Messenger Ribonucleic Acid Stability in Rat Pancreas and Liver*

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ABSTRACT: Messenger ribonucleic acid (mRNA) was characterized in rat pancreas and liver by two different methods: (1) by measuring the rate of valine incorporation into proteins and (2) by a specific labeling in presence of low doses of actinomycin D. Its degradation was then followed after injection of actinomycin D at high doses. The two methods are in good agreement. Hepatic mRNA is more stable than pancreatic mRNA. In both systems, mRNAs coding for basic proteins are more stable than those coding for acidic proteins. The effects of inhibitors of protein biosynthesis were investigated. The results might be interpreted in terms of the effect of the net charge of the nearly completed polypeptide chain on mRNA stability.

Messenger ribonucleic acid (mRNA) has been found very labile in bacterial cells (Levinthal *et al.*, 1962; Woese *et al.*, 1963). At the opposite, animal cell mRNAs are a 100 times more stable (Revel and Hiatt, 1964; Trakatellis *et al.*, 1964). However the stability range is wide in both systems (Arnonson, 1966; Yudkin, 1965; McClatchy and Rickenberg, 1967; Pitot *et al.*, 1965; Marchis-Mouren and Cozzone, 1966; Wilson and Hoagland, 1967), so that the control of protein biosynthesis might take place at the level of mRNA degradation. Our aim was (1) to establish the existence of differences in lifetimes between specific mRNAs and (2) to investigate why such similar structures are broken down at different rates.

Actinomycin D given at high doses blocks all RNA biosynthesis in rat pancreas and inhibits valine incorporation into specific purified proteins and groups of proteins. The biosynthesis of basic proteins including amylase and ribonuclease is less sensitive to the antibiotic than that of acidic proteins including chymotrypsinogen and trypsinogen. These data have been discussed in terms of mRNA stability in a previous report (Marchis-Mouren and Cozzone, 1966). The present paper extends these experiments to the case of liver. Moreover confirmation of these results was obtained by measuring total mRNA stability after specific labeling. Experiments made with pancreas and liver either by the valine incorporation technique or by ^{32}P incorporation are in good agreement. Hepatic mRNA is more stable than pancreatic mRNA. As previously shown in the case of pancreas, hepatic mRNAs coding for basic proteins are more stable than those coding for acidic proteins. Finally, in order to interpret the relation which may exist between the nascent protein and the stability of its template, the effects of inhibitors of protein biosynthesis on mRNA stability have been studied.

For convenience in formulating experimental conditions and results, any change in the amount of a specific messenger (m*) coding for the biosynthesis of a protein (p*) has been described by eq 1

$$d(m^*)/dt = k_1 - k_2(m^*) \quad (1)$$

and any change in the amount of protein is given by eq

$$d(p^*)/dt = k_3(m^*) - k_4(p^*) \quad (2)$$

2, where k_1 and k_3 are, respectively, the rate constants of transcription and translation, k_2 and k_4 the rate constants of degradation.

Materials and Methods

Wistar male rats weighing between 200 and 250 g and fed *ad libitum* on a balanced diet were used in these experiments. Actinomycin D, hereafter called actinomycin, was dissolved in propylene glycol. Treated rats received high or low doses of the antibiotic in solution. Control rats were injected with the same volume of solvent. L-[^{14}C]Valine (10 μc) dissolved in 0.4 ml of 25 mM HCl was injected intraperitoneally and, after 10 min, each rat was sacrificed by decapitation. [^{32}P]Orthophosphate (0.5–2 mc) dissolved in 0.5 ml of water, puromycin, and cycloheximide dissolved in 0.9% sodium chloride were given by the same route. Control rats received the same volume (1 ml/kg) of saline.

