STUDIES ON THE MECHANISM OF HYDROGEN TRANSPORT IN ANIMAL TISSUES

VII. INHIBITION BY RIBONUCLEASE*

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Earlier in this series of investigations, it was pointed out that succinic dehydrogenase and cytochrome oxidase are associated with the particulate components of cytoplasm (1), and attention was called to the similarity between these particles and the mitochondria which Bensley (2) separated from broken cell preparations by centrifugal means. Szent-Györgyi (3) as well as Stern (4) had previously called attention to the fact that these enzymes are apparently attached to some macromolecular entity. The enzyme which we have called "coenzyme I-cytochrome c reductase" (5) is also in this category (5, 6), and Kabat (7) has shown that alkaline phosphatase is associated with the particulate matter.

During this same period, Claude (8) has been perfecting the method of separation of the morphological components of cytoplasm by centrifugal techniques and has obtained discrete granules of material having "the general constitution of a phospholipid-ribonucleoprotein complex" with diameters ranging from 50 to 150 m. The particles were considered to pre-exist in the original material and were shown to be similar in size and chemical composition to the particles of the Rous tumor virus.

These independent observations provide no proof, however, that the enzyme systems include a ribonucleoprotein, since the particulate components might consist of a mixture of ribonucleoprotein particles and enzyme particles. Furthermore, there is thus far no evidence that any given particle contains more than one enzyme (9). Isolation of the enzymes in crystalline form does not provide an answer to the question since it seems likely that the highly active enzymes which have been isolated as soluble proteins of low molecular weight may not occur as such in the cell, but rather (4,7) that they are attached to the larger particles which Claude has shown to contain ribonucleic acid and phospholipid.

Kunitz (10) has recently isolated ribonuclease in crystalline form and has demonstrated that it splits ribonucleic acid into smaller acidic groups without

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liberating free phosphoric acid. It has no effect upon deoxyribonucleic acid. However, it apparently is able to combine with the ribonucleic acid contained in crystalline tobacco mosaic virus to give an inactive complex as Loring has shown (11). Loring's experiments suggested the investigation herein reported in which it is shown that ribonuclease inactivates the succinoxidase system associated with the particulate components of liver homogenates. A number of other enzymes were also tested in an attempt to learn whether the effect is specific for the succinoxidase system.

EXPERIMENTAL

The succinoxidase system was set up on the basis of previous work (1, 9), and its over-all activity was studied by measuring the rate of oxygen uptake in a conventional Warburg apparatus. Both rat and mouse livers and kidneys were used to prepare dilute homogenates, and the homogenization was invariably carried out in ice-cold water which had been recently redistilled from glass, in contrast to our previous method of homogenizing in isotonic phosphate at pH 7.4. In most cases, the distilled water contained sufficient NaOH to give pH 7.8-8.0 so that the dilute homogenates had a pH of 7.0-7.2. Metal contamination was carefully avoided. Two components of the succinoxidase system, succinic dehydrogenase and cytochrome oxidase, were studied in a Cenco photoelectric spectrophotometer on the basis of the change in extinction at 550 mμ. Coenzyme I-cytochrome c reductase was likewise studied on the basis of the change in the cytochrome spectrum, using sodium dihydrocoenzyme I (12) as a substrate. The cytochrome oxidase system was also studied manometrically, using a test system worked out in this laboratory by W. C. Schneider. The ascorbic acid used as a substrate in this work was a gift from Merck and Company. The urease was a commercial preparation, sold by the Arlington Chemical Company, and was studied on the basis of the rate of CO₂ evolution under conditions modified from those previously described (13). Catalase was studied with dilute liver homogenates as the source of the enzyme. The evolution of oxygen was measured in the Warburg apparatus under conditions employed in this institute by B. E. Kline and H. P. Rusch. Alkaline phosphatase was studied essentially according to Kabat (7), using dilute homogenates as the source of enzyme. We are indebted to K. P. DuBois for testing the adenosine triphosphatase, using liver homogenates under conditions which he has worked out. Xanthine oxidase was studied in the Warburg apparatus, using a highly purified preparation which was a gift from S. W. Schwartzman. Specially purified xanthine was a gift from J. A. Bain. Crystalline, salt-free ribonuclease was obtained through the kindness of Dr. M. Kunitz of The Rockefeller Institute.

