5. The antiphage component of crude papain has been separated by zone electrophoresis and found to have no thiolesterase activity.

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REFERENCES

- Campbell, W. R. & Hanna, M. I. (1937). *J. biol. Chem.* **119**, 1.
 Fildes, P. & Kay, D. (1957). *Brit. J. exp. Path.* **38**, 563.
 Fildes, P. & Kay, D. (1959). *Brit. J. exp. Path.* **40**, 71.
 Johnston, R. B. (1956). *J. biol. Chem.* **221**, 1037.
 Kalmanson, G. M. & Bronfenbrenner, J. (1943). *J. Immunol.* **47**, 387.
 Kay, D. (1959). *Biochem. J.* **73**, 149.
 Kimmel, J. R. & Smith, E. L. (1954). *J. biol. Chem.* **207**, 515.
 Kozloff, L. M., Lute, M. & Henderson, K. (1957). *J. biol. Chem.* **228**, 511.
 Kunkel, H. G. (1954). *Meth. biochem. Anal.* **1**, 141.
 Lipmann, F. & Tuttle, L. C. (1945). *J. biol. Chem.* **159**, 21.
 Strecker, H. J., Mela, P. & Waelsch, H. (1955). *J. biol. Chem.* **212**, 223.

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Parapepsinogen II: The Zymogen of Parapepsin II

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The isolation and characterization of two new enzymes, parapepsins I and II, from crude porcine pepsin has already been described (Ryle & Porter, 1959).

In seeking to determine whether these enzymes might be derived from pepsinogen or whether they arose from other precursors partly purified extracts of porcine fundic mucosa have been chromatographed on diethylaminoethylcellulose and a zymogen for parapepsin II has been found. The isolation and some of the properties of this zymogen are described in this paper.

EXPERIMENTAL

Abbreviations. The following abbreviations will be used throughout this paper: diethylaminoethyl, DEAE; haemoglobin, Hb; acetyl-DL-phenylalanyl-L-di-iodotyrosine, APD; 1-fluoro-2:4-dinitrobenzene, FDNB; dinitrophenyl, DNP.

Instruments. Extinction coefficients were determined with either the Hilger Uvispek or a Unicam spectrophotometer.

Determinations of pH were made either with an E.I.L. direct-reading pH meter or a Cambridge pH meter, the meters being standardized against buffer solutions made from Cambridge buffer tablets.

Buffer solutions. Except where otherwise stated buffer solutions were made from sodium salts. Their concentrations are stated with respect to the anionic component.

Activity determination with haemoglobin. Assays of activity against haemoglobin were performed in duplicate by a method based on that of Northrop, Kunitz & Herriott

(1948) as described by Ryle & Porter (1959). In the earlier part of the work, when zymogens were assayed 0.1 ml. of the zymogen solution in 0.02 M-phosphate buffer, pH 6.9, was mixed with 0.1 ml. of 0.035 N-HCl and allowed to stand at 35.5° for 10 min. to allow activation to occur before addition of 1 ml. of acid-denatured haemoglobin (2%, w/v). Later it was found that no significant difference appeared in the assay of either pepsinogen or parapepsinogen II if this activation period were omitted, and 0.2 ml. samples were then assayed by the addition of 1 ml. of acid-denatured

Fig. 1. Standard curve for parapepsin II. A solution (0.2 ml.) of enzyme in 0.1 M-acetate, pH 5.6, was incubated with 1 ml. of acid-denatured haemoglobin (4 vol. of 2.5% Hb + 1 vol. of 0.39 N-HCl) for 10 min. at 35.5°. The reaction was stopped with 5 ml. of 4% (w/v) trichloroacetic acid. Units were chosen as described in the text.

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haemoglobin [0.8 ml. of 2.5% (w/v) Hb + 0.2 ml. of 0.315N-HCl]. In all the haemoglobin assays the total amount of acid used was such as to give a digestion mixture of pH 1.7.

