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CONDITIONS AFFECTING THE PURIFICATION AND SOLUTION OF A UTERINE EPINEPHRINE-OXIDIZING ENZYME*

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Abstract—Purification and characterization of a uterine epinephrine-oxidizing enzyme have been continued in the present studies. It was reported previously that the oxidation of epinephrine by this enzyme resulted in the formation of an actomyosin adenosine triphosphatase (ATPase) inhibitor. Adrenochrome was identified as an intermediate in the reaction. New data have been obtained which confirm the formation of adrenochrome in the reaction. Progressively greater disintegration of the myometrium has resulted in increased yields and purity of the enzyme. The most striking effect, however, was the separation of a water-soluble form of the enzyme through disruption of a high-purity fraction with ultrasonic sound. Yields of water-soluble enzyme with present procedures were increased approximately 200-fold over that possible with the former methods. In addition, the activity of the water-soluble protein in terms of ATPase inhibitor formation was increased approximately 9-fold. Although the uterine oxidase was soluble in water, it was found to be insoluble at physiological ionic strengths. These observations suggest that the enzyme probably exists in association with a membrane or organelle. Reference is made to the possible role of this enzyme in relation to the negative inotropic effects of epinephrine.

A RECENT report described an epinephrine-oxidizing enzyme from bovine uterine muscle which resulted in the formation of an actomyosin adenosine triphosphatase (ATPase) inhibitor from epinephrine.[1] Adrenochrome was identified as an intermediate in the oxidation of epinephrine by the uterine enzyme from spectral analyses of the reaction mixture. The ATPase inhibitor was isolated and tentatively identified as a zwitterion isomer of adrenochrome (ultraviolet absorption spectrum and tentative structure presented in reference 1).

The oxidation of norepinephrine and isoproterenol to noradrenochrome and isopropylnoradrenochrome, respectively, was also catalyzed by the uterine enzyme, while tyrosine, dihydroxy-L-phenylalanine (Dopa) and cytochrome *c* were not substrates.[1] It was reported earlier that a uterine actomyosin ATPase inhibitor was formed in the oxidation of epinephrine by the uterine oxidase.[2, 3] The more recent study concerned the inhibition of cardiac actomyosin by the ATPase inhibitor.[1]

In the present studies, confirmation of the oxidation step, epinephrine to adrenochrome, was obtained by comparison of the intermediate formed in the enzyme-catalyzed reaction and crystalline adrenochrome in three solvent systems, using paper chromatography. Also, this report describes further advances in the conditions

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for the extraction and purification of the epinephrine-oxidizing enzyme, particularly in regard to formation of the ATPase inhibitor. One of the most significant results of this work was the development of techniques for separation of a water-soluble enzyme.

The information gained from the past and present studies has made possible the design of a rapid survey procedure which combines the essential requirements for the extraction, isolation and assay of the epinephrine-oxidizing enzyme. The distribution of the epinephrine oxidase in a number of mammalian tissues, as determined by the modified procedure, is described separately.[4]

METHODS AND MATERIALS

Water distilled from alkaline $KMnO_4$ in an all-glass still, after a preliminary distillation and deionization, was used in these experiments.[5]

The uteri were stored at $-20°$ prior to use. Freezing the tissue had no apparent effect on the uterine oxidase.

Purification of uterine enzyme. The initial steps in the extraction and purification of the uterine oxidase are the same as those described in the previous paper and will only be summarized here. (1) Disintegration of the tissue with the Latapie mincer or Waring blendor using as extraction solution, 0·3 M KCl, 0·15 M in K_2HPO_4 and 0·15 M in KH_2PO_4. In all of the previous studies the Latapie mincer was used to disintegrate the tissue. The Latapie apparatus results in an extremely fine mince. The blendor was introduced in the present studies to obtain a true homogenate of the tissue. The total blending time was 50 sec. (2) After removal of the insoluble residue by centrifugation, the oxidase was repeatedly (three times) precipitated by dilution of the extract to an ionic strength of 0·1 and redissolved with 2 M KCl between each precipitation. (3) The final precipitate, dissolved with an equal volume of 2 M KCl, was centrifuged at 35,000 *g*. More than 90 per cent of the activity was shown previously to remain in the supernatant solution.[1] (4) Adjustment of the supernatant solution to pH 5·25 resulted in isolectric precipitation of uterine actomyosin which contaminated the preparation at this stage. Actomyosin was removed by centrifugation at 35,000 *g*, and again, over 90 per cent of the activity which was present before the adjustment to pH 5·25 was recovered in the supernatant solution.[1]

In the present studies, the pH 5·25 supernatant protein has been fractionated further. The pH 5·25 supernatant solution was dialyzed against water to an ionic strength of approximately 6×10^{-5} and centrifuged at 10,000 *g*. Depending on the technique used in the initial disintegration of the tissue, varying amounts of the oxidase activity were recovered in the supernatant solution, as described below. Also, it was found that the yield of water-soluble protein was markedly increased when the pH 5·25 supernatant solution was sonicated prior to dialysis against water. A Bronwill Biosonik (BPI) was used for the sonication treatments. The apparatus delivers a constant frequency of 20 kc/sec. The output was set at approximately 1 acoustical watt; the protein concentration of the solutions ranged between 220 and 240 μg N per ml. The protein solutions were sonicated in a beaker immersed in a slurry of wet ice. Thirty-sec periods of irradiation were alternated with 1-min rest periods to avoid a temperature rise in the solutions. The sonication times described in the experiments represent the total irradiation times.

