CORRELATION BETWEEN CO_2 AND H_2S PRODUCTION BY *ENDAMOEBA HISTOLYTICA**

by

ERNEST KUN**, JOHN L. BRADIN, JR. AND J. M. DECHARY

Departments of Medicine (Division of Infectious Diseases), Tropical Medicine and Public Health, Tulane University, New Orleans, La. (U.S.A.)

It has been previously reported (KUN AND BRADIN[1]) that the anaerobic fermentation of glucose by suspensions of *Endamoeba histolytica* is greatly stimulated by cysteine. Simultaneously an increased reduction of S to H_2S occurs when glucose is added. It was suggested that the explanation of this unusual type of anaerobic oxidation process is a coupling of the reduction of a reactive form of sulfur with the dehydrogenation of triose phosphate. This type of reaction may be of general biological interest; therefore a more detailed presentation of experimental methods and results appears to be justified.

It is possible that technical difficulties, so often encountered during the culturing of *E. histolytica* will make it impractical to use this protozoon as the source of isolated enzyme systems, which would permit a study of enzymic reactions in detail. The mechanism of reactions proposed in this paper may be considered as a working hypothesis suggesting further investigations along these lines in other organisms or tissues.

EXPERIMENTAL

1. *Preparation and properties of Amoeba suspensions*

Endamoeba histolytica of clone 22[2] were cultured in aqueous egg yolk infusion medium[3] in the presence of a mixed bacterial flora, associated with this strain of amoebae. This method of culturing yielded the largest number of protozoa. For comparison, metabolic studies were also carried out with strain 103, obtained through the courtesy of Dr. C. W. REES (National Microbiological Institute, Bethesda 14, Maryland, U.S.A.) and occasionally with bacteria free cultures grown on embryonic fluid[4]. There was no biochemical difference among these various amoeba cultures.

The cultures were flushed with N_2 (pure or containing 5% CO_2) 24 hours prior to the manometric experiment and kept in the incubator at 37° C. At the time of the experiment the native cultures were quickly transferred into chilled centrifuge tubes (0° to 4° C) and the starch granules separated by immersing the tubes into an ice bath for 10 minutes. The supernatant fluid, containing the amoebae and associated bacteria, was carefully pipetted off and centrifuged at 6000 × *g* for 15 minutes at 0 to 4° C. The supernatant fluid after this centrifugation consisted of the nutrient medium and almost all of the associated bacteria. The sedimented amoebae were resuspended in ice cold salt solution (100 parts per volume of 0.15 *M* NaCl, 4 parts of 0.15 KCl, 1 part of 0.15 *M* $MgSO_4$ and 21 parts of 0.1 *M* phosphate buffer of pH 7.4) and washed free of

* This investigation was supported by research grants (HG-980) from the National Heart Institute of the National Institutes of Health, Public Health Service, Bethesda 14, Maryland, and from the Lilly Research Laboratories (Eli Lilly Company, Indianapolis 6, Indiana).

** Present address: The University of Wisconsin, Institute for Enzyme Research, Madison 5, Wisconsin, U.S.A.

References p. 159.

adsorbed bacteria by centrifugation at 0 to 4° C. Microscopic examination of the sedimented amoebae showed that together with the intact protozoa a variable amount of amoeba fragments settled at the centrifugal force used for separation. These cell fragments contained measurable amounts of enzymic activity. Centrifugation at lower speeds resulted in the retention of cell fragments in the amoeba free supernatant fluid; consequently misleading results may be obtained[5].

When centrifugal washings are carried out at 37° or even at room temperature, the washed amoebae, although morphologically intact, do not exhibit glucose fermentation; glucose fermentation appears only after 10–15 hours of anaerobic incubation with cysteine. This reversible inhibition is due to the inactivation of hexokinase. Centrifugation does not alter the physiological behavior of *E. histolytica*[6].

