enechlorophosphite were added at about −10° after which the mixture was allowed to come to room temperature. On the following day, the solid reaction mixture was diluted with water (600 ml.) and 1 ml. of acetic acid was added to bring the *p*H to about 6. The precipitate was separated, washed well with water and dried in air; wt. 12.2 g. (92%), m.p. 223–227° dec. 6.1 g. was suspended in 150 ml. of methanol and stored at room temperature for 1 hr., when it was filtered, washed with a further 150 ml. of methanol and dried *in vacuo* at room temperature over P_2O_5. The yield was 4.8 g. (72%), m.p. 235–236°. The avian depressor activity of this product, after reduction with sodium in liquid ammonia, was approximately 200 u./mg.

Removal of the Protective Groups from the Nonapeptide Derivative IX and Preparation of Biologically Active Material.—1.34 g. of IX was dissolved in about 450 ml. of liquid ammonia and sodium was added in small portions until a blue color lasting for at least 15 minutes was obtained. 0.35 g. of sodium was required. Addition of 0.82 g. of ammonium chloride caused the white precipitate formed during the reaction to disappear. The ammonia was allowed to evaporate, the last 30–40 ml. being removed by evaporation from the frozen state *in vacuo*. Five hundred ml. of water was added to the residue, the *p*H of the solution was adjusted to 6.5 with acetic acid, and air was bubbled through until the nitroprusside sodium test for sulfhydryl groups disappeared (about 4 hr.). The solution was acidified to about *p*H 4 with acetic acid, filtered, evaporated *in vacuo* below room temperature to a small volume and then dried from the frozen state. The solid residue from two such reductions was extracted with 60 ml. of ethanol in several portions and filtered, and the solution was diluted with 500 ml. ethyl acetate. The precipitate thus obtained was filtered, washed with ethyl acetate and dried *in vacuo* over $CaCl_2$. It was extracted with pyridine in several portions, a total of 50 ml. being used. The filtered solution was diluted with 500 ml. of ethyl acetate, and the precipitated product was filtered, washed with ethyl acetate and dried over $CaCl_2$ *in vacuo*. One g. of amorphous powder was obtained with a total activity of approximately 300,000 units.

0.40 g. of this material was dissolved in 40 ml. of water saturated with butanol and placed in the first four tubes of a countercurrent distribution apparatus.[33] After 340 transfers in the solvent system 1:1 butanol–water,[34] the contents of Tubes 101–125 were pooled, evaporated *in vacuo* below room temperature to a small volume and lyophilized. The solid was dissolved in 12 ml. of ethanol and precipitated with 150 ml. of ethyl acetate, filtered, washed with ethyl acetate and dried *in vacuo* over P_2O_5 at room temperature. 0.10 g. of white powder was obtained with an avian depressor activity of approximately 500 u./mg.[35]

Anal. Calcd. for $C_{43}H_{66}N_{12}O_{12}S_2$: C, 51.27; H, 6.60; N, 16.69. Found: C, 51.51; H, 6.81; N, 16.36.

Acknowledgments.—The authors wish to thank Mr. Robert L. Tostevin and Miss Dade W. Tull for carrying out the bioassays and Mrs. Lorraine S. Abrash for technical assistance.

(33) L. C. Craig, *Anal. Chem.*, **22**, 1346 (1950).

(34) In subsequent experiments the solvent system butanol–ethanol–0.05% acetic acid (4:1:5) was used with advantage. The distribution coefficient of oxytocin in this system at 24° is about 0.43.

(35) Biological assay of oxytocic activity was made following "The Pharmacopeia of the United States of America," 14th revision, Mack Printing Co., Easton, Pa., 1950, p. 475.

NEW YORK, NEW YORK

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL SCIENCES, SOUTHEAST LOUISIANA HOSPITAL]

Peroxidase Catalyzed Oxidations in Essentially Non-aqueous Media: The Oxidation of Phenothiazine and Other Compounds

BY D. J. CAVANAUGH[1]

RECEIVED AUGUST 18, 1958

The peroxidase catalyzed oxidations of unsubstituted phenothiazine and several common donors for the reaction were carried out in water-poor media in which the primary solvent was propylene glycol. Demonstrable reactions occurred at water concentrations as low as 2 to 3% of the total volume. The reaction rates were dependent to a considerable extent upon the nature of the electron donor and were, therefore, not determined solely by such factors as hydration of the enzyme itself.

It has been shown that the phenothiazine derivative chlorpromazine serves very well as a donor in the horseradish peroxidase system.[2] It was desirable to determine the effect of substitution by comparison of the behavior of the parent compound with its derivatives in the peroxidase reaction. The low water solubility of phenothiazine prompted the examination of various organic solvents as media for the enzymic system. Propylene and ethylene glycol–buffer solutions supported the reactions even when the water volume was restricted to about 2% of the total volume of the reaction mixtures. The glycols had the further advantage that highly insoluble compounds such as phenothiazine could be retained in solution at fairly high concentrations after dilution of the glycol solution with water to give final water concentrations in excess of 20% of the total volume. These properties of the glycol solutions permitted study of the behavior of the enzyme over a wide range of water concentrations and the use of various organic reagents normally precluded by poor water solubility. This report is concerned with the oxidation of phenothiazine and several of the more common donors in buffered propylene glycol solutions.

Since the reactions were most conveniently followed by the polarographic determination of hydrogen peroxide, a brief addendum on peroxide polarography in glycol solutions has been included.

Experimental

Reagents.—All standard reagents were reagent grade or the best obtainable. Phenothiazine was purified by recrystallization from benzene after treatment of the solution with activated charcoal. Purpurogallin was synthesized by the peroxidase system, extracted into ether and recrystallized from alcohol. Stock solutions of autoxidizable compounds were stored in a freezer without deterioration on some occasions. Horseradish peroxidase and crystalline beef liver catalase were obtained commercially.[3] The enzyme stock solutions were stored frozen between experiments.

To determine qualitatively what the nature of the impurities in the enzyme were samples were studied by means

(1) Pharmacology Branch, Directorate of Medical Research, Army Chemical Center, Maryland.

(2) D. J. Cavanaugh, *Science*, **125**, 1040 (1957).

(3) Worthington Biochemicals, Inc., Freehold, New Jersey.

