- Randle, P. J. & Smith, G. H. (1958a). *Biochem. J.* **70**, 490.
 Randle, P. J. & Smith, G. H. (1958b). *Biochem. J.* **70**, 501.
 Segal, S., Blair, A. & Weinberg, A. (1960). *Metabolism*, **9**, 1033.
 Seltzer, H. S. (1962). *J. clin. Invest.* **41**, 289.
 Shaw, W. N. & Stadie, W. C. (1959). *J. biol. Chem.* **234**, 2491.
 Smith, M. J. H. & Jeffrey, S. W. (1956). *Biochem. J.* **63**, 524.
 Smith, M. J. H. & Moses, V. (1960). *Biochem. J.* **76**, 579.
 Smith, M. J. H. & Moses, V. (1961). *Biochem. J.* **79**, 275.
 Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques*, p. 149. Minneapolis: Burgess Publishing Co.

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Some Non-Reducing Carbohydrates in Animal Tissues and Fluids

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The presence of sorbitol in ram seminal vesicles and sheep foetal blood was reported by Hers (1957a, b, 1960a, b), and evidence was also obtained that sorbitol is an intermediate in the conversion of glucose into fructose, the pathway being

The present investigation confirms the presence of sorbitol, and a preliminary account has been given (Britton, 1958, 1960).

METHODS

Deproteinization and desalting. Samples were deproteinized with ZnSO_4 and Ba(OH)_2 (Somogyi, 1945), a 1:10 dilution being used, or with 5% trichloroacetic acid, which was extracted with ether before desalting. Desalting before chromatography was effected with Bio-Deminrolit (The Permutit Co. Ltd., London, W. 4). When it was not desired to remove the reducing sugars the resin was saturated with CO_2 (Woelf, 1953) and made into a column (vol. 2 ml., length 25 cm.). A portion (2–5 ml.) of the deproteinized solution was run slowly through the column, which was then washed with 3–4 ml. of water. Recoveries of glucose, fructose and sorbitol were quantitative. To remove reducing sugars fresh CO_2 -free Bio-Deminrolit was used and 2–5 ml. of the deproteinized solution run slowly through the column in about 1 hr. The column was then washed with CO_2 -free water to give a total volume of eluate of 20 ml. With this technique all but traces of reducing sugar (less than 5% of glucose and fructose appeared in the eluate when 100–1000 μg . of the sugars was put through the column) were removed, but the recovery of glycerol and sorbitol was quantitative. Samples of glycerol, mannitol,

sorbitol and dulcitol remained chromatographically and electrophoretically homogeneous after this treatment. All columns were well washed before use: this was especially necessary to remove formaldehydogenic material.

Chromatography. Paper chromatography was carried out with the solvent systems A, B, C and D of Table 1 on Whatman no. 1 paper. The solutions to be chromatographed were concentrated in a vacuum desiccator and volumes of 20–100 μl . were dried at the origin in a cold air draught. The amounts of extract applied were equivalent to between 1 and 7.5 ml. of the deproteinized filtrate. Non-reducing and reducing carbohydrates were localized by the method of Trevelyan, Procter & Harrison (1950) as modified by Smith (1960) for systems A, B and C. For system D, the method of Britton (1959a) was employed, 40% HF-acetone (1:40, v/v) proving suitable. Periodate-benzidine (Cifonelli & Smith, 1954) was also used to detect non-reducing carbohydrates in the presence of borate but it was much less sensitive and gave no indication of the amount of material on the spot. For reducing carbohydrates aniline-diphenylamine and naphtharesorcinol were used (Smith, 1960).

