cidin-treated leucocyte are made on cells in free suspension. Under these conditions the localization of the extruded material will not be as effective as in the pancreas or adrenal medulla and the restrictions on the movements of the cell surface of an individual cell due to the resistance to deformation of its neighbours will be absent.

In the pancreas, the adrenal medulla, the platelet and the leucocidin-treated leucocyte, the transfer from the cell of material contained within cytoplasmic particles is associated with fusion of the cytoplasmic particles with the surface of the cell. The subsequent extrusion of the material can be by rupture of the cell surface or by a permeability change at the site of fusion. The relative contribution of these two processes is not known in any secreting tissue.

SUMMARY

1. Leucocytes treated with leucocidin have been examined with the light-microscope and electron microscope.

2. When calcium is present in the medium the cells lose their granules and vesicles can be seen in the cytoplasm and in the medium. Some of the vesicles are fused to the surface of the cell.

3. In the absence of calcium the random brownian motion of the granules persists for at least 30 min. There is no degranulation and vesicles cannot be observed. When calcium is added to the leucocidin-treated cells after incubation in calcium-free medium the brownian motion stops, some of the granules disappear and vesicles can be seen in the cytoplasm.

4. The specific activities of some enzymes in the granules and the vesicles have been measured. It is suggested that the vesicles are produced from granules, some of which have discharged part of their contents. The conversion of the granules into vesicles may be simultaneous with the fusion of the granule and the cell surface.

5. It is suggested that the release of protein from the vesicles follows rupture of the membrane at the site of fusion, or occurs by a permeability change at the site of fusion or by both of these processes.

A.M.W. is a Member of the Scientific Staff of the Medical Research Council.

REFERENCES

Caulfield, J. B. (1957). *J. biophys. biochem. Cytol.* **3**, 827.
Cohn, Z. A. & Hirsch, J. G. (1960). *J. exp. Med.* **112**, 983.
de Robertis, E. D. P., Nowinski, W. W. & Saez, F. A. (1960). *General Cytology*, p. 488. Philadelphia: W. B. Saunders Co.
Ekholm, R., Zelander, R. T. & Edlund, Y. (1962). *J. Ultrastruct. Res.* **7**, 61.
Florey, H. W. & Grant, L. H. (1961). *J. Path. Bact.* **82**, 13.
French, J. E. & Poole, J. C. F. (1963). *Proc. Roy. Soc.* B, **157**, 170.
Gladstone, G. P., Mudd, S., Hochstein, H. O. & Lenhart, N. (1962). *Brit. J. exp. Path.* **43**, 295.
Gladstone, G. P. & van Heyningen, W. E. (1957). *Brit. J. exp. Path.* **38**, 123.
Marchesi, V. T. (1961). *Ciba Found. Study Group: Biological Activity of the Leucocyte*, **10**, p. 76.
Millonig, G. (1961). *J. biophys. biochem. Cytol.* **11**, 736.
Palade, G. E. (1959). *Symp. Subcellular Particles, Woods Hole, Mass.* Ed. by Hyashi, T. New York: Ronald Press Co.
Woodin, A. M. (1961). *Biochem. J.* **80**, 562.
Woodin, A. M. (1962). *Biochem. J.* **82**, 9.
Woodin, A. M. (1963). *Symp. biochem. Soc.* **22**, 126.
Woodin, A. M. & Wieneke, A. (1963). *Biochem. J.* **87**, 480.

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Isolation of Phosvitin from the Plasma of the Laying Hen

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(*Received* 19 *December* 1962)

Heald (1962) has shown that phosphorylserine can be isolated from the plasma phosphoproteins of the laying hen and presented evidence suggesting that all of the phosphoprotein phosphorus was present in this form. It was also pointed out that the overall nature of the phosphoproteins of plasma had not been firmly established. Thus, although Mok, Martin & Common (1961) isolated a small quantity of phosvitin from the plasma of the laying hen after treatment with oestrogens, they were apparently unable to do so from the plasma of the untreated laying hen.

