of ammonium acetate. Losses of activity occurred in all the CM-cellulose columns used and these could have been due to irreversible adsorption.

It was surprising to find that bradykinin contained magnesium after passage twice through CM-cellulose. Accurate data are not available for the magnesium content of pure bradykinin obtained from EDTA-treated columns, owing to the relatively large amount needed for flame-spectrographic analysis. Qualitative results show that it is still present in the purest material, although in decreased amount compared with P5 prepared without the aid of EDTA.

Recent work has shown (Elliott, Horton & Lewis, 1960) that bradykinin exerts all the actions which have been attributed to one or another of the plasma kinins (smooth-muscle stimulation, vasodilatation, increased capillary permeability, pain production), but the question whether or not the plasma kinins constitute several biologically active peptides or a single peptide (bradykinin) must remain open until other plasma kinins have been isolated and their structures determined.

SUMMARY

1. Bradykinin was formed by trypsin digestion of the fraction of ox-serum proteins which precipitated between 33 and 45 % of saturation with ammonium sulphate.
2. Bradykinin was isolated in the pure state by application of the techniques of countercurrent distribution, chromatography on carboxymethyl-cellulose and paper electrophoresis.
3. Bradykinin contained the five amino acids

glycine, serine, proline, phenylalanine and arginine in the molar proportions 1:1:3:2:2 and one of the arginine residues was *N*-terminal.

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REFERENCES

- Andrade, S. O. & Rocha e Silva, M. (1956). *Biochem. J.* **64**, 701.
 Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
 Durrum, E. L. (1950). *J. Amer. chem. Soc.* **72**, 2943.
 Elliott, D. F., Horton, E. W. & Lewis, G. P. (1960). *J. Physiol.* **153**, 473.
 Elliott, D. F., Lewis, G. P. & Horton, E. W. (1960). *Biochem. biophys. Res. Commun.* **3**, 87.
 Frey, E. K., Kraut, H. & Werle, E. (1950). *Kallikrein (Padutin)*. Stuttgart: Ferdinand Enke.
 Gaddum, J. H. & Horton, E. W. (1959). *Brit. J. Pharmacol.* **14**, 117.
 Hilton, S. M. & Lewis, G. P. (1956). *J. Physiol.* **134**, 471.
 Holdstock, D. J., Mathias, A. P. & Schachter, M. (1957). *Brit. J. Pharmacol.* **12**, 149.
 Jepson, J. B. & Smith, I. (1953). *Nature, Lond.*, **172**, 1100.
 Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
 Lewis, G. P. (1958). *J. Physiol.* **140**, 285.
 Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
 Mathias, A. P. & Schachter, M. (1958). *Brit. J. Pharmacol.* **13**, 326.
 Mills, G. L. (1952). *Biochem. J.* **50**, 507.
 Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
 Rocha e Silva, M., Beraldo, W. T. & Rosenfeld, G. (1949). *Amer. J. Physiol.* **156**, 261.
 Werle, E. (1953). *Arch. int. Pharmacodyn.* **92**, 427.

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Preparation of Purified 3-Hydroxyanthranilic Acid Oxidase from Rat and Ox Liver

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Hepatic 3-hydroxyanthranilic acid oxidase is an enzyme which has been studied hitherto without any previous and proper purification, except the only consistent attempt by Stevens & Henderson (1959), who obtained a fourfold purification from an extract of acetone-dried powder of ox liver.

The present work deals with the conditions for

obtaining higher purification of the oxidase. The specific activity we have obtained is 50-fold that obtained by Stevens & Henderson (1959). Some properties of the purified enzyme are also reported.

MATERIALS AND METHODS

Chemicals. 3-Hydroxyanthranilic acid (Hoffman-La-Roche, Switzerland), ethanethiol (Schuchardt, Germany), γ -collidine (Merck, Germany); other products were obtained

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as chemically pure compounds from local commercial sources. Ammonium sulphate had been recrystallized from solutions containing 1% of ethylenediaminetetraacetate. Glass-distilled water was used.