Purification of RNA from Pancreas and Liver. Fresh tissue (2–3 g) was homogenized for 2.5 min in a Waring Blendor with nine volumes of 0.1 M phosphate buffer (pH 6.0), containing 1% sodium dodecyl sulfate plus 0.17 volume of a sodium magnesium lithofluorosilicate suspension in 0.05 M Tris buffer (pH 7.6) and 0.01 M magnesium chloride, together with the same volume of a phenol–cresol mixture consisting of 500

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† Most of this work was done in partial fulfillment of the requirements for the degree of Docteur ès Sciences at the Université d'Aix-Marseille.

TABLE I: Inhibition by Actinomycin D of [^{14}C]Valine Incorporation into Different Protein Fractions from Pancreas and Liver.[a]

Fraction	Actinomycin Exposure (hr)	Pancreas (cpm)	Liver (cpm)	Inhibn (%) in Pancreas	Inhibn (%) in Liver
Total	0	2100	833	60	20
Proteins	6	840	670		
Soluble	0	1380	624	58	22
Proteins	6	587	486		
Basic	0	744	125	38	11
Proteins	6	461	111		
Acidic	0	570	453	70	26
Proteins	6	173	337		

[a] After 0- or 6-hr exposure to actinomycin (2 mg/kg), each rat was injected with 10 μc of L-[^{14}C]valine. Animals were killed 10 min later. All the radioactivity values should be multiplied by 10^3. Incorporation values are expressed as counts per minute per milligram of DNA–phosphorus. Per cent of inhibition is given as compared to the control. In spite of the low degree of inhibition in liver, the results are statistically significant.

g of crystalline phenol, 55 ml of water, 70 ml of *m*-cresol, and 0.5 g of 8-hydroxyquinoline. The suspension was stirred for 20 min at 6° and centrifuged for 20 min at 14,000*g*. The aqueous phase and the interphase were removed and extracted twice with an equal volume of the phenol–cresol mixture. At the end, the interphase was discarded. The clear solution was made 0.2 M in acetate buffer (pH 5.0), and RNA was precipitated with 2.5 volumes of cold 96% ethanol, then kept overnight at −15°. After centrifugation, RNA was dissolved in 2 M potassium acetate and precipitated with cold 24% ethanol. This procedure was repeated twice at −10°. Total RNA was then extracted three times with 1 M sodium chloride at 0° in order to remove sRNA, and the pellet was finally dissolved in 0.1 M sodium chloride and 0.01 M acetate buffer (pH 5.0).

Sucrose gradient (5–20%) analysis was carried out in a swinging-bucket SW 25 at 25,000 rpm for 14 hr at 0°. Fractions of 1.5 ml were collected and analyzed.

Base Composition Analysis. RNA was digested by 0.3 M potassium hydroxide for 18 hr at 37°. The nucleotides were separated by high-voltage electrophoresis (30 v/cm for 2 hr) according to the technique of Davidson and Smellie (1952).

Assays and Counting Procedure. Total, soluble, acidic, and basic proteins were precipitated in hot 5% trichloroacetic acid and dissolved in a small volume of 0.1 M sodium hydroxide. They were assayed by the method of Lowry (1951) using horse serum albumin as a standard. RNA was measured at 260 mμ taking K = 25 cm^2 mg^{-1} or by the orcinol method. DNA was assayed by the diphenylamine method.

An aliquot of each radioactive sample was added to 10 ml of scintillation counting fluid (Bray, 1960) in a glass vial and the radioactivity was measured in Packard Tri-Carb spectrometer. The radioactivity of the valine incorporated into protein fractions is given as counts per minute per milligram of DNA–phosphorus of the homogenate. The nucleotide spots were located by ultraviolet light and their positions were identified on the paper as compared to standard. They were then cut out and the radioactivity of each was counted as such in a Packard vial containing 10 ml of scintillation counting fluid.

L-[^{14}C]Valine (95 mc/mmole) and [^{32}P]orthophosphate (20 mc/mg) were purchased from the Commissariat à l'Energie Atomique. Actinomycin D was a gift from Merck Sharp and Dohme. Cycloheximide was a gift from the Upjohn Co. Puromycin dihydrochloride was purchased from the Nutritional Biochemicals Corp. Macaloid was a gift from the National Lead Co.

