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A Study of the Inhibition of Catalase by 3-Amino-1:2:4-Triazole

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(Received 7 June 1957)

Heim, Appleman & Pyfrom (1955, 1956) have shown that the injection of 3-amino-1:2:4-triazole into rats causes a rapid decrease of the catalase activity of the liver and kidney to very low levels, but has no effect on the catalatic activity of blood. These authors found that crystalline catalase was partly inhibited *in vitro* only at high concentrations of 3-amino-1:2:4-triazole ($> 0.05M$); such concentrations were far above those necessary for the inhibition of catalase by 3-amino-1:2:4-triazole *in vivo*. The inhibition *in vitro* was reversible by dilution, whereas that obtained *in vivo* was not.

Sugimura (1956) showed that the incubation of rat-liver suspensions with 3-amino-1:2:4-triazole at 37° caused similar decreases in the catalatic activity of the suspensions, that the incubation of a purified crystalline preparation of ox-liver catalase with 3-amino-1:2:4-triazole did not result in any decrease of activity, but that when a liver extract was incubated with purified catalase in the presence of 3-amino-1:2:4-triazole the inhibition of catalatic activity occurred. In every case the inhibition required the presence of oxygen.

The present paper is concerned with the further investigation of these effects with simple systems *in vitro* and with the properties of the catalase inhibited by 3-amino-1:2:4-triazole.

MATERIALS AND METHODS

Catalase preparations. Two preparations of catalase were used. One was a commercial recrystallized ox-liver catalase (Worthington Biochemical Corp., St Louis, Mo., U.S.A.) with Kat.f. (Euler & Josephson, 1927) 26 000, and the

other was a crystalline catalase (Kat.f. 58 000) prepared from human erythrocytes according to Herbert & Pinsent (1948). The concentration of the haemoprotein solutions was determined by the pyridine haemochromogen method of Keilin & Hartree (1951), and throughout this paper is expressed in terms of haemoprotein haematin.

Enzymic activities. Catalatic activity was estimated by the sodium perborate method of Feinstein (1949), at 37° , and the results were expressed in moles of perborate destroyed/ μ mole of catalase haematin under the conditions of the test. The concentration of catalase haematin required for this determination is of the order of $10^{-9}M$. Kat.f. values were determined according to Euler & Josephson (1927). The rate of oxidation of ribose 5-phosphate (R 5-P) (Francoeur & Denstedt, 1954) was determined at pH 8.5, with 0.025M-2-amino-2-hydroxymethylpropane-1:3-diol-HCl (tris) buffer, with 0.02M-R 5-P as hydrogen donor and 0.002M- $K_3Fe(CN)_6$ as hydrogen acceptor in Thunberg tubes under N_2 at 37° . The reduction of the $K_3Fe(CN)_6$ was followed colorimetrically in a Klett-Summerson photoelectric colorimeter (filter 42) in which the Thunberg tubes could fit directly.

Spectrophotometry. Hilger Uvispek and Beckman DU spectrophotometers were used. Anaerobic spectra were measured in 1 cm. quartz cells fused to quartz Thunberg tubes.

Tissue preparations. Male albino rats (100 g.) of a local strain, bred randomly, were decapitated and the liver and kidneys were removed to cooled moist Petri dishes and weighed rapidly. Suspensions (1:5) were prepared in water with a Potter glass homogenizer (Umbreit, Burris & Stauffer, 1949). Erythrocyte haemolysates from rat blood obtained by heart puncture under ether anaesthesia, or from human blood, were prepared by defibrinating the blood with glass beads, washing the erythrocytes with 0.85% NaCl soln. five times on the centrifuge, haemolysing the cells with an equal volume of water and centrifuging

down the cell debris. All tissue preparations were made at 4° and used within 1 hr.

Materials. The yeast ribonucleic acid (RNA) and sperm deoxyribonucleic acid (DNA) were ordinary depolymerized commercial preparations. Similarly, the purines and pyrimidines, their derivatives and the R 5-P were commercial preparations not specially purified. R 5-P was obtained from the barium salt by passing it through a column of Dowex-50 (H⁺ form).

Hydrogen peroxide was estimated by titration with 0.05N-KMnO₄. Nitrogen was purified by passing the gas through a column of activated copper, deposited on kieselguhr, at 180° (Brauer, 1954).

