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SOME ASPECTS OF METHIONINE METABOLISM BY *TRYPANOSOMA EQUIPERDUM, IN VITRO**

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Abstract—Homogenates of *Trypanosoma equiperdum* contained approximately 2·3 g methionine per 100 g crude protein, as determined by two independent methods. Radioactivity derived from both L-methionine-$^{14}CH_3$ and D,L-methionine-$^{14}COOH$ was recovered in the acid-soluble, lipid, nucleic acid, and protein fractions of trypanosomes after their incubation in the presence of these labeled substrates. Methionine served as the source of methyl groups for the biosynthesis of choline, and possibly also for methylated purine and pyrimidine bases present in RNA and DNA.

Most of the radioactivity derived from L-ethionine-ethyl-1-^{14}C was recovered in the acid-soluble fraction of *T. equiperdum* incubated in the presence of this labeled substrate, but small amounts were also detected in the protein and nucleic acid fractions. L-Ethionine depressed the uptake of methionine by the trypanosomes to a greater extent than did the D-isomer.

Trypanasoma equiperdum possesses a relatively high rate of phospholipid synthesis[1] and a closely related species, *T. rhodesiense*, was found to contain significant amounts of acetylcholine.[2] It seemed likely, therefore, that the biosynthesis of choline could figure prominently in the lipid metabolism of the *brucei-evansi* group of trypanosomes. Since it has been established[3, 4] that the three methyl groups present in choline are derived from S-adenosylmethionine, it was considered of interest to investigate this and other aspects of methionine metabolism in *T. equiperdum*.

Pizzi and Taliaferro[5] had previously found that radioactivity derived from ^{35}S-labeled methionine was incorporated into proteins by *T. equiperdum in vivo*. On the other hand, Williamson and Desowitz[6] were unable to isolate methionine from protein hydrolysates of several related trypanosomal species belonging to the *brucei-evansi* group. Aronson[7] found that *T. equiperdum, in vitro* was unable to synthesize methionine from ^{14}C-labeled glucose but was able to assimilate this amino acid when it was present in the surrounding medium.

Data are presented to show that, in *T. equiperdum*, methionine is a constituent of proteins as well as a source of substituents found in the lipid and, possibly, the nucleic acid fractions. There is evidence suggesting that methionine serves as a source of methyl groups for the biosynthesis of choline.

In addition, the disposition of the ethyl analog of methionine, ethionine, as well as its effects upon the uptake of methionine, are described.

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MATERIALS AND METHODS

In order to obtain sufficiently large numbers of trypanosomes for studies *in vitro*, male Wistar rats (500–600 g) were inoculated i.p. with approximately $4{\cdot}5 \times 10^6$ viable parasites (determined by hemocytometer count). When parasitemia levels reached approximately $1{\cdot}5 \times 10^9$/ml (usually 45–50 hr after inoculation), the rats were anesthetized with ether and, after thoracotomy and left ventricular puncture, 15–18 ml blood was slowly withdrawn into a 20-ml glass syringe containing 2 ml of heparin solution (1 mg/ml). Eight-milliliter aliquots of blood were transferred to 12-ml centrifuge tubes at 0° and diluted with 4 ml of a modified Krebs phosphate buffer (pH 7·6, 0·116 M).[8] The diluted blood was centrifuged at 850 *g* for 13 min. The whitish-colored interphase between the packed erythrocytes and the supernatant containing the trypanosomes was carefully removed by aspiration and diluted with additional buffer to ensure an adequate supply of glucose and accommodate the pyruvic acid excreted by the parasites. The suspensions of trypanosomes were recentrifuged at 1500 *g* for 5 min, the supernatants discarded, and the pellets resuspended in fresh buffer containing 2 per cent fraction V bovine serum albumin.

