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Fructose and Glucose in the Blood of the Foetal Sheep

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(Received 15 July 1947)

Little is known of the distribution of D-fructose in animal tissues, apart from its demonstration in certain embryonic fluids (see, e.g., Needham, 1931, 1942). Recently it has been positively identified by Mann (1946) as the reducing sugar in the seminal plasma; the fact that hitherto it had been taken for granted that this was glucose suggests that fructose may have escaped detection in other instances.

There is no excuse for this, for a characteristic, though not entirely specific, colour reaction, due to Seliwanoff (1887), has been available, and in the methylphenylosazone Neuberg (1902; Neuberg & Strauss, 1902) found a derivative by which it could be distinguished from accompanying glucose, provided always that a sufficiently concentrated solution was available. Our own attention was drawn to the problem of identifying fructose by the late Sir Joseph Barcroft and Mr S. W. Cole, who in the course of recent work on the foetus of the sheep had confirmed an observation of Paton, Watson & Kerr (1907). The latter authors detected by the Seliwanoff test a fructose-like substance in the amniotic and allantoic fluids of sheep, cow, pig, cat, dog, rabbit, ferret and guinea pig, and in foetal blood. They investigated the blood more closely: 'by extracting large quantities of foetal blood [of the sheep?] with alcohol, evaporating with charcoal, and taking up in a small quantity of water, we were able to extract a sugar which was laevorotatory and which gave Seliwanoff's reaction distinctly...'. Orr (1924) obtained similar results using lead acetate instead of charcoal, with blood from a newly born kid, and blood taken from the human placenta after birth.

The observations of Cole & Hitchcock (1946) indicated that appreciable quantities of fructose (up to 100 mg./100 ml.) were present in the blood of the

foetal sheep, and at Sir Joseph Barcroft's request we attempted (a) to prove that the substance responsible for the colour reaction was in fact fructose, and (b) to isolate a derivative of the sugar.

EXPERIMENTAL

Methods

Standard fructose and glucose solutions. These were made in saturated benzoic acid solution (Folin, 1929); a concentrated solution (c. 2%) was made for polarimetric measurement of the concentration, and this was diluted with more saturated benzoic acid solution to give a final sugar concentration of 0.5 mg./ml. In our experience a solution of either sugar at this, or a lower concentration, does not deteriorate when kept for several years at room temperature in a well-stoppered bottle.

Total reducing substances (t.r.s.). (More strictly, reducing substances estimated by the ferricyanide-cerium method.) The procedure used was that of Miller & Van Slyke (1936), the solutions being heated in boiling tubes without stoppers, and titrated to the nearest drop (0.02 ml.) after cooling and acidification, using xylene cyanole FF as an internal indicator. Instead of standardizing the ceric sulphate solution and using a fixed time of heating, a standard fructose, and occasionally a glucose solution, containing 100 µg. of sugar, was included in each set of estimations, and heating was carried out for 10–12 min. in a boiling water bath. The ceric sulphate solution was of such a concentration that 1 ml. was equivalent to about 100 µg. of reducing substance, calculated as hexose. Contrary to the findings of Miller & Van Slyke (1936) we found no discrepancy greater than the experimental error, between the reducing values of glucose and fructose, nor did we find any appreciable variability in the reducing value of fructose.

Fructose. For the estimation of those substances giving the Seliwanoff reaction, called here 'fructose' for the sake of brevity, a modification of Roe's method (1934) was used. This was devised by Mr S. W. Cole, who very kindly gave

us the details and provided the facilities for carrying it out. A full account of the application of the method to the estimation of fructose and inulin in blood and urine will be published at a later date.

To the test material contained in 4 ml. of aqueous solution (in the case of the blood filtrates, diluted 1 in 10, 1 ml. plus 3 ml. of water was usually sufficient), in glass-stoppered test tubes, were added first 6 ml. of resorcinol solution (A.R., 0.15% (w/v) in absolute ethanol), thereafter at 1 min. intervals 6 ml. of conc. HCl containing 7.5 mg. $\text{FeCl}_3/1$. After thorough mixing of the contents, the tubes were placed at 1 min. intervals in a thermostatically controlled water bath at 75°, and left for 30 min. They were then cooled in jars of cold water, and the colour developed read with suitable filters in 2 cm. cells in a Spekker photo-electric absorptiometer, against a 'blank' on the reagents plus water. Standard fructose solutions containing 50 or 100 μg . were included in each set of estimations; these gave densities of about 0.32 and 0.65 respectively. The majority of readings were made with a spectrum blue filter (Ilford Ltd., no. 602), but in some experiments a Wratten no. 75 (Kodak Ltd.), which gave rather smaller densities, was used instead. (In a very few experiments the bath temperature was 80° and the time of heating then either 20 or 30 min.) Mr Cole tells us that under these conditions the specificity of the method is such that glucose gives between 0.6 and 0.8% of the colour given by an equal amount of fructose, and that the relationship of density to concentration over the range 20–100 μg . is for all practical purposes a straight line.

