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The Catabolism of Glucose by Strains of *Trypanosoma rhodesiense*

BY P. T. GRANT* AND J. D. FULTON

National Institute for Medical Research, Mill Hill, London, N.W. 7

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The African trypanosome, *Trypanosoma rhodesiense*, which in man causes sleeping sickness, has been maintained by blood passage in rats for many years. When isolated from rat blood, glucose or another carbohydrate is essential for the *in vitro* survival of the parasites and under these conditions the catabolism of glucose results in the formation of a number of extracellular end products (Fulton & Stevens, 1945), although the amounts of pyruvate and glycerol account for almost all the glucose utilized (Ryley, 1956). We have now used ¹⁴C-labelled glucose to study the metabolic pathways involved in the formation of pyruvate and glycerol and in the further catabolism of pyruvate by the same strain of *T. rhodesiense*, its drug-resistant variants and a new strain freshly isolated from a human patient. In addition, experiments are described which indicate that pyruvate is also a major end product of the growing trypanosome *in vivo*.

* Present address: Department of Biological Chemistry, University of Aberdeen.

Some of these results have already been reported in a preliminary communication (Grant & Fulton, 1956).

MATERIALS AND METHODS

Strains of trypanosomes. Two distinct strains of *T. rhodesiense* were maintained in the rat as host.

The Liverpool strain ('L' strain) was isolated in 1923 from a human patient and has subsequently been maintained in either mice or rats by blood inoculation. The atoxyl-resistant variant of this strain was obtained from the Liverpool School of Tropical Medicine (Yorke & Murgatroyd, 1930). The stilbamidine-resistant variant was prepared as described by Fulton & Grant (1955).

A new strain, termed 'Maun' ('M' strain), which had been isolated from a human patient only a few weeks before the present experiments, was obtained through the courtesy of Dr W. E. Ormerod, London School of Hygiene and Tropical Medicine.

In the experiments which are to be described, the 'L' strain was used exclusively except where indicated.

Direct estimation of trypanosome numbers. Trypanosomes in infected blood or in a saline suspension were determined

by a direct count in a Thoma-Zeiss haemocytometer by the method of Voegtlin & Smith (1920).

Isolation of trypanosomes from rat blood. (a) 'L' strain and its drug-resistant variants: at the height of infection, when the blood was swarming with parasites, the rats (250–300 g.) were anaesthetized and bled by cardiac puncture into citrated saline [1% (w/v) NaCl and 2% (w/v) sodium citrate] containing 0.2% of glucose. After centrifuging at about 1400 g for 10 min. the serum was removed and the trypanosomes which had separated into a white layer above the red cells were removed as completely as possible with a Pasteur pipette and resuspended in buffered saline (Krebs & Eggleston, 1940) containing 10% (v/v) of horse serum and 0.2% of glucose. Residual red cells in this preparation were then removed by the technique of Moulder (1948), in which the serum of a rabbit immunized against rat red cells was used to agglutinate them. The final suspension of trypanosomes was examined microscopically and then centrifuged in a fine-bore graduated centrifuge tube, at exactly 900 g, for 5 min. In this way reproducible volumes of the packed trypanosomes could be removed for inoculation of the incubation medium. (b) 'M' strain: heavy infections of rats did not usually occur and abnormally large numbers of platelets and white cells were present, which proved difficult to eliminate from trypanosome preparations by the technique described above. The following procedure was therefore adopted. Platelets were removed by defibrination of the blood with glass beads. The blood was then centrifuged and the upper layer of trypanosomes and white cells removed and resuspended in buffered saline containing glucose. Residual red cells were agglutinated as described above and each 20 ml. of the remaining thin suspension was percolated through a column of filter-paper powder (1 g. of Whatman ashless cellulose powder for chromatography per column; W. and R. Balston Ltd., Maidstone) supported on a pad of cotton wool. Although there was some loss of trypanosomes by this procedure, the eluate was essentially free from white cells. A known volume of packed trypanosomes was then prepared from the combined eluates as described above.

In vitro incubation of trypanosome suspension. In all cases the medium consisted of buffered saline containing 10% (v/v) of rat serum and 0.2% of glucose. Each 100 ml. was inoculated with 1 ml. of the packed trypanosomes. These suspensions were then dispensed into suitable conical flasks so that the volume ratio of medium to residual air-space was 1.3. The gas phase was either air-CO₂ (95:5) or N₂-CO₂ (95:5). The flasks were stoppered with tight-fitting rubber bungs and incubated at 37° for 2 hr. with gentle agitation on a reciprocating shaker.

