^{13}C-NMR reveals glycerol as an unexpected major metabolite of the protozoan parasite *Trichomonas vaginalis*

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Received 3 September 1985; revised version received 16 September 1985

^{13}C-NMR has been used to study the kinetics of the formation of metabolites from [1-^{13}C]glucose in intact cells of *Trichomonas vaginalis* during anaerobic incubation. As well as the expected metabolites lactate and acetate, this technique revealed glycerol as an additional major product, present in amounts equimolar with acetate. The formation of glycerol is readily explained in terms of the need to maintain redox balance. This protozoan now joins the small group of organisms which are known to produce glycerol as a result of normal metabolic activities.

Trichomonas vaginalis *Glucose metabolism* *^{13}C-NMR* *Metabolite formation* *Glycerol* *[^{13}C]Glucose*

1. INTRODUCTION

Trichomonas vaginalis is an aerolerant anaerobic flagellate protozoan which causes the major human sexually transmitted disease of vaginal trichomoniasis. It, and the related species *Tritrichomonas foetus*, a sexually transmitted cattle pathogen, have interested biochemists because of the novel method of carbohydrate dismutation they employ. In most aerobic eukaryotes the oxidation of pyruvate, generated by glycolysis, is carried out in the mitochondrion; the ultimate products being CO_2 and water, and at the same time energy is conserved as ATP through the process of oxidative phosphorylation. In trichomonads there are no mitochondria and haem-iron proteins present [1]. The role of the mitochondrion is taken over by a different organelle, the hydrogenosome, which catalyses the dismutation of pyruvate to acetate, CO_2 and molecular hydrogen, with conservation of energy into ATP by substrate level phosphorylation. Although lacking haem-iron, the hydrogenosome has a number of iron-sulphur proteins associated with electron transfer terminating in a hydrogenase, including a [2Fe-2S] ferredoxin [2]. The biochemistry of the hydrogenosome has been reviewed by Müller [3]. The formation of pyruvate (and lactate) by *T. vaginalis* has been ascribed to a conventional glycolytic sequence and the presence of an active lactate dehydrogenase [4,5], and lactate and acetate have been claimed to be the sole organic end products in this organism [6]. This would be surprising since though the production of lactate is in redox balance, formation of acetate produces a net surplus of reducing power equivalent to 2 mol NADH per mol glucose [7] (assuming that the dismutation of pyruvate to acetate in the hydrogenosome is in net redox balance). Accordingly it seemed likely that a metabolite whose production involved net consumption of reducing power was being overlooked. We report here that the missing product has been identified during a study on the kinetics of metabolite accumulation from [1-^{13}C]glucose by NMR.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

2. MATERIALS AND METHODS

2.1. *Organisms*

Two strains of *T. vaginalis* were used in this study, Bushby [8] and the American type culture collection (ATCC) strain 30001. They were grown in a modified Diamond's medium [9] containing 10% heat-inactivated horse serum at 37°C for about 48 h. Cells were harvested in late log phase (1×10^6 organisms $\cdot$ ml^{-1}) by centrifugation at 1200 $\times$ *g* for 15 min at room temperature. The pellet was gently resuspended in Hanks' balanced salt solution (pH 7.4) without glucose and washed twice by centrifugation.

The final pellet of cells was resuspended in 150 mM K-phosphate buffer, pH 7.4.

2.2. *Preparation of samples for NMR*

The suspension of organisms was diluted with 150 mM K-phosphate buffer (pH 7.4) to a cell density of 5×10^7 organisms $\cdot$ ml^{-1} and 2.9 ml was transferred to a 10 mm NMR tube. The suspension was extensively bubbled with O_2-free nitrogen and warmed to 35°C. [1-^{13}C]Glucose (MSD Isotopes, Montreal, Canada; 99.5% enrichment) was dissolved in buffer at 900 mM and 100 μl added to the cells in the NMR tube to give a final glucose concentration of 30 mM. The sample was briefly re-gassed with O_2-free nitrogen; the tube was then sealed and transferred to the NMR spectrometer. Timing was commenced from the moment of addition of glucose. For quantitation of ^{13}C label in metabolic products a cell-free supernatant was prepared by incubating the cells under identical conditions for the stated length of time followed by centrifugation at 1200 $\times$ *g* for 15 min to remove the cells. The supernatant was then examined by NMR or other analytical procedures.