Yeast fermentation. This was carried out with a commercial (Distillers Company Ltd.) baker's yeast. A sample of the pressed yeast was suspended in ten times its weight of tap water, left for an hour or more at room temperature, centrifuged, and resuspended in water. In some experiments the washing was repeated; in others the yeast was resuspended in phosphate buffer at pH 6. An equal volume of the suspension was added to the test solution and left, with occasional mixing, for three or more hours at room temperature, or for a shorter period at 25°. Under these conditions, as the results below show, fructose is completely removed from the solution.

Estimation of glucose by the use of glucose oxidase. For these estimations we are greatly indebted to Dr T. Mann (cf. Mann, 1946) who provided a solution of the enzyme and assisted in the carrying out of the experiments. The oxidation was allowed to proceed at room temperature in the presence of a trace of catalase, after which appropriate estimations were made, usually without preliminary deproteinization.

Source and collection of blood

All the foetal blood used was obtained for us by Sir Joseph Barcroft, and was collected from a cannula in the carotid artery of the foetus, or, occasionally, by a syringe from the umbilical vessels, when the maximum quantity was wanted. The foetus was in every case still attached by the placenta to its mother who was anaesthetized with spinal Duracaine before the operation, and both were partly immersed in a warm bath. Observations had been made on the foetus and mother during the 1–1.5 hr. between the opening of the uterus and the taking of blood, but these were not considered to have disturbed the metabolism of the foetus unduly. It is likely, however, that the blood

sugar level of the mother was in these circumstances higher than before anaesthetization (Hitchcock, 1948), and the glucose level in some of the foetal blood samples may have been raised correspondingly.

The blood was allowed to run through a rubber tube into a convenient volume of distilled water (see below) contained in a stoppered measuring cylinder which had been chilled on ice, the time for collection, even of large samples, not exceeding 5 min. In this way glycolysis was reduced to a minimum. The partly haemolyzed blood was mixed thoroughly, the increase in volume noted, and samples withdrawn for cadmium deproteinization (see below). The amount of blood collected was inferred from the increase in volume. Immediately after the withdrawal of samples more water and 97% ethanol were added, and the haemolysis thereby completed. The mixture was then transferred to bottles of litre capacity stoppered by rubber bungs, and kept cold until the next step in solvent deproteinization.

Deproteinization

With cadmium hydroxide. This was used for the samples of diluted blood, which were used to measure the t.r.s. and fructose contents of the blood, and as a check on the recovery of the sugars in the bulk deproteinization by organic solvents. The method used was essentially that of Fujita & Iwatake (1931). The precipitated material was removed by centrifuging because of the possibility that heating in alkaline solution might convert glucose to fructose. The supernatant fluids obtained were water-clear; for convenience they are referred to as 'cadmium filtrates'.

With ethanol-chloroform. This method is an adaptation by Keilin & Mann (1940) of that used by Tsuchihashi (1923), and we are grateful to Dr Mann for the guidance which he gave us in its use. A typical treatment was as follows: 160 ml. of blood were collected into 200 ml. of water. After withdrawal of two 1 ml. samples for cadmium deproteinization, haemolysis was completed by the addition of a further 250 ml. of water, and 300 ml. of 97% ethanol. To the haemolyzed mixture 150 ml. of chloroform were added and the mixture shaken *very gently*. The resulting precipitate did not flocculate completely. 2 ml. of approx. 20% acetic acid were added to make the suspension orange to methyl red, and after very gentle agitation the precipitate separated rapidly, leaving a water-clear supernatant fluid. The material was left in the refrigerator for 2 hr. and centrifuged. (Centrifuging at this stage gives three layers, an upper aqueous one, an intermediate layer of coagulated protein, and a lower layer of chloroform. When the aqueous layer is decanted the chloroform sometimes runs out as well, but can easily be separated from it.) 755 ml. of supernatant fluid were decanted through glass wool. The protein and chloroform layers were shaken up vigorously with 300 ml. of an emulsion made by shaking a 2:1 water-ethanol mixture with an excess of chloroform. The suspension was again centrifuged and 300 ml. of water-clear supernatant fluid decanted through glass wool into the main bulk. The colourless solution was stored in the refrigerator.

In our experience it was necessary to adjust the pH carefully, and not to shake too vigorously at any stage, otherwise the coagulated protein formed a fine suspension which made opalescence in the aqueous phase difficult to see. Ideally, a water-clear fluid should be obtained on centrifuging; slight opalescence, however, did not appear to

interfere with subsequent operations. For convenience, the deproteinized solutions obtained by this procedure will be referred to as 'ethanol-chloroform filtrates'.

Concentration of ethanol-chloroform filtrates. This was done under reduced pressure with a bath temperature not exceeding 50°. When the volume had been reduced to about 20 ml., representing usually a fivefold concentration of the original blood, solid separated, but filtration in the presence of kieselguhr or Hyflo Supercel readily gave a clear pale-yellow solution. Alternatively the last stages of evaporation were done in the presence of a relatively large volume of Hyflo Supercel (e.g. 2 g.) and the blood concentrate was thereby obtained in a dry finely divided form. In a few experiments the concentrate was taken to complete dryness and extracted with absolute ethanol (see below). (All these are called 'ethanol-chloroform concentrates'.)