Apart from this work there is no clear evidence to show whether the phosphoprotein of the plasma

is in fact phosvitin. In the present work the isolation of phosvitin, in good yield, from the plasma of the untreated laying hen is described.

MATERIALS AND METHODS

Collection of plasma

The birds used were mainly single-comb White Leghorns of an inbred line purchased from Moncrieff's Animal Station, Godstone, Surrey. Two further groups of a Rhode Island Red × Light Sussex cross, bred at the National Institute for Research in Dairying, Shinfield, near Reading, were also used. All birds were in full lay. Plasma was obtained as described by Heald (1962) and stored at 2° until required, but not for a period exceeding 6 hr. Blood samples up to 10 ml. were collected from the wing vein.

Isolation of phosvitin from plasma

Precipitation of calcium-phosphoprotein complex. To the cold plasma (490 ml.) 0·1 vol. of $CaCl_2$ solution (20 mg. ions of Ca^{2+}/ml.) was added. After mixing, the solution was diluted to 5 l. with ice-cold water and left at 2° for 1–2 hr. to allow the precipitate to flocculate. As much as possible of the supernatant was decanted off and the precipitate was collected by centrifuging the remainder. This precipitate was sometimes gelatinous and if so was first broken by stirring before centrifuging.

The precipitate was mixed with 0·25 M-NaCl (120 ml.), by using a Vibromix (Shandon Scientific Co., Cromwell Road, London) for 1 hr., and filtered. The fibrous residue was washed with approx. 50 ml. of 0·25 M-NaCl and again filtered. The combined filtrates, which were pale-yellow, were diluted with 9 vol. of water and, after standing for 1–2 hr. at 2°, the precipitate was collected by centrifuging. It was dissolved in 100 ml. of 0·2 M-NaCl and dialysed against two changes of 0·05 M-EDTA (sodium salt), pH 7·4, and finally against two changes of water (2 l. each).

Extraction of lipid and removal of protein. To the dialysed solution, which was filtered if necessary, was added M-sodium acetate solution, pH 4·0, to give a final concentration of 0·05 M, and solid $(NH_4)_2SO_4$ to give a final concentration of 0·4 M. The solution was then shaken vigorously with 1 vol. of diethyl ether and left overnight. The ether layer was discarded, the opaque lower phase was removed and kept, and the gelatinous interface was centrifuged to remove as much solution as possible. The residue was extracted twice with 0·4 M-$(NH_4)_2SO_4$ in 0·05 M-sodium acetate, pH 4·0, 0·5 vol. of diethyl ether being added to the first extraction.

The combined lower phases were shaken with 1 vol. of diethyl ether and, after standing for 1 hr., the resulting lower phase was filtered and dialysed against three changes of water (2 l. each), and finally freeze-dried.

Fractionation on diethylaminoethylcellulose. Diethylaminoethylcellulose (DEAE-cellulose) (Whatman DE50), treated as described by Peterson & Sober (1962), was equilibrated with 0·1 M-NaCl in 5 mM-tris–HCl buffer, pH 8·0 (Connelly & Taborsky, 1961), and poured to form a column 30 cm. × 2·5 cm.^{2}. A sample (80–100 mg.) of the freeze-dried material was dissolved in 5 mM tris–HCl buffer, pH 8·0, and loaded on the column. Fractions were eluted with a NaCl gradient in 5 mM-tris–HCl buffer, pH 8·0. The reservoir contained 500 ml. of 0·5 M-NaCl in 5 mM-tris–HCl buffer, pH 8·0, and the mixing chamber contained 250 ml. of 5 mM-tris–HCl buffer, pH 8·0. Fractions (10 ml.) were eluted at a rate of 50–70 ml./hr. and analysed for protein (extinction at 280 mμ) and phosphorus. On such a column authentic phosvitin prepared from egg yolk was eluted in fractions 28–38. The bulk of the phosphorus present in plasma fractions, when separated on a similar column, was also found in the eluate fractions 28–38. The combined fractions were dialysed against two changes of 5 mM-EDTA (sodium salt) in 0·01 M-sodium acetate, pH 4·0, and then against two changes of 2–3 l. of water, and finally freeze-dried. The final product was a white fluffy powder readily soluble in water.

