the placenta, it appears that one important factor in maintaining its concentration continuously at such high levels must be the specific impermeability of the placenta to this sugar which prevents its passage into the maternal circulation. Evidence has been reported (Karvonen & Rih, 1954; Davies, 1955) that a similar relative placental impermeability to fructose may occur also in the rat, rabbit and guinea pig, and similar observations have been made independently in this Laboratory. None of these species normally contains appreciable concentrations of fructose in the foetal blood. This suggests that the phenomenon of a specific impermeability of the placenta to fructose may be a quite general one. If this is so, it is perhaps related only somewhat fortuitously to the phenomenon of placental fructose formation, which is peculiar to the ungulates (Goodwin, 1956), in that, by helping to maintain high concentrations of fructose once it is formed, it accentuates the effect.

The remarkable disparity in placental permeability between fructose and other naturally occurring monosaccharides raises many fascinating problems about the nature of the transport mechanisms involved. The work reported in this paper offers more information on the specificity of the placental barrier but does not attempt to explain the mechanism of transfer or barrier effect. At the moment, virtually nothing is known about the details of such processes although the problems have been discussed (Widdas, 1952; Chinard, 1954). The only other monosaccharide which at all resembles fructose in this respect is the ketohexose sorbose. It may be that there is some peculiarity in the detailed nature of the transport processes which favours aldose rather than ketose groupings.

SUMMARY

1. The goat placenta has been shown to act in a similar manner to that of the sheep in that it is the

site of formation of foetal-blood fructose from foetal-blood glucose.

2. The goat placenta is freely permeable to glucose, mannose, galactose and xylose but is impermeable to fructose, sucrose, maltose and lactose. Sorbose can traverse the placenta but only at a slow rate.

3. The role of the remarkable relative impermeability of the placenta to fructose is discussed with reference to the occurrence of fructose in goat foetal blood and the mechanism of sugar transmission.

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The Preparation of Soluble Vasopressinase from Human Placenta

By K. C. HOOPER

Medical Research Council Clinical Endocrinology Research Unit, University of Edinburgh

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Hooper & Jessup (1959) indicated that oxytocin and vasopressin were destroyed by separate enzyme systems present in human placenta. Previously, Hawker (1956) showed that homogenate of placenta destroyed both hormones. Some properties of the enzymes were revealed by means of inhibitors, and it appeared likely that both oxytocinase and vasopressinase were peptidases, the former, in addition,

possessing esterase activity (Hooper, 1959). A study of the distribution of the enzymes in the placenta (Hooper & Jessup, 1959) showed that oxytocinase was present in the supernatant obtained by centrifuging the homogenate at 113 000 g and thereby removing particulate matter. Vasopressinase, on the other hand, was found to be predominantly in the mitochondrial and microsomal

fractions, with some activity in the soluble fraction. The distribution of vasopressinase between mitochondria and microsomes was such that activity associated with microsomes might be due to contamination by particles of disrupted mitochondria formed by grinding placenta with sand.

If a soluble preparation of vasopressinase could be obtained, it would facilitate further characterization of the enzyme, and would also permit sterilization by filtration. Attempts have been made to extract the enzyme, and results presented here describe the effects on vasopressinase of certain procedures which dissociate other enzymes from particulate matter.

EXPERIMENTAL

Preparation of fraction containing enzyme. Placentae containing the minimum amount of infarcted tissue were obtained from healthy women. Membranes were removed and pieces of washed, blotted tissue were mixed with one part (w/v) of cold 0.25 M-sucrose solution and ground with sand in a mortar. The homogenate was squeezed through muslin, and debris plus nuclei were removed by centrifuging at 600 g for 30 min. The greater part of vasopressinase activity was in the particulate fraction obtained by centrifuging the supernatant at 20 000 g for 40 min. Sedimented particles were drained and suspended in buffer solutions, giving concentrations of approx. 2 vasopressinase units/ml., the unit of activity defined by Hooper & Jessup (1959) being used. All operations were carried out near 2°.

