$5 \times 10^{-4}$ M concentration, chlorpromazine did not produce any observable lysis, and at this preincubation concentration maximal PFC production was observed. With lower preincubation concentrations of chlorpromazine, less enhancement of PFC was seen. At $5 \times 10^{-6}$ and $5 \times 10^{-7}$ M, the lowest drug concentrations used, PFC production was equivalent to control values. However, when treated and washed SRBC were allowed to remain at 4° for 3 days, additional lysis of SRBC occurred at $1 \times 10^{-4}$ M. As shown in Fig. 2b, no enhancement of PFC production occurred at this concentration. Inhibition of PFC was seen at $5 \times 10^{-4}$ M and higher concentrations of the drug. On the other hand, enhancement of PFC occurred at $5 \times 10^{-6}$ and $5 \times 10^{-7}$ M. Similarly, Braun *et al.*[15,16] found that concomitant administration of chlorpromazine with sheep red cells produced enhancement of PFC production *in vivo*.

These findings point out that alterations of the membrane structure of either the erythrocyte antigen or the developmental cell type of the antibody-producing system are determinants in the differentiation of the antibody-producing cell.

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### Purification and substrate specificity of uterine catecholamine oxidase

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PREVIOUS reports from this laboratory have described progress in the isolation, purification and characterization of an epinephrine-oxidizing enzyme which is present in a number of mammalian smooth muscles.[1–5] It was also observed that a product of the oxidation reaction inhibited uterine and cardiac actomyosin adenosine triphosphatase (ATPase) activity. The ATPase inhibitor was identified as a zwitterion, indole isomer of adrenochrome.[1,2] This enzyme system was shown to catalyze both the oxidation of epinephrine to adrenochrome and the isomerization of adrenochrome to a zwitterion isomer.[2,3]

Norepinephrine and isoproterenol were also oxidized by the uterine enzyme to their respective aminochromes; L-tyrosine, dihydroxy-L-phenylalanine (Dopa)[2] and phenylephrine[5] were not substrates for the enzyme.

The present study concerns a further purification of the bovine uterine catecholamine oxidase using gel-filtration chromatography. A water-soluble form of the enzyme[3] was used as starting material. Data have also been obtained which further demonstrate a distinction between the two catalytic activities of the enzyme preparation (oxidation of epinephrine to adrenochrome; isomerization of adrenochrome to the ATPase inhibitor). The substrate specificity of the oxidase reaction has also been extensively investigated.

*Preparation and purification of the bovine uterine enzyme.* The initial steps in the extraction and purification of the uterine oxidase were described previously by Inchiosa[2] and Inchiosa and Rodriguez[3] and are summarized here: (1) Uterine myometrial tissue was homogenized with 0·3 M KCl, 0·15 M in $K_2HPO_4$ and 0·15 M in $KH_2PO_4$; (2) The insoluble residue was removed by centrifugation. The crude enzyme was precipitated by dilution to ionic strength, 0·1. The precipitate was redissolved with 2 M KCl. The precipitation and solution procedure were repeated two additional times (the final precipitate was termed, "3 × precipitate"). (3) Uterine actomysin, which is a contaminant of the preparation, was isoelectrically precipitated at pH 5·25 and removed by centrifugation. The enzyme was retained in the supernatant solution; the "pH 5·25 supernatant solution" was sonicated for a total period of 5 min at approximately one acoustical watt. (4) The sonicated sample was dialyzed against water to an ionic strength of approximately $6 \times 10^{-5}$ and centrifuged at 10,000 *g*; the 10,000 *g* supernatant solution is the water-soluble form of the enzyme.[3]

*Gel-filtration column chromatography.* Gel-filtration chromatography of the water-soluble enzyme was carried out in 0·9 M KCl using an agarose* gel with a molecular weight exclusion limit of 1·5 million. The water-soluble enzyme was concentrated prior to chromatography. Solid KCl was added to yield a final ionic strength of 0·9 (the enzyme preparation is soluble at this ionic strength). A 1·2 × 40 cm column was used for most of the separations; 300–1000 μg N was applied to the column; 2-ml fractions were collected. Prior to analysis for enzymatic activity, the fractions were dialyzed against water to an ionic strength of approximately $6 \times 10^{-5}$ and subsequently concentrated 5- to 10-fold by evaporation at 4°.