Phosvitin

An authentic sample of phosvitin was prepared from egg yolk by the method of Mecham & Olcott (1949). It contained 10·3% of P and 11·0% of N. When subjected to chromatography on DEAE-cellulose as described above a single peak was obtained. No other protein component was detected.

Analytical methods

Determinations. Total nitrogen, total phosphorus and phosphorus labile in alkali were determined by methods described by Heald (1962).

Ultracentrifuging. Sedimentation constants were determined in the Spinco model E centrifuge with the standard cell. Material was dissolved in 0·5 M-NaCl at concentrations of 3·76 and 8·8 mg./ml.

RESULTS

Phosphoprotein in the plasma of the domestic fowl has previously been precipitated by adjusting the pH to 5·0 before diluting with water (Laskowski, 1935; Mok *et al.* 1961), or by diluting the plasma with 9 vol. of water without a preliminary pH adjustment (Laskowski, 1935; McIndoe, 1959). Comparisons of the effectiveness of these treatments (Table 1) showed that precipitation by dilution at pH 4·9 resulted in the bulk of the plasma phosphoprotein being found in the precipitate, whereas dilution of the untreated plasma yielded a precipitate containing only 44–45% of the plasma phosphoprotein (cf. McIndoe, 1959; Heald, 1962). Complete precipitation of the plasma phosphoprotein was also obtained if the Ca^{2+} ion concentration was adjusted to at least 2·0 mg./ions/ml. before dilution. This procedure yielded a product containing less protein than was found in the acid precipitate. The precipitate could also be dissolved in 0·25 M-sodium chloride and reprecipitated by dilution without further loss of phosphoprotein. Precipitation by adjusting the concentration of Ca^{2+} ions was therefore chosen as a simple method of obtaining the phosphoprotein of plasma in a purer form for further treatment.

Removal of lipid and contaminating protein. The precipitate was insoluble in water and contained appreciable quantities of ash. It became soluble

when dialysed against EDTA (sodium salt) solution, presumably owing to the removal of Ca^{2+} ions. Extraction of lipid and removal of the bulk of the contaminating protein from solution was carried out under the conditions described by Mecham & Olcott (1949) for the preparation of phosvitin from egg yolk. Even so the material finally obtained on freeze-drying of the dialysed solutions contained variable amounts of phosphorus ranging from 5·2 to 8·1% of the dry weight. This phosphorus was completely labile in N-sodium hydroxide when incubated for 18 hr. at 37°. A typical recovery of the plasma phosphoprotein phosphorus up to this stage is shown in Table 2. Three other experiments gave recoveries of 48·3, 64·5 and 77%.

Further purification was achieved by chromatography on DEAE-cellulose under the conditions described by Connelly & Taborsky (1961). A typical elution pattern for the plasma phosphoprotein is shown in Fig. 1. The bulk of the phosphorus-containing fraction was eluted between 0·34M- and 0·38M-sodium chloride, and was collected and dialysed against 0·04M-sodium acetate, pH 4·0, to reverse any intramolecular displacement of phosphate groups that may have occurred during elution at the alkaline pH (Taborsky & Allende, 1962). After dialysis against water the material was freeze-dried. When rechromatographed on a similar column the material was eluted as a single peak in a position similar to that of the original sample. The product contained 10·0% of P and 11·02% of N on a dry-weight basis (N:P molar ratio, 2·44). The recovery in the presumptive phosvitin fraction of phosphorus added to the column was 81·5% in this experiment. Two other experiments gave recoveries of 64·8 and 87·5%.

Amino acid composition. The amino acid composition of the plasma phosphoprotein is shown in Table 3. In the same table, for comparison, are presented analytical values obtained by other workers using phosvitin isolated from egg yolk. The composition of the plasma phosphoprotein was almost identical with that of the yolk phosvitin. The values obtained for serine were slightly lower than those reported by Mecham & Olcott (1949) and Taborsky & Allende (1962), who applied corrections for losses of pure serine or phosphorylserine when these were hydrolysed at the same time as the protein.