Estimation of enzyme activity. Vasopressin (Pitressin, Parke, Davis and Co. Ltd.) was incubated with enzyme at 37° in pH 7.5 buffer (Hooper & Jessup, 1959) with an initial substrate concentration of 400 International pressor m-units/ml. After 30 min., 0.1 vol. of N-HCl was added and the reaction stopped by boiling. The boiled mixture was neutralized with NaHCO₃ and the NaCl concentration adjusted to 0.9% by addition of water (Hooper & Jessup, 1959). The amount of enzyme used for incubation was chosen so as to leave a residual concentration of approximately 10–30% of hormone for assay.

Vasopressin was measured by pressor activity on the anaesthetized rat (Dekanski, 1952). Atropinized animals were used in instances where solutions could have been

contaminated with di-isopropyl phosphorofluoridate (DFP). A bracketing type of assay was used. Two dose levels of a standard hormone solution were given before and after two dose levels of the unknown; the unknown was diluted until responses were approximately equal to those produced by the standard. The sequence was repeated, giving a total of ten injections per assay. Residual substrate concentrations were converted into enzyme units by means of a reference graph (Hooper & Jessup, 1959). Fiducial limits at a probability of 0.95 were calculated according to Burn (1952).

Extraction of enzyme from particulate fraction. The fraction containing vasopressinase activity was subjected to the following procedures. (1) Freezing and thawing: suspensions of particles were frozen at –30° and thawed at room temperature. (2) Lysis: particles were suspended in water at 2°. (3) Treatment with ultrasonic oscillations; these experiments were kindly done by Dr G. G. Selman with the apparatus described by Selman & Wilkins (1949). (4) Acetone-drying: suspensions of particles were run into 5 vol. of acetone at –20°, stirred for 30 min. and centrifuged and washed with acetone before drying at room temperature. (5) Treatment with butan-1-ol: both the one- and two-phase systems were used (Morton, 1955a). (6) Treatment with detergent: suspensions of particles in 0.1 M-phosphate buffer, pH 8.0, were treated with sodium cholate for varying periods of time. (7) Autolysis and use of enzymes: suspensions of particles were kept at 2°, or treated with lipase (L. Light and Co. Ltd.) or trypsin (Armour salt-free material, stated activity 3530 units/mg.) both in the presence and the absence of cholate. Trypsin was inhibited by making the preparation mM with respect to DFP.

Buffers. Buffers were of the phosphate type (Sørensen, 1909) prepared by mixing 0.2 M-Na₂HPO₄ and 0.2 M-NaH₂PO₄. With the exception of the buffer used for incubation, the desired molarities were obtained by diluting with water.

RESULTS

Methods involving mechanical disruption

Effect of lysis. Suspension of particles in a volume of glass-distilled water approximately equal to $\frac{1}{2}$ vol. of the original homogenate for 2 hr. at 2° caused particles to aggregate and sediment more easily than they did originally. After centrifuging

Table 1. *Effect of lysis and freezing and thawing on placental vasopressinase*

Particles containing vasopressinase were treated by methods shown in first column. Soluble and insoluble fractions were separated and the insoluble fraction was suspended in pH 7.5 buffer to give a volume equal to that of the soluble fraction. Preparations were incubated with vasopressin (2 international pressor units) for 30 min. at 37°, total volume 5 ml. Enzyme was inactivated and residual hormone assayed as described in the text. Fiducial limits are shown in parentheses.

Preparation	Vol. of preparation incubated (ml.)	Residual vasopressin (%)	Enzyme (units/ml.)	Enzyme present (%)
Particulate fraction	1.0	5 (2–9)	1.8 (1.5–2.4)	100
Lysed, supernatant	2.0	8 (6–10)	0.8 (0.7–0.9)	44
Lysed, debris	2.0	5 (3–8)	0.9 (0.8–1.1)	50
Particulate fraction	0.8	27 (23–31)	1.0 (0.9–1.1)	100
Frozen and thawed, supernatant	2.0	55 (51–58)	0.2 (0.18–0.21)	20
Frozen and thawed, debris	2.0	8 (4–12)	0.8 (0.7–1.0)	80

at 20 000 *g* for 15 min., activity was estimated in the optically clear supernatant and in the debris. Table 1 shows that some 40 % of total activity was obtained in solution. The loss of substrate occasioned by incubation with supernatant was due to enzyme action and not to loss by adsorption, since incubation with boiled supernatant did not cause a significant loss of vasopressin.

