THE INTRACELLULAR ACCUMULATION OF METABOLITES BY RAM SPERMATOZOA DURING INCUBATION IN THE PRESENCE OF EXOGENOUS FRUCTOSE

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Summary

A method is described by which sufficient spermatozoa can be separated rapidly from their incubation medium to assay individual metabolites accumulating from [U-^{14}C]fructose. Acetate and lactate made up 60–80% of the substrate carbon accumulated within the cell. Acetate was the main intracellular metabolite and its concentration in the cell was 10–20 times its extracellular level. The intracellular accumulation of lactate varied with the period of incubation but its concentration in the cell was always below that in the medium. The remaining substrate carbon in the cell was made up of fructose and at least three other unidentified compounds. No accumulation of substrate carbon was detected in citric acid cycle intermediates.

The intracellular accumulation of metabolites was unaffected by the presence or absence of metabolic carbon dioxide during incubation. While lactate production was decreased by a restriction in the availability of substrate to the cell, acetate accumulation was unaffected. It is suggested that acetate acts as an intracellular substrate pool for entry into the citric acid cycle.

I. INTRODUCTION

Considerable information is available on the utilization of fructose by ram spermatozoa under various conditions (see Mann 1964; Wallace and Wales 1964; O'Shea and Wales 1965, 1966; Wales and O'Shea 1966*a*, 1966*b*; Murdoch and White 1968). Most of this has been gained by measuring such parameters as oxygen uptake, substrate disappearance, and end-product formation. Although some information is available about the accumulation of metabolic intermediates from various substrates by mammalian spermatozoa (Hines and Smith 1963; Graves, Lodge, and Salisbury 1966; Szepesi and Hopwood 1966), little is known about the intracellular accumulation of metabolites during incubation and their relationship to extracellular components.

Lack of information on the intracellular accumulation of metabolites is due in part to the difficulty of rapidly separating cells from media prior to analysis. Wales and O'Shea (1966*a*) published a method for collecting spermatozoa free of medium, by centrifuging through a lactose rinse. This method gives too few cells to allow positive identification of separate intracellular metabolites. The present paper describes modifications of the method using larger numbers of spermatozoa, and the major intracellular metabolites under different conditions of incubation have been measured.

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II. Materials and Methods

(a) *General Methods*

Ram semen was collected by electrical stimulation and washed twice with 12 vol. of a phosphate–saline diluent [20 mM mono- and disodium phosphate buffer (pH 7·1), 127 mM sodium chloride] and finally reconstituted at the original volume of the ejaculate. Incubation was carried out in 5-ml single-side-arm Warburg flasks at 37°C. The main compartment of the Warburg flasks contained 0·75 ml spermatozoal suspension and 0·45 ml incubation diluent (20 mM phosphate buffer, 1 mM potassium chloride, 2 mM magnesium chloride, 123 mM sodium chloride). The D-(−)-fructose substrate (0·3 ml), made isotonic with the addition of the appropriate amount of sodium chloride and containing isotopically labelled fructose, was added to the side-arm of the flasks and tipped into the main compartment after 10 min equilibration at 37°C.

At the completion of incubation, spermatozoa were isolated from the incubation diluent by a modification of the technique described by Wales and O'Shea (1966*a*). The modification is described in full in the following section. After centrifugation, an aliquot of the spermatozoal plug was taken for radioactive assay. The remainder of the spermatozoal plug was deproteinized with 2 vol. of 4% perchloric acid. After centrifuging, the precipitate was removed and the supernatant was neutralized to pH 8·5 with potassium hydroxide. The precipitated potassium perchlorate was then removed by centrifugation and the resultant extracts reduced in volume under a stream of air before chromatography for the separation of metabolic products as described in Section II(*c*).

Assay of radioactivity was carried out by liquid scintillation techniques using a Nuclear Chicago liquid scintillation spectrometer. The scintillation mixture consisted of 0·01% w/v 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene (dimethyl POPOP) and 0·4% w/v 2,5-diphenyloxazole (PPO) in a 2:1 mixture of toluene–Triton X100 detergent (Patterson and Green 1965). All counts were corrected for background and for efficiency of counting.