Elution from the paper after use of solvents A, B and C was effected by conventional techniques with water as the eluting agent. For system D a modified method was employed to remove the boric acid present. The area to be eluted (10–25 cm^2) was cut out and dipped into a solution of 40% HF-acetone (1:20, v/v). The paper was allowed to dry at room temperature for about 1 hr. and was then eluted in the conventional way, contact with glass being avoided. To the eluate (1–2 ml.) was added an approximately equal volume of aq. 0.01% HF and the solution was run slowly through a column of Bio-Deminrolit saturated with CO_2 (vol. 10 ml., length 25 cm.). The column was further washed with 0.01% HF to give a total volume of eluate of 12 ml., the whole procedure taking about 1 hr. This removed boric acid but gave a quantitative recovery of glucose, fructose and sorbitol. The method appears to be generally applicable, but might not be suitable for highly acid-labile substances owing to the formation of fluoroboric acids on the paper.

Electrophoresis. To separate the hexitols paper electrophoresis was carried out in basic lead acetate (Frahn & Mills, 1959). A solution of basic lead acetate was prepared by shaking 58 g. of basic lead acetate (AnalaR; British Drug Houses Ltd.) with 1 l. of water and filtering. The electrophoresis paper (Whatman no. 1 paper, 57 cm. long) was dipped into the solution, well blotted and placed in a horizontal electrophoresis tank in which the paper hung freely, supported by transverse glass rods. After 30–60 min. had been allowed for capillary rise of solvent, spots containing 10–20 μ g. of material were applied to the origin with a wire loop. Electrophoresis was carried on for 48 hr. at 3° with a voltage gradient of about 10 v/cm. along the paper. The material was applied asymmetrically towards the negative pole; the position was important since movements of the solvent due to evaporation and endosmosis affect the relative separations obtained. Electrophoresis at room temperature gave a more rapid separation but the results were less reproducible and for rapid separation a totally enclosed strip as employed by Frahn & Mills (1959) is probably desirable. Detection of the sugar alcohols was effected by a modification of the method of Trevelyan *et al.* (1950). After the paper had dried at room temperature, it was dipped twice into a solution of AgNO_3 in aqueous acetone (prepared by adding 0.2 ml. of a saturated solution of AgNO_3 in water to 40 ml. of acetone and adding water drop by drop until the precipitate redissolved). The paper was then dipped into a solution of 40% HF-acetone (1:10, v/v) so that the paper was well saturated. After allowing the paper to dry for 15–20 min. at room temperature it was dipped into a solution of aqueous 50% NaOH-ethanol (1:10, v/v). The paper was hung in the dark or in subdued light for 1–2 hr. for the spots to develop and then photographed, since although fixation could be effected with ammonia or sodium thiosulphate these procedures led to a loss of density. If the impregnation with lead acetate had been heavy a second dip into the HF solution was sometimes required, but it was essential that most of the excess of HF should volatilize before the alkaline dip, although for maximum sensitivity it was necessary for a small amount of HF to be present. To accelerate the development of the spots the alkaline dip was repeated or heat applied. About 5 μ g. of sorbitol was detected by the method.

For identification of glycerol, electrophoresis was carried out in 0.05 M-sodium tetraborate on Whatman paper no. 1 at room temperature. Spots were made visible by the modified silver method of Britton (1959*a*).

Estimation of total hexitol. In foetal sheep blood the total hexitol present was estimated by the method of Britton (1959*b*), with Whatman paper no. 1 for chromatography.

Identification of sorbitol. Sorbitol dehydrogenase was prepared from sheep liver by the method of Todd (1954) except that the last acetone fractionation was omitted. To show reduction of NAD, 1.8 ml. of 0.2 M-glycine-NaOH buffer, pH 8.5, was placed in a cuvette, followed by 0.2 ml. of NAD solution (C. F. Boehringer und Soehne; NAD 75%, 25 mg./ml.) and 0.2 ml. of the unknown solution. A blank was set up at the same time. The extinctions were read at 340 $m\mu$ and 0.2 ml. of suitably diluted enzyme solution was added to both cuvettes. The change in *E* of the blank was used to correct the change in the unknown. To show the formation of fructose the total volume was kept to a minimum owing to the relatively high K_m of the enzyme: the volume of buffer was therefore brought to 0.5 ml. and

0.5 ml. of undiluted enzyme was added. About 100 μ g. of the unknown dissolved in 0.2 ml. of water was taken and the mixture left overnight at room temperature. Under these conditions virtually complete conversion of sorbitol into fructose took place.

