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# ENZYMIC OXIDATION OF EPINEPHRINE WITH FORMATION OF AN ACTOMYOSIN ADENOSINE TRIPHOSPHATASE INHIBITOR*

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**Abstract**—An epinephrine-oxidizing enzyme from bovine uterine muscle has been further purified and characterized. Adrenochrome is an intermediate in the oxidation of epinephrine by this enzyme. Adrenochrome also activates the enzyme, resulting in an autocatalytic oxidation of epinephrine. Another product of this reaction, which has been isolated and tentatively identified as a zwitterion isomer of adrenochrome, strongly inhibits actomyosin ATPase.

OXIDATION of epinephrine by an extract of bovine uterine muscle was observed earlier to result in the formation of oxidation products which inhibited uterine actomyosin ATPase activity.[1] Although the enzyme was not found in the heart, cardiac actomyosin ATPase was also inhibited by the products of the uterine-catalyzed oxidation of epinephrine.[2] Epinephrine had no effect on actomyosin ATPase activity.[1] This report describes the further purification and characterization of the epinephrine-oxidizing enzyme and the isolation and tentative identification of the ATPase inhibitor.‡

## METHODS AND MATERIALS

*Assay of epinephrine oxidation.* Oxidation of epinephrine to adrenochrome was determined by measuring the rate of absorbance change at 485 mμ per min. The substrate was *l*-epinephrine-*d*-bitartrate or *l*-epinephrine HCl. A Bausch and Lomb model 505 recording spectrophotometer equipped with an external strip chart recorder was used for these measurements. The usual procedure was to add buffer to the reaction mixtures 30 sec before epinephrine. Epinephrine was added to start the assays, and the recorder was started immediately thereafter. Chart speeds of up to 24 in./min were necessary in some cases to record the rapid absorbance changes. The cell compartment temperature was maintained at 25° except where noted. In all cases, the rates of epinephrine oxidation were corrected for the autoxidation of epinephrine under identical conditions but in the absence of enzyme.

*Purification of uterine enzyme.* Bovine uteri were collected at the abattoir as the animals were slaughtered. The tissue was chilled during transit to the laboratory,

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‡ A preliminary report of this work was presented at the 50th Annual Meeting of the Federation of American Societies for Experimental Biology, 1966.[3]

and all subsequent work with the tissue or the extracts was carried out at 5°. The separation of uterine myometrium from endometrium was begun approximately 2 hr after death of the animals. However, no change was observed in the yield or activity of the enzyme when the work was started as much as 24 hr later.

Thirty grams of uterine myometrium was minced in a Latapie mincer with 100 ml of a solution, 0·3 M in KCl, 0·15 M in $K_2HPO_4$, and 0·15 M in $KH_2PO_4$. The homogenate was centrifuged at 11,000 *g* for 30 min. The supernatant solution was diluted with 12 volumes of water and transferred to separatory funnels for collection of the precipitate. Water distilled from an all-glass apparatus after a preliminary deionization was used throughout. The precipitate was allowed to settle for about 2 hr and was then drawn off and collected by brief centrifugation at 500 *g* and dissolved with an equal volume of 2 M KCl. This material was designated as "1× precipitated protein" and was dialyzed against 1 M KCl before assays of epinephrine oxidase activity.

The 1× precipitated protein solution was purified further by dilution with water to 0·6 M KCl concentration and centrifugation at 500 *g* for 10 min to remove any precipitated protein. The supernatant solution was diluted with water to a KCl concentration of 0·1 M, and the precipitate was collected by brief centrifugation at 500 *g*. The purification steps were repeated again, and the final precipitate was dissolved in an equal volume of 2 M KCl and designated as "3× precipitated protein."

Centrifugation of the 3× precipitated protein solution at 35,000 *g* for 30 min increased the specific activity of the supernatant solution with only a small loss of active protein to the pellet. Additional centrifugation at 100,000 *g* for 1 hr caused no loss of activity in the supernatant solution.

After centrifugation at 35,000 *g* the supernatant solution was diluted with 1 M KCl and water to give a final protein concentration of 560 μg N/ml and a KCl concentration of 1 M. Uterine actomyosin which contaminated the preparation at this stage was removed by isoelectric precipitation. The solution was adjusted to pH 5·25 with 0·01 N HCl and the actomyosin was removed by centrifugation at 35,000 *g*. Most of the epinephrine oxidase was recovered in the supernatant solution which was now almost water-clear.

The supernatant protein (pH 5·25) has been fractionated on a carboxymethylcellulose* column. The protein solution was dialyzed against 0·01 M $KH_2PO_4$–NaOH buffer (pH 7·6), 0·225 M in KCl, and 5·6 ml was applied to a 12 × 1·2 cm column (2 g of cellulose exchanger) which had been previously washed with the same buffer. Elution was carried out with solutions of increasing KCl concentration. Three-milliliter fractions were collected. The fractions were dialyzed against 1 M KCl prior to assay for oxidase activity.

*Preparation of cardiac actomyosin.* The preparation of rabbit cardiac actomyosin and the assays of ATPase activity were carried out as described previously.[4, 5]

*Preparation of epinephrine derivatives.* Crystalline adrenochrome (2,3-dihydro-3-hydroxy-N-methylindole-5,6-quinone) was prepared from epinephrine (Eastman) by the method described by MacCarthy[6] and Harley-Mason.[7]

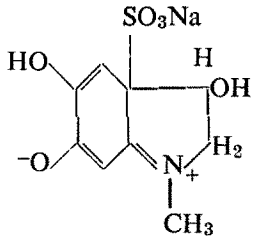
Crystalline adrenolutine (5,6-dihydroxy-N-methylindoxyl) was prepared from adrenochrome by the method of Fischer.[8]

Adrenochrome bisulfite addition compound (I) was prepared essentially as described by Tse and Oesterling.[9] To a solution of adrenochrome containing 3·2 μmoles/ml

* Cellex CM, Bio-Rad Laboratories.

was added, with constant stirring, an exactly equal number of $\mu$moles of $NaHSO_3$. The initial red solution gradually turns yellow with complete formation of the addition compound.