Suspensions containing 2×10^9 trypanosomes ($\pm$ 10 per cent) were added to 250-ml Erlenmeyer flasks and the volume made up to 39·9 ml with buffer. The flasks were then placed in a Dubnoff shaking incubator and were equilibrated in air for 15 min at 37° before the addition of appropriate radioactive substrates (0·1 ml). Incubation proceeded for an additional 60 min and was terminated by decanting the suspensions into 50-ml centrifuge tubes at 0° and centrifuging at 1500 *g* for 15 min. After centrifugation, the supernatants were discarded, and the trypanosomal pellets were washed twice more with ice-cold fresh buffer.

L-Methionine-$^{14}CH_3$, D,L-methionine-$^{14}COOH$, adenine-^3H, uracil-^3H, and orotic acid-7-$^{14}COOH$ were purchased from the New England Nuclear Corp., Boston, Mass., and L-ethionine-ethyl-1-^{14}C was purchased from Calbiochem, Los Angeles, Calif.

After incubation of the trypanosomes, the acid-soluble fraction was obtained by extracting the pellet three times with 1 N perchloric acid at 0°. Lipids were removed from the acid-insoluble residue by vigorous extraction with alcohol:ether (3:1).[9] Fractions containing either RNA or DNA were prepared by the method of Ogur and Rosen.[10] The remaining protein pellet was dissolved in 0·5 N NaOH.

One-tenth-ml aliquots of each fraction were added to 10 ml of a toluene:absolute ethanol mixture (14:5) containing 0·23 per cent of 2,5-diphenyloxazole (PPO).[11] Radioactivity was measured by liquid scintillation counting (Packard Tri-Carb liquid scintillation spectrometer, model 3214). All counts were corrected for background and represent the mean of several experiments. Samples were checked for quenching by addition of internal standards.

Purine and pyrimidine bases isolated from *T. equiperdum* by the method of Marshak and Vogel[12] were separated by paper chromatography on Whatman 1 filter paper with Wyatt's[13] isopropanol:HCl (12 N):water solvent (120:33:37 by volume). The areas corresponding to adenine and cytosine were located by photography with u.v. light at 260 mμ,[14] eluted with glass-distilled water, and redeveloped in a solvent mixture consisting of *n*-butanol and 0·1 N NH_4OH (6:1 by volume).[15] Purine and pyrimidine bases were assayed for u.v. absorption from 220–320 mμ with a Cary model 15 recording spectrophotometer.

Trypanosomal choline was isolated by homogenizing the washed pellet with

chloroform:methanol (2:1 by volume), formation of choline reinekate, and conversion to the chloroplatinate derivative.[16] Radioactivity was measured by liquid scintillation counting.

Pyruvate excreted into the medium by the trypanosomes during the incubation period was measured colorimetrically by formation of a hydrazone complex with 2,5-diphenylhydrazine;[17] the amount of glucose remaining in the medium at the end of the incubation period was determined by the glucose oxidase method.[18, 19]

To measure evolution of $^{14}CO_2$, 2·9-ml suspensions of 10^8 trypanosomes were placed in 15-ml Warburg vessels containing 0·3 ml of 2 N NaOH in the center wells to trap liberated gas. After equilibration in air for 15 min at 37°, 0·1 ml of appropriate radioactive substrate was added, the vessels quickly capped with rubber stoppers of the type used on serum bottles, and the incubations carried out in a Dubnoff incubator for an additional 60 min. Incubations were terminated by injecting 0·4 ml of 50% trichloracetic acid through the stoppers into the suspensions. After allowing 30 min for complete absorption of $^{14}CO_2$, 0·1-ml aliquots of the NaOH solution were withdrawn for assay of radioactivity by liquid scintillation counting.

The presence of methionine in trypanosomal homogenates was determined by these two methods: (1) a pellet of thoroughly washed trypanosomes was homogenized with chloroform:methanol (2:1), filtered, and a sample of the air-dried residue analyzed by the colorimetric method of McCarthy and Paille;[20] (2) a second homogenate prepared with glass-distilled water was subjected to performic oxidation at − 10°,[21] after which the residue was hydrolysed in 6 N HCl for 22 hr at 110° before being assayed with a Beckman amino acid analyser, model 120.