RESULTS

Inhibition of the catalatic activity of tissue suspensions by 3-amino-1:2:4-triazole

Rat-liver and -kidney suspensions. Fig. 1 (A) and (B) show that the incubation at 37° of rat-liver and rat-kidney suspensions with 3-amino-1:2:4-triazole (AT), in O₂-N₂ (5:95), resulted in a rapid loss of catalatic activity. Within 2 hr. the residual catalatic activity was less than 5% of the original activity, whereas the tissue suspensions similarly incubated without AT showed practically no loss of activity. The addition of substances which, as will be shown below, could cause the inhibition of

purified preparations of catalase by AT, such as R 5-P, RNA, ascorbic acid or cysteine, did not increase the rate of inhibition of catalase in these tissue suspensions.

Human- and rat-blood haemolysates. With blood haemolysates prepared from human and rat erythrocytes it could be shown that incubation with AT did not cause any diminution of the catalatic activity, in contradistinction to what happened with liver and kidney suspensions (Fig. 2). However, the addition of R 5-P or RNA, which could effectively diminish the catalatic activity of purified preparations of catalase in the presence of AT, resulted in the inhibition of the enzyme. Similar inhibitions could be obtained by dialysing the haemolysates against low concentrations of H₂O₂ in the presence of AT (see below).

Inhibition of the catalatic activity of purified preparations of catalase by 3-amino-1:2:4-triazole

Requirements for the inhibition of purified catalase by 3-amino-1:2:4-triazole. The incubation at 37° of a recrystallized preparation of ox-liver catalase with AT did not cause any significant inhibition of the catalatic activity of the preparation within 3-6 hr. At the concentration of AT used (0.02M)

Fig. 1 (A). Effect of AT on the catalatic activity of rat-liver suspension. Final concentrations: 1:10 rat-liver suspension; 0.02M-AT; 0.02M-R 5-P; 0.02M-NaHCO₃. Atmosphere O₂-CO₂-N₂ (5:5:90). pH 7.4. Total vol., 3 ml. Incubated at 37° in stoppered 25 ml. flasks. Liver suspension alone (1); liver suspension with R 5-P (2); liver suspension, R 5-P and AT (3); liver suspension with AT (4). (B). Effect of AT on the catalatic activity of rat-kidney suspension. Final concentrations and conditions were as for (A). Kidney suspension alone (1); kidney suspension with R 5-P (2); kidney suspension, R 5-P and AT (3); kidney suspension with AT (4).

Table 1. *Effect of various substances on the inhibition of purified ox-liver catalase by 3-amino-1:2:4-triazole*

Final concentrations in incubation mixtures were: 2.3×10^{-6} M-recrystallized ox-liver catalase haematin; 0.02 M-AT, 0.02 M added substances, unless otherwise stated. Total vol., 3 ml. Unless otherwise stated, the incubation was carried out at 37° under 5% of O_2 in N_2 with the addition of 5% of CO_2 for the bicarbonate buffer. Buffers: 0.02 M- $NaHCO_3$, pH 7.4; 0.025 M-tris buffer, pH 8.5; 0.05 M-phosphate buffer, pH 7. Concentrations of the RNA and DNA were calculated on the basis of the mol.wt. of the mononucleotides.

Added substance	pH	Percentage of original catalase activity after incubation for 2 hr.
R 5-P	7.4	5
R 5-P	8.5	5
R 5-P (under N_2)	8.5	99
Yeast RNA	7.4	17
Yeast RNA	8.5	5
Yeast RNA (incubation at 25°)	8.5	60
Yeast RNA (incubation at 0°)	8.5	95
Sperm DNA	7.4	9
Guanylic acid	7.4	29
Guanylic acid	8.5	25
Guanine	7.4	3
Ascorbic acid	8.5	8
Ascorbic acid (0.02 mm)	7.0	25
Ascorbic acid (0.02 mm) with EDTA (mm)	7.0	64
Cysteine	8.5	3
Cysteine (0.02 mm)	7.0	42
Cysteine (0.02 mm) with EDTA (mm)	7.0	101
Cytidylic acid	7.4	91
Adenosine 3-phosphate	7.4	91
Adenosine 5-phosphate	7.4	88
Adenosine 5-phosphate	8.5	102
Adenosine	7.4	89
Adenine	7.4	102
Ribose	7.4	83
Sodium perborate	8.5	86