To measure the evolution of ¹⁴CO₂ from ¹⁴C-labelled glucose 25 ml. of a trypanosome suspension was pipetted into a 100 ml. wide-necked conical flask which also contained a 10 ml. flat-bottomed conical flask in place of the conventional centre well. The main flask was tightly stoppered with a rubber bung, through which two glass tubes passed so that the end of one tube was just above the surface of the suspension and the other tube ended just inside the neck of the 10 ml. conical flask. After the flasks had been flushed with the air-CO₂ mixture for 10 min., the exterior end of each glass tube was sealed by a rubber vaccine cap and the flask was incubated as described above. Potassium hydroxide solution [1 ml. of 20% (v/v)] was then injected through the vaccine cap into the small conical flask with the aid of a fine-bore needle and syringe, and the suspension was

acidified by the injection of 2 ml. of 2N-H₂SO₄ through the other vaccine cap. After shaking for a further 15 min. the vaccine caps were removed and traces of alkali on the interior surfaces of the tube into the small conical flask were rinsed into the latter by means of a few millilitres of CO₂-free water. The small flask containing the alkali and washings was then removed, the contents were quantitatively transferred to a small centrifuge tube and the carbonate was precipitated as BaCO₃ for radioassay.

Labelled compounds. Uniformly labelled glucose ([U-¹⁴C]-glucose) and [2-¹⁴C]acetate were purchased from the Radiochemical Centre, Amersham. [1-¹⁴C]Glucose was a synthetic sample kindly given by Dr H. R. V. Arnstein (see Arnstein & Keglević, 1956). [3-¹⁴C]Glucose was prepared biosynthetically *in vitro* from rabbit-liver slices as described by Topper & Hastings (1949). Sodium [¹⁴C]bicarbonate was prepared by liberating ¹⁴CO₂ from Ba¹⁴CO₃ with 10% (v/v) perchloric acid in an evacuated system and trapping it in a calculated volume of 2N-NaOH.

Radioactivity measurements. Samples of 'infinite thickness' were plated on 1 cm.² polythene disks (Popják, 1950) and counted with a bell-shaped helium-filled Geiger-Müller counter. All radioactivity measurements made directly on iodoform samples were corrected for back-scattering, which was determined experimentally to cause errors of +9% on the particular counter used. The combined errors due to variation in sample preparation and statistical errors in counting were estimated to be ±5%.

Isolation of lipid fractions and protein-bound amino acid from trypanosome material. At the end of an incubation trypanosomes were isolated by centrifuging as described previously, and washed twice in buffered saline containing glucose; the final sedimented material was freeze-dried.

The lipids of freeze-dried trypanosomes were quantitatively isolated and fractionated as described by Gidez & Karnovsky (1954) into acetone-soluble and acetone-insoluble lipid. After hydrolysis each fraction was extracted with ether, the solvent was removed *in vacuo* and the fatty acids were oxidized to CO₂ by the procedure of Van Slyke & Folch (1940) for radioassay as BaCO₃. The residues which remained after ether extraction of the hydrolysate, containing mainly glycerol in the acetone-soluble fraction and glycerophosphate in the insoluble fraction, were evaporated nearly to dryness *in vacuo* at 40° and then converted into BaCO₃ as described above.

The freeze-dried trypanosome material (about 2 g.) was freed of lipid and then extracted three times with trichloroacetic acid (6%, w/v) at 90°, then with ethanol and ether, and dried at about 80°. The crude protein was hydrolysed with glass-distilled 6N-HCl (10 ml./g.) at 105° for 18 hr. in a sealed tube. The required amino acids were isolated by chromatography on Zeo-Karb 225 resin (The Permutit Co. Ltd.) from this hydrolysate as described by Fulton & Grant (1956). The purity of the isolated amino acids was confirmed by paper chromatography and by their constant radioactivity after recrystallization.

Isolation and degradation of metabolic products present in the medium after incubation

Glucose. The rat serum used in the incubation medium contained variable amounts of glucose, so that when [¹⁴C]-glucose was added the final radioactivity was determined by isolation of glucosazone from a sample of the trypanosome

suspension immediately before incubation, as described by Feller, Strisower & Chaikoff (1950).

Pyruvate. Protein was precipitated from the medium by the addition of 10% (w/v) metaphosphoric acid, so that the final concentration of this acid was 2%. From the filtrate pyruvate was precipitated as the 2:4-dinitrophenylhydrazone by the addition of a saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl, and after standing overnight at +4° the hydrazone was filtered off, washed with water and recrystallized from ethanol to constant radioactivity. The location of ¹⁴C in some of these hydrazone samples was determined as described below. Pyruvate 2:4-dinitrophenylhydrazone (usually about 200 mg.) and an equal weight of Adam's platinum oxide catalyst (Johnson, Matthey and Co. Ltd., London) were suspended in 25 ml. of water and hydrogenated at room temperature and pressure for 4 hr. The catalyst was then filtered off, the almost colourless filtrate turning yellow and then green when brought into contact with air. The reaction products, alanine and 2:4:6-triaminobenzene, were absorbed from solution by a column of Zeo-Karb 225 (4 cm. × 2 cm.) in the hydrogen form, alanine being subsequently eluted with 0.15M-NH₃ aq. soln. The alkaline eluate was evaporated to dryness *in vacuo*, the amino acid being recrystallized by adding acetone to an aqueous solution and its purity checked by paper chromatography with phenol-NH₃ as solvent. Yield, based on hydrazone used, was usually 50–60%. Alanine was subsequently degraded with ninhydrin as described by Gilvarg & Bloch (1951) to yield C₍₁₎ as CO₂ and C₍₂₎ plus C₍₃₎ as acetaldehyde. A portion of the acetaldehyde was further degraded with hypiodite (see Hatcher & Mueller, 1929) to give C₍₃₎ as iodoform.