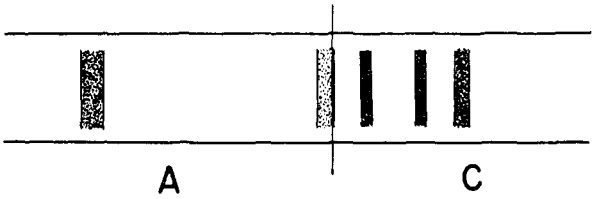

Fig. 1.—Starch gel electrophoresis of peroxidase and hemoglobin. Bands represent benzidine staining reaction. The origin was at the center line, regions A and C were anodic and cathodic, respectively, with respect to the origin. Region A bands were due to hemoglobin; C bands were due to peroxidase. The test solution contained 2 mg./ml. peroxidase and 3 mg./ml. hemoglobin. A total of 20 μl. of solution was used in a gel strip. Runs were made at 20°, 5 ma., *p*H 8.5 (borate buffer), ionic strength 0.1 and were of 26 hr. duration. The drawing is to scale.

of starch gel electrophoresis with the aid of an apparatus built in this Laboratory.[4] The enzyme contained at least two components in addition to the main fraction which were capable of oxidizing benzidine in the presence of H_2O_2 (Fig. 1). In concentrations optimal for electrophoretic resolution of most proteins the secondary components were nearly undetectable, but upon elevating the enzyme concentration to 0.5 mg./ml. and using at least 25 μl. of solution per gel strip the secondary bands were easily seen and the color formed in the benzidine reaction was about as intense in these bands as in the primary band, which appears in the figure at about 73 mm. from the origin. All three components migrated in the direction of the cathode at *p*H 8.4. The runs were of 24 hr. duration at a constant current of 5 ma. and at constant temperature at 20°. In the experiments illustrated in Fig. 1 hemoglobin was added to the peroxidase solution for comparison purposes. The hemoglobin migration was about 93 mm. in the direction of the *anode*, and there was no possible confusion of the peroxidase reaction with peroxidase-like molecules electrophoretically similar to hemoglobin. In view of the electrophoretic results it appeared reasonable to assume that the principal peroxidase component was responsible for virtually all of the enzymic activity observed in these experiments and that peroxidatic heme compounds similar to hemoglobin were probably absent. It also seemed likely that all three detectable peroxidases were actually related fractions, possibly representing small differences between batches of the plant material from which the enzyme was prepared.

For polarographic purposes it was desirable to use a lithium salt as the supporting electrolyte and this was done by preparing lithium acetate–acetic acid buffers in concentrated form and diluting to the desired volume with propylene glycol. The final *p*H in the presence of the glycol was taken to be the true *p*H of the buffer. The buffer stock solutions were made to give a final concentration of lithium in the polarographic cell of 0.078 *M*.

Hydrogen peroxide solutions were initially standardized by permanganate titration and standard curves under the conditions achieved in the actual enzymic reaction mixtures with respect to water concentration were obtained polarographically (see addendum).

Spectrophotometry.—A Perkin-Elmer model 4000 recording spectrophotometer was used throughout. The instrument was equipped with a time drive attachment and was modified by the addition of a manually operated key which was wired into the pen amplifier circuit to permit an overriding signal to be made to appear as a vertical deflection during operation of the instrument. Timing marks which appear in the figures showing direct traces were made by this means. The spectrophotometric method was used in some cases for recording time courses, but at high glycol concentrations the solution viscosity permitted only slow mixing in the cuvettes, and there was sufficient lag between mixing and recording that any attempt to extrapolate to zero time entailed considerable error. This error was less serious when large amounts of water were present and rapid mixing was possible. The polarographic method in which peroxide consumption was measured was much less sensitive but greater reliability was achieved than was possible photometrically. Ideally, substrate (H_2O_2) and donor consumptions would be measured in parallel with product appearance. In practice this was not possible since the photometric technique necessary for donor disappearance and product appearance required conditions which could not be paralleled polarographically in the measurement of substrate consumption, *i.e.*, extreme color intensity of the product formed and overlap of ultraviolet absorptions for product and parent donor limited the usable concentration ranges to levels which were sub-optimal for the polarographic method. An additional severe limitation of the photometric method was the appearance of several products once the *p*H was elevated above about *p*H 4.5. In the polarographic experiments it was possible to work within a wide *p*H range although this was not necessary or desirable in the work reported below.

Polarography (see also addendum).—A model XXI Sargent polarograph was used in conjunction with standard H-cells in which the anode was a saturated calomel electrode. The cell contents were deaerated with tank nitrogen which was passed through vanadous chloride solution and water and nitrogen was passed over the surface of the reaction mixture during runs. After deaeration of all components except peroxide and the enzyme the residual current at −1.4 volt was compensated for and the peroxide was added. The pen was allowed to come to its equilibrium position (mid-point of oscillation) and, with vigorous stirring by nitrogen, the enzyme was introduced by injection from a simple syringe system. It was possible to deflect the gas stream and begin recording of the current within 0.2 minute of enzyme addition and from the time elapsed after adding the enzyme (an electric timer calibrated in hundredths of minutes was used) and the known chart speed satisfactory time-course curves of peroxide consumption could be obtained. Because the polarographic wave in glycol solutions was much reduced as compared to waves obtained in strictly aqueous solutions, the method was relatively insensitive and considerable uncertainty was introduced when the peroxide concentrations fell to very low levels during the reactions. However, the concentration range during the first two or three minutes of the reactions in most cases could be considered reliably high. Following the cessation of peroxide consumption, complete polarograms of the reaction mixture was obtained over the range +0.3 → −1.4 volt. In no case were waves from oxidation products well developed or sufficiently large to have had the effect of increasing the height of the peroxide wave height (that is, products formed did not contribute significantly to the residual current as they were accumulated during the reaction). Accordingly, no correction was applied to the observed peroxide currents. Failure of new waves to appear was probably the result of the wave suppression effect of the glycol rather than absence of substances reducible at the dropping electrode.

Results and Discussion

Part I. Phenothiazine Oxidation.—In the presence of peroxidase the oxidation of phenothiazine could be obtained under several sets of conditions: (a) by shaking a butanol solution of phenothiazine with hydrogen peroxide and peroxidase in an acetate buffer solution, (b) by mixing the reactants in propylene or ethylene glycol solutions in the presence of at least 1.5% by volume of water, or, (c) by adding the reactants to phenothiazine in a dimethyl sulfoxide solution. No reaction occurred when benzene solutions of phenothiazine were shaken with enzyme and peroxide. In dimethyl sulfoxide it was necessary to use relatively large amounts of the enzyme and precipitates occasionally appeared. In the glycols it was possible to use enzyme concentrations comparable to those normally required

(4) The electrophoretic apparatus details were presented at the Xth International Congress of Genetics, Montreal, 1958 by Dr. D. D. Watt to whom the author is indebted for the use of the apparatus and valuable advice concerning the technique (see D. D. Watt and H. W. Kloepfer, Proc. Xth Int. Congress of Genetics, II: 309, 1958) and to Jacques Vandevoorde and R. Galatas for the execution of the electrophoresis experiments.

in purely aqueous media. No side reactions or precipitations were detected, and, since ethylene glycol provided no advantages over propylene glycol, the latter was used throughout.