Acetylation of sorbitol was carried out with acetic anhydride and perchloric acid. A sample of the unknown (0.5–1 mg.) was evaporated to dryness in a small test tube and 0.5–1 ml. of a reagent prepared by mixing 0.1 ml. of 60% HClO_4 and 10 ml. of acetic anhydride was added. After about 20 min. NaHCO_3 solution and solid NaHCO_3 were added until the solution was neutral. The acetylated derivative was then extracted with CHCl_3 or ethylene dichloride. Crystallization was carried out by allowing some of the extract to dry on a glass slide. One drop of ethylene dichloride was added to dissolve the acetate and then a drop of redistilled amyl alcohol was added. By enclosing the slide in a chamber containing the solvents the rate of evaporation was slowed. Crystals were removed with a needle under a lower-power microscope and recrystallization was effected by the same technique. Alternatively the extract to be acetylated was dried on a glass slide and several drops of the acetylating reagent were added. The slide was placed in a chamber saturated with the vapour of the acetylating reagent and left for 20 min. The reagent was removed with a cold air draught and crystallization carried out as described above. By this technique the hexa-acetate from 100 μ g. of sorbitol could be prepared.

Melting points were determined in a Kofler apparatus. X-ray crystallography on single crystals was carried out by Dr T. Lomer of the Physics Department, St Mary's Hospital Medical School.

Experiments with radioactive compounds. The compounds were obtained from The Radiochemical Centre, Amersham, Bucks. and used without further purification. Approximately 150 mg. of slices of seminal vesicle were incubated with 1 ml. of Krebs solution ('medium III', Krebs, 1950) in a Warburg apparatus at 37° for 2 hr. The reaction was stopped by adding 10% (w/v) trichloroacetic acid. In some experiments the supernatant was decanted and the slices were washed several times with Krebs solution. The supernatant and the tissue were then examined separately.

Rapid freezing. For observation of post-mortem changes the tissues were dropped into liquid N_2 and ground in a stainless-steel mortar and 1–2 g. of the powdered frozen material was triturated in 10 ml. of 10% trichloroacetic acid at 0° (LePage, 1948).

Specificity of methods. Glycerol and the hexitols lack specific colour reactions by which they may be detected on chromatograms. However, it could be shown that they did not react with aniline-diphenylamine or with naphtharesorcinol, whereas reaction occurred rapidly with periodate-benzidine and slowly with silver reagents. The failure of these substances to be removed by strongly basic resins also pointed to their non-reducing character. It is important to distinguish sorbitol from the other isomeric hexitols and their R_F values relative to sorbitol are listed in Table 1. Sorbitol in solvent systems A and B gave a complete separation from allitol and a partial separation from all the isomers with the exception of dulcitol. System D, on the other hand, gave a partial resolution from dulcitol.

A separation of the hexitols was possible by paper electrophoresis in basic lead acetate but relatively large quantities were required and these were not always available. In

typical electrophoresis the distances (cm.) of migration from the origin towards the negative pole were: talitol 11.8, mannitol 13.5, galactitol 16.4, sorbitol 17.8, iditol 21.6. The spots remained compact at the low temperature and these differences in migration were sufficient for a clear separation to be obtained.

The specificity of sorbitol dehydrogenase from sheep liver has been reported by Todd (1954) to be similar to that of rat liver (McCorkindale & Edson, 1954), except that a slight reaction (3% of the rate with sorbitol) took place with mannitol. Thus, among hexitols, the only substrates that react at a substantial rate are D-sorbitol and L-iditol, and these could be distinguished by chromatography of the products fructose and sorbose in solvent system C.

Glycerol is well separated from other lower sugar alcohols by electrophoresis in 0.05 M-borate (Frahm & Mills, 1959).

