ENZYMIC FORMATION OF THE DISULFIDE BRIDGES OF RIBONUCLEASE

By

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An enzyme was found in the pancreas of several animal species which is able to catalyze the reactivation of reduced bovine pancreatic ribonuclease.

The enzyme was partially purified from chicken and pig pancreas.

A heat-stable factor was essential to the activity of the enzyme. This substance could be replaced by dehydroascorbic acid.

The possible significance of these results in the problem of protein biosynthesis is discussed.

Introduction

ANFINSEN and HABER [1], and WHITE [2] described the reversibility of the reduction of ribonuclease. These authors reduced the disulfide groups of bovine pancreatic RNase*, and after removing the reducing agent reoxidized them with atmospheric oxygen. The reoxidized enzyme proved to be identical with the original, both in respect to physico-chemical properties, and enzyme activity. Consequently the reformation of the disulfide bridges was a specific and not a random process. Since then analogous reactivation of several other reduced proteins has been observed [3—8].

The significance of these results is obvious, considering the probable identity of the reduced protein and the polypeptide chain which is formed on the ribosomes during protein biosynthesis. Accordingly such reoxidation experiments may serve as *in vitro* modells in studying the final steps of the biosynthesis — *i.e.* the formation of the properly folded protein molecule. Indeed, it is generally believed on the basis of these experimental facts, that no new information is needed for this final step, the tertiary structure being perfectly determined by the primary, *i.e.* the correct pairing of cysteine residues is coded in the amino acid sequence itself. However, the rate of reactivation is too slow to account for the *in vivo* observed biosynthesis, even under optimal conditions. Moreover it must be kept in mind that the optimal conditions (pH 8.2 24° C) [9] are not physiological.

At the start of this work we assumed therefore, that ribonuclease-synthesizing tissues may contain an enzyme, or enzyme system which catalyzes the final step of the biosynthesis, *i.e.* the reactivation of reduced RNase. In this paper we describe the existence of this enzyme in the pancreas, its partial

* RNase: ribonuclease; tris: trishydroxymethylaminomethane; DHA: dehydroascorbic acid; EDTA: ethylenediaminotetraacetic acid; GSH, GSSG: reduced and oxidized glutathione; NAD: nicotinamide adeninedinucleotide (DPN); NADP nicotinanideadenine dinucleotide phosphate (TPN).

purification, and some properties of the reaction. A preliminary report on this subject was already published [10]. Recently GOLDBERGER *et al.* [11] reported a rat liver system capable of accelerating the reactivation of reduced ribonuclease. Considering the similarities we believe that this system and the pancreatic enzyme described by us are identical.

Materials and methods

"*Reanal*" bovine pancreatic RNase was purified by chromatography on IRC—50 column. The protein peak corresponding to RNase A was lyophilized, Purified RNase was reduced with 2-mercaptoethanol in 8 *M* urea according to the method of ANFINSEN and HABER [1]. The reagents were removed by means of a Sephadex G—25 column, with 0.1 *N* acetic acid as eluent. Reduced RNase could be stored for several weeks in the acid solution without considerable reactivation. It was neutralized immediately before use.

RNase activity was assayed by the method of SCHUCHER and HOKIN [12] with one modification. The incubation time was 10 min. instead of 30 min. In the calibration of the assay method the concentration of the standard enzyme solution was determined by measuring the optical density at 275 mμ. At this wavelength, the molar extinction coefficient of the native as well as the reduced RNase is 9200 [13].

SH groups were determined by the method of BOYER [14], protein concentration was measured by the method of LOWRY *et al.* [15].

In the assay of the reactivating enzyme the following procedure was employed. The enzyme preparation to be tested, was shaken at 37° C in pH 7.5 tris-HCl buffer with eventual additions as noted in the figures. After 5 min. preincubation, the reaction was started with the addition of reduced RNase. At intervals 0.1 ml aliquots were taken out from the reaction mixtures and pipetted into 1 ml 0.25 *N* H_2SO_4. Before RNase assay these solutions were neutralized with 1 *N* NaOH and appropriately diluted with 0.04 *M* pH 7.5 phosphate buffer. In work with pancreatic homogenates or crude extracts this sulfuric acid treatment was essential in order to release the particle-bound RNase. When purified enzyme preparations were tested, the 0.1 ml aliquots were pipetted into cold 0.04 *M* phosphate buffer and immediately assayed for RNase activity.