RESULTS

The presence of approximately 2·3 g crude protein methionine/100 g in homogenates of *T. equiperdum* was confirmed by two independent methods of analysis (Table 1).

TABLE 1. COMPARISON OF THE AMOUNT OF METHIONINE IN HOMOGENATES OF *T. equiperdum* MEASURED BY TWO INDEPENDENT METHODS

Type of extract*	Methionine (g/100 g crude protein)	Method of analysis
Homogenate prepared with chloroform–methanol	2·3 ± 0·2†	McCarthy and Paille
Homogenate subjected to performic oxidation	2·4	Beckman amino acid analyzer

* A pellet of thoroughly washed trypanosomes was homogenized with chloroform:methanol (2:1), the homogenate filtered, and a sample of the air-dried residue taken for analysis according to the method of McCarthy and Paille.[20] The second homogenate was prepared with double-distilled water (see text), subjected to the performic oxidation of Harris and Ingram,[21] hydrolyzed with 6 N HCl, and the determination performed on a Beckman model 120 amino acid analyzer.

† Standard deviation of the mean.

T. equiperdum, in vitro was able to assimilate preformed methionine from the surrounding medium and to incorporate this amino acid or radioactive portions of it ($-^{14}CH_3$ or $-^{14}COOH$) into various cellular components (Table 2). Radioactivity derived from L-methionine-$^{14}CH_3$ was recovered to a greater extent in trypanosomal

TABLE 2. UPTAKE AND DISTRIBUTION OF RADIOACTIVITY DERIVED FROM ^{14}C-LABELED METHIONINE AND ETHIONINE BY *T. equiperdum, in vitro*

Compound	Acid soluble (%)	Lipid (%)	Total nucleic acids		Protein (%)
			RNA (%)	DNA (%)	
L-Methionine-$^{14}CH_3$ (sp. act. = 45·6 μc/μmole)	42·4 ± 2·6†	8·0 ± 0·3	10·3 ± 0·8	9·8 ± 4·3	29·5 ± 2·3
D,L-Methionine-^{14}COOH (sp. act. = 3·2 μc/μmole)	65·3 ± 3·3	3·8 ± 0·2	3·5 ± 0·7	5·4 ± 0·7	21·9 ± 2·3
L-Ethionine-ethyl-1-^{14}C (sp. act. = 2·0 μc/μmole)	93·3 ± 2·5	0·0	1·7 ± 1·3	2·1 ± 0·9	3·2 ± 0·4

* Approximately 2 × 10^9 trypanosomes were placed in a final volume of 39·9 ml of modified Krebs phosphate buffer (pH = 7·6, 0·116 M) and incubated at 37° for 15 min before addition of the isotope (0·1 μc). After addition of the isotope, the incubation was continued for an additional 60 min.
† Standard deviation of the mean.

lipid, RNA, and DNA than that derived from D,L-methionine-^{14}COOH. Incorporation of both labeled compounds into protein occurred to approximately the same extent.

After its uptake by the trypanosomes *in vitro*, L-ethionine-ethyl-1-^{14}C exhibited a completely different pattern of distribution from that of methionine (Table 2). The acid-soluble fraction contained much higher amounts of radioactivity derived from this labeled substrate than was the case with labeled methionine. On the other hand, incorporation of radioactivity from labeled ethionine into other cellular components was uniformly lower than that derived from either methyl- or carboxyl-labeled methionine. Evidently, after its uptake, L-ethionine is utilized to only a very limited extent by the trypanosomes.

When *T. equiperdum* was incubated in the presence of D,L-methionine-^{14}COOH, a small amount of radioactivity was recovered as $^{14}CO_2$. In contrast, no $^{14}CO_2$ was produced when L-methionine-$^{14}CH_3$ served as substrate (Table 3).