Results

Effect of Actinomycin on Valine Incorporation into Liver Proteins. mRNA stability in liver cells has been investigated by different authors in two ways. (1) Polysomes were isolated by sucrose gradient centrifugation at various times after injection of actinomycin and the ratio polysomes:ribosomes was measured (Staehelin *et al.*, 1963). (2) The amount of mRNA was determined by the pulse incorporation of a labeled amino acid into proteins at different times after the injection of antibiotic (Revel and Hiatt, 1964). These two types of experiments indicate rather stable mRNA. In our experiments, high doses (2 mg/kg) of actinomycin were given at zero time (k_1 = 0) and the rate of valine incorporation $d(p^*)/dt = k_3(m^*) - k_4(p^*)$ was determined as a function of time in liver and in pancreas for comparison. Since the incorporation time is short, $k_4(p^*)$ was then neglected. The liver was quickly excised, weighed, homogenized, and filtered through gauze. Only 28% of the hepatic proteins is soluble

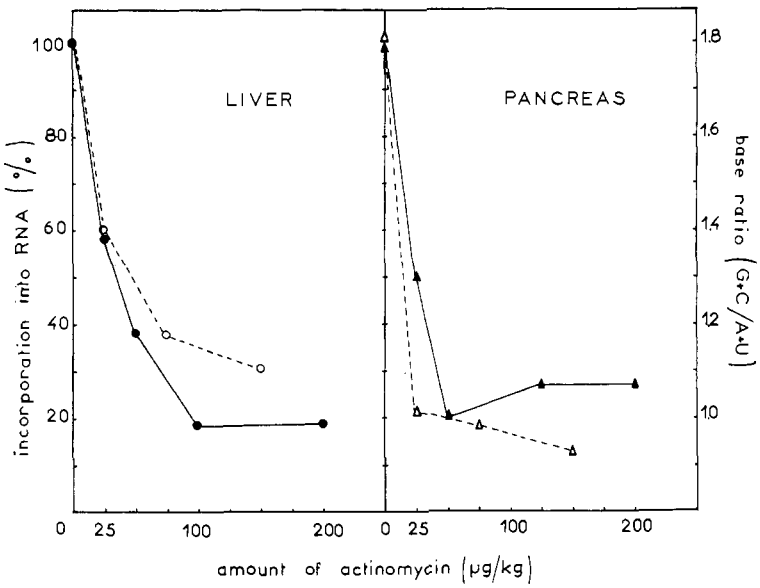

FIGURE 1: Effect of actinomycin D on RNA synthesis in pancreas and liver. Incorporation into purified RNA (fraction d) was measured at different actinomycin doses and base composition (plain line) of the labeled material (dotted line) was determined as indicated in Methods.

when the gland is homogenized with nine volumes of 0.1 M phosphate buffer (pH 8.0 or 9.0). In order to make soluble a greater fraction of proteins, the liver homogenate was treated with 8 M urea. This homogenate was then centrifuged for 1 hr at 105,000*g* and dialyzed against 5 mM phosphate buffer (pH 8.0) containing 8 M urea. This fraction was referred to as the soluble fraction. Basic and acidic proteins were obtained by chromatography of the soluble fraction on a DEAE-cellulose column (9 × 0.9 cm) equilibrated with the same buffer. The basic proteins are not adsorbed and are obtained in the break-through peak while the acidic proteins (65% of the soluble fraction) are eluted by 0.3 M phosphate buffer (pH 8.0) containing 8 M urea. The radioactivity was counted in each fraction. The results are presented in Table I.

The effect of actinomycin on valine incorporation into total and soluble proteins is much smaller in the case of liver as compared to pancreas. This difference is not due to a lower concentration of the antibiotic in liver (Marchis-Mouren and Cozzone, 1967). In both tissues, basic protein biosynthesis is less inhibited than for the acidic proteins.