(b) *Separation of Spermatozoa from the Incubation Medium*

The centrifuge tubes used to recover spermatozoa had an internal diameter of 1·1 cm and were 10 cm long. Approximately 2 cm from the bottom of the tube, a taper began and continued until the tube had an internal diameter of 0·3 cm at the base (Fig. 1). For reclamation of spermatozoa after incubation, tubes were prepared immediately before use. A 5-ml aliquot of 11% lactose solution was pipetted into the centrifuge tubes. Then 0·5 ml of 15% lactose solution containing 5% formalin was injected into the bottom of the tube under the 11% lactose layer with the aid of a long, round-pointed hypodermic needle attached to a tuberculin syringe. With sufficient care it was possible to obtain a distinct interface between the two lactose layers and thus any mixing of the two layers during centrifuging could be detected. Only tubes with such a clearly defined interface were used.

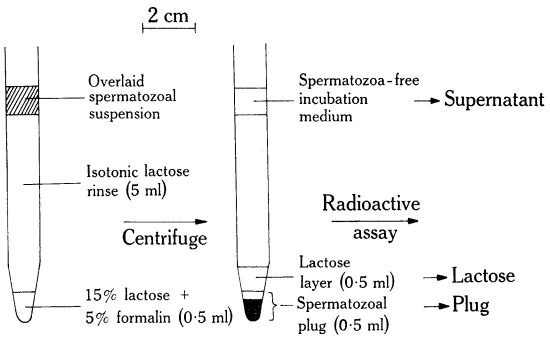

Fig. 1.—Procedure for collecting spermatozoa free of incubation medium.

After incubation, a known volume of flask contents was pipetted carefully onto the lactose layer and the cells centrifuged for 10 min at 2500 *g*. The incubation medium remained at the top of the isotonic lactose as a clearly defined supernatant while the cells formed a plug in the bottom of the tube. After removal of the medium and most of the isotonic lactose, a 0·5-ml sample of the lactose layer immediately above the lactose–formalin layer containing the spermatozoa was taken for radioassay (see Fig. 1). The spermatozoal plug, including the overlying lactose–formalin,

was made to 1·5 ml with water and a 0·5-ml aliquot taken for radioactive assay. Net counts in the spermatozoa were then calculated by subtracting the counts in the overlying lactose from the total gross counts in the spermatozoal plug.

To test the assumption that the counts in the lactose solution overlying the spermatozoal plug gave an estimate of the contamination of the spermatozoal plug with extracellular isotope, spermatozoa (4–21 $\times 10^8$ per flask) were incubated for 30 min in diluents containing sodium [2,5-^{14}C]citrate (7·5 μmoles/ml, specific activity 80 mCi/mole) and centrifuged as above. The results were compared with aliquots incubated in fructose (7·5 μmoles/ml and 67 mCi/mole) and treated in a similar fashion. As citrate does not readily enter spermatozoa (Flipse 1966), its presence in the plug and overlying lactose indicates contamination with medium. In addition, if the isotope found in the lactose is due to contamination with medium rather than leaching of intracellular isotope, the estimate of contamination of this region should be similar whether labelled citrate or fructose were used. The results for four ejaculates showed that the amount of citrate in the uncorrected plug did not differ significantly from that in the overlying lactose ($t_3 = 1{\cdot}41, P > 0{\cdot}05$), and there was a highly significant correlation between counts in the plugs and counts in the corresponding lactose layers ($r_3 = 0{\cdot}97, P < 0{\cdot}01$). In addition, the estimate of contamination of the overlying lactose with medium when fructose was used (3·4%) was not significantly different from the estimate using citrate (4·0%) ($t_3 = 1{\cdot}20, P > 0{\cdot}05$).

Another preliminary experiment involving four replicates, showed that the addition of unlabelled fructose to isotonic lactose rinse at the concentration in the medium resulted in a 25% increase in the level of isotope in the spermatozoal plug. As the difference was significant ($t_3 = 3{\cdot}86, P < 0{\cdot}05$), fructose was included in the lactose rinse in all the subsequent experiments.