Fig. 2. Effect of AT on the catalytic activity of human-erythrocyte haemolysate. Final concentration of blood haemolysates: four times the volume of packed erythrocytes. Other concentrations and conditions were as in Fig. 1 (A). Blood haemolysate alone (1); blood haemolysate with R 5-P (2); blood haemolysate, R 5-P and AT (3); blood haemolysate with AT (4).

prolonged incubations (18–24 hr.) caused relatively small but consistent inhibitions, resulting in catalatic activities that were 65–80% of those of

the control enzyme, incubated under the same conditions without AT. However, the addition of a number of substances to the incubation mixture led to a rapid progressive inhibition of activity, which was practically complete with 2 hr. (Table 1). Of the various substances tested the following were effective: R 5-P, ascorbic acid, cysteine, a commercial yeast RNA, a commercial sperm DNA, guanylic acid and guanine. With limiting concentrations of ascorbic acid and cysteine, the addition of ethylenediaminetetra-acetic acid (EDTA) to the incubation mixture resulted in smaller inhibitions than those obtained without the chelating agent (Table 1). The following substances were ineffective: adenosine 3-phosphate, adenosine 5-phosphate, adenosine, adenine, ribose, cytidylic acid, sodium perborate.

A second requirement for the rapid inhibition of catalase by AT was the presence of O_2 during the incubation. When the incubation was carried out under purified N_2 no inhibition was observed, and in a standard test the atmosphere was always O_2 - N_2 (5:95) (Table 1).

Incubation at 0° prevented the inhibition of catalase by AT, whereas incubation at 37° was

effective and incubation at 25° gave intermediate values, with RNA as added substance (Table 1).

Similarly, Sugimura (1956) found that O_2 was required for the inhibition of catalatic activity in

liver suspensions by AT, and that incubation at 0° was not effective.

Effect of the concentration of reactants on the inhibition of purified catalase by 3-amino-1:2:4-triazole. Fig. 3 shows the effect of the concentration of AT, RNA and catalase respectively on the rate of inhibition of the catalatic activity of recrystallized ox-liver catalase. It can be seen that the lower the concentration of AT and RNA and the higher the concentration of catalase, the smaller the inactivation rate.

Lack of effect of pre-incubation of catalase with ribose 5-phosphate, 3-amino-1:2:4-triazole or both on the inhibition of catalase by 3-amino-1:2:4-triazole. In order to test whether, during the incubation of catalase with R 5-P and AT, an excess of free inhibitor was produced or whether the incubation with R 5-P *per se* was necessary to enable the AT to combine with the enzyme, the following experiment was performed. Mixtures containing respectively catalase alone, AT alone, catalase and R 5-P, catalase and AT, and catalase, R 5-P and AT were incubated for 4 hr. under O_2-N_2 (5:95) at 37°. The catalatic activities of these incubation mixtures were determined. Equal quantities of them were then mixed together and the catalatic activity was determined at once. The results presented in Table 2 show that under these conditions there was no excess of free inhibitor produced during the incubation of catalase, R 5-P and AT that could inactivate either catalase or catalase pre-incubated with R 5-P. Moreover, catalase pre-incubated with R 5-P was not susceptible to an immediate action of AT. The inhibition of catalase by AT could be obtained only when all the required components were incubated together.

Sugimura (1956) was also unable to detect the presence of an inhibitory metabolite in incubation mixtures containing total liver suspensions and AT.

Fig. 3. Effect of the concentration of AT, yeast RNA and catalase on the inhibition of catalatic activity by AT. Curve 1: 2.3 μ M-ox-liver catalase haematin, 20 mM-RNA and varying concentrations of AT. Curve 2: 2.3 μ M-ox-liver catalase haematin, 20 mM-AT and varying concentrations of RNA. Curve 3: 20 mM-AT, 20 mM-RNA and varying concentrations of ox-liver catalase haematin. All flasks were incubated for 2 hr. at pH 8.5 (0.025 M-tris buffer) and 37° under O_2-N_2 (5:95). The concentration of RNA was calculated on the basis of the average mol.wt. of the mononucleotides.

