values increased toward, but did not exceed, those observed following intraperitoneal injection. In the case of Ca^{45}–Sr^{++} the variability which accompanied the high values attained suggested a high degree of uncertainty.

Discussion.—The results shown in Table I indicate that intraperitoneal injection of non-radioactive calcium has no perceptible effect on deposition of tracer quantities of Ca^{45} or Sr^{89} in femur. The value of 2·71% for Ca^{45} was not statistically different from the 2·85% obtained with Sr^{89} in the presence of stable calcium, a result which may be related to the chemical similarity of these metals. The very small tracer amount of Sr^{89} may be readily incorporated under these experimental conditions into bone concurrently with, and similarly to, the normal calcium uptake.

Injection of very large amounts of stable strontium did not apparently affect the overall processes associated with calcium uptake enough to be reflected in any alteration of the amounts of tracer Ca^{45} deposited. This is indicative of a close regulation of bone calcium uptake. Although Ca^{45} uptake was not influenced in this way, femur strontium uptake decreased following injection of massive doses of stable strontium by about 30% from the value obtained with carrier-free solutions. This reduction may have some analogy to isotopic dilution effects where Sr^{89} may compete with stable strontium in the processes associated with bone deposition of this metal. The results do not preclude the possibility of similar competition as between stable calcium and Ca^{45} but small effects produced in this way may have been undetectable within the limits of experimental error, especially in consideration of the recent demonstration[4,5] that calcium supplementation of a diet adequate in calcium results in minimal decreases in bone deposition of Ca^{45}, even over extended experimental periods.

The amounts of Ca^{45}–Sr^{89} deposited in femur following oral administration appeared to reflect some reduced availability of the radioisotopes, for with the exception of the Ca^{45}-stable strontium series, the values of Table II were less than those observed after intraperitoneal injection. This exception which attained values as great or greater than after intraperitoneal injection, was also in accord with the general trend of the femur radioisotope deposition to increase with increasing stable metal.

The positive dependency of radioisotope depositions on amount of stable metal orally administered suggests that mixing and dilution with intestinal contents as well as excretion may have had some effects to alter the amounts of isotopic mixtures available for gastrointestinal absorption.

If absorption of the isotopic mixtures from the gastrointestinal tract is related to their concentration gradient across the intestinal barriers as recent considerations of absorption and membrane transfer suggest[6], dosage effects of stable calcium or strontium administered orally might also be reflected in absorption differences. These factors may account indirectly to some extent for the generally lower, but increasing positive proportionality observed between oral dosage and femur deposition of calcium and strontium. Variabilities in quantities of metals absorbed from the gastrointestinal tract would in effect influence their femur deposition more directly than the values of total oral dosage of the isotopic mixtures. Further and more extensive study would be required to establish this possibility.

Acknowledgement.—The able assistance of D. S. DECHANT and other members of the Metabolism Operation is gratefully acknowledged. Statistical analysis of the data was performed by W. L. NICHOLSON of the Operations Research and Synthesis Operation.

B. KAWIN*

Biology Operation, Hanford Laboratories, Richland (Washington), April 6, 1959.

Zusammenfassung

Nach einmaliger intraperitonealer Verabreichung von stabilem Kalzium oder Strontium in Mengen bis zu 3000 µg/g Körpergewicht war der Bruchteil des gleichzeitig verabreichten Ca^{45}, das sich im Rattenschenkel ablagerte, unverändert. Unter denselben Umständen verabreicht, verminderte stabiles Strontium im Schenkel abgelagertes Sr^{89} ein wenig, aber stabiles Kalzium hatte keine solche Wirkung auf Sr^{89}.

Während sich die Konzentration des Radioisotops nach einmaliger peroraler Verabreichung im entsprechenden Verhältnis zur Dosis des stabilen Kalzium- oder Strontiumgehalts vermehrte, waren im allgemeinen die Konzentrationen im Schenkel nach einmaliger peroraler Verabreichung kleiner als nach intraperitonealer Verabreichung.

* Present Address: Space Medicine Branch, Systems Management Office, Boeing Airplane Company, Seattle (Washington).

