Evidence has been obtained which indicates the possibility that diphosphopyridine nucleotide (DPN) and flavine adenine dinucleotide (FAD) are constituents of the succinoxidase complex of *Ascaris*. This system was inactivated by incubation with a nucleotide pyrophosphatase*; following this treatment, reactivation of succinate oxidizing capacity could be observed when DPN and FAD were added. The addition of FAD alone generally did not produce any observable reactivation, while both FAD and DPN usually were required for maximal stimulation. Nicotinamide brought about inhibition of succinate oxidation in concentrations comparable to those found to inhibit some known DPN- and TPN-linked dehydrogenases [11,12]. Furthermore, incubation of the succinoxidase system with succinate and purified lactic dehydrogenase [13] resulted in a significant reduction of pyruvate to lactate.

So far, no metal requirement of the system has been detectable. Succinic dehydrogenase activity was stimulated by manganese as well as by ethylenediaminetetraacetate suggesting that manganese displaces an ion which has an inhibitory effect on the enzyme.

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* The source of the nucleotide pyrophosphatase preparation was the venom of *Crotalus adamantus*. The enzyme was prepared according to an unpublished method of L. ASTRACHAN AND N. O. KAPLAN and was kindly supplied to us by Mr. F. STOLZENBACH. The enzyme has been used for cleavage of bound DPN from triosephosphate dehydrogenase by L. ASTRACHAN (*Federation Proc.*, 13 (1954) 172) and for cleavage of bound FAD from D-amino acid oxidase by C. DE LUCA and N. O. KAPLAN (personal communication).

Deuterium exchange between ribonuclease and water

As a link in a recent series of isotope-exchange studies carried out in the Carlsberg Laboratory[1-6] an investigation has been made of the exchange of deuterium between ribonuclease (RNase) and water. The results will be briefly reported below and compared with those found for insulin[2,4,6] to which protein RNase bears a rather close resemblence.

The sample used (Armour 38159) was presented to us by Dr. C. B. ANFINSEN. The method applied in the exchange experiments is described in[3,4,5]. 200 μl of a well-defined 1–2% aqueous solution of RNase are lyophilized and the water replaced by 200 μl 99.73% D_2O. After complete exchange the D_2O is removed by cryosublimation and the sample dried for 3 hours by heating to 60° against a trap at −60° (see [4]). The dry deuterium-loaded sample of protein is then dissolved in 200 μl H_2O for back-exchange and the exchange reaction followed by taking out 15 μl samples at suitable intervals, removing their water by cryosublimation and determining its concentration of deuterium by density determinations in the gradient tube.

According to analysis by HIRS, MOORE AND STEIN[7], RNase, Mw 13895, consists of 126 amino acids arranged in one peptide chain, with four intrachain S-S bridges. It has the all over composition: Asp_{16} Glu_{12} Gly_{13} Ala_{12} Val_9 Leu_2 $Ileu_3$ Ser_{15} Thr_{10} Cys_8 Met_4 Pro_5 Phe_3 Tyr_6 His_1 Lys_{10} Arg_4 $(CONH_2)_{17}$ and at the isoelectric point the molecule contains 238 theoretically exchangeable oxygen- and nitrogen-bound hydrogen atoms, out of which 120 belong to the –CO–NH–

groups in the backbone. To bring isoelectric RNase to pH 4.7 where the first series of experiments were carried out, 5 hydrogen ions have to be added per mole, so that the number of theoretically exchangeable hydrogen atoms at this pH is 243.

In Fig. 1 the number, n, of deuterium atoms exchanged per mole RNase is plotted against time. The exchange was followed at pH 4.7 and two different temepratures, 0° and 38°. In both cases an instantaneous reaction of 185 atoms per mole was found, followed by a slower exchange which was complete after 20 hours at 38°. As reported earlier[2,5,6] all the O- and N-bound hydrogen atoms in shorter peptides without internal hydrogen bonding exchange instantaneously even at 0° —those of the –CO–NH–groups included—and we therefore take the slow part of the exchange in the case of RNase as evidence for the existence of some kind of hydrogen-bonded, folded structure in the molecule, shielding part of the hydrogen atoms from instantaneous exchange. This point of view is supported by the fact that—as shown in Fig. 1—the exchange is much more rapid in the presence of guanidine chloride (2.5 molal), a compound which like urea has an unfolding effect upon proteins (see[8]). If RNase is oxidized by performic acid the 4 intrachain S–S bridges are broken, and a molecule is obtained which exchanges all its hydrogen atoms instantaneously (Fig. 1). Hence the hydrogen bonded structure in native RNase seems to be greatly stabilized by the S–S bridges, a conclusion which is in conformity with theoretical consideration by SCHELLMAN[8]. In fact the situation is quite similar to that met with in the case of insulin and the isolated A-chain[2,5,6]. Unpublished data by HARRINGTON AND SCHELLMAN indicate that the oxidized RNase is actually unfolded in aqueous solution.