Characterization of Hepatic and Pancreatic mRNA by Base Composition. The ability of actinomycin to combine with the guanosine groups of native DNA has been well established (Goldberg and Reich, 1964). On the other hand, rRNA synthesis has been shown to be specifically inhibited in animal cells by low doses of actinomycin (Georgiev *et al.*, 1963; Roberts and Newman, 1966). These results are consistent with the high G content of rRNA cistrons in such cells. We found similar results in the cases of rat pancreas and liver. Different doses of actinomycin were injected intraperitoneally and nucleic acid biosynthesis followed after injection of radioactive orthophosphate (0.5–2 mc/rat). The rats were killed 1–4 hr later, liver and pancreas were excised and homogenized, and RNA was extracted with cold phenol as described earlier. As much RNA was extracted as was found by hot 5% trichloroacetic acid extraction of the homogenate. Total RNA purity was estimated from absorbancy at 260 mμ/absorbancy at 280-mμ ratio. tRNA was removed by molar sodium chloride extraction (fraction e) and the per cent of inhibition of incorporation into the insoluble fraction (d) was measured before digestion by alkali. Nucleotides were separated by high-voltage electrophoresis, counted, and the base ratio was calculated (Figure 1).

Analysis of total RNA (Figures 2 and 3a–c) and of the two fractions obtained after NaCl extraction (Figures 2 and 3d,e) was carried out on sucrose gradient. The control pattern (a) shows, after 4 hr, an even distribution of the radioactivity under the ribosomal and the transfer peaks. In presence of high doses of actinomycin (b) the radioactivity is found associated with the tRNA peak, probably due to the pCpCpA sequence turnover. With low doses, an heterogeneous peak of radioactivity appears at the ribosomal level (gradient c). Molar sodium chloride extraction was shown to eliminate only low molecular weight RNA (especially tRNA), radioactivity, and absorbancy (e). The remaining fraction (d) was taken to be mRNA because of its base composition.[1]

Similar results were obtained with pancreas and

[1] At 75 μg of actinomycin, only rRNA biosynthesis is blocked, while total mRNA biosynthesis appears unaffected since incorporation of valine into hepatic and pancreatic proteins was found not to be inhibited.

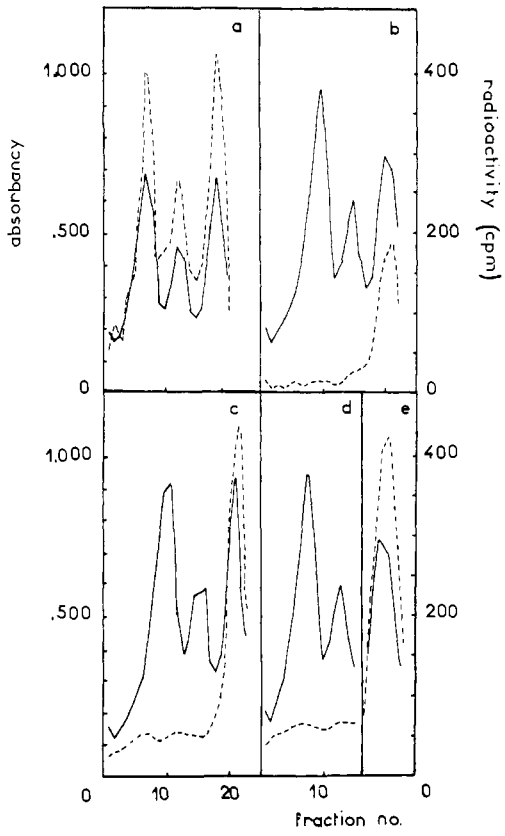

FIGURE 2: Sucrose gradient analysis of pancreatic RNA. Plain line: absorbancy at 260 mμ; dotted line: ^{32}P radioactivity. (a) Total RNA control; (b) total RNA at high dose of actinomycin (2000 μg/kg); (c) total RNA at low dose (75 μg/kg); (d) RNA remaining after 1 M NaCl extraction. Neither counts nor absorbancy was found in the 4S region; (e) 1 M NaCl extracted RNA. Neither counts nor absorbancy was found in the ribosomal region.