In general, the ribonuclease was incubated with the various enzyme systems for 1 to 2 hours at 38° in unbuffered solutions. Since the ribonuclease solutions were acidic, they were brought to pH 6.8-7.0 with 0.002 N NaOH immediately before use. The stock solution of ribonuclease was kept in the cold. The pH of the various enzymes was measured with and without ribonuclease; no change occurred either on mixing or upon standing. Since incubation in dilute solution tends to emphasize the destructive action of oxidation and metal contaminants especially in the case of urease
and succinic dehydrogenase, we routinely used two types of controls: a "fresh control" to give the original activity and an "incubated control" to show the effect of incubation in high dilution per se. The activity of the “fresh control” was measured simultaneously with that of the other mixtures, but during the incubation period it was kept at 0°C and in more concentrated solution.

The details of the experimental test systems will be reported with the results, which will be considered under separate headings.

Cytochrome Systems

Succinoxidase System, Manometric Technique—Ribonuclease completely inactivates the over-all activity of the succinoxidase system when sufficient time is allowed, if the proper amounts of ribonuclease relative to the amount of homogenized tissue are used, and if the two enzymes are allowed to act in unbuffered solution. Fig. 1 shows that the inhibition becomes progressively greater over a 2 hour period. This is in contrast to the results of Loring who reported that the loss in virus activity on standing in contact with ribonuclease was not significantly different from that immediately after mixing. However, it is not clear how the time of incubation could be stated in the virus experiments since the virus-ribonuclease mixture must have remained together for a time after the test leaves were inoculated. Fig. 1 also shows that the percentage inhibition varies according to the amount of succinoxidase used since the activity of a small amount of liver is completely destroyed much sooner than the activity of a larger amount. The results indicate that the ribonuclease-succinoxidase relationship is that of an enzyme to its substrate. Fig. 2 shows that very small amounts of ribonuclease are able to inhibit the succinoxidase system in unbuffered solution, while much larger amounts are required to produce inhibition in the presence of buffer. In both cases, the inhibition appears to be proportional to the log of the ribonuclease concentration. The effect of buffering is in harmony with Loring's results on the precipitation of a crystalline ribonuclease-virus complex. He stated that the precipitate was insoluble in distilled water but could be dissolved readily in phosphate buffer at pH 7.0, in which solution, nevertheless, inactivation of the virus occurred.

Cytochrome Oxidase, Manometric Technique—Since the results in Figs. 1 and 2 clearly demonstrated the fact that ribonuclease inhibits the succinoxidase system, it was of interest to determine whether the results were due to the inhibition of succinic dehydrogenase or cytochrome oxidase or of both. It was found that both components are inhibited. Cytochrome oxidase was tested by both manometric and spectrophotometric techniques. The manometric tests were done with ascorbic acid, neutralized to pH 7.0, as the source of electrons for the reduction of cytochrome c. Otherwise, the technique was essen-
Fig. 1. The progressive inactivation of the succinoxidase system by ribonuclease. Each flask contained 1.0 ml. of 0.1 M sodium phosphate buffer pH 7.4, 0.4 ml. of 10^{-4} M cytochrome c, 0.3 ml. of 4 \times 10^{-4} M CaCl_2, 0.3 ml. of 4 \times 10^{-4} M AlCl_3, 0.3 ml. of 0.5 M sodium succinate, plus water to give a final volume of 3.0 ml. after addition of the liver homogenate and ribonuclease. The succinoxidase, in the form of a 5 per cent mouse liver homogenate, was placed in the side arms of the flasks and kept in a warm room for 2 hours prior to the activity measurements. At appropriate times, 0.2 ml. of 0.2 per cent ribonuclease was added to the side arms so that the succinoxidase was incubated for 2 hours in all cases but only for the indicated time in the presence of ribonuclease. At the end of the 2 hour period, the flasks were connected to manometers, placed in a thermostat at 38\(^\circ\), the enzyme mixture was tipped into the main flask, and the rate of oxygen uptake was observed for four 10 minute periods. The values at zero time are the controls: the incubated control is on the continuous line, and the fresh control (see text) is on the dotted line. The upper curve is for 10 mg. of liver, and the lower curve is for 5 mg. of liver.