I




*Paper chromatography.* Ascending chromatography was carried out with the organic phase of a mixture of *n*-butanol:glacial acetic acid:water (4:1:5) as solvent.

Prior to use, Whatman 1 paper was washed with 0·5 N HCl followed by water rinses, and then washed with 0·1 % Na-EDTA solution (pH 7·4) followed by extensive rinsing with distilled water. Samples to be chromatographed were applied 3 cm from the base of the paper.

*Protein determination.* Protein N was determined by a modified biuret assay suitable for translucent actomyosin solutions.[10]

## RESULTS AND DISCUSSION

### *Route of epinephrine oxidation*

It was established that epinephrine was oxidized to adrenochrome by the uterine enzyme from measurements of the absorption spectrum of the reaction mixture in the early minutes of oxidation (Fig. 1). The reactions were stopped at the designated times by addition of acetic acid which lowered the pH to 4·7. The absorption spectra of the reaction mixtures showed excellent agreement with the absorption spectrum for crystalline adrenochrome and formed the basis for the assay of epinephrine oxidation. With extended oxidation, the spectra lost the close agreement with the adrenochrome spectrum.

### *Purification of epinephrine-oxidizing enzyme*

Table 1 summarizes the steps in the purification of the uterine enzyme. The first precipitate (1 × precipitated protein) obtained from the extract of the tissue was used as a reference, since the oxidase activity of the extract was always found to be considerably inhibited. The cause of this inhibition is not known but is presumed to be due to the protective action of the large amount of nonspecific protein on the oxidation of epinephrine. Dialysis of the extract against 1 M KCl did not alter the inhibition. Thus, purification of the enzyme per unit of protein relative to the tissue extract of the homogenate is many times that shown in Table 1.

The results of a typical chromatographic separation of the supernatant solution (pH 5·25) on a carboxymethylcellulose column are shown in Fig. 2. A fraction eluted with 0·4 M KCl, 0·01 M in $KH_2PO_4$–NaOH (pH 7·6), showed the most oxidase activity per unit of protein. The highest purification obtained for this fraction has been a 60 per cent increase over the supernatant protein (pH 5·25). This fraction comprises 4 per cent of the total activity applied to the column.

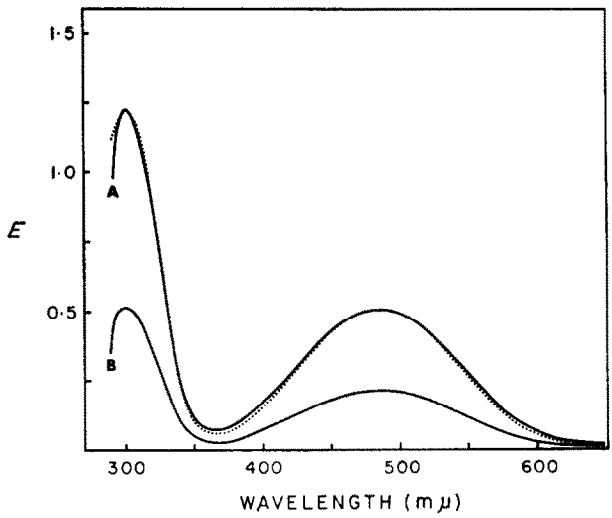



FIG. 1. Demonstration of adrenochrome as an intermediate in the oxidation of epinephrine by the uterine enzyme. A: Absorption spectrum of reaction mixture after 2 min of epinephrine oxidation. B: After 1·5 min of epinephrine oxidation. The dotted line is the absorption spectrum for crystalline adrenochrome at a concentration of 21 μg (117 mμmoles) per ml. The reaction mixtures contained, in a final volume of 1·2 ml: tris-HCl buffer, pH 9·70, 50 μmoles; KCl, 600 μmoles; enzyme (3 × precipitated protein), 107 μg N; epinephrine, 2·4 μmoles. Final pH = 8·8. At the times designated, 0·1 ml of 1 N $CH_3COOH$ was added to stop the reactions. This resulted in precipitation of the protein which was removed by centrifugation at 35,000 *g*. The absorption spectrum of the supernatant solution was recorded with, as a reference, the same reaction mixture as for the oxidized sample but with the addition of acid before epinephrine.

TABLE 1. PURIFICATION OF EPINEPHRINE-OXIDIZING ENZYME

| | Total protein N per g tissue (mg) | $\Delta E_{485}$ mμ/min per 75 μg N/ml | Relative activity | Recovery (%) |
|---|---|---|---|---|
| 1 × Precipitated protein | 1·20 | 0·076 | 1 | |
| 3 × Precipitated protein | 0·74 | 0·116 | 1·5 | 92 |
| 35,000 *g* Supernatant solution | 0·63 | 0·129 | 1·7 | 86 |
| 100,000 *g* Supernatant solution | 0·59 | 0·133 | 1·8 | 84 |
| pH 5·25 Supernatant solution (after isoelectric precipitation of actomyosin) | 0·33 | 0·223 | 2·9 | 79 |

The reaction mixtures contained, in 1·2-ml final volume: tris-HCl buffer, pH 9·70, 150 μmoles; KCl, 600 μmoles; enzyme preparation, 90 μg N; adrenochrome bisulfite addition compound, 320 mμmoles; epinephrine, 2·4 μmoles. Final pH = 9·5.