Assay of ATPase inhibitor formation. Complete details were described previously

for the oxidation of epinephrine by the uterine oxidase using a buffer system (pH 8·5) of low ionic strength, which allowed direct paper chromatography of the oxidation products at the end of the assay period.[1] The only modification in the procedure was the use of 27 mm by 150 mm test tubes for reaction vessels rather than Warburg flasks. Before the assays, the enzyme preparations were dialyzed against water to a KCl concentration of approximately 6×10^{-5} M. The reaction mixtures (2–3 ml) were flushed with O_2 to remove dissolved CO_2. The flushing with O_2 and the addition of epinephrine to start the reaction were carried out by the use of hypodermic needles which pierced a Parafilm* cap that sealed the assay tube. The reactions were stopped by addition of HCl to a final concentration of 0·001 N, flushing the reaction mixture with 95% N_2–5% CO_2, and lowering the temperature to 0°. The precipitated protein was removed by centrifugation at 35,000 *g* and an aliquot of the supernatant solution was chromatographed. The above procedure was used for all experiments in which oxidation of epinephrine was followed by paper chromatography of the products (experiments pertaining to data of Tables 1–3 and Figs. 1, 2 and 4).

In experiments in which the yield of ATPase inhibitor was quantified, the oxidation period was 10·5 min. A 0·70-ml portion of the mixture of epinephrine oxidation products was applied to a strip of Whatman 1 paper, 5 cm in width, under a current of 95% N_2–5% CO_2. The preparation of the chromatography paper, the solvent system (4:1:5, butanol:acetic acid:water), and the chromatographic procedure were the same as those used before.[1] The development period was 2·5 hr; the strips were dried at room temperature with N_2–CO_2, then placed in a cylinder and flushed with N_2–CO_2 for 1 hr to remove solvent vapors. The ATPase inhibitor bands (R_f approximately 0·16, bright yellow fluorescence under u.v. light)[1] were cut out, placed in beakers and flushed for an additional 1·5 hr with N_2–CO_2. The sections were eluted for 1 hr at 5° in tubes containing 1·3 ml water, which was flushed with a 95% N_2–5% CO_2 mixture before and after addition of the paper sections. At the end of the elution period, the absorbance of the undiluted eluates was measured at 348 mμ. The concentration of the ATPase inhibitor was calculated on the basis of the spectral data we reported for this compound.[1] The yields of inhibitor presented below (data of Tables 1–3) are corrected for the small amount of ATPase inhibitor which is formed non-enzymically as a result of autoxidation of epinephrine.[6, 7] The control reaction mixtures were the same as for the experimental samples, except that the enzyme was omitted; the assay, chromatography, and elution procedures were identical. The nonenzymic formation of the inhibitor was 0·66 mμmole per ml of reaction mixture for a 10·5-min assay time.

Spectrophotometric assay of epinephrine oxidation. For one experiment described in the text below, the enzymic oxidation of epinephrine to adrenochrome was assayed at the pH optimum for the uterine enzyme by measuring the rate of absorbance change at 485 mμ, as described previously.[1] The reaction mixtures contained, in a final volume of 1·2 ml: Tris-HCl buffer, pH 9·70, 150 μmoles; KCl, 600 μmoles; enzyme (pH 5·25 supernatant protein), 90 μg N; adrenochrome bisulfite addition compound, 320 mμmoles; *l*-epinephrine-*d*-bitartrate, 2·4 μmoles. Final pH, 9·5; temperature, 25°.

The oxidation of epinephrine by the uterine enzyme was shown to be an autocatalytic reaction.[1] The addition of catalytic amounts of crystalline adrenochrome or of the bisulfite addition compound of adrenochrome to the reaction mixture before

* American Can Company, Marathon Products.

the addition of epinephrine eliminated the latent period.[1] The bisulfite addition compound does not interfere with the spectrophotometric assay of epinephrine oxidation, since it has no absorbance at 485 mμ;[8] it should be emphasized that the bisulfite addition compound was not a part of the reaction mixtures used for the chromatographic assays of ATPase inhibitor described in the preceding section. The rates of epinephrine oxidation presented below are corrected for the autoxidation of epinephrine under identical conditions but in the absence of enzyme (Δ $E_{485\ m\mu}$/min = 0·050).