Fig. 2. The effect of various amounts of ribonuclease on the succinoxidase system in presence and absence of buffer. Each flask contained phosphate, cytochrome c, CaCl_2, AlCl_3, succinate, and water as in Fig. 1. All flasks contained 5 per cent mouse liver homogenate equivalent to 10 mg. of fresh liver, and 0.2 per cent ribonuclease was added to the flasks in the amounts indicated in the graph. In the "buffered" series, the ribonuclease and succinoxidase were in the main part of the flask with the phosphate, cytochrome, CaCl_2, and AlCl_3, while the succinate was in the side arms. In the "unbuffered" series, the succinate, etc., were in the main compartment, and the ribonuclease and succinoxidase were in the side arms. The flasks were connected to manometers and shaken in the thermostat for 2 hours. The side arms were then tipped, and the rate of oxygen uptake measured as in Fig. 1.
tially similar to that used for the over-all system as in Figs. 1 and 2. In a typical experiment, the main flasks contained phosphate, A1C4, and water as in Fig. 1, plus 0.5 ml. of 1 per cent ascorbic acid and 0.6 ml. of $4 \times 10^{-4}$ M cytochrome $c$, and the side arms contained 0.3 ml. of 1 per cent mouse liver homogenate plus 0.2 ml. of 0.2 per cent ribonuclease in appropriate flasks. After 90 minutes in the thermostat, during which time the autoxidation rate of the ascorbate was noted, the side arms were tipped into the main compartments, and the rate of oxygen uptake was measured for 10 minute intervals. The results, expressed in $\mu$1. $O_2$ per 10 minutes, were as follows: autoxidation rate, 3.6; fresh control, 58.9; incubated control, 56.8; incubated with ribonuclease, 4.0. When the rates are corrected for the autoxidation rate, it is apparent that ribonuclease completely inactivated cytochrome oxidase.

**Cytochrome Systems, Spectrophotometric Technique**—The oxidation and reduction of cytochrome $c$ was used as a basis for studying cytochrome oxidase, coenzyme I-cytochrome $c$ reductase, and succinic dehydrogenase, following the principles and method of calculation described earlier in this series (14). All these enzymes are inactivated by ribonuclease. Dilute mouse liver homogenates were used as the source of enzyme and were filtered through bolting silk (Schindler Standard No. 20 obtained from the Allis-Chalmers Co.) to eliminate small shreds of connective tissue. The test is so sensitive that only 0.02 cc. of a 1 per cent homogenate is sufficient to reduce the cytochrome $c$ in a period of several minutes. Since the dry weight of this amount of liver is only 60 $\gamma$, the weight of active enzyme required must be very small. In order to study the effect of ribonuclease, tubes were made up with liver homogenate alone and with ribonuclease, with concentrations and incubation times determined from Figs. 1 and 2 and with water added to give a final liver concentration of 1 per cent. Fresh and incubated controls were used.

