Sex Accessory Fructose: An Evaluation of Biochemical Techniques* (32837)

J. A. THOMAS, M. MAWHINNEY, AND W. MASON, (Introduced by W. W. Fleming)
Department of Pharmacology, West Virigina University Medical Center, Morgantown, West Virginia 26506

Sex accessory organs of several species are known to contain fructose, but the chemical form in which this hexose exists has received very little attention. While seminal plasma is reported to contain 'free' fructose, seminal vesicles per se are reported to contain sizeable amounts of phosphorylated derivatives of this keto sugar (1). No attempts have been made to determine the relative amounts of phosphorylated and nonphosphorylated fructose in seminal vesicles, but they are presumed to possess significant amounts of phosphofructose. Furthermore, no efforts have been undertaken to examine the relative amounts of these chemical forms of fructose in other sex accessory organs.

The analyses of fructose in biological materials has been carried out primarily by either the Seliwanoff's resorcinol reaction adapted by Roe (2), and subsequently modified (3,4), or by the cysteine–carbazole reaction initially described by Dische and Borenfriend (5). The method employed by Roe has been modified for fructose analysis of sex accessory tissues using either heavy metals or trichloroacetic acid (TCA) (6,7). Later this same colorimetric assay was applied to tissues extracted with ethanol, barium hydroxide, and zinc sulfate (Ba–Zn) (1). Improved sensitivity and specificity of the resorcinol reaction has been reportedly achieved by the addition of 1,1-diethoxyethane (8,9). The original cysteine–carbazole test has been slightly modified by Bartlett (1). Other methods or extraction procedures have also been used for the chemical determination of fructose (cf. 6, 11–14). Clearly there are several methods or modifications of methods for the biochemical determination of fructose in sex accessory organs. In addition, the means of extracting this sugar or its derivatives from these tissues have employed either organic acid or heavy metals. Still other means of extracting have utilized ethanol and heavy metal procedures.

Some criticism has been voiced against using TCA or perchloric acid to homogenize tissues since it reportedly results in the measurement of total fructose (1,12). Grinding sex accessory organs in organic acid consequently would not distinguish between phosphorylated and nonphosphorylated forms of fructose. Previous reports seem to suggest that only ethanol, barium hydroxide, and zinc sulfate are suitable precipitating steps for the subsequent analyses of fructose in sex accessory organs of reproduction. The purpose of the present studies was to determine if precipitating procedures (*viz.* TCA vs ethanol-barium hydroxide–zinc sulfate) affected the subsequent measurement of sex accessory fructose. Furthermore, it was of interest to determine whether or not the resorcinol reaction would yield fructose values similar to those obtained from the cysteine–carbazole test.

Methods. Sex accessory organs were obtained from normal mature mice. It was necessary to pool sex accessory organs in order to obtain sufficient amounts for biochemical analyses. Seminal vesicles (empty) and lobes of anterior prostate glands (coagulating glands) were removed, blotted on moist filter paper, and weighed on a torsion balance. Tissues were immediately homogenized in either cold 10% TCA or in cold 0.154 *M* potassium chloride. Aliquots (equivalent to 4 mg of tissue) of homogenate suspended in TCA were diluted with distilled water and analyzed for fructose by either the resorcinol reaction or by the carbazole–cysteine method. Similar aliquots of KCl homogenates were treated with 1.8% barium hydroxide and 2% zinc sulfate and assayed for fructose. In some experiments, KCl treated tissues were sus-

* This investigation was supported in part by research Grant RO1 AM 09011 from the National Institutes of Health and by a general research support grant from the West Virginia University School of Medicine.

TABLE I. Comparison of Anterior Prostate Fructose Levels (mean ± SEM) (mg/100 mg of wet wt.) in Normal Mice.[a]

Extraction procedure	Biochemical method	
	Resorcinol[b]	Cysteine–carbazole
10% Trichloroacetic acid	0.438 ± 0.02	0.379 ± 0.06
Ethanol–heavy metals	0.318 ± 0.01[c]	0.361 ± 0.01
Heavy metals only	0.316 ± 0.01[c]	0.338 ± 0.01

[a] Each group consists of 6 or more paired mice.
[b] Method described by Roe, J. Biol. Chem. **107**, 15 (1934).
[c] Significantly lower than TCA–resorcinol group.

pended in 80% ethanol and then precipitated with heavy metals. Aliquots of KCl homogenates, after first being subjected to alcohol and/or heavy metal precipitation, were analyzed by either the resorcinol reaction or by the cysteine–carbazole method.