*Spectrophotometric assay of epinephrine oxidation to adrenochrome.* Oxidation of epinephrine to adrenochrome by the catecholamine oxidase was monitored spectrophotometrically at 485 nm as described previously.[2] Oxidase activity is optimal at pH 9·3–9·5; however, the enzyme is active at physiological pH.[4] The rate of oxidation of epinephrine by the cow uterine oxidase varies with different animals from approximately 0·3 to 3·0 μmoles substrate oxidized per min per mg of protein N.[2] The assays were carried out at saturating substrate concentrations of epinephrine; the nonenzymatic oxidation (autoxidation) of epinephrine under the conditions of the assay was 0·01 μmoles/min.[2] Enzymatic oxidation of catecholamines other than epinephrine (Table 3) has been expressed relative to the rate of oxidation of epinephrine. The extinction coefficients of the aminochromes of the other substrates at 485 nm were used to calculate the rate of enzymatic oxidation for each substrate.[2] The activities which are presented have been corrected for any nonenzymatic oxidation which the substrates may have displayed.

*Assay of ATPase inhibitor formation.* Earlier reports contain the details of the procedure which was used for the determination of ATPase inhibitor formation from adrenochrome.[2,3] Briefly, the oxidation of epinephrine by the catecholamine oxidase was carried out in a low ionic strength buffer system (pH 8·5); this procedure allowed direct paper chromatography of the oxidation products at the end of the assay period. After chromatography (4:1:5, butanol–acetic acid–water), the inhibitor zone ($R_f$ 0·16) and adrenochrome ($R_f$ 0·47) were eluted with water and the absorbancies of the solutions were measured at 348 and 485 nm respectively. The concentrations of the adrenochrome isomer and adrenochrome were calculated on the basis of the spectral data reported previously.[2] Corrections for any nonenzymic formation of ATPase inhibitor were applied as in earlier studies; this amounts to 0·66 nmole/ml of reaction mixture.[3]

*Studies on the relative lability of the oxidase and isomerase activities.* The enzyme preparation used for these studies was the fraction remaining after isoelectric precipitation of actomyosin at pH 5·25. An aliquot of the "pH 5·25 isoelectric supernatant solution" was acidified with 0·01 N HCl to give the desired pH at 5°. Approximately 20 min after the acid addition, the samples were dialyzed against water to yield an ionic strength of $6 \times 10^5$. The dialyzed protein was assayed for oxidase and isomerase activity as described above.

*Chemical assays.* Total acid-soluble phosphorus was determined colorimetrically by the Fiske–SubbaRow method.[6] The samples were digested with 1:1 $H_2SO_4$ followed by oxidation with 30% hydrogen peroxide. The orcinol reaction of Albaum and Umbreit[7] was used for determination of

* Bio-Gel A-1·5 m (100–200 mesh), Bio-Rad Laboratories.

pentose; hexose concentrations were determined by a modification of the anthrone reaction as described by Scott and Melvin.[8] Protein nitrogen was determined by a modified biuret assay as described by Inchiosa.[9]

*Gel-filtration chromatography.* Gel-filtration chromatography of the water-soluble enzyme was first attempted with gels having molecular weight exclusion limits ranging from 100,000 to 1·5 million. Using water-equilibrated salt-free systems, these gels excluded all of the protein of the water-soluble enzyme preparation. A typical result is presented in Fig. 1A. The exclusion of the protein in this experiment was clearly evidenced by the fact that the protein peak coincided with the elution characteristics of Blue Dextran* (mol. wt, 2·0 million) on the same column. However, if the chromatography was carried out in 0·9 M KCl, approximately 54 per cent of the protein was still excluded (fraction I), but the remainder of the protein was within the molecular weight operating range of the gel (Fig. 1B). This second peak of protein will be referred to as fraction II. All subsequent gel-filtration chromatography was carried out at ionic strength 0·9 with a 1·2 × 40 cm column.

