 10-g livers. The actual ranges of liver weights were 6 to 12 g. The top curves represent glucose concentration and amylase activity in the perfusate, and the bottom curve represents bile production.

accumulation of the pancreatic enzymes, trypsin or trypsinogen, in the perfusate under conditions in which small quantities of added trypsin could be readily detected.

Complete elimination of the pancreas in the perfusion system was achieved by a "backward" perfusion method. The hepatic portal vein and bile duct were removed up to their junction with the liver. Perfusion was accomplished by directing the blood flow through the vena cava and allowing it to flow freely out of the liver into a container through which recirculation was accomplished. The results of two "backward" perfusions are shown in Table I. Amylase accumulated in both experiments, but the net increase was especially low for Experiment 41. The mottled appearance of the liver after Experiment 41 indicated impaired function during perfusion. The results obtained in Experiment 44 are more representative of the normal state. In this experiment, the accumulation of amylase in the medium was compared with the incorporation of radioactive L-leucine into trichloroacetic acid-precipitable material, presumably protein. The fact that there was a net gain in amylase units by the system in the absence of pancreatic tissue suggested that the pancreas was not responsible for amylase accumulation, and incorporation of labeled leucine into plasma proteins indicated that synthesis of proteins was occurring under these experimental conditions.

An additional perfusion experiment was performed with the liver of the guinea pig since the pancreas of this animal is more localized and can be completely removed from the liver. Under conditions similar to those employed in the rat liver perfusions, amylase accumulated in the medium during perfusion of the

TABLE I
Amylase production and C¹⁴ incorporation during "backward" perfusion

Details concerning the methods of perfusion and enzymatic assays are discussed in "Results." In Experiments 25 and 44, 5 μ c of L-leucine-C¹⁴ were added to the medium at the beginning of perfusion. Radioactivity of the precipitated protein in 1 ml of perfusate is expressed as counts per minute after correction for self-absorption and background.

Condition	Experiment	Time	Amylase in perfusate	Loss in liver amylase	Net gain	Perfusate
			units			
Normal	25	240	90	19	71	1000
Backward	41	240	88	48	40	363
	44	165	81	15	66	

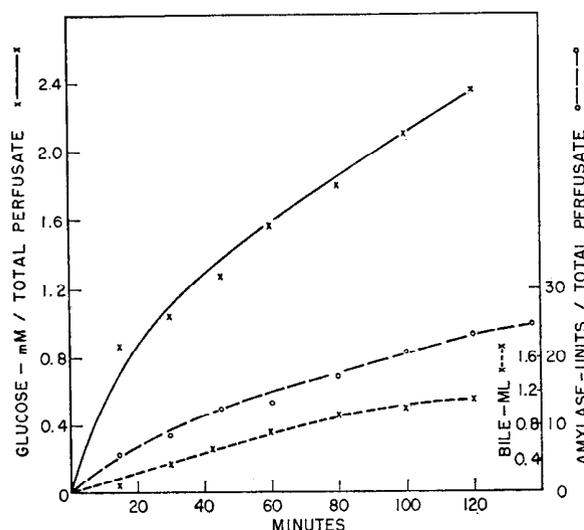


Fig. 2. Glucose, amylase, and bile production by perfused guinea pig liver. Standard perfusion medium was employed, except that washed guinea pig red cells replaced rat red cells. An 840-g guinea pig was the liver donor, and the liver weighed 30.6 g.

guinea pig liver (Fig. 2). The relative quantity of amylase produced was not as large as with the rat livers, but optimal conditions for the perfusion of guinea pig liver may not have been achieved in these experiments.

Inhibition of Amylase Accumulation in Perfusates and of Incorporation of Radioactive Amino Acids into Perfusate Proteins—Evidence for amylase synthesis was obtained by comparing amylase accumulation and incorporation of radioactive amino acids into perfusate proteins under varying conditions. As indicated in Table II, the deletion of amino acids in the perfusate medium, or of a single required amino acid, tryptophan, resulted in a lowered accumulation of amylase and also a lowered incorporation of radioactive leucine into perfusate protein. In addition, the replacement of phenylalanine by fluorophenylalanine in the perfusate medium resulted in a similar depression of amylase accumulation and perfusate protein synthesis. The fact that only partial depressions in these activities is achieved in the absence of added amino acids is not surprising since considerable quantities of free amino acids accumulate in the per-

fusion system during incubations. This endogenous amino acid pool no doubt influences the response of the system to *p*-fluorophenylalanine.

A more dramatic depression in amylase accumulation is seen with puromycin, a specific inhibitor of protein synthesis (17, 18). Kinetic data are presented in Fig. 3, correlating amylase production with protein synthesis as measured by amino acid incorporation into trichloroacetic acid-precipitable material. The net change in amylase units during the perfusion experiments is shown in Table III.

