Qualitative observations on early postnatal changes in several blood sugars of calves by the use of gas-liquid chromatography

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Fructose is known to be a major component of the fetal blood sugars in all species of *Ungulata* and *Cetacea* which has been examined.

GÜBER and GRÜNBAUM¹⁴⁾ found that fructose was contained in both the amniotic and allantoic fluids of the cow, pig and goat, and produced the methylphenylosazone in several cases. PATON, WATSON and KERR²⁸⁾ detected by the SELIWANOFF test a fructose like substance in the amniotic and allantoic fluids of the sheep, cow, pig, cat, dog, rabbit, ferret and guinea-pig, and in their fetal blood. COLE and HITCHCOCK¹¹⁾ indicated that appreciable amount of fructose was present in the blood of the fetal sheep. BACON and BELL⁸⁾ attempted to prove that the substance responsible for the colour reaction was in fact fructose, and to isolate a derivative of the sugar, and established that the SELIWANOFF-positive material in the blood of the fetal sheep was free *D*-fructose.

These early workers engendered an assumption that fructose was a universal feature of fetal life. However, this assumption was difficult to follow, because this was conducted by the results of a tendency to seek fructose even at very low concentration.

It was first shown by AINSWORTH, PARE and WARREN¹⁾ that sheep and goats were fructogenic, whereas rats, guinea-pigs and rabbits were not. WALKER²⁸⁾ extended this to three species of deer, roe and fallow. With the advent of experimental work on sheep fetus, the fetal blood was examined by several workers,^{10,11,16,20,21)} and they reported average values for fructose concentration of about 70–100 mg/dl. KARVONEN²²⁾, however, examined human cord blood taken at delivery and found fructose concentrations of 1 mg/dl or less. GOODWIN^{12,13)} examined the presence of fructose in fetal blood from the horse, pig, sheep, ox, goat, whale, dog, cat, ferret, guinea-pig, rabbit and rat, and demonstrated that SELIWANOFF-positive material (taken as fructose) was present in high concentration in the samples from the horse, pig, sheep, ox, goat and whale, but was present only in trace in the remaining species. He suggested that, pending a much wider survey of the mammalian orders, it may be assumed that the presence of fructose at high concentration in the fetal blood of land mammals at term was a peculiarity of ungulates. HUGGETT¹⁷⁾ extended his studies on fructose and showed that the whale, the hippopotamus and the antelope were fructogenic. HUGGETT and NIXON¹⁹⁾ added to this list the rhinoceros, the camel and the llama. But in the edentata, the sloth only had trace of fructose in the fetal blood. HUGGETT¹⁸⁾ reviewed the comparative distribution of blood sugars of the fetus.

The purpose of this study was to know qualitative changes in several blood sugars of the calf, which is known to be fructogenic, during early postnatal times by the use of gas-liquid chromatography.

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The application of gas-liquid chromatography for the separation of carbohydrates has recently progressed. As carbohydrates are not sufficiently volatile for gas-liquid chromatography, many studies have been directed to preparing suitable volatile derivatives by a method which is simple and applicable to a wide variety of compounds. Among three types of derivatives, polymethyl ethers, polyacetyl esters and polymethylsilyl ethers (TMS), which have been reported up, the last was concluded by SWEeley²⁷⁾ to be ideally suitable for routine determination. The reaction of trimethylsilylation proceeds to completion in a very short time, and a distinct advantage of the method is that injections may be made directly from the reaction mixture into the gas-liquid chromatography²⁷⁾.

Materials and Methods

Blood of the jugular vein was obtained immediately after birth, and then the blood samples were taken at suitable time intervals.

A dual-column gas chromatograph (Shimadzu Corporation, Kyoto, model GC-1C) fitted with a hydrogen flame ionization detector was used. The stainless steel columns (3 mm i.d.), 262.5 cm in length, were packed with SE-52. Solid support was Shimalite W, 60-80 mesh. The injection block, column oven and detector oven were maintained at 330°C, 190°C and 350°C respectively. A nitrogen carrier gas was used at flow rate 60 ml/min. The hydrogen flow to the flame jet was 45 ml/min. Aliquots, 1-2 μ l, of appropriate solutions of the TMS ethers were injected into the gas-chromatograph with the 10 μ l syringe (No. 701, Hamilton Company).