Sources of material. The sheep foetal blood was obtained from the umbilical vessel shortly after exposure of the foetus by Caesarian section, the mother being anaesthetized

with thiopentone or a spinal anaesthetic. For the surgical procedures the writer is indebted to Dr D. A. Nixon of the Physiology Department, St Mary's Hospital Medical School. The foetal rabbit, rat and guinea-pig blood was obtained by cardiac puncture under ether anaesthesia. Whale blood was collected 17-36 hr. *post mortem* from sperm whales caught in the Azores, by arrangement with the National Institute of Oceanography, Godalming, Surrey. The samples were deproteinized on collection with trichloroacetic acid. Foetal bloods of the llama, hippopotamus and rhinoceros were collected by Mr E. H. Tong and his staff at Whipsnade Zoological Park from the placentae at various times after delivery. The samples (2-30 ml.) were placed in bottles containing 100 mg. of a mixture of NaF and potassium oxalate (1:3, w/w) and refrigerated pending delivery to the Laboratory, where deproteinization was effected with ZnSO_4 and Ba(OH)_2 .

RESULTS

Results of a chromatographic survey of some bloods and tissues are shown in Table 2. Traces of sorbitol and fructose were found in the foetal and maternal blood of the rabbit, guinea pig and rat and also in the maternal blood of the sheep. The quantities did not exceed 0.5 mg./100 ml. and the identification can only be considered tentative. With these species a third spot was also visible, on two-dimensional chromatograms in the position of mannose, the quantity involved being similar.

Further identification of sorbitol. Sufficient quantities of sorbitol-like material were available

Table 1. R_{sorbitol} values of hexitols in paper chromatography

Solvent systems: A, amyl alcohol-pyridine-water (4:3:2, by vol.) (Kawerau, 1956); B, butanol-acetic acid-water (4:1:5, by vol.); C, phenol-water (5:2, w/v); D, butan-2-one-acetic acid-saturated aqueous boric acid solution (9:1:1, by vol.) (Rees & Reynolds, 1958).

Solvent system	...	A	B	C	D
Allitol		1.15	1.13	1.0	1.03
Dulcitol		1.03	1.02	0.97	0.81
Iditol		0.92	0.97	1.0	0.91
Mannitol		1.08	1.07	0.97	0.84
Talitol		1.10	1.09	1.02	0.96

Table 2. *Sorbitol, glycerol, fructose and inositol in some animal fluids and tissues*

Approximate concentrations were estimated from the density of the silver spot on the chromatograms, with the exception of the sorbitol content of sheep foetal blood which was estimated by the method of Britton (1959b).

	No. of specimens	Concentrations (mg./100 ml. or mg./100 g. of wet tissue)			
		Sorbitol	Glycerol	Fructose	Inositol
Foetal blood					
Sheep	6	7-22*	4	50-100†	10-31†
Llama (domestic breed)‡	1	15*	4	43†	10-20
Hippopotamus amphibius‡	1	15	4	60†	10-20
Rhinoceros unicornis‡	1	15	4	31†	10-20
Whale (sperm)§	4	10-20	4	30-100	10-20
Rabbit	3	Trace	1-2	Trace	3.2-13.9†
Guinea pig	3	Trace	1-2	Trace	2-10
Rat	3	Trace	1-2	Trace	2-10
Maternal blood					
Sheep	4	?Trace	1-2	Trace	0.6-2.7†
Rabbit	3	Trace	1-2	Trace	0.8-3.2†
Guinea pig	3	Trace	1-2	Trace	2-5
Rat	3	Trace	1-2	Trace	2-5
Ram seminal vesicle					
Post mortem	5	200	40	300	100
Frozen	3	10	—	300	100
Ram semen	4	10-20	0	247†	40

* Not homogeneous in solvent system D.

† Collected results of Huggett & Nixon (1961); Nixon (1954); Mann (1954).

‡ Blood from placenta after delivery.