Recoveries

Fructose. A preliminary experiment showed that fructose added to maternal blood could be recovered in good yield in the ethanol-chloroform filtrate. The comparison of fructose contents of ethanol-chloroform filtrates and cadmium filtrates prepared from the same samples of foetal blood showed that the recovery at this stage was nearly complete (Table 1).

T.r.s. Recovery of t.r.s., though less significant, indicates no great discrepancy between the two methods of deproteinization (Table 1).

The reducing substances in cadmium filtrates of triplicate diluted samples of blood 19 were analyzed for us by Mr M. W. S. Hitchcock, who used the copper-reduction method of Somogyi (1945). Corrected for dilution, these gave an average of 133.3 mg. reducing substances/100 ml. of

blood, as against our value of 130.4 mg./100 ml. by the ferricyanide-cerium method.

Ethanol extraction. Table 2 shows the percentage recoveries (in all fractions) of fructose and t.r.s. at several stages of the working up of a sample of the pooled blood filtrates 3, 4, 5, 6. The losses on removal of the ethanol and chloroform are probably mainly fictitious, since these solvents increase slightly the values obtained by both methods (e.g. in the fructose estimation the addition of 1 ml. of the clear supernatant fluid from shaking together 10 ml. water, 5 ml. 97% ethanol, and 2 ml. chloroform increased the 'fructose' value of 50 µg. fructose to 52.4 µg., and of 100 µg. to 103.2).

From these results it was concluded that the ethanol-chloroform filtrates could be taken as reasonably representative of the original bloods, in so far as fructose and t.r.s. were concerned.

RESULTS

Composition of the original foetal blood samples as measured on the cadmium filtrates

The figures in Table 3 give the age of the foetus, and the fructose and t.r.s. contents of the blood sample, measured on the cadmium filtrate (cf. Cole & Hitchcock, 1946). In some cases the amount of blood used was not the whole of that obtained from the foetus. It will be seen that variation in the fructose and t.r.s. contents is apparently at random, although Hitchcock (1948), who took samples from younger foetuses, suggests that the fructose content falls towards the end of term (147 ± 3 days), while the t.r.s.-fructose (which he assumes to be glucose) rises.

Table 1. *Total reducing substances (t.r.s.) and fructose (F) in ethanol-chloroform and cadmium filtrates compared*

Blood sample	Amount in sample (mg.)							
	3, 4, 5, 6*		3, 4, 5, 6, 7*		11		19	
	T.r.s.	F	T.r.s.	F	T.r.s.	F	T.r.s.	F
Cadmium filtrate	147	30	105	56	326	158	196	124
Ethanol-chloroform filtrate (evaporated to small bulk)	157	28	98	52	311	137	201	104

* Pooled ethanol-chloroform filtrates from several blood samples; the figures refer to a portion of the combined filtrates. F=fructose.

Table 2. *Recovery of fructose and t.r.s. at successive stages of concentration and ethanol extraction of an ethanol-chloroform filtrate*

Stage	Recovery of fructose (%)		Recovery of t.r.s. (%)	
	Between stages	Overall	Between stages	Overall
Initial ethanol-chloroform filtrate	—	(100)	—	(100)
Evaporated to dryness <i>in vacuo</i> below 50°	94.8	94.8	88.3	88.3
Extracted with boiling absolute ethanol	86.2	81.8	97.3*	86.0
Ethanol extract evaporated to dryness <i>in vacuo</i>	97.7	80.0	89.9	77.3

* Part in extract, part in residue.

Table 3. *Composition and other data on blood samples, measured on cadmium filtrates*

No.	Age of foetus (days)	Fructose (mg./100 ml.)	T.r.s. (mg./100 ml.)	Vol. used (ml.)	No.	Age of foetus (days)	Fructose (mg./100 ml.)	T.r.s. (mg./100 ml.)	Vol. used (ml.)
1	107	142	157	70	11	142	71	145	224
2	112	97	—	60	12	141	75	155	300
3	120	123	142	62	13	141	51	83	184
4*	108	53	84	69	14†	142	56	114	142
5	129	34	61	71	15	141	76	150	180
6	129	25	57	85	16	141	69	107	160
7	132	69	159	178	17†	141	69	109	250
8†	139	66	—	110	18	124	—	—	50
9†	139	81	—	178	19	141	82	130	150
10	144	35	86	—	20	—	55	82	—

* Twin died, not sampled.

— Not estimated.

† 10–70 ml. of blood taken for other purposes. The bracketed fetuses are twins.

