Irreversible Reaction of 3-Amino-1:2:4-triazole and Related Inhibitors with the Protein of Catalase

By E. MARGOLIASH*, A. NOVOGRODSKY and A. SCHEJTER
Department of Experimental Medicine and Cancer Research, Hebrew University-Hadassah Medical School, Jerusalem, Israel

(*Received* 2 *February* 1959)

The common catalase inhibitors such as cyanide, azide, fluoride, etc., all give rise to inhibitions which are fully reversible on dilution or dialysis. 3-Amino-1:2:4-triazole, however, can produce both an immediate reversible inhibition of the enzyme at rather high concentrations of the inhibitor (Heim, Appleman & Pyfrom, 1956) and a slowly developing irreversible inhibition of catalatic activity in the presence of a low and constant concentration of hydrogen peroxide (Margoliash & Novogrodsky, 1958*a*). This irreversible inhibition explains the decrease in catalatic activity of the liver and kidney of rats and mice observed by Heim, Appleman & Pyfrom (1955, 1956) within a few hours after the injection of 3-amino-1:2:4-triazole.

The present paper reports the minimal structural requirements of compounds able to cause such irreversible inhibitions of catalase, demonstrates that the irreversible inhibition reaction is a reaction between catalase–hydrogen peroxide complex I and the inhibitors and that although these inhibitors react with catalase–hydrogen complex II the product of this reaction is free uninhibited catalase. A study of the kinetics of the reactions with both catalase–hydrogen peroxide complexes is presented. It is, moreover, shown that in the irreversible inhibition the essential binding of the inhibitor is with the protein part of catalase, since denaturation of the enzyme and removal of the haematin prosthetic group leaves the inhibitor attached to the protein.

EXPERIMENTAL

The catalase preparations used, the methods for determining the concentration of the catalase solutions in terms of haematin and the catalatic activity as well as the Kat. f. (Euler & Josephson, 1927) values of the enzyme preparations were the same as those previously given (Margoliash & Novogrodsky, 1958*a*). In those kinetic experiments in which accurate determinations of catalatic activity were required, the concentrations of the partially irreversibly inhibited catalase solutions were adjusted so that the actual permanganate-titration readings in the perborate test (Feinstein, 1949) fell within a narrow range (10–14 ml. of 0·05 N-$KMnO_4$). This procedure gave values for the catalatic activity which were quantitatively comparable one to another. In all cases the dilution of the incubation mixtures used for estimations of catalatic activity of the irreversibly inhibited enzyme was sufficient to render negligible the reversible inhibition of the enzyme by the inhibitors used.

Microspectroscopic observations were carried out with a Hartree microspectroscope (Beck Ltd., London). Catalase–H_2O_2 complex II (Chance, 1951) was prepared by incubating the catalase solution with 4 mM-ascorbate or cysteine at pH 7·0 in air at 37° (Chance, 1950). The incubation was continued until all the catalase had been transformed into catalase–H_2O_2 II as judged by the Soret-band spectrum (Chance, 1950) measured with a Beckman DU spectrophotometer. The reaction of catalase–H_2O_2 II with inhibitors was followed by estimating the extinction every 15 sec. at 440 mμ with a Beckman DU spectrophotometer in which the temperature of the cell chamber could be kept constant by a fluid-circulation system.

Materials. The materials used were commercial preparations except for the following: methyl hydroperoxide (Rieche & Hitz, 1929); *S*-methyl*iso*thiosemicarbazide iodide (Freund & Paradies, 1901); *N*-methylthiourea (Näf, 1891); *N*-methyl-*S*-methyl*iso*thiourea iodide (Schenck, 1912); 3:3′-azo-1:2:4-triazole (Thiele & Manchot, 1898); acetone semicarbazone (Thiele & Stange, 1894): acetone thiosemicarbazone (Freund & Schander, 1902).

3-Amino-[^{14}C]1:2:4-triazole, labelled in C-5, was prepared from sodium [^{14}C]formate and aminoguanidine bicarbonate according to Sjostedt & Gringas (1955), exchanging the [^{14}C]formate between the sodium formate and the free formic acid in the reaction mixture. The crude product, dissolved in 0·01 N-HCl, was adsorbed on a 6 cm. × 1 cm. Dowex-50 column (H^+ form), the excess of formate was washed away with 1 mN-HCl and the 3-amino-1:2:4-triazole then eluted with aq. 5 N-NH_3 soln. On evaporating the eluate to dryness over H_2SO_4, pure crystalline 3-amino-[^{14}C]1:2:4-triazole, giving 18 990 counts/min./μmole, was obtained. The radioactivity of the catalase preparations inhibited by 3-amino-1:2:4-triazole was estimated with an end-window β-counter, thin layers being used. The required correction for absorption of radioactivity by the solids was determined in separate experiments with known amounts of unlabelled protein and of 3-amino-[^{14}C]1:2:4-triazole. The haematin was separated from the protein of catalase by the acid–acetone technique of Sumner & Dounce (1939) by washing the protein precipitate three times on the centrifuge with acid–acetone.

* Present address: Laboratory for the Study of Hereditary and Metabolic Disorders, College of Medicine, University of Utah, Salt Lake City, Utah, U.S.A.

RESULTS

Reversible inhibition of catalase by 3-amino-1:2:4-triazole. Heim *et al.* (1956) showed that relatively high concentrations of 3-amino-1:2:4-triazole caused a reversible inhibition of catalatic activity as determined by the rate of H_2O_2 breakdown. The effect of the concentration of 3-amino-1:2:4-triazole on this type of inhibition was determined at 22° and concentrations of H_2O_2 equivalent to those used in the kinetic study of the irreversible inhibition reaction described below. The method of Euler & Josephson (1927) for the determination of the first-order reaction constant (k_0) for the decomposition of H_2O_2 was used. From the results given in Fig. 1 it was possible to calculate the equilibrium constants for the possible reversible reactions of 3-amino-1:2:4-triazole with either free catalase (K_5) or catalase–H_2O_2 I (K_6) according to the equations developed by Beers (1955). Considering 33 % of the catalase haematin to be bound by H_2O_2 in complex I (n 0·33), as determined by Beers & Sizer (1952, 1953) for ox-liver catalase, the average value obtained for K_5 was 40 mM, and that obtained for K_6 was 20 mM.