Small amounts of radioactivity derived from L-methionine-$^{14}CH_3$ were recovered in both the free choline fraction (isolated as choline chloroplatinate) (Table 4) and the nucleic acid fraction of *T. equiperdum* incubated in the presence of this labeled substrate (Table 2). Except to ascertain that methionine also served as a source of methyl groups for the synthesis of choline by this species, no further investigation of this area was carried out. On the other hand, the possibility that methylated purine and pyrimidine bases were present in trypanosomal nucleic acids was more actively explored.

After incubation of the trypanosomes in the presence of L-methionine-$^{14}CH_3$, the purine and pyrimidine bases were isolated from RNA and DNA by the method of

TABLE 3. PRODUCTION OF $^{14}CO_2$ FROM METHIONINE BY *T. equiperdum**

Compound	Total counts/min† recovered as $^{14}CO_2$
D,L-Methionine-^{14}COOH (sp. act. = 3·2 μc/μmole)	3413
L-Methionine-$^{14}CH_3$ (sp. act. = 45·6 μc/μmole)	0

* Approximately 10^8 trypanosomes were placed in a final volume of 2·9 ml of modified Krebs phosphate buffer (pH = 7·6, 0·116 M) containing 0·01 M glucose and incubated at 37° for 15 min in a 12-ml capacity Warburg vessel (which contained 0·3 ml of 2 N NaOH in the center well) before addition of the isotope (0·1 μc). Immediately after addition of the isotope, the vessel was capped and the incubation allowed to continue for an additional 60 min, after which the incubation was terminated and the amount of radioactivity collected as $^{14}CO_2$ was determined (see text).

† Counts/min after correction for background.

Marshak and Vogel[12] and then subjected to chromatographic analysis. As shown in Fig. 1, most of the recovered radioactivity was associated with those areas of the chromatogram occupied by adenine and cytosine, while the remainder was detected in the areas occupied by guanine, uracil, and thymine. The adenine and cytosine moieties were removed by elution and rechromatographed. Upon development with

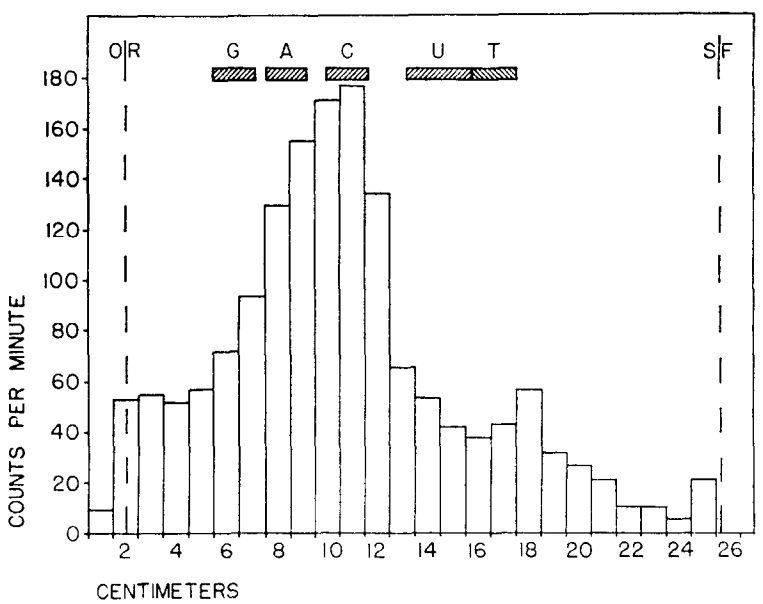

FIG. 1. Distribution of radioactivity derived from L-methionine-$^{14}CH_3$ on a chromatogram of purine and pyrimidine bases isolated from *T. equiperdum*. The chromatogram was developed on Whatman 1 paper in the isopropanol-HCl solvent describe by Wyatt,[13] for approximately 15 hr by the ascending technique at room temperature (20–25°).

