APPLICATION OF A LIQUID-SCINTILLATION TECHNIQUE TO THE STUDY OF AEROBIC $^{14}\text{CO}_2$ PRODUCTION FROM GLUCOSE AND FRUCTOSE BY BOAR, RAM AND BULL SPERMATOOZOA

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Summary. A technique which depends on absorbing $^{14}\text{CO}_2$ on paper strips containing small amounts of KOH, and ‘counting’ the strips directly in a liquid-scintillation system, has been applied to studies on spermatozoal metabolism. A small quantity of uniformly-labelled [^{14}C]-glucose or [^{14}C]-fructose has been added to washed spermatozoal suspensions which already contained non-labelled glucose or fructose and the $^{14}\text{CO}_2$ production assessed at the end of a 1 hr incubation period in an air atmosphere, at 38° C. Boar spermatozoa, like those of ram, bull and man, have been shown to utilize glucose preferentially if presented with a mixture of these two sugars.

INTRODUCTION

Mann (1951), van Tienhoven, Salisbury, VanDemark & Hansen (1952), and Freund & MacLeod (1958), respectively, reported that ram, bull and human spermatozoa preferentially utilize glucose when incubated in a mixture of glucose and fructose. Lorenz (1958) noted fructose utilization by cock spermatozoa, but only in the absence of glucose. Aalbers, Mann & Polge (1961) found that, in the presence of fructose as the sole carbohydrate, motility of boar spermatozoa was greater in aerobically than in anaerobically incubated samples (1 to 3 hr at 37° C). In aerobic suspensions, containing either glucose or fructose or mannose alone, any of these three sugars was utilized at about the same rate.

Metabolism of ^{14}C -labelled substrates by spermatozoa has been previously studied by spreading washed cells on planchets and ‘counting’ to establish ‘uptake’ (Flipse, 1954), by absorbing CO_2 on paper containing KOH and then measuring the radioactivity of the $\text{Ba}^{14}\text{CO}_3$ precipitate subsequently prepared (Flipse & Almquist, 1955) and by autoradiographic techniques (Pickett & Merilan, 1957).

Chiriboga & Roy (1962) reported on another technique for measuring $^{14}\text{CO}_2$ after absorption on paper containing KOH and then ‘counting’ dis-

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integrations within a liquid-scintillation system. This appeared to be a technique worth applying to studies on spermatozoal metabolism. It also offered the opportunity to verify whether boar spermatozoa would preferentially utilize glucose as has been previously noted for spermatozoa of certain other species.

MATERIALS AND METHODS

Semen was obtained from three Yorkshire boars and three Suffolk rams with the artificial vagina, and from three Hereford bulls with the electro-ejaculator. Samples sufficient to provide 7.5×10^9 spermatozoa from individual bulls and boars, and pooled samples from rams were washed twice with calcium-free Krebs'-Ringer-phosphate (KRP), and the spermatozoa were resuspended in KRP to a concentration of 10^9 cells/ml.

Flasks (in duplicate) containing 0.5 ml of the spermatozoal suspension and 2 ml of substrate were set up as shown in Table 1, and shaken in an air atmosphere at 38° C at a rate of 110 strokes/min with a 2 cm length. Pre-trial experiments established that, under these conditions, motility was the same irrespective of which sugar was used; therefore, the contents of one flask only were used for evaluating motility. Motility was expressed as the percentage of spermatozoa which exhibit progressive movement when a suspension on a glass slide and under a cover slip is examined microscopically ($\times 100$ magnification).

Incubation flasks were as described by Chiriboga & Roy (1962) except for the elimination of the trichloroacetic acid dipper, and the inclusion of a rubber-covered hole in the flask through which trichloroacetic acid could be injected with a syringe (Chiriboga, 1963). As shown in Table 1, the spermatozoal suspensions were pre-incubated with non-labelled sugars for 20 min at 38° C prior to the inclusion of the ^{14}C -labelled sugar because it was thought that the direct addition of labelled substrate to a spermatozoal suspension which had been 'starved' by washing in the sugar-free KRP might give less reliable results. The $^{14}\text{CO}_2$ was measured at the end of a further 60 min incubation period. Following deproteinization, an additional 60 min was allowed for the $\text{CO}_2 + \text{KOH}$ reaction to go to completion. The paper-strip was then transferred with forceps into a liquid-scintillation vial, dried (vial not capped) for 5 min at 60° C, and 5 ml of scintillation fluid added. The 'counting' procedure and scintillation fluid composition were as described by Chiriboga & Roy (1962). In order to relate the amount of evolved $^{14}\text{CO}_2$ to the amount of added labelled sugar, both $[^{14}\text{C}]$ -glucose and $[^{14}\text{C}]$ -fructose were 'counted' after applying 0.01 to 0.05 ml of the sugar solution to paper-strips which, however, were not impregnated with KOH; these strips were then dried and subsequently handled in scintillation vials as described above. A direct addition of labelled sugar to trichloroacetic acid-treated samples resulted in negligible radioactivity of the paper-strips, indicating that the $^{14}\text{CO}_2$ production was really due to the action of the live cells in the incubation mixtures. Water-elution of paper-strips having relatively high radioactivity (by reason of exposure to the atmosphere of incubating flasks) completely removed radioactivity from the strips as determined by subsequent 'counting' in the liquid-scintillation system.