*Time course of epinephrine oxidation*

The oxidation of epinephrine by the uterine enzyme is an autocatalytic reaction (Fig. 3). The time course of the reaction is similar to that for the oxidation of tyrosine by mushroom tyrosinase.[11] The addition of catalytic amounts of adrenochrome to the reaction mixtures before the addition of epinephrine eliminates the latent period (Fig. 3). This is similar to the catalytic effect of dihydroxy-L-phenylalanine (DOPA) on tyrosinase.[11] Activation of the uterine enzyme by adrenochrome took place at a

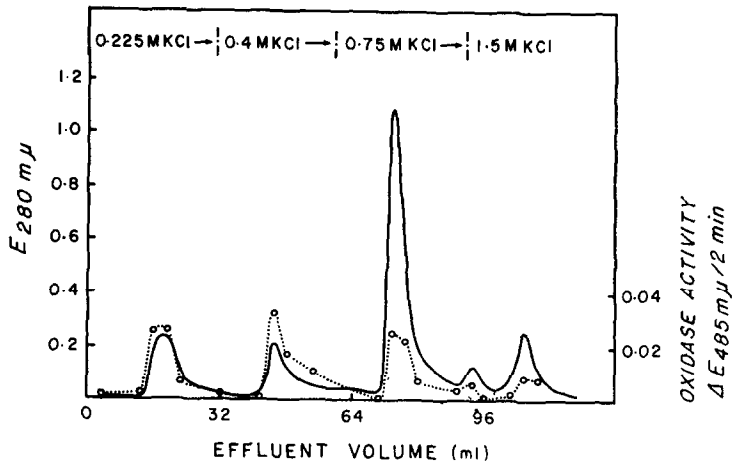



FIG. 2. Column chromatography of pH 5·25 supernatant protein on carboxymethylcellulose. The eluting solutions were 0·01 M in $KH_2PO_4$–NaOH (pH 7·6). The solid line represents absorbance of the fractions at 280 mμ. Epinephrine-oxidizing activity (○) was assayed in reaction mixtures which contained, in a final volume of 1·2 ml: tris-HCl buffer, pH 9·70, 150 μmoles; 0·65 ml of dialyzed column fraction (1 M in KCl); adrenochrome bisulfite addition compound, 320 mμmoles; epinephrine, 2·4 μmoles. Final pH = 9·5.

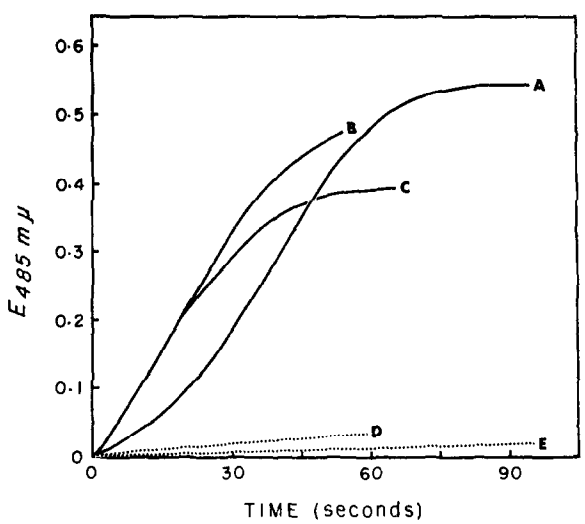



FIG. 3. Effect of added adrenochrome on the time course of epinephrine oxidation by the uterine enzyme. A: Time course of oxidation in the absence of added adrenochrome. B: Time course with addition of 96 mμmoles adrenochrome before epinephrine. C: Time course with addition of 160 mμmoles adrenochrome before epinephrine. D: Autoxidation of epinephrine in the absence of enzyme but with the addition of 120 mμmoles adrenochrome before epinephrine. E: Autoxidation of epinephrine in the absence of enzyme and with no added adrenochrome. The reaction mixtures (1·2 ml final volume) contained: tris-HCl buffer, pH 9·35, 150 μmoles; KCl, 600 μmoles; enzyme (3× precipitated protein), 90 μg N; epinephrine, 2·4 μmoles.

molar ratio of adrenochrome to epinephrine of 0·04. The adrenochrome bisulfite addition compound activated the uterine epinephrine-oxidizing enzyme to the same extent as adrenochrome. Optimal activation took place at molar ratios of the addition compound to epinephrine of 0·07–0·12. Because of its greater stability, the adrenochrome bisulfite addition compound was more convenient to use than adrenochrome.

pH *Optimum*

The pH optimum for the uterine enzyme is 9·4–9·6 (Fig. 4). Axelrod[12] has described a salivary gland enzyme that oxidizes epinephrine to adrenochrome. Optimal activity

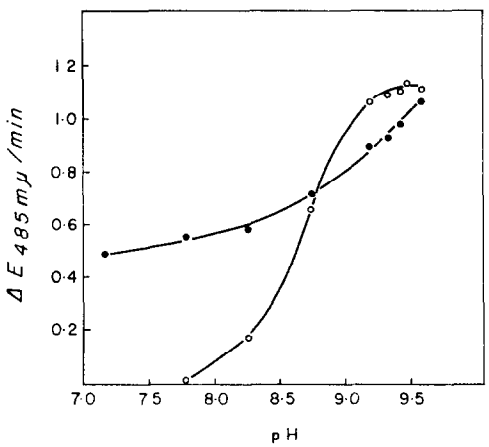



FIG. 4. Effect of pH on the oxidation of epinephrine by the uterine enzyme (○) and by mushroom tyrosinase (●). Reaction mixtures for the uterine enzyme assays (1·2 ml final volume) contained: tris-HCl buffer, 150 μmoles; KCl, 600 μmoles; enzyme (3× precipitated protein), 90 μg N; adrenochrome bisulfite addition compound, 320 mμmoles; epinephrine, 2·4 μmoles. Commercial (Sigma) mushroom tyrosinase (5 μg N) replaced the uterine protein in the tyrosinase assays. The adrenochrome bisulfite addition compound was omitted for the tyrosinase assays, as the time courses showed no latent period. The oxidation rates were corrected for the autoxidation of epinephrine at the various pH values.

for that enzyme is at pH 7. Mushroom tyrosinase also oxidizes epinephrine to adrenochrome.[13] Data for the oxidation of epinephrine by mushroom tyrosinase are presented in Fig. 4. Although both enzymes catalyze the same reaction, the activity profiles relative to pH are markedly different.