Preparation of epinephrine derivatives. The methods used for the preparation of crystalline adrenochrome (2,3-dihydro-3-hydroxy-*N*-methylindole-5,6-quinone) and of the bisulfite addition compound of adrenochrome were cited previously and specific details were described.[1]

Protein determination. Protein nitrogen was determined by a modified biuret assay.[9]

RESULTS

Chromatography of epinephrine oxidation products

The formation of adrenochrome as an intermediate in the oxidation of epinephrine by the uterine enzyme was confirmed by comparison of the products of the enzyme reaction to crystalline adrenochrome using paper chromatography. The water-soluble epinephrine-oxidizing enzyme was used in this experiment. The results are shown in Fig. 1. The solvent systems used for these comparisons were 4:1:5, 4:2:4, and 4:0·2:5·8, *n*-butanol: glacial acetic acid: water. For the 4:1:5 and 4:0·2:5·8 mixtures, the organic phases were used as developing solvents with the respective aqueous phase present in a beaker in the chamber. The 4:2:4 mixture did not separate into phases. The development was ascending for all of the solvents. A zone present in each chromatogram of the enzyme products, which was fluorescent in u.v. light and bright pink in visible light, had chromatographic properties identical to crystalline adrenochrome in all three solvent systems. The R_f values for adrenochrome were 0·47, 0·66, and 0·35 for the 4:1:5, 4:2:4, and 4:0·2:5·8 solvent mixtures respectively. The u.v.-fluorescent zone seen at lower R_f values in the chromatograms of the enzyme products (Fig. 1) is the ATPase inhibitor. The R_f values for the ATPase inhibitor were 0·16, 0·52, and 0·04 for the same solvents respectively. An R_f value of 0·16 was reported previously for the ATPase inhibitor with the 4:1:5, butanol:acetic acid:water solvent system.[1] The 4:1:5 solvent was used for all of the experiments described below.

The marked catalytic effect of the water-soluble enzyme on both the oxidation of epinephrine to adrenochrome and the formation of ATPase inhibitor is demonstrated by the results presented in Fig. 2. A concentrated enzyme preparation was incubated aerobically with epinephrine for 3·5 min before the reaction was stopped and the oxidation products were chromatographed (chromatogram A). One control assay (chromatogram B) was identical except for the absence of the enzyme. A second control assay (chromatogram C) also contained no enzyme, but the nonenzymic, autoxidation of epinephrine was allowed to proceed for 30 min. Adrenochrome (R_f 0·47) and large amounts of ATPase inhibitor (R_f 0·16) were formed in the presence of the enzyme (A). The 3·5-min control assay (B) showed insignificant formation of either adrenochrome or ATPase inhibitor. In the second control assay, clearly detectable amounts of adrenochrome were formed after 30 min of autoxidation of