The oxidation of phenothiazine proceeded in at least two spectrophotometrically distinct stages. The initial phase was characterized by the appearance of a deep green color which, depending upon the *p*H, was somewhat unstable and was ultimately replaced on the appearance of red products. The reaction was evidently rather complex and intermediate stages could be detected (Fig. 2). Above

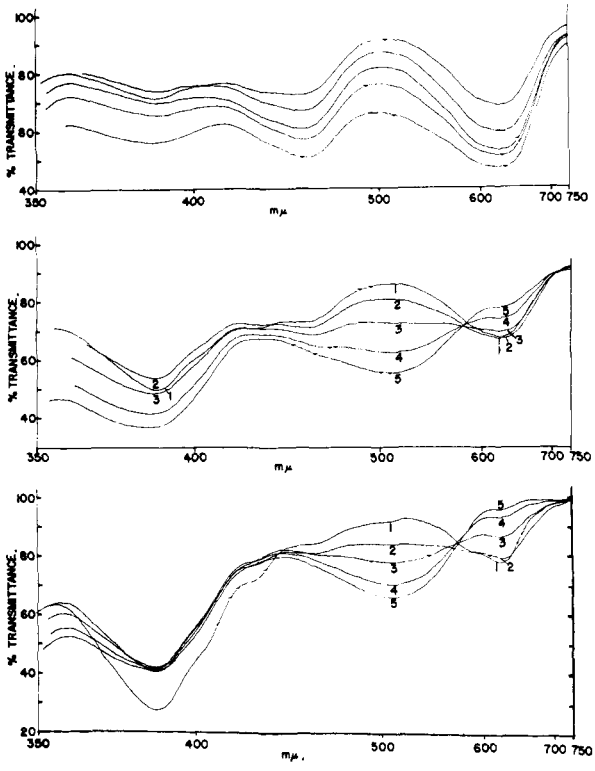

Fig. 2.—Sequential appearance of absorption bands during phenothiazine oxidation showing *p*H dependency. Upper, center and bottom sequences, respectively, *p*H 4.2, *p*H 5.28 and *p*H 9.0. In all cases: $10^{-3}M$ phenothiazine, 3.45×10^{-3} *M* H_2O_2, 0.016 mg./ml. peroxidase (1.47 units). Below *p*H 9.0, 0.2 *M* acetate buffers. For *p*H 9.0, 0.03 *M* borate buffer. Total water content in all cases 35% v./v. Upper sequence: scans from top to bottom at 1, 5, 10, 20, 30 minutes after starting reaction. Two lower sequences: numbers indicate same time relations. Scanning time slightly less than one minute. Enzyme unit = activity required to produce 1 mg. purpurogallin in 20 seconds under standard conditions at *p*H 6.0 and 20°.

*p*H 5.0 or in unbuffered solutions the transience of the green phase was markedly higher than at relatively low *p*H values (in the range *p*H 4.0–*p*H 5.0). The addition of base to bring an unbuffered solution of the initial green product to about *p*H 10.0 instantly converted it to the final red product. In a few trials the development of an absorption maximum at 590 mμ was observed after the red product had been allowed to stand for a long time in the presence of base in excess of 0.01 *N* NaOH. Otherwise this final product was stable for months.

When carried out below *p*H 5.0 the enzymic oxidation produced only the green product initially and this was stable for several hours. At higher *p*H or in unbuffered solution the transformation green → red took place readily in a nitrogen atmosphere, indicating that atmospheric oxygen was not involved in the conversion. Because the sole product at low *p*H appeared to be the green compound and since this substance appeared initially regardless of *p*H, it was likely that the only enzyme catalyzed reaction was the first step of the over-all sequence. This was verified by comparison of the production of color and the disappearance of hydrogen peroxide. It was found (see Part II) that peroxide disappearance corresponded only to the green phase and that the red product appearance occurred long after the peroxide concentration had fallen to immeasurably low levels.

Dilution of the red product with alcohol to give a final alcohol concentration of about 98% of the total volume resulted in a shift (Fig. 3) in the principal absorption maximum to 505 mμ, which was characteristic[5] of the spectrum of phenothiazone-3 in alcohol. Since the thionol maximum of 590 mμ in alkaline solution[6] was obtained only under unusual circumstances, this compound was not regarded as a normal primary product. However, in view of the strong probability that the end products represented the results of a non-enzymic reaction, the most interesting product was the initial green substance. Efforts to isolate this material have failed. Some indirect indication of the nature of the first reaction step may be obtained by noting that in the reductive titration of thionol (7-hydroxy-phenothiazone-3) in the range *p*H 1.0–*p*H 1.85 DeEds and Eddy[7] obtained a series of color stages proceeding from the red of the fully oxidized

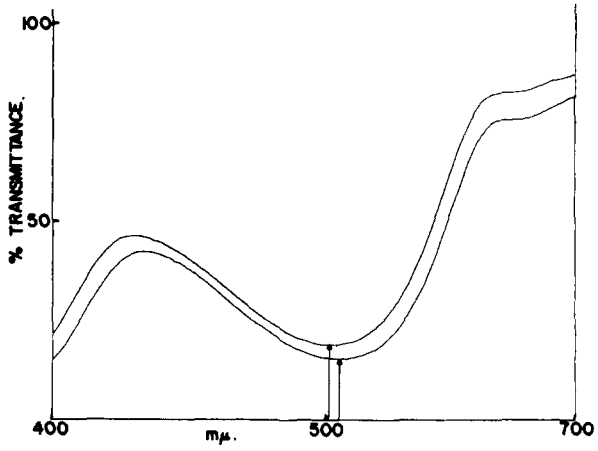

Fig. 3.—Absorption spectra of final red product in propylene glycol and alcohol. System: 20 mg. of phenothiazine in 5 ml. of propylene glycol incubated with 0.2 ml. of 1.036 *M* H_2O_2, 0.5 ml. of enzyme solution (1 mg. peroxidase/ml.) and 0.55 ml. of water for several hours. For spectra 0.05 ml. of reaction mixture was diluted with 2.95 ml. of propylene glycol or ethanol. Lower trace, glycol; upper trace, alcohol.

(5) D. F. Houston, E. B. Kester and F. DeEds, THIS JOURNAL, **71**, 3816 (1949).

(6) S. Granick and L. Michaelis, *ibid.*, **69**, 2983 (1947).

(7) F. DeEds and C. W. Eddy, *ibid.*, **60**, 2079 (1938).

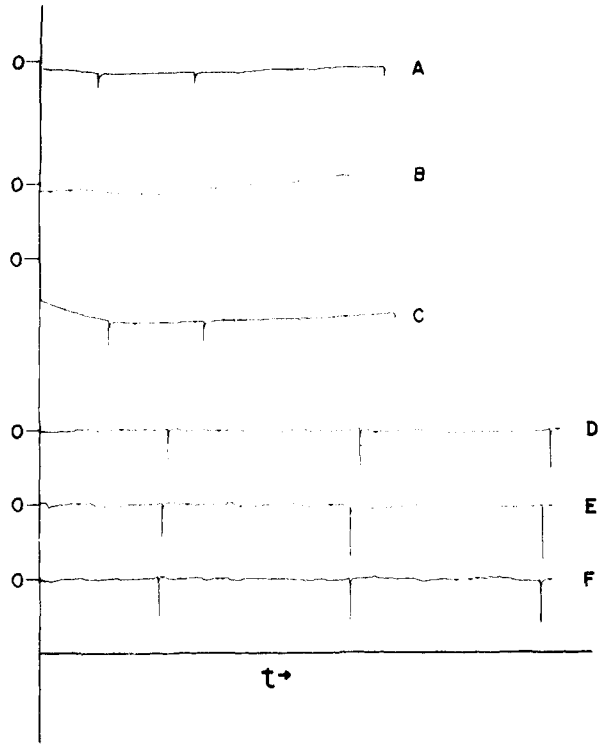

Fig. 4.—Photometric traces of phenothiazine oxidation at 640 mμ. Curves A, B and C, respectively, hemoglobin, catalase and peroxidase effects. Curves D, E and F, respectively, Cu^{+2}, Co^{+2} and Fe^{+2} activities. System curves A, B, C: catalyst 0.013 mg./ml., phenothiazine 5×10^{-4} *M*, H_2O_2 4.5×10^{-3} *M* acetate buffer 0.07 *M* and *p*H 4.2. Water content 20% of volume. Curves D, E, F: all conditions identical, metal ions equimolar at 3.3×10^{-5} *M*. Total time lapse from addition of catalyst for curves A, B, C minutes, timing marks at 0.5, 1.0 minute. For D, E, F timing marks at 1.0, 2.0 and 3.0 minutes. Ordinate is transmittance, base-line zeros set arbitrarily, 2 mm. corresponds to 1% of full scale.