Analysis of concentrates of ethanol-chloroform filtrates

(a) *Optical rotation.* This was first measured on an aqueous solution of the absolute ethanol-soluble fraction of the material in an ethanol-chloroform filtrate. In a 2 dm. tube the rotation was -0.73° ; that calculated from its fructose content was -0.52° , suggesting that other laevorotatory substances were present, and that they were ethanol-soluble. Subsequently, rotations were read of several ethanol-chloroform concentrates; these are summarized in Table 4 with the values calculated from the fructose contents.

and could be read after filtration without the need for decolorization.

In most experiments 6.0 ml. of the concentrate were mixed with 5.0 ml. of a yeast suspension prepared as described above, and the fermentation was allowed to go to completion. The yeast was then centrifuged out, leaving sufficient clear supernatant fluid for the rotation to be read in a 2 dm. tube (capacity c. 9.5 ml.). Estimations of fructose were done on the solution before and after fermentation, and the rotation due to the fructose fermented was calculated. When these experiments and those with glucose oxidase indicated the possible presence of glucose, fermentable t.r.s. were estimated also, and the difference between this result and the fructose

Table 4. *Rotations of concentrates from ethanol-chloroform filtrates compared with the rotations calculated from their fructose contents*

Blood sample	...	3, 4, 5, 6, 7	11	19	12, 14, 16, 37
		(α_D)	(α_D)	(α_D)	(α_D)
Rotation in 2 dm. tube		-0.58°	-1.54°	-1.40°	-2.10°
Rotation calculated from fructose content		-0.32°	-1.00°	-0.75°	-1.42°

These results indicate clearly the presence of laevorotatory material which is not fructose, and illustrate the danger of assuming that laevorotation in itself is evidence for the presence of fructose (cf. Orr, 1924).

(b) *Change in optical rotation on treatment with yeast.* If the other laevorotatory material were not yeast-fermentable it should be possible to show that the change in rotation accompanying fermentation is equivalent to the fructose disappearing. To this end a series of experiments was performed. It was essential to begin with a relatively large optical rotation, and fortunately the concentrates of ethanol-chloroform filtrates were almost colourless,

content taken as glucose; the change in rotation expected on its disappearance was calculated and subtracted from that for the fructose. The results are summarized in Table 5.

These results make it clear that non-fermentable laevorotatory material is present. They also show fairly good agreement between the observed change in rotation and that calculated on the assumption that the fermentable reducing substance is a mixture of fructose and glucose. It will be noticed that in the absolute ethanol-soluble fraction the amount of non-fructose fermentable material is smaller, probably because of the limited solubility of glucose in that solvent.

Table 5. *Effect of yeast treatment on the optical rotation of ethanol-chloroform concentrates: comparison of the observed change in rotation with that calculated from the fructose and t.r.s. disappearing*

Sample	Vol. (ml.)	Losses on fermentation				Optical rotation		Change	
		Fructose		Non-fructose		Before	After	Observed	Calculated
		(mg.)	(α_D)	(mg.)	(α_D)				
11*	25	134.6	-1.00°	115.6	+0.49°	-1.54°	-0.97°	+0.57°	+0.51°
19	25	100.2	-0.75°	13.0	+0.06°	-1.40°	-0.67°	+0.73°	+0.69°
Control	10	120.1	-1.48°	58.6	+0.41°	-1.11°	0.0°	+1.11°	+1.07°
Ethanol extract 11†	25	112.0	-0.82°	15.4	+0.07°	-0.98°	-0.31°	+0.67°	+0.75°

* 10 ml. of the concentrate used for this experiment were evaporated to dryness *in vacuo* below 50°, pH 5, in the presence of 0.5 g. barium acetate and a little acetic acid. The residue, dried exhaustively over H_2SO_4 , was extracted with a total of about 100 ml. absolute ethanol, the extract being filtered through Hyflo Supercel on a G4 filter and evaporated to dryness, with the same precautions as to temperature. The residue was extracted with exactly 14 ml. of approx. 2% Na_2SO_4 , the $BaSO_4$ being removed by centrifuging. The clear supernatant liquid was used for the second experiment (†).

(c) *The action of glucose oxidase on concentrates.* Positive evidence for the presence of glucose was obtained through experiments with glucose oxidase.

A mixture of five ethanol-chloroform filtrates (500 ml.) (3, 4, 5, 6, 7) was evaporated to small bulk under diminished pressure with a bath temperature never higher than 52°. The pH was checked by means of methyl red throughout the concentration, which took 5 hr. A little kieselguhr was added and the suspension filtered by pressure on a G3 sintered-glass filter. The volume of filtrate was made to 30 ml. Samples (2.0 ml.) of this were taken for the estimation of yeast-fermentable t.r.s. and fructose. Other 2.0 ml. samples were taken to study the effect of glucose oxidase (plus catalase) on the t.r.s. and fructose. 200 μ g. of enzyme were used in a total volume of 2.9 ml., which included 0.5 ml. of m -acetate buffer (pH 5.2). The solutions were shaken for 1.5 hr. in the cups of Barcroft manometers. Estimations of t.r.s. and fructose were done without deproteinization. A control on the enzyme and buffer was not distinguishable from the blank, and the buffer had no effect on the results of either estimation when added to 100 μ g. of fructose. The significant findings are summarized in Table 6.