The increments of E found for pepsinogen were converted into milliproteolytic units ($m[P.U.]^{Rb}$) by reference to a standard curve obtained with a preparation of crystalline pepsin which had been standardized by the original method of Anson (1938). For parapepsinogen II a new standard curve, prepared with a solution of parapepsin II, was used (Fig. 1). The units were chosen so that with an increment of E of 0.28 the activities determined by the two curves are equal (0.0855 $m[P.U.]$). Since the assays previously performed (Ryle & Porter, 1959) were as far as possible restricted to this region the difference between the units found with the curve for parapepsin II and those obtained with the pepsin curve is small. With the new curve assays have usually been restricted to the range 0.05–0.12 $m[P.U.]^{Rb}$, where the reproducibility is maximal.

Activity determinations with acetyl-DL-phenylalanyl-L-di-iodotyrosine. Determinations of activity against APD were carried out on the zymogens in a manner similar to that used for the enzymes by Ryle & Porter (1959). At first, as in the haemoglobin assay, a 10 min. period was allowed for activation to occur, but after it had been found that omission of this period made no difference to the activity the assays were performed without previous activation. Instead of the ninhydrin reagent containing acetate and citrate buffers and stannous chloride the modified reagent of Moore & Stein (1954) was used at first, and was found to give higher colour yields than the stannous chloride reagent. It was later found that the small amount of phosphate present in the assay mixtures did not cause precipitation of the stannous chloride and the reagent containing this reducing agent has also been used. To 0.5 ml. of solution to be assayed 0.25 ml. of 0.088N-HCl is added at 35.5°, followed 15 sec. later by 0.25 ml. of 2 mM-APD in 0.01N-NaOH to give a solution pH 2.0. After 20 min. at 35.5° the digestion is stopped by the addition of 1 ml. of ninhydrin reagent and the colour is developed and read in the usual way.

Milk-clotting activity. Determinations of milk-clotting activity were made as in the earlier work (Ryle & Porter, 1959) except that, following Berridge (1955), the milk solution contained 0.01M-CaCl₂. Under these conditions parapepsin II clotted the milk more rapidly than it did when CaCl₂ was not added so that this enzyme showed a ratio $[P.U.]^{Ren} : m[P.U.]^{Rb}$ higher than 0.47, found in the absence of added calcium. Since these activity ratios were found to be somewhat variable the ratio for parapepsin II was determined at the same time as that for the zymogen when a comparison was to be made. One $[P.U.]^{Ren}$ is the amount of enzyme which will clot 11 ml. of enzyme-milk mixture in 1 min. at 35.5°.

Preparation of crude pepsinogen solution. Pig stomachs were collected, rinsed and packed in ice for transport to the Laboratory. The fundic parts of the mucosae were removed and either used at once or frozen at -10° for storage. No difference was observed between preparations made from mucosae used at once or from those stored for several weeks.

The pepsinogen was extracted and partly purified by the method of Herriott (1948) up to the stage of precipitation of the zymogen with 0.68-saturated (NH₄)₂SO₄, filtration and re-solution in water (about 50 ml./100 g. of mucosa).

The recovery of activity up to this step has been close to that found by Herriott (80%) and close to 100% in preparations made from single mucosae.

The solutions thus obtained were concentrated by ultrafiltration under 60 cm. Hg pressure through $\frac{3}{8}$ in. dialysis tubing (Visking Corp., obtained from Viscose Ltd., 40, Chancery Lane, London, W.C. 2) supported by a sleeve of silk net and dialysed against 0.02M-phosphate buffer, pH 6.9.

Chromatography of crude pepsinogen. The dialysed solutions were fractionated by ion-exchange chromatography on columns of DEAE-cellulose (Peterson & Sober, 1956) in 0.02M-phosphate buffer, pH 6.9, with a rising gradient of NaCl concentration obtained by passing a solution containing 0.45M-NaCl and 0.02M-phosphate buffer, pH 6.9, into a mixing vessel of fixed volume containing 0.02M-phosphate buffer, pH 6.9.