2.3. *Acquisition of NMR spectra*

^{13}C-NMR proton decoupled spectra were measured at 90.56 MHz on a Bruker AM360 spectrometer using a 10 mm broad-band probe. To reduce effects from dielectric heating and to maintain a sample temperature of about 35–37°C power-gated proton decoupling was used, switching from 2 W for decoupling during the acquisition time (0.4 s) to 0.5 W for nuclear Overhauser enhancement (NOE) during a 3 s delay. Free induction decays (FIDs) were acquired into 16K data points covering a spectral width of 20000 Hz using 45° pulses and a repetition rate of 3.4 s for qualitative data. FIDs were stored sequentially every 10 min over 2–3 h. Each FID was multiplied by a line broadening of 5 Hz before transformation. Chemical shifts were measured with respect to C-1 of β-glucose at 96.7 ppm relative to tetramethylsilane at 0 ppm [10]. Deuterium oxide (3.3%, v/v) was used as an internal lock in all samples. Identity of signals was established from coupling patterns in proton coupled ^{13}C spectra and from spiking extract solutions with known compounds.

For quantitative analysis of extracts, ^{13}C-NMR spectra with inverse gated proton decoupling were measured using a 60 s delay. This minimised NOE and ensured full relaxation of the signal from acetate C-2. The distribution of the label was estimated from the integrated signal areas, making the assumption that all the label present was detected.

2.4. *Assay of products*

The production of lactate in the supernatant fluid after incubation was quantitated by GLC. The apparatus used was a Pye 106 gas chromatograph equipped with a hydrogen detector. Separation was effected on a glass column of 1.5 m length packed with Chromosorb 101, 80–100 mesh. The column was operated at an oven temperature of 200°C and detector temperature of 240°C. A carrier gas flow of 10 ml $\cdot$ min^{-1} nitrogen was employed. Glycerol was determined by a glycerol dehydrogenase method [11] using the enzyme from *Cellulomonas* sp. (Sigma). Glucose was measured using the *o*-toluidine reagent according to Feteris [12]. Acetate was assayed using the test kit supplied by Boehringer Mannheim according to the manufacturer's instructions.

3. RESULTS AND DISCUSSION

The use of ^{13}C-NMR in the non-invasive monitoring of metabolism in vivo in microorganisms is now well established [13–15]. The technique can be employed to obtain information about the nature and kinetics of formation of metabolites in intact biological systems. ^{13}C-NMR for metabolite identification has the advantage that, unlike conventional biochemical analysis, it

makes no assumptions about the likely nature of the products present. All compounds which receive the ^{13}C label from the initial substrate supplied will be visible in subsequent NMR spectra, provided they are present at levels which exceed the detection limit set by the experimental conditions and the instrument. To monitor the time course of metabolite formation ^{13}C spectra had to be acquired during short consecutive time intervals. To achieve a satisfactory signal-to-noise ratio in that time the rate of pulsing was increased, accepting some saturation of signals where relaxation time (T_1) was long. This was significant for acetate C-2 which has a much longer T_1 than α, β C-1 of glucose, C-3 of lactate, C-1,3 of glycerol and C-3 of alanine ([15]; J. Williams, unpublished), and its signal was therefore low in intensity in spectra acquired under these conditions. For quantitative assessment by NMR, saturation and NOE of signals must be avoided [15]. To achieve this much longer times are required and thus only supernatants taken at fixed time points could be examined.

