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Inhibition by Actinomycin D of Valine Incorporation into Specific Proteins of Rat Pancreas *in Vivo**

G. Marchis-Mouren and A. Cozzone

ABSTRACT: Valine incorporation was used to study protein biosynthesis in rat pancreas. Incorporation into pancreas proteins was reduced to 50% by actinomycin D within 4 hr; meanwhile incorporation into nuclear proteins was drastically diminished. Cytoplasmic material was separated into acidic and basic proteins. Inhibition by actinomycin D of valine incorporation into acidic proteins (chymotrypsinogen and trypsinogen 2) was two to three times as great as the inhibition of incorporation into basic proteins (amylase and ribonuclease). Results were interpreted in terms of messenger ribonucleic acid stability.

Ribonucleic acid biosynthesis in bacterial and animal cells has been shown to be inhibited by actinomycin D (Reich *et al.*, 1961; Levinthal *et al.*, 1962; Revel and Hiatt, 1964; Penman *et al.*, 1963; Scott and Bell, 1964). This antibiotic appears to react by forming a stable complex with some of the guanosine groups of deoxyribonucleic acid (DNA) (Goldberg and Reich, 1964), thereby impairing the transcription process. In bacteria, the inhibition of ribonucleic acid (RNA) synthesis produces a rapid decrease in protein biosynthesis. The messenger ribonucleic acid (m-RNA) that governs this synthesis is quite unstable; its half-life is measured in minutes (Levinthal *et al.*, 1962; Kepes, 1963). It soon becomes the limiting factor in protein biosynthesis, which stops after about 15 min in *Bacillus subtilis*. In mammals, RNA biosynthesis is also blocked by actinomycin D, but protein biosynthesis is less rapidly affected. The biosynthetic system (polysomes) is much more stable and can, in certain instances, remain operative for several days.

In order to study polysome stability in the absence of RNA synthesis, two types of experiment have been performed using actinomycin D. In the first set of experiments (Penman *et al.*, 1963; Staehelin *et al.*, 1963) polysomes were isolated by sucrose gradient centrifugation at various times after injection of antibiotic, and the ratio of polysomes to ribosomes was measured. In the second type of experiment (Revel and Hiatt, 1964; Bloom *et al.*, 1965), the amount of polysomes was determined by the incorporation of labeled amino acids into proteins.

Ribosomal and transfer ribonucleic acids (t-RNA's) are stable both in bacterial and animal cells (Levinthal *et al.*, 1962; Scherrer *et al.*, 1963). The amount of polysomes in such cells is thought to be determined by the amount of available m-RNA that serves as a connecting link between the ribosomes. Penman *et al.* (1963) working with HeLa cell cultures and Staehelin *et al.* (1963) with rat liver have shown that the heavy polysome fraction decreases after exposure to actinomycin D. This is accompanied by an increase in the 73S ribosome peak, suggesting the breakdown of polysomes into ribosomes. In HeLa cells, m-RNA is unstable; its half-life is about 3–4 hr. The m-RNA of liver is reported to have a longer half-life.

The experiments using amino acid incorporation

* From the Institut de Chimie Biologique, Faculté des Sciences, Marseille, France.

into proteins as a measure of m-RNA stability may be more difficult to interpret. At present m-RNA is thought to be the limiting factor in protein biosynthesis. Hemoglobin biosynthesis in reticulocytes (Nathans *et al.*, 1962) and protein biosynthesis in developing sea urchin eggs (Gross *et al.*, 1964) are not sensitive to actinomycin D, in contrast to collagen biosynthesis in mouse fibroblast tissue cultures which are inhibited within a few hours (Bloom *et al.*, 1965). Liver cell polysomes have different lifetimes depending on the type of protein to be synthesized; for example, tyrosine transaminase biosynthesis is inhibited by actinomycin D in a few hours whereas the synthesis of tryptophan pyrrolase continues for several days (Pitot *et al.*, 1965).

These differences in stability of messengers from the same cell type have been of interest to us. Our purpose has been to investigate factors related to the stability of polysomes. Pancreas was selected because this organ can synthesize large amounts of relatively few specific proteins. The principal proteins synthesized are secretory enzymes which have been extensively studied (Marchis-Mouren, 1965) and can be easily detected by their enzymatic activities.