Fructose and Fructolysis in Human Semen Determined Chromatographically

The work of DAVIS and MACUNE[1], BIRNBERG, SHERBER, and KURZOK[2], and VAISHWANAR[3] has shown that the rate of fructolysis of human spermatozoa can give an indication of their activity. MANN[4] has shown that fructose is metabolised in bull semen incubated *in vitro* and that the rate of fructolysis as assessed by the colorimetric estimation of disappearing fructose gives an accurate measure of the activity of spermatozoa. Most workers have employed the colorimetric estimation of fructose using the resorcinol method as a simple and convenient procedure for the estimation of the content of fructose and the rate of fructolysis in semen. However, MANN[5] has pointed out that, unlike bull semen, that of man contains a large amount of reducing material other than fructose which can seriously interfere with the conventional methods of sugar determinations. He has further pointed out that these reducing substances are not only present in fresh human semen but that their content increases during incubation of semen *in vitro*. If however fructose could be separated from other reducing substances occurring in human semen and then estimated, such values would be more accurate. With this view in mind, an attempt was made to apply to semen a chromatographic procedure for the estimation of fructose based in principle, on the method of GIRI and NIGAM[6].

A 1% potassium hydroxide solution was prepared in 95% alcohol. The triphenyl tetrazolium chloride solution was prepared by dissolving 2 g of the substance in 100 ml of butanol saturated with water. Both solutions were kept in the cold in brown bottles. The tetrazolium reagent (T.T.C.) was

[1] M. E. DAVIS and W. W. MACUNE, Fert. Ster. *1*, 362 (1950).

[2] C. H. BIRNBERG, D. A. SHERBER, and R. L. KURZOK, Amer. J. Obstetr. Gynecol. *63*, 877 (1952).

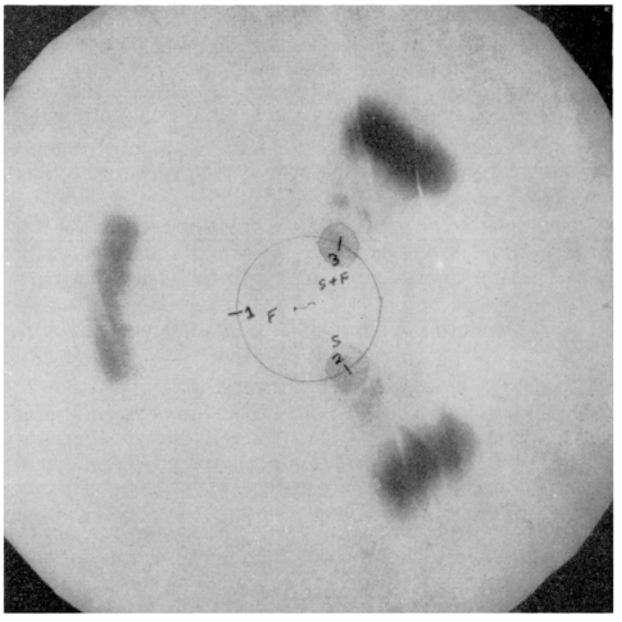
[3] VAISHWANAR, Amer. J. Obstetr. Gynecol. *75*, 139 (1958).

[4] T. MANN, Lancet *1948/I*, 446.

[5] T. MANN, *Biochemistry of Semen* (Methuen and Co. Ltd., London 1954), p. 48.

[6] K. V. GIRI and V. N. NIGAM, J. Indian Inst. Sci. *36*, 49 (1954).

[6] T. ROSENBERG and W. WILBRANDT, Exp. Cell Res. *9*, 49 (1955).



prepared by mixing these two solutions in equal proportions just before use. The actual estimation of fructose in semen was carried out as follows. A circular disk 18 cm in diameter, was cut from Whatman filter paper No.1. A circle of 2 cm radius was drawn at the centre of disk. Four equidistant points were marked along the circumference of the circle. 10 µl of semen was applied at one of these points, and varying amounts of a standard solution of fructose (0·1%) were applied at the remaining points. The spots were allowed to dry in air. The chromatogram was run in the usual way by irrigating with butanol/acetone/water (2:7:1). After the solvent had travelled a distance of about 8 cm from the centre, the paper was dried at room temperature and treated with the T.T.C. reagent by placing the paper on a glass plate and applying the T.T.C. reagent with a cotton applicator. The chromatogram was then placed in an incubator at 37·5°C in a moist atmosphere for 1 h during which time intense red bands appeared. It was then washed with water and dried at room temperature. The zones corresponding to the red bands were cut out and the colour extracted from them with 10 ml of 95 % alcohol. The intensity of the colour was measured by means of a Klett-Summerson colorimeter using a green filter. The quantity of fructose was evaluated from the readings by the help of a calibration curve which was prepared as follows. Varying amounts of a 0·1% standard solution of fructose were spotted on the Whatman No. 1 paper and the experiment was carried out as described above. When the concentrations of fructose were plotted against the optical density given by the alcoholic extract, a straight line graph was obtained as shown in Figure 1. The fructose value of human semen would usually lie within the range plotted.