form to green and finally colorless forms. These authors concluded that the non-red forms represented semiquinone formation. Stabilization was greatly improved by increasing the acidity. The reactions encountered in the enzymic oxidation of phenothiazine would appear to have been the reverse of those obtained by DeEds and Eddy in starting with thionol Tentatively, then, it was not unlikely that the initial enzymic reaction involved the formation of a semiquinone which was stabilized by the glycol solvent, thus circumventing the need for a highly acid medium. In this regard it may be noted that in studies on the photochemical production of radicals from compounds related to phenothiazine Lewis and Bigeleisen[8] pointed out that in their solvents and at low temperatures radicals which could not be observed in the extremely acid solutions used by Michaelis, *et al.*,[9] were readily obtained.

Since it has been recognized that spurious results may be obtained in the oxidation of such donors as pyrogallol[10] when rigid precautions to account for non-enzymic factors have been neglected, several types of control experiments were necessary to establish the strictly enzymic character of the reactions studied. In general, strong oxidants such as ferric chloride and lead peroxide readily oxidized phenothiazine through the same reaction sequence as peroxidase, providing that water was present. An important distinction between the action of these agents and the enzyme was that hydrogen peroxide was required in the enzymic case and was not necessary in the inorganic systems. This automatically eliminated this class of oxidants from consideration as possible enzyme contaminants. A further important consideration was that in the presence of the acetate buffer used in most of this work even high concentrations of ferric ion failed to oxidize the donor. This was probably due to formation of the well known ferric acetate complex. The ferric ion was therefore eliminated conclusively. Several other metal ions known to decompose hydrogen peroxide or to induce peroxidase-like reactions were tested directly under the same conditions shown to be highly favorable to the enzymic reaction, *i.e.*, at relatively high water concentration and at *p*H 4.2. The metals selected as being most likely to interfere were Cu^{+2}, Co^{+2} and Fe^{+2}. The ferrous ion was particularly interesting because with hydrogen peroxide it has often been studied as a peroxidase model (Fenton's reagent) and could be regarded as having potentially the greatest effect as a contaminant of all possible metal ions. In amounts which could be assumed to exceed the likely *trace* metal concentrations that would occur in these studies none of the ions tested induced color formation with phenothiazine (curves D, E, F of Fig. 4). Furthermore, in a polarographic experiment in which the three metals were successively added to the same solution so that in the final solution all three were present in equimolar (approximately) concentration there was no measurable peroxide consumption, but addition of the enzyme following the last metal addition induced immediate color formation and rapid peroxide consumption (Fig. 5). Evidently there was neither peroxidatic or catalytic activity during the times of observation in the cases of substantial amounts of three metal ions having known peroxide decomposing activity. These results did not eliminate all possible metal contaminants, but it could be concluded that Fe^{+3}, Fe^{+2}, Cu^{+2} and Co^{+2} contributed negligibly if at all to the observed reactions and that since fairly large amounts of PbO_2 were required to obtain color intensities comparable to those produced by extremely small amounts of enzyme (milligrams compared to micrograms) that lead could also be eliminated. Since in the polarographic work Hg and Hg^+ were present and no reactions occurred in the absence of the enzyme, mercury was also eliminated. Qualitative tests with Ni^{+2} and Mn^{+2} in concentrations of the order of 0.01 *M* produced no color. It was doubtful that metal contamination was responsible for the peroxidase effects.

The presence of more than one peroxidase com-

(8) G. N. Lewis and J. Bigeleisen, THIS JOURNAL, **65**, 2419 (1943).

(9) L. Michaelis, S. Granick and M. Schubert, *ibid.*, **63**, 351 (1941).

(10) A. C. Maehly in "Methods of Biochemical Analysis," ed. D. Glick, Vol. I, Interscience Publishers, Inc., New York, N. Y., 1954, p. 390.

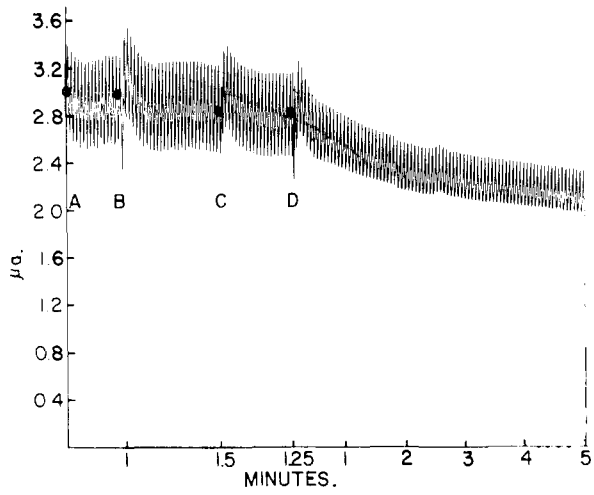

Fig. 5.—Effects of metal ion and enzyme on peroxide consumption. Circles indicate mid-points of oscillation and correspond to consecutive additions of Cu^{+2}, Co^{+2}, Fe^{+2} and peroxidase to a solution of 1.1×10^{-3} *M* H_2O_2 which was 1.6×10^{-4} *M* in phenothiazine and contained 20% by volume of water. The metal ion concentrations were 6×10^{-6} *M*, enzyme concentration was 0.06 mg./ml. (5.52 units).

ponent in the electrophoretic experiments suggested the possibility that non-specific heme catalysis may have been of some importance. Although this possibility was not conclusively eliminated, it was shown that when either crude hemoglobin or crystalline catalase was used in equivalent concentration (weight basis) in comparison with peroxidase, neither compound produced appreciable color, and it was doubtful that catalase had any effect since the photometric trace (curves A, B and C of Fig. 4) showed transmittance variations within the 1–2% of full scale error of the instrument. There was also no detectable catalase activity in polarographic experiments (next section). However, if peroxide consumption had been apparent polarographically, the result would have been ambiguous since it could have been indicative of *peroxidatic* glycol oxidation. The failure to produce colored products from phenothiazine, however, could be taken as a clear indication that any contaminating catalase was not acting peroxidatically if it was present. The result with catalase was of some interest, for it was shown previously[2] that catalase was fairly active in the oxidation of chloropromazine. The possible contamination with peroxidatically active compounds similar to hemoglobin has been discussed (Experimental part), but it was clear from the result (curve A of Fig. 4) of this experiment that even rather large amounts of a contaminant heme having general properties similar to hemoglobin would contribute only slightly to the peroxidase activity in the buffered glycol system.