2. Chemical methods and preparations

Manometric measurements were carried out in conventional Warburg vessels of 15 to 18 ml volume under N_2 atmosphere (or 95 % N_2 + 5 % CO_2) at 37° C. The length of experiments varied between 30 minutes and a maximum of 2–3 hours. At the end of the manometric measurement 0.3 ml 100 % trichloroacetic acid was tipped in from the side arm in order to liberate dissolved H_2S from the main compartment. Hydrogen sulfide was trapped as CdS in the center well (containing 0.2 ml saturated sol. of Cd acetate adsorbed on a strip of filter paper). The CdS was quantitatively transferred to 50 ml graduated tubes (with stoppers), then 5 ml of a freshly prepared solution of *p*-aminodimethylaniline hydrochloride (0.4 g dissolved in 100 ml of 1 volume of 36 % HCl + 1 volume of H_2O) is added together with 1 ml of fresh solution of $FeCl_3$ (0.27 g dissolved in 5 ml of 1:5 diluted HCl of the above-mentioned HCl solution). The volume is made up to the mark with H_2O, the tubes quickly stoppered, shaken and left standing for 1 hour. The optical density of the methylene blue solution[7] is read at 670 mμ. The method was calibrated with an accurately standardized solution of Na_2S, placed into the main compartment of the Warburg flasks and treated as the experimental ones. Recoveries run between 80 to 90 %. Lactic acid was determined according to BARKER AND SUMMERSON[8], pyruvate as described by FRIEDEMANN AND HAUGEN[9], thiols by a slightly modified procedure of ANSON[10].

Unless specified, reagents of analytical purity were used. The calcium salt of fructose-1,6-diphosphate (HDP) was purified by ion exchange according to MCCREADY AND HASSID[11]. Bis-(*o*- and *p*)-carboxyphenyl disulfide[12], bis-(*m*)-carboxyphenyl disulfide[13], dithiobenzene[14], and N bis-(*o*)-aminophenyl disulfide[15] were synthesized as described in the literature. Coenzyme I (DPN 90 %), coenzyme II (TPN, 60 %), ATP, cocarboxylase, pyridoxine, and coenzyme A were obtained from Sigma Chemical Company.

RESULTS

The evolution of CO_2 and H_2S

When suspensions of *E. histolytica*, treated with the precautions described above, are incubated with glucose (55 μ*M*) and cysteine (32 μ*M* of freshly neutralized cysteine hydrochloride) under N_2 atmosphere, a rapid evolution of CO_2 and H_2S occurs. As shown in Table I, the rates are roughly proportional to the number of protozoa. With very large numbers of amoebae ($5–7 \cdot 10^5$ per flask) the CO_2/H_2S ratio is greatly in favor of CO_2, while with dilute suspensions it approaches that of 2 to 3 μ*M* CO_2 per 1 μ*M* H_2S.

TABLE I

CORRELATION BETWEEN THE NUMBER OF AMOEBAE AND THE RATES OF CO_2 AND H_2S EVOLUTION

*Number of Amoebae**	*Micromoles of* CO_2	H_2S
	Per 60 minutes	
$2 \cdot 10^4$	2.8	0.90
$4 \cdot 10^4$	7.0	1.92
$8 \cdot 10^4$	15.1	3.62

* The amoebae were sedimented by one centrifugation and used immediately after resuspension in saline-phosphate.

The isolated bacteria did not ferment glucose to CO_2 nor did they evolve H_2S from cysteine. When the mixed bacterial flora after separate culturing were reinoculated to a culture of amoebae, they again supported amoebic growth. It appears to be evident that the growth-supporting properties of certain bacteria constitute a separate problem, which has no apparent connection with the biochemical reactions studied in the course of this work.

The CO_2 yielding reaction was found to be the decarboxylation of pyruvate, which is the product of glucose fermentation. The CO_2 evolved could not

References p. 159.

be derived from bicarbonate since it occurred equally well in its absence. In suspensions of amoebae pyruvate was reduced to lactate only to a negligible extent (0.5 to 1.5%) and even this slight reduction could be due to residual bacterial contaminants. The reduction of pyruvate to lactate by protozoon free bacteria was observed but not studied in detail.

The rate of decarboxylation of pyruvate was dependent on the type of amoeba preparation used. While $2.5 \cdot 10^4$ amoebae (washed 3 times) evolved 18.3 μM CO_2 from glucose, under identical conditions (with cysteine present) only 10.1 μM CO_2 was produced from equimolar amounts of pyruvate. When the suspension of amoebae was ground in a tight fitting glass homogenizer (at 0°) the resulting "broken cell" preparation decarboxylated pyruvate at the same rate as it produced CO_2 from glucose (21.6 μM CO_2 from glucose, 19.5 μM CO_2 from pyruvate). With intact cells 2 μM of pyruvate disappeared per 1 μM CO_2 while in broken cells a 1:1 ratio was approached. There is no evidence which would indicate that in *E. histolytica* pyruvate is metabolized by an enzyme system similar to the Krebs cycle of many aerobic organisms. The products of decarboxylation were isolated as the 2,4-dinitrophenylhydrazine derivatives of acetaldehyde and biacetyl. No other α-keto acid besides pyruvate was decarboxylated by *E. histolytica*. The accumulation of pyruvate from glucose could not be readily measured in these preparations, since most of pyruvate was decarboxylated as soon as it was formed.