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 at a final concentration of 2 mg/ml; 2 mg/kg of body weight was given intraperitoneally at zero time. Control animals received 0.25 ml of propylene glycol. The time interval between the injection of actinomycin and of valine is given in Results. L-[^{14}C]Valine (10 μc) dissolved in 0.4 ml of 0.0025 N HCl was injected into the femoral vein, and after 10 min, each rat was bled. The pancreas was quickly excised, weighed, minced with scissors, and homogenized with nine volumes of either water or 0.1 M phosphate buffer, pH 8.0. A Potter–Elvehjem homogenizer was used with a difference in diameter between pestle and glass of 0.3 mm. All experiments were carried out at 0°.

In the experiments of [^{14}C]adenine incorporation into pancreas RNA, each rat (250 g) received a single dose of actinomycin intraperitoneally at zero time, and 3 hr later, 20 μc of [^{14}C]adenine was injected. The animal was killed after 7 hr. Total RNA's were prepared from pancreas homogenate using the method of Schmidt and Thannhauser (1945).

Purification of Proteins. The proteins were extracted from the homogenate using the method of Schneider (1957). Amylase was purified as previously described (Marchis-Mouren *et al.*, 1963). Chymotrypsinogen and trypsinogen 2 were separated by chromatography through a SE-cellulose column (Reboud *et al.*, 1964). RNAase was prepared by a method the detailed description of which is in preparation. The main steps of the procedure for isolation of RNAase and amylase are: (a) homogenization of the gland; (b) chromatography of the 105,000*g* supernatant fraction on a DEAE-cellulose column at pH 8.0; RNAase and amylase are not retained and are eluted with the break-through peak; (c) formation of a glycogen–amylase complex insoluble in 12% ethanol; RNAase remains in solution; (d) acidification to pH 2.2; denatured proteins are removed by centrifugation; (e) chromatography on CM-cellulose at pH 6.0; (f) chromatography on Amberlite IRC-50; the enzyme is eluted by using a linear pH gradient.

Purification of Valine. "Free intrapancreatic valine" is the valine found in the 5% cold TCA[1] supernatant fraction. To determine it, the TCA was extracted with ether and the aqueous phase was concentrated and analyzed with a Technicon amino acid analyzer (B. Abdeljlil, personal communication).

Extraction of Nuclei. Pancreatic nuclei were prepared following a method similar to the one described by Hubert *et al.* (1962). The gland was homogenized with nine volumes of 0.04 M Tris buffer containing 0.25 M sucrose, 5×10^{-4} M $CaCl_2$, and 10^{-3} M $MgCl_2$. The solution was brought to pH 6.9 with 2 N HCl. Cemulsol NPT 12 detergent was added to make a final concentration of 0.15%. The suspension was homogenized and layered on the top of 20 ml of 0.5 M sucrose in the same medium in a centrifuge tube; it was then sedimented at 600*g* for 5 min at 0°. Only the nuclei pass through this density barrier to form a colorless and gelatinous pellet. They appear intact on examination with the optical microscope ($G = 1200$).

Assays. The following previously described assays (Reboud *et al.*, 1966b) were used: chymotrypsinogen and trypsinogen 2 were activated with trypsin, then assayed for activity using, respectively, acetyl-L-tyrosine ethyl ester and benzoyl-L-arginine ethyl ester as substrates (titrimetric method). Amylase was assayed by the colorimetric method using dinitrosalicylic acid. RNAase was assayed by the spectrophotometric method of Kunitz using yeast RNA as a substrate. Yeast RNA was purified by precipitation in 50% acetic acid and was then extensively dialyzed against water. Proteins were assayed by the method of Lowry (1951) using horse serum albumin as a standard. DNA was assayed by the diphenylamine method, and RNA by the orcinol method.

Counting Procedure. 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 a Packard Tri-Carb spectrometer. The radioactivity incorporated into enzyme fractions is given as counts per minute per enzyme unit per milligram of DNA-P, units of enzyme activity and DNA-P of the total homogenate serving as reference.