Effect of freezing and thawing. Freezing and thawing of suspensions of particles in 0.1M-phosphate buffer, pH 8.0, caused a release of 20 % of enzyme (Table 1). The resulting physical changes were similar to those caused by lysis. Repeated freezing and thawing did not liberate additional enzyme.

Effect of ultrasonic oscillations. Particles were suspended in water and subjected to oscillations of approximately 1 megacyc./sec. for times between 5 and 15 min. at densities of 10 or 15 w/cm.². The enzyme was not inactivated by 15 w/cm.² for 15 min., and of the 1.6 enzyme units/ml. present in the suspension, 0.6 unit went into solution. A release of 38 % indicated that ultrasonic oscillations did not cause a released of enzyme in addition to that already achieved by lysis. Electron microscopy showed progressive particle disintegration with increasing severity of treatment.

Methods involving dissociation of lipoprotein

Effect of acetone-drying. A suspension of particles treated with acetone resulted in a powder containing 19 % of the initial activity. In view of the loss of activity incurred during preparation, no effort was made to determine whether or not the remaining activity could be extracted by buffer solutions.

Effect of butan-1-ol. Dissociation of lipoprotein by butan-1-ol appears to be greater at pH values somewhat removed from neutrality than at pH 7.0 (Morton, 1955*b*). Suspensions of particles in a variety of buffers were treated with 20 % (v/v) butan-1-ol, centrifuged and enzyme distribution in aqueous phase and debris was determined. The dissociation of lipoprotein in acid media was more effective than it was in alkali, as judged by the colour of the organic phase. However, the large volume of butan-1-ol employed inactivated the

greater part of the enzyme. Treatment by addition of butan-1-ol to particles in 0.1M buffer, pH 8.0, to give a 3 % solution was less harsh than in the previous method, and released 23 % of enzyme with a destruction of some 35 % of total activity (Table 2). These conditions were approximately optimum for the release of enzyme with minimum loss of activity.

Effect of sodium cholate, autolysis and enzymes. Suspension of particles for 2 hr. at 2° in 0.1M buffer, pH 8.0, containing 0.5 % of sodium cholate released 46 % of activity (Table 3). It was found that the proportion of enzyme in the supernatant increased with autolysis in the presence of sodium cholate. Autolysis in the absence of cholate was not so effective. The release of vasopressinase appeared to depend therefore on digestion of lipoprotein or protein of the particles. Digestion with a lipase was only as effective, approximately, as mechanical disruption (Table 3). In experiments with trypsin, particles were digested with the enzyme at 2° for periods of time up to 5 hr. and at enzyme concentrations between 1:20 and 1:150. Some 40 % of vasopressinase was obtained in solution by this procedure. Trypsin neither destroyed vasopressinase nor liberated all the enzyme. The digestion by trypsin was conveniently terminated by addition of DFP, since it has been shown that organophosphorus inhibitors are ineffective on vasopressinase (Hooper, 1959). It seemed probable therefore that proteolysis in the presence of sodium cholate would be more effective, and in a 0.5 % cholate medium trypsin frequently liberated 85 % or more of vasopressinase in 2 hr. at 2°. Yields were not consistent, however, and occasionally they were no higher than those obtained by use of cholate or trypsin alone. Treatment of particles suspended in 0.01M phosphate buffer, pH 8.0, containing 0.5 % of sodium cholate for 16 hr. at 2° before incubation with 1:40 trypsin for 2 hr. at 2° caused a more reproducible release of enzyme. Optically clear supernatants were obtained only by centrifuging the tryptic digest at high speeds (25 000 *g* or more). Table 3 also shows the effect of incubating vasopressin with enzymes in the absence and the presence of DFP. The preparation containing DFP, i.e. inhibited trypsin,

Table 2. *Effect of butanol*

Method was as described in Table 1.