Hydrolysis of the plasma phosphoprotein. When the plasma phosphoprotein was hydrolysed in hydrochloric acid the rate of release of inorganic phosphorus was identical with that from an authentic specimen of phosvitin (Fig. 2).

Table 1. *Precipitation of phosphoprotein from the plasma of the laying hen*

Plasma was obtained, and the precipitates were analysed for phosphoprotein phosphorus and total nitrogen, as described in the Materials and Methods section. Samples (0·5 ml.) of plasma together with the various additions were brought to a final volume of 5·0 ml. with water. After being chilled in ice for 1 hr. the precipitates were centrifuged and analysed. The values are given as percentages of similar values obtained when plasma samples were precipitated with 10% (w/v) trichloroacetic acid.

Addition	Final concn. in plasma	Recoveries (%) Phospho-protein	Nitrogen	N:P molar ratio
None	—	11·9	10·8	1·1
		25·5	42·2	1·65
Sodium acetate (pH 4·9)	0·2M	92·0	17·7	0·47
		99·5	16·8	0·41
		93·0	14·8	0·36
Ca^{2+} ions	2·5 mg.ions/ml.	92·6	7·7	0·23
		94·8	8·8	0·23
		86·0	6·2	0·16

Table 2. *Recovery of phosphoprotein phosphorus during isolation from the plasma of the laying hen*

Plasma was collected and processed as described in the Materials and Methods section. Samples of each fraction were analysed for phosphoprotein as described in that section.

Fraction	Volume (ml.)	Phosphoprotein phosphorus: Total/ fraction (mg.)	As % of that in original plasma
Plasma	360	14·44	100
Ppt. with $CaCl_2$ after extraction with NaCl and dialysis	122	15·4	106
Ether-extracted $(NH_4)_2SO_4$ soln.	134	8·8	59·0