L-[^{14}C]Valine (67 mc/mmole) and [^{14}C]adenine (1 mc/mmole) were purchased from the Commissariat à l'Energie Atomique. Actinomycin D was a gift from Merck Sharp and Dohme. Yeast RNA and glycogen were obtained from Nutritional Biochemicals Corp.

[1] Abbreviation used: TCA, trichloroacetic acid.

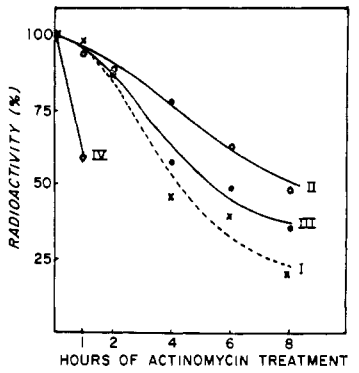


FIGURE 1: Effect of actinomycin on valine incorporation into: I, total proteins (×); II, amylase (○); III, RNAase (●); IV, nuclear proteins (◇). Six to twelve rats were used for each time point.

Starch was purchased from Etablissements Fluka. Cemulsol NPT 12 was generously supplied by Société des Produits Chemiques de Synthèse. Acetyl-L-tyrosine ethyl ester and benzoyl-L-arginine ethyl ester were synthesized in this laboratory.

Results

Actinomycin Inhibition of Adenine Incorporation into Pancreatic RNA. RNA biosynthesis was measured using [^{14}C]adenine. Adenine incorporation into RNA is inhibited by actinomycin depending on the dose. RNA (320 cpm/mg) is incorporated into control animals. Maximum inhibition (75–80%) is reached with about 1 mg of drug/kg of body weight; at this level, the adenine incorporation is probably due to the turnover of the terminal CCA sequence of RNA (Merits, 1963; Hurwitz *et al.*, 1962; Tamaoki and Mueller, 1962) rather than to *de novo* synthesis. Thus, with 1 mg or more of the antibiotic, the DNA-dependent RNA synthesis appears to be completely blocked. All subsequent experiments were carried out using a dose of 2 mg of actinomycin/kg of body weight or twice the lowest dose necessary for complete inhibition. Under these conditions the effect of the antibiotic on valine incorporation into total cell proteins, into different groups of proteins, and into certain purified enzymes was measured.

Actinomycin Inhibition of Valine Incorporation into Total Proteins. At zero-time actinomycin was given to five groups of rats; control animals received an equivalent volume of propylene glycol. After 1, 2, 4, and 6 hr, [^{14}C]valine was injected and the animals were killed 10 min later. The amount of radioactivity incorporated into total proteins was calculated as counts per minute per milligram of DNA-P. For all experiments, inhibition was expressed as per cent of valine incorporation into the pancreas of control animals. At this short time of labeling it has been shown that losses of proteins by excretion or degradation should be negligible (Reboud *et al.*, 1966a). Figure 1 shows the effect of actinomycin on valine incorporation into total proteins. Each point of the curve represents the mean value obtained from pooled pancreas. Other experiments have shown that the differences observed are greater than the variations among randomly selected animals. The effect of actinomycin upon pancreatic proteins is more marked than its effect upon liver proteins (Revel and Hiatt, 1964; A. Cozzone and G. Marchis-Mouren, unpublished data). Incorporation of valine into total proteins decreases as the time interval after antibiotic treatment increases. There is a 50% reduction of incorporation after 4 hr, and an 80% reduction after 8 hr. The above values represent valine incorporation into total proteins. The individual proteins will be discussed below.