Addition of 5 mg of puromycin had little effect on amylase accumulation or incorporation of radioactive amino acids into protein. However, when 10 mg of puromycin were added to the perfusion medium, both amylase activity and incorporation of labeled leucine were depressed below normal values. The initial lag period in C¹⁴ incorporation into protein is no doubt a reflection of the time required for equilibration of the added C¹⁴ with the liver amino acid pools. A similar lag in the incorporation of radioactive amino acids into plasma proteins has been observed by Miller *et al.* (19) and Jensen and Tarver (20) in their perfusion studies and by Green and Anker (21) during studies *in vivo* of plasma protein synthesis. As would be expected, the accumulation of amylase activity occurs without a lag since no distinction can be made between newly and previously synthesized protein by this assay.

In the presence of 20 mg of puromycin during perfusion, there was a net loss of amylase from the system, even though there was accumulation of this enzyme in the perfusate. When 15 mg of puromycin were injected intraperitoneally into a rat 1 hour before the liver was placed on the perfusion apparatus, leucine incorporation continued *in vitro* after a considerable lag period. There was a similar but less pronounced lag in amylase accumulation. This suggests that the processes involved in secretion of amylase and other plasma proteins exhibit different kinetic characteristics or different degrees of inhibition by puromycin.

When the liver was subjected to doses of puromycin both *in vivo* and *in vitro*, the incorporation of leucine-C¹⁴ into proteins was arrested completely whereas the amylase accumulation in the medium was lowered only to one-quarter of the normal.

TABLE II

Effects of amino acids and analogues on amylase synthesis

In Experiment 25, basal medium was used; in Experiment 30, phenylalanine was omitted and 150 mg of *p*-fluorophenylalanine were added; in Experiment 17, tryptophan was omitted; in Experiment 33, all amino acids were omitted from the medium. Total perfusate volume in all experiments was 250 ml. L-Leucine-C¹⁴, 5 μ c, was added in Experiments 25, 30, and 33 at the beginning of perfusion. The amount of leucine present in the perfusion medium of Experiment 33, presumably because of catabolism of protein, was determined in an amino acid analysis and found to be 4.5 mg, which was 0.26 of the amount present in Experiments 25 and 30. The counts per minute for Experiment 33 before correction by this value were 164.

Experiment	Condition	Net gain in amylase	Relative leucine-C ¹⁴ content
		units/10 g liver	c.p.m./ml/g liver
25	Normal	74	106
30	<i>p</i> -Fluorophenylalanine	33	29
17	Minus tryptophan	26	
33	Minus all amino acids	37	43

There was no incorporation of leucine- C^{14} into the perfusate protein, and in addition, as shown in Table III, there was no significant increase in the total amylase of the system; therefore it is concluded that the amylase was not synthesized during the perfusion experiment. The slow release of amylase must represent a redistribution of the enzyme in the system—probably a release of glycogen-bound amylase, since the glycogen content is declining during this period.

Demonstration of Incorporation of Radioactive Amino Acids into Amylase during Perfusion—The tentative conclusion that amylase was synthesized by liver during perfusion experiments was confirmed by direct demonstration of the incorporation of labeled free amino acids into amylase protein. The specific isolation of the enzyme was achieved with the aid of precipitating antibodies prepared against crystalline hog pancreatic amylase. The results are shown in Table IV. The small amount of radioactivity found in the 15- and 30-minute samples of isolated amylase may represent trapped or bound C^{14} unrelated to protein synthesis, since similar protein samples precipitated with trichloroacetic acid contained no significant radioactivity. The incorporation of radioactive amino acids into the antibody-precipitable protein increases steadily with time after the initial lag period.

A variant protocol was employed to eliminate the possibility of non-amylase protein cross-reacting with the antiserum or of physical adsorption or trapping of free leucine. Samples at the