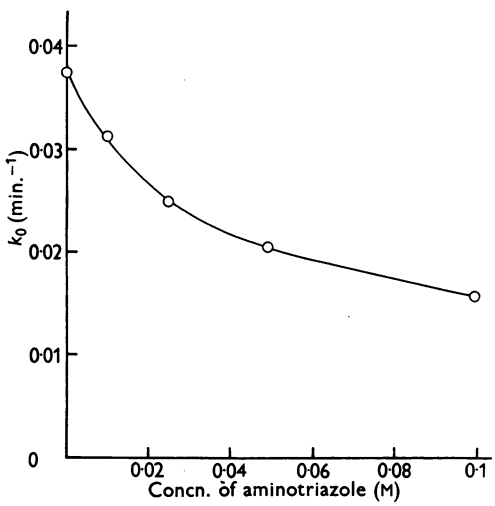


Fig. 1. Reversible inhibition of catalatic activity by 3-amino-1:2:4-triazole. The first-order reaction constant for the decomposition of H_2O_2 (k_0) was determined according to Euler & Josephson (1927) at 22° and in the presence of varying concentrations of aminotriazole. Initial H_2O_2 concentration: 4 mM; 1·4 μmM ox-liver catalase haematin; 6·6 mM-phosphate buffer, pH 7·0. The equilibrium constants for the reversible reaction of 3-amino-1:2:4-triazole with free catalase (K_5) or the reversible reaction of aminotriazole with catalase–H_2O_2 I (K_6), calculated according to Beers (1955) from the first-order reaction constants for the decomposition of H_2O_2 in the absence (k_0^c) and in the presence (k_0^*) of inhibitor, were respectively 40 and 20 mM.

Reaction of the 3-amino-1:2:4-triazole type of inhibitors with catalase–hydrogen peroxide complex II. Microspectroscopic observations indicated that when the compounds that could cause the irreversible inhibition of catalase in the presence of H_2O_2 (see below) were added to catalase–H_2O_2 II or catalase–methyl hydroperoxide II the spectrum of complex II rapidly disappeared, to be replaced by a spectrum nearly identical to that of free catalase. The kinetics of these reactions could be followed at appropriate wavelengths in the ordinary spectrophotometer since their rate was not very large. The reactions followed first-order kinetics with all the effective inhibitors tested at any particular inhibitor and enzyme concentration, as shown in Fig. 2 for 3-amino-1:2:4-triazole.

These kinetics were, however, pseudo-first-order, since the first-order reaction constant (k_8') was found to vary linearly with the inhibitor concentration

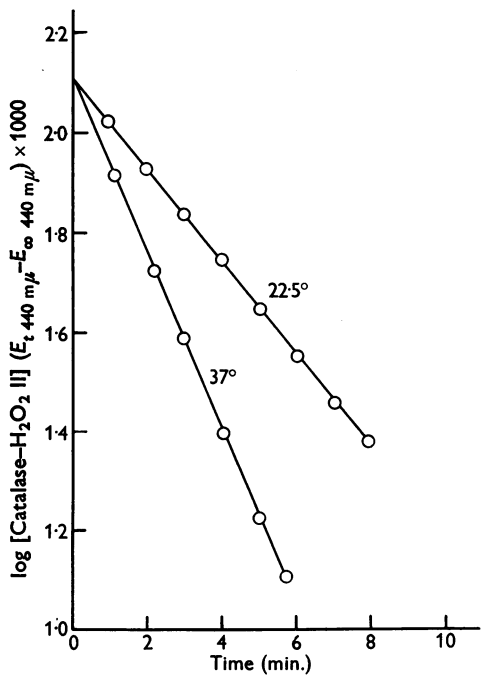


Fig. 2. First-order kinetics of the reaction between catalase–H_2O_2 II and 3-amino-1:2:4-triazole. Final concentrations: 9 μM ox-liver catalase haematin; 0·1 M-phosphate buffer, pH 7·0; 4 mM-ascorbate. Total volume 3 ml. Incubated in spectrophotometric cells at 37° until the formation of catalase–H_2O_2 II was maximal. The cells were then equilibrated in the spectrophotometer at the required temperature, 0·02 M-aminotriazole was added and extinction readings were taken, at short intervals, at 440 mμ. The concentration of catalase–H_2O_2 II was estimated from the difference between the extinction reading at any time and that after the completion of the reaction.

(*i*), for 3-amino-1:2:4-triazole and *S*-methyl*iso*-thiosemicarbazide at 37°. Plots of the logarithm of the first-order reaction constant against the logarithm of the inhibitor concentration gave straight lines with a slope of 45°, indicating that the reaction was first order with respect to the inhibitor (Fig. 3). From Fig. 3 and similar plots the second-order reaction constant (k_8) could be calculated for the various inhibitors.

A similar plot of the logarithm of the first-order reaction constant against the logarithm of the concentration of catalase–H_2O_2 II, from data obtained in experiments in which the inhibitor concentration was kept constant and the concentration of catalase was varied, also gave straight lines with slopes of 45°, indicating that the reaction was of the first-order with respect to catalase–H_2O_2 II (Fig. 4). Hence the overall reaction was of the second order.

When catalase–H_2O_2 I donors (i.e. substances that can be oxidized by catalase–H_2O_2 I) such as ethanol and propan-1-ol having widely differing reaction constants with catalase–H_2O_2 I (1000 M^{-1} sec.$^{-1}$ for ethanol and 17 M^{-1} sec.$^{-1}$ for propan-1-ol; Chance, 1947) were added in great excess to catalase–H_2O_2 II under the conditions used for the above kinetic studies, it was found that the rate of disappearance of catalase–H_2O_2 II did not vary with the concentration of the catalase–H_2O_2 I donor, at the large concentrations used, and was slower than the rate of disappearance of complex II on addition of the inhibitors of the 3-amino-1:2:4-triazole series. These results, shown in Fig. 3, indicated that the inhibitors of the 3-amino-1:2:4-triazole series actually reacted with catalase–H_2O_2 II, and that the disappearance of the spectrum of catalase–H_2O_2 II in these reactions was not due to a shift of the equilibrium between complex II and complex I such as occurs when a catalase–H_2O_2 I donor is added to their mixture (Chance, 1949).