Both the uterine epinephrine-oxidizing enzyme and mushroom tyrosinase were stable over the pH range studied (Fig. 5). These data indicate that there are no pH artifacts in the curves presented in Fig. 4.

*Kinetic data*

The effect of epinephrine concentration on the velocity of the enzyme reaction followed the Michaelis–Menten relationship,[14] as shown by the data in Fig. 6. Plots of the reciprocal oxidation velocities vs. the reciprocal substrate concentrations in the Lineweaver–Burk form[15] gave straight-line relationships in the case of those reactions that were assayed in the presence of catalytic amounts of adrenochrome or the adrenochrome bisulfite addition compound. An enzyme preparation that was assayed in

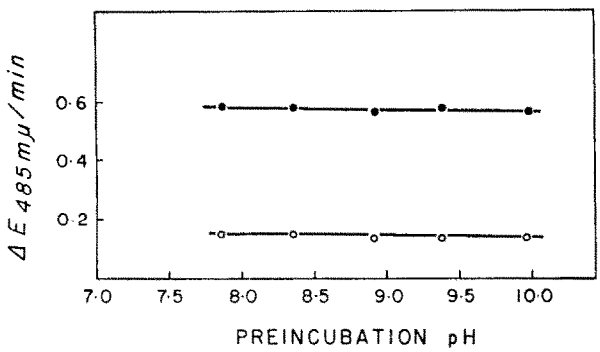



FIG. 5. Effect of preincubation of the uterine enzyme (○) and mushroom tyrosinase (●) at various pH values on the subsequent activity when assayed at pH 8·25. The uterine enzyme or tyrosinase was preincubated with 30 μmoles tris-HCl buffer in a volume of 0·4 ml for 2 min prior to addition of 120 μmoles of appropriate tris-HCl buffer to adjust the pH to 8·25. The preincubation period of 2 min was slightly more than the assay period for the data of Fig. 4. The complete reaction mixture for the assays of epinephrine-oxidizing activity at pH 8·25 was the same as for Fig. 4.

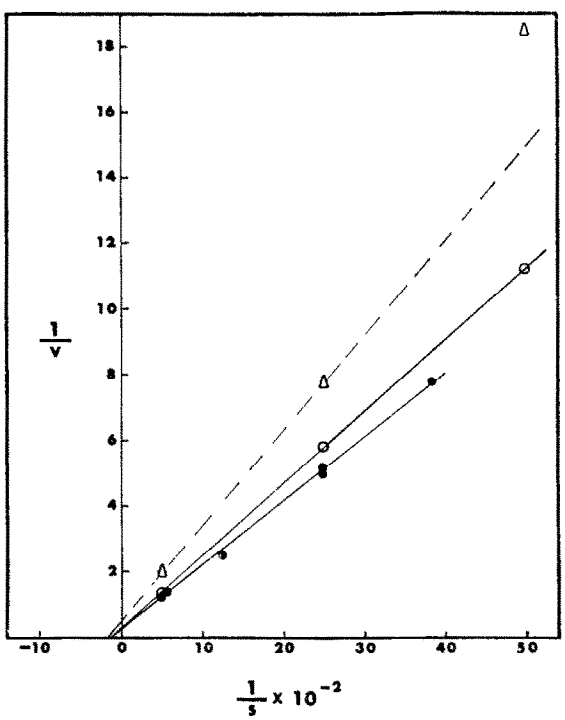



FIG. 6. Lineweaver–Burk plots of the reciprocal of the rate of oxidation of epinephrine ($\Delta E_{485}$ mμ per min) vs. the reciprocal of the molar concentration of epinephrine under the following conditions: ○, 120 mμmoles adrenochrome added before epinephrine; ●, adrenochrome bisulfite addition compound added before epinephrine (molar ratio of addition compound to epinephrine = 0·07); △, no activator added before epinephrine. The reaction mixtures (1·2 ml final volume) contained, in common, KCl, 600 μmoles; tris-HCl buffer, 150 μmoles (final pH of assays, 9·3 or 9·6). Three different enzyme preparations (3× precipitated protein) were used for the three assays. In the assays in which adrenochrome was added as activator the reaction mixtures contained 90 μg N. In the two other assays 180 μg N was used.

the absence of an activator (Fig. 6) showed a departure from linearity at the lowest substrate concentration. This observation is reproducible and is believed to result from the fact that the slow reaction at low substrate concentration does not produce adrenochrome rapidly enough to fully activate the enzyme before the reaction rate starts to decline.

The graphical analysis of the kinetic data (Fig. 6) indicates a $K_m$ of approximately $8 \times 10^{-3}$ M for the oxidation of epinephrine by the uterine enzyme at the pH optimum.

The rate of epinephrine oxidation was found to be proportional to enzyme concentration. The 3× precipitated protein fraction has been assayed at protein concentrations up to 300 μg N/ml.

