with benzoquinone, supporting the suggestion of a hydroquinone-quinone system operating. Methylene blue caused an increase in [1—^{14}C] glucose utilisation and in the proportion converted to $^{14}CO_2$, indicating that mere addition of NADP did not extend the capacity of the pentose phosphate pathway to its limit. The addition of certain compounds normally present in biological fluids, such as oxidised and reduced glutathione, cystine, cysteine and ascorbic acid, has been reported to increase the proportion of glucose metabolised via the pentose phosphate pathway in intact human erythrocytes.[8-10] The latter compounds all decreased [1—^{14}C] glucose utilisation in the haemolysates, and all but cystine increased the proportions of glucose converted to $^{14}CO_2$. The effects of glutathione, both oxidised and reduced forms, were consistent with its envisaged role in an oxidation-reduction cycle linking $NADPH_2$ oxidation with molecular oxygen.[11]

The results confirm that gentisate, although possessing certain modes of action resulting from the two hydroxyl groups being in the para-positions on the benzene ring, acts in a similar manner to salicylate as an inhibitor of certain vital groups of cellular enzymes.

Acknowledgements—I should like to thank Professor M. J. H. Smith for continuous encouragement during this work, and the King's College Hospital and Medical School Research Committee for financial support.

Department of Chemical Pathology,
King's College Hospital Medical School,
London, S.E.5

J. A. Sturman

REFERENCES

1. R. A. Steggle, A. K. Huggins and M. J. H. Smith, *Biochem. Pharmac.* **7**, 151 (1961).
2. W. J. W. Hines, *J. Pharm. Pharmac.* **18**, 256 (1966).
3. W. J. Hines, C. Bryant and M. J. H. Smith, *Biochem. Pharmac.* **12**, 1109 (1963).
4. J. A. Sturman and M. J. H. Smith, *Biochem. Pharmac.* **16**, 220 (1967).
5. V. R. Potter and K. P. Dubois, *J. gen. Physiol.* **26**, 391 (1943).
6. L. F. Hewitt, *Oxidation-Reduction Potentials in Bacteriology and Biochemistry*, p. 21. London County Council (1937).
7. M. J. H. Smith and J. A. Sturman, *J. Pharm. Pharmac.* **19**, 108 (1967).
8. W. C. J. de Loecker and T. A. J. Prankerd, *Clin. chim. Acta*, **6**, 641 (1961).
9. A. Szeinberg and P. A. Marks, *J. clin. Invest.* **40**, 914 (1961).
10. G. Fornaini, P. Segni, G. Leoncini, A. Fantoni and O. Chieffi, *Ital. J. Biochem.* **12**, 374 (1963).
11. P. W. Spear and M. D. Sass, *Metabolism* **13**, 911 (1964).

Biochemical Pharmacology, Vol. 16, pp. 1634–1636. Pergamon Press Ltd. 1967. Printed in Great Britain

The depressor activity of trypsin-like enzymes purified from rat submandibular gland

(*Received* 6 *February* 1967; *accepted* 16 *March* 1967)

Earlier we have reported the purification and characterization of three alkaline trypsin-like proteases from the rat submandibular gland. They were trivially named salivain, glandulain and kallikrein-like peptidase.[1-4] All of the enzymes were capable of hydrolyzing synthetical ester or amide substrates typical for trypsin, e.g. *N*α-benzoyl-DL-arginine ethyl ester (BAEE) or *N*α-benzoyl-DL-arginine 2-naphthylamide. Salivain and glandulain, but not the kallikrein-like peptidase (all of the four isozymic forms), hydrolyzed readily also proteins. The high esterase activity of the enzymes resembles that of kallikrein, a depressor substance known to be present also in the submandibular gland. and

saliva.[5,6] Thus it was of interest to study whether one or all of the purified enzymes have an influence on blood pressure.