TABLE 1
DETAILS OF THE EXPERIMENTAL PROCEDURE

Time period	Description	Treatment flasks				Motility flask
		I	II	III	IV	
Pre-incubation (0 to 20 min)	Spermatozoal suspension added to solution of non-labelled sugar in flasks	Glucose	Fructose	Fructose	Glucose	V
Incubation (20 to 80 min)	Labelled sugar added to flasks	$[^{14}\text{C}]$ -glucose	$[^{14}\text{C}]$ -fructose	$[^{14}\text{C}]$ -glucose	$[^{14}\text{C}]$ -fructose	Glucose None

Pre-incubation period: Flasks I to V each containing 2 mg non-labelled sugar/2 ml KRP (Krebs'-Ringer-phosphate, calcium-free) + 0.5 ml twice-washed spermatozoal suspension (5×10^8 cells) in KRP were incubated aerobically at 38°C .

Incubation period: At the end of the 20 min pre-incubation period, 0.1 ml of ^{14}C -labelled sugar solution was added to flasks I to IV (0.28 $\mu\text{C}/\text{flask}$, specific activity of labelled sugar 2.5 mC/mM) and all flasks incubated aerobically for further 60 min at 38°C . At the end of that period 0.4 ml of 50% trichloroacetic acid was injected into flasks I to IV. The contents of flask V were used for evaluating spermatozoal motility.

RESULTS

Data from the incubation experiments are portrayed in Text-fig. 1. When the [^{14}C]-sugar added to an incubation flask was identical with the non-labelled sugar (see Table 1, flasks I and II) the following quantities of $^{14}\text{CO}_2$, expressed as $\mu\text{C} \times 10^{-3}$, were produced by 5×10^8 spermatozoa in 60 min from [^{14}C]-glucose and [^{14}C]-fructose respectively: boar 2.9 and 3.3; bull 8.2 and 7.3; ram 10.5 and 9.5. Since $280 \times 10^{-3} \mu\text{C}$ of labelled sugar was added originally, the percentage of radioactivity recovered as $^{14}\text{CO}_2$ may be easily calculated, e.g. $(2.9 \times 100)/280$ for CO_2 produced from labelled glucose by boar spermatozoa. The standard deviations of samples, as graphically portrayed in Text-fig. 1, can be seen to be relatively small and of the same order as are usually encountered in spermatozoal metabolism studies; within species the differences between conversion rates of labelled glucose and fructose to CO_2 were not

TEXT-FIG. 1. The evolution of $^{14}\text{CO}_2$ by 5×10^8 boar, bull and ram spermatozoa incubated 1 hr at 38°C in 2.5 ml of calcium-free Krebs'-Ringer-phosphate. Incubation flasks contained 2 mg non-labelled sugar + $0.28 \mu\text{C}$ of labelled sugar (specific activity 2.5 mc/mm). Boar spermatozoa were from three samples of semen from each of three Yorkshire boars; bull spermatozoa were from two samples of semen from each of three Hereford bulls; and ram spermatozoa were from five samples of pooled semen, each pooled sample from three Suffolk rams. $^{14}\text{CO}_2$ evolved from: glucose + [^{14}C]-glucose (open columns); fructose + [^{14}C]-fructose (cross-hatched columns); fructose + [^{14}C]-glucose (stippled columns); glucose + [^{14}C]-fructose (solid black columns). Standard deviations (samples) are shown by the vertical bars.

significant. Although the number of individuals was too small to characterize the conversion rate for each species, the data suggest that boar spermatozoa convert sugar to CO_2 at a lesser rate than those of either bull or ram.

When labelled glucose was mixed with non-labelled fructose (see Table 1, flask III), or when labelled fructose was mixed with non-labelled glucose (see Table 1, flask IV) then the amounts of $^{14}\text{CO}_2$, expressed as $\mu\text{C} \times 10^{-3}$ were, respectively: boar 27.6 and 0.7; bull 27.0 and 2.5; ram 64.9 and 0.7. In the mixtures of fructose and glucose there were thus marked preferences by spermatozoa of all three species for glucose.

The results of spermatozoal motility evaluation are given in Table 2. It

would seem that both the initial motility of the washed spermatozoa, and the decline in motility which occurs during the aerobic incubation period of 1 hr at 38° C are similar for all three species.

TABLE 2

PROGRESSIVE MOTILITY OF SPERMATOZOA AT THE
END OF (a) THE PRE-INCUBATION PERIOD (20 MIN)
AND (b) THE INCUBATION PERIOD (1 HR LATER)
AT 38° C

Incubation	Spermatozoa (% motile)		
	Boar	Bull	Ram
(a)	55	55	58
(b)	45	45	50

Flasks contained 5×10^8 washed spermatozoa in 2.5 ml Krebs'-Ringer-phosphate (calcium-free) + 2 mg non-labelled glucose.

DISCUSSION

The use of liquid-scintillation as a counting technique where $^{14}\text{CO}_2$ is produced from a ^{14}C -labelled substrate offers distinct advantages in studies of spermatozoal metabolism: it is possible to follow metabolic changes affecting small amounts of substrate and, in addition, the liquid-scintillation system has a much higher counting efficiency than one involving the use of $\text{Ba}^{14}\text{CO}_3$ precipitates in a gas-flow GM counter. The technique has another advantage: it involves minimum time for the preparation of samples for incubation and subsequent 'counting' of radioactivity.

An example illustrating the value of the technique is provided by the results of our experiments on the aerobic CO_2 evolution by spermatozoa from glucose and fructose. Glucose and fructose are utilized at about the same rate by washed spermatozoa. Our data should not be interpreted as indicating that the *rate* of glucose utilization is affected by the presence of fructose. The correct interpretation is that when the two sugars are presented to a washed spermatozoal suspension together, glucose is utilized before fructose.

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