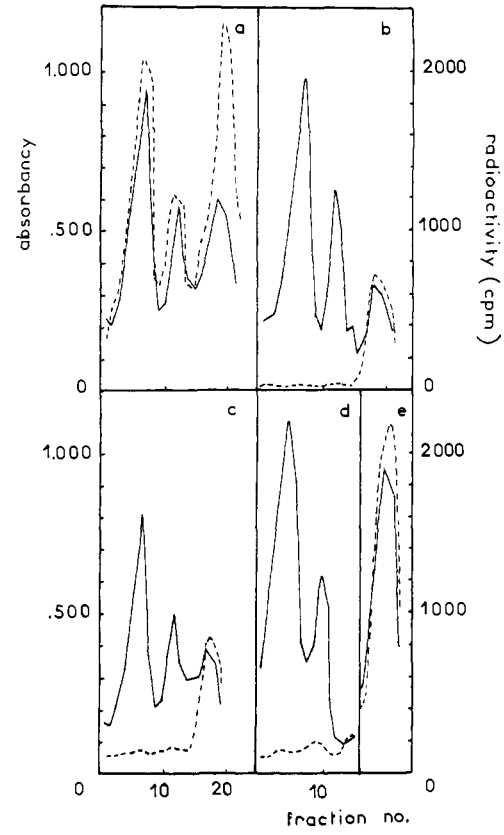

FIGURE 3: Sucrose gradient analysis of hepatic RNA. Legend is the same as in Figure 2.

liver except that the low molecular weight RNA peak appears bigger in the case of pancreas. So, in order to show that this difference was not due to degradation products, the fractions (e) were analyzed by polyacrylamide gel electrophoresis in Tris buffer (pH 8.9) and 7 M urea (J. Scott, personal communication). After staining, only one main band was obtained both in pancreas and in liver. This band migrated at the same position as pure *Escherichia coli* 4S RNA. The gel was cut into 2–3-mm thick slices; each slice was minced and counted directly in a scintillating fluid. Most of the radioactivity was found to coincide with 4S RNA (Figure 4). Almost no radioactive material was counted at the position of nucleotides and RNA of molecular weight lower than 3000.

Decay of Prelabeled mRNA after Injection of High Doses of Actinomycin. A low dose of actinomycin (75 μg/kg) and 1 mc of [^{32}P]orthophosphate were injected into rats nearly at the same time. After 1-hr labeling, all RNA synthesis was blocked by the injection of a high dose of actinomycin (2000 μg/kg). The decrease of prelabeled DNA-like RNA was then followed as a function of time (Figure 5). Since $k_1 = 0$, eq 1 gives $d(m^*)/dt = -k_2(m^*)$. Liver mRNA decay is rather slow as compared to pancreas mRNA. The half-life of pancreas mRNA extracted under our conditions is about 4 hr while only 20% of liver mRNA is broken down after 6 hr. The results given by this technique are quite the same as those obtained previously by measuring valine incorporation into proteins (*cf.* Table I).

Effect of Puromycin and Cycloheximide on mRNA Stability. Puromycin (Nathans, 1964) is known to inhibit protein biosynthesis by competing with the incoming aminoacyl-tRNA; protein biosynthesis is then interrupted by the release of puromycyl peptides while ribosomes keep moving. Cycloheximide (Colombo *et al.*, 1965), by inhibiting the polymerizing enzymes, prevents the transfer of aminoacyl-tRNA to polypeptides; then no new chain is initiated and no peptides are released; the biosynthetic machinery is blocked. Therefore the use of these two inhibitors might shed some light on the relation both between protein biosynthesis and mRNA degradation, and on the protection effect of polypeptides on the template. First, the amount of puromycin and cycloheximide necessary to block protein biosynthesis was determined in pancreas and