Further corroboration of the enzymic character of the phenothiazine oxidation in a water-poor system was obtained in the classical manner using heat inactivation as a criterion of enzymic character. Peroxidase solutions were heated for varying periods of time at boiling water temperature in a sealed vessel and tested for activity photometrically. As in the normal fully aqueous system, there

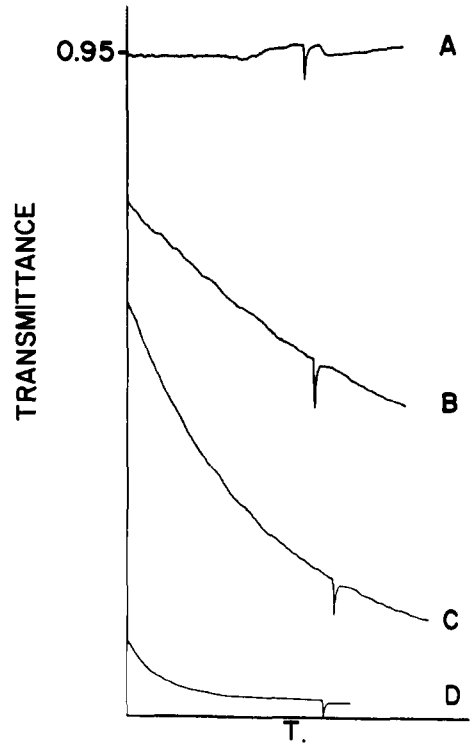

Fig. 6.—Heat inactivation of peroxidase. Curves A, B, C, D, respectively, 60, 30, 17 and no minutes heating at 100°. Timing marks in each case made 30 seconds after start of reaction. Recording at 640 mμ. In all cases: phenothiazine 5×10^{-4} *M*, H_2O_2 4.5×10^{-3} *M*, enzyme 0.016 mg./ml. (1.47 units), 0.1 *M* acetate buffer at *p*H 4.2. Water 24% of volume. Ordinate of zero base for all four curves was set at 0.95 transmittance.

was progressive inactivation (Fig. 6) until finally after 60 minutes of heating all activity was lost. This behavior would be uncharacteristic of an inorganic catalyst having a high degree of heat stability in aqueous solution and would usually be interpreted as an indication of denaturation of the enzyme protein.

To eliminate interference from a source other than the enzyme solution itself, it was shown that the *extrapolated* initial rate of peroxide consumption varied proportional to the enzyme concentration as in the usual fully aqueous type of enzymic system (Fig. 7).

The results of these experiments supported the conclusion that the medium used supported the normal peroxidase reactions and that until further tests of highly purified or crystalline peroxidase could be undertaken the tentative assumption that the observations were concerned essentially exclusively with the enzyme itself was quite well justified.

Since at very low water concentrations the reaction rates fell off markedly, the possible solvent inactivation of the enzyme and the question of enzyme solubility in the various solvent mixtures required study. To examine the effects of the solvent alone on peroxidase concentrated enzyme solutions were made in water and then diluted with propylene glycol and permitted to stand in the refrigerator for 24 hr. The activities of the solvent-

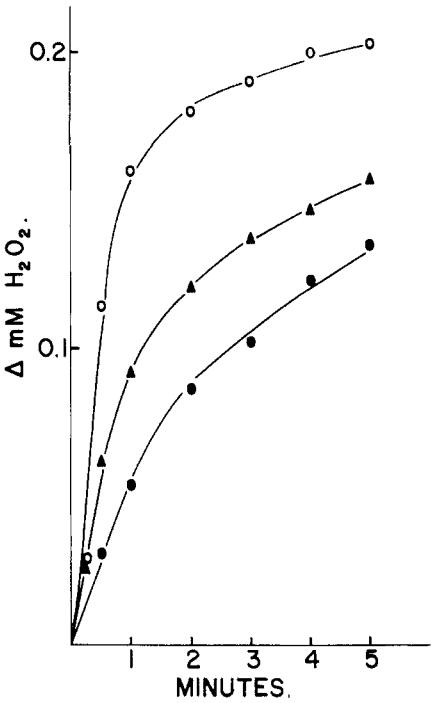

Fig. 7.—Variation of reaction rate with peroxidase concentration. For all three curves phenothiazine 1.6×10^{-4} *M*, H_2O_2 0.9 m*M*, peroxide consumption computed from polarographic data. Open circles, 0.016 mg./ml. enzyme (1.47 units); triangles, 0.0066 mg./ml. (0.607 unit), closed circles, 0.0033 mg./ml. (0.304 unit). Respective rates by extrapolation to zero time, 0.2, 0.11, 0.06 m*M* H_2O_2/minute. Water 29.6% of volume.

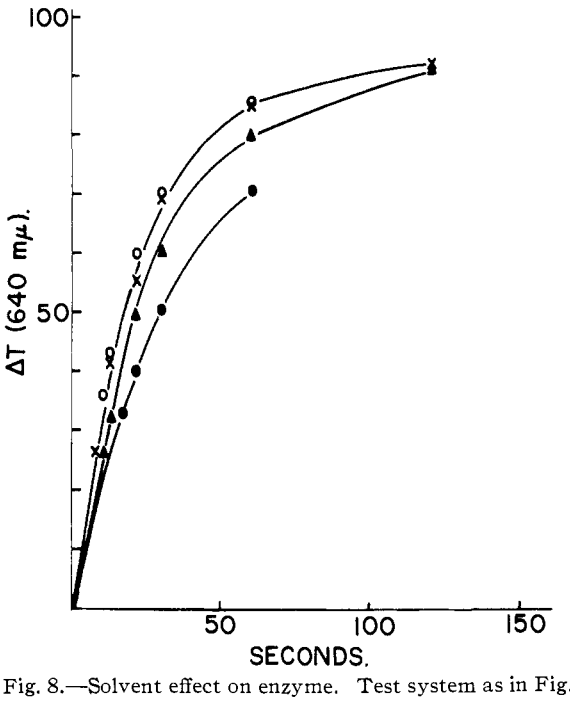

Fig. 8.—Solvent effect on enzyme. Test system as in Fig. 6. Stock enzyme solution containing 5 mg./ml. peroxidase diluted with water (open circles) or glycol–water mixtures to same final volume and permitted to stand in cold 24 hours before use. Crosses, water only after 24 hr., triangles, 90% propylene glycol immediately on mixing; closed circles, 90% glycol after 24 hr. Final enzyme concentration in reaction mixtures 0.0033 mg./ml. (0.304 unit).

treated enzyme solutions were compared with those of solutions of the same stock diluted with water alone and permitted to stand an equal length of time in the cold before use. In the presence of glycol concentrations below 90% (v./v.) there was negligible inactivation and at 90% propylene glycol there was a remarkably small amount of inactivation (Fig. 8). Since the enzyme stood up well under this rather severe test of solvent effect, it was unlikely that the role of water in the reaction was merely one of protection against denaturation of the enzyme by the solvent. It has been noted that even oxidizing agents of the ferric ion or lead dioxide types required water in order to function in the glycol media, and it would thus appear that water participation was a general requirement possibly extending to all catalysts of oxidations of the type under consideration. It has been considered that water may normally be bound to the iron of the hydroperoxidases[11] and that a displacement reaction involving the coördinated water takes place in the formation of the enzyme–substrate complex. A solvent capable of replacing some or all of this water would be expected to interfere with the normal course of the reactions catalyzed by the enzyme. If it could be assumed that the affinity of the enzyme for water were very much higher than for a glycol the necessity for an extremely high glycol concentration to exist before all loss of activity occurred would be readily explained. If this should prove to be the case it would be of considerable interest for it might be unique in enzymology as an example of a solvent effect directly associated with the enzymic mechanism rather than with some side effect such as protein denaturation.

