were appreciably lower in the presence of catalase than they were when peroxidase was used. At very low peroxide concentrations and fairly high chlorpromazine concentration, the bleaching was retarded, and the absorption spectrum of the red material could be examined (Fig. 1*A*). When bleaching was complete, the ultraviolet absorption spectra of the enzymic reaction mixture, chlorpromazine, and chlorpromazine sulfoxide were compared. The spectral comparison (Fig. 1*B*) indicates that the sulfoxide was probably present in the product mixture.

Descending paper chromatograms were developed with the solvent mixture recommended by Salzman and Brodie (*2*). Aliquots of the enzymic reaction mixture were taken at several intervals after the reaction was started. Within 10 minutes, a spot identified as that of the sulfoxide was detectable, and within 30 minutes the sulfoxide spot was strongly colored, while the chlorpromazine spot showed a greatly reduced color intensity. Sampling of the completely bleached reaction mixture several hours later resulted in the appearance of a very strong sulfoxide spot. In no case did more than two spots appear. Either the red product was not detectable with the sulfuric acid spray, or its migration was identical with that of chlorpromazine. Catalase oxidation produced the same result, giving spots having R_f values of 0.82 to 0.84 and 0.93 to 0.96. Control R_f values for the sulfoxide and chlorpromazine run under the same conditions were, respectively, 0.77 to 0.86 (0.82 average) and 0.89 to 0.97 (0.93 average).

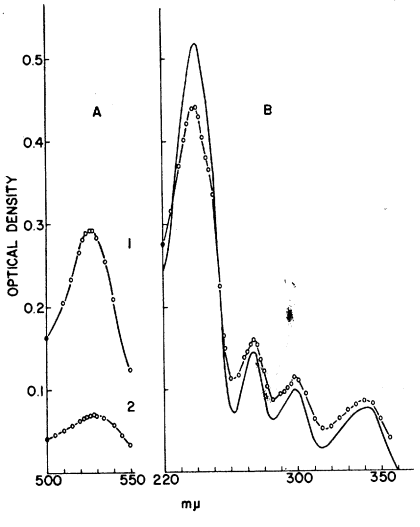

Fig. 1. Absorption spectra of enzymic oxidation products. (*A*) Curve 1: H_2O_2 ($1.7 \times 10^{-5}M$), chlorpromazine hydrochloride ($9.2 \times 10^{-4}M$), and horse radish peroxidase (Worthington; 1.3 μg/ml) in 0.1*M* acetate buffer at *p*H 4.5. Curve 2: H_2O_2 and chlorpromazine as for curve 1 and crystalline beef liver catalase (Worthington; 13 μg/ml) in 0.06*M* acetate buffer at *p*H 4.5. The blanks contained all components except H_2O_2. (*B*) Solid line: chlorpromazine sulfoxide ($10^{-5}M$). Curve showing experimental points: the initial reaction mixture contained chlorpromazine · HCl ($1.6 \times 10^{-4}M$), H_2O_2 ($3.8 \times 10^{-3}M$), and peroxidase (3 μg/ml) in 0.06*M* acetate buffer at *p*H 5.5. After 30 minutes, a sample was diluted 1/15 with buffer for spectrophotometry. Both curves represent measurements made in the same buffer. The blanks contained all components except chlorpromazine.

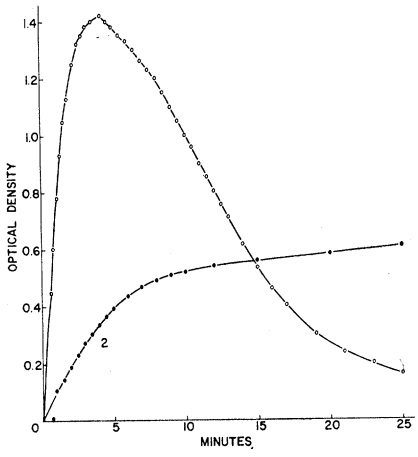

Fig. 2. Time courses of formation and decay of enzymic oxidation products. Curve 1: red intermediate; chlorpromazine · HCl ($9.7 \times 10^{-4}M$), H_2O_2 ($1.76 \times 10^{-4}M$), and peroxidase (2.3 μg/ml) in 0.1*M* acetate at *p*H 4.5. Measurement was made with the photomultiplier attachment at 527 mμ, slit 0.09 mm, and approximately 25°C. The blank contained all components except the enzyme. Curve 2: same conditions as in curve 1 except that chlorpromazine and enzyme concentrations were reduced to $9.7 \times 10^{-5}M$ and 0.23 μg/ml, respectively. Measurements were made at 274 mμ, slit 2.1 mm, no photomultiplication. The blank contained all components except the enzyme.