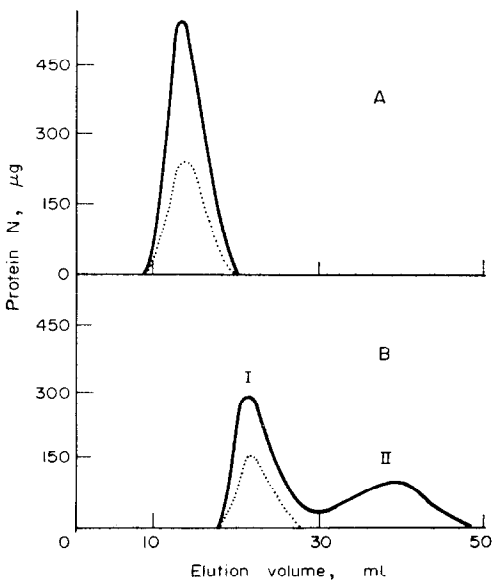



FIG. 1. Gel-filtration column chromatography of the water-soluble enzyme preparation. Agarose gel (Bio-Gel A-1·5 m); mol. wt. exclusion limit 1·5 million. (A) 1·2 × 20 cm column, equilibrated with $H_2O$; 1·72 mg protein N applied to column. (B) 1·2 × 40 cm column, equilibrated with 0·9 M KCl; 1·87 mg protein N applied to column. Solid lines represent protein concentration; broken lines indicate relative Blue Dextran 2000 concentrations.

The water-soluble enzyme preparation from uterine smooth muscle of seven different animals was chromatographed by this procedure. In all cases, the protein retarded by the gel (fraction II) showed only one peak of concentration, as in the experiment presented in Fig. 1B. The elution pattern for fraction II in these seven experiments was consistent; the $V_e/V_0$ ratio (elution volume of peak for fraction II/elution volume for peak of Blue Dextran 2000) for fraction II showed a mean value of 1·70 and a range of 1·66–1·78.

The elution characteristics of fraction II protein were compared with those of three crystalline proteins using identical column conditions (Fig. 2). Aldolase, ovalbumin and chymotrypsinogen were selected for this comparison because their molecular weights encompassed the estimated average molecular weight of fraction II protein. A plot of $V_e/V_0$ vs. the log of the molecular weight of the crystalline enzymes (inset, Fig. 2) yielded an estimate of the average molecular weight for fraction II protein of approximately 80,000.

* Pharmacia Fine Chemicals, Inc.

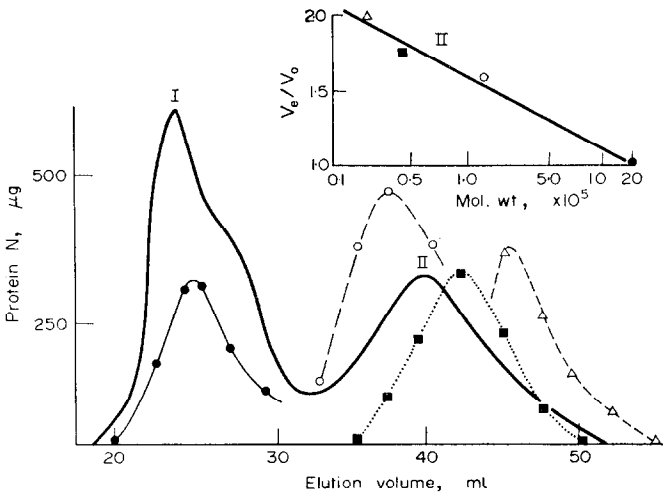



FIG. 2. Molecular weight estimation of fraction II protein. Bio-Gel A-1·5 m; 1·2 × 40 cm column eluted with 0·9 M KCl. Water-soluble enzyme preparation, solid line; aldolase, ○— —○; ovalbumin, ■- - -■; chymotrypsinogen, △— —△; relative Blue Dextran 2000 concentration, ●——●. Inset presents plot of $V_e/V_o$ vs. molecular weight for the three crystalline proteins; the symbols are the same as for the elution patterns.