Throughout this work it has been implied that the enzyme was soluble in the non-aqueous solvents used. Since visually transparent solutions of enzyme–water–glycol mixtures were obtained and since such mixtures gave characteristic protein absorption spectra in the ultraviolet with no undue "noise" commonly associated with solution heterogeneity, this implication was justified. However, further tests were carried out by mixing concentrated protein solutions in water with the organic solvents. Catalase, crystalline trypsin, peroxidase, hemoglobin, crystalline bovine serum albumin and bovine serum all gave clear solutions. Mixture of dry peroxidase with propylene or ethylene glycols resulted in the extremely slow dissolution of the solid enzyme and this was considerably hastened by the addition of 1 or 2% by volume of water. Solubility in the absence of water was about the same in dimethyl sulfoxide. Precipitation of the protein mentioned from the glycol solutions as used here has not been observed.

Part II. Generality of the Peroxidase Reaction in Propylene Glycol.—To determine whether the peroxidase system functioned generally in a water-poor environment, several donors in addition to

(11) B. Chance in the "Enzymes," Part 1, Vol. II, 1st Ed., ed. J. B. Sumner and K. Myrback, Academic Press, Inc., New York, N. Y., 1951, p. 444; H. S. Mason, "Advances in Enzymology," Vol. 19, ed. F. F. Nord, Interscience Publishers, Inc., New York, N. Y., 1957, p. 111–117.

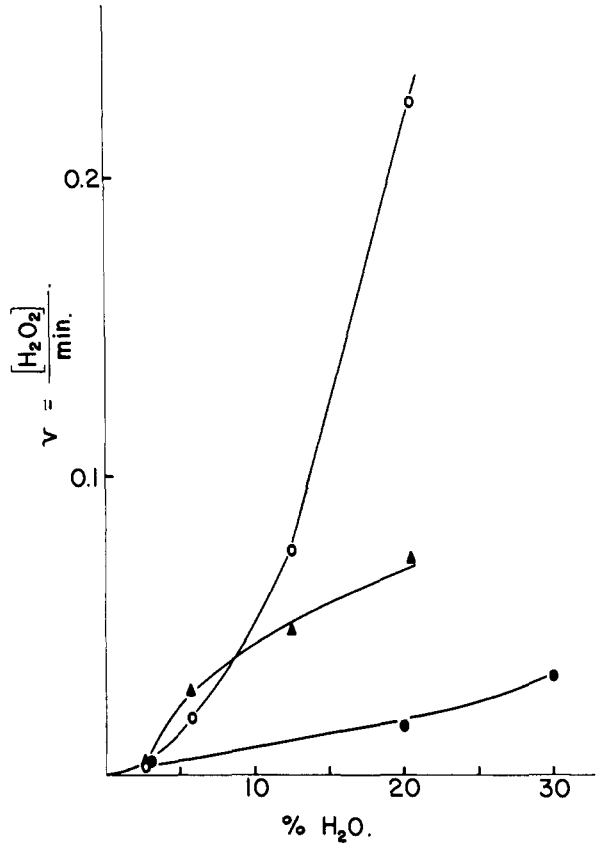

Fig. 9.—Initial rates of hydrogen peroxide consumption for phenothiazine, pyrogallol and resorcinol oxidations as functions of water concentration. Ordinates: decrease in H_2O_2 per minute, straight portion of curve extrapolated to zero time. System: 8.3×10^{-4} *M* phenothiazine or pyrogallol, 1.66×10^{-3} *M* resorcinol, 6.9×10^{-4} *M* H_2O_2 for phenothiazine and pyrogallol, 3.93×10^{-3} *M* H_2O_2 for resorcinol. Enzyme: 66 μg./ml. (6.07 unit) of resorcinol, 40 μg./ml. (3.68 units) of pyrogallol and phenothiazine. Buffer: *p*H 4.2, resorcinol; *p*H 4.45, pyrogallol and phenothiazine. Open circles, phenothiazine; triangles, pyrogallol; closed circles, resorcinol. Results computed from polarography at −1.4 volt.

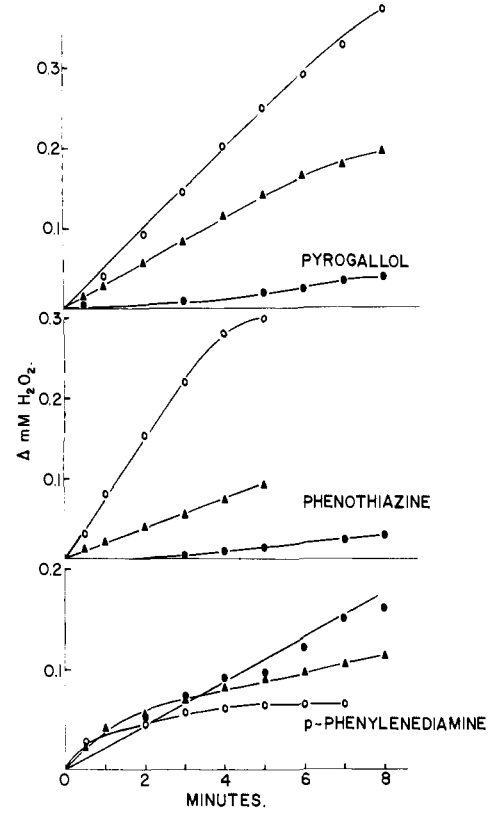

Fig. 10.—Rates of peroxide consumption with three donors under identical conditions. Ordinates in units of H_2O_2 and represent computation from cumulative decreases in the current at −1.4 volt. In all cases run at *p*H 4.45, donor concentration 8.3×10^{-4} *M*, H_2O_2—6.9×10^{-4} *M*, peroxidase—40 μg./ml. (3.68 units). Open circles, 12.48% water; triangles, 5.81%; closed circles, 2.48%.

phenothiazine were tested. It was found qualitatively that pyrocatechol, resorcinol, pyrogallol, *m*- and *p*-phenylenediamines, leucomalachite green and *p*-aminobenzoic acid were all readily oxidized under the same conditions in which phenothiazine reacted. Comparison of the rates of hydrogen peroxide consumption with phenothiazine, pyrogallol, resorcinol and *p*-phenylenediamine as the donors were made. In all cases except that of *p*-phenylenediamine the rate of peroxide consumption increased markedly with increasing water concentration (Fig. 9). The abnormal behavior of *p*-phenylenediamine (Fig. 10) was the result of several factors of which the most important were the super-position of a small but measurable rate of uncatalyzed oxidation in the presence of peroxide on the enzymic rate and a change in character of the reaction with increasing water concentration. At very low water concentrations of the order of 2% of the total volume a single yellow product appeared when *p*-phenylenediamine was oxidized. As the water concentration was increased, the initial yellow product became increasingly unstable and was converted to a greenish substance. Finally, at about 12.5% water the reaction slowed considerably and the yellow color rapidly gave way to a final violet product which was characteristic of the fully aqueous reaction product. A detailed study of this series of reactions has not been made, but it was clear that variation in solvent composition might have the very useful feature of separating stages in a reaction sequence of this or the phenothiazine type to permit detailed study of the steps.