Pure fructose and the seminal sugars exhibited the same R_f value. This was shown in an experiment where the paper was spotted with 10 µl 0·1% fructose, 10 µl 0·1% fructose + 10 µl of semen, and 10 µl of semen, respectively. The chromatogram was developed as described above, and all three colour bands showed the same R_f value.

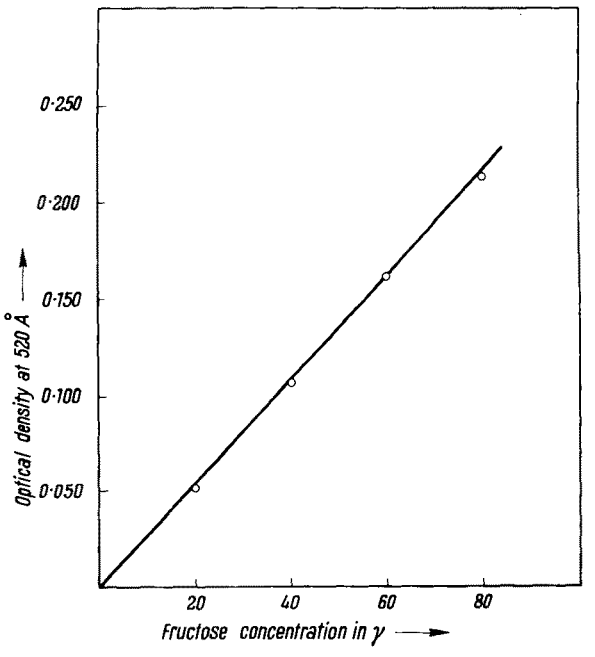
Simultaneous assays of fructolysis were carried out by the chromatographic method and by resorcinol method of Roe[7]. A sample of semen was obtained from a healthy donor. It had a pH of 8·2, a sperm density of 48 million/ml and a motility of 50%. It was incubated at 37·5°C. At intervals of $1^1/_2$ h, 10 µl samples were withdrawn for determinations of fructose, by the chromatographic method and 100 µl for the resorcinol method. The results are given in the Table.

[7] J. H. Roe, J. biol. Chem. *107*, 15 (1934).

Fructose content of semen incubated at 37·5°C determined by the resorcinol method and by the chromatographic method

Time in h after ejaculation	Percentage motility	Resorcinol method: Fructose (mg/100 ml semen)	Chromatographic method: Fructose (mg/100 ml semen)
0·5 h	50	675	477
2·0 h	35	675	367
3·5 h	20	760	313
5·0 h	10	900	272

As can be seen from the Table, fructose values calculated by the resorcinol method tended to increase during the incubation. This is in all probability due to the increase in the content of interfering reducing substance and does not depict the behaviour of true fructose. Fructose as such could not have increased, since it was being metabolised by the spermatozoa all the time during incubation. However, the fructose values obtained by the chromatographic method decreased on incubation, as might be expected.



The results reported in the present study suggest that for the estimation of fructose in human semen, the chromatographic method has considerable advantages over the conventional resorcinol method.

Acknowledgements—We gratefully acknowledge all the valuable help and encouragement given by our Director, Dr. V. R. Khanolkar. We are deeply indebted to the Raptakos Medical Research Board for the award of a fellowship to one of us (A.R.S.) and for giving a grant in aid for work. We wish to thank Dr. T. Mann for going through the manuscript and giving his valuable comments.

A. R. Sheth and Shanta S. Rao

Contraceptive Testing Unit at the Indian Cancer Research Centre, Parel, Bombay, April 6, 1959.

Résumé

Le fait que le fructose disparaît de la semence incubée *in vitro* est utilisé par plusieurs chercheurs pour déterminer l'activité métabolique des spermatozoaires. La quantité du fructose est dosée par le Résorcinol. Ce dosage est très pratique pour mesurer la teneur en fructose et la fructolyse dans le cas de la semence du Bœuf. Mais, puis-que la semence humaine contient d'autres produits aussi réductibles que le fructose, cette méthode ne convient pas au sperme humain.

Nous avons essayé de séparer le fructose de la semence humaine par la chromatographie circulaire, et de le doser ensuite quantitativement en utilisant le tetrazoliumchloride du triphényl. Cette méthode donne des résultats précis pour déterminer la teneur en fructose et la fructolyse dans la semence humaine.

Studies on the Swelling of Rat-Liver Mitochondria in Relation to Tumor Incidence During Feeding of Aminoazo Dyes

It was reported previously by one of us[1] that the swelling ability of rat liver microsomes decreases during feeding of 3'-methyl-*p*-dimethylaminoazobenzene (3'-Me-DAB), reaching a minimum level at 4 weeks. Even though the feeding of the dye is continued, this swelling ability eventually recovers and reaches a normal level at about 20 weeks. The microsomes from hepatoma, induced with 3'-Me-DAB, swell to an extent comparable to those of the liver after 4 weeks of feeding of this same dye. The non-carcinogenic isomer, 2-Me-DAB, however, does not produce these effects.