Glycerol. Usually 200 ml. of protein-free medium was adjusted to pH 8 with 20% (w/v) KOH and evaporated to dryness *in vacuo* at 50°. The residue was kept overnight *in vacuo* over P₂O₅ and then extracted with three portions of ethanol (15 ml.) under reflux conditions. The combined extracts were filtered, 1 ml. of water was added and the volume was reduced to about 1 ml. in a stream of air. The residual solution was applied as a streak 10 in. long to Whatman 3 MM paper and, after air-drying, the chromatogram was developed in the ascending manner for 16 hr. with a solvent system consisting of butanol-ethanol-NH₃ aq. soln. (1.5M) (45:50:5). After development thin strips were cut off the air-dried sheet, sprayed with ammoniacal AgNO₃ and heated at 110°. The area on the untreated part of the chromatogram containing glycerol (*R_F* 0.43) was cut out and eluted with water. This eluate was then benzoylated in alkaline solution and the tribenzoyl derivative recrystallized from ligroin to constant radioactivity; m.p. 72° (uncorr.). In some cases the tribenzoyl derivative was hydrolysed at reflux temperature with ethanolic NaOH (2N-NaOH-ethanol, 1:1) for 40 min. The solution was cooled, acidified with H₂SO₄ and benzoic acid extracted with light petroleum (b.p. 60–80°). The residual solution was cooled, filtered and treated with periodic acid (Reeves, 1941) to yield C₍₁₎ and C₍₂₎ as formaldehyde, which was isolated as the dimedone derivative.

Succinate. In one instance (see Table 5) this acid was isolated as described below and degraded by the method of Phares & Long (1955).

Analytical methods

Glucose was estimated by the procedure of Nelson, the modified colorimetric reagents described by Somogyi (1952)

being used. Pyruvate and α-oxoglutarate were estimated in blood samples by the specific paper-chromatographic technique (El Hawary & Thompson, 1954). In one instance, total keto acids were determined by the procedure of Friedemann & Haugen (1943). The small amount of succinate produced from [U-¹⁴C]glucose (see Table 2) was estimated manometrically by the procedure of Krebs, Smyth & Evans (1940). In order to determine the total radioactivity associated with this latter acid, a known amount of unlabelled pure sodium succinate was added to a known volume of the suspension and, after thorough mixing, the non-volatile acids were isolated by continuous ether extraction. These acids were separated by partition chromatography (Swim & Krampitz, 1954) and succinic acid in the appropriate fractions was isolated as the silver salt and oxidized to CO₂ for radioassay as BaCO₃ as described above.

RESULTS

Utilization of [U-¹⁴C]glucose by *T. rhodesiense* in vitro

The essential nature of a suitable carbon source for the *in vitro* survival of *T. rhodesiense* made it desirable to determine to what extent such compounds are utilized for cell synthesis as well as for energy. From the results in Table 2 it can be seen that under aerobic conditions only a small amount of the glucose utilized is converted into cell carbon and CO₂, the major part being catabolized to pyruvate and to a lesser extent glycerol. Under anaerobic conditions, however, approximately equimolar amounts of these latter compounds are formed.

The use of [U-¹⁴C]glucose in these experiments made it possible to estimate metabolic products by isotopic dilution (Table 1). This is of particular value with glycerol, which is difficult to estimate accurately in biological fluids. The determination of pyruvate by this procedure is valid in these experiments because we have found by paper chromatography that it is the only keto acid produced under these conditions.