a solvent system capable of separating 5-methyl cytosine from cytosine, only single spots corresponding to adenine and cytosine were identified (Fig. 2). The area just ahead of the cytosine spot, which would contain 5-methyl cytosine, was eluted, con-

TABLE 4. RECOVERY OF CHOLINE-^{14}C AFTER INCUBATION OF *T. equiperdum* IN THE PRESENCE OF L-METHIONINE-$^{14}CH_3$

Compound	Total counts/min† recovered as the choline chloroplatinate derivative
L-Methionine-$^{14}CH_3$ (sp. act. = 45·6 μc/μmole)	330 (equivalent to 3·8 × 10^{-6} μmole/mg dry tissue/hr)

* Approximately 2 × 10^9 trypanosomes were placed in a final volume of 399 ml of modified Krebs phosphate buffer (pH = 7·6, 0·116 M) and incubated at 37° for 15 min before addition of the isotope (0·2 μc). After addition of the isotope, the incubation was continued for an additional 60 min. Free choline was isolated as the reineckate and converted to the chloroplatinate.[16]

† Counts/min after correction for background.

centrated, and its ultraviolet spectrum determined. No characteristic u.v. absorption peak for 5-methyl cytosine was detected (Fig. 3), indicating that this methylated base was absent or was present in such limited amounts as to be undetectable by the methods employed.

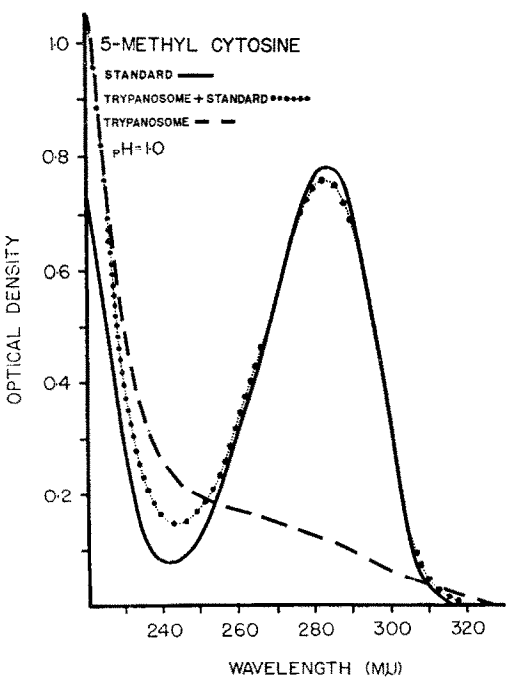

FIG. 3. Ultraviolet spectrum of 5-methyl cytosine.