EXPERIMENTAL

Adult rabbits weighing 2·5–3 kg were anaesthetized with urethane (1 g/kg). The arterial blood pressure was measured from the carotic artery with a mercury manometer. Blood clotting was inhibited with heparin. The substances were injected into the cannula in the jugular vein. The method was essentially the same as described by Frey *et al.*[7]

The purified enzymes were those described earlier. Twice crystallized salt-free trypsin preparation was from Nutritional Biochemicals Ohio (U.S.A.). Depot-Padutin® was a product of Bayer, Leverkusen, kindly supplied by Berner Oy., Helsinki (Finland). The BAEE-splitting fraction of Depot-Padutin® was separated using DEAE-cellulose chromatography as will be described elsewhere[8] and was used immediately. Dilutions were made in physiological saline. Protein concentrations were determined according to the method of Lowry *et al.*[9]

RESULTS

The results are presented in Figs. 1–8.

It is shown in Fig. 1 that the injection vehicle, *Tris-HCl buffer* as well as small doses of *bovine trypsin* did not produce any significant and lasting decrease in the blood pressure of the experimental animals. Trypsin was tested up to doses 70 μg/kg.

Depot-Padutin® in a dose of one biological unit (60 μg/kg) caused readily a depression of 25 mmHg in our experimental conditions as is shown in Fig. 2. The effect was over in 2 min.

The BAEE splitting fraction of Depot-Padutin® was much more effective. A very low dose, 1 μg/kg, caused a decrease of 20 mmHg as is shown in Fig. 3. The effect was over in about 1 min. The proteinase fraction separated from Depot-Padutin® in chromatography on DEAE-cellulose,[8] was inactive biologically.

Kallikrein-like peptidase caused a very effective decrease in blood pressure. Fig. 4 demonstrates that the isoenzyme C caused an immediate and long-lasting depression of 40 mmHg. An equal effect was observed by isozymes B and A as shown in Figs 5 and 6.

Salivain was the most active of the enzymes tested. A long-lasting (15 min) severe hypotension was effected by 1 μg/kg. After the pressure had retured to the normal level the test animal was tachyphylatic to repeated doses of salivain at least half an hour.

Glandulain effected hardly any change in blood pressure when used in doses up to 300 μg/kg (Fig. 8).

DISCUSSION

These results demonstrate clearly that both salivain and all of the four isozymic forms of the kallikrein-like peptidase have a potent depressor activity on the rabbit blood pressure. This effect is even more marked than that observed using Depot-Padutin® in the same experimental conditions. Trypsin and glandulain, on the other hand, failed to show any depressor effect when used at the same or even in considerably higher doses.

Earlier we have shown that both glandulain and salivain are very active toward protein substrates as well as toward synthetic substrates of trypsin, the hydrolysis rates being comparable to those found with trypsin. The BAEE splitting fraction of Depot-Padutin® and the kallikrein-like peptidase, on the other hand, have only minimal capacity to hydrolyze proteins. Therefore, even while the depressor activity is by and large a characteristic of the enzymes hydrolyzing readily BAEE but not proteins, salivain is a potent proteinase and still it is also a potent depressor. Recent studies by Habermann[10] have shown that serum kallikrein hydrolyzes casein although the activity was about one sixteenth of that of trypsin. Trypsin and glandulain (and the proteinase fraction present in Depot-Padutin®), the other proteinases now tested, have no depressor activity. Trypsin is, however, known to have a depressor activity in high doses (200 μg/kg) in dogs[11] and the same could possibly be found in rabbits if comparable doses were used. This may be the case also with glandulain.

It is to be noted that all of the enzymes tested are capable of hydrolyzing BAEE at a very high rate. Therefore, the BAEE splitting activity and the depressor activity are not correlated in all of these enzymes; the esterase activity of glandulain and trypsin[1,3] is remarkably high in comparison to their low depressor activity.

Salivain, but not glandulain and the kalliktrein–like peptidase, was found by us to be present in saliva at high concentrations. Since salivain is a secretory enzyme[12] it is apparently located in the secretory (zymogen) granules. Bhoola and Ogle (1965)[13] have, in fact, demonstrated that the kallidin, releasing activity of the guinea-pig submaxillary gland homogenate is located in the fraction containing the zymogen granules. The activity was taken as kallikrein even while the distribution of the protease activity was not tested. It seems likely, that the activity tested by these authors has been the proteinase called salivain by us. Werle *et al.* (1960)[14] have stated that rat submandibular gland contains 3000 kallikrein units/g wet tissue, while e.g. human gland contains 1–2 units. In the light of the present study it seems clear that both salivain and kallikrein-like peptidase are included in the kallikrein activity measured by Werle *et al.* Whether both of these enzymes are real kallikreins depends on the definition of kallikrein.