A comparison of various sugars as sources of CO_2 is given in Table II. All experimental conditions being constant, the rate of CO_2 evolution from glucose was taken as 100 and all other rates were compared as per cent activities.

TABLE II

COMPARISON OF THE RATES OF CO_2 EVOLUTION FROM VARIOUS SUBSTRATES

Substrate	*Activity*
Glucose	100
Mannose	100
Galactose	88
Sucrose	86
Fructose	58
Raffinose	57
Arabinose	43
Ribose	36
Xylose	29
Trehalose	10
Sorbitol	9
No added substrate	9

Each flask contained $3 \cdot 10^5$ amoebae separated by centrifugation.

The mechanism of sugar fermentation cannot be studied in suspensions of intact protozoa. Homogenized amoebae still fermented glucose to pyruvate, but cells which were completely disrupted by gently freezing and thawing (in acetone dry ice mixture) did not act upon glucose. Addition of ATP, however, temporarily activated fermentation, although the high phosphatase activities of these preparations interfered with the measurement of hexokinase. Suspensions of *E. histolytica*, which were subjected to lysis by freezing and thawing, still decarboxylated pyruvate and fermented fructose-1,6-diphosphate (HDP) at a fair rate. The appearance of alkali-labile P from HDP was considered evidence for aldolase. This enzyme was also assayed by the method of SIBLEY AND LEHNINGER[16]. The fermentation of HDP via pyruvate to CO_2 required the presence of cysteine (or cystine), while the production of H_2S from cysteine (or cystine) occurred at a maximal rate only upon addition of HDP. Pyruvate did not stimulate H_2S formation. Similarly the decarboxylation of pyruvate was unaffected by cysteine. The mutually activating effect of cysteine and glucose (intact cells) or HDP in lyzed cells is summarized in Table III. Since the suspensions of *E. histolytica* used in these experiments were freshly harvested from native cultures, they contained considerable amounts of endogenous substrates. It could therefore not be expected

References p. 159.

that "all or none" interdependence between H_2S evolution from added cysteine and CO_2 evolution from glucose (or HDP) would be demonstrable. When suspensions of washed amoebae were kept for some time (0.5 to 2 hours) under anaerobic conditions, most of the endogenous substrate was exhausted. Such cell suspensions did not ferment glucose unless cysteine (or cystine) was present, and did not produce H_2S from cysteine in the absence of glucose. The drawback of this "starvation" was a considerable loss in intact amoebae (up to 70% in 1 hour).

TABLE III

THE MUTUALLY ACTIVATING EFFECT OF GLUCOSE (OR HDP) AND CYSTEINE ON CO_2 AND H_2S EVOLUTION

Substrates	*Intact cells*		*Lysed cells*	
	CO_2	*H_2S*	*CO_2*	*H_2S*
None	0.2	0.32	0.0	0.01
Glucose	9.5	0.60	0.2*	0.31*
Cysteine	0.4	2.10	1.1	1.80
Glucose + cysteine	16.0	3.30	3.6*	2.42*

* HDP (10^{-2} *M* as Na salt) as substrate.
The results in Tables III to V are expressed in terms of micromoles.

As seen in Table III, both CO_2 and H_2S evolution by lysed amoeba preparations were considerably below those of the intact cells. Since these experiments were carried out simultaneously with amoebae harvested from the same culture ($8 \cdot 10^4$ cells per flask) they are exactly comparable. The activity of lysed preparations could be raised almost to the level of the intact cells by a boiled extract of an acetone powder of rat liver homogenate (60 mg of acetone powder was extracted with 5 ml 0.1 *M* phosphate buffer, then deproteinized by immersion into boiling H_2O for 10 minutes, followed by filtration). This effect is shown in Table IV, which represents an experiment fully

TABLE IV

THE EFFECT OF RAT LIVER EXTRACT ON H_2S AND CO_2 EVOLUTION BY LYSED CELL PREPARATIONS

Substrates	*CO_2*	*H_2S*
None	0.0	0.0
HDP	0.3	0.2
HDP + extract*	10.0	1.0
Cysteine	1.0	1.6
Cysteine + extract*	8.0	2.5
Cysteine + HDP	4.0	2.4
Cysteine + HDP + extract*	14.0	2.4

Lysed preparation as in Table III.
* 0.3 ml of the liver acetone powder extract was added to each vessel.

comparable to those summarized in Table III. The components of the liver extract responsible for the reactivation of lysed cells have not been identified yet. There is some indication that water-soluble substances related to amino and keto acids may be involved. Our work was limited by the rather small number of amoebae available for

References p. 159.

test systems. The activating component(s) cannot be replaced by any of the known coenzymes (DPN, TPN, ATP, cocarboxylase, flavin nucleotides, pyridoxal-phosphate coenzyme A).