Pathway of glucose utilization by *T. rhodesiense* in vitro

The results in Table 3 for the [U-¹⁴C]glucose experiment with the 'L' strain show that pyruvate is formed exclusively from the glucose utilized, since the radioactivity of glucose is, within the limits of experimental error, double that of the pyruvate. In other words, pyruvate has the same specific activity/mole of carbon as the original glucose supplied. On incubation with [1-¹⁴C]glucose, pyruvate labelled only in methyl carbon was obtained, whereas carboxyl-labelled pyruvate was obtained from [3-¹⁴C]glucose (Table 3). These experiments were repeated with [1-¹⁴C]glucose and the atoxyl-resistant and stilbamidine-resistant variants of the 'L' strain, both in the presence and

Table 1. *Estimation of pyruvate and glycerol formed by incubation of T. rhodesiense with [U-¹⁴C]glucose by the technique of isotope dilution*

Identical trypanosome suspensions (400 ml.) each containing [U-¹⁴C]glucose (800 mg., 1.04 μ C) were incubated either aerobically or anaerobically for 2 hr. as described in the Materials and Methods section. From portions of each suspension, samples of pyruvate and glycerol were isolated as the 2:4-dinitrophenylhydrazones and the tribenzoyl derivative respectively to determine the specific activity (x) of these compounds. Known amounts (M') of pure pyruvic acid (added as sodium pyruvate) and anhydrous glycerol were then added to measured volumes of these suspensions (70 ml.) and, after thorough mixing, pyruvate and glycerol were isolated as before. The specific activity of these diluted samples (y) completes the information required to calculate the mass of labelled compound (M) present at the end of the incubation (cf. Rittenberg & Foster, 1940).

Atmosphere	Compound isolated	Specific activity of compound isolated from suspension		Mass (M') of unlabelled compound added to 70 ml. of suspension (g.)	Specific activity (y) of compound isolated after addition of its unlabelled counterpart (μ C/g.)	Mass (M) of labelled compound originally present in 70 ml. of suspension $M'y/x-y$ (g.)
		x (μ C/g.)	μ C/m-mole			
Air-CO ₂	Pyruvic acid	0.42	0.11	0.0274	0.26	0.0445
	Glycerol	0.29	0.11	0.0442	0.031	0.0053
N ₂ -CO ₂	Pyruvic acid	0.41	0.11	0.0274	0.17	0.0194
	Glycerol	0.28	0.11	0.0442	0.082	0.0182

Table 2. *A comparison of the products formed by incubation of T. rhodesiense with [U-¹⁴C]glucose under aerobic or anaerobic conditions*

Experimental details are given in Table 1. Evolution of ¹⁴CO₂ was measured in duplicate trypanosome suspensions (25 ml.) which were identical with those described in Table 1. Glucose and succinic acid were estimated as described in the Materials and Methods section; - signifies value not determined.

Material	Air-CO ₂ expt.		N ₂ -CO ₂ expt.	
	m-moles	Yield of ¹⁴ C from glucose utilized (%)	m-moles	Yield of ¹⁴ C from glucose utilized (%)
[U- ¹⁴ C]Glucose utilized	1.75	-	1.17	-
Pyruvate	2.89	82.6	1.26	53.9
Glycerol	0.33	9.4	1.13	48.3
Succinate	0.02	0.6	-	-
Respiratory CO ₂	-	1.0	-	-
Freeze-dried trypanosomes	-	1.5	-	-

Table 3. *Percentage of the total radioactivity in glucose utilized by T. rhodesiense which was converted into carbon dioxide and the distribution of radioactivity in pyruvate formed during the same incubation*

Trypanosome suspensions (200 ml.) containing [U-¹⁴C]glucose (212.6 mg., 1.41 μ C), [3,4-¹⁴C₂]glucose (214 mg., 0.091 μ C) or [1-¹⁴C]glucose (212 mg., 0.068 μ C) were incubated under aerobic conditions for 2 hr. at 37°. Pyruvate was isolated as the 2:4-dinitrophenylhydrazones and degraded as described in the Materials and Methods section. Glucose was estimated colorimetrically at the beginning and end of each incubation, carbon dioxide evolution being measured concurrently in identical 25 ml. suspensions in duplicate.

Strain of trypanosome	Type of labelling in glucose added	Molar radioactivity ($\mu\text{C}/\text{m-mole}$)					Isotope in the ^{14}C glucose utilized evolved as $^{14}\text{CO}_2$ (%)
		Glucose	Pyruvate				
			$\text{C}_{(1)} + \text{C}_{(2)} + \text{C}_{(3)}$	$\text{C}_{(1)}$	$\text{C}_{(2)} + \text{C}_{(3)}$	$\text{C}_{(3)}$	
'L'	[U- ^{14}C]	1.12	0.59	0.21	0.39	0.20	1.12
'L'	[3,4- $^{14}\text{C}_2$]	0.074	0.036	0.035	Nil	Nil	0.74
'L'	[1- ^{14}C]	0.051	0.025	Nil	0.025	0.025	0.59
Atoxyl-resistant*	[1- ^{14}C]	0.055	0.028	Nil	0.027	0.027	0.46
Stilbamidine-resistant*	[1- ^{14}C]	0.055	0.027	Nil	0.026	0.027	0.54
'M'	[1- ^{14}C]	0.056	0.028	Nil	0.028	0.029	0.62

* The same results were obtained by incubation in the presence and absence of drug (see text).

absence of drug (Table 3). With the atoxyl-resistant variant the final concentration of drug in the medium was 20 $\mu\text{g./ml.}$, which had no observable effect on motility, whereas this concentration was lethal to the parent strain within 10 min. Stilbamidine had no observable effects on the motility of the parent strain or its resistant variant (Fulton & Grant, 1955).