When the catalatic activity of the reaction mixtures was determined at the end of the reaction between the inhibitors and catalase–H_2O_2 II, after suitable dilution, it was found that nearly the entire original catalatic activity was retained. There was, in fact, very little or no difference in the rate and kinetics of the irreversible inhibition of catalatic activity whether the reaction was started with free catalase, ascorbate and 3-amino-1:2:4-triazole, or with catalase–H_2O_2 II, ascorbate and 3-amino-1:2:4-triazole (Fig. 5). This indicated that the product of the reaction of the inhibitor with complex II was free catalase. The lack of difference between the rate of the inhibition reaction when the reaction was started with free catalase and with catalase–H_2O_2 II, as shown in Fig. 5, was due to the fact that the inhibition reaction was much slower than the reaction with complex II. It could, for example, be calculated that 150 sec. after the start of the reaction with complex II at 37° the rate of the inhibition reaction was already over 60% of that when the

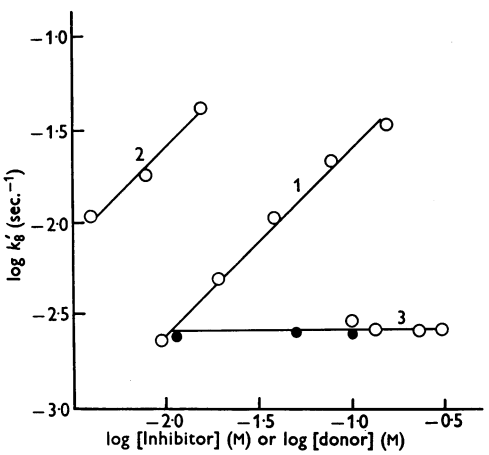


Fig. 3. Variation of the logarithm of the first-order reaction constant (k_8') for the reaction of inhibitors and catalase–H_2O_2 II with the logarithm of the inhibitor concentration. The final concentrations and conditions were as in Fig. 2. Temp., 37°. The concentrations of inhibitors varied. The second-order reaction constant (k_8) calculated by extrapolation from the value of the first-order reaction constant when the log [inhibitor] = 0 was 0·26 M^{-1} sec.$^{-1}$ for 3-amino-1:2:4-triazole (curve 1) and 2·4 M^{-1} sec.$^{-1}$ for *S*-methyl*iso*thiosemicarbazide iodide (curve 2). Curve 3 shows the first-order reaction constant for the disappearance of catalase–H_2O_2 II on the addition of ethanol (○) or propan-1-ol (●) at varying concentrations of the donors.

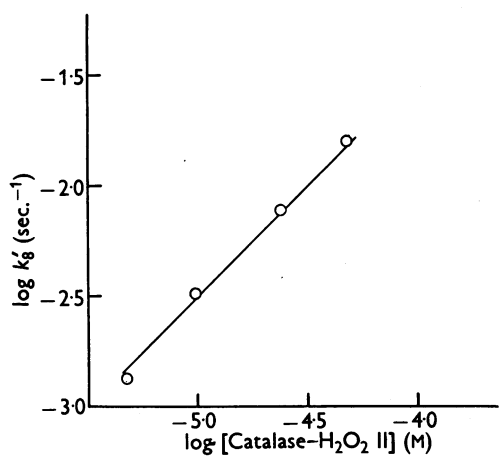


Fig. 4. Variation of the logarithm of the first-order reaction constant (k_8') for the reaction of 3-amino-1:2:4-triazole with catalase–H_2O_2 II with the logarithm of the concentration of catalase–H_2O_2 II. Final concentrations and conditions were as given in Fig. 2, except that the reactions were carried out with 0·02 M-aminotriazole and varying concentrations of catalase–H_2O_2 II. Temp., 37°.

reaction was started with free catalase. Such a small difference could not be detected under the experimental conditions available.

The same conclusion could be reached by estimating the percentage of catalase haematin free to react with cyanide (mM) as shown by the change in the Soret-band spectrum. At the end of the reaction between catalase–H_2O_2 II and 3-amino-1:2:4-triazole nearly the entire catalase haematin reacted with cyanide, whereas in the preparations of irreversibly inhibited 3-amino-1:2:4-triazole–catalase the percentage of haematin able to combine with cyanide varied from 16 to 5%.

Compounds causing the irreversible inhibition of catalase in the presence of hydrogen peroxide. Margoliash & Novogrodsky (1958*a*) had demonstrated that the presence of a relatively low and constant concentration of H_2O_2 was required to cause the irreversible inhibition of catalase by 3-amino-1:2:3-triazole. The H_2O_2 could be introduced by using auto-oxidizable substances in the presence of air, or by dialysis against a large volume of solution containing a low concentration of H_2O_2. With both these systems a series of compounds were tested for their ability to cause the irreversible inhibition of catalase, in order to establish the minimal structural characteristics required.

The results given in Table 1 indicated that: (*a*) the ring system of 3-amino-1:2:4-triazole as such

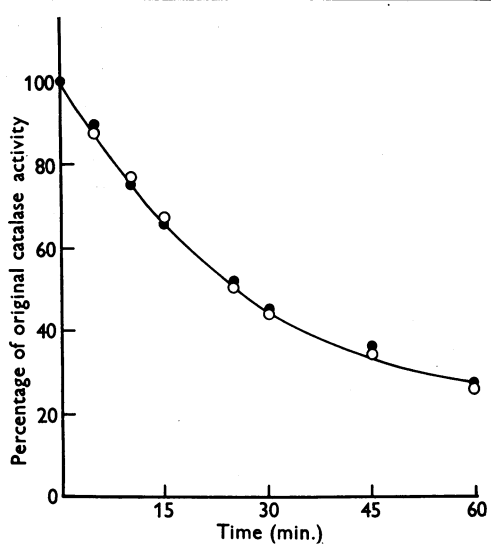


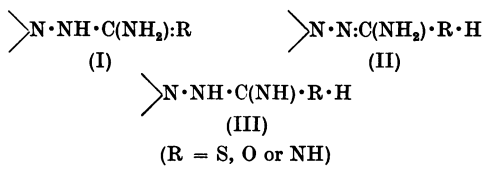
Fig. 5. Kinetics of irreversible 3-amino-1:2:4-triazole inhibition of catalase, starting with free catalase and with catalase–H_2O_2 II. Final concentrations: 2·5 μM recrystallized ox-liver catalase haematin; 20 mM-aminotriazole; 50 mM-phosphate buffer, pH 7·0; 4 mM-ascorbate, pH 7·0. Total volume, 3 ml. Atmosphere, air. Temp., 37°. ●, Catalatic activities obtained when starting with free catalase; ○, catalatic activities obtained when the aminotriazole was added after a period of pre-incubation at 37° sufficient to transform the catalase maximally into catalase–H_2O_2 II, as determined spectrophotometrically. The catalatic activity was estimated according to Feinstein (1949), after suitable dilution of samples in the presence of 50 mM-ethanol.