The contents of the tubes from the active peaks were suitably pooled, concentrated by ultrafiltration through Visking $\frac{3}{8}$ in. tubing under 60 cm. Hg pressure, dialysed against distilled water and freeze-dried. These operations were all carried out at about 2° and solutions were kept saturated with toluene.

Nitrogen determination. Determination of total nitrogen in solutions of parapepsin II and parapepsinogen II were made by digestion with H₂SO₄ and estimation by ninhydrin of the NH₃ formed (Jacobs, 1959).

Determination of free amino groups. N-Terminal amino acid residues and lysine residues were determined by the fluorodinitrobenzene method [details of the techniques used are described by Porter (1957)]. Duplicate samples of the protein were allowed to react with FDNB in the presence of trimethylamine and, after removal of excess of FDNB and dinitrophenol by ether extraction, were hydrolysed in 5.7N-HCl for 20 hr. at 105° under N₂; to one sample known amounts of DNP-serine and ϵ -DNP-lysine were added before hydrolysis so that correction could be made for the breakdown of these DNP-amino acids during the hydrolysis. The ether-soluble DNP-amino acids were separated by chromatography on paper in *tert*-amyl alcohol (Blackburn & Lowther, 1951), estimated by their E at 360 m μ in 1% (w/v) NaHCO₃ soln. and, where necessary, had their identities confirmed by paper chromatography in phosphate buffer (Levy, 1954).

Portions of the aqueous fraction of the hydrolysates were chromatographed in butanol-acetic acid-water (1260:200:540, by vol.) on Whatman no. 3MM paper, together with standard amounts of ϵ -DNP-lysine. The yellow spots were cut out, eluted with 5 ml. of N-HCl at 60° for 15 min., and the amounts of ϵ -DNP-lysine were determined by E at 350 m μ .

Analysis in the ultracentrifuge. The work with the ultracentrifuge was performed by Dr P. A. Charlwood of this Institute with a Spinco model E centrifuge. Determinations of molecular weight [by the method of Archibald (1947) as used by Charlwood (1957)] and of sedimentation coefficient were made in phosphate buffer, pH 6.9, I 0.20 (1.47 g. of NaH₂PO₄·2H₂O, 4.85 g. of Na₂HPO₄·12H₂O and 11.18 g. of KCl/l.).

RESULTS

Chromatographic fractionation of crude pepsinogen. Fig. 2 shows a chromatogram of about six-sevenths of the crude pepsinogen obtained from a

single mucosa, containing potential activity against haemoglobin equivalent to about 870 mg. of pepsin. The large active peak has activity against both haemoglobin and acetyl-DL-phenylalanyl-L-di-iodo-tyrosine and is presumably pepsinogen. This material has not been studied further. The peak emerging at 4.4 l. after the application of the gradient shows activity against haemoglobin but little against the synthetic substrate. Because of this specificity, and the apparent stability of the activity at pH 6.9 (at which pH parapepsin II undergoes inactivation), it was tentatively concluded that the material in this peak was the zymogen of parapepsin II, parapepsinogen II. Further results have supported this conclusion.

The recovery of enzyme activity against haemoglobin from this column was 90% and the recovery of E at 280 $m\mu$ was 100%. The parapepsinogen II accounts for 7% of the total activity against haemoglobin.

Specific activity of parapepsin II and parapepsinogen II. Freeze-dried samples of the enzyme have had the specific activity $[P.U.]_{mg. \text{ of } N}^{Hb}$ 0.31–

0.34. The specific activity of the zymogen has varied from 0.28 to 0.32 $[P.U.]_{mg. \text{ of } N}^{Hb}$.

Stability of parapepsinogen II at pH 6.9. At 25° and pH 6.9 parapepsin II loses about half its activity in 1 hr. (Ryle & Porter, 1959). During the handling of parapepsinogen II solutions at this pH in the cold no definite losses of activity were observed and further experiments showed that solutions of the zymogen (4.5 and 2.5 $m[P.U.]^{Hb}/ml.$) in 0.02M-phosphate buffer, pH 6.9, retained their potential activity for days at room temperature.