The fractionation of the water-soluble enzyme using the gel technique resulted in the elimination or marked reduction of several nonprotein contaminants in fraction II. The contaminants were proportionally concentrated in fraction I (Fig. 3).

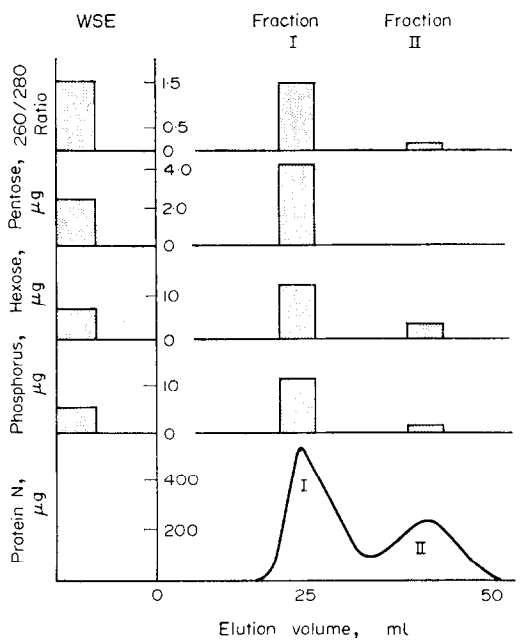



FIG. 3. Elimination of nonprotein contaminants from one fraction (fraction II) of the water-soluble enzyme preparation (WSE).

The enzymic activity of fraction II was also found to be greater than that of the water-soluble enzyme preparation from which it was derived. This comparison was carried out with enzyme preparations obtained from the uteri of four different animals. The oxidation of epinephrine to adrenochrome and the isomerization of adrenochrome to the ATPase inhibitor were catalyzed most rapidly by fraction II protein. These results are presented in Table 1. In six of the seven assays, fraction II protein demonstrated the highest enzymic activity.

TABLE 1. ENZYMIC ACTIVITY OF THE WATER-SOLUBLE ENZYME PREPARATION COMPARED WITH FRACTION I AND FRACTION II PROTEIN*

| Enzyme preparation | Products | WSE† (n-mole/ml)‡ | Fraction I (n-mole/ml)‡ | Fraction I Ratio frac. I/WSE | Fraction II (n-mole/ml)‡ | Fraction II Ratio frac. II/WSE |
|---|---|---|---|---|---|---|
| 1 | Adrenochrome | | | | | |
| | inhibitor | 6·26 | 2·00 | 0·32 | 13·67 | 2·18 |
| 2 | Adrenochrome | 20·00 | 10·35 | 0·52 | 31·49 | 1·57 |
| | inhibitor | 2·00 | 0·66 | 0·33 | 1·48 | 0·75 |
| 3 | Adrenochrome | 6·68 | 5·75 | 0·86 | 21·61 | 3·24 |
| | inhibitor | 3·95 | 1·15 | 0·29 | 15·32 | 3·88 |
| 4 | Adrenochrome | 15·63 | 10·35 | 0·66 | 22·99 | 1·47 |
| | inhibitor | 5·60 | 1·15 | 0·21 | 14·83 | 2·65 |

* Assay conditions: the reaction mixtures contained per milliliter: $NaHCO_3$, 1 μmole; enzyme protein, 75 μg N; *l*-epinephrine-*d*-bitartrate, 0·25 μmole. Oxidation period, 10·5 min; temp. 37·5°; pH 8·5.

† Water-soluble enzyme.

‡ Millimicromoles of adrenochrome or ATPase inhibitor formation per milliliter of reaction mixture (75 μg protein N/ml).

*Acid lability of uterine enzyme activity.* An attempt was made to determine whether the two catalytic activities of the uterine enzyme preparation (oxidation of epinephrine to adrenochrome; isomerization of adrenochrome to ATPase inhibitor) were related to a single enzymatic site or to two different sites. A small reduction in the pH of the enzyme preparation resulted in a distinctly different effect on the two activities (Table 2). ATPase inhibitor formation was consistently and markedly decreased by the reduction in pH. In contrast to this, the same degree of acid exposure did not produce comparable effects on the oxidation of epinephrine to adrenochrome. These findings were true for both the water-soluble enzyme and the supernatant enzyme obtained following isoelectric precipitation of actomyosin.