Table 2. *Lack of effect of pre-incubation of recrystallized ox-liver catalase with ribose 5-phosphate, 3-amino-1:2:4-triazole or both on the inhibition of catalase by 3-amino-1:2:4-triazole*

Final concentrations in mixtures pre-incubated were: 2.3×10^{-6} M recrystallized ox-liver catalase haematin; 0.02 M R 5-P; 0.02 M-AT; 0.025 M-tris buffer, pH 8.5. Total vol., 3 ml. Incubated for 4 hr. at 37°, under O_2-N_2 (5:95). At the end of the period of pre-incubation equal volumes of the original incubation mixtures were mixed, as indicated, and the catalatic activity was determined at once.

Mixtures pre-incubated	Catalatic activity (moles of perborate/ μ mole of catalase haematin)		Secondary mixtures	Catalatic activity (moles of perborate/ μ mole of catalase haematin)	
	Before pre-incubation	After pre-incubation		Expected, if no effect of pre-incubation	Observed
A. Catalase	37.2	35.5	A + B	35.5	32.5
B. AT	0	0	A + E	18.0	17.0
C. Catalase, R 5-P	34.2	30.5	B + C	30.5	30.0
D. Catalase, AT	34.7	31.0	C + E	15.5	15.5
			C + D	30.7	31.4
E. Catalase, R 5-P, AT	34.5	0.5	D + E	15.7	14.8

Inhibition in the presence of low and constant concentrations of hydrogen peroxide. The dialysis of H_2O_2 through a cellophan membrane has been used by Laser (1955) to obtain the gradual release of small amounts of H_2O_2 into a solution of catalase, thus obtaining high yields of the products of the peroxidatic activity of the enzyme. By the same principle, solutions containing either crystalline human-erythrocyte catalase (Fig. 4) or recrystallized ox-liver catalase, and AT at pH 6.8, were dialysed at 37° against small concentrations of H_2O_2 (0.2–2 mm). It was found that the inhibition of the catalatic activity proceeded as fast or faster than in total liver suspensions or with purified liver catalase together with substances mentioned above. Under the same conditions there was no significant inhibition in the absence of either AT or H_2O_2 within 3 hr. (Fig. 4). However, in the absence of H_2O_2 , inhibitions of up to 50 % could be obtained if the incubation was continued for 18 hr. When sodium perborate or H_2O_2 was added directly to solutions containing catalase and AT, the resulting inhibition was small and did not differ significantly from that obtained with AT alone (see Table 1).

Protective effect of ferricyanide on the inhibition

Fig. 4. Inhibition of crystalline human-erythrocyte catalase by dialysis of H_2O_2 into the catalase solution in the presence of AT. Final concentrations: $2.1 \mu\text{M}$ crystalline human-erythrocyte catalase haematin; 0.02 M -AT; 0.033 M -phosphate buffer, pH 6.8. Total volume of catalase-containing solution in dialysis bag: 6 ml. Outer solution (50 ml.) contained 2 mm- H_2O_2 with the same concentrations of AT and buffer as the solution in the dialysis bag. Temp., 37° . Catalase dialysed against H_2O_2 (1); catalase with AT dialysed against AT (2); catalase with AT dialysed against H_2O_2 and AT (3).

of catalase by 3-amino-1:2:4-triazole. In the systems in which AT could cause the inhibition of catalase the addition of ferricyanide prevented this inhibition. Ferricyanide had no effect. When the system RNA-AT-catalase in $\text{O}_2\text{-N}_2$ (5:95) was used, concentrations of ferricyanide of the order of mm were fully effective (Fig. 5). Experiments with the H_2O_2 dialysis technique, designed to show whether the protective effect of ferricyanide was accompanied by the formation of a ferricyanide-inhibitor complex leading to a change in the ferricyanide spectrum or whether ferricyanide was reduced to ferrocyanide, were consistently negative.