Chromatographic behaviour of activated parapepsinogen II. When parapepsinogen II which had been allowed to stand for 5 min. at pH 2.0 at room temperature was chromatographed on a column of DEAE-cellulose in 0.1M-acetate buffer, pH 4.0, with rising NaCl gradient, an active peak emerged in the position parapepsin II was expected to occupy. Similarly, when parapepsinogen II which had been allowed to stand for 10 min. at pH 2.0 at room temperature was mixed with parapepsin II only one active peak appeared on the chromatogram, in the position of parapepsin II. This peak accounted for 65% of the activity applied, or 112% of the activity of the parapepsin II, so that both activated zymogen and enzyme must have been present in the single peak found. The recovery of parapepsin II from this type of column has usually been about 70–80%.

Study of parapepsinogen II in the ultracentrifuge. In a single run, the molecular weight found (the mean of five values determined at different times) was 40 500, and the sedimentation coefficient was 3.36s at a concentration of 0.7%. The material showed only a single symmetrical sedimenting boundary.

Free amino groups in parapepsinogen II. In a preliminary experiment performed to determine which amino acid was *N*-terminal in parapepsinogen II, but in which internal breakdown controls were not used, the quantities of DNP-amino acids found per mole of protein, corrected for breakdown during hydrolysis by the figures of Porter (1957), were 0.9 mole of DNP-serine and about 10 moles of ϵ -DNP-lysine. Only very faint traces of other DNP-amino acids were found.

In a second experiment with a different batch of zymogen, the breakdown of DNP-serine and ϵ -DNP-lysine was determined by internal controls. The corrected amounts of these DNP-amino acids found, per 40 500 g. of protein, were: DNP-serine 0.9 mole, ϵ -DNP-lysine 8.9 moles. In addition, about 0.2 mole of fast-running DNP-amino acids, shown by chromatography in phosphate buffer to be mainly DNP-phenylalanine, were found.

Activation of parapepsinogen II. In the assay with haemoglobin it was found that a preliminary

Fig. 2. Chromatography of partly purified pepsinogen, 20 $[P.U.]^{Hb}$ in 28 ml., on a 5 cm. $\times$ 26 cm. column of DEAE-cellulose in 0.02M-phosphate, pH 6.9. The gradient was 0.045M-NaCl in the same buffer with a mixing vessel of 2240 ml. ●, Activity against Hb; ○, activity against APD. For clarity experimental points have been omitted from the curve of E at 280 $m\mu$ and the curves of activity against Hb and APD have not been drawn.

period of incubation of parapepsinogen II at pH 2.0 and 35.5° for 5 or 10 min. in the absence of substrate did not increase the extent of digestion of haemoglobin subsequently added. At pH 1.7, therefore, the zymogen must be very rapidly activated.

For this reason it was not possible to follow the activation of the zymogen by the haemoglobin assay and so the assay by milk-clotting activity was used. This activity is measured at pH close to 5.6, and it was found that quantities of zymogen ten times as great as those which, after complete activation, would clot milk in 1.5 min., only brought about clotting in about 1 hr. The activation of the zymogen at pH 5.6 was therefore sufficiently slow to allow the milk-clotting method to be used to follow the conversion of zymogen into enzyme.