The results obtained on incubating the 'M' strain with $[1-^{14}\text{C}]$ glucose (Table 3) were the same as those obtained for the other strain and its variants.

The equal radioactivities of pyruvate and glycerol formed by incubation of *T. rhodesiense* aerobically and anaerobically with $[\text{U}-^{14}\text{C}]$ glucose (Table 1) indicate that both compounds are exclusively formed from glucose. With $[1-^{14}\text{C}]$ glucose as substrate, however, the radioactivity of glycerol is equal to that of pyruvate under aerobic conditions (Table 4), in which only a small amount of glycerol is formed (Table 1), whereas under anaerobic conditions, in which almost equal amounts of pyruvate and glycerol are produced, glycerol contains a markedly greater radioactivity than pyruvate (Table 4). Moreover, under these latter conditions the specific activity of glycerol/mole of carbon is actually greater than that of the glucose from which it was formed.

*Further metabolism of pyruvate by
T. rhodesiense in vitro*

A large-scale incubation of the parasite with $[\text{U}-^{14}\text{C}]$ glucose permitted a sufficient amount of trypanosome material to be harvested for the isolation of specific compounds or fractions (Table 5).

Table 4. *A comparison of the molar radioactivities of pyruvate and glycerol formed by incubation of T. rhodesiense with $[1-^{14}\text{C}]$ glucose under aerobic and anaerobic conditions*

Identical trypanosome suspensions (300 ml.), each containing $[1-^{14}\text{C}]$ glucose (600 mg., 0.23 μC), were incubated either aerobically or anaerobically at 37° for 2 hr. At the end of the experiment, pyruvate and glycerol were isolated and the glycerol was degraded with periodic acid as described in the Materials and Methods section.

Compound	Molar radioactivity ($\mu\text{C}/\text{m-mole}$)	
	Air- CO_2 expt.	N_2 - CO_2 expt.
Glucose utilized	0.065	0.065
Pyruvate 2:4-dinitrophenyl- hydrazone	0.031	0.020
Glycerol tribenzoate	0.032	0.042
Formaldehyde dimedone derivative ($\text{C}_{(1)}$ + $\text{C}_{(3)}$ or glycerol)	0.015	0.020

Table 5. *Radioactivity in fractions or specific compounds isolated from trypanosomes incubated with $[\text{U}-^{14}\text{C}]$ glucose*

Trypanosome suspensions (3×400 ml.) containing $[\text{U}-^{14}\text{C}]$ glucose (2.4 g., 5.04 μC) and two suspensions (400 ml.) containing unlabelled glucose and either $[2-^{14}\text{C}]$ acetate (5 mg., 1.35 μC) or sodium $[^{14}\text{C}]$ bicarbonate (8 mg., 1.3 μC) were incubated for 2 hr. under aerobic conditions. The parasites were then harvested and the fractions or specific compounds were isolated as described in the Materials and Methods section.

Labelled compound used	Material isolated	Total radioactivity (μmC)	Specific radioactivity*
$[\text{U}-^{14}\text{C}]$ Glucose	Acetone-soluble trypanosome lipid:		
	Glycerol fraction	21.0	—
	Fatty acid fraction	0.046	—
	Acetone-insoluble trypanosome lipid:		
	Glycerophosphate fraction	9.0	—
	Fatty acid fraction	0.002	—
	Amino acids in trypanosome protein:		
	Aspartic acid	—	89.0
	Glutamic acid	—	0.6
	Glycine	—	29.0
$[2-^{14}\text{C}]$ Acetate	Serine	—	34.0
	Alanine	—	104.0
	Fatty acids of trypanosome lipid	—	169.0
$[\text{U}-^{14}\text{C}]$ Bicarbonate	Extracellular pyruvate	—	0.9
	Respiratory CO_2	—	Nil
	Pyruvate	—	1.9
	Succinate†	—	46.2

* Counts/min. above background at 'infinite thickness'.

† Isolated after addition of unlabelled succinate to the medium.

Glutamic acid was essentially unlabelled, and this is of particular interest in view of the significant radioactivity found in alanine, serine, glycine and aspartic acid.

The incorporation of isotopic glycerol derived from [$U-^{14}C$]glucose into trypanosome lipids and the utilization of [$2-^{14}C$]acetate for lipid fatty acids (Table 5) indicate that fatty acid and triglyceride synthesis take place under these *in vitro* conditions. The relative lack of incorporation of ^{14}C from [$U-^{14}C$]glucose into fatty acid fractions of lipid thus suggests that the conversion of pyruvate into acetate does not occur to a significant extent. Moreover, the negligible incorporation of ^{14}C from [$2-^{14}C$]acetate into extracellular pyruvate indicates that the reverse reaction does not occur.