TABLE 2. EFFECT OF INCREASING HYDROGEN ION CONCENTRATION ON ENZYMIC ACTIVITY OF THE UTERINE ENZYME*

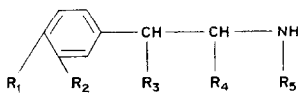
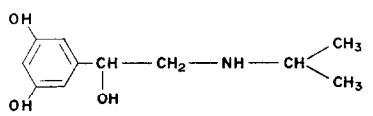
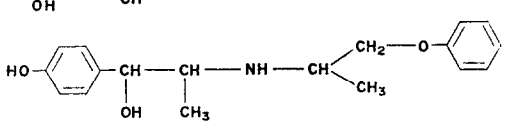
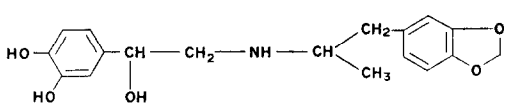
| Enzyme preparation | | pH | Adrenochrome formation (n-moles/ml)† | Change in activity (%) | ATPase inhibitor formation (n-moles/ml)† | Change in activity (%) |
|---|---|---|---|---|---|---|
| Isoelectric supernatant enzyme | I | 5·25 | 22·30 | | 18·62 | |
| | | 4·75 | 22·99 | +3 | 6·59 | −65 |
| | II | 5·25 | 32·18 | | 1·98 | |
| | | 4·90 | 45·98 | +43 | 1·32 | −33 |
| Water-soluble enzyme | | 5·25 | 2·30 | | 8·90 | |
| | | 5·00 | 4·60 | +100 | 2·31 | −74 |

* Assay conditions: same as Table 1, except that the enzyme preparations were assayed at 150 μg N/ml of reaction mixture.

† Millimicromoles of adrenochrome or ATPase inhibitor formed per 150 μg N/ml.

*Substrate specificity of the uterine enzyme.* A number of amines were tested for substrate activity in an attempt to determine the structural requirements of the initial enzymic step (oxidation of epinephrine to adrenochrome). The choice of compounds was made to allow evaluation of the influence of subtle structural differences; the selection also was intended to test certain compounds of markedly different adrenergic activity. The structures for the amines which were tested as substrates are listed in Table 3. Of the new compounds tested in the present study, only dopamine, epinine, alpha-methylnorepinephrine and protokylol were substrates.

TABLE 3. SUBSTRATE SPECIFICITY OF CATECHOLAMINE OXIDASE*

| Compounds |  | | | | | Relative activity (%) |
|---|---|---|---|---|---|---|
| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | |
| Epinephrine | OH | OH | OH | H | $CH_3$ | 100 |
| Norepinephrine† | OH | OH | OH | H | H | 18 |
| Isoproterenol† | OH | OH | OH | H | $CH(CH_3)_2$ | 53 |
| Phenylephrine† | H | OH | OH | H | $CH_3$ | 0 |
| Tyrosine† | OH | H | H | COOH | H | 0 |
| Dopa† | OH | OH | H | COOH | H | 0 |
| Dopamine | OH | OH | H | H | H | 47 |
| Epinine | OH | OH | H | H | $CH_3$ | 22 |
| α-Methylnorepinephrine | OH | OH | OH | $CH_3$ | H | 18 |
| Octopamine | OH | H | OH | H | H | 0 |
| Tyramine | OH | H | H | H | H | 0 |
| Hydroxyamphetamine | OH | H | H | $CH_3$ | H | 0 |
| Methamphetamine | H | H | H | $CH_3$ | $CH_3$ | 0 |
| Mephentermine | H | H | H | $(CH_3)_2$ | $CH_3$ | 0 |
| Ephedrine | H | H | OH | $CH_3$ | $CH_3$ | 0 |
| Amphetamine | H | H | H | $CH_3$ | H | 0 |
| *meta*-Proterenol |  | | | | | 0 |
| Isoxuprine |  | | | | | 0 |
| Protokylol |  | | | | | 63 |
| Dichloroisoproterenol‡ | | | | | | 0 |
| Propranolol | | | | | | 0 |
| Phentolamine | | | | | | 0 |
| Phenoxybenzamine | | | | | | 0 |
| Dibenamine | | | | | | 0 |

* Assay conditions: the reaction mixtures contained, in 1·2 ml final volume: Tris-HCl buffer, pH 9·7, 150 μmoles; KCl, 600 μmoles; enzyme (3 × precipitated protein), 180 μg N; substrate, 2·4 μmoles. Final pH, 9·5.