A solution of parapepsinogen II in 0.02M-phosphate buffer, pH 6.9, was diluted tenfold with 0.1M-acetate buffer, pH 4.0, at 35.5°, and the milk-clotting activities of samples taken at intervals were determined. The pH of the activation mixture, determined by the glass electrode at the end of the experiment, was 4.1. Fig. 3 shows that the activation of the zymogen follows first-order kinetics. However, the ratio $[P.U.]^{Ren}:m[P.U.]^{Hb}$ for the activation mixture at 60 min. was only about 0.5, whereas the ratio for parapepsin II determined on

the same milk substrate was 0.77. A portion (1 ml.) of the activation mixture was added to 0.028 ml. of N-HCl to reduce the pH to 2.0, and allowed to stand for 5 min. at 35.5°. It was then diluted tenfold with 0.1M-acetate, pH 4.0. The milk-clotting activity of this acid-treated solution, calculated back to the concentration of the activation mixture, is shown in Fig. 3. The ratio $[P.U.]^{Ren}:m[P.U.]^{Hb}$ was now 0.8. Fig. 4 shows the course of the activation at pH 4.7. The kinetics here are not simply first-order or autocatalytic but appear to lie somewhere between.

Mixed chromatogram of parapepsin II and parapepsinogen II. Fig. 5 shows the result of chromatographing about 10 mg. each of parapepsin II and parapepsinogen II at pH 5.6, at which pH the

Fig. 3. Activation of parapepsinogen II at pH 4.1 and 35.5°. The activation mixture contained 1 ml. of a solution (3 mg./ml.) of parapepsinogen II in 0.02M-phosphate, pH 6.9, and 9 ml. of 0.1M-acetate, pH 4.0. ●, Milk-clotting activity; ○, milk-clotting activity after 5 min. incubation at pH 2.0 and 35.5°. The curve is the theoretical curve for a first-order reaction, K 0.06, taking the initial concentration of zymogen as 4.17 potential $[P.U.]^{Ren./ml.}$

Fig. 4. Activation of parapepsinogen II at pH 4.7 and 35.5°. The activation mixture contained 0.6 ml. of a solution (5 mg./ml.) of parapepsinogen II in 0.02M-phosphate, pH 6.9, +9.4 ml. of 0.1M-acetate, pH 4.6. ●, Milk-clotting activity; ○, milk-clotting activity after 5 min. incubation at pH 2.0 and 35.5°.

Fig. 5. Chromatography of mixed parapepsinogen II and parapepsin II. About 10 mg. each of zymogen and enzyme on a DEAE-cellulose column (1.2 cm. $\times$ 33 cm.) equilibrated with 0.1M-acetate, pH 5.6. Elution was initially with a gradient obtained by passing 0.4M-NaCl in 0.1M-acetate, pH 5.6, into a mixing vessel (140 ml.) containing 0.1M-acetate, pH 5.6. At the point indicated by the arrow the 0.4M-NaCl soln. was replaced by M-NaCl in the same buffer. The line represents E at 280 $m\mu$ (experimental points omitted); ●, activity against Hb; ▲, milk-clotting activity.

enzyme is stable and the zymogen only very slowly activated. The zymogen appeared first, and when it became clear that the enzyme was emerging as a trailing peak in the gradient to 0.4 M-NaCl, changing the reservoir to M-NaCl in 0.1 M-acetate, pH 5.6, resulted in rapid elution of the enzyme. The recovery of activity of the zymogen was 92 % and of the enzyme 90 %.

DISCUSSION

The results reported above answer one of the questions posed by the similarity of pepsin and parapepsin II: that of the origin of the new enzyme. The enzyme is derived from a zymogen, which in its similarity to pepsinogen reflects the similarity of the enzymes. There seems little doubt that the material obtained here is in fact the zymogen of parapepsin II, since at low pH it shows the same feature of activity against haemoglobin unaccompanied by activity against acetylphenylalanyldi-iodotyrosine, and, after standing at pH 2.0, it behaves on chromatography at pH 4.0 in exactly the same way as parapepsin II. It has, moreover, one *N*-terminal amino acid, serine, the same as one of the two *N*-terminal amino acids of parapepsin II (serine and leucine or isoleucine) and its molecular weight (40 500) is of the same order as that found for the enzyme (40 700). Its ratio of milk-clotting to haemoglobin-digesting activity, after activation at pH 2.0, is the same as that of parapepsin II.