When cytochrome oxidase was to be studied, the solution of cytochrome in phosphate buffer was treated with one or two minute grains of solid Na$_2$S$_2$O$_4$, which immediately reduced all of the cytochrome to the ferrous state. Following this treatment, the liver homogenate was added with a drawn-out pipette. The solution was mixed, and the zero time was noted. The solution was then transferred to an absorption cell, placed in the spectrophotometer, and the first reading was taken exactly 30 seconds from the zero time. Values for $I_0$ and $I$ at 550 $\mu\mu$ were then taken at 15 second intervals until the reaction appeared to be definitely slowing down, and the initial rate at which log $I_0/I$ changed was used to calculate enzyme activities. Fig. 3 shows that cytochrome oxidase was inhibited by ribonuclease, in confirmation of the manometric experiments. Since the inactivation of cytochrome oxidase would provide adequate explanation for the results with succinoxidase, it was of particular interest to determine whether the succinic dehydrogenase was also inactive.

Succinic dehydrogenase was studied by measuring the rate of cytochrome $c$ reduction in the presence of $M/3,000$ cyanide, which was sufficient to prevent
completely the reoxidation of cytochrome c by cytochrome oxidase but was a low enough concentration to avoid appreciable combination with cytochrome c itself in the time covered (14). In these tests, the homogenate was added to the buffered cytochrome c solution and allowed about a minute in order to oxidize completely the cytochrome c. Cyanide was then added to block the oxidase, and the reaction catalyzed by succinic dehydrogenase was initiated by adding sodium succinate. Readings were then taken every 15 seconds in the spectrophotometer to obtain the data shown in Fig. 3. Determinations of cytochrome oxidase and succinic dehydrogenase made on the same incubation mixtures showed that both enzymes were inactivated to the same extent by ribonuclease.

Cytochrome c can be reduced not only by succinic dehydrogenase but also by enzymes which transport electrons from the reduced coenzymes I and II. In order to distinguish between the two latter enzymes, which are specific for their respective substrates (5), we have called them coenzyme I-cytochrome c reductase and coenzyme II-cytochrome c reductase, following the nomenclature of Haas, Horecker, and Hogness (15) who applied the general name of cytochrome reductase to the enzyme which we prefer to call coenzyme II-cytochrome reductase. It appears that there is a family of cytochrome reductases and that succinic dehydrogenase may be considered as a member of this group, at least until it has been shown to consist of more than one component. In the present work, we studied the coenzyme I-cytochrome c reductase by using sodium dihydrocoenzyme I as a substrate (5). The tests were made in a manner identical with that used for succinic dehydrogenase, except that the reduced coenzyme was used in place of succinate. Experiments run parallel with succinic dehydrogenase are shown in Fig. 4. Both enzymes were completely inactivated by ribonuclease in this experiment. According to the earlier ideas of the mechanism of hydrogen transport, the oxidation of CoH2I was mediated by succinic dehydrogenase and, if this concept were correct, the inhibition of CoI-cytochrome c reductase by ribonuclease could be explained on the basis of the inhibition of succinic dehydrogenase. Although this concept was considerably weakened by the demonstration that the oxidation of a coenzyme I system could proceed under conditions in which succinate could not be oxidized (16), it seemed desirable to show that under the conditions employed in the experiment shown in Fig. 4, the results achieved earlier in the over-all system could still be obtained. Therefore, simultaneous tests of the rate of reduction of cytochrome c by CoH2I and by succinate in the presence of malonate were made. Fig. 4 shows that, in the presence of sufficient malonate to block succinic dehydrogenase completely, the coenzyme I-cytochrome c reductase is completely unaffected. From this fact it may be concluded that the oxidation of coenzyme I is not mediated by the intermediate oxidation and reduction of the succinate-fumarate system and therefore that ribonuclease
Fig. 3. Inhibition of succinic dehydrogenase and cytochrome oxidase by ribonuclease. A 5 per cent mouse liver homogenate was incubated with an equal volume of 0.2 per cent ribonuclease for 90 minutes and then diluted with water to give a liver concentration of 1 per cent. Fresh and incubated controls were also diluted to 1 per cent. When cytochrome oxidase was tested, each absorption cell contained 1.0 ml. of sodium phosphate pH 7.4, 0.2 ml. of $3.62 \times 10^{-4} \text{M}$ cytochrome $c$, and 1.78 ml. of water. After adding a grain or two of solid Na$_2$S$_2$O$_4$ to reduce the cytochrome $c$, 0.02 ml. of 1 per cent liver homogenate was added, and readings were begun. When succinic dehydrogenase was measured, each cell contained phosphate and cytochrome as above, plus 1.58 ml. of water and 0.02 ml. of 1 per cent liver homogenate. The reaction was initiated by adding 0.2 ml. of a 1:1 mixture of 0.01 m NaCN and 0.5 m sodium succinate. In the chart, increases in $E_{560}$ show succinic dehydrogenase activity, and decreases in $E_{560}$ show cytochrome oxidase activity. Incubated controls are shown in solid lines, and samples incubated with ribonuclease are shown in dotted lines. Fresh controls were not significantly different from incubated controls and are therefore not shown.