Dehydroascorbic acid was always freshly prepared by oxidizing ascorbic acid in dilute solution with bromine and removing excess bromine with a current of air. Crystalline DHA was prepared according to KENYON and MUNRO [16].

Results

1. *Experiments with pigeon pancreas*

Pigeon pancreas was homogenized in 0.25 *M* sucrose. After removal of cell debris and the nuclear fraction with centrifugation (1000 *g*, 10 min.), the supernatant was incubated with reduced RNase. As shown on Fig. 1 the homogenate greatly accelerates the reactivation of RNase. This effect disappears after heating the homogenate for 5 min. at 100° C.

The extract of an acetone-dry powder of pigeon pancreas exhibited similar catalytic effect, indicating that the activity was not necessarily bound to intact structures.

2. *Experiments with chicken pancreas*

Owing to the fact that pigeon pancreas was not available in sufficiently large quantities to purify the catalytic factor, we tried other species. Attempts to demonstrate the activity in extracts of beef and pig pancreas powder were

unsuccessful. However, a slight activity was observed in chicken pancreas. The addition of a boiled (*i.e.* in itself inactive) pigeon pancreatic extract enhanced the rate of reactivation of reduced RNase. The boiled extract could be replaced by yeast extract. These data, summarized in Table I suggest that a heat-stable factor is the necessary component of the reactivation system. The concentration of this factor(s) is rate limiting in chicken pancreas.

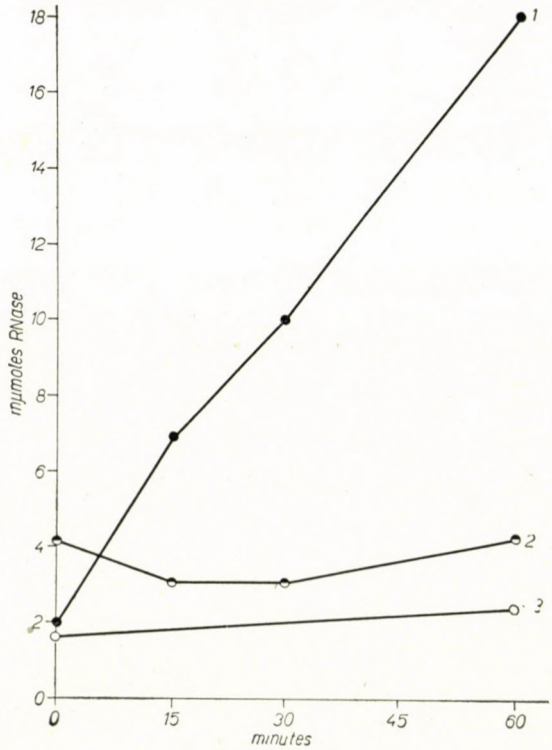

Fig. 1. The effect of pigeon pancreas homogenate on the reactivation of reduced RNase Reaction mixtures: 0.03 *M* pH 7.5 tris-HCl; 23 mμmoles reduced RNase; plus additions. Final volume: 3.2 ml
Curve *1*: 0.5 ml 20% pigeon pancreas homogenate. *2 :* 0.5 ml boiled (5 min 100° C) homogenate, *3 :* no addition (control)

3. *Purification of the reactivating enzyme from chicken pancreas*

When testing the purification procedure, a heat-stable factor *(Difco* lyophilized yeast extract) was always added to the assay mixtures. The unit of activity was defined arbitrarily as follows: one unit of enzyme is that which reactivates 1 millimicromole (13.7 microgram) reduced bovine pancreatic RNase in 1 min., based on the initial velocity. The exact quantitative evalu-

ation of the purification procedure was not possible because the conditions were not always optimal, and the crude extracts contained inhibitory substances (see later).

Step 1. The pancreas of 6 weeks-old chickens were homogenized in Waring blendor in 10 vol. twice destilled —10° C cold acetone. After centrifugation, the homogenization was repeated, the homogenate filtered, and the cake dried *in vacuo.* This powder could be stored for several months without loss of activity.