When pepsinogen is activated to pepsin the molecular weight of the protein falls from about 42 000 (Herriott, 1938*a*) to 35 500 and among the basic peptides split off is the pepsin inhibitor (Van Vunakis & Herriott, 1956). With parapepsinogen II the change in weight appears to be smaller, and this is supported by the small change in specific activity. Comparison of the behaviour of the zymogen and enzyme on chromatography at pH 5.6 shows that the zymogen is more basic; presumably basic fragments are split off on activation. The experiments on activation at pH 4.1 and 4.7 provide an indication that with parapepsinogen II, as with pepsinogen, an inhibitor may be among the fragments split off. Although the activity produced at pH 4.1 had reached its maximum after 1 hr., treatment of this solution at pH 2.0 for 5 min. caused a 50 % increase in milk-clotting activity; a similar increase appeared with zymogen activated at pH 4.7. This effect could be explained by activation of a second component, not activated at pH 4.1 or 4.7, but the evidence of the chromatography, the ultracentrifuge and end-group analysis all indicated that the protein was homogeneous. It is possible that during the activation an inhibitor is produced, so that the enzyme is only partly active, and that at pH 2.0 the enzyme digests the inhibitor

(as pepsin digests its inhibitor) so that on return to pH 4.0 the full activity is demonstrable. This explanation of the phenomenon accords well with the fact that parapepsinogen II is inhibited by the pepsin inhibitor.

There is a difference in the kinetics of activation from the behaviour of pepsinogen, which Herriott (1938*b*) found to be activated by an autocatalytic process at all pH values, although the kinetics were complicated at low pH by the interaction of the pepsin and inhibitor. With parapepsinogen II the activation is first-order at pH 4.1, and at pH 4.7 apparently proceeds by both autocatalytic and first-order mechanisms. It may be that the zymogen is activated by H^+ ions and by parapepsin II, and that at pH 4.7 both mechanisms are important, whereas at pH 4.1 the activation by H^+ ions masks the autocatalytic reaction.

Since parapepsinogen II has been found with pepsinogen in extracts of single mucosae it is clear that the zymogen is not a genetically controlled aberrant form of pepsinogen. This still leaves unanswered the question of the biological function of parapepsin II. As a digestive enzyme it seems that it can do little that pepsin cannot do, since its specificity is slightly narrower than that of pepsin (Ryle & Porter, 1959), and its pH optimum, at least for the later stages of digestion of haemoglobin, is the same as that of pepsin. It is slightly more stable in alkaline solution than pepsin but it is not easy to see how this could be significant. It is perhaps most likely that it has some specific action against a peptide structure, resistant to pepsin, which does not appear in any of the substrates against which it has been tested.

Tang, Wolf, Caputto & Trucco (1959) have isolated a crystalline enzyme, gastricsin, from human gastric juice which has properties not unlike those of parapepsin II (though they have not studied its specificity) and it is possible that their enzyme is the human version of parapepsin II.

No evidence has been found for the presence in extracts of pig fundic mucosa of a zymogen corresponding to parapepsin I or for the presence of parapepsin I itself, which is stable at the pH of the eluting buffer. The recovery of protein from the chromatograms (measured by *E* at 280 m μ) has usually been close to 100 %, so that large quantities of the enzyme or zymogen cannot have been left on the columns. It is possible that the material is not extracted from the mucosa under the conditions used here, or that it occurs in the mucosa of only a few pigs.

SUMMARY

1. The zymogen of parapepsin II has been isolated from extracts of porcine gastric mucosa.
2. The zymogen is rapidly converted into the

enzyme at pH 2.0 and more slowly at pH values above 4.0.