To compare the present modified method with the published method (Wales and O'Shea 1966*a*), 1-ml aliquots of ejaculates containing 7–16 $\times 10^8$ spermatozoa were pipetted from the same Warburg flasks on to the surface of isotonic lactose either in tubes prepared as described by Wales and O'Shea (1966*a*) or in tubes prepared as described above. After centrifuging, the spermatozoal plug and an equal volume of overlying lactose were assayed for radioactivity.

TABLE 1

EFFECT OF METHOD OF SEPARATION OF SPERMATOZOA FROM THE INCUBATION MEDIUM ON THE LEVEL OF ISOTOPE IN THE SPERMATOZOA-CONTAINING FRACTION AND IN THE OVERLYING LACTOSE LAYER

Values are means ± standard error for four replicates (7–16 $\times 10^8$ cells per flask), and are expressed as counts/min/sample

Experimental Details	Measurement	Method of Separation	
		Published Method*	Present Modification
Spermatozoa were incubated 30 min in presence of [U-^{14}C]-fructose (6·8 μmoles/ml, specific activity 73 mCi/mole) before being separated from their incubation medium	Total counts in spermatozoal fraction	69,558 ± 6,033	56,374 ± 4,438
	Total counts in overlying lactose	20,044 ± 3,368	8,877 ± 912
	Adjusted counts in spermatozoal fraction	49,514 ± 5,933	47,496 ± 4,370

* Wales and O'Shea (1966*a*).

The results for four replicates are shown in Table 1. Using the method of Wales and O'Shea (1966*a*) separation was incomplete after centrifuging the large numbers of spermatozoa and, as a result, the total counts in the spermatozoa-bearing fraction were significantly higher than those obtained when the modified method was used ($t_3 = 3{\cdot}87, P < 0{\cdot}05$). This elevated level of isotope in the spermatozoal plug was due to higher and more variable counts in the overlying lactose. Adjustment of the counts in the spermatozoa for contamination with medium, by subtracting the counts in the overlying lactose layer, gave the same net counts in the spermatozoa with each method.

(c) Isolation and Identification of Metabolic Products

The initial isolation of metabolic products in the spermatozoal plug was made by fractionating the organic acids produced from the added fructose by liquid–liquid partition chromatography using butanol–hexane solvents (O'Shea and Wales 1968). The radioactive compound identified tentatively as acetate was recovered and steam distilled at pH 4·0. Recovery of the isotope in the distillate was 100%. The presence of acetate was also confirmed by paper chromatography of the spermatozoal extracts on formic-acid-washed Whatman No. 1 paper using ethanol–ammonia–water (8:1:1 v/v) as solvent in a descending system. The radioactive peak corresponding to lactate from the partition chromatography column was distinguished from succinate by paper chromatography in ethanol–ammonia–water (8:1:1). In this system, the R_F values of lactate and succinate were 0·6 and 0·2 respectively. A peak corresponding to lactate was also obtained on paper chromatograms developed with n-butanol–acetic acid–water (4:1:5 v/v).

The extracellular accumulation of organic acids was measured by liquid–liquid partition chromatography of an aliquot of incubation medium. In some cases, the accumulation of lactic acid was confirmed by enzymic assay (Barker and Britton 1957). Fructose was determined by the resorcinol–HCl method as used by White (1959).

From the total net counts in the spermatozoal plug, counts per millilitre of packed spermatozoa were calculated using spermatocrit values obtained by centrifuging cold-shocked spermatozoa at 4200 *g* until a constant reading was obtained. The number of gram-atoms of substrate carbon accumulated in the cell was then calculated from the specific activity of the ^{14}C isotope used.