When using resorcinol, samples were heated for 15 min at 80°C. This laboratory routinely uses a 30% solution of HCl for the resorcinol reaction, since concentrated HCl enhances the formation of color. After cooling the reaction mixture in cold tap water, the tubes were analyzed for their fructose content at a wavelength of 515 mu on a Beckman DU spectrophotometer.

The cysteine–carbazole method used was very similar to that employed by Bartlett (10). The cysteine solution was made fresh daily, but preliminary experiments revealed that the solution was stable overnight. Samples were incubated for 15 min at 60°C. After cooling the final reaction mixture, samples were read within the hour at 560 mu. No demonstrable color changes were noted up to 4 hours following completion of the reaction.

In a limited number of experiments the resorcinol–acetal method (8) for fructose determination was carried out in both tissues and synthetic derivatives of this sugar.

Fructose was routinely calculated from a standard curve using crystalline *beta*-D(—)-fructose (Sigma Biochemicals) although some experiments were run using both fructose-6-phosphate (barium salt, Grade IV, Sigma Biochemicals) and fructose-1,6-diphosphate (sodium salt, Sigma Biochemicals). Comparisons of the resorcinol and the cysteine–carbazole reactions using equimolar concentrations of various fructose standards were also performed.

All other reagents were obtained commercially and were not subjected to further purification procedures. Resorcinol, thiourea, and hydrochloric acid were purchased from Fisher Scientific while carbazole, cysteine hydrochloride, and 1,1-diethoxyethane (acetal) were obtained from Eastman Chemicals. Carbazole reagent was prepared with absolute ethanol while the resorcinol reagent was mixed with 95% ethyl alcohol. Glacial acetic acid was used for resorcinol–thiourea reagent.

Results. Table I reveals that various analytical techniques produced differences in the levels of measurable amounts of prostate gland fructose. Extraction procedures likewise caused notable differences in the amounts of this sugar. Differences arising from extraction procedures were more evident in the resorcinol method than they were in the cysteine–carbazole determination of fructose. Regardless of the biochemical method, trichloroacetic acid extraction procedures consistently produced higher mean fructose values than those tissues extracted with heavy metals and/or ethanol.

Although ethanol has been reported to produce a hyperchromic action upon the resorcinol reaction for fructose, Table I reveals no difference between prostate fructose values obtained from ethanol–heavy metal extraction procedures compared to those obtained from only heavy metal extraction procedures. Both final reaction mixtures contained 0.1% ethanolic resorcinol solution yet no hyperchromic effect was noted in the assay utilizing additional amounts of alcohol for precipitating

TABLE II. Comparison of Seminal Vesicles (Empty) Fructose Levels (mean ± SEM) (mg/100 mg of wet wt.) in Normal Mice Using Two Separate Tissue Extraction Procedures and Two Different Biochemical Determinations.[a]

Extraction procedure	Biochemical method: Resorcinol[b]	Biochemical method: Cysteine–carbazole
10% Trichloroacetic acid	0.341 ± 0.03	0.229 ± 0.01
Heavy metals only	0.216 ± 0.02[c]	0.321 ± 0.05
Ethanol–heavy metals	0.108 ± 0.01[c]	0.194 ± 0.01

[a] Each group consists of 6 or more paired mice. See results section for further statistical analyses.

[b] Method described by Roe, J. Biol. Chem. **107,** 15 (1934).

[c] Significantly lower than TCA–resorcinol group.

proteins. Unexpectedly, extraction procedures involving ethanol enhanced the cysteine-carbazole assay for fructose. However, this enhancement is not statistically greater than that recorded for similar assays without ethanol extraction procedures.