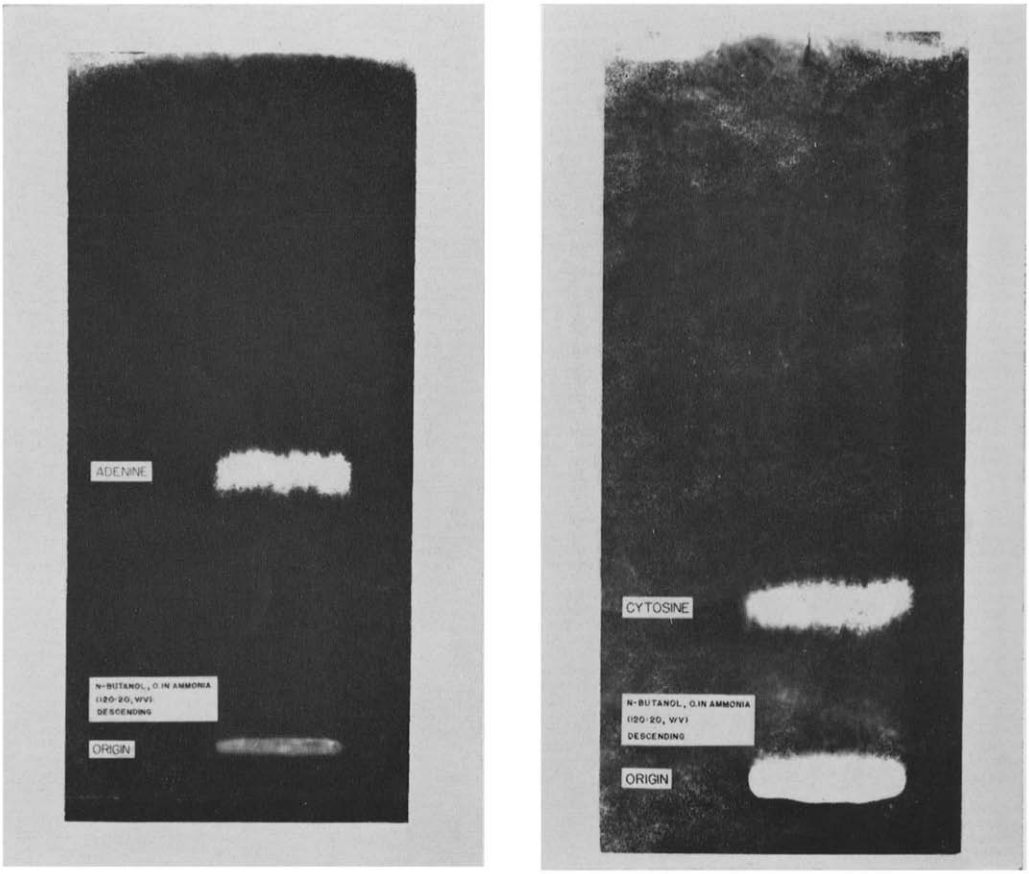

FIG. 2. Chromatographic purification of purine and pyrimidine bases isolated from *T. equiperdum*. Left, trypanosomal adenine. Right, trypanosomal cytosine.

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The presence of small amounts of radioactivity derived from D,L-methionine-^{14}COOH in the nucleic acid fraction suggested that $^{14}CO_2$ produced during metabolic alteration of methionine by *T. equiperdum* might become fixed during the biosynthesis *de novo* of purine or pyrimidine nucleotides. Since the ability of trypanosomatids, including *T. equiperdum*, to synthesize purine nucleotides *de novo* is very much more limited than their ability to synthesize pyrimidine nucleotides *de novo*,[5, 22, 23] the latter pathway was chosen for study of this question.

In order for -$^{14}CO_2$ to be incorporated into the pyrimidine ring, it must be first converted to carbamyl phosphate which, after carbamylation of L-aspartic acid and subsequent ring closure, becomes carbon-2 of orotic acid. Orotic acid is eventually converted to the key pyrimidine nucleotide intermediate, uridylic acid, in the presence of orotidylic acid decarboxylase. Uridylic acid is anabolized to various polyphosphorylated derivatives that are incorporated into RNA and DNA.

When *T. equiperdum* was incubated in the presence of D,L-methionine-^{14}COOH and 6-azauracil (0·5 μmole/ml), a known inhibitor of orotidylic acid decarboxylase in this species,[24] the latter did not significantly affect the incorporation of radioactivity into either RNA or DNA (Table 5). Thus at this time, the presence of radioactivity derived

TABLE 5. EFFECT OF 6-AZAURACIL ON THE INCORPORATION OF D,L-METHIONINE-^{14}COOH BY *T. equiperdum**

Fraction	Control (counts/min†)	Azauracil (0·5 μmole/ml) (counts/min)
Acid-soluble	43,200	43,650
Lipid	2,200	2,000
RNA	1,350	1,050
DNA	2,850	2,100
Protein	10,470	10,080

* Approximately 2×10^9 trypanosomes were placed in a final volume of 39·9 ml of modified Krebs phosphate buffer (pH = 7·6, 0·116 M) containing 0·01 M glucose and the drug being studied, and incubated for 15 min at 37° before addition of the isotope (0·1 μc, sp. act. = 3·2 μc/μmole). After the addition of the isotope, the incubation was allowed to continue for an additional 60 min.