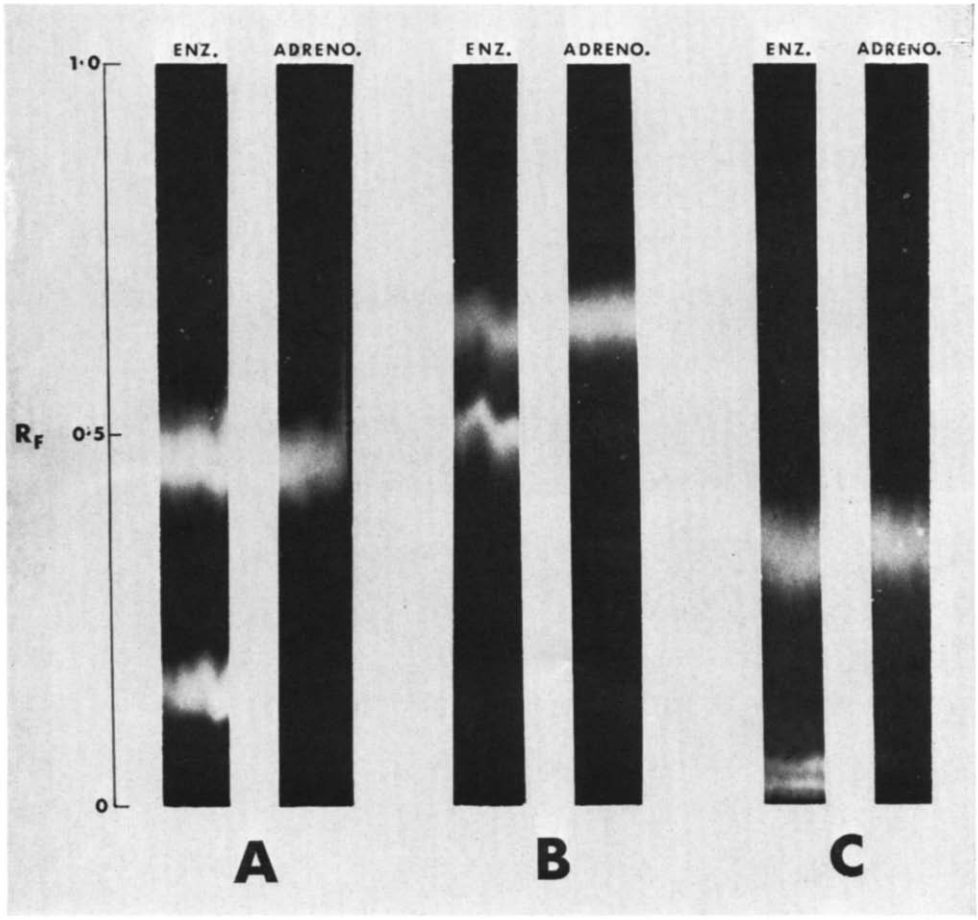

FIG. 1. Chromatograms of enzymic oxidation products of epinephrine compared with crystalline adrenochrome in three solvent systems: A, 4:1:5; B, 4:2:4; C 4:0·2:5·8 (*n*-butanol:acetic acid:water). The reaction mixtures for the enzyme assays contained, per ml: $NaHCO_3$, 1 μmole; water-soluble enzyme, 75 μg N; *l*-epinephrine-*d*-bitartrate, 0·25 μmole. Oxidation period, 7 min; temp. 37·5°; pH 8·5. Each chromatogram (1·5 cm in width) represents products from 0·2 ml of reaction mixture. Chromatograms of adrenochrome contained 1·5 μg of the crystalline compound. The chromatograms were photographed while being irradiated with u.v. light (320–400 mμ).

The double zone seen for the ATPase inhibitor (R_f 0·04) in the products of the enzyme reaction of comparison C, results from the fact that during application at the origin the products are concentrated into an upper and lower margin. Upon development, this results in the appearance of two lines at very low R_f values.

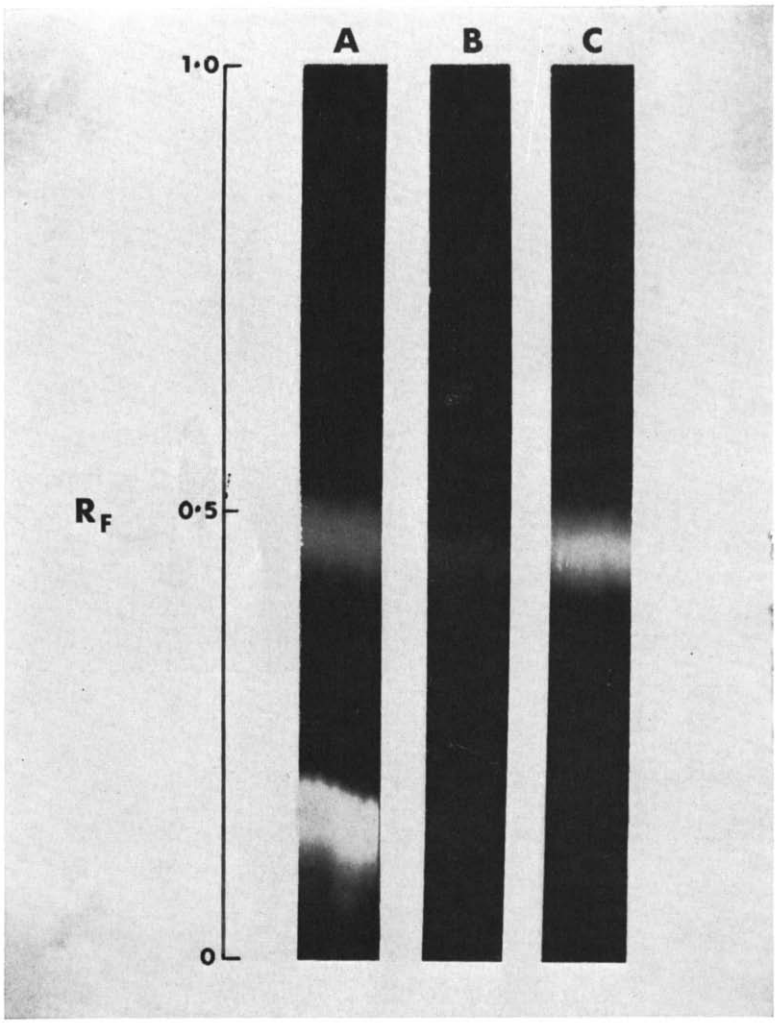

FIG. 2. Comparison of products of enzymic oxidation of epinephrine and autoxidation controls. A, chromatogram of enzymic oxidation products of epinephrine (3·5 min oxidation period); B, autoxidation control (3·5 min oxidation); C, autoxidation control (30·0 min oxidation). The reaction mixture for the enzyme assay was the same as for Fig. 1, except that the protein concentration was 200 μg N per ml. The reaction mixtures for the control assays were identical, except that the enzyme was omitted. Each chromatogram (1·5 cm in width) represents products from 0·2 ml of reaction mixture.