Fig. 4. Inhibition of succinic dehydrogenase and coenzyme I-cytochrome reductase by ribonuclease. Inhibition of succinic dehydrogenase but not of coenzyme I-cytochrome $c$ reductase by malonate. A 2 per cent mouse liver homogenate was diluted with water and ribonuclease solution to give a final concentration of 1 per cent liver and 0.067 per cent ribonuclease. Fresh controls yielded results identical with those of the incubated controls and therefore are not shown. Each absorption cell contained 2.5 ml. of 0.04 m sodium phosphate pH 7.4, 0.1 ml. of $3.62 \times 10^{-4} \text{M}$ cytochrome $c$, and 0.1 ml. of 0.01 m NaCN, plus water to make a final volume of 3.0 ml. after all additions, which included various combinations of the following: liver homogenate, 0.02 ml.; 0.1 m malonate, 0.1 ml.; 0.5 m succinate, 0.1 ml.; and 0.005 m CoH$_2$I, 0.1 ml. The curves shown correspond to the following combinations: curve 1, liver + CoH$_2$I; curve 2, liver + CoH$_2$I + malonate; curve 3, ribonuclease-treated liver + CoH$_2$I; curve 4, liver + succinate; curve 5, liver + succinate + malonate; curve 6, ribonuclease-treated liver + succinate; curve 7, CoH$_2$I only; curve 8, liver only.
inactivates CoI-cytochrome c reductase per se. The data also show that cytochrome c is not the locus of ribonuclease action.

**Non-Cytochrome Systems**

**Catalase.**—The enzyme catalase resembles the cytochrome system insofar as it contains an iron porphyrin prosthetic group. Nevertheless, it appears to be completely unaffected by ribonuclease. A 5 per cent mouse liver homogenate was diluted to a final concentration of 0.02 per cent, and 0.4 ml. of this solution (equivalent to 24γ of dry liver) was placed in the side arms of Warburg flasks. The main compartment contained 1.0 ml. of 0.1 M sodium phosphate pH 7.4, 0.6 ml. of H₂O, and 1.0 ml. of 0.075 per cent H₂O₂ which was freshly prepared from Merck’s superoxol. After equilibration at 38°, the side arm was tipped, and the evolution of oxygen was measured by taking readings at 2 minute intervals. In the first tests, the liver homogenate was incubated with ribonuclease under conditions comparable to those in Fig. 1, following which the mixture was diluted to give 0.02 per cent liver. Since this procedure might facilitate dissociation of any complex which might have been formed between catalase and ribonuclease, the final procedure was to incubate a mixture containing 0.02 per cent liver and 0.1 per cent ribonuclease, so that the ratio of the dry weights was 24γ of liver to 400γ of ribonuclease instead of 3,000γ of liver to 400γ of ribonuclease (which gave nearly complete inhibition in 2 hours in the case of succinoxidase as was shown in Fig. 1). In an experiment in which the liver was incubated for 2 hours with ribonuclease, using both the 5 per cent and 0.02 per cent homogenates and employing both fresh and incubated controls, the ribonuclease was found to exert no effect upon the catalase in either instance. The results, expressed in μl. O₂ evolved in the first 10 minutes per 24γ of dry liver, were as follows: for the ratio 400γ ribonuclease/24γ liver: fresh controls, 103 and 99; incubated controls, 101 and 98; incubated with ribonuclease, 97 and 96; for the ratio 400γ ribonuclease/3,000γ liver: fresh controls, 117 and 128; incubated controls, 100 and 104; incubated with ribonuclease, 131 and 129. As a further control, a homogenate was incubated with ribonuclease, and aliquots were simultaneously tested for catalase and cytochrome oxidase. The cytochrome oxidase was completely inactivated, while the catalase was unaffected.