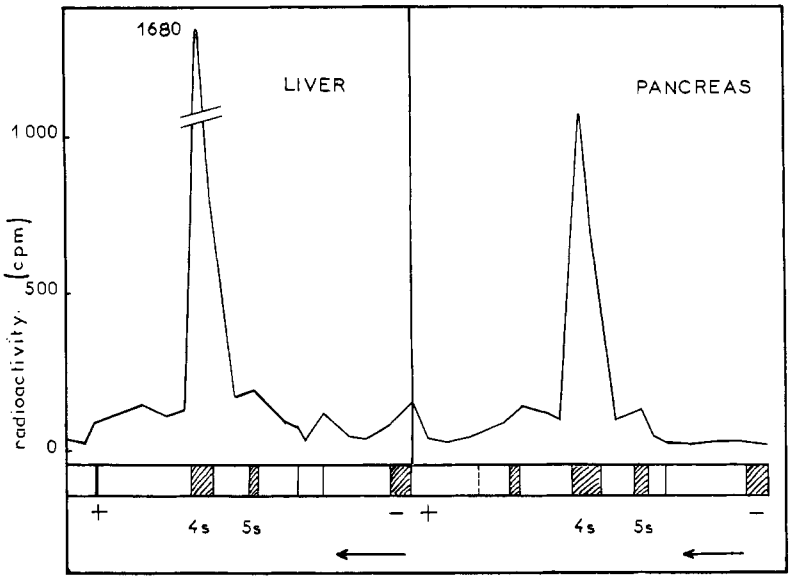

FIGURE 4: Polyacrylamide gel electrophoresis of soluble pancreatic and hepatic RNA. The migration through the gel is given in abscissa.

TABLE II: Effect of Protein Synthesis Inhibitors on the Rate of mRNA Decay.

		Liver		Pancreas	
	Time (hr)	Cpm/mg of RNA	% of Decay	Cpm/mg of RNA	% of Decay
Control	0	50,031	0	11,766	0
Actinomycin	3	44,028	12	7,178	39
	4	42,043	16	5,979	49
Actinomycin + cycloheximide	3	30,188	40	6,312	46
	4	23,334	53	4,491	62
Actinomycin + puromycin	3	27,635	45	5,336	55
	4	28,213	44	4,637	61

[a] Experiment a: each rat received at zero time 1 mc of [^{32}P]orthophosphate plus actinomycin (low dose). After 1-hr labeling, a high dose of antibiotic was injected alone or with inhibitors as indicated. Animals were killed 3 or 4 hr later.

in liver (Figure 6). Different doses were injected into rats and, 4 hr later, the rate of valine incorporation into proteins was measured. Puromycin (70 mg/kg) and cycloheximide (2 mg/kg) are sufficient doses to inhibit more than 90% of valine incorporation. These doses were used in the two following experiments.

EXPERIMENT A. The experimental procedure is the same as in the study of prelabeled mRNA decay, except that puromycin and cycloheximide were given at the same time as high dose of actinomycin. The decay of labeled mRNA was then compared after 3 and 4 hr between inhibitor-treated rats and controls. As shown in Table II, the rate of mRNA decay is increased both by puromycin and cycloheximide as well in pancreas as in liver, and to a larger extent in the case of liver.

EXPERIMENT B. Labeled orthophosphate plus a low dose of actinomycin plus puromycin or cycloheximide were injected into each rat at zero time. Incorporation into DNA-like RNA was then followed as a function of time. The kinetics obtained account both for biosynthesis and degradation of newly synthesized mRNA. If any change in the amount of labeled mRNA is given by $d(m^*)/dt = k_1 - k_2(m^*)$, then, if k_2 is increased by specific agents, $d(m^*)/dt$ will diminish and the plateau will be reached earlier assuming that these agents