Spectral changes during the inhibition of purified ox-liver catalase by 3-amino-1:2:4-triazole. Concentrated solutions of recrystallized ox-liver catalase (final concentration in incubation mixture: 0.031 mm -haematin) were dialysed in the presence of AT (0.02 M) against H_2O_2 (4 mm) at 37° and pH 6.8. Controls included catalase dialysed against H_2O_2 in the absence of AT, catalase dialysed against water in the presence of AT and catalase dialysed against water in the absence of AT. When after $2\frac{1}{2}$ hr. the catalatic activity of the solution con-

Fig. 5. Protective effect of ferricyanide on the AT inhibition of recrystallized ox-liver catalase in the presence of yeast RNA and O_2 . Final concentrations: $2.3 \mu\text{M}$ -ox-liver catalase haematin; 0.02 M -AT; 0.02 M -RNA; 0.025 M -tris buffer, pH 8.5; varying concentrations of $\text{K}_3\text{Fe}(\text{CN})_6$ and $\text{K}_4\text{Fe}(\text{CN})_6$. Total vol., 3 ml. Atmosphere $\text{O}_2\text{-N}_2$ (5:95). Temp., 37° . Ferricyanide concentrations: 1.28 mm (1); 0.385 mm (2); 0.128 mm (3); 0.0385 mm (4); 0.0128 mm (5). Ferrocyanide concentration: 5.2 mm (6). Concentration of the RNA was calculated on the basis of the average mol.wt. of the mononucleotides.

taining all three components had decreased to 3% of the original activity, the activity of the control solutions was unchanged. The spectrum of the contents of each dialysis bag was then recorded at once. The results are presented in Fig. 6 (A) and (B). There was no change in the spectrum of catalase incubated under the above conditions with either H_2O_2 or AT; catalase incubated with both H_2O_2 and AT showed a broadening of the α -band and a shift of its maximum towards the red end of the spectrum, as well as a slight decrease in the height of the Soret band. Under similar conditions, with appropriate concentrations of catalase and

AT, it was not found possible to demonstrate any consistent changes in the concentration of AT, the spectrum of AT in the far ultraviolet region being used as a measure of its concentration.

*Properties of the catalase inhibited
by 3-amino-1:2:4-triazole*

Preparation of inhibited catalase. Catalase inhibited by AT was prepared by incubating at 37° recrystallized ox-liver catalase (0.031 mM) with R 5-P and AT (both 0.02M) in O_2 - N_2 (5:95), or by dialysing at 37° H_2O_2 (4 mM) into a solution of catalase and AT. When inhibition was complete (3–4 hr.) the preparations were dialysed against 0.85% NaCl soln. (changed twice daily) for 5 days to get rid of the excess of reagents. In order to crystallize the inhibited enzyme these solutions were then dialysed against glass-distilled water (changed daily). The crystals obtained, examined under the microscope with ordinary and polarized light, appeared to be identical with those of the original uninhibited ox-liver catalase.

Spectrum of inhibited catalase. The spectrum of the preparations of catalase inhibited by AT was identical with that given in Fig. 6 (A) and (B). Addition of H_2O_2 to these preparations under an atmosphere of pure N_2 did not cause any change in the spectrum.

Enzymic activity of inhibited catalase. The preparations of catalase inhibited by AT were devoid of any detectable catalatic activity in concentrations 100 times those used for testing the original preparation. However, when tested for the oxidation of R 5-P with $\text{K}_3\text{Fe}(\text{CN})_6$ as hydrogen acceptor (Francoeur & Denstedt, 1954), as given under Methods, these preparations showed the full activity of the original uninhibited catalase. During the oxidation of R 5-P the inhibited catalase did not recover its catalatic activity, remaining fully inhibited, whereas the control uninhibited enzyme retained its full catalatic activity (Table 3).

Lack of reversal of inactivation of inhibited catalase by dialysis. Dialysis of preparations of ox-liver catalase inhibited by AT against 0.85% NaCl soln. (changed daily) for 15 days did not result in any increase of the catalatic activity of the preparation.

DISCUSSION

The experiments described above showed that the presence of hydrogen peroxide was required for the inhibition of catalase by 3-amino-1:2:4-triazole. In the absence of hydrogen peroxide only a relatively small degree of inhibition could be obtained after prolonged incubation. The inhibition observed was of the type described by Heim *et al.* (1956) after the injection of 3-amino-1:2:4-triazole into rats, and not the one obtained by these authors with high

Fig. 6 (A) and (B). Spectrum of ox-liver catalase inhibited by AT. Recrystallized ox-liver catalase (final concn. 0.031 mM-catalase haematin) dialysed at 37° against H_2O_2 (2 mM) in the presence of AT (0.02M) at pH 6.8 (0.033M-phosphate buffer) for $2\frac{1}{2}$ hr. (curve 1). At the end of the dialysis period the catalatic activity of the solution had decreased to 3% of its original value. Controls included catalase solutions dialysed under the same conditions as above, against water in the absence of AT, against water in the presence of AT and against H_2O_2 in the absence of AT (curve 2). At the end of the dialysis period the spectra of all three controls were identical and their catalatic activity had not varied significantly from the original value.