**Phosphatase.**—Kabat has reported that the particulate matter in the cytoplasm of kidney cells contains an active alkaline phosphatase (7); one might expect this enzyme to be inhibited by ribonuclease, following the results with the succinoxidase system. However, a series of tests revealed no significant inhibition of the phosphatase by ribonuclease. The activity of the enzyme was measured in the following way: a 5 per cent kidney homogenate was incubated with varying amounts of ribonuclease and for varying time intervals. Concentrations of ribonuclease were used ranging from 500γ/25 mg. of kidney
to 2,000γ/25 mg. of kidney. The incubation times were 30, 60, 90, and 120 minutes. In some experiments the ribonuclease was incubated with kidney for as long as 6 hours. At the end of the incubation periods, 0.1 cc. samples of the incubation mix were removed and pipetted into tubes containing 0.9 ml. H₂O; 1.0 ml. of 0.1 M veronal buffer, pH 9.0; and 0.5 ml. of 0.03 M MgCl₂. At zero time, 0.5 ml. of 0.3 M sodium β-glycerophosphate was added, and the whole was incubated for time intervals ranging between 20 and 30 minutes at 37°. The reactions were stopped by the addition of 1.0 ml. of 20 per cent trichloracetic acid which also precipitated the protein. 1 ml. aliquots were used for the determinations of inorganic phosphorus. The results of a typical experiment expressed as γ of inorganic phosphorus liberated in 20 minutes by 2.5 mg. of liver were as follows: fresh control, 7.7; incubated control, 8.8; incubated with ribonuclease, 7.7. The incubation time with the ribonuclease in the above experiment was 2 hours; the ribonuclease concentration was 950γ/25 mg. liver. In the same experiment, using the same controls and the same ribonuclease mix, succinoxidase and cytochrome oxidase assays were also carried out. The succinoxidase and cytochrome oxidase activities were inhibited completely.

Eight experiments were carried out in all, testing the action of ribonuclease on phosphatase. The average inhibition by ribonuclease for the whole group was 10 per cent.

Adenosine Triphosphatase.—The experiments with alkaline phosphatase are subject to the criticism that the significance of this enzyme in the relation to the hydrogen transport mechanism is obscure. However, the enzyme adenosine triphosphatase is probably closely integrated with the respiratory enzymes since its substrate is synthesized by the esterification of inorganic phosphate using the energy of both glycolysis and respiration, and, in fact, the dehydrogenation of succinate has been related to phosphate esterification (17). The activity of the enzyme was measured on the basis of the rate of liberation of inorganic phosphate from pure adenosine triphosphate (ATP) by dilute liver homogenates. Each flask contained 0.5 ml. of 0.1 M KCl-glycine buffer pH 9.1, 200γ of ATP in a volume of 0.4 ml., 0.45 ml. of 1 per cent liver homogenate, 1.35 ml. of water. After incubating for 45 minutes, 0.3 ml. of 100 per cent trichloracetic acid was added, and inorganic phosphate was determined on the filtrate. The liver homogenates were incubated with ribonuclease as in Fig. 1; both fresh and incubated controls were used. The results, expressed in γ of inorganic phosphorus liberated per flash were as follows: fresh control, 25; incubated control, 23; incubated with ribonuclease, 22; ribonuclease alone, 0.6. As a further control, a homogenate was incubated with ribonuclease, and aliquots were simultaneously tested for succinic dehydrogenase and adenosine