† Data obtained from references 2 and 5.

‡ These last five compounds are adrenergic blocking agents which were tested for possible substrate activity.

In the process of determining the best conditions for gel-filtration column chromatography of the water-soluble enzyme preparation, it was observed that the protein was completely excluded from a water-equilibrated (salt-free) agarose gel system (mol. wt. exclusion limit, $1{\cdot}5 \times 10^6$; Fig. 1A). At ionic strength 0·9, 46 per cent of the protein was retarded by the same gel (Fig. 1B). The elution pattern at ionic strength 0·9 was the same for the water-soluble enzyme prepared from the uteri of a number of different animals. The protein retarded by the gel (fraction II) showed an approximate, average molecular weight of 80,000 based on the elution characteristics of protein of known molecular weight in the same system; however, no conclusion can be drawn about the homogeneity of the protein in fraction II.

Fraction II protein demonstrated a marked reduction in nonprotein contaminants (Fig. 3). The ultraviolet absorbance measurements showed a change from a 260/280 nm ratio of 1·57, for the water-soluble enzyme, to only 0·09 for fraction II protein. The 260/280 nm ratio for the water-soluble enzyme (and also for fraction I) suggested a large contamination by nucleic acid; the higher absorbance at 280 than at 260 nm for fraction II protein indicated marked reduction or elimination of the nucleic acid. The presence of nucleic acid in the water-soluble enzyme was also demonstrated by a pentose contamination (Fig. 3). The pentose was completely eliminated from fraction II by the gel-filtration procedure. Ninety per cent of the phosphate contaminating the water-soluble enzyme and 87 per cent of a hexose contaminant were also removed by gel-filtration (Fig. 3). It is probable that the small amount of these contaminants remaining in fraction II could have been eliminated by lengthening the column, since the column dimensions which were used did allow some overlap of fractions I and II (Fig. 3).

Fraction II protein from gel filtration also showed a consistent purification in enzymic activity (Table 1). Studies by Inchiosa and Rodriguez[3] demonstrated that the water-soluble enzyme was a 9-fold purification in enzymic activity compared with earlier preparations. The present studies have demonstrated that 46 per cent of this protein (fraction II) shows still further increased enzyme activity.

Inchiosa and Rodriguez[3] also demonstrated that the two catalytic activities of the uterine enzyme preparation existed in different ratios at different stages in the purification procedure. The present study has shown that the isomerizing activity of the uterine enzyme is decreased after exposure to weakly acidic solutions (Table 2). In comparison, the acid treatment did not produce comparable effects in oxidase activity. The decrease in isomerase activity ranged from 33 to 74 per cent with relatively small reductions in pH; the same treatment resulted in an increased adrenochrome formation ranging from 3 to 100 per cent. Thus the two enzymatic activities of the catecholamine oxidase are apparently related to two different sites. Whether or not these sites are on the same enzyme or two different enzymes is not known at present.

The substrate specificity studies have contributed considerable information regarding the structural requirements of the uterine oxidase reaction. Epinephrine was again found to be oxidized the most rapidly of all compounds studied (Table 3). Inchiosa[2] showed that norepinephrine, a compound that does not have the methylamine group ($R_5$ in Table 3) was oxidized 18 per cent as rapidly as epinephrine. Alpha-methylnorepinephrine was included in the present series of compounds. The addition of an alpha-methyl group to norepinephrine did not alter its activity as a substrate; it was also oxidized at 18 per cent of the epinephrine rate.