In these experiments [1-^{13}C]glucose was consumed after about 180 min incubation with no anomeric specificity detectable in the rate of disappearance of the 2 anomer signals. Cells retained their integrity throughout the experiment as judged by microscopy, though motility declined as glucose was used up. Three main products of

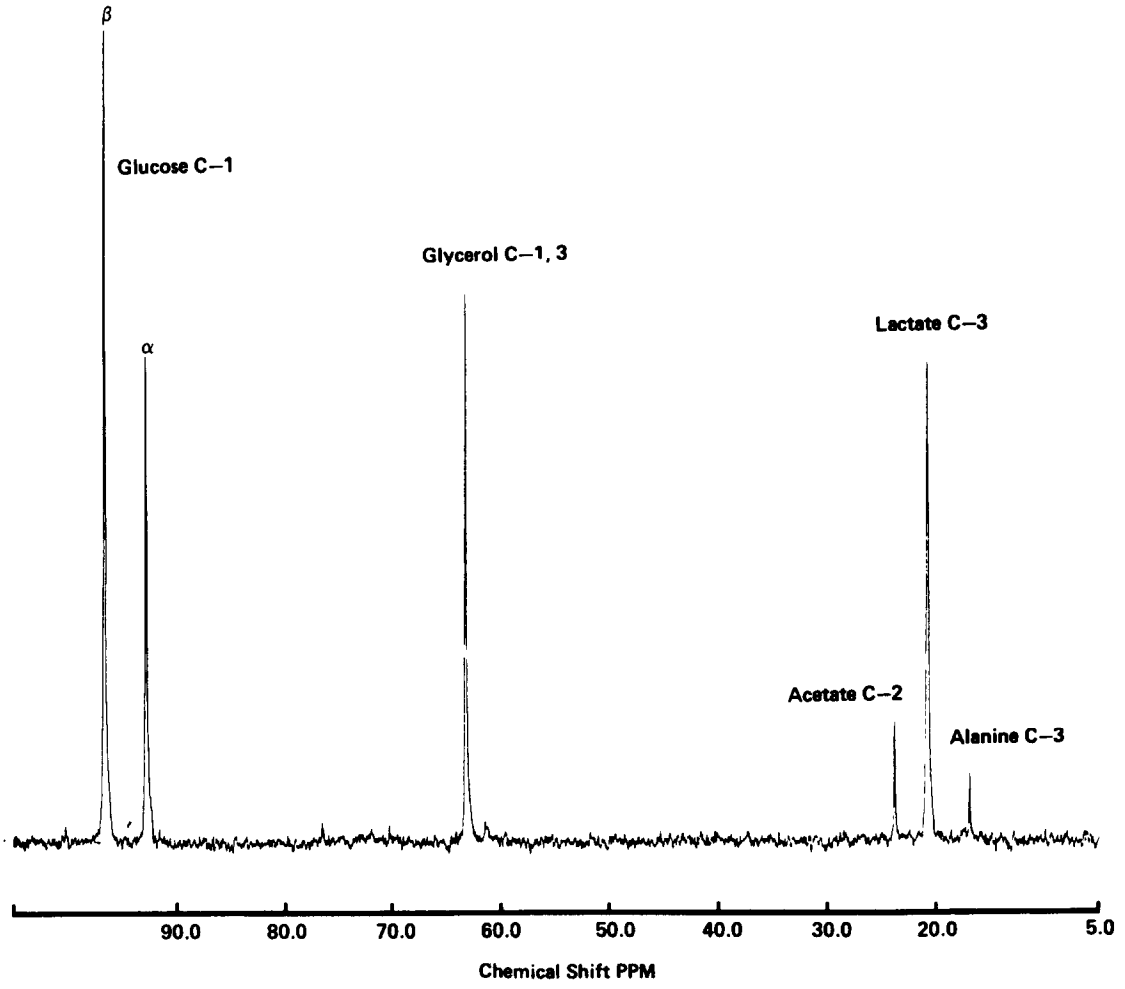

Fig.1. ^{13}C-NMR spectrum of whole cells of *T. vaginalis* Bushby strain after 120 min of incubation with 30 mM [1-^{13}C]glucose. For conditions of incubation and acquisition see text.