Activity test. The 3-hydroxyanthranilic acid-oxidase activity was measured spectrophotometrically by following the increase in extinction at 360 m μ (E_{360}) due to the formation of the primary oxidation product (Bokman & Schweigert, 1951). All measurements were carried out in 0.033M-collidine buffer prepared according to Gomori (1946) and at room temperature. The temperature is indicated in the graphs; usually it was about 20°.

Definition of activity. The activity unit used by Stevens & Henderson (1959) is the amount giving an initial rate such that the increase in E_{360} is 0.01/min. in 3 ml. of an incubation mixture containing 0.1 μ mole of substrate. We preferred, however, to use a different unit based on μ m-moles of primary oxidation product formed/min. in the incubation mixture; for this compound we assumed a molar extinction coefficient of 4.7×10^4 at 360 m μ , as reported by Wiss & Weber (1958). This enzyme unit corresponds to 1.56 Stevens & Henderson (1959) units.

The specific activity is given as units/mg. of protein. Proteins were determined by the biuret method according to Gornall, Bardawill & David (1949).

Apparatus. Spectrophotometric and spectrofluorometric measurements were carried out in a Beckman model DK 2 spectrophotometer and in a Farrand double-grating spectrofluorometer respectively.

Extraction of the enzyme. Previous studies on 3-hydroxyanthranilic acid oxidase were carried out either on homogenates (Schweigert & Marquette, 1949; Priest, Bokman & Schweigert, 1951; Rabinowitz, Fineberg & Greenberg, 1953) or on acetone-dried-powder extracts (Bokman & Schweigert, 1950; Long, Hill, Weinstock & Henderson, 1954; Mehler, 1956; Stevens & Henderson, 1959). We observed, however, that it is more convenient to start purification from the supernatant of cell fractions. Rat- and ox-liver homo-

genates show a specific activity of about 20 whereas the cytoplasmic fraction shows a specific activity of about 100 for rat liver, ox liver being slightly less active. The presence of the 3-hydroxyanthranilic oxidase in the cytoplasmic fraction was also reported by Singal, Sydenstricker & Littlejohn (1953).

Rat-liver homogenates in 0.25M-sucrose were obtained and fractionated according to Schneider (1948). After the mitochondria had been separated at 24 000 g according to Schneider's method, the supernatant was acidified to pH 5.0 by the dropwise addition of 0.1N-HCl with constant stirring at 0°. The resultant precipitate was centrifuged at 24 000 g for 20 min. The supernatant was our 'cytoplasmic fraction'.

Protection of the enzyme. The generally reported instability of 3-hydroxyanthranilic acid oxidase caused the failure of all previous purification attempts (Mehler, 1956; Stevens & Henderson, 1959). Table 1 shows that the removal of oxygen, presence of substrate, addition of Fe²⁺ ions and ethanethiol prevent enzyme inactivation. The activating role of Fe²⁺ ions and reduced glutathione was previously reported by Stevens & Henderson (1959).

Consequently, the purification of 3-hydroxyanthranilic acid oxidase was performed in the presence of 0.1 mM-FeSO₄, mM-ethanethiol, 0.2–0.5 mM-3-hydroxyanthranilic acid and in the absence of molecular oxygen.

Purification procedure. Table 2 shows a typical purification procedure in detail. It appears that the precautions described above result in a good yield, the increase in total units being due both to good preservation of the enzyme and the removal of inhibitory substances.

The maximal specific activity was 2700; the purification obtained was 40-fold as compared with the activity of the cytoplasmic fraction.