Table 1. *Compounds causing the irreversible inhibition of catalase in the presence of hydrogen peroxide*

In the system with ascorbic acid as a source of hydrogen peroxide (*a*) the final concentrations in the incubation mixtures were: 2·4 μM recrystallized ox-liver catalase haematin; 20 mM compound tested, unless otherwise stated; 50 mM-phosphate buffer, pH 7·0; 1 mM-ascorbic acid, pH 7·0. Total volume, 3 ml. Atmosphere, air. Temperature, 37°. In the system in which hydrogen peroxide was dialyzed into the incubation mixture (*b*), the concentrations and conditions were similar except that ascorbic acid was absent and the incubation mixture (total volume 3 ml.) was dialysed in cellophan tubing against 50 ml. of phosphate buffer (50 mM) containing 20 mM compound tested, unless otherwise stated, and 4 mM-H_2O_2. In each case, samples were removed at intervals, were suitably diluted and the catalatic activity was determined according to Feinstein (1949).

Compound tested	System used	Percentage of original catalatic activity after incubation for 2 hr.
3-Amino-1:2:4-triazole	*a*, *b*	7
3:3′-Azo-1:2:4-triazole	*a*	100
Aminoguanidine bicarbonate	*b*	58
Guanidine hydrochloride	*a*	100
Creatine	*a*	100
Arginine	*a*	100
Semicarbazide hydrochloride	*a*	64
Semicarbazide hydrochloride	*b*	20
Semicarbazide hydrochloride (0·2M)	*a*	41
Acetone semicarbazone (40 mM)	*a*	55
Urea	*a*	93
Glycinamide	*a*	100
Acetamide (0·2M)	*a*	95
Ethylenediamine	*a*	100
Ethyl urethane	*a*	100
Carbohydrazide	*a*	98
Dicyandiamide	*a*	100
Thiosemicarbazide	*a*	36
Thiosemicarbazide	*b*	6
Acetone thiosemicarbazone	*a*	34
S-Methyl*iso*thiosemicarbazide iodide	*a*	0
Potassium iodide	*a*	96
Thiourea	*a*	100
N-Methylthiourea	*a*	76
N-Methyl-*S*-methyl*iso*thiourea iodide	*a*	98
Thiocarbohydrazide	*a*	86
Isonicotinic acid hydrazide	*a*, *b*	100
Histidine	*b*	100
Allyl*iso*propylacetylurea	*a*	96
2:4-Dinitrophenol	*b*	82
Iodoacetic acid	*a*	96

was not required; (*b*) the primary amino group attached to the carbon atom had to be free; the substitution of one of its H atoms by even as small a substituent as an amino group, as in carbohydrazide or thiocarbohydrazide, destroyed the activity; (*c*) in non-cyclic compounds the terminal amino group (corresponding to position 1 in 3-amino-1:2:4-triazole) did not have to be free, but a N atom was necessary, since substituting a nitrile or a methyl group for this amino group caused a complete loss of inhibitory activity; (*d*) the N atom corresponding to position 2 in 3-amino-1:2:4-triazole could not be replaced by a C (as in glycinamide) without loss of activity; (*e*) the atom corresponding to position 4 in 3-amino-1:2:4-triazole could be N, O or S, the inhibitory activity relations being S > O ⩾ N. The most active compound found in this series was *S*-methyl*iso*thiosemicarbazide iodide, which was considerably more active than the original 3-amino-1:2:4-triazole. In conclusion it may be stated that, as far as it was tested, the probable minimal structural requirements of a compound of this series to enable it to cause an irreversible inactivation of catalase in the presence of H_2O_2 was one of the isomeric structures I, II or III.



Moreover, since substitution of the H atom on the R group by a methyl group, forcing the compound into either the form II or III, considerably increased the inhibitory activity, as in *S*-methyl*iso*thiosemicarbazide, it is probable that the active species in all the effective compounds was either II or III.

This conclusion does not in any way imply that compounds of other organic or inorganic series may not be able to show the same type of activity.

Effect of catalase–hydrogen peroxide complex I donors on irreversible inhibition of catalase. Catalase–H_2O_2 I donors such as ethanol, nitrite, formate, ethylene glycol, etc., were found to prevent the irreversible inhibition of catalase by inhibitors of the 3-amino-1:2:4-triazole series. Similar effects occurred in systems containing auto-oxidizable substances as H_2O_2 source, and when dialysis was used to introduce the H_2O_2. Some typical results are presented in Table 2. However, in all cases when irreversible inhibition had occurred, incubation with catalase–H_2O_2 I donors did not cause any recovery of the catalatic activity.

These results confirm the observations of Nelson (1958), who showed that the injection of ethanol into rats prevented the decrease of liver-catalase activity caused by 3-amino-1:2:4-triazole.

Effect of pH *on the irreversible inhibition of catalase by 3-amino-1:2:4-triazole.* The lack of effect of changing the pH on the inhibition of catalase by 3-amino-1:2:4-triazole, in the presence of H_2O_2, is shown in Fig. 6. The rate of inhibition did not vary

Table 2. *Effect of catalase–hydrogen peroxide complex I donors on irreversible inhibition of catalase*

Conditions and concentrations were similar to those given in Table 1 for both the system with ascorbic acid (*a*) and that with dialysis against H_2O_2 (*b*). Unless otherwise stated concentration of catalase inhibitors was 20 mM and of donors 0·1 M.

Inhibitor	System used	Donor	Percentage of original catalatic activity after incubation for 2 hr.
3-Amino-1:2:4-triazole	*a*	None	10
	a	Ethanol	97
	a	Nitrite	100
	a	Formate	88
	a	Formaldehyde	84
	a	Ethylene glycol	71
	a	Pyrogallol	78
Thiosemicarbazide	*a*	None	36
	a	Ethanol	100
	b	None	6
	b	Ethanol	88
Semicarbazide hydrochloride (0·2 M)	*a*	None	57
	a	Ethanol	100
Aminoguanidine bicarbonate	*b*	None	56
	b	Ethanol	98

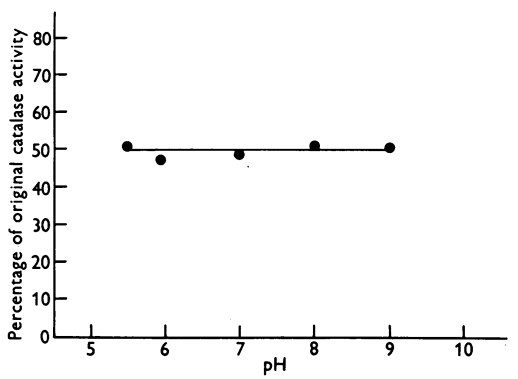


Fig. 6. Effect of pH on the inhibition of catalatic activity by 3-amino-1:2:4-triazole. Hydrogen peroxide was dialyzed into the catalase solution. Final concentrations: 2·6 μM ox-liver catalase haematin; 10 mM-aminotriazole; 50 mM-phosphate–phosphoric acid buffer, pH 5·50; 50 mM-phosphate buffer, pH 5·95 and 8·00; 50 mM-2-amino-2-hydroxymethylpropane-1:3-diol–HCl buffer, pH 7·0 and 9·0. Total volume in dialysis bag: 3 ml. The outer solution (50 ml.) contained 4 mM-H_2O_2 and the same concentrations of aminotriazole and buffer as the solution in the dialysis bag. Incubated for 1·5 hr. at 37°. The catalatic activity was estimated according to Feinstein (1949) after suitable dilution of samples.

from pH 5·5 to 9·0. Lower pH values could not be tested because of precipitation of the catalase under the required experimental conditions.