Table 3. *Oxidation of ribose 5-phosphate by AT-inhibited catalase, with potassium ferricyanide as hydrogen acceptor*

Final concentrations in pre-incubation mixtures were: 0.46 μ M recrystallized ox-liver catalase haematin; 0.02 M-R 5-P; 0.02 M-AT; 0.025 M-tris buffer, pH 8.5. Pre-incubation was for 2 hr. at 37° under O_2-N_2 (5:95). Total vol., 6 ml. Final concentrations in R 5-P oxidation test mixture: 0.384 μ M-catalase haematin; 0.016 M-R 5-P; 0.002 M- $K_3Fe(CN)_6$; 0.025 M-tris buffer, pH 8.5. Oxidation of R 5-P carried out after pre-incubation in Thunberg tubes, under N_2 , at 37°.

Pre-incubation mixture	Catalatic activity (moles of perborate/ μ mole of catalase haematin)		R 5-P oxidation rate after pre-incubation [m-moles of $K_3Fe(CN)_6$ reduced/hr./ μ mole of catalase haematin]	Catalatic activity after R 5-P oxidation (moles of perborate/ μ mole of catalase haematin)
	Before incubation	After incubation		
A. Catalase, R 5-P	28.5	25.3	2.11	25.0
B. Catalase, R 5-P, AT	29.0	1.7	2.16	1.4

concentrations of 3-amino-1:2:4-triazole *in vitro*, since it could not be reversed by prolonged dialysis. The effect of substances other than hydrogen peroxide which were found to be effective in causing the inhibition of catalase by 3-amino-1:2:4-triazole, such as ribose 5-phosphate, nucleic acids, ascorbic acid and cysteine, required the presence of oxygen, and therefore probably acted by producing small amounts of hydrogen peroxide by auto-oxidation. This conclusion was further supported by the fact that with ascorbic acid and cysteine the addition of ethylenediaminetetraacetic acid which, like other chelating agents, inhibits the auto-oxidation of these compounds, was found to decrease considerably their effectiveness in causing the inhibition of catalase by 3-amino-1:2:4-triazole.

The inhibition of catalase by 3-amino-1:2:4-triazole could thus be due either to the oxidation of 3-amino-1:2:4-triazole by catalase-hydrogen peroxide to an inhibitory metabolite under conditions favouring the peroxidatic activity of the enzyme (Laser, 1955), or to the reaction of unchanged 3-amino-1:2:4-triazole with the enzyme under these conditions. It has so far not been possible to eliminate either of these two possibilities. The absence of an excess of free inhibitor in incubation mixtures containing catalase, 3-amino-1:2:4-triazole and hydrogen peroxide, and the slow partial inhibition of catalase by 3-amino-1:2:4-triazole in the absence of hydrogen peroxide (see also Heim *et al.* 1956), seem to favour the second mechanism, but cannot be considered as conclusive evidence against the first. It should be noted that both possible mechanisms proposed for the rapid inhibition of catalase by 3-amino-1:2:4-triazole require hydrogen peroxide. In other cases in which hydrogen peroxide has been shown to play a role in the inhibition of the enzyme, such as with azide and hydroxylamine, an inhibitor (nitric oxide) is

produced by the peroxidatic action of the enzyme, and the original substances are themselves strong immediate inhibitors (Keilin & Hartree, 1954). With 3-amino-1:2:4-triazole, even in the presence of hydrogen peroxide, the inhibition occurred much more slowly.