The agreement between (3) and (4) suggests that all the fermentable reducing substances can be accounted for by fructose and glucose.

A repetition of this experiment with blood sample 19, in which all the solutions were deproteinized before estimations were done, gave rather less satisfactory agreement; the ratio of fructose to glucose in this sample was much higher, about 8 : 1.

(d) *Other constituents of the concentrates.* The concentrate used for the experiment of Table 6 contained 82 mg. of lactic acid in 30 ml., corresponding to 91 ml. of mixed blood. (This is not enough to account, as L-lactate, for the non-fermentable laevorotatory material; see (b) above, cf. Bell, 1932.) In addition the concentrates gave a strong nitroprusside reaction which was removed by cadmium deproteinization. However, there is evidence that this treatment does not remove all the non-fermentable reducing material (see below). It was thought that extraction with absolute ethanol in the presence of barium acetate might leave behind some of these substances, yet the extract so obtained from

Table 6. *Effect of glucose oxidase and yeast on fructose and t.r.s. contents of a pooled ethanol-chloroform concentrate*

(All results expressed as mg./30 ml. of the original concentrate.)

	Untreated concentrate		After glucose oxidase treatment		After yeast treatment
	Alone	With buffer	No addition	2 mg. glucose added	
Fructose	50.9	51.6	51.0	50.3	1.2
T.r.s.	97.3	98.6	73.7	73.0	22.3

These results demonstrate that the enzyme had no effect on fructose, and that it had worked to completion. From them were calculated:

	(mg.)
(1) Fermentable reducing substances	(97.3-22.3)=75.0
(2) Fermentable fructose	(50.9-1.2)=49.7
(3) Fermentable non-fructose reducing substances	(75.0-49.7)=25.3
(4) Glucose from the effect of glucose oxidase	(98.6-73.7)=24.9

blood sample 11 still contained 50 mg. of non-fermentable t.r.s. ('fructose' content, 4.2 mg.) as against 56.5 before this treatment. The fermented extract still had a strong negative rotation (see Table 5).

We are indebted to others for certain additional information: Miss V. Moyle kindly estimated allantoin for us, in blood concentrate 19, by the method of Young, MacPherson, Wentworth & Hawkins (1944), and found an

amount corresponding to 0.3 mg./100 ml. of the original blood (cf. 1.9 mg./100 ml. in adult sheep's blood, and similar values for cattle and pigs; Fosse, Brunel & Thomas, 1931). Dr S. M. Partridge (Partridge, 1948) has shown that sample 18, and pooled samples (3, 4, 5, 6, 7, 13, 15) and (8, 9, 10) contain inositol. Inositol gives no reduction under the conditions of the ferricyanide-cerium method for t.r.s.

Isolation of fructose derivatives

Methylphenylosazone. A preliminary experiment on maternal blood with added fructose showed that the osazone could be prepared from ethanol-soluble material in an ethanol-chloroform filtrate. This procedure was applied to the first sample of foetal blood.

The ethanol-soluble fraction (0.6 g.) of the material in the ethanol-chloroform filtrate, estimated to contain 57 mg. of fructose, was dissolved in 2.5 ml. of water, in which it was completely soluble, 1 ml. of freshly distilled methylphenylhydrazine added, with 2.2 ml. of 96% ethanol to render the mixture homogeneous, and finally 1 ml. of 50% acetic acid. The darkening solution was warmed in a water bath for 10 min. and left at room temperature for 1.5 hr. 10 ml. of *N*-acetic acid were then added, and the osazone extracted with successive portions of 20, 15 and 15 ml. of chloroform. The combined extracts were shaken with 25 ml. of *N*-acetic acid, then with dilute NaHCO_3 solution until no more CO_2 was given off, and finally with water. After being dried over anhydrous Na_2SO_4 , the chloroform solution was boiled with a little charcoal, filtered, and evaporated to dryness under reduced pressure. The 0.8 g. of residue (*N*-methylaniline was present) was recrystallized from a mixture of chloroform-ligroin (b.p. 40–60°). The melting point was raised to 151–152° by recrystallization from 20% ethanol-water. Yield 37.8 mg. (31% of theory). Under the same conditions 134.5 mg. of pure D-fructose gave 84.6 mg. of osazone (29% of theory). 170 mg. of glucose gave no crystallizable product.

The tetraacetyl derivative was prepared from the osazone from foetal blood: 23.2 mg. were dissolved in 2 ml. of 2 : 1 pyridine-acetic anhydride mixture and left 20 hr. at 40°. 20 ml. of water were added, the mixture shaken vigorously, left 4–5 hr. and filtered. The thoroughly washed material was dissolved in the minimum of hot ethanol, and water was added at boiling point until crystallization began. Yield 27.9 mg. of lemon yellow crystals; m.p. 125–126° (cf. Percival & Percival, 1937).

The yields of the methylphenylosazone are much lower than those claimed by Neuberg in his earlier papers. The highest which we have obtained by following his methods exactly are about 50% of theoretical. We have not applied

the new modification of the procedure which he published after the present work was completed (Neuberg & Mandl, 1946).