*Substrate specificity of uterine enzyme*

Table 2 presents the results of tests of several related compounds as substrates for the uterine enzyme. As in the case of Axelrod's salivary gland enzyme,[12] *l*-epinephrine

TABLE 2. SUBSTRATE SPECIFICITY

| Substrate | Relative activity (%) |
|---|---|
| *l*-Epinephrine | 100 |
| *d*-Epinephrine | 90 |
| Norepinephrine | 18 |
| Isopropylarterenol | 53 |
| Tyrosine | 0 |
| DOPA | 0 |

The reaction mixtures contained, in 1·2 ml final volume: tris-HCl buffer, pH 9·70, 150 μmoles; KCl, 600 μmoles; enzyme (pH 5·25 supernatant protein), 90 μg N; substrate, 2·4 μmoles. Final pH = 9·5. The rates of oxidation of norepinephrine and isopropylarterenol were determined by comparison of the absorbance change at 485 mμ to the extinction values of the aminochromes of these compounds. The aminochromes were prepared in dilute solutions by oxidation of the catecholamines with potassium ferricyanide as described previously.[16] Assays of tyrosine and DOPA oxidation were followed at 475 mμ[11] at pH 9·5 and at pH 7.

was also the best substrate for the uterine enzyme. Both the uterine and salivary gland enzymes[12] oxidize *d*-epinephrine.

The supernatant fraction (pH 5·25) was tested for cytochrome *c* oxidase activity by procedures described by Smith,[17] but showed no activity.

The enzyme preparation also showed no peroxidase activity when assayed as described by Polis and Shmukler.[18]

*Activation and inhibition experiments*

The enzyme showed a marked temperature dependence, with optimal activity at 37° (Table 3). Preincubation of the enzyme at high temperatures resulted in complete inactivation.

Also, the enzyme was markedly inactivated by preincubation at moderately high (9·80) and low (4·17) pH. The preincubations for tests of pH sensitivity took place at room temperature or below.

As in the case of tyrosinase[19] and the salivary gland enzyme,[12] cyanide inhibited the uterine epinephrine-oxidizing enzyme; $Ca^{2+}$ stimulated the oxidation of epinephrine but $Mg^{2+}$ caused a marked inhibition. The inhibition by $Mg^{2+}$ is of interest, since the epinephrine-oxidizing enzyme from salivary gland[12] is activated almost 100 per cent by $Mg^{2+}$.

TABLE 3. ACTIVATION AND INHIBITION OF UTERINE ENZYME

| Conditions | Relative activity (%) |
|---|---|
| Enzyme assayed at 25° (control) | 100 |
| Temperature effect on activity | |
| Assay temp. 10° | 51 |
| Assay temp. 20° | 91 |
| Assay temp. 30° | 107 |
| Assay temp. 37° | 129 |
| Assay temp. 42° | 109 |
| Preheated enzyme | |
| 80°, pH 7 (20 min) | 27 |
| 60°, pH 8·5 (15 min) | 0 |
| pH Pretreatment | |
| pH 9·80, 25° (15 min) | 92 |
| pH 9·80, 25° (30 min) | 66 |
| pH 4·83, 5° (1 hr) + 25° (8 min) | 100 |
| pH 4·54, 5° (1 hr) + 25° (8 min) | 94 |
| pH 4·17, 5° (1 hr) + 25° (8 min) | 20 |
| Ion additions | |
| $1 \times 10^{-4}$ M KCN | 36 |
| $5 \times 10^{-3}$ M $MgCl_2$ | 54 |
| $5 \times 10^{-3}$ M $CaCl_2$ | 139 |
| $1 \times 10^{-4}$ M K-citrate | 103 |

The reaction mixtures were the same as for Table 2. Epinephrine was substrate for all the assays. For assays of the effect of temperature on oxidase activity, the reaction mixture also contained 320 mμmoles adrenochrome bisulfite addition compound. The addition compound was omitted in the assays testing the effect of various ions and citrate on oxidase activity.

Citrate had no influence on the enzymic oxidation of epinephrine. Ceruloplasmin, which also oxidizes epinephrine to adrenochrome, is completely inhibited by $1 \times 10^{-4}$ M citrate.[20]

Various aspects of the data presented above led to the conclusion that the oxidation of epinephrine by the uterine extract was an enzymic reaction. A progressive increase was observed in the specific activity of the active protein through a number of purifications steps which involved marked changes in ionic strength, repeated precipitation and re-solution of the protein, and extensive dialysis (Table 1). In addition to dialysis against 1 M KCl as mentioned above, the specific activity of the protein remained constant during extensive dialysis against water or phosphate buffer (pH 7·6) of varied concentrations (0·01–0·30 M). Dialysis has been carried out for periods up to 72 hr. Column chromatography of the uterine protein extract on carboxymethylcellulose yielded several protein fractions which had some variation in specific activity

(Fig. 2). The relationship observed between the concentration of substrate and the rate of oxidation of epinephrine also supports an enzymic basis for the reaction (Fig. 6). The data fitted the Michaelis–Menten equation, which has been found to describe the majority of enzyme reactions.[21] Also, the catalytic activity demonstrated lability to heat and pH in a manner typical for most enzymes (proteins).[21] The decrease in the rate of epinephrine oxidation when the assay temperature exceeded 37° (Table 3) is probably related to thermal inactivation, since preincubation of the protein at higher temperatures prior to assays at 25° demonstrated gross inactivation. Moderate extremes of pH also caused marked inactivation (Table 3).