The levels of fructose in seminal vesicles varied depending upon the particular biochemical procedure (Table II). If TCA extracts total fructose and the heavy metal–ethanol protocol results in the extraction of free fructose, then the values obtained from mouse seminal vesicles analyzed by the resorcinol method seem to agree with that reported for bull seminal vesicles (1). It is not clear, however, why heavy metals alone resulted in somewhat higher resorcinol-determined fructose than those subjected to the combination of ethanol and heavy metals.

The cysteine–carbazole fructose analyses of seminal vesicles revealed concentrations approximately the same as those obtained by the resorcinol methods (Table II). However, the manner in which the proteins were precipitated exerted a marked effect upon the subsequent cysteine–carbazole analyses of fructose. There was no statistical difference between TCA treated samples and those precipitated with ethanol and Ba–Zn, but when only heavy metals were used, fructose concentrations were significantly lower ($p \leq 0.1\%$). Despite the fact that the cysteine-carbazole method per se cannot distinguish between free and phosphorylated fructose (10), the manner in which the tissues themselves were treated influenced the amount of measurable hexose.

Table III shows the effects of changing the Seliwanoff reaction by the addition of thiourea to the resorcinol reagent. The original method of Roe (2) was later modified (3,4) to improve its sensitivity. The TCA extraction procedures resulted in higher fructose levels than those obtained by heavy metal precipitation. This difference was most marked in the resorcinol–thiourea determination of fructose and was particularly evident in the seminal vesicles.

Discussion. These studies have made no specific effort to distinguish between the various fructose derivatives that are responsible for the chromogenic reactions with either resorcinol or cysteine–carbazole, but rather they have been concerned with variations in the already existing colorimetric procedures arising from the manner in which sex accessory tissues are initially extracted. Earlier reports by Hers *et al.* (11) indicated that total fructose could be extracted by either TCA or by perchloric acid and that free fructose could be measured from zinc–barium filtrates. Lindner and Mann (1) indicated that discrepencies in fructose levels may arise from the use of organic acids for the extraction of this hexose from the seminal vesicles. These same investigators found that bull seminal vesicles contain, apart from free fructose, a small but nevertheless significant amount of phosphofructose. Unfortunately the quantity of this significant amount of phosphofructose was not specifically reported. The sex accessory glands of the mouse revealed higher fructose levels with TCA extracts than with either those of heavy metals or heavy

TABLE III. Effect of Thiourea on Sex Accessory Fructose Levels (mean ± SEM) (mg/100 mg of wet wt.) in Normal Mice Using Two Different Extraction Procedures.

Extraction procedure	Biochemical method: Resorcinol[a]	Resorcinol–thiourea[b]
	Anterior prostate	
10% Trichloroacetic acid	0.438 ± 0.02	0.355 ± 0.01
Heavy metals only	0.316 ± 0.01[c]	0.223 ± 0.01[c]
	Seminal vesicles	
10% Trichloroacetic acid	0.341 ± 0.03	0.247 ± 0.03
Heavy metals only	0.216 ± 0.02[c]	0.089 ± 0.01[c]

[a] Method described by Roe, J. Biol. Chem. **107**, 15 (1934).
[b] Method described by Roe, *et al.*, J. Biol. Chem. **178**, 839 (1949).
[c] Significantly lower than respective acid extracted groups ($p \leq 5\%$).

metals and ethanol (Table III). Thus these results would appear to be in agreement with the contention of Lindner and Mann (1). It may be noted, however, that while TCA extracted tissues assayed for fructose by the resorcinol method consistently yielded different fructose levels, the particular biochemical determination also produced differences in mean values. For example, consideration of Ba–Zn filtrates of seminal vesicles revealed mean fructose concentrations to be cysteine–carbazole>resorcinol>resorcinol–thiourea. A similar consideration of TCA filtrates shows resorcinol>resorcinol–thiourea>cysteine–carbazole. It would seem that not only are precipitating procedures influencing the amount of measurable fructose, but different modifications of the Seliwanoff reaction and the cysteine–carbazole reaction are likewise responsible for differences in the amount of detectable sugar.