The diacetone compound. Following our experience of the somewhat unsatisfactory osazone derivative, one of us (D.J.B.) has devised a method by which fructose can be isolated in high yield as its 2:3-4:5-di-isopropylidene derivative (Bell, 1947).

The procedure adopted for use with the deproteinized blood was as follows (all concentrations and evaporations were done under reduced pressure, below 35°). The ethanol-chloroform filtrate (1 to 5 l.) was evaporated to small bulk, 2 g. of Hyflo Supercel added and evaporation continued, the solid residue being dehydrated by distillation with ethanol-benzene, and finally kept for some hours in a high vacuum over H_2SO_4 and KOH. The dry mass resulting from the above procedure was broken up, if necessary, and shaken with 50 ml. of acetone (dried over CaCl_2) to which had been added 2.5 ml. of conc. H_2SO_4 . After 6 hr. anhyd. Na_2CO_3 was added with vigorous stirring until the original dark brown colour of the fluid had changed to a very pale yellow. The solids were filtered off, washed with acetone, and the combined filtrates evaporated to small bulk in the presence of a few drops of 40% NaOH solution. (This ensures that acidic condensation by products of acetone and H_2SO_4 do not attack the labile isopropylidene groups in the sugar derivatives.) 100 ml. of water were then added to the syrupy residue and the whole distilled until all the mesityl oxide (a by-product) had passed over. The residue in the flask (c. 50 ml.) was shaken with a little charcoal to remove insoluble acetone by-products, and filtered into a separating funnel. The filtrate was three times extracted with its own volume of chloroform, the extract dried over anhyd. Na_2SO_4 and evaporated to dryness. The residue consisted of 2:3-4:5-diacetone fructose, a little of its 1:2-4:5-diacetone isomer, and 1:2-5:6-diacetone glucose. The former compound is completely stable to 0.1N- H_2SO_4 at room temperature, while the two latter substances undergo quantitative partial hydrolysis to the 1:2-monoacetone derivatives. The mixture was therefore dissolved in cold 0.1N- H_2SO_4 and the solution kept for 6 hr., after which time it was rendered alkaline (pH 9) by addition of 40% NaOH solution, shaken with charcoal if deemed necessary, and filtered into a separating funnel. Threefold extraction of the filtrate with its own volume of chloroform quantitatively extracted the 2:3-4:5-diacetone fructose only. After drying over Na_2SO_4 the extract was evaporated to dryness and the resulting crystalline residue recrystallized from ligroin (b.p. 60–80°).

The results of three experiments are summarized in Table 7.

Table 7. Yields of diacetone fructose from ethanol-chloroform concentrates

Blood samples	Vol. of filtrate (ml.)	Fructose content of blood sample (mg.)	Crude yield of diacetone compound		After recrystallization	
			(mg.)	% of theory	m.p.	$[\alpha]_D$ (H_2O)
8, 9, 10	1200	136	140	71	94–95°	–32.95°
3, 4, 5, 6, 7, 13, 15	2500	280*	353	87	95°	–32.80°
12, 14, 16, 17	4900	470†	480	71	95°	–32.90°

Fructose : non-fructose ratios in cadmium filtrates: *1.3 : 1, †1.2 : 1.

Bearing of these results on the analyses of cadmium filtrates

Relation of results on ethanol-chloroform concentrates to those on cadmium filtrates. The main difference between the two types of deproteinization is in the degree to which non-fermentable reducing substances are removed. The non-fermentable reducing substances in the ethanol-chloroform filtrates are a considerable fraction of the t.r.s. With human blood and adult sheep blood the cadmium method removes effectively all this material, which includes such substances as glutathione, uric acid, etc. The question therefore arises as to the validity of assuming that the same is true of the foetal blood, and that the difference between fructose by Seliwanoff, and t.r.s. by ferricyanide-cerium is to be attributed to glucose.

That this might not be the case was shown by the fructose : glucose ratios obtained by Seliwanoff and glucose oxidase with ethanol-chloroform concentrates of bloods 3, 4, 5, 6, 7 (pooled) and 19. In the first this was rather greater than 2 : 1 (52.0 : 24.3) and in the second 8 : 1 (104.1 : 13.2). The fructose : non-fructose t.r.s. ratios of the cadmium filtrates were respectively 56.0 : 48.8, and 123.6 : 39.2. In each case the recoveries of fructose are good (see Table 1), and the discrepancy could be due either to an incomplete recovery of glucose in the ethanol-chloroform filtrate, or to the presence of non-fermentable reducing substances in the cadmium filtrate. The latter possibility was tested directly on other blood filtrates.

Glucose oxidase treatment and yeast fermentation of cadmium filtrates. It was found in preliminary experiments that both glucose oxidase and yeast would work in the presence of cadmium filtrates to which phosphate buffer had been added.