Heating was performed in a water bath at 55° for 5 min. at pH 6.4, in the absence of oxygen and in the presence of the mentioned protecting substances. Acetone precipitation was carried out at a temperature below 0°, the fraction

Table 1. *Protective effect of various substances on 3-hydroxyanthranilic acid-oxidase activity*

Rat-liver homogenate was kept at 4° for 7 hr., in 0.033M-collidine buffer, pH 6.5, with or without additions. The 3-hydroxyanthranilic acid-oxidase activity at zero time was 480 units/ml. Reported values are expressed in terms of activity/ml. Temperature of the test, 20°. +, Addition; –, no addition.

N ₂	3-Hydroxyanthranilic acid	Ethanethiol	FeSO ₄	O ₂	Units/ml.
–	–	–	–	–	430
+	–	–	–	–	540
–	+	–	–	–	310
–	+	+	–	–	400
–	+	+	+	–	960
+	+	+	+	–	1090
–	–	–	–	+	310

Table 2. *Purification procedure of 3-hydroxyanthranilic acid oxidase from ox liver*

Step	Units/ml.	Volume (ml.)	Total units	Proteins (mg./ml.)	Total proteins (mg.)	Specific activity
Cytoplasmic fraction	480	260	125 000	7.7	2 000	60
After heating at 55° for 5 min. (pH 6.4)	740	250	185 000	5.5	1 380	130
After acetone treatment	2 440	89	218 000	3.6	317	680
After (NH ₄) ₂ SO ₄ precipitation	10 110	27.5	279 000	4.1	119	2 470

precipitated between 45 and 60 % being collected. Dialysis was subsequently performed for 8 hr. at 4° in the absence of oxygen, against 0.033 M-collidine buffer, pH 6.5, containing 0.1 mM-FeSO₄ and mM-ethanethiol.

Ammonium sulphate fractionation was performed by collecting the precipitate obtained between 35 and 60 % saturation. A second 8 hr. dialysis followed, done under the same conditions as above.

Since this enzyme preparation was sometimes observed to contain traces of reduced haemoglobin, a precipitation procedure with ethanol and chloroform (Tsuchihashi, 1923) was often included after the heating step. The precipitation of haemoglobin was then followed by centrifuging and acetone precipitation.

The ethanol-chloroform step generally caused an increase in specific activity, though the final values were not higher than those given in Table 2.

RESULTS

Reaction rate and 3-hydroxyanthranilic acid concentration. By plotting the reciprocal of $\Delta E_{360}/\text{min.}$ against the reciprocal of the molarity of 3-hydroxyanthranilic acid (Fig. 1) it appears that the relationship between 3-hydroxyanthranilic acid-oxidase activity and substrate concentration cannot be expressed simply in terms of the Michaelis & Menten (1913) equation. Hence, K_m values cannot be calculated from this plot. However, Fig. 2 shows that a plot of $[S]/v$ versus $[S]$ (as according to Haldane & Stern, 1932) gives

Fig. 1. Relationship between 3-hydroxyanthranilic acid-oxidase activity and 3-hydroxyanthranilic acid concentration. Lineweaver & Burk (1934) reciprocal plots. Temperature 21-22°.

Fig. 3. Relationship between 3-hydroxyanthranilic acid-oxidase activity and oxygen pressure in the incubation mixture. Oxidase activity is expressed as $\Delta E_{360}/90 \text{ sec.}$ Temperature 20-21°.

Fig. 2. The experimental values of Fig. 1 in a plot $[S]/v$ versus $[S]$ (according to Haldane & Stern, 1932).

Fig. 4. Absorption spectrum of the purified 3-hydroxyanthranilic acid-oxidase solution (enzyme concentration, 1 mg./ml.).

increased accuracy in a region where the Lineweaver & Burk (1934) plot is least accurate. From Fig. 2 a value of $7 \mu\text{M}$ can be obtained for K_m .

Reaction rate and oxygen partial pressure. Fig. 3 shows the effects of oxygen partial pressure on the reaction rate measured as ΔE_{360} in 90 sec. Different oxygen pressures were obtained by equilibrating the reaction mixture with different gaseous mixtures before the addition of the enzyme, according to the method used by Boeri & Tosi (1954) in the study of the auto-oxidation of cytochrome *c*. The shape of the curve seems to indicate an inhibition by excess of oxygen.