Table I

The effect of the heat-stable factor(s)

Incubation mixture: 0.1 *M* pH 7.5 tris-HCl; 10 mμ moles reduced RNase; + additions as noted in the table. Final volume: 0.5 ml. Pancreatic extracts: acetone-dried pancreas powder was extracted with 10 volumes of buffer as described in the text.

Boiled extract: pigeon pancreatic extract was heated at 100° C for 5 min.

Yeast extract: 2 mg *Difco* powdered yeast extract.

Exp.	Additions	Reactivation rate mμ mole RNase/min.
1	No addition (control)	0.003
	0.1 ml pigeon extract + 0.05 ml boiled extract	0.051
	0.1 ml pigeon extract	0.033
	0.05 ml boiled extract	0.003
2	No addition	0
	0.01 ml chicken extract	0.041
	0.01 ml chicken extract + 0.05 ml boiled extract	0.056
3	No addition	0.002
	0.1 ml chicken extract + 0.05 ml boiled extract	0.039
	0.1 ml chicken extract + 0.05 ml boiled dialyzed extract	0.011
	0.1 ml chicken extract + 2 mg yeast extract	0.056

Step 2. The dry powder was extracted with 0.05 *M* pH 7.5 tris-HCl buffer for 30 min. in the cold room. The buffer contained 0.1 mg/ml diisopropylfluorophosphate to inhibit proteolytic enzymes. The extract was centrifuged with 20 000 *g* for 10 min. and the insoluble residue was discarded. All subsequent operations were carried out in the cold.

Step 3. Solid ammonium sulfate was added slowly to the supernatant to 54 per cent saturation. It was centrifuged after standing overnight, the precipitate was discarded and ammonium sulfate was added to the supernatant to 75 per cent saturation. After centrifuging the supernatant was discarded and the precipitate was dissolved in a minimal amount of the extracting buffer. This solution was dialyzed against 0.01 *M* pH 7.5 tris-HCl buffer.

Step 4. The dialyzed solution was submitted to chromatography on DEAE-cellulose with a gradient elution technique. (Gradient from 0.01 *M* pH 7.5 tris-HCl to 0.7 *M* NaCl.) The active fractions were pooled and dialyzed

against saturated ammonium sulfate. The precipitated protein was centrifuged, dissolved in a minimal amount of tris buffer and dialyzed.

The results of some purification experiments are summarized in Table II. It is remarkable that in some cases the crude extract was completely inactive, activity appeared only after the ammonium sulfate fractionation. Probably the crude extract contained inhibitory substances which had to be removed. This observation led us to try a similar purification from pig pancreas which appeared to be inactive in earlier experiments.

Table II

Purification of the reactivating enzyme from chicken pancreas

Exp.	Specific activity: mμmole RNase/min./mg protein		
	Crude extract	After $(NH_4)_2SO_4$ fractionation	After DEAE chromatography
1	0.009	0.041	0.352
2	0	0.089	0.390
3	0	0.095	0.476

4. *Purification of the enzyme from pig pancreas*

After removal of the fat and connective tissue, pig pancreas was chopped up with scissors and homogenized in a Waring blendor for 2 min. in five volumes of 0.025 *M* pH 7.5 phosphate buffer which contained EDTA (0.002 *M*) and diisopropyl-fluorophosphate (0.1 mg/ml). This, and all subsequent operations were carried out in the cold. The homogenate was centrifuged for 30 min. with 1000 *g*, the supernatant was decanted and filtered through glass wool to remove fat. Ammonium sulfate was added slowly to 54 per cent saturation. After standing overnight the precipitate was removed by centrifugation. The clear pink supernatant was already active. Ammonium sulfate was added slowly to 70 per cent saturation. The solution was centrifuged, the supernatant discarded and the precipitate dissolved in 0.01 *M* pH 7.2 tris-HCl buffer. This solution was dialyzed against the same buffer overnight. The white insoluble material which precipitated during dialysis was removed by centrifugation. The activity remained in the supernatant. The next step was the DEAE chromatography, with a gradient from 0.01 *M* pH 7.2 tris-HCl to 0.6 *M* NaCl in the same buffer. The active fractions were concentrated as described above. The solution of the enzyme at this stage was light yellow, and always contained slight amounts of nucleic acid besides protein. The data of the purification procedure are summarized in Table III.