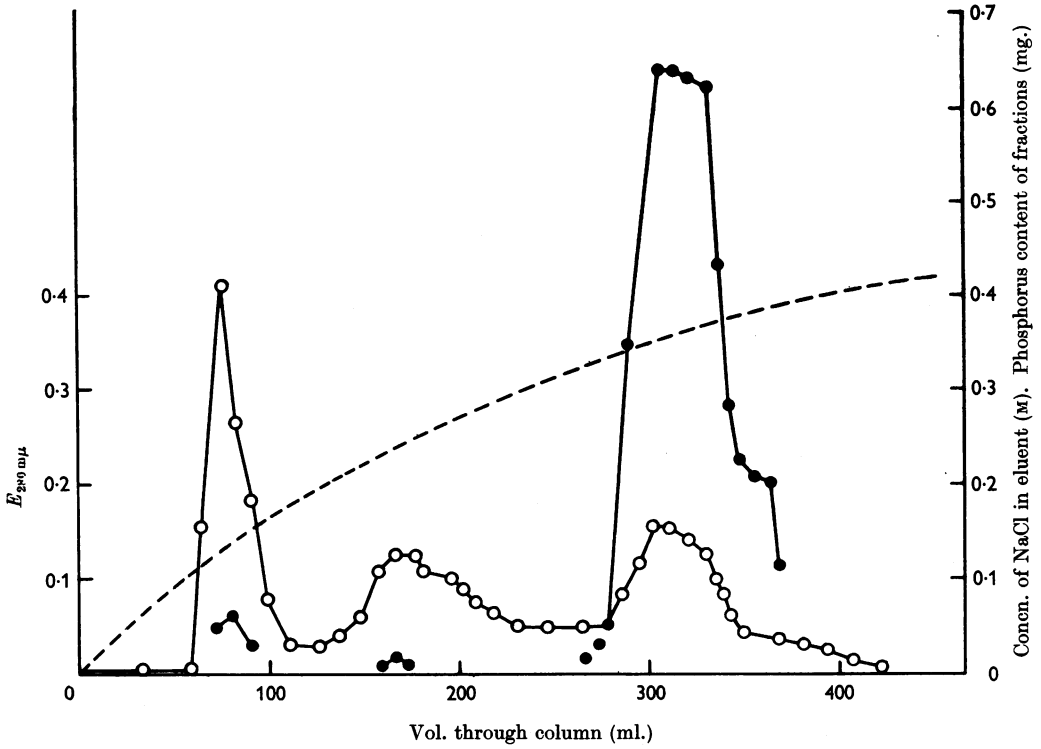

Fig. 1. Separation of phosphoproteins in a fraction from laying-hen plasma on DEAE-cellulose. The column and fractions were prepared as described in the Materials and Methods section. The broken line indicates the concn. of NaCl in the eluent. ○, Extinction at 280 mμ; ●, total phosphorus in the fractions.

Sedimentation constant. The sedimentation constant of the plasma fraction in 0·5 M-sodium chloride was determined at two concentrations of 3·76 mg./ml. and 8·8 mg./ml. Extrapolation to zero concentration gave a $S^0_{20,w}$ value of 3·11 s, in good agreement with that of 3·14 s reported for egg-yolk phosvitin in 0·5 M-sodium chloride (Joubert & Cook, 1958) and that of 3·17 s for phosvitin from the plasma of the oestrogen-treated hen (Mok *et al.* 1961).

DISCUSSION

There seems little doubt that the material isolated from the plasma of the laying hen is phosvitin. Thus the phosphorus and nitrogen contents, sedimentation constant and rate of hydrolysis agree with those found for phosvitin prepared from egg yolk. More strikingly the amino acid composition of the two materials is in close agreement. Phosvitin is a unique protein in which serine accounts for slightly more than half of the total amino acid present. The similarity between the amino acid distribution of the two materials is therefore good evidence of their identity. The equivalence of the phosphorus and serine contents of both phosphoproteins re-emphasizes that in the plasma fraction, as in the yolk phosvitin, phosphorus is present solely as *O*-phosphorylserine (Heald, 1962).

The recovery of phosvitin over the whole procedure can be calculated from the results given in Table 1 and from the quantities recovered from the DEAE-cellulose columns. Thus in three experiments the overall calculated recoveries were 39·5, 41·5 and 67·5 % of the phosphoprotein phosphorus originally present in the plasma. Since major losses could not be attributed to any individual step in the isolation procedure and since no other major phosphorus-containing protein was detected in eluates from the DEAE-cellulose columns, it is considered that the overall loss represents the summation of small losses normally occurring in fractionations of this type. There is at present no reason to believe that a phosphoprotein other than phosvitin is present in the plasma of the laying hen.

The identity of the plasma and yolk phosvitins appears to establish that the yolk constituent is synthesized elsewhere in the body and is transferred to the yolk from the plasma without further modification. Nevertheless, phosvitin as such is

Table 3. *Amino acid composition of phosvitin from the plasma of the laying hen*

Samples were hydrolysed at 100° in 6N-HCl for 24 and 48 hr. respectively and, after removal of the HCl by evaporation at reduced pressure over KOH and P_2O_5, the residues were taken up in water and analysed by the method of Moore, Spackman & Stein (1958). The final values were obtained by plotting the analytical values on graph paper and extrapolating to zero-time to correct for loss incurred during hydrolysis. Values are given in moles/kg. of protein; the results for egg-yolk phosvitin are from Mecham & Olcott (1949) and Taborsky & Allende (1962).

Amino acid	Plasma phosphoprotein	Egg-yolk phosvitin
Serine	2·86	3·1–3·7
Lysine	0·31	0·40–0·45
Arginine	0·30	0·28–0·31
Histidine	0·28	0·30–0·31
Aspartic acid	0·35	0·32–0·36
Glutamic acid	0·34	0·23–0·32
Glycine	0·17	0·16–0·21
Alanine	0·10	0·20
Valine	0·03	0·06
Leucine	0·15	0·07–0·08
Isoleucine	0·09	0·04
Proline	0·0	0·09
Threonine	0·07	0·12
Phenylalanine	0·06	0·07
Tyrosine	—	0·01–0·07
Tryptophan	—	0·03
Methionine	0·14	0·0–0·02
Cysteine + cystine	0·0	0·0
Phosphate	3·22	3·10–3·23
Phosphorus:serine ratio	1·13	0·87–1·0