3. The zymogen has one N-terminal residue and a molecular weight close to that of the enzyme.

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REFERENCES

- Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
 Archibald, W. J. (1947). *J. phys. Chem.* **51**, 1204.
 Berridge, N. J. (1955). In *Methods in Enzymology*, vol. 2, p. 69. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Blackburn, S. & Lowther, A. G. (1951). *Biochem. J.* **48**, 126.
 Charlwood, P. A. (1957). *Trans. Faraday Soc.* **53**, 871.
 Herriott, R. M. (1938*a*). *J. gen. Physiol.* **21**, 501.
 Herriott, R. M. (1938*b*). *J. gen. Physiol.* **22**, 65.
 Herriott, R. M. (1948). In *Crystalline Enzymes*, 2nd ed., p. 259. Ed. by Northrop, J. H., Kunitz, M. & Herriott, R. M. New York: Columbia University Press.
 Jacobs, S. (1959). *Nature, Lond.*, **183**, 262.
 Levy, A. L. (1954). *Nature, Lond.*, **174**, 126.
 Moore, S. & Stein, W. H. (1954). *J. biol. Chem.* **211**, 907.
 Northrop, J. H., Kunitz, M. & Herriott, R. M. (1948). *Crystalline Enzymes*, 2nd ed., p. 305. Ed. by Northrop, J. H., Kunitz, M. & Herriott, R. M. New York: Columbia University Press.
 Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
 Porter, R. R. (1957). In *Methods in Enzymology*, vol. 4, p. 221. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Ryle, A. P. & Porter, R. R. (1959). *Biochem. J.* **73**, 75.
 Tang, J., Wolf, S., Caputto, R. & Trucco, R. E. (1959). *J. biol. Chem.* **234**, 1174.
 Van Vunakis, H. & Herriott, R. M. (1956). *Biochim. biophys. Acta*, **22**, 537.

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Effect of Ethanol on Respiration of Rat-Brain-Cortex Slices

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Potassium ions and some other agents enhance the respiration and glycolysis of nervous tissue *in vitro* (Ashford & Dixon, 1935; Dickens & Greville, 1935), as do applied electrical pulses McIlwain, 1951*a*). The respiration of stimulated tissue is affected by narcotics at pharmacologically active concentrations (Bronk & Brink, 1951; McIlwain, 1953*a*; Buchel, 1953), whereas that of unstimulated tissue is not.

Heavy ethanol intoxication in human subjects is associated with diminished cerebral oxygen consumption (Hine, Shick, Margolis, Burbridge & Simon, 1952; Battey, Heyman & Patterson, 1953). Recent work with potassium-stimulated brain-cortex tissue indicates that its oxygen consumption is slightly but significantly depressed at 'physiological' ethanol concentrations (Ghosh & Quastel, 1954; Sutherland, Hine & Burbridge, 1956; Fischer, 1957; Beer & Quastel, 1958*b*), whereas glucose consumption and lactic acid accumulation appear to be enhanced (Sutherland *et al.* 1956). Ethanol in corresponding concentrations slightly elevates unstimulated respiration, at least in the initial phase of the experiment (Fuhrman & Field, 1948; Ghosh & Quastel, 1954; Sutherland *et al.* 1956; Beer & Quastel, 1958*b*).

Here we report studies *in vitro* on the oxygen and glucose consumption and lactic acid formation in stimulated and unstimulated slices of cerebral cortex from the rat. The first aim was to determine whether ethanol had any effect at all in our experimental conditions. A depression of the respiration was observed in tissues stimulated electrically or with potassium chloride. Since changes in glucose concentration did not influence the ethanol effect, it was concluded that there cannot be any competition between ethanol and glucose for access to the enzymes. The nature of the non-competitive inhibition could not be defined.

MATERIAL AND METHODS

Apparatus. All experiments were performed with conventional Warburg constant-volume respiratory manometers and vessels of 13–16 ml. volume. The flasks for electrical stimulation of tissue were modified from the type described by McIlwain (1951*b*) as 'conical electrode vessels E' (Fig. 1). Before experiments, silicone grease was applied to the base of the platinum wires. After each experiment, the electrodes were removed, washed with detergent, rinsed with water, dipped for 15 sec. into dichromate-H₂SO₄ solution, rinsed with water and dried. To facilitate the application and removal of the electrodes, the