In the experiment with $NaH^{14}CO_3$, the $^{14}CO_2$ remaining at the end of the incubation was recovered by acidification of the medium and trapping the evolved CO_2 in alkali as described in the

Materials and Methods section. The total radioactivity recovered in this way was found to be, within the limits of experimental error, the same as that originally added, although succinate isolated after the addition of carrier and pyruvate were both radioactive (Table 5). Degradation of this succinate sample showed that the isotope was located only in the carboxyl carbon atoms. It is thus clear that CO_2 fixation had taken place, although the amount involved must have been too small to detect by the technique adopted to measure it.

Correlation of pyruvate production by T. rhodesiense in vitro and in vivo. It seemed possible that the accumulation of extracellular pyruvate under aerobic conditions *in vitro* might be due to a deficiency of some essential cofactor or substrate in the incubation medium. However, the addition of cofactors such as coenzyme A, thioctic acid, diphosphopyridine nucleotide (oxidized, DPN $^+$, or

Fig. 1. Amounts of pyruvate and α -oxoglutarate present in normal and infected rat blood. Keto acids were determined in the blood of seven normal and nineteen infected rats by the quantitative paper-chromatographic method of El Hawary & Thompson (1954). At the same time the degree of infection was estimated by a direct count of trypanosome numbers. Individual results for each infected rat are shown. The spread of the values obtained from normal rats was 0.75–1.25 mg. of pyruvate and 0.32–0.64 mg. of α -oxoglutarate/100 ml. 'L' Strain: $\circ$, pyruvate; $\square$, α -oxoglutarate. 'M' Strain: $\bullet$, pyruvate; $\blacksquare$, α -oxoglutarate.

Fig. 2. Effect of Mapharside (3-amino-4-hydroxyphenyl-arsenoxide) on the total keto acid level in the blood of normal and infected rats (*T. rhodesiense*, 'L' strain). All infected animals had approximately the same degree of parasitaemia as judged by microscopic examination of tail blood. Total keto acids were determined by the method of Friedemann & Haugen (1943) on individual blood samples obtained from groups of normal and infected rats. Similar estimations were then made at intervals after treatment of each rat with 5 mg. of Mapharside (intraperitoneal injection in water). Each point in the figure is the mean of values obtained from at least four animals. $\circ$, Infected rats; $\square$, normal rats.

reduced, DPNH), cocarboxylase or yeast extract added singly or together to the medium did not bring about any significant change in the amount of pyruvate formed during an incubation. Altering the serum content of the medium to a final concentration of 50% (v/v), and the addition of NaHCO_3 , were likewise ineffective.

Quantitative analyses of the keto acids in the blood of normal and infected rats (Fig. 1) showed that abnormally large amounts of pyruvate and α -oxoglutarate were present in the blood of rats infected with either the 'L' or the 'M' strain of the parasite. The blood pyruvate levels were correlated with the degree of infection and the role of the parasite was more conclusively demonstrated by treating infected rats with the trypanocidal drug Mapharside (3-amino-4-hydroxyphenylarsenoxide). The parasites had completely disappeared from the blood 90 min. after treatment. At this time the total keto acid levels in these rats had returned to near normal (Fig. 2), and at 120 min. were almost the same as those found in the blood of normal rats treated with Mapharside at the same time as the infected animals.

DISCUSSION

It has been estimated that the highly motile bloodstream forms of the pathogenic African trypanosomes consume glucose at a rate corresponding to about 50–100% of their dry weight in 1 hr. under *in vitro* conditions (Brand, 1951), and in its absence they rapidly become motionless and disintegrate. With *T. rhodesiense* the carbon balance given by Ryley (1956) and the results presented here would indicate that the glucose utilized is largely catabolized to pyruvate and glycerol (Table 1), only minor amounts being used for cell synthesis (Tables 1 and 4) or converted into respiratory carbon dioxide (Table 1).

The distribution of isotope in pyruvate formed by incubation of *T. rhodesiense* with specifically ^{14}C -labelled glucose (Table 3) is consistent with that predicted by the Embden–Meyerhof–Parnas (EMP) scheme (see Wood, 1955). In this classical glycolytic pathway one mole of glucose is cleaved to 2 moles of triose phosphate, which are equivalent to one another in so far as pyruvate is concerned, so that $\text{C}_{(3)}$ and $\text{C}_{(4)}$ of glucose become the carboxyl carbon atoms of pyruvate, whereas $\text{C}_{(1)}$ plus $\text{C}_{(6)}$, and $\text{C}_{(2)}$ plus $\text{C}_{(5)}$ become the β and α carbon atoms respectively. The oxidative pathway of glucose utilization in which the $\text{C}_{(1)}$ of glucose is evolved as CO_2 would not appear to be of any importance to the trypanosome under these conditions, since only 0.59% of the $[1\text{-}^{14}\text{C}]\text{glucose}$ utilized was evolved as respiratory CO_2 (Table 3). The possibility that this latter estimate is low owing to the use of endo-