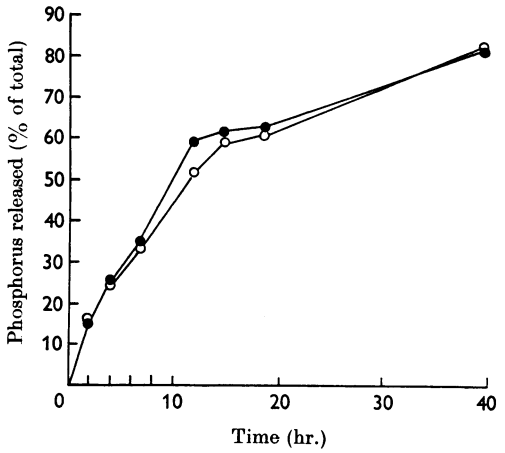

Fig. 2. Release of orthophosphate from plasma and egg-yolk phosvitins on hydrolysis in 2N-HCl at 100°. ○, Egg-yolk phosvitin; ●, plasma phosvitin.

unlikely to exist in the plasma since the presence in the molecule of numbers of negative phosphate groups make it probable that such a material would form a complex with other plasma constituents. Thus McKully & Common (1961) showed that, although a presumptive phosvitin could be separated from egg yolk by electrophoresis on paper in veronal buffer, no such similar component was distinguishable when whole serum of laying hens was similarly treated. It was further shown that chelation or removal of Ca^{2+} ions from the serum led once again to the appearance of a phosphoprotein zone, presumably owing to the dissociation of the phosphoprotein from the lipoproteins of the serum. It has also been shown, by ultracentrifuging techniques, that the plasma of the laying hen contains a complex consisting of phosphoprotein, lipid and Ca^{2+} ions (Schiede & Urist, 1960).

The nature of such complexes is not clear, but those in plasma differ in certain chemical respects from those in yolk. Thus, apart from the findings of McKully & Common (1961), attempts to prepare phosvitin from 400 ml. of plasma from laying hens by applying the method of Mecham & Olcott (1949) were unsuccessful. No precipitate of a magnesium–lipovitellin complex was obtained in the first stage. Further, when plasma containing radioactive phosphoprotein (Heald, 1962) was mixed with egg yolk, and phosvitin isolated from this mixture by the method of Mecham & Olcott (1949), the phosvitin so obtained was not radioactive. These findings parallel those of Mok *et al.* (1961), who were unable to isolate phosvitin from the plasma of the untreated laying hen by another method involving the initial precipitation of the magnesium–lipovitellin complex (Joubert & Cook 1958). Taken together they indicate that, although the plasma and yolk phosvitins are chemically identical, the complexes in which they exist in the two sources are not.

SUMMARY

1. Phosvitin has been isolated from the plasma of untreated laying hens by a simple procedure. The overall yields varied from 39 to 67% of the phosphoprotein phosphorus present in the original plasma samples.

2. The material isolated contained 10·0% of P and 11·02% of N. The amino acid composition was identical with that of egg-yolk phosvitin in which serine represented more than half of the total amino acids. The sedimentation constant, $S^0_{20,w}$, of 3·11S agreed with that of authentic yolk phosvitin. The rate of release of phosphate on acid hydrolysis was also similar to that of phosvitin.

3. Phosvitin in the plasma probably exists in a complex chemically different from that in the yolk.

The authors thank Dr J. L. Bailey and Mr L. Lester for carrying out the amino acid analyses and Dr M. E. Coates for supplying some of the birds used in these experiments.