§ Collected 17-36 hr. *post mortem*.

from the ram seminal vesicle and whale foetal blood to permit further identification. Thus it was shown that reaction occurred with sorbitol dehydrogenase (as judged by the reduction of NAD) at a rate compatible with the amount of material estimated chromatographically. Furthermore, the reaction was shown to go to virtual completion with the formation of a substance chromatographically indistinguishable from fructose. As a further verification the hexa-acetates were prepared and the single-crystal X-ray-diffraction patterns were indistinguishable from authentic sorbitol hexa-acetate. The melting points after recrystallization were 99°, not depressed on mixture with authentic sorbitol hexa-acetate.

Presence of other hexitols. When extracts of foetal sheep blood and foetal llama blood were chromatographed on system D a second spot could be seen running just behind the sorbitol position. This spot remained after the reducing sugars had been removed with Bio-Deminrolit and was still present when these extracts were chromatographed on system A and the hexitol area was eluted. It seemed probable therefore that other hexitols were present in addition to sorbitol and paper electrophoresis of the eluate on basic lead acetate showed three spots migrating in the positions of sorbitol, galactitol and mannitol. The concentrations of sorbitol and galactitol were similar, whereas the concentration of mannitol was rather smaller. Three independent samples of foetal sheep blood gave the same picture on electrophoresis and the chromatographic pattern of a further four samples was similar. One set of umbilical and arterial venous samples, which had been taken simultaneously, were chromatographed and there was no obvious difference between the arterial and venous samples. The specimen of foetal llama blood was a single one; the amniotic fluid, however, was similar in composition.

Further identification of glycerol. The glycerol fraction separated by chromatography was not adsorbed by CO₂-free Bio-Deminrolit. This and its reaction with the silver reagent on chromatograms and its partial volatility in steam indicated that it was probably a polyhydric alcohol. Electrophoresis in sodium borate buffer was carried out as a confirmatory measure except with the llama, hippopotamus and rhinoceros blood, where insufficient material was available.

Hexitol content of foetal sheep blood. Umbilical arterial and venous samples were taken from six foetal sheep and the total hexitol was estimated by the method of Britton (1959*b*). The results are shown in Table 3. The close correspondence of the arterial and venous concentrations suggests that there is no large production of hexitols by the placenta. The high values for sheep 5 are of

interest, as this animal had been grossly anoxic during the operative procedure and had a high concentration of lactic acid. It was therefore thought that the sorbitol concentration might have been increased by a reversal of the sorbitol into fructose conversion which occurs in the foetal liver (Hers, 1957*a, b*; Andrews, Britton & Nixon 1959), due to a change in the NAD⁺/NADH + H⁺ ratio. However, chromatography of blood obtained from a number of foetuses that had been asphyxiated to foetal death showed no gross increase in sorbitol content, though with the technique used a small rise would not have been noticed.

Production of hexitol by the placenta. Blood obtained from the placenta after removal of the foetus contained larger quantities of hexitol, and subsequent studies on the perfused placenta (Andrews *et al.* 1959; H. G. Britton, A. St G. Huggett & D. A. Nixon, unpublished work) showed a production of hexitol. Chromatography of the samples obtained showed that the increase in concentration took place in the sorbitol fraction, although a small increase in the other isomers could not be excluded. Concomitant with the production of sorbitol an increase in the glycerol content was also noted.

Accumulation of sorbitol in the ram seminal vesicle post mortem. Since the conversion of sorbitol into fructose in the seminal vesicle is NAD-linked it was thought possible that the reaction might be reversed by anoxia. The seminal vesicles were removed from an anaesthetized animal and cut into portions. One piece was frozen immediately in liquid N₂, a second was frozen after 5 min. at room temperature, a third was frozen after 20 min. and the fourth was frozen after 2 hr. Equal quantities of the extracts were placed on a chromatogram and run with solvent system A. The results in Fig. 1 show that there is an increase in the sorbitol fraction on keeping at room temperature. The material in the post-mortem specimen appears to be largely, if not entirely, sorbitol. Unfortunately the sample that was frozen immediately was not examined in system D and the homogeneity of the sorbitol fraction is uncertain. To exclude the