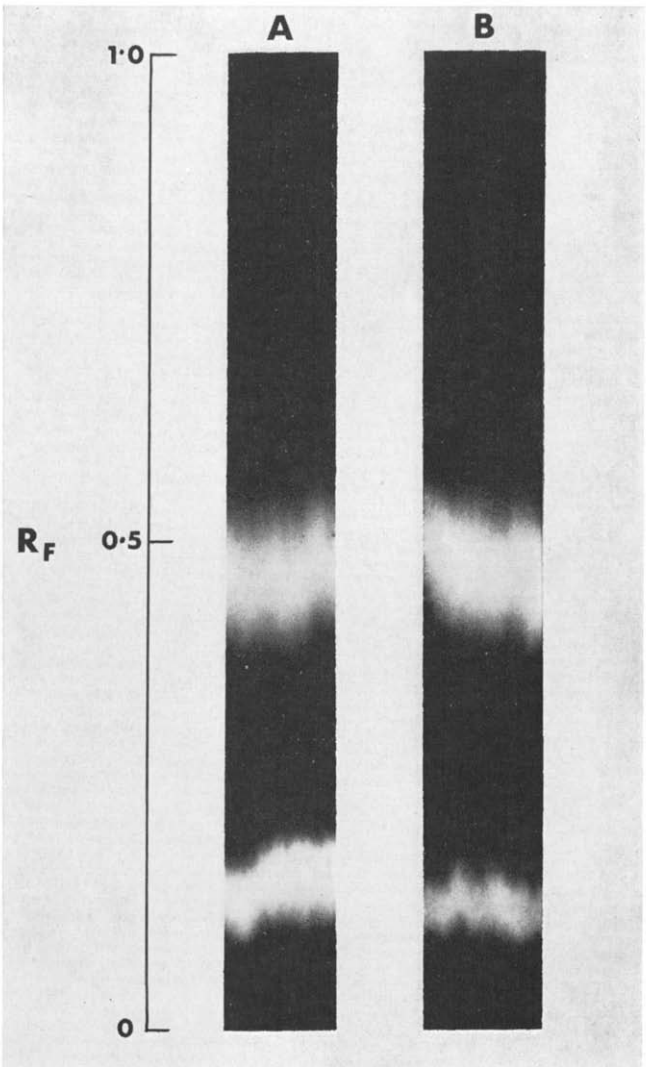

FIG. 4. Chromatograms of oxidation products of epinephrine from reaction catalyzed by water-soluble enzyme (A) and by water-insoluble enzyme (B). The reaction mixtures were the same as those described in Table 1. Each chromatogram (1·5 cm in width) represents products from 0·2 ml of reaction mixture.

epinephrine (C); however, there was insignificant isomerization of this adrenochrome to ATPase inhibitor in the absence of the uterine protein.

Enzyme extraction and purification

The effects of homogenization of the uterine muscle and sonication of the pH 5·25 supernatant fraction were compared with our former techniques in regard to the yield of protein in the fractions and the enzyme activity expressed as millimicromoles of ATPase inhibitor formed (Table 1). Tissue from the same animal was used for the

TABLE 1. EFFECT OF HOMOGENIZATION AND SONICATION ON ENZYME YIELD AND PURIFICATION*

Tissue disintegration technique	Protein yield /g muscle (μg N)	Relative yield	Inhibitor (mμmoles formed/ml reaction mixture)	Relative activity
Fractions:				
Minced tissue (Latapie mincer)				
(a) pH 5·25 supernatant protein (after isoelectric precipitation of actomyosin)	172·2	1·0	4·9	1·0
(b) water-soluble fraction	0·9	1·0	†	
Homogenized (Waring blendor)				
(a) pH 5·25 supernatant protein	266·4	1·6	19·9	4·1
(b) water-soluble fraction	38·4	42·7		
(c) water-soluble fraction after sonication of "a"	173·0	192·2	46·1	9·4

* The reaction mixtures for ATPase inhibitor formation contained, per ml: $NaHCO_3$, 1 μmole; enzyme, 75 μg N; *l*-epinephrine-*d*-bitartrate, 0·25 μmole. Oxidation period, 10·5 min; temp., 37·5°; pH, 8·5.

† Insufficient protein for assay.

comparison of mincing with the Latapie apparatus versus homogenization. The yield and activity of the pH 5·25 supernatant protein were increased as a result of the homogenization procedure. The most striking increases were seen, however, in the yield and activity of a clear water-soluble fraction of the enzyme.

The effect of disintegration of the tissue on enzyme yield was further emphasized by the results of another experiment in which uterine muscle from one animal was minced with scissors as compared with blending the tissue. Mincing the tissue with scissors resulted in a much coarser mince than that produced with the Latapie mincer. The yield of pH 5·25 supernatant protein for the minced tissue was 76 μg N/g muscle, while the yield of protein in the same fraction derived fromt he blended tissue was 343 μg N/g. Although the tissue used in this experiment was from a different animal, a comparison of these data with those presented in Table 1 clearly shows the progressive increase in yield of purified protein with increasing degree of disintegration of the tissue.

The effect of sonication time on the yield of water-soluble enzyme from the pH 5·25 supernatant protein is demonstrated by the data presented in Fig. 3. On the basis of these data, a 5-min sonication period was used routinely.

Table 2 summarizes our findings on the yield and activity of water-soluble enzyme from several uterine preparations. The muscle was homogenized for all of these

preparations. With one preparation, the homogenate was sonicated prior to the purificati on procedure. The difficulties encountered in the sonication of a concentrated homogenate and the long period of sonication required (approx. 1 hr) did not recommend this procedure over the brief sonication of the pH 5·25 supernatant