REFERENCES

Connelly, C. & Taborsky, G. (1961). *J. biol. Chem.* **236**, 1364.
Heald, P. J. (1962). *Biochem. J.* **83**, 212.
Joubert, F. J. & Cook, W. H. (1958). *Canad. J. Biochem. Physiol.* **36**, 399.
Laskowski, M. (1935). *Biochem. Z.* **278**, 345.
McIndoe, W. M. (1959). *Biochem. J.* **72**, 153.
McKully, K. A. & Common, R. H. (1961). *Canad. J. Biochem. Physiol.* **39**, 1451.
Mecham, D. K. & Olcott, H. S. (1949). *J. Amer. chem. Soc.* **71**, 3670.
Mok, C. C., Martin, W. G. & Common, R. H. (1961). *Canad. J. Biochem. Physiol.* **39**, 109.
Moore, S., Spackman, D. H. & Stein, W. H. (1958). *Analyt. Chem.* **30**, 1185.
Peterson, E. A. & Sober, H. A. (1962). In *Methods in Enzymology*, vol. 5, p. 3. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Schiede, O. A. & Urist, M. R. (1960). *Nature, Lond.*, **188**, 291.
Taborsky, G. & Allende, C. C. (1962). *Biochemistry.* **1**, 406.

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The Post-Natal Development of Hepatic Fructokinase

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(*Received* 19 *December* 1962)

The presence of fructose in the foetal blood and fluids of some species has been indicated for a long time (for review, see Needham, 1931) and was confirmed by Bacon & Bell (1948). Attempts to decide which of two postulated mechanisms is involved in the formation of foetal fructose have been inconclusive (Neil, Walker & Warren, 1961). Relative impermeability of the placenta to fructose is observed in both fructogenic and non-fructogenic species and may only be indirectly related to the presence of foetal fructose by assisting in its accumulation once formed (Walker, 1960).

The accumulation of fructose in the conceptus will also depend on its utilization by the foetal tissues. Absence of liver enzymes involved in the metabolism of fructose is likely to be of major importance. Parks, Ben-Gershom & Lardy (1957) reviewed methods for the assay of specific fructokinase (adenosine triphosphate–D-fructose 1-phosphotransferase, EC 2.7.1.3) and described an improved procedure giving higher activities and zero-order kinetics. Using earlier methods, Kuyper (1955) reported that liver fructokinase is absent in the foetal rat and that it develops after birth, and Hers (1957) briefly noted the absence of the enzyme in the foetal sheep. Andrews, Britton, Huggett & Nixon (1960) demonstrated by a perfusion technique that the foetal-sheep liver is unable to utilize fructose. Andrews, Britton & Nixon (1961) showed that conceptual age and possibly premature delivery are factors determining the commencement of the hepatic metabolism of fructose in sheep as indicated by the perfusion technique, and suggested that this was due to the post-natal appearance and development of hepatic fructokinase. The indirect evidence for this is therefore good. As part of a wider programme of work on carbohydrate metabolism in the developing foetal and new-born mammal (Walker, 1962; Lea & Walker, 1962), direct evidence for the post-natal development of hepatic fructokinase in several species has been obtained and is reported in the present paper. Evidence is also presented indicating that the absence of the enzyme in the new-born rabbit (a non-fructogenic species) lowers the fructose tolerance of the animal compared with the adult.

MATERIALS AND METHODS

Animals. The male and female rats were an ordinary laboratory albino strain, and the rabbits were of no special variety. The guinea pigs were of the Pirbright albino strain (the original stock having been obtained from the Animal Virus Diseases Research Institute, Pirbright, Surrey) and were bred by an intensive polygamous group method (Paterson, 1957). They were maintained on a diet consisting of equal parts (w/w) of Spiller's Intensive Poultry Pellets with added vitamins and Diet R.G.P. (C. Hill Ltd., Poole, Dorset) plus a liberal supply of fresh greenstuff daily and hay and water *ad lib.*

Gestational age was assessed on the assumption of post-partum mating (rats), observed mating (rabbits) and a combination of post-partum mating and the tables (Draper, 1920) of crown–rump length and weight against age (guinea pigs). Gravid guinea pigs were used normally during their second or third pregnancy; the mean gestation period was 68 days.

Chemicals and enzyme. Fructose ('glucose-free') was obtained from British Drug Houses Ltd. ATP (disodium salt) and creatine phosphate (sodium salt) were obtained from Sigma Chemical Co. (through G. T. Gurr Ltd.,