This report[2] describes a correlation that has now been established between microsomal swelling and the appearance of gross tumors. Table I shows that if the feeding of 3'-Me-DAB to rats is continued beyond 4 weeks under our experimental conditions, the animals rapidly reach a point of no return, since the percentage of tumor incidence in the groups shows a sudden steep rise at about 4 weeks.

The fine structural alterations in the cell, that can be detected by the study of swelling, during chemical carcinogenesis are, however, not restricted to the endoplasmic reticulum, origin of the 'microsome' fraction. Of more particular interest in this respect are the findings of EMMELOT and Bos, that the thyroxine-induced swelling of rat liver mitochondria decreases after feeding DAB for 5 months[3] or by incubating these particulates *in vitro* with carcinogens[4].

Table II shows alterations of the mitochondrial swelling, essentially similar to those of microsomal swelling during feeding 3'-Me-DAB[1]. There is a minimum swelling with the liver at about 4 weeks, and low values have been observed with the hepatoma. As with the microsomes[1], no appreciable change in mitochondrial swelling was observed during feeding 0·06% of the non-carcinogenic dye, 2-Me-DAB, for 6 weeks.

Table I

Groups, of 16 male Sprague-Dawley rats each, were fed a riboflavin-low semi-synthetic diet[1], containing 0·06% 3'-Me-DAB, for periods varying from 1 to 12 weeks. After the respective periods of dye feeding the animals were continued to be fed the same diet without dye, and were sacrificed after a total of 7 months[5].

Weeks of feeding	No. of tumor bearers over total survivors	Percentage of tumor incidence
1	0/14	0 %
2	0/15	0 %
3	1/13	7·5%
4	4/16	25 %
5	8/16	50 %
6	11/17	65 %
8	16/16	100 %
10	16/16	100 %

The differences between the macromolecular organization of liver mitochondria and of hepatoma mitochondria are evident not only from the extent of swelling, but also from its pH dependence (cf.[1]). Swelling was studied in 0·17 *M* sucrose, and in 0·30 *M* sucrose in presence of 1×10^{-5} *M*/l thyroxine or of 5×10^{-3} *M*/l $CaCl_2$. The pH range was from 5·0 to 9·6. All curves obtained with liver mitochondria show relatively sharp maxima around pH 7·4, while the curves obtained with the hepatoma were rather flat with no clear-cut maxima.

Table II

Animals and diet were identical to those described in Table I. The method of isolation of the mitochondria and the swelling test (40 min) were essentially those described by TAPLEY[6]. Percentage swelling was calculated as in previous work[1].

'Percentage Swelling'

Weeks of dye feeding	0·17 *M* sucrose	0·30 *M* sucrose 1×10^{-5} thyroxine	0·30 *M* sucrose 5×10^{-3} $CaCl_2$	0·30 *M* sucrose 1×10^{-5} $HgCl_2$
0	50·2	37·2	38·0	65·1
2	44·1	24·1	46·5	55·8
3	—	28·1	41·4	50·2
4	35·0	13·8	29·9	29·4
5	—	9·3	32·3	30·9
8	—	19·2	36·2	35·9
10	38·6	21·2	37·1	—
12	—	29·8	41·4	47·6
Tumor	18·7	12·1	19·7	21·3

The influence of various compounds on the swelling on normal mitochondria was studied with particular emphasis on the role of sulfhydryl groups. Since carbonyl compounds are known to interact with sulfhydryl groups of proteins, the effect of such agents was investigated. The fact, that at the same molar concentration of 1×10^{-2} only alloxan and diacetyl are inhibitors of swelling, while acetone, chloroacetone, acetylacetone, and acetonylacetone are inactive, seems to indicate that at least two vicinal carbonyl groups in the molecule are required for the inhibition of mitochondrial swelling by these com-

[1] J. C. ARCOS and M. ARCOS, Biochim. biophys. Acta *28*, 9 (1958); Naturwissenschaften *44*, 331 (1957).

[2] These investigations are supported by the U.S. Public Health Service Grant C-4351.

[3] P. EMMELOT and C. J. Bos, J. exp. Cell Res. *12*, 191 (1957).

[4] P. EMMELOT and C. J. Bos, Biochim. biophys. Acta *24*, 442 (1957).

[5] The authors wish to thank Dr. V. M. AREAN and Dr. J. SIMON for the histopathological examinations.

[6] D. F. TAPLEY, J. biol. Chem. *222*, 325 (1956).