Considerable differences in susceptibility to oxidation as a function of the water content existed even between members of the same donor type, *e.g.*, phenolic compounds in the cases of pyrogallol and resorcinol.

The possible oxidation of the glycol by peroxidase was considered and control experiments in which the enzyme, peroxide and the glycol medium were mixed were carried out. In the absence of any of the donors mentioned above there was no peroxide consumption, and it was concluded that oxidation of the alcoholic hydroxyl groups did not occur.

While the physiological roles of peroxidase remain obscure, it is interesting that in the cases of

TABLE I

Water, % total vol.	Supporting electrolyte	pH	Id[a]	$E_{1/2}$[b]	$(m^{2/3}t^{1/6})$[c]	Double wave
8.0	0.133 M LiCl	Unbuffered	2.38	−0.845	1.819	Well developed
6.73	$LiOOCCH_3$ (0.078 M Li)	4.45	1.90	−0.865	1.753	Well developed

[a] Total diffusion current (microamperes), 1.035×10^{-3} M H_2O_2 in the unbuffered case and 1.03×10^{-3} M in the buffered solution. [b] Half-wave potential *vs.* S.C.E. at 25° for the first waves only. [c] Capillary constant in terms of mg./sec. Hg(in.) and seconds (t) at −1.4 v. in each medium.

phenothiazine and the derivative chlorpromazine peroxidase induced oxidations gave rise to products which in the former were probably identical in part to *in vivo* detoxication products[12] and in the latter case were identical to the principal detoxication product.[1] A point of more fundamental biological interest may be noted: cellular reaction sites of an

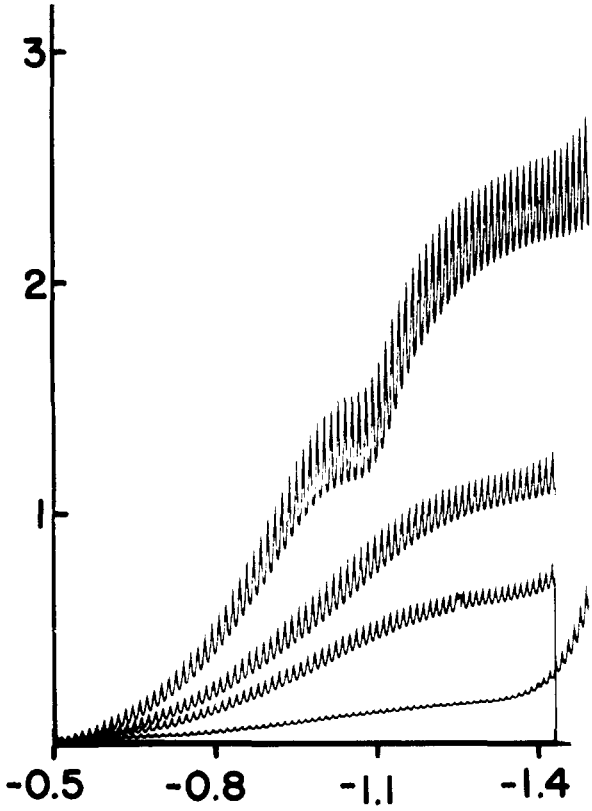

Fig. 11.—Polarogram of hydrogen peroxide in propylene glycol showing double wave. Ordinates microamperes, abscissae volts *vs.* S.C.E. Run at pH 4.45, 25°. Water concentration 15.4%. Lower curve, residual current: upper, center and bottom curves, respectively, 6.86×10^{-4} M, 3.44×10^{-4} M, and 1.72×10^{-4} M hydrogen peroxide.

essentially non-aqueous character, *e.g.*, in cell membranes,[13] have been invoked to account for possible reaction localization. There have been, however, relatively few studies made of enzymic reactions of a non-hydrolytic nature in non-aqueous solvents. Systems of the type described here may serve as interesting models of intracellular or membrane conditions in which local restrictions in water content exist.

(12) H. B. Collier, *Can. J. Research*, **18B**, 272 (1940).

(13) See discussion of physical compartmentation in cells by J. F. Danielli, "Cell Physiology and Pharmacology," Elsevier Publishing Co., New York, N. Y., 1950, pp. 14 *et seq.*

Addendum

Polarography of H_2O_2 in Propylene Glycol Media.—The polarography of hydrogen peroxide in the media containing lithium acetate buffered propylene glycol was marked by two abnormalities: (a) there was a strong wave suppression effect, which was not unusual for organic solvent polarography,[14] and (b) at water concentrations between 2.5 and 30% a double wave was observed at moderately high peroxide concentrations (Fig. 11). The double wave was not due to incomplete deaeration, for it appeared even after extension of the deaeration time to more than an hour, and it was found that the initial oxygen wave was absent in the glycol media after a deaeration period as short as 15 minutes. At all water and peroxide concentrations the height of the first wave was very nearly 50% of the total wave height. It was assumed that little error would result from taking the total diffusion current as the true measure of peroxide concentration and this was verified by the linearity of the current–concentration curves in the double wave concentration region (Fig. 12). The

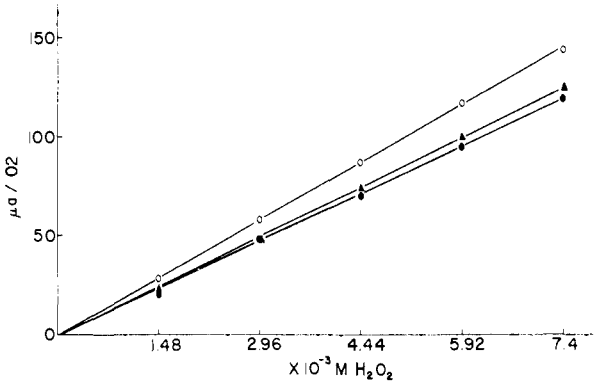

Fig. 12.—Hydrogen peroxide standard curves at various water concentrations and including double wave concentration region. Ordinates are microamperes/0.02. Run at pH 4.2, 25°, −1.4 volt *vs.* S.C.E. Open circles, 30% water; triangles, 20% water: closed circles, 10% water.

hydrogen peroxide waves in glycol media were not strongly dependent upon either pH or the nature of the anions, for the double wave behavior and half-wave potentials were about the same whether unbuffered glycol–lithium chloride media or the buffered acetate solutions were used (Table I). At very low peroxide concentrations the waves were very poorly developed and the suppressive effect of the medium introduced considerable uncertainty into quantitative peroxide determinations at concentrations less than 10^{-4} M.