*Inhibition of actomyosin* ATP*ase activity by oxidized epinephrine*

It was previously reported[1] that a uterine actomyosin ATPase inhibitor is formed in the oxidation of epinephrine by the uterine enzyme. Cardiac actomyosin ATPase is also inhibited by the oxidation products of epinephrine formed by the uterine enzyme.[2] The oxidation of epinephrine and separation of the products from the uterine enzyme were carried out as follows. (1) Dialysis of the uterine enzyme solution (either the 3× precipitated protein solution or the pH 5·25 supernatant solution) against water to a final KCl concentration of approximately $1 \times 10^{-5}$ M. The protein at this step was in a fine suspension. (2) Incubation of the uterine protein (150–200 $\mu$g N/ml) in a suspension containing $2{\cdot}5 \times 10^{-4}$ M *l*-epinephrine-*d*-bitartrate and $1 \times 10^{-3}$ M $NaHCO_3$. The reaction was carried out at 37° in a Warburg flask which was flushed with $O_2$ to remove dissolved $CO_2$. The resulting pH was 8·5. The reaction mixture volume was 1·0 to 6·0 ml. Oxidation was allowed to proceed until the reaction mixture reached a deep pink color (10–15 min). (3) The addition of 0·01 ml of 0·1 N HCl/ml reaction mixture produced a flocculent precipitation of the protein and stopped the reaction. (4) The contents of the flask were transferred to a centrifuge tube, the tube was flushed with a 95% $N_2$–5% $CO_2$ mixture, sealed, and centrifuged at 35,000 *g* (5°). The supernatant solution was tested on cardiac actomyosin ATPase. A time course of the inhibition is presented in Fig. 7.

The inhibition has also been studied at a constant concentration of oxidized epinephrine and increasing concentrations of actomyosin; Fig. 8 shows these results. The per cent inhibition decreased with increasing actomyosin concentration.

Crystalline adrenochrome, crystalline adrenolutine, and a highly purified preparation of the adrenochrome bisulfite addition compound did not inhibit actomyosin ATPase.

*Chromatography of oxidized epinephrine*

The supernatnant solution of epinephrine oxidation products, separated from the uterine enzyme as described above, was chromatographed on paper (butanol–acetic acid–water solvent). A 0·16-ml portion (7·7 $\mu$g) of the oxidized epinephrine solution was applied to a strip of paper 1 cm in width, under a current of 95% $N_2$–5% $CO_2$. For one experiment, DL-epinephrine-*d*-bitartrate-1-$^{14}$C (Calbiochem, 1·1 $\mu$c/$\mu$mole) was oxidized by the uterine enzyme.

After development for approximately 1·5 hr the strips were dried at room temperature with $N_2$–$CO_2$ and placed in a cylinder which was flushed for 3–4 hr with the $N_2$–$CO_2$ mixture to remove solvent vapors. A typical chromatogram (Fig. 9A) was

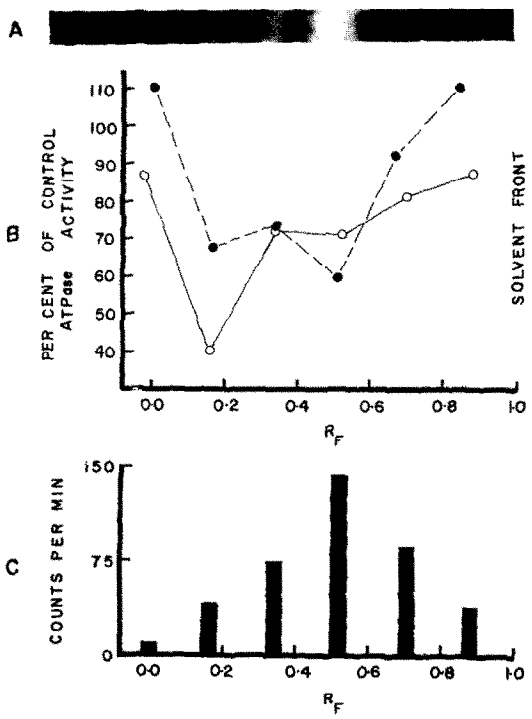



FIG. 9. A: Chromatogram of epinephrine oxidation products photographed under u.v. light. B. Effect of eluates from two chromatograms of oxidized epinephrine on cardiac actomyosin ATPase activity. ○, Results for chromatogram of $^{14}$C-epinephrine oxidation products; ●, Results with oxidation products of nonlabeled epinephrine. The reaction mixtures were the same as in the experiments described in Fig. 6 except that the actomyosin concentration was 25 $\mu$g N/2 ml of reaction mixture. C: Relative radioactivity of the eluates from the chromatogram of $^{14}$C-epinephrine oxidation products described in B, above.

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photographed while being irradiated with ultraviolet light. The chromatograms showed a small amount of u.v.-fluorescent material at the origin and distinct u.v.-fluorescent bands at approximately $R_f$ 0·16, 0·34, and 0·52. Adrenochrome is resolved at about $R_f$ 0·47. It is pink in visible light and is partially superimposed upon the u.v.-fluorescent band at slightly higher $R_f$. The compound at $R_f$ 0·16 has bright yellow fluorescence under u.v. light.

Eluates from the chromatograms were tested upon cardiac actomyosin ATPase activity (Fig. 9B). The chromatograms were cut into sections which included, as far

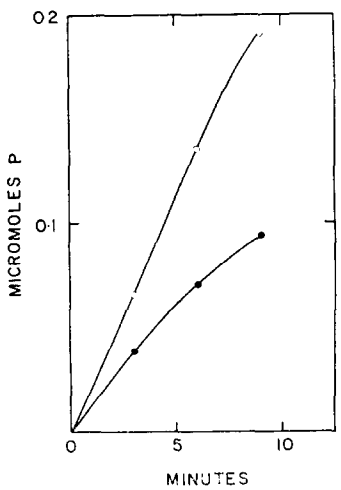



FIG. 7. Time course of the inhibition of cardiac actomyosin ATPase by the oxidation products of epinephrine formed by the uterine enzyme. The ordinate represents μmoles of phosphoric acid hydrolyzed from ATP per 2 ml of reaction mixture; ○, control ATPase activity; ●, ATPase activity in the presence of $1·6 \times 10^{-5}$ M oxidized epinephrine. The reaction mixtures for the ATPase assays contained, in a final volume of 2 ml: tris-HCl buffer, pH 7·4, 50 μmoles; KCl, 1 m-mole; cardiac actomyosin, 37·5 μg N; ATP, 5 μmoles.