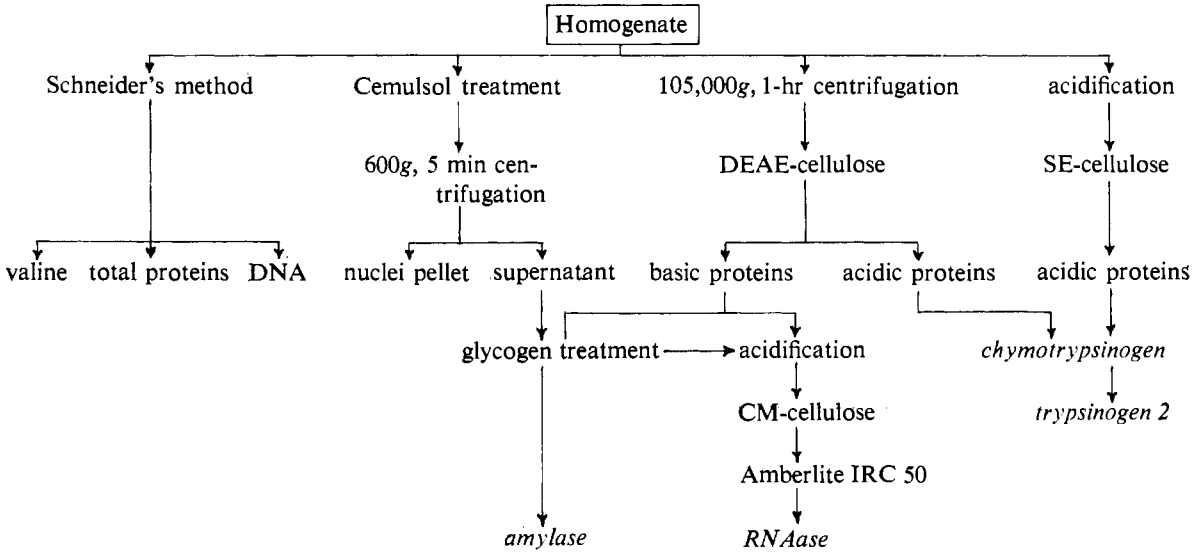
Effect of Actinomycin on the Intrapancreatic Valine Pool. A decrease in valine incorporation is not, in itself, proof that actinomycin has changed the rate of protein biosynthesis. A change in the specific activity of the precursor pool can be responsible for apparent changes in the rate of protein biosynthesis. The radioactivity of the pool changes with time so that it is difficult to measure it exactly. The specific activity of the free valine has been measured at 3, 6, and 10 min after injection (Table I). It is a little higher in the case of treated animals, but the effect of actinomycin on valine incorporation is marked despite the increase

TABLE I: Effect of Actinomycin D on the Intrapancreatic Valine Pool.[a]

Valine Incorp Time (min)	Controls		Treated Rats	
	Valine (cpm/μmole)	Total Protein (cpm/mg of DNA-P)	Valine (cpm/μmole)	Total Protein (cpm/mg of DNA-P)
3	97,000	—	137,000	—
6	56,000	—	88,000	—
10	42,000	2.1×10^6	66,000	0.84×10^6

[a] After 6-hr exposure to actinomycin, the rats were injected with 10 μc of [^{14}C]valine. Animals were killed 3, 6, and 10 min later.

SCHEME I: Fractionation Procedure.



in specific activity of the precursor pool.

Effect of Actinomycin on Amylase. Amylase is a major component of pancreatic proteins; moreover, it is stable and easily purified. Figure 1 shows that the incorporation of valine into amylase is much less affected than the incorporation into total cell proteins. After 4 hr of exposure to actinomycin the inhibition is about 20% in the case of amylase and reached 50% after 8 hr. At all time intervals the per cent inhibition is lower than that found for incorporation into total proteins extracted from the same group of rats. The system for amylase biosynthesis is probably more stable than the systems for the biosynthesis of other pancreatic proteins.

Effect of Actinomycin on RNAase. RNAase is of special interest because its molecular weight is one-third that of amylase. These two proteins were purified from the same pancreas homogenate (Scheme I). RNAase behaves like amylase on a DEAE-cellulose column. Amylase was isolated as the specific glycogen complex. RNAase was purified as described in Methods and in Scheme I. Figure 1 shows the effect on the incorporation of valine into this enzyme; 35% inhibition is seen after 4 hr of actinomycin treatment and 50% after 5 hr. These values are intermediate between those found with amylase and with total proteins. Moreover, valine incorporation into both amylase and RNAase is less affected than incorporation into total proteins. Hence, the biosynthesis of certain proteins must be strikingly inhibited.