The importance of the hydroxyl group in the beta position ($R_3$) was determined by the assay with epinine. This compound differs from epinephrine only in that it lacks the beta-hydroxyl group; it was oxidized 23 per cent as rapidly as epinephrine.

Dopamine was a good substrate for the uterine enzyme (47 per cent relative to epinephrine) even though it lacked both the beta-hydroxyl and amino-methyl groups. It was of interest that the absence of both the beta-hydroxyl and amino-methyl groups was associated with better substrate activity than was the absence of only one or the other group.

Two compounds studied, protokylol* and *meta*-proterenol, are structurally related to isoproterenol (Table 3). Isoproterenol, which contains an isopropyl group on the amino nitrogen ($R_5$), is enzymatically oxidized 53 per cent[2] as rapidly as epinephrine. Protokylol was oxidized at 63 per cent of the epinephrine oxidation rate. The results of the assay with *meta*-proterenol contributed important information regarding the specificity of the uterine enzyme. *meta*-Proterenol is identical to isoproterenol except that the two phenolic hydroxyls are in the 3,5-position of the ring (*meta*) and not in the 3,4-position as with isoproterenol and protokylol. Because of this modification, *meta*-proterenol was not a substrate for the oxidase.

Phenylephrine, octopamine, tyramine, hydroxyamphetamine, ephedrine, amphetamine, methamphetamine and mephentermine (Table 3) showed no substrate activity. This entire series establishes the absolute requirement of dihydroxy substitution on the ring for substrate activity.

* Supplied by Lakeside Laboratories, Milwaukee, Wis., as Caytine.

It was shown by Inchiosa[2] that dopa is not a substrate for the uterine enzyme. Apparently, the presence of the carboxyl group completely precluded substrate activity.

Several adrenergic blocking agents were tested as substrates for the catecholamine oxidase. None of these agents showed substrate activity (Table 3). These same agents have been tested for possible effects on the oxidation of epinephrine by the uterine enzyme. Dichloroisoproterenol and propranolol had no influence on enzyme activity. In preliminary studies, two alpha-adrenergic blocking agents, dibenamine and phentolamine, inhibited the oxidation of epinephrine. Both agents caused a 20 per cent inhibition at $8 \times 10^{-4}$ M; phentolamine, which is more soluble, was also tested at $3 \times 10^{-3}$ M where it produced 50 per cent inhibition. However, phenoxybenzamine, another alpha-adrenergic blocking agent, caused no inhibition. It is not possible with the present information to draw conclusions about the pharmacological importance of the findings with the adrenergic blocking agents.

The principal contribution of the substrate specificity studies has been the confirmation of the fact that the initial enzymatic reaction is carried out by a catecholamine oxidase. Thus far, only 3,4-dihydroxy derivatives of phenylethylamine have been found to be substrates for the oxidase reaction.

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### Studies with alkylating esters—V. The reactions, metabolism and biological activities of some cyclic dimethanesulphonates; the relevance to the mechanism of action of myleran

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DUE TO its clinical importance in the treatment of certain leukemias,[1] myleran ("Busulphan", 1,4-bis(methanesulphonyloxy)-butane, 1) has been the subject of much investigation related to its mode of action. Timmis and Hudson[2] have reviewed the evidence concerning the myleran and dimethyl-myleran series of dimethanesulphonates and although stating that it "remains open to doubt", concluded that a mechanism of cycloalkylation may explain their biological activities. Confirmation of this hypothesis came from the chemical[3] and metabolic[4] studies of Roberts and Warwick showing[5] that myleran can dethiolate or sulphur-strip a number of sulphur containing compounds through the formation of cyclic sulphonium ions. Recent work, however, indicates that a mechanism involving dethiolation may not be valid since a myleran homologue, 1,3-bis(methanesulphonyloxy)-propane (PDS, 11), producing similar effects as myleran both on haemopoiesis and spermatogenesis,[6] does not cycloalkylate either *in vivo* or *in vitro*.[7] Addison and Berenbaum have also questioned this hypothesis due to the immunosuppressive activity of myleran being potentiated by exogenous cysteine rather than being inhibited.[8]

Further evidence arguing against a cycloalkylation mechanism has emerged from structure–activity