Kinetics of the irreversible inhibition reaction. Since a continuous supply of H_2O_2 was required for the irreversible inhibition reaction and this reaction was not with catalase–H_2O_2 II, as shown above, it was probable that catalase–H_2O_2 I was the catalase complex involved. The irreversible inhibition of catalase by 3-amino-1:2:4-triazole in the presence of H_2O_2 was followed by determining the catalatic activity, at suitable intervals, in the system in which H_2O_2 was dialysed into the incubation mixture. Plotting the logarithm of the percentage of the original catalase activity against time gave straight lines only during the early stages of the reaction, and the period of time during which the line obtained remained straight increased with a decrease in temperature and a decrease in the inhibitor concentration. The overall plot was a sigmoid curve (Fig. 7).

For all the kinetic calculations that follow, only the initial slope of such curves was used. The possible reasons for the sigmoid shape of these curves are discussed below. It should also be noted that the data for these kinetic calculations were obtained at 22°, since kinetic data given by Chance, Greenstein & Roughton (1952) and Beers & Sizer (1953) required for these calculations were also obtained at 22°. The derivation of two kinetic equations suitable for the estimation of the second-order reaction constant for the irreversible inhibition reaction (k_7), in this system, assuming the reaction to occur between the inhibitor and catalase–H_2O_2 I (see Appendix, equations 9 and 12), made it possible to show that the experimental data obtained fitted well with these two assumptions.

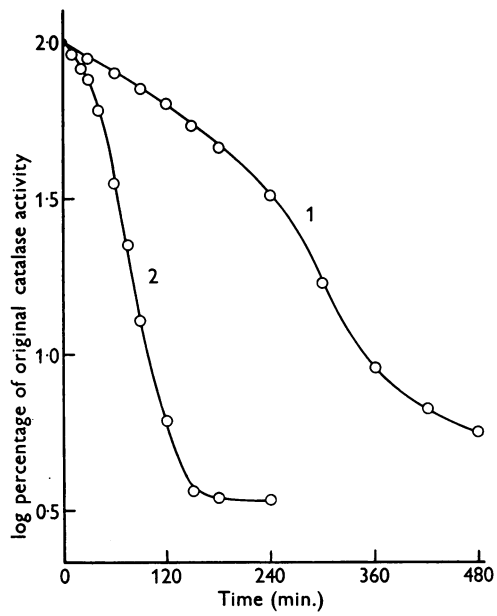


Fig. 7. Kinetics of irreversible inhibition of catalase by 3-amino-1:2:4-triazole. Hydrogen peroxide was dialysed into the catalase solutions. Final concentrations in dialysis bag: 54 μM ox-liver catalase haematin; 0·1 M-phosphate buffer, pH 7·0; 20 mM-aminotriazole (curve 1) and 0·1 M-aminotriazole (curve 2). Volume: 5 ml. The outer solution (100 ml.) contained the same concentrations of buffer and aminotriazole, no catalase and 4 mM-H_2O_2. Temp., 37°. The catalatic activity was estimated according to Feinstein (1949) after suitable dilution of samples.

Thus plotting the inverse of the first-order reaction constant for the irreversible inhibition of catalase by 3-amino-1:2:4-triazole in the presence of H_2O_2 ($1/\lambda$) against the inverse of the inhibitor concentration ($1/i$) gave a straight line (Fig. 8), as expected from equation (9) (see Appendix). From the slope of this line, equal to $1/(k_7 n)$, in which k_7 was the second-order reaction constant for the irreversible reaction of 3-amino-1:2:4-triazole with catalase–H_2O_2 I, and n the ratio of the concentration of H_2O_2–bound haem in the primary complex to that of total active haem (n 0·33 for ox-liver catalase; Beers & Sizer, 1952, 1953), k_7 was estimated to have a value of 25 mM^{-1} sec.$^{-1}$. The intercept of the line in Fig. 8 with the $1/\lambda$ axis was given by equation (9) as equal to $(1-n)/(k_7 K_5 n)$ in the case in which the reversible reaction of 3-amino-1:2:4-triazole occurred with free catalase, and by equation (13) as equal to $1/(k_7 K_6)$ in the case in which the reversible reaction of 3-amino-1:2:4-triazole occurred with catalase–H_2O_2 I. K_5 and K_6 were respectively the equilibrium constants for these two possible alternative reversible reactions. The values of K_5 and K_6 estimated from the intercept in Fig. 8 were respectively 44 mM and 22 mM. These values checked well with the values of 40 mM and 20 mM for K_5 and K_6 respectively, obtained from the direct estimation of the reversible inhibition of catalatic activity by 3-amino-1:2:4-triazole described above (see Fig. 1).

On the other hand, plotting the first-order reaction constant for the irreversible inhibition of catalase by 3-amino-1:2:4-triazole in the presence of H_2O_2 (λ) against the product of the inhibitor concentration and the first-order reaction constant for the decomposition of H_2O_2 at each inhibitor concentration (ik_0^*) gave a straight line passing through the origin, as expected from equation (12) (see Appendix) (Fig. 9). The slope of this line was given in equation (12) as equal to $(k_7 n)/k_0^c$, in which k_0^c was the first-order reaction constant for the decomposition of H_2O_2 in the absence of any inhibitor, and both k_7 and n were the constants defined above. The values for k_0^c and the various values for

k_0^* were estimated directly as described above in the section on the reversible inhibition of catalatic activity by 3-amino-1:2:4-triazole (see Fig. 1). With these values and the slope of the line in Fig. 9, k_7 was estimated from equation (12) at 26 mM^{-1} sec.$^{-1}$, a value which checks well with that estimated from Fig. 8 (25 mM^{-1} sec.$^{-1}$).