Effect of Actinomycin on Nuclear Proteins. It has been shown by Honig and Rabinovitz (1964) that [^{14}C]lysine incorporation into nuclear proteins by sarcoma 37 in the ascites form was rapidly inhibited by actinomycin. This led us to investigate the case of nuclear pancreatic proteins. Nuclei were treated with cemulsol NPT 12, as described in Methods, to get rid of cytoplasmic constituents. Proteins and nucleic acids were extracted using Schneider's (1957)

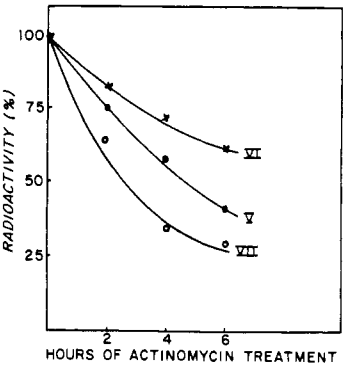


FIGURE 2: Effect of actinomycin on valine incorporation into: V, soluble proteins (●); VI, basic proteins (×); VII, acidic proteins (○). Five to nine rats were used for each time point.

procedure. Valine incorporation into nuclear proteins was affected to a much greater extent than total cell proteins, as shown in Figure 1. Within 1 hr after administration of the antibiotic, labeling was suppressed to about 40% of the control value. However, this striking inhibition is insufficient to explain the effect upon total proteins. Nuclear protein represents no more than 6–7% of the total protein fraction. As shown in Table II, the amount of [^{14}C]valine incorporated into the nuclear fraction is small, and the marked effect of actinomycin on incorporation into pancreatic proteins must be sought elsewhere.

Differential Labeling of Anionic and Cationic Proteins. A systematic study of cytoplasmic proteins was carried out as follows. Pancreas was homogenized in 0.1 M phosphate buffer, pH 8.0; under these conditions zymogen granules are broken down. Subcellular particles and membranes were removed as a pellet by centrifugation at 105,000*g* for 1 hr. The soluble proteins of the

TABLE II: Amounts of Radioactivity Incorporated into Different Fractions after Actinomycin D Treatment.[a]

	Time of Actinomycin Exposure (hr)					
	0	1	2	4	6	8
Total proteins (I)	2100	2080	1860	900	840	420
Nuclear proteins (IV)	67	40	—	—	—	—
Soluble proteins (V)	1380	—	1035	800	587	—
Basic proteins (VI)	744	—	614	527	461	—
Acidic proteins (VII)	570	—	373	197	173	—
Amylase (II)	525	498	467	399	310	252
RNAase (III)	57	—	—	33	28	21
Chymotrypsinogen (VIII)	220	—	115	97	61	—
Trypsinogen 2 (IX)	79	—	43	34	16	—

[a] Incorporated radioactivity is expressed as counts per minute per milligram of DNA-P. Time of incorporation of [^{14}C]valine, 10 min; time of exposure to actinomycin, 0, 1, 2, 4, 6, and 8 hr. All the radioactivity values should be multiplied by 10^3. The numbers in brackets indicate the curves of the figures.

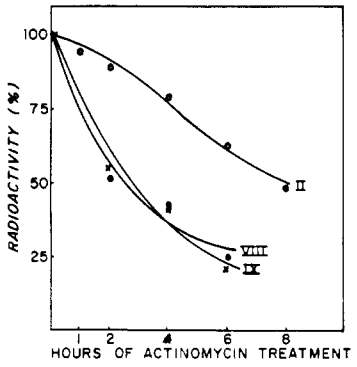


FIGURE 3: Effect of actinomycin on valine incorporation into: II, amylase (○); VIII, chymotrypsinogen (●); IX, trypsinogen 2 (×). Six to twelve rats were used for each time point of curve II and five to nine rats for each time point of curves VIII and IX.

supernatant fraction were then separated into two groups. At a low ionic strength (0.005 M phosphate buffer) and pH 8.0, cationic proteins, negatively charged, were not adsorbed on a DEAE-cellulose column and eluted after the first retention volume. In contrast, anionic proteins, neutral or positively charged, were retained by the same column and could be eluted only by increasing the ionic strength of the solvent. Scheme I summarizes the fractionation procedure. After labeling, two-thirds of the total protein radioactivity was incorporated into soluble proteins. The effect of actinomycin is shown in Figure 2. The amount of radioactive valine incorporated into soluble proteins is reduced by 50% within 4.5 hr. This value is quite similar to that for total proteins. The inhibitory effect is much less pronounced in basic proteins; a 30% decrease occurs after 4 hr. In contrast, acidic protein biosynthesis is drastically diminished: 65% after the same time of exposure to actinomycin.