Spectra of purified enzyme solutions. Fig. 4 shows the spectrum of a solution of purified 3-hydroxyanthranilic acid oxidase. An absorption band at $287 \text{ m}\mu$ (E 0.910) and another at $400 \text{ m}\mu$ (E 0.520) are evident. The solution is free from haem groups since it fails to give any haemochromogen spectrum with pyridine and dithionite in alkaline medium. By treatment of the solution with perchloric acid, followed by centrifuging and neutralization, no spectrophotometric evidence of flavins was obtained in the supernatant fluid. No variation of the

$400 \text{ m}\mu$ absorption maximum was observed either in the absence or in the presence of oxygen. Addition of borohydride irreversibly depressed both enzyme activity and absorption maximum at $400 \text{ m}\mu$.

Fluorescence spectra. The purified enzyme reveals fluorescence with an activation maximum at $310 \text{ m}\mu$ and an emission maximum at $415 \text{ m}\mu$. The intensity of fluorescence is slightly increased upon addition of borohydride (Fig. 5).

After treatment of the enzyme solution with perchloric acid and then centrifuging and neutralizing, the supernatant fluid shows fluorescence with an activation maximum at $340 \text{ m}\mu$ and an emission maximum at $500 \text{ m}\mu$. Also, in this instance, borohydride caused a slight increase of the emission.

DISCUSSION

This work shows the possibility of purifying the oxidase by conventional methods (heating, acetone and ammonium sulphate precipitation). Care must be taken, however, in eliminating molecular oxygen and in operating in the presence of the other substrate, namely 3-hydroxyanthranilic acid, and of Fe^{2+} ions and sulphhydryl-group-containing substances. Evidently combination with oxygen impairs activity of the enzyme as, for instance, with cytochrome *b*₂ (Appleby & Morton, 1958; Boeri & Rippa, 1959).

The purified enzyme functions at a rate which is dependent on the concentration of both substrates (3-hydroxyanthranilic acid and oxygen). K_m for 3-hydroxyanthranilic acid is low ($7 \mu\text{M}$, when the oxygen pressure is that of air), indicating a great avidity of the enzyme for this substrate.

The dependence of the reaction on the oxygen pressure gives some indication of the role of 3-hydroxyanthranilic acid oxidase in the liver. At the physiological partial pressures of oxygen (20–100 mm. mercury) the enzyme operates at a rate which is far from maximal. The oxidation of 3-hydroxyanthranilic acid by this route is therefore slow and it should be markedly depressed when the oxygen supply is insufficient. The rate of oxidation of 3-hydroxyanthranilic acid in the liver should therefore depend on the integrity not so much of the portal circulation, but rather of the supra-hepatic arteries which supply oxygenated blood to the liver.

We do not know as yet whether the $400 \text{ m}\mu$ absorption could be ascribed to a chromophore on the enzyme molecule or to an impurity. This absorption is not due to haem groups since the enzyme solution fails to exhibit any absorption band between 500 and $600 \text{ m}\mu$ upon reduction with borohydride, and since there is no evidence of the presence of haemochromogens after pyridine treatment.

Fig. 5. Fluorescence spectra of the purified 3-hydroxyanthranilic acid-oxidase preparation. Activation spectrum (●—●); fluorescence spectra, with (○—○) and without (○—○) borohydride addition. In ●—● the second monochromator was fixed at $420 \text{ m}\mu$; in ○—○ and ○—○ the first monochromator was fixed at $310 \text{ m}\mu$.

The purified preparations seem to be free from flavins, since no spectrophotometric and fluorometric evidence thereof was obtained.