Thus two procedures, one in which only the rate of the inhibition reaction was estimated, as in Fig. 8, and the other in which both the rate of the inhibition reaction and the separately determined rate of decomposition of H_2O_2 with and without inhibitor were used, as in Fig. 9, gave nearly the same value for k_7. It should, however, be noted that this confirmation of the validity of the kinetic equations derived in the Appendix is not independent of the confirmation obtained above from the equilibrium constants for the possible reversible reactions of 3-amino-1:2:4-triazole with either free catalase or catalase–H_2O_2 I (K_5 and K_6), which gave comparable values whether estimated from the intercept in Fig. 8 or determined directly from the data given in Fig. 1. Indeed it can be readily shown algebraically that since the values for K_5 and K_6 obtained by these two independent experimental procedures were similar, the values for k_7 obtained by one of them or a combination of both must also give similar values.

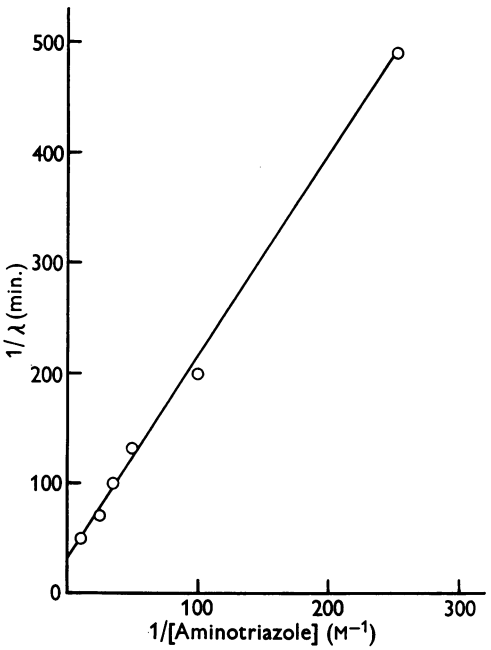


Fig. 8. Variation of the inverse of the first-order reaction constant ($1/\lambda$) for the irreversible inhibition of catalase by 3-amino-1:2:4-triazole with the inverse of the inhibitor concentration ($1/i$). Hydrogen peroxide was dialysed into the catalase solutions. Final concentrations in dialysis bag: 2·4 μM ox-liver catalase haematin; 0·1 M-phosphate buffer, pH 7·0; varying concentrations of aminotriazole. Total volume in dialysis bag: 5 ml. The outer solution (50 ml.) contained the same concentrations of buffer and aminotriazole, no catalase and 4 mM-H_2O_2. Temp., 22°. Samples were removed at short intervals and the catalatic activity was estimated according to Feinstein (1949) after suitable dilution. The first-order reaction constant (λ) for the irreversible inhibition of catalase by aminotriazole was estimated graphically from the initial slope of the plot of log $[(E-q'')/E]$ against time, in which $E-q''$ was the catalatic activity at time t, and E the total initial catalatic activity. The initial slopes only were used since the overall shape of the curves obtained was sigmoid (see Fig. 7). The second-order reaction constant for the reaction of aminotriazole with catalase–H_2O_2 I (k_7), estimated from the slope of the line in this Figure according to equation (9) (see Appendix), was 25 mM^{-1} sec.$^{-1}$ The equilibrium constants for the reversible reaction of aminotriazole with free catalase (K_5) or the reversible reaction of aminotriazole with catalase–H_2O_2 I (K_6) estimated from the intercept of the line in this Figure with the ordinate, according to equations (9) and (13) (see Appendix), were respectively: 44 and 22 mM.

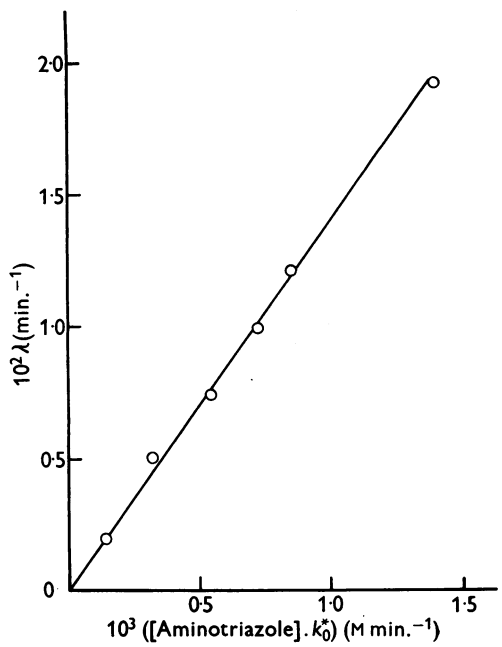


Fig. 9. Variation of the first-order reaction constant for the irreversible inhibition of catalase by 3-amino-1:2:4-triazole with the product of the inhibitor concentration and the first-order reaction constant for the decomposition of H_2O_2 at each inhibitor concentration (ik_0^*). The concentrations of the reactants and the conditions were the same as in Fig. 8; k_0^* was estimated from Fig. 1. The second-order reaction constant for the reaction of aminotriazole with catalase–H_2O_2 I (k_7), estimated from the slope of the line in this Figure, according to equation (12) (see Appendix), was 26 mM^{-1} sec.$^{-1}$.

Table 3. *Incorporation of* 3-*amino*-[^{14}C]1:2:4-*triazole into ox-liver catalase in the presence of hydrogen peroxide*

Concentrations: 86·5 μM recrystallized ox-liver catalase haematin; 40 mM-phosphate buffer, pH 7·0; 10 mM-ascorbate; 16 mM-3-amino-[^{14}C]1:2:4-triazole (18 990 counts/min./μmole). Total volume, 5 ml. Incubated at 37° for 4·5 hr until catalatic activity was 3% of the original activity. Atmosphere, air. The control was identical, except that ascorbate was absent. After incubation, mixtures were dialysed for 5 days against 2 l. of 10 mM-phosphate buffer, pH 7·0, changed daily. A portion (75 ml.) of final outer solution dried on a planchet showed no detectable radioactivity. Moles of 3-amino-[^{14}C]1:2:4-triazole incorporated per mole of reacted haematin were calculated from the total of the compound incorporated and the proportion of free haematin, estimated by the effect of cyanide (mM) on the extinction at 422 mμ. Catalase protein was separated from its haematin by the method of Sumner & Dounce (1939). Control protein separation was carried out with unlabelled catalase to which 3-amino-[^{14}C]1:2:4-triazole was added before the protein precipitation. See Methods section for technique of measuring radioactivity.