For these experiments blood was collected into a glass-stoppered flask containing potassium oxalate. 10, 5 and 1 ml. samples were withdrawn, treated with 80, 40 and 8 ml. of acid cadmium sulphate solution, and shortly afterwards with 10, 5 and 1 ml. of NaOH, of a concentration adjusted to give complete precipitation of the cadmium. The 1 ml. sample was heated in a boiling water bath for 2 min., cooled and filtered. The other two were centrifuged and the clear supernatant liquid decanted through filter papers into stoppered flasks. 5 ml. of 0.5 M-phosphate buffer (pH 6) were diluted to 50 ml. with the unheated cadmium filtrate, and used for the experiments in Table 8. No precipitation was noticed in this solution, which remained perfectly clear on standing for several hours. A second series of experiments was done to compare the heated and unheated cadmium filtrates, particularly with respect to non-fermentable reducing substances. The results of these are summarized in Table 9.

The discrepancy between the t.r.s. estimations on the filtrate with and without added glucose (6, 4), indicates that the glucose oxidase had not worked to completion in the former, and the figures for glucose in the filtrate must therefore be regarded as a minimum. Unfortunately no more foetal blood was available for a repetition of the experiment, this being the last foetus of the 1946-7 season. This was not thought to justify delaying publication for another year, although it detracts from the value of the results here recorded.

Table 8. *Effect of glucose oxidase and yeast on fructose and t.r.s. contents of a cadmium filtrate of blood sample 20*

No. of exp.	Blood filtrate + buffer (ml.)	Water filtrate + buffer (ml.)	Yeast suspension (ml.)	Glucose oxidase + catalase (ml.)	Added sugar	Fructose (mg./100 ml. blood)	T.r.s. (mg./100 ml. blood)
1	—	—	—	—	—	55.2	82.4
2	20.0	—	10.0	—	—	1.1	14.8
3	—	20.0	10.0	—	2 mg. fructose	1.1	8.0
4	20.0	—	—	0.2	—	54.5	63.6
5	—	20.0	—	0.2	—	—	3.4
6	5.0	—	—	0.05	1 mg. glucose*	57.2	76.0

* The added glucose would represent an additional 200 mg./100 ml. of the original blood, i.e. about 10 times that in 4.

Table 9. *Effect of heating cadmium-precipitation mixture on the amount of non-fermentable t.r.s.*

No. of exp.	Cadmium filtrate of blood (ml.)	Cadmium filtrate of water (ml.)	Yeast suspension (ml.)	0.1 M-Phosphate buffer, pH 6 (ml.)	Fructose (mg./100 ml. blood)	T.r.s. (mg./100 ml. blood)
7	5.5*	—	3.0	1.0	2.5	26.4
8	—	6.0	3.0	1.0	—	14.0
9	6.0	—	3.0	1.0	1.4	23.8

* Heated 2 min. at 100°; plus 0.5 ml. water.

From these results the following calculations can be made:

The relation between non-fructose fermentable t.r.s., and glucose by glucose oxidase.

From (1), (2), (3) we have $82.4 - (14.8 - 8.0) = 75.6$ mg./100 ml. fermentable t.r.s.

From (1), (4), (5) we have $82.4 - (63.6 - 3.4) = 22.2$ mg./100 ml. glucose.

From (1), (2), (3) we have $55.2 - (1.1 - 1.1) = 55.2$ mg./100 ml. fermentable fructose.

I.e. non-fructose fermentable t.r.s. $75.6 - 55.2 = 20.4$ mg./100 ml. Glucose by glucose oxidase = 22.2 mg./100 ml.

The difference between these two figures (1.8 mg./100 ml. of the original blood) is within the limits of experimental error.

The non-fermentable t.r.s. in the cadmium filtrate. This is equivalent (as hexose) to 6.8 mg./100 ml. (2, 3), 12.4 mg./100 ml. (7, 8), and 9.8 mg./100 ml. (9, 8). These values, which are similar to those obtained in an earlier experiment, could well be disregarded in the estimation of t.r.s. in the cadmium filtrate, but they are of the same order as the glucose content, and 'inflate' this if it is assumed to be equivalent to (t.r.s. - fructose). This result confirms the earlier supposition based on experiments with ethanol-chloroform concentrates, and it would seem that when a critical investigation of the glucose and fructose levels is being conducted, the use of glucose oxidase or some other method specific at least for aldoses, is desirable.

DISCUSSION

The experiments here described establish beyond doubt that all, or practically all, the substance in the blood of the foetal sheep giving a positive Seliwanoff reaction is D-fructose. They further establish an equally important fact, that this fructose is present mainly in the free state. This could not be inferred from the Seliwanoff reaction, nor from the formation of the diacetone derivative, nor even from its removal by yeast, but is clear from the formation of a methylphenylosazone, and from the changes in optical rotation accompanying yeast treatment. We believe that this is the first unequivocal demonstration of the presence of free fructose in blood.

In addition, the experiments provide strong evidence that apart from free fructose the blood also contains free glucose, but in much smaller concentrations in most of the samples examined. Within the limits of the methods employed there was no evidence for the presence of any other fermentable reducing sugar. This is confirmed by the findings of Partridge (1948) with the paper partition chromatogram; he detected glucose and fructose, but no appreciable quantities of any other reducing sugar.