† Counts/min after correction for background.

from D,L-methionine-^{14}COOH in trypanosomal nucleic acids cannot be accounted for satisfactorily. The possibility of contamination of the nucleic acid fraction with a small amount of tenaciously bound protein labeled with ^{14}C-methionine cannot be excluded.

Ethionine (D and L), at concentrations having no significant effect upon glucose utilization or pyruvate production by *T. equiperdum in vitro* (Table 6), inhibited the uptake of D,L-methionine-^{14}COOH (Fig. 4). It can be seen that the L-isomer of ethionine was a more active inhibitor than was the D-isomer. In contrast to its ability to inhibit total uptake of methionine, L-ethionine did not significantly influence the ntracellular distribution of radioactivity derived from L-methionine-$^{14}CH_3$ (Table 7).

TABLE 6. EFFECT OF D- AND L-ETHIONINE ON PYRUVATE PRODUCTION AND GLUCOSE UTILIZATION BY *T. equiperdum**

Drug	Concentration (μg/ml)	Pyruvate production (μmoles)	Glucose utilization (μmoles)	Ratio, pyruvate/glucose
Control		17·40	9·45	1·84
L-Ethionine	50	17·40	9·45	1·84
	100	17·40	10·65	1·63
	200	17·40	10·65	1·63
	400	19·20	10·95	1·75
D-Ethionine	50	19·20	11·55	1·66
	100	19·20	11·25	1·77
	200	19·20	11·70	1·64
	400	19·20	10·50	1·82

* Approximately 10^8 trypanosomes were placed in a final volume of 3 ml of modified Krebs buffer (pH 7·6, 0·116 M) containing 0·01 M glucose and the drug being studied, and incubated at 37° for 60 min. Pyruvate was determined by a modification of the method described by Friedman,[17] and glucose utilization was determined indirectly by the glucose oxidase method.[18, 19] (Refer to text for details of both methods.)

TABLE 7. EFFECT OF L-ETHIONINE ON THE UPTAKE AND DISTRIBUTION OF L-METHIONINE-$^{14}CH_3$ BY *T. equiperdum**

Fraction	Control	L-Ethionine (μg/ml)		
		250	500	1000
Acid-soluble	36,900	4,200 (88·6)	2,100 (94·3)	1,200 (96·8)
Lipid	5,000	400 (92·0)	0 (100·0)	0 (100·0)
RNA	6,900	1,200 (82·6)	600 (91·3)	450 (93·5)
DNA	8,100	1,200 (85·2)	750 (90·8)	450 (94·5)
Protein	19,500	3,390 (82·6)	2,041 (89·5)	1,380 (92·9)

* Approximately 2×10^9 trypanosomes were placed in a final volume of 39·9 ml of modified Krebs phosphate buffer (pH = 7·6, 0·116 M) containing 0·01 M glucose and the drug being studied, and incubated for 15 min at 37° before addition of the isotope (0·1 μc, sp. act. = 45·6 μc/μmole). After the addition of the isotope, the incubation was allowed to continue for an additional 60 min. The figures in parentheses represent per cent inhibition achieved; the other values signify total counts/min after correction for background.