Preparation	Incorporated amino-[^{14}C]triazole (moles/mole of total catalase haematin)	Incorporated amino-[^{14}C]triazole (moles/mole of reacted catalase haematin)
1. Irreversibly inhibited catalase	0·83	0·88
2. Control uninhibited catalase	0·06	—
3. Catalase protein from irreversibly inhibited catalase	0·77	0·82
4. Supernatant from protein precipitation of inhibited catalase, including haematin	0·04	—
5. Protein from uninhibited catalase with added amino-[^{14}C]triazole	0·00	—
6. Supernatant from protein precipitation of 5, including haematin	0·97	—

Stoicheiometry of the irreversible reaction of 3-*amino*-1:2:4-*triazole with catalase in the presence of hydrogen peroxide.* A preparation of irreversibly inhibited ox-liver catalase was made with 3-amino-[^{14}C]1:2:4-triazole and the system in which the auto-oxidation of ascorbate provided the H_2O_2. The excess of 3-amino-1:2:4-triazole was removed by prolonged dialysis. A control sample was similarly treated except that no ascorbate was present. The radioactivities of the inhibited enzyme and the control enzyme were estimated and used as a measure of the 3-amino-1:2:4-triazole incorporated. Suitable corrections were made for protein absorption of radioactivity and for the amount of free haematin remaining in the inhibited enzyme, as estimated from the percentage of the haematin able to bind cyanide to give a changed Soret-band spectrum. The results given in Table 3 show that in the inhibited enzyme 0·8–0·9 mole of 3-amino-1:2:4-triazole was incorporated for each mole of haematin which had reacted, whereas the control sample showed a small degree of incorporation only. Moreover, on splitting the haematin from the enzyme with acid–acetone the incorporated 3-amino-1:2:4-triazole remained entirely in the protein precipitate. That this was not an artifact due to absorption of 3-amino-1:2:4-triazole on the protein precipitate was shown by adding 3-amino-[^{14}C]1:2:4-triazole to unlabelled free catalase and removing the haematin by the same procedure. In this case all of the 3-amino-1:2:4-triazole was recovered in the supernatant.

DISCUSSION

The experiments presented above showed that 3-amino-1:2:4-triazole was not unique in its ability to cause the irreversible inhibition of catalase in the presence of a continuous supply of hydrogen peroxide. A series of related organic compounds were similarly active. The experiments with amino-[^{14}C]triazole indicated that the essential binding of the inhibitor was to the protein of the enzyme and not to the haematin. Moreover, had the inhibitors of the aminotriazole series, which all contained a free primary amino group, co-ordinated with the haem iron in the irreversibly inhibited catalases one would expect a striking change in spectrum, whereas the spectrum of the 3-amino-1:2:4-triazole-inhibited catalase differed only slightly from that of the original catalase (Margoliash & Novogrodsky, 1958*a*).

The haem iron was nevertheless affected since after irreversible inhibition it could not bind cyanide. Whether this was due to the fixation of the aminotriazole near the iron so as to prevent sterically its reaction with cyanide, or whether the inhibitor although fixed at a distant position changed the whole configuration of the protein so as to make the iron unavailable to cyanide, cannot be decided on the basis of the present data. However, both the ease of crystallization of the irreversibly inhibited catalases and the hydrogen peroxide requirement for the inhibition reaction tended to make the first possibility the probable one, although they did not exclude the second. Similarly, excluding the possibility that the effective inhibitory molecule is an oxidation product of the inhibitor formed and set free by the enzyme–hydrogen peroxide system, a possibility for which no evidence could be found (Margoliash & Novogrodsky, 1958*a*), two possible mechanisms could

have accounted for the irreversible inhibition reaction. In one, the hydrogen peroxide of complex I would 'activate' in a chemical sense an adjacent site on the protein, enabling it to react with the inhibitor, and in the other the hydrogen peroxide would change the spatial arrangement of the protein molecule, making a distant group available for spontaneous reaction with the inhibitor. Experiments designed to distinguish between these two types of inhibition mechanism and their corresponding products are being carried out.

The hydrogen peroxide requirement for the irreversible inhibition reaction indicated that either a catalase–hydrogen peroxide complex was the reactive species involved or that hydrogen peroxide reacted with a catalase–inhibitor complex. However, the irreversible inhibition reaction appeared to be a second-order reaction between the inhibitor and catalase–hydrogen peroxide complex I. This conclusion was supported by the applicability of kinetic equations derived on such an assumption to the experimental data obtained from both measurements of the rate of irreversible inhibition and measurements of the reversible reaction of 3-amino-1:2:4-triazole with the enzyme. The protective effect of catalase–hydrogen peroxide complex I donors against the irreversible inhibition and the lack of effect of pH on the rate of the irreversible inhibition reaction were in agreement with this conclusion. Indeed, catalase–hydrogen peroxide complex I donors decrease the steady-state concentration of the primary complex (Chance, 1953), and complex I is known to be unaffected by pH with respect to both its rate of formation and its rate of reaction with donors (Chance, 1952). It was shown, moreover, that although the inhibitors of the aminotriazole series reacted with catalase–hydrogen peroxide complex II, the product of this reaction was free uninhibited catalase.

A remarkable feature of the irreversible inhibition reaction was that when the logarithm of the catalatic activity was plotted against time a sigmoid curve was obtained, so that for all the kinetic calculations only the initial slopes of such curves could be used. In the kinetic equations developed for this system (see Appendix) two parameters were assumed to remain constant throughout the reaction: the second-order reaction constant for the reaction of catalase–hydrogen peroxide complex I (k_7) and the ratio of haem bound to peroxide in complex I to total active haem (n). If these values actually remained constant then a straight line should have been obtained. A change in k_7 would mean that the incorporation of one molecule of inhibitor near one haem increased the reactivity of a second haem in the same catalase molecule in this reaction. A change of n would mean that the percentage of haem bound by peroxide in complex I to total active haem increased with the number of aminotriazole molecules incorporated. Both phenomena could be interpreted as haem–haem interaction in the irreversible inhibition reaction, and may well indicate that the haems in each catalase molecule are spatially near each other.