One ml of plasma or whole blood was pipetted into a test tube containing 10 ml of distilled water to which was added in the following order, 5.0 ml of 2 g/dl zinc sulfate (7 H₂O) and 5.0 ml of 1.8 g/dl barium hydroxide (8 H₂O), according to the method of the SOMOGYI²⁸⁾. The choice of the SOMOGYI procedure is important, as acidic filtrates inhibit trimethylsilylation. The mixed suspension was centrifuged and 15 ml of the supernatant fluid was frozen in 25-ml pear formed flask, and was lyophilized to dryness. The samples were kept dried on calcium chloride in the desiccator until ready for analyses. The reagent used for trimethylsilylation was a mixture of 10 ml of anhydrous pyridine, 2 ml of hexamethyldisilazane and 1 ml of trimethylchlorosilane. About 0.1 ml of this reagent was added to the dried sample in the pear formed flask, and the mixture was mixed thoroughly. After approximately 10 minutes, injections were made into the gas chromatograph. The materials of standard sugars for the gas chromatograph were prepared from 1 ml of 100 mg/dl aqueous solutions, and were treated exactly as that described for plasma or blood. Sugars used for the standards were the commercial guaranteed grade.

Results

The chromatogram of TMS-derivatives of the several sugars in plasma of the calf at birth are shown in Fig. 1. Five major peaks were separated, and the average retention time of those were 9.4, 12.9, 15.5, 18.6 and 27.6 minutes respectively. The identifications of the peaks were made by reference to a chromatogram of a standard samples, as shown in Fig. 1. The gas chromatographic peaks were two, major one and minor one, for fructose. Glucose showed two large peaks. It was well recognized by SWEeley²⁷⁾ that the former peak of glucose was α -glucose and the latter was β -glucose. One peak was observed for sorbit or inosit. Fructose, glucose, sorbit and inosit could be identified in plasma of the calf at birth, in reference to the

1: fructose, 2: α -glucose, 3: sorbit,
4: β -glucose, 5: inosit

Fig. 1. Trimethylsilyl ethers of plasma sugars from a newborn calf, compared to the corresponding derivatives of fructose, glucose, sorbit and inosit

result of the chromatography of TMS ether obtained from a standard mixture of these four sugars. Five major peaks on the chromatogram of TMS ethers of plasma sugars of the calf at birth were, in latening order of the retention time, fructose, α -glucose, sorbit, β -glucose and inosit. In addition to these four sugars, glycerinealdehyde, erythrose, arabinose, lyxose, xylose, ribose, galactose, mannose, galactit and mannit were examined for the identification of plasma sugars, and were not detected in plasma of the newborn calves.

Blood sugars of newborn calves

1: fructose, 2: α -glucose, 3: sorbit, 4: β -glucose, 5: inositol

Fig. 2. Sugars in plasma of several calves at birth

1: fructose, 2: α -glucose, 3: sorbit, 4: β -glucose, 5: inositol

Fig. 3. Sugars in whole blood and blood plasma of newborn calves

The chromatogram of TMS ethers of plasma sugars of 8 calves at birth are shown in Fig. 2. Fructose, in addition to glucose, was contained in all calves. It could be presumed that the fructose contents against glucose was larger in calf 4206 than in other calves, from the reference of the peak areas of these sugars. Sorbit was found in all calves except for calf 4215, and was less extent in its contents than fructose and glucose, with reference to the standard chromatogram of the equivalent mixture shown in Fig. 1. Inosit was always contained in all calves.

As shown in Fig. 3, there was no difference in the sort of the sugars between whole blood and blood plasma. The pattern of each peak was almost similar in both samples.

The qualitative changes in plasma sugars with the increase of the postnatal time are shown in Fig. 4. Fructose which was contained in the blood plasma at birth disappeared rapidly with

1: fructose, 2: α -glucose, 3: sorbit, 4: β -glucose, 5: inosit

Fig. 4. Disappearance of fructose and sorbit from plasma with the increase of postnatal time

the increase of the postnatal time. In the case of calf 4206, only trace amount of fructose was recognized after 9 hours of birth. The peak of plasma fructose of calf 4214 grew smaller within the first two hours of birth, and eventually it became a mere trace at 4 hours of age followed by the disappearance after 8 hours of birth. No peak of fructose of calf 4215 was observed after 8 hours of birth. Similar results were obtained from the other calves. The

peak of sorbit diminished with the increase of the postnatal time, and it was recognized that the disappearance of sorbit was accompanied by that of fructose.

Discussion

It was observed in this study that blood fructose in the newborn calves disappeared within about 6 to 10 hours of birth. COLE and HITCHCOCK¹¹⁾ reported that fructose level fell in the blood of the sheep to 1 or 2 mg/dl within 24 to 48 hours of birth. SHELLEY²⁴⁾ showed that the blood fructose level in the fetal lambs declined slowly with increasing gestational age and fell to very low values within a few hours of birth. These rapid disappearance of fructose from the blood of the newborn calves or lambs is due to detaching of the fetus from the placenta. It was shown by several workers^{2,8,20)} that the precursor of fructose in fetal blood was probably glucose, and that its formation occurred mainly in the placenta. They postulated that intermediate phosphate esters would participate in the synthesis of the free fructose. However, HERS¹⁵⁾ found that dialysed extracts of ewe placenta were able to convert glucose to sorbit, but contained only small amounts of ketose reductase, and that latter enzyme was present in fetal liver, which readily converted sorbit to fructose but lacked fruktokinase. He clearly demonstrated that fetal fructose could be formed by the same mechanism as seminal fructose, the first enzymic step taking place in the placenta and the second in the liver of the fetus. ANDREWS, BRITTON and NIXON⁷⁾ studied on the perfused sheep placenta and showed that sorbit was formed *in vivo* in sheep placenta in confirmation of the observation of HERS.¹⁵⁾ ANDREWS, BRITTON, HUGGETT and NIXON⁶⁾ showed that sorbit could be converted to fructose by the fetal sheep liver and glucose, galactose and inosit did not increase the fructose concentration in the perfusate.