Catalase inhibited by 3-amino-1:2:4-triazole showed a spectrum somewhat different from that of catalase, with a shift of the visible part of the spectrum towards the longer wavelengths and a slight decrease of the height of the Soret band. Similarly, the spectrum of azide-catalase is not very different from that of the original catalase, but when hydrogen peroxide is added and nitric oxide-catalase is formed there is a gross change in the visible spectrum (Keilin & Hartree, 1945). No such effect could be observed on the addition of hydrogen peroxide under anaerobic conditions to catalase inhibited by 3-amino-1:2:4-triazole.

In the presence of oxygen, but in the absence of added hydrogen peroxide, the effect of 3-amino-1:2:4-triazole on the catalatic activity of tissue suspensions exactly paralleled its effect *in vivo*, described by Heim *et al.* (1956). The catalatic activity of liver and kidney suspensions was rapidly reduced to low levels, whereas that of blood haemolysates was unaffected. The catalatic activity of blood haemolysates could, however, be inhibited if hydrogen peroxide was added under suitable conditions. Purified erythrocyte catalase itself did not differ from purified liver catalase in its behaviour towards 3-amino-1:2:4-triazole. Thus the inhibition of liver and kidney catalase by 3-amino-1:2:4-triazole administered to rats, and its lack of effect on blood catalase (Heim *et al.* 1956), as well as the corresponding effects in tissue suspensions, may possibly be ascribed to the availability of sufficient hydrogen peroxide in liver and kidney and its lack in blood. This does not, however, preclude the possibility that other factors in blood

interfere with the reaction of catalase, hydrogen peroxide and 3-amino-1:2:4-triazole just as ferri-cyanide was shown to do.

SUMMARY

1. 3-Amino-1:2:4-triazole caused a complete, irreversible and relatively rapid inhibition of crystalline purified preparations of liver and erythrocyte catalase in the presence of low and constant concentrations of hydrogen peroxide. Without hydrogen peroxide the inhibition of catalase by 3-amino-1:2:4-triazole was slight and proceeded much more slowly. Other substances that could replace hydrogen peroxide required the presence of oxygen, and it is considered that they acted by producing peroxide by auto-oxidation.

2. Ferri-cyanide, but not ferrocyanide, prevented this inhibition.

3. The spectrum of inhibited catalase differed from that of catalase. The visible part of the spectrum was shifted towards the longer wavelengths, and the Soret band was slightly lowered.

4. Inhibited catalase was obtained in a crystalline form that did not differ from that of uninhibited catalase.

5. The ability of catalase inhibited by 3-amino-1:2:4-triazole to oxidize ribose 5-phosphate with potassium ferri-cyanide as hydrogen acceptor was unchanged.

6. In the presence of oxygen, but in the absence

of added hydrogen peroxide, 3-amino-1:2:4-triazole caused the inhibition of catalase in liver and kidney suspensions, but had no effect in blood haemolysates. The catalatic activity of blood haemolysates could be inhibited by 3-amino-1:2:4-triazole only in the presence of added hydrogen peroxide.

The authors are grateful to Professor D. Appleman for interesting them in the problem and for the gift of the aminotriazole used in this study. They also wish to thank the Hebrew University Lutie Goldstein Fund for a supporting grant.

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The Kinetics of Hydrazinolysis of Simple Peptides in Anhydrous Hydrazine

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(Received 29 April 1957)

The method of Akabori, Ohno & Narita (1952) for the determination of the C-terminal amino acids of peptides and proteins consists in treating with anhydrous hydrazine at 100–120° for about 8 hr. to split the peptide bonds and produce amino acid hydrazides and free amino acids from the C-terminal positions. The latter can be separated from the former by treatment with benzaldehyde, by dinitrophenylation and subsequent extraction with sodium bicarbonate of the dinitrophenyl-amino acid from the dinitrophenyl-amino acid hydrazide (Ohno, 1953). The main disadvantage of this method is that the severe treatment required

for complete hydrazinolysis causes decomposition of cysteine and cystine and poor yields of arginine, aspartic acid and glutamic acid (Locker, 1954; Niu & Fraenkel-Conrat, 1955). The problem of severity of treatment has been largely overcome by the addition of an acid catalyst, hydrazine sulphate, to the reaction mixture, when heating at 60° for 16 hr. brings about complete fission of the peptide bond (in the seven peptides studied) and allows all the common amino acids to be recovered in good yields (Bradbury, 1956).

In order to apply the hydrazine method with confidence it is important to know more about the