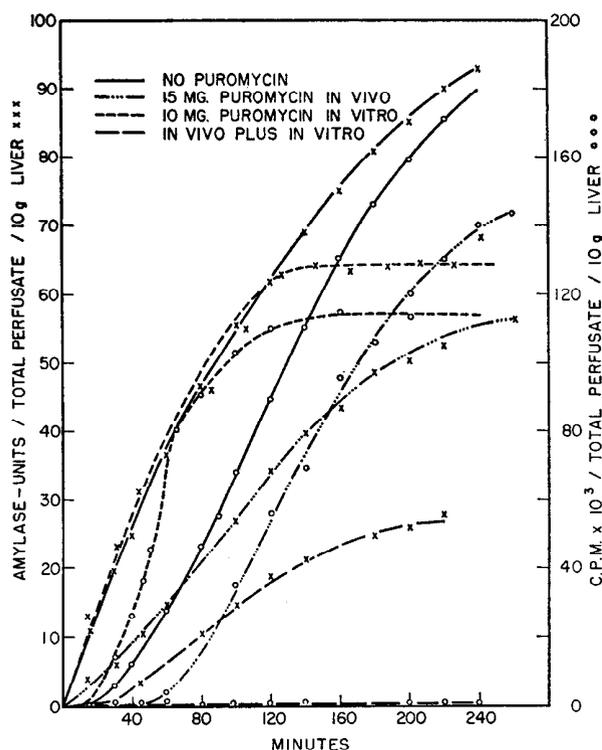


FIG. 3. Effect of puromycin on protein synthesis by perfused livers. L-Leucine- C^{14} , 5 μ c, was added to the perfusion medium at the beginning of perfusion in each experiment. Incorporation of leucine- C^{14} into perfusate protein and amylase activity in the perfusate samples of such experiments have been plotted. The control (no puromycin) represents an average of three experiments. The curves are identified in the figure; conditions for each experiment are explained in "Results."

TABLE III

Effect of puromycin on amylase production

Perfusion experiments were performed under standard conditions as described in "Experimental Procedure." Puromycin was added to the perfusion medium after 20 minutes of perfusion or was given intraperitoneally to the liver donor rat 1 hour before the liver was isolated for perfusion experiments. Results have been normalized for 10-g livers.

Condition	Total perfusate amylase	Net gain in amylase
		units/10 g liver
No puromycin	94	74
Puromycin, 5 mg	114	81
Puromycin, 10 mg	68	29
Puromycin, 20 mg	58	-8
Puromycin <i>in vivo</i> , 15 mg	59	55
Puromycin <i>in vivo</i> , 15 mg, plus 15 mg <i>in vitro</i>	29	9

TABLE IV

 C^{14} measurements in amylase-antibody precipitates

Samples from perfusion Experiment 33 were incubated with equal volumes of rabbit antiserum for $\frac{1}{2}$ hour at 37° followed by 36 hours at 3°. The precipitates were washed three times with 1 ml of 0.9% NaCl and then solubilized with excess hog pancreatic amylase. Aliquots were then removed for determination of radioactivity.

Sample	Total radioactivity in perfusate	Net incorporation into perfusate	Per cent of total incorporation
<i>min</i>	<i>c.p.m./ml</i>		
15	7		
30	7		
80	21	14	1.6
140	33	26	2.3
220	40	33	1.7

end of the perfusion were dialyzed to remove free leucine, incubated with the antiserum, and washed in 0.9% NaCl solution containing bovine serum albumin to solubilize nonspecific reacting precipitate. Since the radioactivity accumulating at the end of the experiment was reduced to 25 c.p.m. per ml from the 39 c.p.m. per ml observed with the first procedure, there may have been some contamination with free leucine- C^{14} or with extraneous non-amylase proteins in the earlier procedure. However, the original conclusion that amylase was synthesized *de novo* was confirmed.

The specific radioactivities of amylase isolated by the above procedure and of the plasma proteins isolated from the perfusate were both 0.078 μ c per mg. This is consistent with the synthesis of these proteins from a common pool of amino acids.

DISCUSSION

With optimal conditions prevailing, amylase is released by the liver into the perfusion medium in a reproducible, linear manner. The experimental results presented here indicate that this accumulation of amylase is primarily due to synthesis of this enzyme by the liver tissue and is not a result of contaminating pancreatic tissue present in the incubation system.

The decreased accumulation of amylase in the perfusate me-

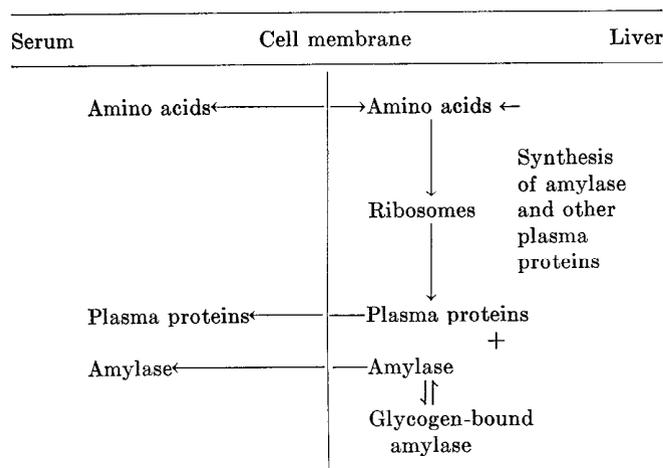
dium in the presence of an inadequate complement of amino acids or of specific inhibitors of protein synthesis, especially puromycin, as well as the direct demonstration of the incorporation of labeled amino acids into perfusate amylase isolated by immunochemical procedures, provides compelling evidence for the synthesis *de novo* of amylase by liver tissue and subsequent release into the surrounding medium. The fact that the kinetics of the amylase release by liver tissue, as indicated by experiments with puromycin, was different from the kinetics of incorporation of L-leucine- C^{14} into serum proteins suggests either that there is a source of amylase which is insensitive to the presence of puromycin (which is unlikely) or that there is a storage form of liver amylase which can then be released into the perfusate.