It will be noticed that neither the glucose nor fructose, nor the two combined, are apparently maintained at a constant level, as one is accustomed to find the blood sugar of some animals. This may be due in part to the fact that the samples were taken from anaesthetized animals (cf. Hitchcock, 1948).

The proof that fructose is a constituent of foetal blood helps to explain the finding of Passmore & Schlossmann (1938) that the blood sugar level of the foetal sheep was higher than that of its mother (also in the case of the goat, and in the cow and pig; Aron, 1924). It seems probable from our results, and those of Cole & Hitchcock (1946) that the blood glucose (which should be if anything less than the non-fructose t.r.s.) of the foetus is lower than that of its mother, which would be more in keeping with results in other animals (cf. Needham, 1931), although of course this is no reason for assuming that fructose does not occur in the foetal blood of these species also. Orr (1924) states that Paton *et al.* (1907) demonstrated the presence of fructose in the blood of both foetal sheep and cow, but we have not been able to find any direct reference to either animal in the original paper. Orr himself showed that there was a fructose-like substance in human foetal blood, and in that of a kid just after birth, but from the feeble negative rotation of his blood concentrates he concluded that more glucose than fructose was present.

By the use of the methods we describe it should be possible to identify and estimate fructose in a variety of foetal bloods, and thus discover whether its occurrence is general. It might be expected that this is so from the general occurrence of Seliwanoff-positive substances, almost certainly fructose, in the amniotic and allantoic fluids of a wide range of species. At birth it would seem that the fructose is quickly lost, a part, or all being eliminated in the urine (cf. Langstein & Neuberg, 1907; Cole & Hitchcock, 1946).

It is too early to attempt an explanation for the presence of fructose in the foetus, or, for that matter, for the equally obscure and abrupt transference of the young animal at birth to a diet containing lactose, although the suggestion of Mann (1946), that fructose provides a source of energy for spermatozoa which is not shared by the somatic tissues, may have some bearing on this problem also.

SUMMARY

1. The Seliwanoff-positive substances in the blood of the foetal sheep have been accounted for by the presence of free D-fructose: they are fermentable, have a negative optical rotation closely corresponding to that of D-fructose, yield a methylphenylosazone under conditions in which glucose forms none, and give the expected high yield (70 %)

of 2:3:4:5-di-isopropylidene-D-fructopyranose on treatment with acid acetone under suitable conditions.

2. The reducing substances other than fructose are partly fermented by yeast, and this fraction has been accounted for by D-glucose, which was estimated by the disappearance of reducing substances on treatment with the specific enzyme, glucose oxidase.

3. A small part of the reducing substances in solutions deproteinized by the use of acid cadmium sulphate and sodium hydroxide, and a greater part of those in solutions deproteinized by chloroform and ethanol, are not fermented by baker's yeast; no evidence was obtained for the presence of any reducing sugar other than D-fructose and D-glucose.

4. The implications of these findings are discussed.

We wish to place on record our gratitude to the late Sir Joseph Barcroft, F.R.S., for the opportunity to investigate this problem, and for his encouragement, given so generously and in so many ways. We are indebted to Dr T. Mann and Mr S. W. Cole, for their advice and help in the course of the work; to Miss V. Moyle, Mr M. W. S. Hitchcock, and Dr S. M. Partridge for analyses which they performed for us; and to the members of the Agricultural Research Council Unit of Animal Physiology for their friendly co-operation.

We wish also to thank Prof. A. C. Chibnall, F.R.S., for his interest in the progress of the research.

One of us (D. J. B.) acknowledges a grant for research from the Agricultural Research Council; the other (J. S. D. B.) was in the full-time employment of the Council when the work was begun.

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Physicochemical Observations on some Glycogens

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(Received 17 July 1947)

The molecular weight of unsubstituted glycogen has, up to the present, been studied in but a few instances. Oakley & Young (1936) have reported, for samples from rabbit liver and muscle, values of $1.2-2.3 \times 10^6$ for the former, and $0.7-1.8 \times 10^6$ for the latter material. These results were obtained by use of a delicate method of osmotic-pressure measurement on solutions in 0.1 N-calcium chloride. These authors claim that both alkaline extraction of the tissue (the so-called Pflüger method) and water extraction followed by precipitation by acetic acid (Bell & Young, 1934) yield products of the same general

molecular size. Oakley & Young (1936) further noted that glycogen solutions when salt-free manifested markedly greater osmotic pressures than in presence of Ca^{++} and Cl^- . Bridgeman (1942) determined the sedimentation and diffusion constants of rabbit-liver glycogen and derived from these an average molecular weight of about 4×10^6 . Glycogen from tubercle bacilli has been shown by Chargaff & Moore (1944), using similar methods, to have a molecular weight of $12-13 \times 10^6$. These are the only reported molecular weight determinations using the ultracentrifuge.