[1-^{13}C]glucose metabolism accumulated extracellularly during this period. These were [3-^{13}C]lactate (20.1 ppm), [2-^{13}C]acetate (24.0 ppm) and [1,3-^{13}C]glycerol (63.4 ppm). In addition minor and variable quantities of [2-^{13}C]alanine (17.0 ppm) were sometimes detected. The assignment of the 63.4 ppm signal to glycerol was based upon 3 factors: (i) the proton coupled spectrum revealed a triplet (J_{C-H} ~ 140 Hz) indicating a methylene carbon, (ii) the chemical shift was characteristic of an oxygen substituted carbon atom and identical with the published value for glycerol C-1,3 [15], (iii) direct addition of glycerol to the sample increased the signal intensity at 63.4 ppm without changing signal shape. An example spectrum illustrating all of these metabolites is shown in fig.1. This spectrum was not acquired under quantitative conditions. The accumulation of the 3 major metabolites with time is illustrated in fig.2. These spectra were also recorded under non-quantitative conditions. This figure demonstrates the lack of anomeric specificity in glucose utilization and the usefulness of the ^{13}C-NMR technique for acquiring a real-time view of changes in metabolite levels. The data for samples of supernatant taken at 3 different time intervals and submitted to NMR spectroscopy under quantitative conditions are shown in table 1. Under these conditions it can be seen that approximately equimolar amounts of glycerol and acetate are formed and that these products slightly exceed the amount of lactate formed. A similar pattern was observed when the products from a similar incubation were assayed by conventional techniques. The results for this are shown in table 2. Essentially no difference was observed when cells of the ATCC strain 30001 were employed instead of cells of the Bushby strain in NMR experiments.

Unlike the earlier postulation of lactate and acetate as the sole organic products of glucose metabolism [6] the findings here are in redox balance. The formation of lactate is self-balancing since this involves the production of 2 mol NADH and the utilization of 2 mol NADH per mol glucose catabolized. The formation of acetate via the hydrogenosome is a net producer of 2 mol NADH per mol glucose from the glyceraldehyde-3-phosphate dehydrogenase reaction. Thus the

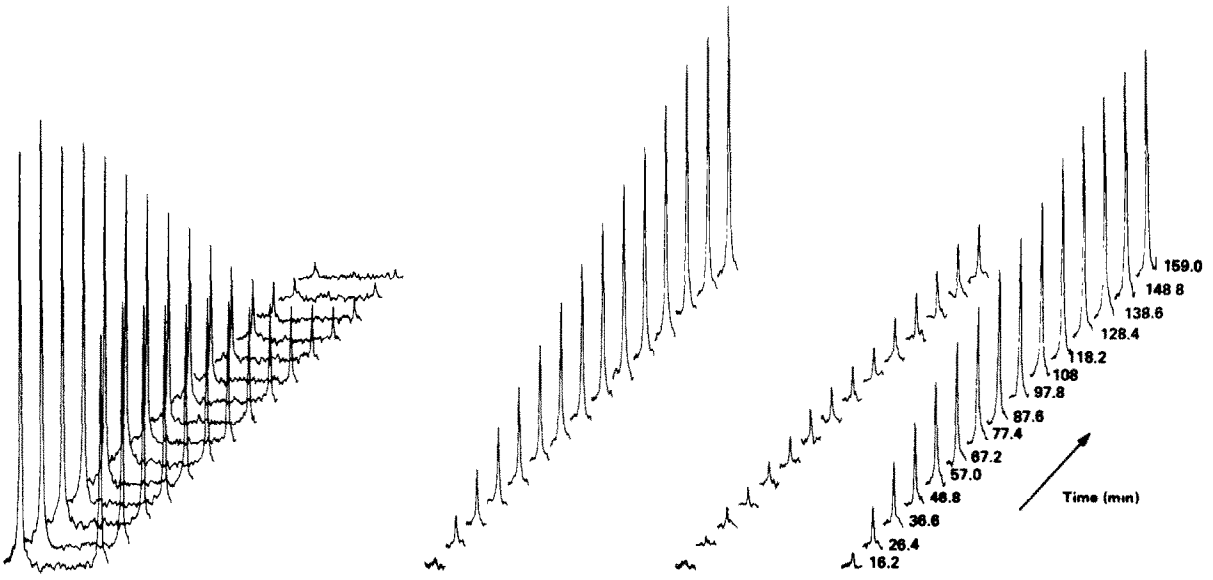

Fig.2. Time course of accumulation of metabolites and disappearance of glucose during the incubation of whole cells of *T. vaginalis* Bushby strain with 30 mM [1-^{13}C]glucose. For conditions of incubation and acquisition and individual chemical shifts see text.