A qualitative indication of the time-courses of formation and decay of the red product and the formation of the sulfoxide was obtained by following absorbancy changes at 527 and 274 mμ (Fig. 2). The rapidity of the former reaction and the relative slowness of the latter suggested that the red product was a precursor of the sulfoxide. Since the blank cuvettes in these experiments contained all components except the enzyme, compensation for any nonenzymic peroxidation was made automatically. Indeed, no slit adjustment was necessary during either experiment, and it was concluded that no measurable uncatalyzed oxidation had occurred. It has been shown *in vivo* (*2*) that the sulfoxide itself undergoes further metabolism. Since the systems described here yielded the sulfoxide as the terminal product, it was clear that, at best, these oxidations could not account for any steps beyond sulfoxide formation in the over-all metabolism.

The metabolic significance of catalase and peroxidase is not entirely clear, nor is it certain that mammalian peroxidases would carry out the reactions described here for the horse radish enzyme. It is doubtful that these reactions represent more than interesting models of the actual mammalian oxidations. It is of interest that Tauber (*4*) has also shown that crystalline catalase is capable of oxidizing various large molecules.

It may be noted that, in tissues that are capable of generating respective amounts of hydrogen peroxide metabolically, the possible effects of catalase or peroxidases (even free hemoglobin) would be difficult to eliminate in studies on phenothiazine oxidation (*5*).

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References and Notes

1. N. P. Salzman, N. C. Moran, B. B. Brodie, *Nature* 176, 1122 (1955).
2. N. P. Salzman and B. B. Brodie, *J. Pharmacol. Exptl. Therap.* 118, 46 (1956).
3. S. P. Massie, *Chem. Revs.* 54, 797 (1954).
4. H. Tauber, *Enzymologia* 16, 311 (1954).
5. Chlorpromazine hydrochloride (Thorazine) and chlorpromazine sulfoxide were generously supplied by the Smith, Kline and French Laboratories, Philadelphia, Pa.

25 February 1957

Sphingosine as an Inhibitor of Blood Clotting

One of us has isolated from brain tissue a powerful clot-delaying substance which has been identified as sphingosine (*1, 2*). This substance is a component of cerebrosides and sphingomyelins, but it is also found free as a contaminant in preparations of the latter, thus acounting for their inhibitory effect in blood clotting (*2*). Pure sphingomyelin is indifferent in blood-clotting reactions.

Sphingosine has also been isolated from the lipid activator of pig brain (*3*), and it seems to be the active principle of antithromboplastin (also called anticephalin) (*4*). According to Tocantins and Carroll (*5*), antithromboplastin appears in the blood of hemophiliacs in abnormally high quantities. With sphingosine and chicken plasma, interesting reactions are observed. With small quantities, clotting times are prolonged to 40 hours or more. However, in the presence of the lipid activator from pig brain, the inhibitory effect of sphingosine is abolished, and the clotting times may even be shorter than those obtained with optimal concentrations of the lipid activator alone. This may be called "sphin-

Table 1. Compensation and incubation effect with physiological and synthetic sphingosine. The composition of the reaction mixtures was as follows: lipid activator, 30 mm^3 (6 mg/1 ml of 0.85-percent NaCl); plasma, 150 mm^3; natural sphingosine, 30 or 60 mm^3 (5 mg/1 ml of H_2O); synthetic sphingosine, 30 or 60 mm^3 (1, 25 mg/1 ml of H_2O); all volumes were made up to 270 mm^3 with either H_2O or NaCl (0.85 percent) as needed to obtain equivalent amounts of each in the final reaction mixture. Chicken plasma obtained from the carotid (method of Carrel) was used throughout. Clotting time was assayed by the method of Hecht (*7*).

Expt. No.	Composition of reaction mixture	Clotting time
1	Physiological NaCl solution + plasma	30 min, 0 sec
2	Lipid activator (30 mm^3) + plasma	4 min, 50 sec
3a	Physiological sphingosine (30 mm^3) + plasma	> 40 hr
	Physiological sphingosine (60 mm^3) + plasma	> 40 hr
3b	Synthetic sphingosine (30 mm^3) + plasma	> 40 hr
	Synthetic sphingosine (60 mm^3) + plasma	> 40 hr
4a	Physiological sphingosine (30 mm^3) + plasma + lipid activator directly	5 min, 0 sec
	Physiological sphingosine (60 mm^3) + plasma + lipid activator directly	15 min, 15 sec
4b	Synthetic sphingosine (30 mm^3) + plasma + lipid activator directly	4 min, 20 sec
	Synthetic sphingosine (60 mm^3) + plasma + lipid activator directly	6 min, 10 sec
5a	Physiological sphingosine (30 mm^3) + plasma + lipid activator added after incubation for 1 hr at 39°C	1 min, 8 sec
	Physiological sphingosine (60 mm^3) + plasma + lipid activator added after incubation for 1 hr at 39°C	1 min, 25 sec
5b	Synthetic sphingosine (30 mm^3) + plasma + lipid activator added after incubation for 1 hr at 39°C	43 sec
	Synthetic sphingosine (60 mm^3) + plasma + lipid activator added after incubation for 1 hr at 39°C	58 sec

gosine compensation." If the chicken plasma is first incubated for 1 hour at 39°C with sphingosine, the clotting times with the lipid activator are one-third to one-sixth of those obtained with the optimal concentration of the lipid activator alone. This is called "incubation effect." This effect is not seen if the plasma is added to the incubated mixture of sphingosine and the lipid activator (*2*). Therefore we assume the presence of an unidentified component in the plasma which reacts with the inhibitor to produce the incubation effect.