Effect of Actinomycin on Chymotrypsinogen and Trypsinogen 2. Amylase and RNAase belong to the group of basic proteins. More than half of the radioactivity incorporated into basic proteins is found in amylase (Table II). Chymotrypsinogen and trypsinogen 2 are the major components of the acidic protein group. As indicated in Figure 3, the incorporation of valine into these two zymogens is rapidly depressed after actinomycin treatment. The degree of inhibition is about the same in both cases, due perhaps to similarity of the two proteins; 65% is reached after 4 hr, and 80–85% after 6 hr, and 50% inhibition within 3 hr. In this respect these two proteins behave similarly to the total acidic proteins (Figure 2).

Discussion

The incorporation into acidic proteins is therefore strikingly diminished as compared to that into basic proteins. But before interpreting these results the following points have to be discussed.

The radioactivity incorporated was counted in well-characterized purified proteins or groups of proteins. The specific activity values of purified enzymes are very close to those observed for the homologous crystallized bovine or porcine enzymes: amylase, 600; RNAase, 950; chymotrypsinogen, 425; and trypsinogen 2, 49. Moreover, amylase and RNAase have been analyzed using disc electrophoresis and shown to give a single band.

Since these are purified proteins, the rates of biosynthesis might be calculated after the determination (1) of the number of valine residues per mole of specific protein, and (2) of the specific radioactivity of valine in the pool used for protein biosynthesis. The number of valine residues is already known (J. Christophe, personal communication) but, as suggested above, the determination of the valine pool is difficult. Moreover,

in single bacterial and yeast cells several cellular amino acid pools have been found (Zalokar, 1961). In pancreas, only the total free intrapancreatic valine has been determined, and slightly higher values have been found after actinomycin treatment. The rate of biosynthesis as measured by the rate of incorporation should be diminished if the radioactive valine uptake into the amino acid pool is taken into account. This would make our results even more dramatic. The true significance of our data, however, depends upon knowledge of the actual effects of actinomycin. Large amounts of this antibiotic are also known to be lethal (Philips *et al.*, 1960). In order to discuss our results in terms of biosynthetic mechanisms with respect to m-RNA, we have to assume that, under our experimental conditions, actinomycin inhibits the transcription process only, without affecting the translation. This hypothesis seems likely since we have reported that the biosynthesis of liver proteins in the same animals remains unimpaired (A. Cozzone and G. Marchis-Mouren, unpublished data). Others have also reported similar results (Revel and Hiatt, 1964).

It seems probable that m-RNA from pancreas is less stable than m-RNA from liver. The stability of the template varies with the nature of the protein synthesized. In terms of the messenger hypothesis, m-RNA for basic proteins would be two or three times more stable than m-RNA for acidic proteins. The half-life of amylase m-RNA would be 8 hr, and the half-life of chymotrypsinogen m-RNA would be only 3 hr. Messenger ribonucleic acid stability has also been studied in bacteria, although the messenger has a far shorter half-life and its stability with respect to its biosynthetic function has been examined. All the experiments do not lead to the same conclusions (Levinthal *et al.*, 1962; Fan *et al.*, 1964; Edlin and Maaloe, 1966); m-RNA of *Bacillus cereus* sporulating cells is stable, a property which could be explained by its firm binding to cytoplasmic membranes (Aronson, 1965, 1966).

In our experiments, an attempt has been made to relate the length of m-RNA to its stability. The length can be estimated from the molecular weight of the polypeptide chain synthesized. In the case of rat pancreas the m-RNA's are listed in order of decreasing stability followed by the molecular weight of the substance synthesized: amylase, 50,000; RNAase, 15,000; chymotrypsinogen and trypsinogen 2, 24,000. No relationship can therefore be established between m-RNA length and stability. On the other hand, it was noticed that m-RNA's coding for basic proteins were much more stable than those coding for acidic proteins. This behavior could be due to different nucleotide sequences. It seems likely that a stabilizing effect of membranes is present. Polysomes synthesizing nascent basic proteins might have a relatively high affinity for membranes and thereby form a stable complex.