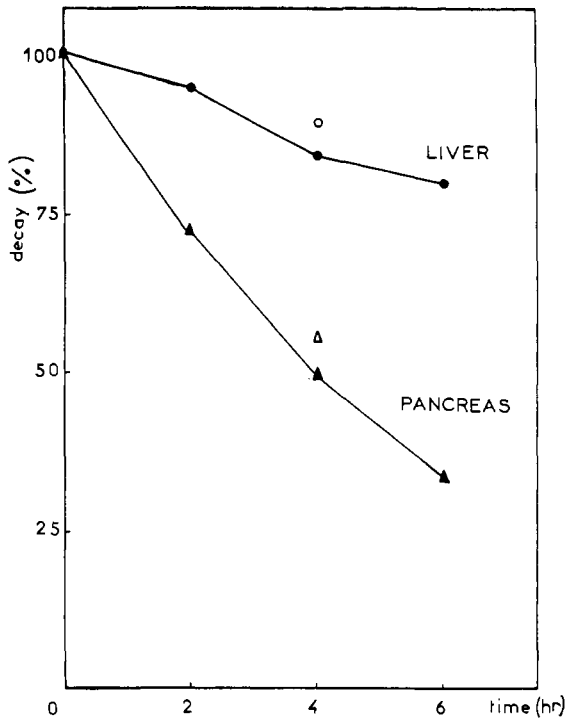

FIGURE 5: Degradation of pancreatic and hepatic RNA. Actinomycin is given at zero time (2 mg/kg) after prelabeling of mRNA by [^{32}P]orthophosphate in the presence of a low dose (75 μg/kg) of antibiotic. Three to four rats were used for each point time. Ordinate: per cent of remaining labeled material. Abscissa: time of action of actinomycin. Open symbols are given when the experiment was made in duplicate.

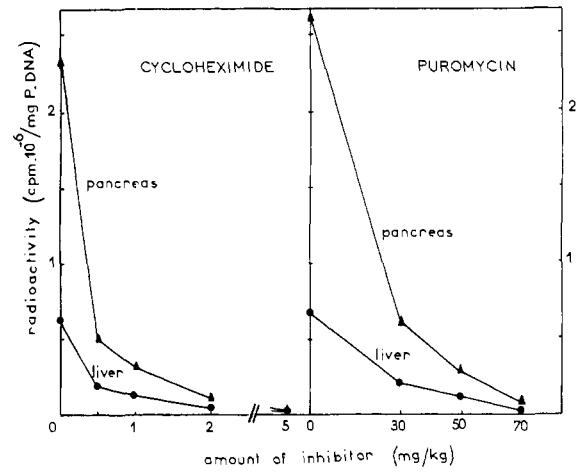

FIGURE 6: Inhibition of [^{14}C]valine incorporation into total proteins of pancreas and liver by puromycin and cycloheximide. Animals were injected intraperitoneally with puromycin (50 mg/kg) or cycloheximide (1.5 mg/kg). Four hours later, each rat received 10 μc of L-[^{14}C]-valine by the same route and was killed after 10 min.

will not modify the rate of transcription (k_1) of mRNA. In HeLa cells, both puromycin and cycloheximide do not modify the synthesis of the total rapidly labeled RNA (Warner *et al.*, 1966). Figure 7 shows the effects of these two inhibitors on the rate of labeled mRNA accumulation. Both of them are inhibitors but the effect of puromycin is much pronounced specially in the case of liver.

Moreover it appears in the control curves that the plateau ($d(m^*)/dt = 0$, $k_1 = k_2(m^*)$) is reached much earlier in the case of pancreas than in liver. This is further evidence that the rate of mRNA degradation (k_2) is higher in pancreas.

Discussion

The goal of the present work has been to study the stability of mRNA coding for different proteins with the same type of cell and compare it with different types of cells. Exact comparisons of total mRNA of pancreas and liver are limited because of the known cell heterogeneity of these tissues. This difficulty has been overcome by purifying proteins synthesized by a specific type of cell. In the case of pancreas the exocrine proteins, amylase, ribonuclease, chymotrypsinogen, and trypsinogen have been purified. Recently Wilson *et al.* (1967) have done a similar measurement in isolated liver albumin. Possible side effects of actinomycin led us to apply two different techniques to pancreas and liver. In both instances, the same values for total mRNA stability were obtained. Therefore the valine incorporation technique, which allows one to measure specific mRNAs half-lives, can be employed with reasonable assurance.