In order to estimate the intracellular concentration of a particular metabolite, the total amount of the metabolite in the plug, estimated from the fraction of the total counts in the plug identified as that metabolite, was adjusted for extracellular contamination by subtracting the amount of the metabolite in the overlying lactose [i.e. net amount of intracellular metabolite = gross amount of the metabolite in the plug − (amount of substrate in the overlying lactose × fraction of incubation medium identified as the particular metabolite)]. The concentration of the metabolite in the spermatozoa was then calculated from this adjusted result using spermatocrit values as described above. For ease of comparison, the concentrations of both intracellular and extracellular metabolites have been expressed as μg-atoms of substrate carbon per millilitre.

III. Results

The partitioning of the total counts in the spermatozoal plug between the perchloric acid (PCA) soluble and insoluble fractions is shown in Table 2. Samples of four washed ejaculates were incubated for 30 min in the presence of 7·5 μmoles of fructose/ml (specific activity 90 mCi/mole) before the spermatozoa were separated from their incubation medium. An aliquot of the plug was assayed to assess the total incorporation of isotope into the plug. The remainder was precipitated with 4% PCA (2 vol.). The precipitate was washed twice with 4% PCA (2 vol.) before being radioassayed. The results (Table 2) showed that almost all of the counts in the plug were accounted for in the PCA supernatant. Only 1–2% of the counts was found in the precipitate.

The accumulation of intracellular metabolites by washed ram spermatozoa (10–16 $\times 10^8$ per flask) incubated in the presence of fructose (7·5 μmoles/ml, 66 mCi/mole) and either in the presence or in the absence of metabolic CO_2 was examined. Aliquots of three washed ejaculates were incubated for 30 min in Warburg flasks with and without the addition of 20% KOH to the centre well, and the results with summaries of the analysis of variance are shown in Table 3. The presence of CO_2 had no significant effect either on the extracellular or intracellular accumulation of lactate or acetate or on the total substrate carbon accumulated in the cell ($t_2 = 0{\cdot}41$, $P > 0{\cdot}05$). Approximately 20% of added fructose was utilized. Most of its breakdown was accounted for by the accumulation of lactate in the medium and

TABLE 2

PROPORTION OF THE TOTAL COUNTS IN THE SPERMATOZOAL PLUG FOUND IN THE ACID-SOLUBLE AND ACID-INSOLUBLE FRACTION FOLLOWING PRECIPITATION WITH TWO VOLUMES OF 4% PERCHLORIC ACID

Values are the means for four replicates incubated for 30 min in the presence of 7·5 μmoles fructose/ml (specific activity 90 mCi/mole)

Fraction Assayed	Total Counts/Min (Mean ± S.E.)	Percentage of Total Counts Recovered
Untreated plug	71,470 ± 18,500	100
PCA supernatant	69,400 ± 17,500	97
First wash of precipitate	2,030 ± 450	2·8
Second wash of precipitate	280 ± 108	0·4
Washed PCA precipitate	1,057 ± 206	1·5

TABLE 3

ACCUMULATION OF METABOLITES IN RAM SPERMATOZOA AFTER A 30-MIN INCUBATION IN THE PRESENCE OR ABSENCE OF METABOLIC CARBON DIOXIDE

Initial fructose concentration 7·5 μmoles/ml. Values are expressed as μg-atoms substrate carbon/ml and are means (± S.E.) for three replicates

Metabolite Accumulated	Site of Accumulation	Concentration of Substrate Carbon	
		$-CO_2$	$+CO_2$
Acetate	Intracellular	13·2	11·4
	Extracellular	0·5	0·5
Lactate	Intracellular	4·0	2·6
	Extracellular	7·1	6·7
Total intracellular substrate carbon		18·9(±5·2)	14·9(±5·2)
Final extracellular fructose concentration		34·5(±1·5)	34·5(±0·7)

Summaries of Analyses of Variance of Acetate and Lactate Data (log transformation)

Source of Variation	Degrees of Freedom	Variance Ratios	
		Acetate	Lactate
Intracellular *v.* extracellular concn. (*A*)	1	120·13**	9·56*
Effect of CO_2 (*B*)	1	0·14	0·51
Interaction $A \times B$	1	0·11	0·40
Replicate difference	2	1·54	0·94
Replicate interactions (error)	6	*0·045*	*0·041*

* $P < 0·05$. ** $P < 0·01$.

little extracellular acetate was found. However, there was a substantial accumulation of acetate in the cell. On the other hand, the concentration of lactate in the cell was significantly lower than that in the medium.