Table III

Purification of the reactivating enzyme from pig pancreas

Exp.	Specific activity: mμmole RNase/min./mg protein		
	Crude extract	After $(NH_4)_2SO_4$ fractionation	After DEAE chromatography
1	0	0.60	2.28
2	0	0.81	4.35

5. *Experiments with the partially purified enzyme*

The reactivating enzyme at this stage of purification appeared to be suitable to study the conditions and requirements of the reaction. Fig. 2 shows the dependence of the reaction rate on the concentration of the sub-

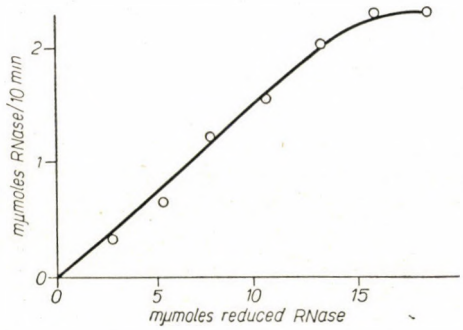

Fig. 2. Effect of the concentration of reduced RNase on the rate of reactivation. The amount of RNase reactivated in the first 10 minutes is plotted against the initial concentration of reduced RNase

Incubation mixtures: 0.05 *M* pH 7.5 tris-HCl; 4 mg *Difco* yeast extract; 1.65 mg purified chicken reactivating enzyme; plus varying amounts of reduced RNase. Final volume: 1 ml

strate, *i.e.* the reduced RNase. This increased velocity with higher concentrations is in sharp contrast with the non-enzymic reactivation [9] where an inverse relationship was observed at similar substrate concentrations.

In contrast to the marked pH dependence of the non-enzymic reoxidation, the enzyme reaction is only slightly influenced by alteration of the pH in the range between 7—8.5. This is in agreement with the results of GOLDBERGER *et al.* [11].

The kinetics of reactivation were compared at 25° C and 37° C. Again in contrast to the non-enzymic reaction [9], the rate was higher at 37° C.

Table IV summarizes data on the effects of various additional compounds on the reactivation. The SH compounds tested are all strongly inhibitory at

higher concentration, whereas at lower values the effect can be slightly stimulatory. Urea and Cu^{++} are strong inhibitors. The marked activating effect of EDTA or cyanide might be explained by assuming that these compounds act by removing the inhibitory heavy metal ions present in the reagents employed. Therefore in later experiments EDTA was always added to the reaction mixtures.

Table IV

Inhibition and activation of the enzymic reactivation

Incubation mixtures in all these experiments contained: 0.1 *M* pH 7.5 tris-HCl buffer; 8—14 mμ moles reduced RNase; 2 mg *Difco* yeast extract; 1—2 mg purified activating enzyme; + additions as noted in the table. Final volume 0.6 ml. Activities are expressed in the percentage of the control values.

Additions	Activity %
No addition (control)	100
5. 10^{-3} *M* cystein	20
5. 10^{-3} *M* GSH	4
5. 10^{-3} *M* 2-mercaptoethanol	20
10^{-4} *M* ,,	107
5. 10^{-3} *M* thioglycolic acid	3
10^{-3} *M* Cu^{++}	0
1.3 *M* urea	21
8. 10^{-4} *M* NaCN	120
8. 10^{-3} *M* NaCN	147
8. 10^{-3} *M* EDTA	155

The reactivating enzyme could be stored even in very dilute solutions in the cold for several weeks without loss of activity. It is sensitive to acid and heat treatment. 5 min. heating at 65° C decreases the activity by 43 per cent. Precipitation at pH 4.8 results in an irreversible decrease in activity. If the enzyme was treated with *p*-chloromercuribenzoate, after removal of the reagent with Sephadex G—25 no decrease of activity was observed. This result suggests that SH groups do not play any role in the activity of the enzyme.