Table 3. *Hexitol content of sheep foetal blood*

Sheep no.	Foetal age (days)	Concn. in umbilical blood (mg./100 ml.)	
		Venous	Arterial
1	90	8.16	8.3
2	120	8.12	10.4
3	120	7.1	6.67
4	121	9.85	9.5
5	121	22.1	19.6
6	135	9.67	9.67

Fig. 1. Paper chromatogram showing post-mortem changes in sorbitol content of ram seminal vesicle. 1, Frozen in liquid N_2 immediately after removal; 2, frozen 5 min. after removal; 3, frozen 20 min. after removal; 4, frozen 2 hr. after removal. M, marker. Solvent system was amyl alcohol-pyridine-water (4:3:2, by vol.). Solvent ran off the paper.

possibility that the sorbitol was released from a readily hydrolysed ester some of the seminal vesicle was hydrolysed for 12 hr. at 100° in 6N-HCl. No increase in the sorbitol content was noted on subsequent chromatography.

Incubation of ram seminal vesicle with radioactive sugars. Slices of ram seminal vesicle were incubated with various radioactive sugars and the extract was chromatographed. The scans of chromatograms run with system D (Fig. 2) show that there is a conversion of glucose into sorbitol and fructose

Fig. 2. Scans of radioactivity of paper chromatograms of extracts of seminal vesicle incubated with (A) $[^{14}C]$ glucose, (B) $[^{14}C]$ sorbitol and (C) $[^{14}C]$ fructose. Chromatograms were run for 30 hr. in solvent system D (Table 1). Solvent ran off the paper. O, Origin of chromatogram; G, F and S, positions of glucose, fructose and sorbitol respectively as determined by parallel markers.

and of sorbitol into fructose but that there is no appreciable conversion of fructose into sorbitol. Experiments in which the supernatant and the tissue were examined separately gave qualitatively similar results. When the incubations were carried on anaerobically there was a decreased labelling of fructose when $[^{14}C]$ glucose or $[^{14}C]$ sorbitol was used but there was still no appreciable conversion of fructose into sorbitol.

DISCUSSION

These observations provide further evidence for the presence of sorbitol in sheep foetal blood (Hers, 1957a, b; 1960a), in ram semen (King, Isherwood & Mann, 1958; King & Mann, 1959) and in some other animal tissues and fluids. The association of this sorbitol with fructose might be expected since Hers (1957b; 1960a) found that fructose is formed from glucose by reduction to sorbitol and subsequent oxidation.

Galactitol and mannitol were only found in foetal sheep and llama blood. However, they might be present in other tissues since the methods of detection would not demonstrate small quantities of these isomers in the presence of sorbitol. The origin of the galactitol and mannitol is unknown but Hers (1957b; 1960b) showed that mannose and galactose are both reduced by ketose reductase. Galactose and mannose have not been observed in many chromatograms of sheep foetal blood but in this connexion the appearance of a faint spot corresponding to mannose in the maternal blood is perhaps relevant. It might also be that these

sugars are only transiently present because of dietary absorption, and the observation of Walker (1960) that the goat placenta (a species that also contains fructose in the foetal blood) is permeable to these sugars is of interest. The lens of the eye of the rat contains ketose reductase and when the animal is fed with galactose or mannose the corresponding sugar alcohol appears in the lens (van Heyningen, 1959), and this may also occur in the placenta.

Glycerol was present in all the tissues and fluids examined except ram semen. In this, however, large amounts of glycerylphosphorylcholine (Dawson, Mann & White, 1957) are present and evidence has been obtained of an esterase in the female genital tract hydrolysing this ester (White & Wallace, 1961). The concentration of inositol is high in fructose-containing fluids and tissues. The large rise in sorbitol content of the seminal vesicle *post mortem* (Table 2) was originally thought to be due to a reversal of the conversion of sorbitol into fructose by sorbitol dehydrogenase, since this enzyme is NAD-linked and anoxia might be expected to alter the NAD⁺/NADH ratio. However, the failure to observe any appreciable labelling of sorbitol when seminal-vesicle slices were incubated with [¹⁴C]fructose makes this unlikely. It therefore seems more likely that the sorbitol is derived from glucose and that under anoxic conditions there is a reduced conversion of sorbitol into fructose. The conversion of sorbitol into fructose must presumably be related to conditions under which the enzyme operates rather than to any cell-permeability phenomenon, since the supernatant and washed tissue slices gave similar chromatograms. However, King & Mann (1959) have shown that ram spermatozoa (which contain a sorbitol dehydrogenase) will reduce fructose to sorbitol under anaerobic conditions.