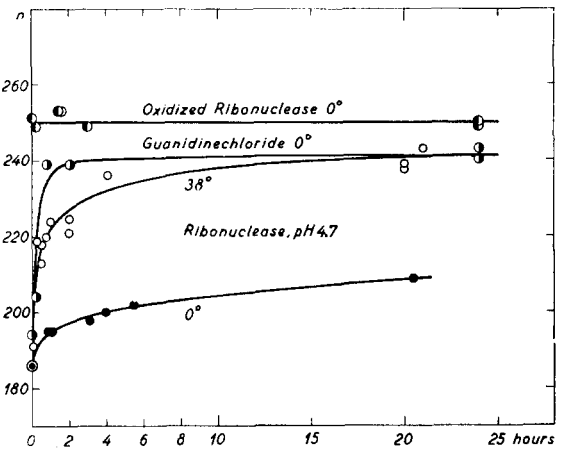

Fig. 1.

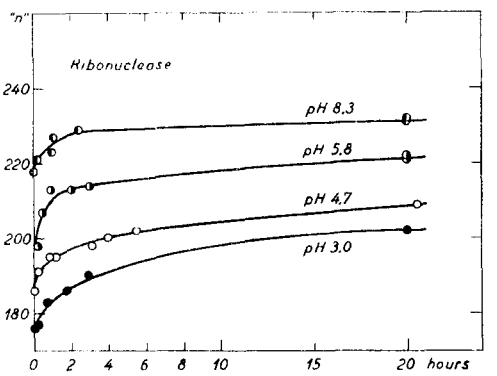

Fig. 2.

The experimentally determined number of exchangeable hydrogen atoms in oxidized RNase is 3% higher than the calculated value. This discrepancy is greater than the usual experimental error, but not so much that a closer inspection was considered necessary. Possibly the sample was not completely dry after the exchange with D_2O, possibly it contained an impurity. The important feature is that all the exchangeable hydrogen atoms reacted instantaneously.

Fig. 2 shows the pH-dependence of the exchange. In this series of experiments the solution of RNase at pH 4.7 was used as a basis, its pH being determined electrometrically. The three other solutions (pH 3.0, 5.8 and 8.3) were made up from this by adding HCl or NaOH. Because of the limited amount of RNase available indicator paper had to be used for the estimation of pH, a not too satisfactory procedure. The results should therefore be taken with some reservation, *i.e.*, they cannot be used for quantitative calculations but give a qualitative demonstration of the fact that the exchange in the pH range from 3 to 8.3 is the slower the lower the pH. Upon the assumption that the titratable groups of RNase exchange their hydrogen atoms instantaneously, the number of hydrogen atoms exchanged have been corrected for the number of hydrogen ions added or removed in the pH adjustment, so that n in Fig. 2 refers to the RNase molecule in its state of ionization at pH 4.7.

At present no simple explanation of the observed pH-dependence can be offered but it is hoped that further work on this problem will throw light on the mechanism of the exchange reaction.

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The pH-dependence of the deuterium exchange of insulin

In continuation of previous experiments[1,2,4] the rate of the reaction of deuterium-loaded pork insulin with H_2O at 0° was studied at different hydrogen ion concentrations. The method applied is outlined in reference[1] and described in detail in references[3,4,5]. The results are seen in Fig. 1 wehre n_i is the number of deuterium atoms exchanged per insulin monomer (Mw 5777). In the experiments at pH 2.66 and 3.50 the protein was loaded with deuterium at these pH values while in that at pH 7.1 the protein was deuterated at pH 3, lyophilized, dried (see [1,3,4]), and dissolved in H_2O + NaHO to bring pH to 7.1. The process of dissolution took about 2 minutes. For the sake of comparison the values experimentally found for the number of deuterium atoms exchanged (n) were corrected by means of the formula

$$n_i = n - i$$

where i is the net charge — reckoned with sign — of the monomer during the loading with deuterium. The justification of this correction is found in the fact that the hydrogen atoms involved in the ionization process must be instantaneously exchangeable[6]. It will immediately be clear that, independent of pH, the values of n_i will all approach 85 valid for isoelectric insulin, the value 89 previously given for insulin with net charge + 4 being used as a basis (see [1,4]). As appears from Fig. 1 the rate of the exchange reaction is strongly dependent upon the hydrogen ion concentration and falls with decreasing pH. At pH 7.1 the exchange is complete after 3 hours. The curves are generally of the same type as those found for ribonuclease[6] and confirm the observations made by LENORMANT AND BLOUT[7] in their study of other proteins (bovine serum albumin and ovalbumin). Since preliminary experiments on β-lactoglobulin have shown the same pH dependence of the exchange of this protein it would appear that the phenomenon is fairly general. Our experiments cannot at present serve as a basis for quantitative calculations of the relationship between pH and rate of exchange. Refined technique and additional experimental material are required to solve this problem. They show, however that the exchange mechanism is more complicated than tentatively assumed in a recent article[2].

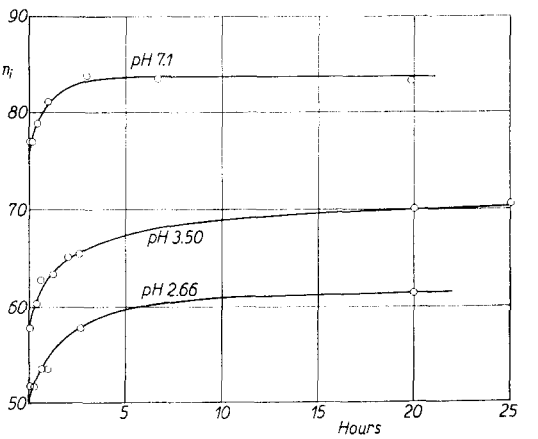

Fig. 1.

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