6. *The heat-stable factor*

Our earlier experiments with crude pancreatic extracts indicated — in agreement with the results of GOLDBERGER *et al.* [11], — thet the heat-stable non-protein factor required in the reactivation process can be removed only

partially by dialysis. Partially purified preparations of the reactivating enzyme were sometimes completely inactive without the addition of yeast or boiled pancreatic extract; other preparations did not require the addition of the heat-stable cofactor. Probably uncontrolled variations in the purification procedure resulted in the loss of bound cofactor in the former case.

Fig. 3 shows the effect of cofactor *(Difco* yeast extract) concentration on the reactivation catalyzed by a cofactor-dependent enzyme preparation. The initial velocity was proportional to the concentration. However, the

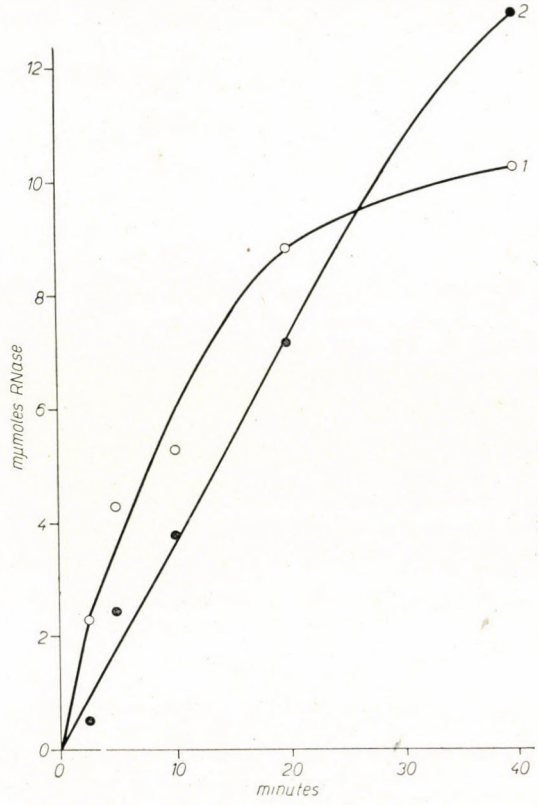

Fig. 3. Effect of the concentration of the yeast extract. Incubation mixtures: 0.1 *M* pH 7.5 tris-HCl; $5 \cdot 10^{-3}$ *M* EDTA; 50 mμmoles reduced RNase; 0.05 mg purified pig enzyme. Final volume: 1 ml. Curve *1 :* 20 mg *Difco* yeast extract, *2 :* 6.6 mg *Difco* yeast extract

maximal extent of reactivation was always higher with lower cofactor concentration. We tried to replace yeast extract with NAD, NADP, GSSG, but these attempts were unsuccessful. GOLDBERGER *et al.* [11] tested a number of other substances but none of them proved to be active. In view of the known hydro-

gen acceptor role of dehydroascorbic acid in plant systems, we tried this substance as cofactor. It was found, that crystalline DHA as well as DHA prepared by the bromine oxidation of ascorbic acid could replace yeast extract in our system. Ascorbic acid was also active but only after a lag period. This is not surprising, owing to the easy oxidizability of ascorbic acid by atmospheric oxygen (Fig. 4).

The kinetics of the reactivation process were therefore studied with DHA. Fig. 5 shows the variation of the kinetics with different DHA concentrations

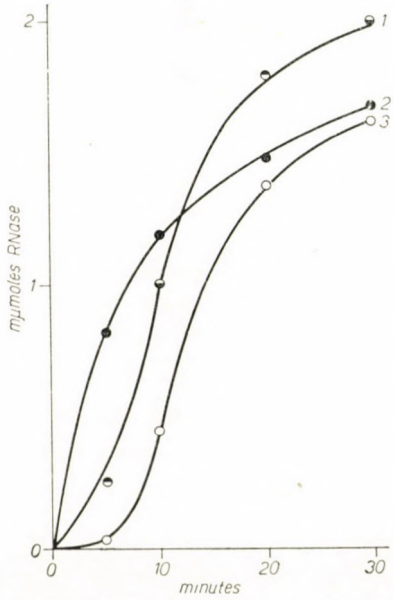

Fig. 4. Replacement of the yeast extract with ascorbic acid and DHA. Incubation mixtures: 0.1 *M* pH 7.5 tris-HCl; $3 \cdot 10^{-3}$ *M* EDTA; 12 mμmoles reduced RNase; 0.12 mg purified pig enzyme. Final vol: 0.8 ml. Curve *1 :* 0.05 ml boiled yeast extract, *2 :* $2 \cdot 10^{-3}$ *M* DHA, *3 :* $6 \cdot 10^{-3}$ *M* ascorbic acid