SUMMARY

1. 3-Hydroxyanthranilic acid oxidase has been purified from rat and ox liver. The most active preparations of the enzyme oxidize 2500 μ m-moles of 3-hydroxyanthranilic acid/min./mg. of protein.
2. The dependence of the reaction rate on the concentration of both substrates (3-hydroxyanthranilic acid and oxygen) was measured. K_m for 3-hydroxyanthranilic acid is low (7 μ M under the conditions of the activity test). At the oxygen pressure of the air the reaction rate is still far from maximal.
3. The purified enzyme solutions present an absorption maximum at 400 m μ and a peculiar fluorescence. There is not yet sufficient evidence to ascertain whether these bands are due to the enzyme itself or to impurities.

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REFERENCES

Appleby, C. A. & Morton, R. K. (1958). *Biochem. J.* **73**, 539.

Boeri, E. & Rippa, M. (1959). *Proceedings of the Symposium on Haematin Enzymes, Canberra*, (in the Press).
Boeri, E. & Tosi, L. (1954). *Arch. Biochem. Biophys.* **52**, 83.
Bokman, A. H. & Schweigert, B. S. (1950). *J. biol. Chem.* **186**, 153.
Bokman, A. H. & Schweigert, B. S. (1951). *Arch. Biochem. Biophys.* **33**, 270.
Gomori, G. (1946). *Proc. Soc. exp. Biol., N.Y.*, **62**, 33.
Gornall, A. G., Bardawill, C. S. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
Haldane, J. B. S. & Stern, K. G. (1932). *Allgemeine Chemie der Enzyme*. Dresden and Leipzig: Steinkopff.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Long, C. L., Hill, H. N., Weinstock, I. M. & Henderson, L. M. (1954). *J. biol. Chem.* **211**, 405.
Mehler, A. H. (1956). *J. biol. Chem.* **218**, 241.
Michaelis, L. & Menten, M. L. (1913). *Biochem. Z.* **49**, 333.
Priest, R. E., Bokman, A. H. & Schweigert, B. S. (1951). *Proc. Soc. exp. Biol., N.Y.*, **78**, 477.
Rabinowitz, M., Fineberg, R. A. & Greenberg, D. M. (1953). *Arch. Biochem. Biophys.* **42**, 197.
Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.
Schweigert, B. S. & Marquette, M. M. (1949). *J. biol. Chem.* **181**, 199.
Singal, S. A., Sydenstricker, V. P. & Littlejohn, J. M. (1953). *Fed. Proc.* **12**, 429.
Stevens, C. O. & Henderson, L. M. (1959). *J. biol. Chem.* **234**, 1188.
Tsuchihashi, M. (1923). *Biochem. Z.* **140**, 63.
Wiss, O. & Weber, F. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **13**, 172.

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The Metabolism of C₂ Compounds in Micro-organisms

6. SYNTHESIS OF CELL CONSTITUENTS FROM GLYCOLLATE BY *PSEUDOMONAS* SP.*

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The growth of micro-organisms on C₂ compounds as the sole source of carbon necessitates the occurrence of reactions whereby the C₂ substrate provides both metabolic energy and the carbon skeletons of cellular constituents. When the C₂ substrate is acetate, both these functions are fulfilled by the tricarboxylic acid cycle, with the glyoxylate cycle operating in addition to maintain the concentrations of intermediates utilized for growth. But this combination of reactions cannot explain growth on C₂ compounds which are more highly oxidized than acetate, such as glycollate.

Moreover, though studies with a mutant of *Escherichia coli* confirmed that the tricarboxylic acid cycle plays an essential role in growth on glycollate, they showed that this C₂ substrate was oxidized via a dicarboxylic acid cycle in the absence of a functional tricarboxylic acid cycle (Kornberg & Sadler, 1960). A different sequence of reactions must therefore occur to replenish from glycollate the supply of intermediates of the dicarboxylic and tricarboxylic acid cycles drained away to provide cell materials for the growing bacteria.

The main purpose of this paper is to present evidence for the nature of such a biosynthetic

* Part 5: Kornberg, Phizackerley & Sadler (1960).