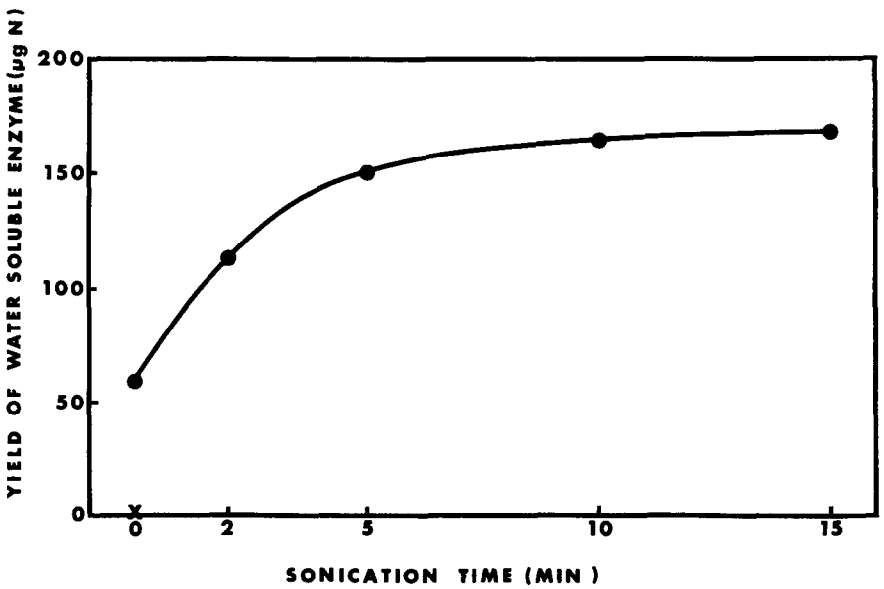

FIG. 3. Effect of sonication of the pH 5·25 supernatant protein on the yield of water-soluble enzyme per gram of muscle. ●, yields of soluble enzyme with varying sonication times; ×, yield of water-soluble enzyme from the same tissue using previous procedures[1] (disintegration of muscle with Latapie mincer; no sonication).

TABLE 2. SUMMARY OF YIELDS OF WATER-SOLUBLE ENZYME AND ACTIVITY*

Preparation	Yield of water-soluble enzyme/g muscle (μg N)	Inhibitor (mμmoles formed/ml reaction mixture)	Activity ratio Water-soluble enzyme/ pH 5·25 super. fraction
1†	38·4		
	173·0‡	46·1	2·3
2	214·9§	18·3	1·5
3	83·4	17·6	2·1
4	151·7‡	41·5	not compared
5‖	59·2	not	
	149·6‡	assayed	
6	78·2	not	
		assayed	
7	151·6‡	not	
		assayed	

* The reaction mixtures and assay conditions were the same as those described in Table 1
† Data from Table 1
‡ Sonication of pH 5·25 supernatant solution for 5 min prior to fractionation.
§ In this preparation, the homogenate was sonicated prior to the purification procedure.
‖ Data from Fig. 3

protein (see Fig. 3). Yields of water-soluble enzyme without sonication ranged from 38·4 to 83·4 μg N per g of muscle. With sonication, yields of water-soluble enzyme ranged from 149·6 to 214·9 μg N per g of muscle.

Solubility characteristics of water-soluble enzyme

It is indicated in the Methods section that the water-soluble enzyme was separated from an insoluble precipitate after the pH 5·25 supernatant solution was dialyzed to an ionic strength of approximately 6×10^{-5}. The water-soluble enzyme has been dialyzed against repeated changes of water to an ionic strength of 6×10^{-11} with no change in the solubility of the protein. It was also found that the enzyme could be quantitatively recovered from its water solution by addition of solid KCl to yield an ionic strength of 0·1. Thus, the enzyme is soluble at the relatively high ionic strengths used in the extraction and is soluble in water, but it is insoluble at physiological ionic strengths.

Comparison of water-soluble and water-insoluble enzymes

The rate of inhibitor formation with oxidation of epinephrine by the water-soluble enzyme was compared with the amount formed in the presence of the water-insoluble precipitate. The pH 5·25 supernatant solution was dialyzed against water as described above (see Methods). After centrifugation at 10,000 *g* and removal of the supernatant solution containing the water-soluble enzyme, the precipitate was washed with the dialysate, recovered by centrifugation at 10,000 *g*, and resuspended in water. A comparison of inhibitor formation by the water-soluble and water-insoluble enzymes is demonstrated by the chromatograms presented in Fig. 4. The fluorescent zone at an R_f of approximately 0·16 is the ATPase inhibitor.[1] The adrenochrome band is seen at a higher R_f (0·47).

The greater formation of ATPase inhibitor by the water-soluble enzyme compared with the precipitate enzyme is obvious from the chromatograms (Fig. 4). In a separate experiment the inhibitor formation was quantified (Table 3). The protein concentration

TABLE 3. ADRENOCHROME AND ATPase INHIBITOR FORMATION BY WATER-SOLUBLE AND WATER-INSOLUBLE ENZYME FRACTIONS

Enzyme preparation	Adrenochrome eluted at R_f 0·47* (mμmoles)	ATPase inhibitor eluted at R_f 0·16* (mμmoles)	Totals (mμmoles)
Water-soluble fraction	13·8	12·8	26.6
Water-insoluble fraction	18·3	8·1	26·4

* The values represent the total amounts of compound eluted from the chromatograms. The volume of reaction mixture applied to each chromatogram was 0·70 ml. The reaction mixtures and assay conditions were the same as those described in Table 1.

in each case was 75 μg N per ml. The adrenochrome bands at R_f 0·47 were also eluted in this experiment and the concentration of adrenochrome was estimated by absorbance measurements at 485 mμ and by comparison with spectral data for crystalline adrenochrome.[1] A smaller amount of adrenochrome was present in

association with a greater formation of ATPase inhibitor in the reaction catalyzed by the water-soluble enzyme. These results may be interpreted as an indication of a more rapid isomerization of adrenochrome to the ATPase inhibitor by this fraction.