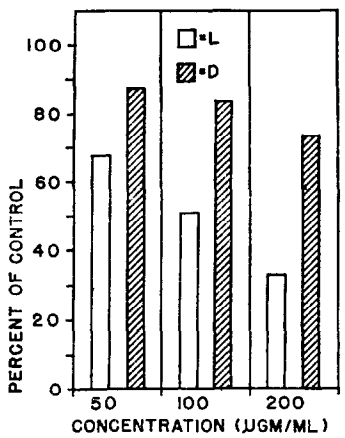

FIG. 4. Comparative ability of D- and L-ethionine to inhibit the uptake of D,L-methionine-^{14}COOH by *T. equiperdum in vitro*. Approximately 2×10^9 trypanosomes were placed in a final volume of 39·9 ml of modified Krebs phosphate buffer (pH 7·6, 0·116 M) containing 0·01 M glucose and the drug being studied, and incubated for 15 min at 37° before addition of the isotope (0·1 μc, sp. act. = 3·2 μc/mole). After addition of the isotope, the incubation was allowed to continue for an additional 60 min.

DISCUSSION

The presence of approximately 2·3 g methionine/100 g crude protein in homogenates of *T. equiperdum*, as demonstrated by two independent methods, is consistent with the finding of Pizzi and Taliaferro[5] that radioactivity derived from ^{35}S-labeled methionine was incorporated into the proteins of this species. The inability of Williamson and Desowitz[6] to isolate methionine from the proteins of related trypanosomal species of the *brucei-evansi* group may be due to differences in methodology. While the colorimetric method of McCarthy and Paille[20] is specific for methionine, the ninhydrin method in combination with paper chromatography depends for its accuracy upon complete separation of the amino acids and subsequent elution of the ninhydrin–amino acid complexes.

It is interesting to note that Wu and Hogg[25] found the content of methionine in the protozoan *Tetrahymena geleii* to be around 1·7–1·8 g/100 g crude protein, whereas Stokes and Gunness[26] found the methionine content of various microorganisms, excluding algae, to range between 0·8 and 2·1 g/100 g crude protein.

The finding that, while methionine-^{14}COOH and methionine-$^{14}CH_3$ were incorporated to about the same extent into proteins, the latter contributed significantly more radioactivity to the lipid and nucleic acid fractions suggests that, as has been found in other biological systems,[27–31] methionine is an important source of methyl groups for transmethylation reactions in *T. equiperdum*. One of these reactions leads to the biosynthesis of choline, and it is of interest that significant amounts of acetylcholine were found in the related species, *T. rhodesiense*,[2] and phosphatidylcholine in several trypanosomatids.[32] The biological roles of these choline-containing substances remain to be ascertained.

The presence of radioactivity derived from carboxy- or methyl-labeled methionine in the nucleic acid fraction cannot, at this writing, be satisfactorily explained. It is conceivable that the $^{14}CO_2$ produced to a small extent by the metabolic alteration of methionine by the trypanosomes could have become fixed during the biosynthesis *de novo* of pyrimidine and purine nucleotides. However, attempts to demonstrate this phenomenon in the more active pathway of pyrimidine biosynthesis *de novo* were unsuccessful. It is also conceivable that the methyl group of methionine, found to be metabolically stable in *T. equiperdum*, could be used to methylate purine and pyrimidine bases in this species, since the work of Littlefield and Dunn[33] indicates that such methylated bases occur widely in nature. Again, attempts to demonstrate the presence of at least one such base, 5-methyl cytosine, were unsuccessful. Therefore, although methionine may yet be found to participate in nucleic acid metabolism in the manner suggested, it is possible that the recovery of radioactivity from methionine in the nucleic acid fraction may be only artifactual.

The ethyl analog of methionine, ethionine, is an established metabolic inhibitor, acting as an antagonist of methionine.[34, 35] The data in Fig. 4 suggest that the pathway by which ethionine enters *T. equiperdum* is governed by steric requirements, and that L-ethionine is the more efficient inhibitor. While the main effect of ethionine in *T. equiperdum* appeared to be upon the uptake of methionine, a small amount of the analog was incorporated into protein and possibly also into nucleic acids. Such incorporation could be biologically significant. It should be reported here that L-ethionine was found to prolong the lives of mice infected with *T. equiperdum*, but the dose required to achieve a significant effect was so high that the potential usefulness of this analog as a chemotherapeutic agent is questionable.

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