genous $^{14}\text{CO}_2$ by the trypanosome for synthetic reactions is unlikely to be of importance in view of the limited synthesis of succinate and cellular components observed in a similar incubation with $[U\text{-}^{14}\text{C}]\text{glucose}$ (Table 2). The EMP scheme is thus the major, if not the only, pathway for glucose utilization by *T. rhodesiense* under these *in vitro* conditions, and it is of interest that other trypanosomes such as *T. hippicum* (Harvey, 1949) and *T. equiperdum* (Chen, 1948) contain glycolytic enzymes. The observation that the molar radioactivities of pyruvate and glycerol formed by incubation of *T. rhodesiense* with $[1\text{-}^{14}\text{C}]\text{glucose}$ can be varied by aerobic or anaerobic conditions may also be explained in terms of the EMP scheme. In this scheme glucose yields equimolar amounts of dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G 3-P) which are in equilibrium with one another. This equilibrium state, which is catalysed by triose phosphate isomerase, has been estimated to be 96% in favour of DHAP (Meyerhof & Junowicz-Kocholaty, 1943). Reduced diphosphopyridine nucleotide formed in the conversion of G 3-P into pyruvate (reaction rate k_5 , see Fig. 3) can be reoxidized by molecular oxygen via a hydrogen-transport system (reaction rate k_6) or by the enzymic conversion of DHAP into glycerophosphate (reaction rate k_4), and the amounts of pyruvate and glycerol formed by *T. rhodesiense* under aerobic and anaerobic conditions (Table 1) are consistent with this explanation, on the assumption that $k_6 > k_4$. Under aerobic conditions, however, it was found that the small amount of glycerol formed from $[1\text{-}^{14}\text{C}]\text{glucose}$ had the same radioactivity as pyruvate, indicating that in the equilibrium mixture of $[1\text{-}^{14}\text{C}]\text{DHAP}$ and unlabelled G 3-P the rate of conversion of G 3-P into DHAP (reaction rate k_2) must be very much greater than the rate of formation of pyruvate and glycerol from these compounds, i.e. $k_2 > k_5 > k_4$. In contrast, under anaerobic conditions equimolar amounts of these end products are formed (Table 1) and the molar radioactivity of glycerol was markedly greater than that of pyruvate (Table 3). Thus, the rate of conversion of DHAP into

Fig. 3. Scheme for the utilization of glucose by *T. rhodesiense*.

glycerol and G 3-P into pyruvate must be equal, and greater than that of G 3-P into DHAP, that is, $k_5 = k_4 > k_2$.

These observations are of more general significance. Studies on the quantitative importance of alternative pathways of glucose catabolism are usually made on the assumption that the two triose phosphates formed from glucose by the EMP scheme are isotopically equivalent for pyruvate formation (see Wood, 1955), so that pyruvate or a related compound formed from [1- ^{14}C]glucose by the EMP pathway should have the same radioactivity/mole of carbon as that of the original sugar. The present results would indicate that this assumption is not necessarily true and suggest that the query of Schambye, Wood & Popják (1954) may be justified. These latter workers found that glucose of rat-liver glycogen is not symmetrically labelled when [1- ^{14}C]glycerol is fed to rats, the C_3 , containing one-third more ^{14}C than the C_4 . More recently, Marks & Horecker (1956) have shown that whereas $^{14}\text{CO}_2$ was fixed into rat-liver glycogen *in vitro*, under certain conditions the C_4 of the glucose unit contained a greater radioactivity than the C_3 .

The incorporation of ^{14}C from [U- ^{14}C]glucose into succinate and cell carbon (Table 2) would show that a small amount of pyruvate is metabolized by the trypanosome, and the results presented in Table 5 are indicative of one pathway that is involved. The similar radioactivities of alanine and aspartic acid in trypanosome protein from the [U- ^{14}C]glucose experiment suggest that there is a direct synthesis of oxaloacetate from pyruvate and carbon dioxide. The incorporation of isotope from $^{14}\text{CO}_2$ into the carboxyl carbon of succinate is in agreement with this view. This latter result is, moreover, only consistent with the formation of succinate from pyruvate by the pathway demonstrated in the ciliated protozoan, *Tetrahymena geleii* (van Niel, Thomas, Ruben & Kamen, 1942) and in other organisms (see Wood, 1946), namely, pyruvate + $\text{CO}_2 \rightarrow$ oxaloacetate $\rightarrow$ malate $\rightarrow$ fumarate $\rightarrow$ succinate. Nevertheless, other intermediates of the tricarboxylic acid cycle such as acetate (Fulton & Stevens, 1945), succinate and citrate (Ryley, 1956) have been found to accumulate during the *in vitro* incubation of *T. rhodesiense*, and the dehydrogenase of malate, citrate, α -oxoglutarate and succinate have been demonstrated in preparations of the same parasite (Williamson, 1953). In the present experiments (Table 5) the insignificant radioactivity in either fatty acids and protein-bound glutamic acid from the [U- ^{14}C]glucose experiment or pyruvate and respiratory carbon dioxide from the [2- ^{14}C]acetate experiment would indicate that there was a negligible formation or utilization of acetate by reactions of the tricarboxylic acid cycle.