Fortunately, sphingosine and certain derivatives have been synthesized (*6*). The structure of sphingosine is as follows: $CH_3—(CH_2)_{12}—CH=CH—CH(OH)—CH(NH_2)—CH_2OH$. DL-sphingosine has been studied in connection with chicken plasma, and all the reactions that occur with the use of concentrates from biological sources are seen with the synthetic preparation. However, smaller quantities suffice.

The intensity of the reactions with sphingosine depends largely on its concentration, on the concentration of the lipid activator, and on individual properties of the plasma. Synthetic *erythro-* and *threo*-dihydrosphingosine are weak inhibitors and give only a slight sphingosine compensation. Both substances may prolong slightly the clotting time of chicken plasma. They raise the activity of the lipid activator to a slight degree and, when incubated with plasma, produce very short clotting times. N-benzoyldihydrosphingosine is completely inactive, as is also the tri-acetyl derivative of sphingosine.

Our experiments give the information that the presence of the double bond, as well as the presence of the free functional hydroxyl and the amino groups, is essential for the clot-delaying activity.

We were also able to synthesize the two isomers of threoninol, which are the lowest homologs of dihydrosphingosine and which have the following structure; $CH_3—CHOH—CHNH_2—CH_2OH$. The free threoninols are indifferent in every respect. Their oxalates have a powerful inhibitory influence on the clotting of chicken plasma. This can be abolished by the lipid activator, but not after preliminary incubation of the oxalates with the plasma. Table 1 summarizes the clotting times of chicken plasma that has been treated with natural and synthetic sphingosine only.

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References

1. E. Hecht, *Nature* **167**, 279, 633 (1951).
2. ——, *Acta Haematol.* **9**, 237 (1953).
3. A. Fischer and E. Hecht, *Biochem. Z.* **269**, 115 (1934).
4. E. Hecht, *Acta Physiol. et Pharmacol. Neerl.* **2**, 134 (1951).
5. L. M. Tocantins and R. T. Carroll, *Blood Clotting and Allied Problems* (Josiah Macy, Jr., Foundation, New York, 1949), pp. 11–28.
6. D. Shapiro and K. Segal, *J. Am. Chem. Soc.* **76**, 5894 (1954); D. Shapiro, H. M. Flowers, E. Hecht, *J. Org. Chem.*, in press.
7. E. Hecht, *Acta Med. Scand.* **102**, 79 (1939).

14 January 1957

Reaction of 8-Quinolinol and 2-Methyl-8-Quinolinol with Rare-Earth Elements

In an attempt to improve the selectivity of 8-quinolinol as an analytic precipitant, various substituted compounds have been proposed. Merritt and Walker (*1*) found that 2-methyl-8-quinolinol did not precipitate aluminum ion; thus it offered some selectivity over 8-quinolinol. Irving, Butler, and Ring (*2*) showed that this increased selectivity was the result of the steric hindrance effect of the methyl group at the 2-position. This effect would also slightly alter the sensitivity of the reaction of this reagent with other metal ions.

From the results of this previous work, one would predict that 2-methyl-8-quinolinol would perhaps be less sensitive in the detection of the trivalent rare earth metal ions than 8-quinolinol. Under the same conditions of hydrogen-ion and chelating-agent concentrations, this has been found to be true.

To determine the sensitivity of the chelating agents, a modification of the Irving, Butler, and Ring (*2*) method was used. An NH_4Cl-NH_4OH buffer was used because, with the buffers recommended in the original method, only 8-quinolinol gave precipitates with the metal ions (*p*H 8.4). The new buffer consisted of 100 g of NH_4Cl, 60 ml of concentrated NH_4OH, and 440 ml of water. The *p*H of a solution containing 2 ml of this buffer in a total volume of 6.2 ml was 9.5. Concentrations of the rare-earth chloride solutions were 0.01 and 0.001*M*. The chelating agents were 0.1*M* in 95-percent ethanol. Test solutions were heated 10 minutes at 70°C, then after they had cooled to room temperature, they were observed visually for signs of a precipitate.

The results of the sensitivity tests are given in Table 1. The concentrations of metal ions are expressed in micrograms of M^{+3} per milliliter. It can readily be seen that under the same conditions, the sensitivity of 2-methyl-8-quinolinol in precipitating the rare-earth metal ions is much less than that of 8-quinolinol. Quantitatively, the former chelating agent averaged about one-eighteenth as sensitive as the latter.