Since its discovery, kallikrein has been taken as a circulatory hormone. It has been suggested that salivary kallikrein might be the mediator of the cholinergic nerve-induced vasodilatation in the submandibular gland. Hilton and Lewis[15] concluded that both cholinergic and adrenergic vasodilatation in this organ is mediated by plasma kinins liberated by kallikrein. It remains to be explained whether the submandibular kallikrein (producing plasma kinin) in their experiments has been salivain, the kallikrein-like peptidase or some additonal enzyme. It could perhaps be suggested that salivain, being a secretory enzyme, has a function in the digestive processes while the non-secretory kallikrein-like peptidase would be responsible for the possible circulatory control mechanism.

Department of Anatomy
University of Turku,
Turku 3,
Finland

T. O. EKFORS
T. MALMIHARJU
P. J. RIEKKINEN
V. K. HOPSU-HAVU

REFERENCES

1. P. J. RIEKKINEN, T. O. EKFORS, and V. K. HOPSU, *Biochem. biophys. Acta* **118**, 604 (1966).
2. P. J. RIEKKINEN, T. O. EKFORS, T. HOLLMEN, and V. K. HOPSU-HAVU, Enzymologia, **32**, 97 (1967).
3. P. J. RIEKKINEN, T. O. EKFORS, and V. K. HOPSU-HAVU, *Enzymologia*, **32**, 110 (1967).
4. T. O. EKFORS, P. J. RIEKKINEN, T. MALMIHARJU and V. K. HOPSU-HAVU, *Hoppe-Seyler's Z. physiol Chem.* **318**, 111 (1967).
5. E. WERLE and P. RODEN, *Biochem. Z.* **286**, 213 (1936).
6. S. M. HILTON and G. P. LEWIS, *Br. Med. Bull*, **13**, 189 (1957).
7. E. K. FREY, H. KRAUT and E. WERLE, *Kallikrein (Padutin)*. F. Enke, Stuttgart (1950).
8. T. O. EKFORS, T. MALMIHARJU, P. J. RIEKKINEN and V. K. HOPSU-HAVU, unpublished data.
9. O. H. LOWRY, N. J. ROSEBROUGH, A. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
10. E. HABERMANN and W. KLETT, *Biochem. Z.* **346**, 133 (1966).
11. E. WERLE, M. M. FORELL and L. MAIER, *Arch. exp. Path. Pharmak.* **225**, 369 (1955).
12. P. J. RIEKKINEN and T. O. EKFORS, *Acta chem. Scand.* **20**, 2013 (1966)
13. K. D. BHOOLA and C. W. OGLE, *J. Physiol.* **184**, 663 (1966).
14. E. WERLE, R. VOGEL and J. LENDRODT, *Archs int. Pharmacodyn Thér.* **126**, 187 (1960).
15. S. M. HILTON and G. P. LEWIS, *J. Physiol.* **134**, 471 (1956).

Biochemical Pharmacology. Vol. 16, pp. 1636–1641. Pergamon Press Ltd. 1967. Printed in Great Britain

Histamine and spermidine in tissues of the guinea pig*

(*Received* 3 *November* 1966; *accepted* 25 *June* 1967)

THE *n*-BUTANOL extraction of tissues with low histamine content is inadequate for the fluorometric determination of histamine[1] because of the presence of the interfering substance.[2–6] Spermidine,[5,6] one of the major contaminants in the extract made by organic solvents, can be separated from

* Supported by Grant 5-R01-NB-06053 from the National Institute for Neurological Diseases and Blindness. The authors gratefully acknowledge the suggestions made by Dr. R. A. Demovsky during the course of these investigations.