Other evidence has been obtained for variations with different preparations in the relative rates of oxidation of epinephrine to adrenochrome and isomerization of adrenochrome to the ATPase inhibitor. The pH 5·25 supernatant protein fractions for Latapie-minced vs. homogenized tissue (Table 1) showed 4·9 vs. 19·9 mμmoles of inhibitor formed per ml of reaction mixture for a 10·5-min incubation period. These same fractions were assayed spectrophotometrically for rate of oxidation of epinephrine to adrenochrome at the pH optimum for the oxidase reaction (see Methods). The $\Delta E_{485\ m\mu}$ per min values were 0·270 and 0·129 for the protein derived from Latapie-minced and homogenized tissue respectively. Thus, the enzyme from Latapie-minced tissue showed a higher oxidase activity compared with the same fraction from homogenized tissue, but a much lower rate of ATPase inhibitor formation.

DISCUSSION

Spectral data were presented in an earlier report which demonstrated that the absorption spectrum of the reaction mixture, in the early seconds of oxidation of epinephrine by the uterine oxidase, was identical to that of crystalline adrenochrome.[1] Confirmation of adrenochrome formation in the reaction was obtained in the present studies by chromatographic identification of the intermediate using three solvent systems (Fig. 1).

The evidence for the enzymic nature of the oxidation of epinephrine to adrenochrome by the uterine enzyme was also presented in the earlier report. Kinetic data for the effect of epinephrine concentration on the velocity of the reaction followed the Michaelis–Menten relationship; the K_m for the oxidase reaction at the pH optimum was approximately 8×10^{-3} M. Similar data have not yet been obtained for the isomerization of adrenochrome to the ATPase inhibitor. However, the catalytic effect of the uterine protein on this reaction is clearly seen in the results presented in Fig. 2. Although adrenochrome was formed in the prolonged autoxidation of epinephrine in the control assay (chromatogram C), this adrenochrome did not spontaneously isomerize to ATPase inhibitor to any significant extent. In marked contrast, the incubation of epinephrine with the water-soluble enzyme (chromatogram A) showed rapid formation of adrenochrome and ATPase inhibitor.

The relationship of the isomerization reaction to specific characteristics of the uterine protein is also demonstrated by certain aspects of the purification data. In the experiments which compared the activities of the water-soluble and water-insoluble forms of the epinephrine-oxidizing enzyme (Table 3 and Fig. 4), oxidation of epinephrine to adrenochrome by the water-insoluble enzyme was at least as great as that observed with the water-soluble enzyme, but the water-soluble enzyme was considerably more active in catalyzing the isomerization reaction. In another experiment, described in the text, the pH 5·25 supernatant fractions derived from Latapie-minced and homogenized tissue were compared for rate of oxidation of epinephrine to adrenochrome. The enzyme from minced tissue showed more oxidase activity than the enzyme from homogenized tissue, but only one-fourth as much ATPase inhibitor formation (Table 1).

It is apparent from all of these results that the formation of ATPase inhibitor is related to catalytic properties of the uterine enzyme and is not the simple consequence of the generation of adrenochrome in the reaction mixtures.

The proposed reaction sequence for the oxidation of epinephrine by the uterine enzyme is presented in Fig. 5. The structure for the ATPase inhibitor is that which was tentatively proposed,[1] and was based on spectral data.

Additional findings of the present studies concern the importance of the degree of disintegration of the tissue and the value of ultrasonic disruption of the extracts on the yield and activity of the uterine epinephrine-oxidizing enzyme. In the previous study, the pH 5·25 supernatant fraction remaining after isoelectric precipitation of

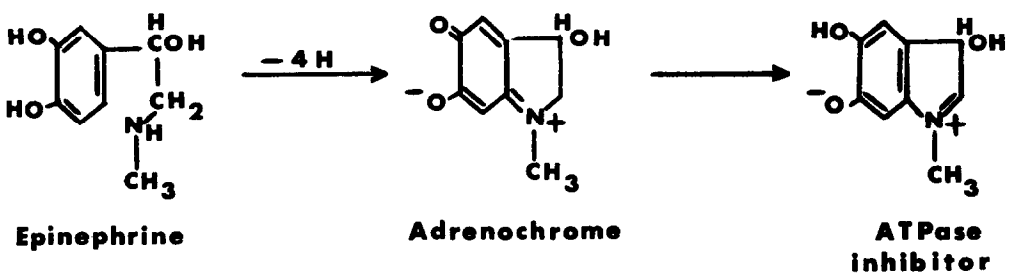

FIG. 5. Structural formula sequence for the oxidation of epinephrine by the uterine enzyme.

uterine actomyosin was the most useful state of purification of the enzyme in terms of yield and activity.[1] The homogenization of the tissue in the present studies resulted in a 60 per cent increase in the protein yield of this fraction and an approximately 4-fold increase in ATPase inhibitor formation per unit of protein (Table 1).