Preparation	Vol. of preparation incubated (ml.)	Residual vasopressin (%)	Enzyme (units/ml.)	Enzyme present (%)
Particulate fraction	1.0	6 (4-9)	1.7 (1.5-2.1)	100
Butanol, aqueous	3.0	17 (12-23)	0.4 (0.3-0.4)	23
Butanol, aqueous plus debris	1.0	17 (12-21)	1.1 (1.0-1.3)	65

Table 3. *Effect of cholate, lipase and trypsin*

Method was as described in Table 1.				
Preparation	Vol. of preparation incubated (ml.)	Residual vasopressin (%)	Enzyme (units/ml.)	Enzyme present (%)
Particulate fraction	1.0	12 (10-14)	1.3 (1.2-1.4)	100
Cholate, supernatant	2.0	18 (7-29)	0.6 (0.4-0.8)	46
Cholate, debris	2.0	14 (10-18)	0.6 (0.5-0.7)	46
Particulate fraction	1.0	13 (10-16)	1.3 (1.2-1.3)	100
Lipase, supernatant	2.0	45 (29-61)	0.3 (0.2-0.4)	26
Lipase, debris	2.0	12 (7-17)	0.7 (0.6-0.9)	54
Particulate fraction	0.5	19 (9-28)	2.1 (1.6-3.0)	100
Particles + cholate + trypsin*	0.5	8 (6-10)	3.1 (2.8-3.5)	147
Particles + cholate + trypsin + DFP	0.5	16 (9-23)	2.3 (1.8-2.5)	109
Supernatant from cholate + trypsin + DFP	0.5	26 (11-41)	1.7 (1.1-2.9)	81

* Particles were treated with 0.5% cholate for 16 hr. at 2° before incubation with trypsin for 2 hr. at 2°; enzyme activity was assayed before and after addition of DFP. DFP-treated material was centrifuged and enzyme content of supernatant measured.

assayed at 2.3 vasopressinase units/ml. whereas untreated material contained 3.1 units/ml. The destruction of vasopressin by trypsin is well known (Lawler & du Vigneaud, 1953).

The proteolytic digestion of tissue presents a hazard when end products contaminate material assayed by blood-pressure techniques, e.g. digestion of blood serum with trypsin releases bradykinin (Rocha e Silva, 1951), which has a pronounced vasodepressor effect. No interference from peptides formed by tryptic digestion of placental vasopressinase was observed in the present work. This was shown by using the assay design and calculation described by Gaddum & Lembeck (1949). *P* values > 0.7 indicated that there was no significant divergence in parallelism between the effects caused by untreated vasopressin and vasopressin contaminated with tryptic digest of particulate matter.

DISCUSSION

Several procedures which disrupt mitochondria cause the release of a small percentage of the vasopressinase activity present in the particulate fraction of placenta. The greater part of the enzyme, however, can be obtained in solution only by preliminary treatment with anionic detergent followed by a short digestion with trypsin. It appears probable therefore that vasopressinase is associated with lipoprotein. Tryptic digestion is aided in some manner by sodium cholate. Presumably the detergent partially dissociates lipoprotein or assists its dispersion in the medium. No evidence is available to show whether or not vasopressinase liberated by mechanical disruption of particles is different from the enzyme liberated by tryptic digestion.

In the work described, it was desirable to minimize bacterial contamination, and for this reason

the use of trypsin was preferred to prolonged autolysis.

SUMMARY

1. Attempts have been made to prepare soluble vasopressinase from a particulate fraction of human placenta.
2. Mechanical disruption of particles liberated a small proportion of enzyme.
3. Treatment of particles with 0.5% sodium cholate solution at pH 8.0, followed by digestion with trypsin, liberated the major part of vasopressinase activity.

I wish to thank Professor R. J. Kellar for his interest, and Dr D. C. Jessup for his collaboration in the early phase of this work. I also thank Dr D. R. Davies, Porton, for a gift of DFP, and Dr J. K. Grant for use of an ultracentrifuge. The skilled technical assistance of Miss M. Cruickshank and Mr J. Caird is gratefully acknowledged.

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