as possible, single u.v. bands. The sections were eluted in individual tubes with 1·0 ml water which was flushed with 95% $N_2$–5% $CO_2$ mixture before and after addition of the paper. The paper was allowed to elute for about 2 hr in the cold. The entire eluate from each section was used in the assays. Control paper strips were carried through the entire procedure (chromatography, elution, and ATPase assay). Thus, the ATPase activity in the presence of each eluate from the experimental strip was compared to that obtained in the presence of an eluate at the same $R_f$ in the control strip. Better recovery of inhibitory activity was obtained with the relatively short chromatographic period (approximately 1·5 hr) which was used for these experiments than with prolonged development (16 hr).

The use of $^{14}C$-labeled epinephrine allowed a relative comparison of the amount of labeled compound in the various eluates from the chromatogram (Fig. 9C). The short physical length of this chromatogram (9 cm) did not permit a continuous scan of the radioactivity. However, chromatograms of oxidized epinephrine have been developed

for 16 hr when optimal recovery of inhibitory activity was not a consideration. Continuous radioactivity scans of these chromatograms show distinct peaks of radioactivity associated with the three fluorescent bands.

The ease of detection of the yellow-fluorescent compound at $R_f$ 0·16, and its inhibitory activity relative to concentration (Fig. 9B), directed our attention to further studies of this material.

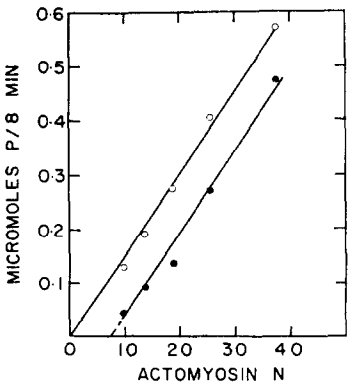



FIG. 8. Effect of concentration of actomyosin on the inhibition of ATPase activity by oxidized epinephrine; ○, phosphate liberated from ATP per 2 ml of reaction mixture (control samples); ●, hydrolysis in the presence of $1·6 \times 10^{-5}$ M oxidized epinephrine. The reaction mixtures were the same as for the experiment described in Fig. 6 except that protein concentration was varied.

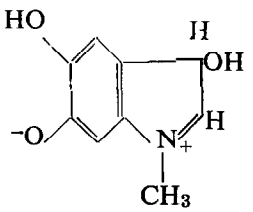
*Studies with the $R_f$ 0·16 inhibitor*

Large-scale paper chromatography was used to obtain enough of the inhibitor for spectral analysis and studies of its inhibitory effect. The reaction mixtures for the oxidation of epinephrine and the procedure for the separation of the oxidation products from the uterine protein were the same as for the small-scale chromatography. $^{14}$C-Labeled epinephrine was used in tracer amounts to allow the calculation of the molar concentration of the inhibitor. The ratio of stable epinephrine to labeled compound was 50:1. Five milliliters of the mixture of epinephrine oxidation products was applied on 30 cm of paper with $N_2$–$CO_2$ gassing and developed for 2 hr. The $R_f$ 0·16 yellow-fluorescent zone was cut out, the vapors blown off with $N_2$–$CO_2$, the strip eluted with water, and the eluate dried from the frozen state, *in vacuo*. The dried sample was reconstituted with 0·2 ml of water (flushed with $N_2$–$CO_2$) and applied to a 2-cm wide strip of paper and rechromatographed for 4 hr. A single intensely fluorescent zone was obtained at $R_f$ 0·16. This sample was handled as for the first chromatography, and the frozen, dried samples were reconstituted with water immediately before use. A strip of control chromatography paper was carried through the entire procedure and was used as a reference sample for the spectral analysis and as a control for tests of the inhibitor on cardiac actomyosin ATPase.

The absorption spectrum of the ATPase inhibitor is presented in Fig. 10. The inhibitor appears to have marked structural similarity to the adrenochrome bisulfite

addition compound. On the basis of the spectral data, structure II is suggested for the ATPase inhibitor; it must be considered tentative.

II




This structure, which presents the inhibitor as a zwitterion isomer of adrenochrome, was proposed by Harley-Mason as one of the products formed in the reduction of adrenochrome.[7] It is presumably formed by rearrangement of adrenochrome.

It should be emphasized that the adrenochrome bisulfite addition compound was not a part of the reaction mixture used for the enzymic oxidation of epinephrine prior to paper chromatography.

Table 4 presents data for the increasing inhibition of cardiac actomyosin ATPase with increasing concentrations of the inhibitor.

The effects of various metals and cysteine on the degree of ATPase inhibition are shown in Table 5. As the affinity of ATP for myosin ($1/K_m$) was increased by the metal, the inhibitory effect was decreased, suggesting a competitive component to the inhibition. Cysteine reversed the inhibitory effect by approximately 75 per cent.

On the basis of these data, a speculative schema for the mechanism of inhibition is shown in Fig. 11. It is suggested that there is a reversible binding of the inhibitor

TABLE 4. RELATION OF INHIBITOR CONCENTRATION TO ATPASE INHIBITION

| Inhibitor concentration (M) | Inhibition (%) |
|---|---|
| $2{\cdot}1 \times 10^{-6}$ | 3·6 |
| $4{\cdot}3 \times 10^{-6}$ | 18·9 |
| $8{\cdot}6 \times 10^{-6}$ | 46·2 |
| $2{\cdot}1 \times 10^{-5}$ | 100·0 |

The reaction mixtures contained, in a volume of 1 ml: tris-HCl buffer, pH 7·4, 50 μmoles; KCl, 500 μmoles; cardiac actomyosin, 18 μg N; ATP, 2·5 μmoles. Aliquots of a water solution of the frozen, dried sample of inhibitor were added to the reaction mixtures to give the desired concentrations.

to myosin sulfhydryl groups which results in the inhibition of ATPase activity. The linkage to sulfhydryl groups is postulated to be the same as in the case of the linkage to bisulfite in the formation of the adrenochrome bisulfite addition compound.