To test the effects of restricted substrate on the accumulation of substrate carbon from fructose, washed ram spermatozoa (8–18 $\times 10^8$ per flask) were incubated for 10 and 30 min in the presence of 1 μmole of fructose per millilitre. The specific activity of the added fructose was increased to 667 mCi/mole to increase the precision of estimating the intracellular constituents following the short period of incubation. The results for three ejaculates and the statistical analysis are shown in Table 4.

TABLE 4

EFFECT OF INCUBATION FOR 10 OR 30 MIN IN THE PRESENCE OF A LOW CONCENTRATION OF FRUCTOSE (1 μmole/ml) ON THE ACCUMULATION OF METABOLITES BY RAM SPERMATOZOA

Values are the means (±S.E.) for three replicates and are expressed as μg-atoms of substrate carbon/ml

Metabolite Accumulated	Site of Accumulation	Time of Incubation	
		10 min	30 min
Acetate	Intracellular	1·6	4·7
	Extracellular	0·1	0·6
Lactate	Intracellular	0·4	0·0
	Extracellular	2·3	2·9
Total intracellular substrate carbon		3·2(±0·6)	6·6(±2·1)
Final extracellular fructose concentration		2·7(±0·4)	0·7(±0·3)

Summaries of Analyses of Variance of Acetate and Lactate Data (log transformation)

Source of Variation	Degrees of Freedom	Variance Ratios	
		Acetate	Lactate
Intracellular *v.* extracellular accumulation (*A*)	1	105·14**	23·19**
Effect of incubation time (*B*)	1	30·34**	1·91
Interaction $A \times B$	1	0·58	9·63*
Replicate differences	2	7·24*	0·02
Replicate interactions (error)	6	*0·028*	*0·199*

* $P < 0·05$. ** $P < 0·01$.

During the 10-min incubation, half the fructose was utilized with the production of lactate. Incubation for 30 min caused the breakdown of almost all the fructose, and lactate production accounted for only half this breakdown; presumably the remainder was oxidized. Little extracellular acetate was produced, but the intracellular accumulation of this metabolite trebled between the 10- and 30-min period of

incubation and accounted for 70% of the intracellular substrate carbon after incubation for 30 min. Practically no lactate accumulated in the cell. Although total intracellular substrate carbon doubled between 10 and 30 min, the difference was not significant ($t_2 = 2{\cdot}08, P > 0{\cdot}05$) mainly because after 30 min of incubation, when most of the extracellular fructose was utilized, the total amount of intracellular substrate carbon that accumulated varied considerably.

A comparison of the accumulation of metabolites by spermatozoa incubated in the presence of 7·5 or 1·0 mM fructose is shown in Table 5. Three samples of pooled ram semen were washed and incubated (10–16 $\times 10^8$ per flask) in the presence of 1 mM fructose as above or in the presence of 7·5 mM fructose (specific activity 312 mCi/mole) for 10 min before being separated from their incubation medium. Lactate production from fructose in the presence of the higher initial substrate concentration was three times that at the lower concentration. There was also some increase in the oxidation of fructose in the presence of the higher initial concentration, but the magnitude of the response varied between replicates and was not significant in a paired t-test ($t_2 = 2{\cdot}13, P > 0{\cdot}05$). The difference in fructose concentration did not affect the accumulation of intracellular metabolites and acetate constituted 80% of the intracellular substrate carbon. Little or no lactate was present in the cell.