These investigations revealed that sex accessory fructose levels vary with the particular biochemical procedures. Both the seminal vesicles and the anterior lobes of the prostate gland yielded higher fructose levels when extracted with organic acid compared to those treated with heavy metals. Extraction procedures seemed to exert a more marked effect upon resorcinol–thiourea determined sex accessory fructose than in those analyzed with only resorcinol reagent (Table III).

If the addition of thiourea enhances the specificity of the Seliwanoff reaction, then this cannot be conclusively determined from the results obtained from mouse sex accessory organs (Table III). Confirmation of this increased specificity would require chromatographic analyses since color forming nonfructose keto groups might be reflected to a greater extent in the Seliwanoff's reaction utilizing only resorcinol. More recent modification of the Seliwanoff reaction indicate that 1,1-diethoxyethane (acetal) further improves the sensitivity of fructose determination, but as yet this modification has been applied only to synthetic mixtures of sugars and not to sex accessory organs (8,9). In preliminary studies, this laboratory has observed that the sex accessory fructose levels are slightly lower when determined by the acetal-resorcinol method (unpublished).

This laboratory has routinely measured sex accessory fructose (3) from TCA filtrates. Indeed fructose levels measured by this method can be reduced by castration (16), estrogens (17,18), and synthetic steroids (19). On the basis of the present studies it appears that sex accessory fructose can be determined from either TCA or heavy metal tissue extraction procedures. Although analyses of sex accessory fructose leads to differences in absolute values depending upon the method of tissue extraction, the importance of these differences cannot be appreciated until the extent to which androgens influence either free or total fructose can be ascertained.

More exhaustive investigations pertaining to factors which modify the colorimetric determination of fructose have been reported (4,8,9,14), but unfortunately many of these studies have been undertaken using synthetic mixtures of sugars and polysaccharides and have not always been extended to biological materials. This laboratory has consistently observed that equimolar solutions of fructose-6-phosphate, fructose-1,6-diphosphate, or free fructose produce different colorimetric responses depending upon the particular biochemical determination (unpublished). Furthermore, subjecting these same purified fructose derivatives to extraction procedures ordinarily reserved for precipitating proteins in sex accessory organs causes alterations in the colorimetric response. Fructose-6-phosphate produced the least amount of color when determined by the cysteine-carbazole method or by certain resorcinol modifications, but in the acetal–resorcinol method (8) this compound yielded higher colors than either fructose-1,6-diphosphate or free fructose. Of the three fructose derivatives, free fructose was generally more consistent in causing the greatest development of color in the various biochemical methods used for the determination of this keto sugar (unpublished). Such findings would suggest that using a single derivative of fructose (usually free) for the construction of a standard calibration curve might also have to be taken into consideration when determining a mixture of these hexoses ordinarily found in sex accessory organs.

There seems to be little doubt that TCA filtrates more frequently result in higher sex accessory fructose values than those determined from heavy metal filtrates. Needless to say, many other factors (exclusive of those caused by the biological material) can affect the determination of fructose. The concentration of the hydrochloric acid, the time and the temperature of incubation, and the inclusion of different constituents into the resorcinol reagent are all factors which affect the Seliwanoff reaction.

Although many known factors (chemical and biological) exert an affect upon sex accessory fructose, the fact remains that this metabolic process is under the primary control of androgens. While the enzymatic determination of fructose would unquestionably result in greater specificity, it seems doubtful that this would achieve any greater insight into factors that control this hormonal dependent biochemical event.

Summary. Sex accessory fructose was analyzed from both trichloroacetic acid and ethanol–heavy metal filtrates using either the resorcinol reaction or the cysteine–carbazole method. Sex accessory fructose levels (mg/100 mg of wet wt.) were consistently higher in tissues homogenized in trichloroacetic acid than in those tissues extracted with ethanol and heavy metals. Several factors were demonstrated to affect the measurable amount of sex accessory fructose. Not only does the manner of tissue extraction affect levels, but the particular method of chemical determination leads to differences in the detectable amounts of this sugar.

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Received Sept. 11, 1967. P.S.E.B.M., 1968, Vol. 127.