1 The assay technique is still being developed and will be reported in detail later.
The succinic dehydrogenase was completely inactivated while the adenosine triphosphatase was unaffected.

Urease.—A large number of diverse compounds have been shown to inhibit succinic dehydrogenase on the basis of reaction with an essential SH group on the enzyme (18). These compounds also inhibit urease by the same mechanism (13). Traces of copper ions and non-specific oxidants fall in this category, and it is apparent that if ribonuclease contained a few tenths of a per cent of such substances as an impurity, succinic dehydrogenase would be inactivated in much the same manner as shown in Fig. 1. However, in such an event, urease would be inhibited in a similar fashion, and tests have shown that it is not. The tests were carried out as follows: the side arms contained 400 ug of ribonuclease in 0.2 ml. of water and 40 ug of commercial urease in 0.2 ml. water; the main flasks contained 1.0 ml. of 0.1 M pyrophosphate buffer at pH 5.0 plus 0.3 ml. of 2 M urea and water to give 3.0 ml. after the urease and ribonuclease were added. The ribonuclease was added to the flasks at various times as in the succinoxidase experiment described in Fig. 1 and, at the end of the 2 hour period, the side arms were tipped and the CO₂ evolution was measured. The results, expressed as CO₂ values, were as follows: fresh control, 9,250; incubated control, 9,620; incubated with ribonuclease: for 30 minutes, 8,800; for 60 minutes, 9,040; for 90 minutes, 8,840; and for 120 minutes, 8,690. None of these figures differs significantly from the mean, showing that ribonuclease does not inhibit urease. From this fact, it may be inferred that the inhibition of succinic dehydrogenase is not due to a non-specific oxidation of sulfhydryl groups either by ribonuclease or by any impurity which might be present.

Xanthine Oxidase.—It has been suggested that succinic dehydrogenase may be a flavoprotein (19, 20), and it is almost certain that xanthine oxidase is a flavoprotein (21). It was, therefore, of interest to test the effect of ribonuclease on xanthine oxidase. The tests were carried out as follows: the side arms contained 120 ug of partially purified xanthine oxidase in a volume of 0.1 ml. plus 400 ug of ribonuclease in a volume of 0.2 ml.; the main flasks contained 1.0 ml. of 0.1 M sodium phosphate pH 7.4, 0.2 ml. of 0.8 per cent sodium xanthate, and water to make a final volume of 3.0 ml. The ribonuclease and xanthine oxidase were added to the various flasks at the same times, and, after various periods of time, the side arms were tipped and the oxygen uptake was measured in flasks containing ribonuclease and in parallel control flasks. The results, expressed in the basis of the oxygen uptake during the first 10 minutes, were as follows after 30, 60, 90, and 120 minute periods of incubation, respectively: controls, 97, 94, 90, and 96; with ribonuclease, 101, 103, 97, and 101. It is apparent that the xanthine oxidase was unaffected by ribonuclease.