The fate of blood fructose after birth, remains unknown, which rapidly disappeared within 6 to 10 hours of birth. There may conceivably little ability that fructose is metabolized by the liver of the newborn calves. HERS¹⁵⁾ was unable to demonstrate fruktokinase activity in the fetal sheep liver. ANDREWS, BRITTON, HUGGETT and NIXON⁶⁾ showed that the fetal liver appeared to be unable to metabolize fructose, but that rapid utilization could not be detected in the perfused neonatal liver before the fifth day after birth. BALLARD and OLIVER⁹⁾ demonstrated that fructose metabolism, as measured by the rates of incorporation of labeled fructose into glycogen and glucose in liver slices and assays of liver ketohexokinase, was barely detectable in the liver of fetal sheep and appeared soon after birth. ALEXANDER, BRITTON and NIXON⁴⁾ studied the magnitude of glucose and fructose metabolism by perfusing the isolated sheep fetus, and showed that the fructose concentration of the perfusate declined very slowly. They also showed that whereas the excretion of glucose was very slight the excretion of fructose was considerable, and suggested that no rapid utilization of fructose occurred in the sheep fetus, and the slight fall in concentration was largely attributable to renal loss. ALEXANDER, BRITTON and NIXON⁵⁾ showed, in similar experiments, that the renal excretion accounted for about half of the fructose which disappeared from the apparent fructose space. SHELLEY and DAWES,²⁵⁾ using the fetal lambs near the term which were delivered by the CAESAREAN section, showed that the amount of fructose excreted in the urine was more than sufficient to account for the apparent loss of fructose from the blood, and suggested that the urinary excretion of fructose probably accounted for the greater part of the fall of blood fructose in normal newborn lambs.

It appears that sorbit, observed in the blood of the jugular vein of the newborn calves, may conceivably be ascribed to the relatively large amounts of sorbit to by-pass into the posterior

vena cava through the ARANTIUS duct in the fetal life, if sorbit in portal blood is completely converted to fructose by the liver. Some doubt, that the chromatographic peak of sorbit might represent the peak of mannit, remained, as the retention time of sorbit was same as that of mannit. However, ANDREWS, BRITTON and NIXON⁷⁾ isolated, crystallized and identified sorbit from the fetal blood of the sheep by X-ray crystallography, and showed to react with purified sorbit dehydrogenase from guinea-pig liver.

Summary

Blood plasma or whole blood of the jugular vein of Holstein calves was sampled immediately after birth and thereafter at the suitable time intervals. Samples were deproteinized by SOMOGYI's method, lyophilized, trimethylsilylated and were qualitatively identified for sugars by gas-liquid chromatography.

1. Fructose, glucose, sorbit and inosit were contained in the blood of the calf at birth.
2. Fructose and sorbit were rapidly disappeared from the blood with the increase of the postnatal time within 6 to 10 hours of birth. Inosit remained in blood.
3. There was no difference in the sort of sugars between whole blood and blood plasma, and the pattern or each peak of sugars was almost similar in both samples.

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References

- 1) AINSWORTH, I.B., C.W. PARR and F.L. WARREN (1951) J. Endocrin., 7: lxiii.
- 2) ALEXANDER, D.P., R.D. ANDREWS, A. ST G. HUGGETT, D.A. NIXON and W.F. WIDDAS (1951) J. Physiol., 129: 352-366.
- 3) ALEXANDER, D.P., A. ST G. HUGGETT, D.A. NIXON and W.F. WIDDAS (1955) J. Physiol., 129: 367-383.
- 4) ALEXANDER, D.P., H.G. BRITTON and D.A. NIXON (1964) J. Physiol., 171: 45-46 p.
- 5) ALEXANDER, D.P., H.G. BRITTON and D.A. NIXON (1966) J. Physiol., 185: 382-399.
- 6) ANDREWS, W.H.H., H.G. BRITTON, A. ST G. HUGGETT and D.A. NIXON (1960) J. Physiol., 153: 199-208.
- 7) ANDREWS, W.H.H., H.G. BRITTON and D.A. NIXON (1959) J. Physiol., 146: 41-42 p.
- 8) BACON, J.S.D. and D.J. BELL (1948) Biochem. J., 42: 397-405.
- 9) BALLARD, F.J. and I.T. OLIVER (1965) Biochem. J., 95: 191-200.
- 10) BARKLAY, H., P. HAAS, A. ST G. HUGGETT, G. KING and D. ROWLEY (1949) J. Physiol., 109: 98-102.
- 11) COLE, S.W. and M.W.S. HITCHCOCK (1946) Biochem. J., 40: li-iii.
- 12) GOODWIN, R.F.W. (1952) Nature, 170: 750.
- 13) GOODWIN, R.F.W. (1956) J. Physiol., 132: 146-156.
- 14) GÜRBER, A. and D. GRÜNBAUM (1904) Münch. med. Wschr., 51: 377-378. cited by Good-