In the final experiment the effects of longer times of incubation on the accumulation of metabolites by ram spermatozoa were studied. Aliquots of four washed ejaculates (10–19 $\times 10^8$ cells per flask) were incubated for 30 or 90 min in the presence of 7·5 mM fructose (specific activity 89 mCi/mole) and the results are shown in Table 6. Oxidative metabolism of the spermatozoa as measured by oxygen uptake and fructose oxidation was constant during the 90-min period of incubation. On the other hand, the rate of lactate production decreased with time and its production after incubation for 90 min was only 30% higher than after 30 min. The accumulation of extracellular acetate showed a linear increase with time, but within the cell its concentration after incubation for 90 min was similar to that at the end of 30 min. Intracellular lactate increased substantially over the longer incubation period, and after a 90-min incubation it constituted 25% of intracellular substrate accumulated from fructose.

In all the foregoing experiments, a portion of the total intracellular substrate carbon could not be accounted for as lactate or acetate. Overall, this fraction equalled 20% of the total intracellular substrate carbon and could be identified as a diffuse peak (R_F 0·02–0·25) when samples of the PCA extracts of the spermatozoal plug were chromatographed on Whatman No. 1 paper for 16 hr, using butanol–acetic acid–water (4:1:5) as solvent. In an attempt to increase the separation in this area of the chromatogram in this solvent, deproteinized samples with and without the addition of authentic [U-^{14}C]fructose were allowed to overrun the edge of the paper for 5 days before the radioactive peaks were identified. Using this method the original peak could be separated into at least two peaks, one being a peak corresponding to fructose and the other a slightly larger, slower moving peak.

Further separation of the unknown was achieved by eluting the unidentified peak from the paper after chromatography in butanol–acetic acid–water, then reducing the volume of the eluate under a stream of air and rechromatographing in propanol–ammonia–water (6:3:1) and ethanol–ammonia–water (8:1:1). In the first

solvent a peak corresponding to fructose was present plus a second peak with an R_F of 0·18. In the second solvent system the unknown peak could be separated into at least four peaks, one of which corresponded to fructose or sorbitol.

TABLE 5

EFFECTS OF CONCENTRATION OF FRUCTOSE ON ACCUMULATION OF METABOLITES BY RAM SPERMATOZOA

Values are the means (±S.E.) for three replicates incubated in the presence of [U-^{14}C]fructose for 10 min and, unless otherwise stated, are expressed as μg-atoms of substrate carbon/ml

Metabolic Parameter Measured	Initial Fructose Concn. (mM)	
	7·5	1·0
Intracellular acetate	9·9	7·1
Extracellular acetate	0·4	0·3
Intracellular lactate	0·4	0·0
Extracellular lactate		
By radioassay	9·4	2·9
By enzyme assay	10·8	3·9
Total intracellular substrate carbon	12·6 (±3·2)	8·8 (±1·7)
Fructose carbon remaining	34·0 (±4·0)	2·9 (±0·8)
Fructose oxidized (μmoles/10^{10} cells)	0·18 (±0·07)	0·10 (±0·04)

Summary of Analysis of Variance for Acetate Data†

Source of Variation	D.F.	Variance Ratio
Intracellular *v.* extracellular concn. (*A*)	1	6·48*
Effect of fructose concn. (*B*)	1	0·66
Interaction *A* × *B*	1	0·05
Replicate differences	2	1·11
Replicate interactions (error)	6	*0·142*

* $P < 0·05$. † Log transformation.

TABLE 6

EFFECT OF TIME OF INCUBATION ON THE ACCUMULATION OF METABOLITES IN RAM SPERMATOZOA INCUBATED IN THE PRESENCE OF 7·5 mM FRUCTOSE

Values are the means (±S.E.) for four ejaculates and unless otherwise stated are expressed as μg-atoms of substrate carbon/ml

Metabolic Parameter Measured	Time of Incubation	
	30 min	90 min
Intracellular acetate	19·8	23·7
Extracellular acetate	0·9	2·4
Intracellular lactate	3·2	11·7
Extracellular lactate		
By radioassay	16·5	22·0
By enzyme assay	15·2	20·0
Total intracellular substrate carbon	29·5(±0·9)	45·8(±1·6)
Fructose carbon remaining	27·3(±1·3)	19·5(±1·0)
O_2 uptake (μmoles/10^{10} cells)	28·0(±6·0)	63·0(±9·0)
Fructose oxidation (μmoles/10^{10} cells)	2·9(±0·9)	8·3(±1·3)