DISCUSSION

The data presented above show that of the eight enzymes tested, ribonuclease specifically inhibits only CoI-cytochrome reductase, succinic dehydro-
genase, and cytochrome oxidase. On the basis of Loring's work, this fact may be taken as an indication that a ribonucleic acid is in some way associated with these enzymes. If this is indeed the explanation for the results, then the failure of ribonuclease to inhibit urease and xanthine oxidase is reasonable enough: these enzymes are highly purified and almost certainly do not contain any ribonucleic acid. But in the case of the alkaline phosphatase, there is evidence that the enzyme is attached to particles (7) and that these particles may be no different from those described by Claude (8). The present evidence would seem to indicate that the phosphatase is not associated with ribonucleic acid. The evidence obtained from negative ribonuclease experiments becomes inconclusive, however, when it is realized that all of the enzymes which we have found to be inhibited by ribonuclease are able to act only when they are associated with cytochrome c. Since ribonuclease does not act on cytochrome c directly, it is possible that succinic dehydrogenase, CoI-cytochrome c reductase, and cytochrome oxidase are components of a complex ribonucleoprotein of macromolecular dimensions and that, when ribonuclease acts on the ribonucleic acid contained therein, it prevents cytochrome c from approaching the enzyme components with the result that those enzymes whose action is dependent upon cytochrome c appear to be inactivated. Other enzymes like phosphatase and catalase could conceivably be in the same or in a similar complex but they do not require cytochrome c for their action, and molecules such as β-glycerophosphate and hydrogen peroxide which are relatively small compared with cytochrome c (molecular weight 13,000) might reach their activating centers unhindered. The CoII-cytochrome c reductase in a homogenate should also be inhibited by ribonuclease, but the CoII-cytochrome c reductase which has been isolated from yeast following autolysis (15) should not be affected by ribonuclease according to this concept.

The experiments on CoI-cytochrome c reductase seem to merit special attention, since the fact that this enzyme and succinic dehydrogenase appear to be closely associated might be taken as evidence in support of the Szent-Györgyi theory. However, the evidence is explained adequately on the basis of their rôle as cytochrome c reductases and implies nothing which can be used to prove that the oxidation of CoH2I is mediated by the succinate-fumarate system. The experiments with malonate, shown in Fig. 4, indicate quite strongly that the activating center for succinate is distinct from that which activates CoH2I and provide striking confirmation for the earlier demonstration of this fact (16). This is the third experimental demonstration of this point; the second was provided by Straub (22) of the Szent-Györgyi school. Straub was able to inactivate the succinic dehydrogenase contained in a muscle pulp by keeping it at pH 9 for an hour, then neutralizing back to pH 7.3. The resulting preparation was completely unable to oxidize succinate but showed scarcely any loss in the ability to oxidize reduced CoI (malate system). As a result of this experiment Straub stated that reduced flavoprotein (which we identify
as CoI-cytochrome c reductase) "does not react with succinic dehydrogenase but has its direct connection with the oxidizing system." This statement is much more far-reaching than that of Potter (16, 23) whose experiments with malonate, which were analogous to Straub's experiments with alkaline incubation, led to the conclusion that there is at least a path alternate to the Szent-Györgyi mechanism. Straub's statement appears to renounce the Szent-Györgyi mechanism completely. The other data pertinent to the Szent-Györgyi theory have been previously discussed (24), and there now seems to be no reason for its continued application in the absence of new supporting evidence.

**SUMMARY**

1. The effect of ribonuclease on various enzyme systems was studied as one approach to the problem of whether or not these enzymes are contained in macromolecules of ribonucleoprotein nature in protoplasm.

2. Ribonuclease inhibited CoI-cytochrome c reductase, succinic dehydrogenase, and cytochrome oxidase, all of which require cytochrome c in order to function. Ribonuclease did not act on cytochrome c.

3. Ribonuclease did not inhibit urease, xanthine oxidase, catalase, alkaline phosphatase, or adenosine triphosphatase under the conditions employed.

4. It was suggested that ribonuclease acted sterically by preventing contact between cytochrome c and its activating centers.

5. It was suggested that the enzymes inhibited may be contained in a ribonucleoprotein of macromolecular dimensions but that the enzymes not inhibited are not necessarily excluded from such a complex by the data presented.

6. Further evidence against the Szent-Györgyi theory of hydrogen transport was presented and discussed.

**BIBLIOGRAPHY**