Of special interest is the fact that in liver as well as in pancreas, in spite of differences in the stability range, messengers coding for basic proteins appear more stable than messengers coding for acidic proteins. Such results have previously been discussed considering the nature of the mRNA (length, base composition, and affinity for membranes). The mRNA length does not seem to be a major factor. Others are just speculative. From our results we prefer to propose an indirect stabilizing effect by the mRNA product due to the polypeptide charge on the polysomal structure. A relation between mRNA stability and protein biosynthesis has already been proposed by Fan *et al.* (1964) and Nakada and Fan (1964) in *Bacillus subtilis* and *E. coli*. Puromycin has no effect on mRNA breakdown but anaerobiosis, cyanide, or azide confer protection on preexisting messenger. It was already proposed that the discrepancy between puromycin and the respiratory inhibitors might be due to the stripping of nascent peptide chain by puromycin thus removing the protective effect. From our data mRNA stability in eucaryote cells is diminished when protein biosynthesis is stopped either by puromycin or by cycloheximide. In order to explain the differences between our results and those of Nakada, it should be pointed out (1) that the stability range between bacterial and animal cells is not in the same order of magnitude and (2) that puromycin

might have side effects (Villa-Trevino *et al.*, 1964). However, both in pancreas and liver, puromycin makes mRNA more labile than when protein biosynthesis is blocked in another way. The nascent protein or the almost finished protein might contribute to the stability of the messenger and of the polysomal structure. The differential effect obtained between messengers coding for basic and acidic proteins might then be due to a more effective protection by basic polypeptides. This hypothesis then favors a nucleolytic attack at the 3′ end since translation proceeds from the 5′- to the 3′-terminal side. We plan to investigate other systems such as animal cells cultures and bacteria in order to see if these results are of general significance.

Acknowledgments

The authors wish to thank Dr. P. Desnuelle for his continued interest throughout the course of this work, Dr. J. F. Scott for helpful advice in the preparation of the manuscript, and Miss C. Teissier for her skillful technical assistance.

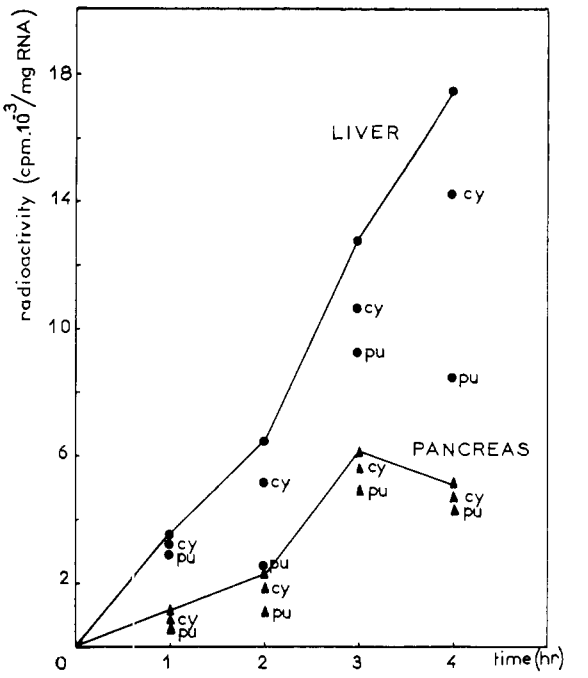

FIGURE 7: Effect of protein biosynthesis inhibitors on [^{32}P]orthophosphate incorporation into pancreatic and hepatic mRNA. Experiment b: zero time, control rats were injected intraperitoneally with 1 mc of radioactive orthophosphate plus 75 μg/kg of actinomycin. In addition, treated rats received either 70 mg/kg of puromycin or 2 mg/kg of cycloheximide. Animals were killed as indicated. Cy, cycloheximide-treated rats; Pu, puromycin-treated rats.

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