The possible physiological or pharmacological importance of the transformation of epinephrine to an actomyosin ATPase inhibitor cannot be assessed at this time.

Kety[22] has recently reviewed the question of the possible importance of oxidation of epinephrine to adrenochrome in relation to certain types of mental illness. Although the question of adrenochrome formation *in vivo* is still controverted,[22] new observations of the pyschotomimetic properties of adrenochrome have been reported.[23]

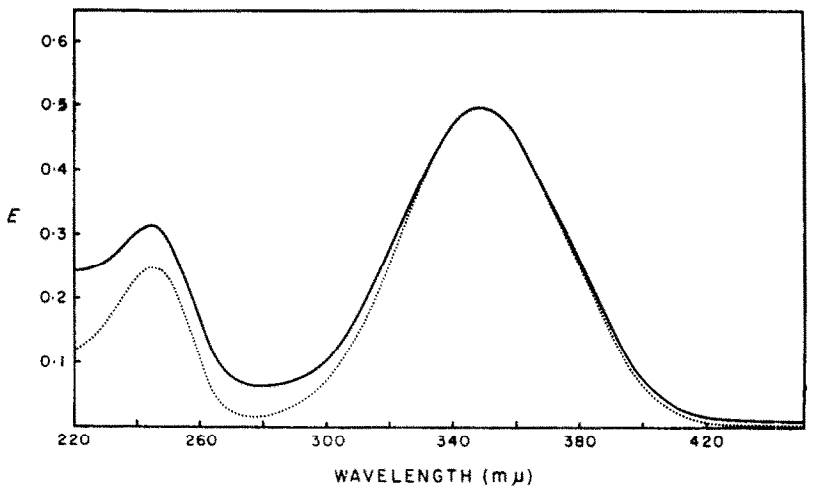



FIG. 10. The absorption spectra for the actomyosin ATPase inhibitor (solid line) and the adrenochrome bisulfite addition compound[8] (dotted line). Both spectra show maxima at 348 and 245 mμ. The spectrum for the adrenochrome bisulfite addition compound represents a concentration of $3{\cdot}4 \times 10^{-5}$ M. The concentration of the ATPase inhibitor, determined from the $^{14}C$ content of the sample, was $4{\cdot}4 \times 10^{-5}$ M. The spectrum for the inhibitor represents the final yield from one chromatogram after rechromatography. The frozen, dried eluate was reconstituted in 1·2 ml of water for the spectral measurements.

TABLE 5. EFFECT OF METALS AND CYSTEINE ON DEGREE OF ATPASE INHIBITION

| Conditions | $1/K_m$ for uninhibited enzyme | Inhibition (%) |
|---|---|---|
| 0·5 M $K^+$ | 970 | 65·5 |
| 0·5 M $K^+$<br>0·008 M $Na^+$ | 3,590 | 44·8 |
| 0·5 M $K^+$<br>0·005 M $Ca^{2+}$ | 13,700 | 26·3 |
| 0·5 M $K^+$<br>0·0001 M cysteine | | 15·5 |

The basic reaction mixtures were the same as for Table 4. The inhibitor concentration for all the assays was $8{\cdot}8 \times 10^{-6}$ M. In the test with $Na^+$, a portion of the $Na^+$ (5 μmoles/ml) was added by the use of the disodium salt of ATP for the assays in place of the potassium salt. The remainder of the $Na^+$ was added as NaCl. $Ca^{2+}$ was added as $CaCl_2$. The $K_m$ values for rabbit cardiac actomyosin ATPase activity were determined at the specified concentrations of the metals.

With the first report of the formation of an actomyosin ATPase inhibitor from epinephrine,[1] it was suggested that if oxidation of epinephrine should proceed *in vivo* in certain muscles with the formation of a derivative that inhibited the contractile apparatus, such a system would represent a mechanism for the relaxing effect of

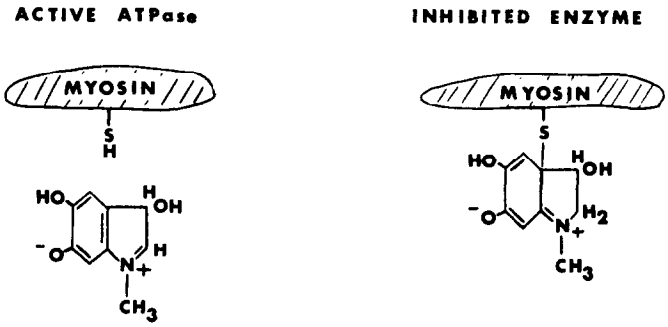



FIG. 11. Speculative schema for the mechanism of inhibition of cardiac actomyosin ATPase by the inhibitor derived from epinephrine.

epinephrine in these muscles. The new information gained from the present work concerning the purification and characterization of the uterine epinephrine-oxidizing enzyme should facilitate the study of the presence of this enzyme in other involuntary muscles. The techniques developed for isolating and concentrating the ATPase inhibitor, and the knowledge that has been gained concerning its identity, may provide a new approach to detecting oxidation of epinephrine via adrenochrome *in vivo*. In this regard it is important that the ATPase inhibitor appears to be a more stable compound than adrenochrome.

The present techniques for preparing the ATPase inhibitor will provide enough of the compound to begin studies of its pharmacological effects.

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