Table 1

Estimation of [^{13}C]glucose and labelled metabolites by quantitative ^{13}C-NMR

Incubation time (min)	Concentration of labelled metabolite (mM)			
	Glucose	Glycerol	Acetate	Lactate
50	21.9	2.6	2.9	2.6
100	14.4	7.0	6.1	2.4
150	7.3	8.8	7.8	6.2

When the glucose molecule labelled at C-1 is cleaved only one of the trioses produced will receive label; values for glycerol, acetate and lactate should accordingly be doubled for direct comparison with table 2. Error is estimated as ± 0.5 mM from repeated processing of FIDs

production of acetate and lactate alone is out of redox balance. The formation of glycerol however is a net utilizer of 2 mol NADH per mol glucose through the glycerol-3-phosphate dehydrogenase reaction and therefore the production of equimolar amounts of glycerol and acetate is in redox balance. The proposed flow of labelled carbon from glucose to the detected metabolic products is shown in fig.3.

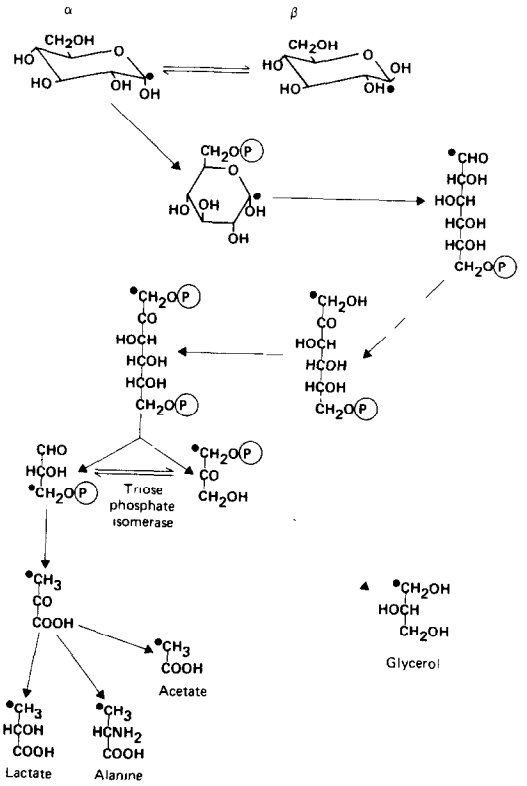

Fig.3. The proposed pathway of carbon flow from (1-^{13}C)-labelled glucose to metabolic end products detected by ^{13}C-NMR in *T. vaginalis*. (●) Labelled carbon atoms.

Table 2

Estimation of changes in glucose concentration and metabolite levels during incubation with unlabelled glucose

Time (min)	Concentration of metabolite (mM)			
	Glucose	Glycerol	Acetate	Lactate
0	31.93 ± 0.66	0.00	0.00	0.00
30	25.75 ± 0.30	4.00 ± 0.50	1.96 ± 0.81	2.83 ± 0.17
50	23.73 ± 1.97	7.19 ± 1.17	6.78 ± 0.98	6.63 ± 1.10
75	18.07 ± 1.91	9.67 ± 0.85	11.48 ± 1.52	9.00 ± 0.46
100	11.80 ± 0.49	13.92 ± 2.08	14.00 ± 1.12	11.40 ± 0.20
125	6.80 ± 0.49	16.33 ± 1.06	15.97 ± 1.08	13.53 ± 2.04
150	3.73 ± 0.47	17.70 ± 1.32	17.49 ± 1.37	14.35 ± 0.72

Results are expressed as mean ± SD for triplicate incubations of a single batch of *T. vaginalis* Bushby strain cells. Conditions of incubation and substrate assay are described in the text

The formation of glycerol as a major metabolite under normal conditions is unusual amongst eukaryotes although it is a feature of anaerobic glucose metabolism in trypanosomatid flagellates [14,15]. In these organisms the enzymes of glycolysis are present in a specific organelle, the glycosome which does not occur in trichomonads. The nature and location of the enzymes producing glycerol in *T. vaginalis* remain to be investigated.

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