The most marked increases, however, were seen in the yield and activity of a water-soluble form of the enzyme which was of insignificant quantity from minced tissue. Homogenization of the tissue, coupled with brief sonication of the pH 5·25 supernatant protein (Fig. 3), resulted in an almost 200-fold increase in the yield of water-soluble protein. Formation of ATPase inhibitor by the water-soluble enzyme per unit of nitrogen was increased more than 9-fold (Table 1). The availability of a water-soluble enzyme should facilitate further characterization of its enzymic and physicochemical properties.

A property of the enzyme described in the text, that of its insolubility at ionic strengths of approximately 0·1, provides a convenient means for concentrating the protein and also appears to have implications regarding its localization in the tissue. The fact that the degree of disruption of the tissue progressively increases the yield of the water-soluble enzyme, together with the observation that the enzyme is insoluble at physiological ionic strengths, would indicate that the oxidase is not soluble in the cytoplasm, but probably exists in association with a membrane or organelle.

Data were presented (Fig. 4, Table 3 and text) which pointed out the variations we have observed, with different enzyme fractions, in the relative rates of the oxidation of epinephrine to adrenochrome and isomerization of adrenochrome to the ATPase inhibitor. Under no conditions have we observed complete separation of the two activities; however, this is still a possibility.

Our observations of varying ratios of two activities do not necessarily indicate two enzymes. As an example, a considerable body of information exists relative to the enzyme, tyrosinase, which indicates that its two enzymic activities, tyrosine hydroxylase and catecholase, are related to two different active sites of one enzyme.[10] Also, tyrosinase from several plant species and human melanoma tyrosinase have all been shown to occur in multiple forms having varying ratios of monophenolic and diphenolic activities. Electrophoretic evidence has been obtained which suggests that combinations of unlike subunits (isoenzymes) of tyrosinase in various proportions may be responsible for the different ratios of the two enzymic activities.[11] There may be a similar basis for our observations with the uterine epinephrine oxidase. This comparison with tyrosinase is of particular interest, since it was pointed out previously that the uterine enzyme was analogous to tyrosinase in another characteristic. Both tyrosinase and the uterine oxidase show autocatalytic reactions. Dopa catalyzes the oxidation of tyrosine by tyrosinase, and adrenochrome catalyzes epinephrine oxidation by the uterine enzyme.[1]

The results of the present study, which have demonstrated the presence of substantially larger amounts of the epinephrine-oxidizing enzyme than were previously recognized, have enforced our interest in the hypothesis, which was previously suggested,[1, 2] that oxidation of epinephrine to an actomyosin ATPase inhibitor in certain smooth muscles may represent the mechanism or a part of the mechanism for its relaxing effect in these muscles.

This hypothesis is supported by our recent findings, reported separately,[4] which concern the distribution of the epinephrine-oxidizing enzyme in various mammalian tissues.[4] Without exception, smooth muscles which are classically inhibited by epinephrine (e.g. rat, rabbit and guinea pig ileum; nonpregnant rat and guinea pig uterus; guinea pig tracheal muscle) contained appreciable to large amounts of the epinephrine oxidase; muscles which show either a positive inotropic response to epinephrine or no response contained comparatively small amounts of the enzyme (e.g. rat, rabbit and guinea pig myocardium; nonpregnant rabbit uterus; rabbit skeletal muscle).

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REFERENCES

1. M. A. INCHIOSA, JR., *Biochem. Pharmac.* **16**, 329 (1967).
2. M. A. INCHIOSA, JR. and N. L. VANDEMARK, *Proc. Soc. exp. Biol. Med.* **97**, 595 (1958).
3. M. A. INCHIOSA, JR., *Fedn Proc.* **17**, 77 (1958).
4. M. A. INCHIOSA, JR. and I. B. RODRIGUEZ, *Biochem. Pharmac.* **18**, 2032 (1969).
5. W. J. O'BRIEN and W. GARNER, *Chemist Analyst* **54**, 57 (1965).
6. M. A. INCHIOSA, JR., *Semi-Annual Report of Biological and Medical Research Division*, Argonne National Laboratory, ANL-5916, 133 (1958).
7. M. A. INCHIOSA, JR. and A. S. FREEDBERG, *Fedn Proc.* **23**, 562 (1964).
8. R. L. TSE and M. J. OESTERLING, *Clinica chim. Acta* **8**, 393 (1963).
9. M. A. INCHIOSA, JR., *J. Lab. clin. Med.* **63**, 319 (1964).
10. D. W. BROOKS and C. R. DAWSON, in *Biochemistry of Copper* (Eds. J. PEISACH, P. AISEN and W. E. BLUMBERG), p. 343. Academic Press, New York (1966).
11. R. L. JOLLY, JR. and H. S. MASON, *J. biol. Chem.* **240**, PC 1489 (1965).