Summaries of Analyses of Variance for Acetate and Lactate Data†

Source of Variation	Degrees of Freedom	Variance Ratios	
		Acetate	Lactate
Intracellular *v.* extracellular concn. (*A*)	1	204·21**	76·71**
Effect of incubation time (*B*)	1	12·14**	34·73**
Interaction *A* × *B*	1	6·64*	14·49**
Replicate differences	3	1·80	4·88*
Replicate interactions (error)	9	*0·029*	*0·015*

* $P < 0·05$. ** $P < 0·01$. †Log transformation.

IV. DISCUSSION

In any method for the study of intracellular constituents, it is necessary to separate effectively the spermatozoa from their incubation medium and to ensure that sufficient isotopic label is present in the spermatozoa to measure accurately the

metabolites present. The latter can be accomplished by increasing both the number of spermatozoa and the specific activity of the substrate. The method previously reported from this laboratory for separating spermatozoa from their incubation medium (Wales and O'Shea 1966*a*) suffers from the disadvantage that as the number of spermatozoa that are centrifuged is increased, the efficiency of separation is decreased. Thus corrections for contamination with incubation medium, estimated from the lactose rinse above the spermatozoal plug, become excessive. The changes described in this paper overcome this and allow intracellular constituents to be measured accurately.

In all the experiments, intracellular lactate was substantially lower than extracellular lactate and, during short periods of incubation, little or no lactate could be demonstrated in the cell. Although the size of the intracellular space occupied by a metabolite needs to be considered when comparing intra- and extracellular concentration, the virtual absence of lactate from the cell during short-term incubation and the varying ratios of intracellular to extracellular lactate with time indicates that its intracellular concentration can be controlled and is not, as previously thought (Wales and O'Shea 1966*a*), determined by the total lactate present.

Although there was only a small accumulation of acetate from fructose in the medium, a large intracellular pool of this metabolite was found. This may not reflect a true energy-dependent concentration gradient of the metabolite across the cell membrane, as the intracellular acetate may be bound to some intracellular molecule, such as coenzyme A, and released by the perchloric acid precipitation. As such, it would not be freely diffusible but could act as an intracellular substrate pool for entry into the citric acid cycle.

In addition to lactate and acetate, a number of other compounds accumulate within the cell in small amounts. As reported by Mann (1964), only small amounts of fructose were present in the cell compared with the extracellular fluid. As well as fructose, there were at least another three compounds present. Because of their low concentration, positive identification was not attempted. However, liquid–liquid partition chromatography showed that there was no detectable accumulation of substrate carbon as citric acid cycle intermediates.

In view of these findings, there may be need for some reassessment of earlier studies of substrate transport. Flipse (1962) incubated spermatozoa in [U-^{14}C]fructose for 30–60 min and used incorporation of isotope as an indication of retention of fructose and loss of isotope as an index of fructose efflux. The rapid incorporation of this substrate into other intracellular carbon pools complicates the interpretation of such results.

Although it has been demonstrated that ram spermatozoa are able to fix carbon dioxide (O'Shea and Wales 1967), any effect of this metabolic pathway on the accumulation of intracellular metabolites from fructose is small and could not be detected using the present techniques. As reported in earlier studies (Wales and O'Shea 1966*b*; Wales and Restall 1966) the presence of metabolic carbon dioxide had no significant effect on the extracellular accumulation of metabolites from fructose.

The accumulation of lactate both within the cell and in the incubation medium is affected by changes in the initial fructose concentration and by the utilization of most of the available fructose during incubation. In the final experiment, rate of

lactate production also decreased as the time of incubation increased, but this effect was probably due to a fall in pH in the medium following the production of large amounts of lactic acid rather than to a restriction in substrate availability. Under all these adverse conditions, neither acetate accumulation nor oxidative metabolism were significantly affected. These latter findings strengthen the suggestion that the intracellular pool of acetate is important in maintaining citric acid cycle activity during incubation.

V. Acknowledgments

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