The blood-stream forms of *T. rhodesiense* have not yet been cultivated *in vitro* (Tobie, Brand & Mehlman, 1950), so that it is not easy to determine whether the metabolic reactions which have been observed are really representative of those taking place in the growing parasite. It is known, however, that heavily infected rats have little or no liver glycogen and are hypoglycaemic (Christophers & Fulton, 1938). The present work shows that abnormally high blood-pyruvate levels are correlated with the degree of infection (Fig. 1) and are directly caused by the metabolism of the parasite (Fig. 2). It thus seemed probable that pyruvate is the main end product of glucose catabolism by the trypanosome *in vivo*.

SUMMARY

1. Glucose is an essential substrate for the survival of the African trypanosome (*Trypanosoma rhodesiense*) *in vitro*, and uniformly ^{14}C -labelled glucose has been used to prepare a radiocarbon balance of the products of this catabolism. Under aerobic conditions, pyruvate and, to a lesser extent, glycerol were the main end products; only minor amounts of ^{14}C were incorporated into succinate, respiratory carbon dioxide and cell material. Approximately equal amounts of pyruvate and glycerol were formed under anaerobic conditions.

2. The distribution of isotope in pyruvate and glycerol and the amount of $^{14}\text{CO}_2$ evolved when the trypanosome was incubated with [U- ^{14}C]- or [3,4- $^{14}\text{C}_2$]- or [1- ^{14}C]-glucose was consistent with the view that the Embden-Meyerhof-Parnas scheme is the major if not the only pathway of glucose utilization under *in vitro* conditions. Similar studies with [1- ^{14}C]glucose have also been made with the drug-resistant variants of this strain and also with a new strain recently isolated from a human patient.

3. Pyruvate and glycerol formed from [1- ^{14}C]-glucose under aerobic conditions have equal molar radioactivities. In contrast, glycerol has a greater molar radioactivity than pyruvate under anaerobic conditions. The implications of these results have been discussed.

4. The further metabolism of pyruvate has been studied by comparing the radioactivities in fractions or compounds isolated after incubation of the trypanosome with [U- ^{14}C]glucose or [2- ^{14}C]acetate or $\text{NaH}^{14}\text{CO}_3$. It has been concluded that carbon dioxide fixation, leading to the direct synthesis of oxaloacetate from pyruvate and the reduction of the former compound, can account for the formation of succinate.

5. Abnormally high blood-pyruvate levels of infected rats have been correlated with the degree of infection. These levels can be rapidly returned to normal by treatment of the host with a trypanocidal

drug. It thus seems probable that the amount of pyruvate produced by the parasite is greater than the capacity of the host to metabolize or excrete it.

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On the Determination of the Time Required for Biosynthesis of a Single Protein Molecule: Experiments with Rat Pancreatic Ribonuclease

BY VALDA M. CRADDOCK AND C. E. DALGLIESH
Postgraduate Medical School, Ducane Road, London, W. 12

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It has been pointed out by one of us (Dalgliesh, 1953) that the time required for biosynthesis of a single molecule of protein must be known before conclusions about the mechanism of protein biosynthesis can be drawn from certain kinetic data, or from the occurrence of unequal labelling in proteins (references summarized by Steinberg, Vaughan & Anfinsen, 1956). If the time required for biosynthesis of a single molecule of protein is other than short, there should be a measurable delay between the addition of a labelled amino acid to a system actively synthesizing protein and the first appearance of free labelled protein in that system. There are in the literature several examples of the

occurrence of such delays, e.g. in the synthesis of serum albumin *in vitro* (Peters, 1953), or whole serum proteins *in vivo* (Green & Anker, 1955), of γ -globulins *in vivo* (Askonas, Humphrey & Porter, 1956), of Bence-Jones protein *in vivo* (Putnam, Meyer & Miyake, 1956) and of the proteins of the pancreatic juice *in vivo* (Junqueira, Hirsch & Rothschild, 1955). The time before appearance of the labelled protein in these cases must include other factors, e.g. diffusion and mixing, besides the time of biosynthesis. To determine the true time of biosynthesis of a protein molecule, it seemed desirable to investigate synthesis of a single molecular species in as simple a system as possible. For