SUMMARY

1. Methods are described for the separation and identification of non-reducing carbohydrates.

2. Claims that sorbitol is present in various animal tissues and fluids have been confirmed and extended.

3. Galactitol and mannitol have been tentatively identified in foetal sheep and llama blood.

4. The concentration of sorbitol in the ram seminal vesicle increases rapidly after death. This appears to be due to reduction of glucose to sorbitol, rather than reduction of fructose to sorbitol.

5. The conversion of glucose and sorbitol into fructose by ram seminal vesicle has been confirmed.

6. Glycerol has been tentatively identified in all tissues and fluids examined except ram semen.

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REFERENCES

- Andrews, W. H. H., Britton, G. H. & Nixon, D. A. (1959). *J. Physiol.* **146**, 41 p.
- Britton, H. G. (1958). *Biochem. J.* **69**, 5 p.
- Britton, H. G. (1959a). *Biochem. J.* **73**, 19 p.
- Britton, H. G. (1959b). *J. Physiol.* **146**, 29 p.
- Britton, H. G. (1960). *Biochem. J.* **78**, 26 p.
- Cifonelli, J. A. & Smith, F. (1954). *Analyt. Chem.* **26**, 1132.
- Dawson, R. M. C., Mann, T. & White, I. G. (1957). *Biochem. J.* **65**, 627.
- Frahn, J. L. & Mills, J. A. (1959). *Aust. J. Chem.* **12**, 65.
- Hers, H. G. (1957a). *Biochem. J.* **66**, 30 p.
- Hers, H. G. (1957b). *Le Métabolisme du Fructose*, 119–148. Bruxelles: Éditions Arscia.
- Hers, H. G. (1960a). *Biochim. biophys. Acta*, **37**, 127.
- Hers, H. G. (1960b). *Biochim. biophys. Acta*, **37**, 120.
- Huggett, A. St G. & Nixon, D. A. (1961). *Nature, Lond.*, **190**, 1209.
- Kawerau, E. (1956). *Chromatographic Methods*, vol. 1, no. 2.
- King, T. E., Isherwood, F. A. & Mann, T. (1958). *Abstr. 4th int. Congr. Biochem.*, Vienna, sect. 6, no. 61, p. 77.
- King, T. E. & Mann, T. (1959). *Proc. Roy. Soc. B*, **151**, 226.
- Krebs, H. A. (1950). *Biochim. biophys. Acta*, **4**, 249.
- LePage, G. A. (1948). *Meth. med. Res.* **1**, 337.
- McCorkindale, J. & Edson, N. L. (1954). *Biochem. J.* **57**, 518.
- Mann, T. (1954). *Biochemistry of Semen*, p. 32. London: Methuen and Co. Ltd.
- Nixon, D. A. (1954). *J. Physiol.* **126**, 71.
- Rees, W. R. & Reynolds, T. (1958). *Nature, Lond.*, **181**, 767.
- Smith, I. (1960). *Chromatographic Techniques*, pp. 250–252. London: William Heinemann Ltd.
- Somogyi, M. (1945). *J. biol. Chem.* **160**, 62.
- Todd, C. M. (1954). *Proc. Univ. Otago med. Sch.* **32**, 9.
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
- van Heyningen, R. (1959). *Biochem. J.* **73**, 197.
- Walker, D. G. (1960). *Biochem. J.* **74**, 287.
- White, I. G. & Wallace, J. C. (1961). *Nature, Lond.*, **189**, 843.
- Woolf, L. I. (1953). *Nature, Lond.*, **171**, 841.