using a cofactor-dependent enzyme. The shapes of the curves are very similar to those of Fig. 3, where yeast extract was used as cofactor. Initial velocity is proportional to the DHA concentration, but the maximal extent of reactivation is achieved with low DHA level. The kinetics are even more complicated in the case of cofactor-independent enzyme preparations. Using a concentrated enzyme solution, the effect of DHA is slightly inhibitory even at low concentration level. However, at lower enzyme concentrations DHA acts as a stimulant, its concentration being directly proportional to the initial rate and inversely proportional to the extent of reactivation (Fig. 6).

Discussion

The experiments described above, demonstrate the presence in the pancreas of several animal species of an enzyme which greatly accelerates the reactivation of reduced RNase. The enzymic nature of the catalysis is supported by the facts that: (i) Activity is bound to a heat-labile, non-dialyzable

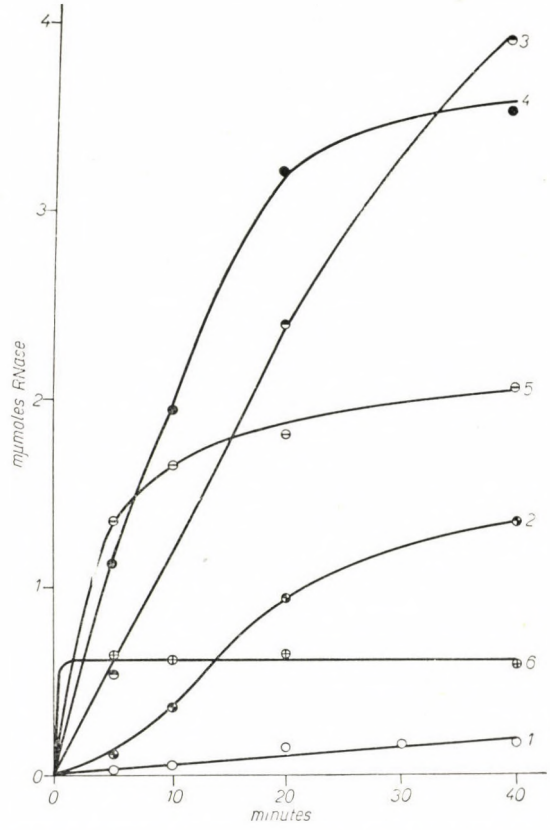

Fig. 5. Effect of DHA concentration. Reaction mixtures: 0.1 *M* pH 7.5 tris-HCl; $3 \cdot 10^{-3}$ *M* EDTA; 18 mμmoles reduced RNase; 0.05 mg purified pig enzyme. Final volume: 0.7 ml. DHA was added in the following concentrations: Curve *1 :* 0 (control), *2 :* $2.1 \cdot 10^{-4}$ *M*, *3 :* $7.1 \cdot 10^{-4}$ *M*, *4 :* $1.7 \cdot 10^{-3}$ *M*, *5 :* $3.4 \cdot 10^{-3}$ *M*, *6.* $1.7 \cdot 10^{-2}$ *M*

factor which gives a well-defined protein peak in DEAE chromatography. (ii) The optimal pH, temperature and substrate concentration values are different from the optimal conditions of the spontaneous reactivation.

Under optimal conditions maximal reactivation may be achieved enzymically within 10 min., while no reactivation occurs in the non-enzymic con-

trols. Our best preparations catalyzed the reactivation of RNase at a rate of 260 μg/minute/mg enzyme.