WIN (1956).

- 15) HERS, H.G. (1957) *Biochem. J.*, 66: 30 p.
- 16) HITCHCOCK, M.W.S. (1949) *J. Physiol.*, 108: 117-126.
- 17) HUGGETT, A. St G. (1959) *J. Physiol.*, 146: 53 p.
- 18) HUGGETT, A. St G. (1961) *Brit. Med. Bull.*, 17: 122-126.
- 19) HUGGETT, A. St G. and D.A. NIXON (1961) *Nature*, 190: 1209.
- 20) HUGGETT, A. St G., F.L. WARREN and N.V. WARREN (1951) *J. Physiol.*, 113: 258-275.
- 21) KARVONEN, M.J. (1949) *Ann. Med. Exp. Fenn.*, 27: 197-214.
- 22) KARVONEN, M.J. (1949) *Acta Paediat.*, Stockh., 37: 68-72.
- 23) PATON, D.N., B.P. WATSON and J. KERR (1907) *Trans. Roy. Soc. Edinb.*, 46: 71-102.
- 24) SHELLEY, H.J. (1960) *J. Physiol.*, 153: 527-552.
- 25) SHELLEY, H.J. and G.S. DAWES (1962) *Nature.*, 194: 296-297.
- 26) SOMOGYI, M. (1930) *J. Biol. Chem.*, 86: 655-663.
- 27) SWEETLEY, C.C., R. BENTLEY, M. MAKITA and W.W. WELLS (1963) *J. Am. Chem. Soc.*, 85: 2497-2507.
- 28) WALKER, D.G. (1954) *Nature*, 173: 309-310.
- 29) WELLS, W.W., T. CHIN and B. WEBER (1964) *Clin. Chim. Acta*, 10: 352-359.

ガスクロマトグラフィーによる新生子牛血糖の 同定とその消長

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成反芻動物の血糖は他の動物種に比較して低レベルにあるといわれているが、子牛のそれは成牛より高濃度に存在し、生後 2~3 ケ令にわたる連続的濃度低下を示して成牛値に達することが知られている。いっぽう、胎児血中にグルコース以外の糖を含有する動物種のあることが認められている。本実験の目的は、出生時におけるウシの血中遊離単糖の種類をガスクロマトグラフィー (GLC) により決定し、同時にそれらの糖の出生後における経時的消長を定性的に検討することである。

東北農業試験場繋養のホルスタイン種を供試し、出生時に頸静脈より採血したのち、適宜、経時的に採血した。全血および血漿の 1 ml を Somogyi の方法により除蛋白し、除蛋白上清を凍結乾燥したのち、乾燥ピリジン：ヘキサメチルジシラザン：トリメチルクロロシランの 10:2:1 (容量) 溶液の適量を添加混和して糖のトリメチルシリルエーテル化を行ない、10 分静置後、反応混液上清の 1~3 μ l を GLC に注入した。GLC 操作条件は、

島津 GC-1C 型, SE-52 (5% on Shimalite W, 60~80 メッシュ, 3 ϕ ×2625 mm), カラム恒温槽温度 190°C, 注入口温度 330°C, 水素炎イオン化検出器温度 350°C, キャリヤーガス N_2 60 ml/min, H_2 45 ml/min である。

1. 市販の特級試薬を標準にして血中遊離単糖類を推定した結果、出生時において、グルコース以外にかなりの量のフラクトースが含有されること、およびソルビット、イノシットの存在を認知することができた。また、これらの糖よりきわめて保持時間の小なる 2 個の未同定小ピークが観察された。

2. フラクトースは出生後の時間経過とともに急激に減少し、生後およそ 6~10 時間で血中より消失することが判った。ソルビットはフラクトースの消失にしたがい、同様に血中より消失した。いっぽう、イノシットはグルコースと同様に、実験観察期間における血中よりの消失は認められなかった。

3. これらの糖は全血および血漿中に同様に含有されたと推定された。

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