(14) I. M. Kolthoff and J. J. Lingane, "Polarography," 2nd Ed., Vol. I, Interscience Publishers, Inc., New York, N. Y., 1952, pp. 99–101.

The double wave tended to disappear with increasing water concentration, and at intermediate peroxide concentrations it was either absent or the first wave was very poorly developed as the water concentration approached 30% of the total volume. The reason for the double wave was unclear, but it appeared likely that slow diffusion of the reduction products from the region of the dropping electrode may have been responsible since even vigorous mixing for several seconds was often insufficient to prevent layering when additions were made to the glycol solutions. In fact, a fairly persistent "streamer" due to the passage of the mercury droplet through the solutions could be visualized by adding a small amount of a dye to the more concentrated glycol solutions. A rotating microelectrode might have been superior to the dropping electrode in the media used.

ARMY CHEMICAL CENTER, MD.

[CONTRIBUTION FROM THE LABORATORIES OF THE SLOAN-KETTERING DIVISION, CORNELL UNIVERSITY MEDICAL COLLEGE

Synthesis of Purine-6-carboxaldehyde and Related Derivatives[1]

BY ALFREDO GINER-SOROLLA, ISAAC ZIMMERMAN AND AARON BENDICH

RECEIVED NOVEMBER 10, 1958

Treatment of 6-methylpurine with iodine and pyridine afforded purine-6-methylenepyridinium iodide which upon reaction with *p*-nitrosodimethylaniline gave *p*-phenylenediamine-N,N-dimethyl-N′-(6-purinylmethylene)-N′-oxide. Acid hydrolysis of the latter gave a solution of purine-6-carboxaldehyde from which the aldehyde could be isolated only in low yield. Direct treatment of the acid hydrolysate of *p*-phenylenediamine-N-N dimethyl-N′-(6-purinylmethylene)-N′-oxide with the appropriate carbonyl reagents led to the hydrazone, oxime, thiosemicarbazone, semicarbazone and phenylhydrazone of purine-6-carboxaldehyde. Good yields of purine-6-carboxaldehyde were obtained by treatment of its hydrazone with ethyl nitrite and hydrochloric acid; when ethyl nitrite and acetic acid were used, purine-6-carboxaldehyde azine was obtained. The catalytic hydrogenation of 6-cyanopurine in the presence of semicarbazide afforded purine-6-carboxaldehyde semicarbazone. Similar treatment with hydroxylamine, hydrazine and phenylhydrazine gave N-amino-6-purinylamidine, 6-hydroxyamidinopurine and N-phenyl-6-purinylamidine, respectively. Treatment of N-amino-6-purinylamidine with nitrous acid led to purine-6-carboiminoazide which was converted into 4,5-diamino-6-carboiminopyrimidine with aqueous ammonia. The reaction of purine-6-carbohydrazide with benzenesulfonyl chloride gave purine-6-phenylsulfocarbohydrazide.

Several purines related to purine-6-carboxylic acid and 6-cyanopurine have been synthesized.[2,3] It was of interest to prepare purine-6-carboxaldehyde. As this compound can be considered an oxidation product of the carcinolytic and extremely toxic 6-methylpurine,[4,5] it was of further interest to determine whether the conversion of the methyl group to the carboxaldehyde function would affect the toxicity and anti-tumor properties. Purine carboxaldehydes have not been reported previously, although several aldehydes or their derivatives in the pyrimidine[6–13] and pteridine (see review by Albert[14]) series are known. Little is known of the effect of such aldehydes on biological systems, although 2-amino-4-hydroxypteridine-6-carboxaldehyde is well known as a potent inhibitor of xanthine and pteridine oxidase.[15–17]

The desired compound was an elusive target which could not be prepared by any of the conventional means tried. The synthesis of purine-6-carboxaldehyde was achieved by the application of methods which have recently been developed.

Synthetic Studies.—A total synthesis of purine-6-carboxaldehyde from the corresponding pyrimidine by classical methods[18] was not attempted since it was expected that neither the carboxaldehyde group nor its acetal would withstand the drastic conditions used in the synthesis. Instead, syntheses of purine-6-carboxaldehyde by transformation of substituents at the 6-position of purine were investigated.

An adaptation of the method of preparation used for substituted β-keto-alkylpyridinium iodides by the reaction of methyl ketones with iodine and pyridine[19] was found suitable in the first step of the synthesis, and the preparation of purine-6-methylenepyridinium iodide (II) (Scheme 1) from 6-methylpurine[20] (I) was achieved in 63% yield. This type of reaction was adapted[21] to the preparation of carboxylic acids and esters and extended

(1) This investigation was supported by grants from the National Cancer Institute, National Institutes of Health, Public Health Service (Grant CY-3190); the Atomic Energy Commission (Contract No. AT (30-1)910); the Ann Dickler League and the Damon Runyon Fund.

(2) L. B. Mackay and G. H. Hitchings, THIS JOURNAL, **78**, 3511 (1956).

(3) A. Giner-Sorolla and A. Bendich, *ibid.*, **80**, 3932 (1958).

(4) D. A. Clarke, F. S. Philips, S. S. Sternberg and C. C. Stock, *Ann. N. Y. Acad. Sci.*, **60**, 2, 235 (1954).

(5) F. S. Philips, S. S. Sternberg, L. Hamilton and D. A. Clarke, *ibid.*, **60**, 283 (1954).

(6) T. B. Johnson and E. F. Schroeder, THIS JOURNAL, **53**, 1989 (1931).

(7) H. Kondo and M. Yanai, *J. Pharm. Soc. Japan*, **57**, 747 (1937).

(8) E. Ochiai and M. Yanai, *ibid.*, **58**, 397 (1938).

(9) M. Delepine, *Bull. soc. chim. France*, **5**, 1539 (1938).

(10) M. Delepine and K. A. Jensen, *ibid.*, **6**, 1663 (1939).

(11) D. Price, E. L. May and F. D. Pickel, THIS JOURNAL, **62**, 2818 (1940).

(12) H. Vanderhaeghe and M. Claesen, *Bull. soc. chim. Belg.*, **66**, 292 (1957).

(13) R. Hull, *J. Chem. Soc.*, 4845 (1957).

(14) A. Albert, *Quart. Rev.*, **6**, 197 (1952).

(15) O. H. Lowry, O. A. Bessey and E. J. Crawford, *J. Biol. Chem.*, **180**, 389 (1949).

(16) H. M. Kalckar, N. O. Kjeldgaard and H. Klenow, *Biochim. et Biophys. Acta*, **5**, 586 (1950).

(17) L. S. Dietrich, W. J. Monson, J. N. Williams, Jr., and C. A. Elvehjem, *J. Biol. Chem.*, **197**, 37 (1952).

(18) *Cf.* A. Bendich, in "The Nucleic Acids," Vol. 1, E. Chargaff and J. N. Davidson eds., New York, N. Y., 1955, p. 81.

(19) L. Carroll King, THIS JOURNAL, **66**, 894 (1944).

(20) S. Gabriel and J. Colman, *Ber.*, **34**, 1234 (1901); this compound was supplied by Cyclo Chemical Corp., Los Angeles, Calif.

(21) L. Carroll King, THIS JOURNAL, **66**, 1612 (1944).