Several enzymes are known which catalyze this type of reaction, *i.e.* the —SH → —S—S— redox process, however most of these, for example glutathion reductase or cystine reductase [18—20] are specific for the low molec-

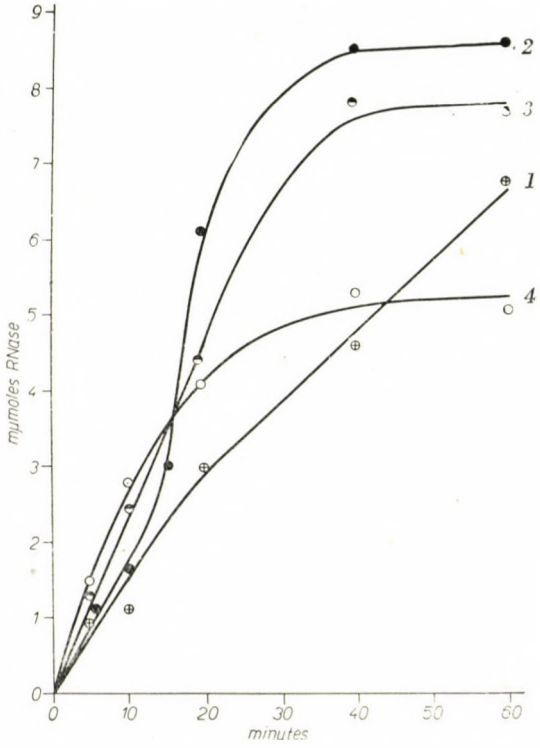

Fig. 6. Effect of DHA concentration. Reaction mixtures: 0.1 *M* pH 7.5 tris-HCl; 3.10^{-3} *M* EDTA; 12 mμmoles reduced RNase: 0.14 mg purified pig enzyme. Final volume 0.7 ml. DHA was added in the following concentrations: Curve *1 :* 0 (control), *2 :* $5 \cdot 10^{-4}$ *M*, *3 :* 10^{-3} *M*, *4 :* $4 \cdot 10^{-3}$ *M*

ular weight substrates. Recently some enzymes have been described in yeast [21], or in plant seeds [22], which specifically reduce protein disulfides. Tomizawa [23] purified an enzyme which specifically catalyzes the reduction of insulin with GSH. Katzen *et al.* [24] demonstrated the reversibility of the transhydrogenation catalyzed by this enzyme. They discussed the possible significance of this fact in protein biosynthesis. The RNase rectivating enzyme appears to be similar in function to this enzyme. However in our case DHA serves as hydrogen acceptor in the transhydrogenation whereas GSSG is inactive. The question whether DHA is the physiological hydrogen acceptor,

or only replaces the unknown cofactor, cannot be resolved as yet. A suitable proof would be the demonstration of protein-bound DHA in purified enzyme preparations which do not require added cofactor. Attempts to do this, have been as yet unsuccessful.

To draw conclusions concerning the mechanism of the catalyzed reaction from the kinetic data, would be premature at this stage of the work. The assay method employed — the determination of RNase activity — can measure only the end-product of a complex process. The question arises: which of the four disulfide bonds is or are formed enzymically. On the basis of the kinetics of the non-enzymic reactivation, ANFINSEN *et al.* [13] assume that in the first stage of the reaction disulfide bonds are formed randomly and the resumption of the correct „native" structure takes place by way of interchange reactions. On the basis of the kinetics of enzymic reactivation reported here, we have attempted to formulate a working hypothesis, compatible with the facts. According to this hypothesis the role of the enzyme would be to connect two specified cystein residues of the reduced RNase chain and oxidize them with the aid of DHA. The new bond facilitates the *correct* pairing of the other six sulfhydryls which takes place rapidly and non-enzymically in the presence of DHA. Without enzyme, or in the presence of excess DHA, a rapid aspecific oxidation occurs, measured by the disappearance of SH groups, but this process is not accompanied by reactivation because the incorrect randomly oxidized structure is formed. Conversely, in the presence of enzyme at low DHA level the correct structure is formed immediately, without interchange.

Finally one may ask what the significance of these results with respect to the problem of protein biosynthesis may be. If the biological function of the enzyme is really to catalyze the specific formation of disulfide bridges as the final step of the biosynthesis, then in contrast to the general speculation, the formation of the tertiary structure does require additional information. This information is not encoded directly in nucleotide triplets but in a specific enzyme protein which catalyzes the formation of one of several possible isomers.

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