A result of the demonstration that the inhibition of catalase by 3-amino-1:2:4-triazole occurred through a reaction with the primary hydrogen peroxide complex was that, since aminotriazole rapidly decreased the catalatic activity of the liver and kidney after its injection into laboratory animals (Heim *et al.* 1956), complex I must be formed *in vivo* in the liver and kidney. Moreover, the experiments of Nelson (1958) showing that the simultaneous injection of ethanol with aminotriazole into rats prevented the decrease in liver-catalase activity can be interpreted to mean that the catalase–hydrogen peroxide complex I formed *in vivo* was fully active towards catalase–hydrogen peroxide complex I donors. On the other hand the inhibition of catalase *in vivo* by aminotriazole indicated that there did not appear to be a sufficient concentration of naturally occurring catalase–hydrogen peroxide complex I donors available to the enzyme in the liver and kidney to prevent this inhibition. Aminotriazole did not affect the catalatic activity of blood *in vivo* (Heim *et al.* 1956) and the catalatic activity of blood haemolysates *in vitro* was decreased slowly and only after the addition of a source of hydrogen peroxide (Margoliash & Novogrodsky, 1958*a*). This probably indicated that blood contained substances that could act as catalase–hydrogen peroxide complex I donors in concentrations high enough to prevent or retard the aminotriazole inhibition of the enzyme.

The 3-amino-1:2:4-triazole–catalase system was sensitive to hydrogen peroxide, and since the inhibitory effect was cumulative and irreversible this system could readily be used to detect the production of hydrogen peroxide at low concentrations over a relatively long period of time. Thus Margoliash & Novogrodsky (1958*b*) have used this system for demonstrating the production of hydrogen peroxide from boiled aqueous tumour extracts and its relation to the catalase inhibitory activity of such extracts (Hargreaves & Deutsch, 1952).

SUMMARY

1. In addition to 3-amino-1:2:4-triazole a series of related compounds could cause the irreversible inhibition of purified recrystallized catalase preparations in the presence of hydrogen peroxide. Of all the compounds tested *S*-methyl*iso*thiosemicarbazide caused the most rapid inhibition. The minimal structure required for inhibitory activity in

this series was $>N\cdot NH\cdot C(NH_2):R$, or an isomer of this structure, in which the primary amino group attached to the C atom had to be unsubstituted and R could be S, O or NH.

2. Substances which can be oxidized by catalase–hydrogen peroxide complex I, such as ethanol, formate, nitrite, pyrogallol, etc., prevented the occurrence of these inhibitions but could not reverse them once they had taken place.

3. The rate of inhibition at any particular enzyme, inhibitor concentration and temperature did not vary from pH 5·5 to 9·0.

4. The effective inhibitors of this series reacted with catalase–hydrogen peroxide complex II or catalase–methyl hydroperoxide complex II to liberate free catalase. The second-order reaction constant of this reaction at 37° was $0{\cdot}26\,M^{-1}$ sec.$^{-1}$ for 3-amino-1:2:4-triazole and $2{\cdot}4\,M^{-1}$ sec.$^{-1}$ for *S*-methyl*iso*thiosemicarbazide.

5. In addition to the irreversible inhibition, 3-amino-1:2:4-triazole reversibly inhibited catalatic activity. The directly determined equilibrium constants for the possible reversible reactions of this inhibitor with either free catalase or the primary complex were respectively 40 mM and 20 mM at 22°.

6. A plot of the logarithm of the catalatic activity against time during the irreversible inhibition of catalase by 3-amino-1:2:4-triazole in the presence of an excess of hydrogen peroxide gave a sigmoid curve. The possible reasons for such a curve are discussed.

7. Two independent kinetic equations for the irreversible reaction of the inhibitors with catalase–hydrogen peroxide complex I were derived. These equations fitted the kinetic data obtained on the initial rates of the irreversible inhibition reaction and on the reversible inhibition of catalatic activity. For 3-amino-1:2:4-triazole the second-order reaction constant for the irreversible reaction with complex I was about 25 mM^{-1} sec.$^{-1}$, at 22°, calculated from either equation.

8. Catalase which had been irreversibly inhibited by 3-amino-1:2:4-triazole had incorporated approximately 1 mole of inhibitor for each mole of catalase haematin which had reacted. On liberation of the haematin by acid–acetone the incorporated inhibitor remained attached to the protein.

The authors are grateful to Professor F. Bergman and Mr G. Levin for some of the compounds used in the present study.

REFERENCES

Beers, R. F., jun. (1955). *J. phys. Chem.* **59**, 25.
Beers, R. F., jun. & Sizer, I. W. (1952). *J. biol. Chem.* **195**, 133.
Beers, R. F., jun. & Sizer, I. W. (1953). *J. phys. Chem.* **57**, 290.
Chance, B. (1947). *Acta chem. scand.* **1**, 236.
Chance, B. (1949). *J. biol. Chem.* **179**, 1341.
Chance, B. (1950). *Biochem. J.* **46**, 387.
Chance, B. (1951). *Advanc. Enzymol.* **12**, 153.
Chance, B. (1952). *J. biol. Chem.* **194**, 471.
Chance, B. (1953). In *Technique of Organic Chemistry*, vol. 8, p. 646. Ed. by Freiss, S. L. & Weissberger, A. New York: Interscience Publishers Inc.
Chance, B., Greenstein, D. S. & Roughton, F. J. W. (1952). *Arch. Biochem. Biophys.* **37**, 301.
Euler, H. von & Josephson, K. (1927). *Liebigs Ann.* **452**, 158.
Feinstein, R. N. (1949). *J. biol. Chem.* **180**, 1197.
Freund, M. & Paradies, Th. (1901). *Ber. dtsch. chem. Ges.* **34**, 3110.
Freund, M. & Schander, A. (1902). *Ber. dtsch. chem. Ges.* **35**, 2602.
Hargreaves, A. B. & Deutsch, H. F. (1952). *Cancer Res.* **12**, 720.
Heim, W. G., Appleman, D. & Pyfrom, H. T. (1955). *Science*, **122**, 693.
Heim, W. G., Appleman, D. & Pyfrom, H. T. (1956). *Amer. J. Physiol.* **186**, 19.
Margoliash, E. & Novogrodsky, A. (1958*a*). *Biochem. J.* **68**, 468.
Margoliash, E. & Novogrodsky, A. (1958*b*). *Biochim. biophys. Acta*, **30**, 182.
Näf, E. (1891). *Leibigs Ann.* **265**, 113.
Nelson, G. H. (1958). *Science*, **127**, 520.
Rieche, A. & Hitz, F. (1929). *Ber. dtsch. chem. Ges.* **62**, 2458.
Schenck, M. (1912). *Hoppe-Seyl. Z.* **77**, 349.
Sjostedt, G. & Gringas, L. (1955). *Org. Synth.* (*Coll.*), **3**, 95.
Sumner, J. B. & Dounce, A. L. (1939). *J. biol. Chem.* **127**, 439.
Thiele, J. & Manchot, M. (1898). *Liebigs Ann.* **303**, 48.
Thiele, J. & Stange, O. (1894). *Ber. dtsch. chem. Ges.* **27**, 32.