Experiments are now being carried out in this laboratory to check this interpretation and extend these results. We intend to characterize m-RNA's in other ways than by their function, and to study their stability using different methods.

Acknowledgments

We wish to thank Dr. P. Desnuelle for his continued interest and stimulating discussions throughout the course of this work, Dr. R. Greif and Dr. R. Monier for helpful advice in the preparation of the manuscript, and Miss C. Teissier and Miss M. Nara for skillful technical assistance and cooperation.

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A Model of Ribonuclease Based on Chemical Evidence*

G. G. Hammes and H. A. Scheraga

ABSTRACT: Available chemical and kinetic data have been used to construct a speculative model of bovine pancreatic ribonuclease A. The model is based on several interactions whose existence is suggested by these data.

Protein chemists have been using chemical modification and physicochemical studies to determine internal interactions in proteins in order to obtain information about molecular structure. Bovine pancreatic ribonuclease A has been extensively investigated, and a wealth of experimental results are at hand (*cf.* Scheraga and Rupley, 1962; Hummel and Kalnitsky, 1964). As this information has been accumulated, several attempts have been made to correlate these data in terms of a model, the most recent being that of Saroff (1965). Recently, several new pieces of information about internal interactions in ribonuclease have become available (Cathou and Hammes, 1965; Li *et al.*, 1966; Erman and Hammes, 1966), and it appeared worthwhile to construct a new model on the basis of these and earlier data, including, among others, the covalent structure (Hirs *et al.*, 1960; Potts *et al.*, 1962; Smyth *et al.*, 1963). Although such an attempt is quite speculative, it represents a convenient summary of the known chemistry; further, the ultimate goal of all structural studies is the determination of a three-dimensional model. In addition, such a model may be useful in suggesting other experiments.

In addition to the four disulfide bonds the model is based on the following interactions: (1) Tyr 25-Asp 14, (2) Tyr 92-Asp 38, (3) Tyr 97-Asp 83, (4) Lys 7-Lys 41, and (5) His 12-His 119 (1–3, Li *et al.*, 1966; 4, Marfey *et al.*, 1965; 5, Crestfield *et al.*, 1963). Kinetic data suggest that His 12, His 119, and Asp 121 may be part of the active site (Erman and Hammes, 1966).

With this information, the structure shown in Figures 1 and 2 was constructed from non-space-filling models. The structure was also put together with Pauling–Corey–Koltun models (not shown here) to demonstrate its steric feasibility. No extended α-helical regions appear in the model.

Besides the interactions listed above, the model has most of the polar side-chain groups on the outside and most of the nonpolar side-chain groups on the inside. No attempt has been made to elucidate the details of side-chain interactions, other than those mentioned above. Tyr 73, 76, and 115 are exposed while the other three are buried (Woody *et al.*, 1966). The C terminal interacts with two of the buried tyrosines (92 and 97), in agreement with the fact that only Tyr 25 is buried in pepsin-inactivated ribonuclease (Fujioka and Scheraga, 1965), a derivative which lacks the C-terminal tetrapeptide (Anfinsen, 1956). The N and C terminals are near each other. However, the location of the first six residues in the N terminal is adjustable. In fact, the over-all shape of the molecule can be varied within limits and still preserve these interactions.

FIGURE 1 (opposite): Non-space-filling model of ribonuclease. See Figure 2 for artist's rendition of model from this view, with essential features indicated. The backbone chain was covered with red crepe paper, and the four disulfide bridges with blue paper. A model of cytidine 2′,3′-cyclic phosphate, covered with blue paper, is placed schematically in the groove at the bottom of the model.

* From the Department of Chemistry, Cornell University, Ithaca, New York 14850. *Received July 25, 1966.* This work was supported by grants from the National Institutes of Health (GM-13292 and AI-01473) and from the National Science Foundation (GB-4766).