It is known that glycogen binds amylase strongly and that considerable quantities of this enzyme are bound to glycogen in well fed livers. Since the glycogen content of the liver rapidly declines during perfusion, it is proposed that intracellular glycogen is the most likely puromycin-insensitive source of perfusate or plasma amylase. This contrasting behavior of the accumulation of amylase and other plasma proteins by perfused liver is schematically represented in Diagram 1, which depicts a more or less direct route from ribosomes to extracellular medium in the case of most plasma proteins but the presence of an additional reservoir for amylase. Of course these experiments do not establish whether some fraction of ribosomal amylase is released directly to the extracellular medium without exposure to the soluble glycogen-containing areas of the cell, or whether all of the amylase formed on the ribosomes is present in the intracellular sap and then is bound or excreted depending simply on the competition of available glycogen and ease of egress into the external medium.

The synthesis and elaboration of amylase by the perfused liver under normal circumstances agree basically with the postulate that liver represents one source of serum amylase. The rate of perfusate amylase synthesis by direct estimate of incorporation of amino acids into amylase is approximately 31 μ g of amylase per hour per 10-g liver. If it is assumed that the specific activity of liver or serum amylase is the same as hog pancreatic amylase, *i.e.* 1200, then the amount of amylase synthesized is estimated to be 17 μ g per hour per 10-g liver. Because

DIAGRAM 1

Schematic representation of amylase and plasma protein production by liver tissue



of the uncertainty in the assumptions involved in these calculations, the values obtained are considered to be in satisfactory agreement. In comparison, the quantity of serum proteins synthesized by the liver has been reported by Jensen and Tarver (20) to be 31 mg per hour, whereas several others have found 7 to 8 mg per hour per liver (19, 22). From these studies, the rate of serum protein synthesis was of the order of 6 mg per hour per liver. The quantity of amylase produced during these perfusions therefore is approximately 0.5% of the total serum protein synthesized. Although this is a small fraction of the total protein produced, the quantity of amylase produced is not small relative to the amount of this enzyme present in the serum. During the course of a 4-hour perfusion, the amount of amylase produced usually approximated the total quantity of serum amylase in the animal: the range of accumulated perfusate amylase was 80 to 100 units in 4 hours of perfusion, whereas a 200-g rat has approximately 80 to 140 units of total amylase in serum (approximately 8 units per ml of blood and 10 to 14 ml of blood). Thus, the time required for complete replacement of the serum amylase by newly synthesized amylase is of the order of 4 hours. In contrast, the time for replacement of serum proteins is approximately 1 to 5 days. These results are consistent with the liver being a major if not the only source of serum amylase under normal circumstances. This conclusion is in agreement with the correlation noted between liver diseases and reduced amylase levels of the serum (23, 24). The data, however, do not eliminate other sources of serum amylase. Under pathological circumstances, in particular, it appears likely that the pancreas contributes to the serum amylase level (25).

The present experimental results suggest that a part of the metabolic economy of the liver cell is specifically directed toward the synthesis of serum amylase. It is assumed that synthesis of serum amylase proceeds by a mechanism similar to that for serum proteins. The present perfused liver preparation appears to present an adequate system for the study of the control of synthesis and elaboration of amylase as well as other serum proteins.

SUMMARY

1. Isolated rat livers were perfused with a defined medium containing washed erythrocytes. The level of amylase was determined in the perfusate and livers and was found to accumulate in the perfusate in a reproducible, linear manner. The total amylase in the system approximately doubled during optimal perfusion conditions.

2. Amylase was produced by perfused systems in the complete absence of pancreatic tissue. It is thus concluded that the liver is the source of amylase in this system.

3. The production of amylase by liver was depressed under conditions such that protein synthesis was decreased. Both serum protein synthesis and amylase production were depressed in the presence of *p*-fluorophenylalanine, and by the omission of amino acids. Almost complete inhibition was achieved under appropriate conditions with puromycin.

4. Amylase produced during perfusion experiments in the presence of L-leucine- C^{14} was isolated with the aid of specific rabbit antiserum and shown to contain the isotope. The quantity of amylase produced was equivalent to about 0.5% of the total serum protein synthesized.

5. It is concluded that the liver is a major, if not the only, source of serum amylase under normal physiological conditions.

Acknowledgments—The authors wish to acknowledge the collaboration of Dr. Rhoda Blostein in the immunochemical experiments and the expert technical assistance of Mrs. Janet Stephenson during various phases of the investigation.

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