The production of H_2S from cysteine by *E. histolytica* is a different reaction than the cysteine desulfhydrase, studied by FROMAGEOT and co-workers[17–21]. There is no net oxidation involved in the reaction catalyzed by the desulfhydrase, while in *E. histolytica* apparently a dehydrogenation with S as hydrogen acceptor occurs. No NH_3 was formed, and the production of alanine was established as determined by paper chromatography. No pyruvate was formed from cysteine alone when added to starved whole cells or lysed amoebae. Both cysteine and cystine were equally effective in supporting H_2S and CO_2 evolution. The thiol and disulfide form of this aminoacid came to a rapid equilibrium, when either of them was added to suspensions of *E. histolytica* under anaerobic conditions. For instance, in a typical experiment (with $8 \cdot 10^4$ cells per flask) with excess glucose present (52 μM) out of 12.7 μM of added cysteine 10.7 μM (determined as –SH compound[10]) disappeared in 60 minutes, while 10 μM of CO_2 and 4 μM of H_2S evolved. Under comparable conditions when cysteine was replaced by the disulfide at the end of 1 hour 15 to 20% was present in the thiol form. A continued linear rate of CO_2 evolution beyond this period indicated that this equilibrium is optimal for conditions of glucose fermentation. Only glutathione was equivalent to cysteine in supporting both H_2S and CO_2 evolution. Dithionate and bis-(*o*-)-carboxyphenyl disulfide were only 60–80% as effective while thioglycolate, cysteic acid, taurine, thiamine, thiourea, homocysteine, ethionine and methionine were ineffective and were not desulfurated. Bis-(*o*-)-aminophenyl disulfide proved to be a powerful inhibitor (80% inhibition at 10^{-2} M concentration), while bis-(*m* and *p*)-carboxyphenyl disulfides inhibited 20 to 30%. A comparison of the reduction of these synthetic aromatic disulfides is summarized in Table V. It is apparent that in order to be reduced and support H_2S as well as activate CO_2 evolution a carboxy substitution in ortho position, with respect to the sulfur substituted C atom is required. Enzymic reduction to the –SH form occurs when the COO^- is in *meta* or *para* position, while CO_2 evolution is unaffected (the same amount of CO_2, *i.e.*, 3 μM, was produced without any added disulfide). Diphenyl disulfide was neither reduced nor it altered endogenous CO_2 evolution.

TABLE V

Disulfide substrate	H_2S	–SH	CO_2
	in micromoles		
Bis(*ortho*-carboxyphenyl)disulfide	1.8	2.0	5.0
Bis(*meta*-carboxyphenyl)disulfide	0	2.7	3.0
Bis(*para*-carboxyphenyl)disulfide	0	2.4	2.5
Diphenyldisulfide	0	0	3.4

Each vessel contained 30 micromoles of disulfide, 50 micromoles of glucose and $8 \cdot 10^4$ of freshly harvested amoebae, suspended in 2.5 ml of saline phosphate. The reaction was stopped at 60 min by tipping in 0.3 ml 100% TCA and at 0′ for initial controls. The initial –SH content at 0′ was 0.01–0.03 micromoles per flask.

References p. 159.

DISCUSSION

It was shown that *E. histolytica*, isolated under certain conditions exhibit a reasonably fast rate of metabolism *in vitro*, which is readily distinguished from that of associated bacteria. It was concluded that a phosphorylative glycolysis takes place and glucose is converted to pyruvate via HDP. The available evidence also suggests that the oxidative step of fermentation, *i.e.*, the dehydrogenation of phosphoglyceraldehyde is coupled with the reduction of S to H_2S. We have already pointed out[1] that the optimal growth of *E. histolytica* occurs in cysteine-containing medium, where E^h is —140 millivolts, an E^h which is exactly that of a system where equilibrium between S and H_2S exists (—141 millivolts). The experimental work described above offers a biochemical interpretation of these observations.

It is noteworthy that a DPN requirement of the triose phosphate dehydrogenase system of *E. histolytica* could not be demonstrated. The H acceptor is a disulfide (cystine, or bis-(*o*-)-carboxyphenyl disulfide) which is reduced to the thiol form. The further steps leading to H_2S must involve several reactions. We suggest that the anaerobic oxidation of the thiols proceeds through a labile intermediate (source of a reactive form of sulfur). This process is similar to the oxidation of cysteine by sulfur, which is derived from the desulfuration of β-mercaptopyruvate as described by MEISTER, FRAZER AND TICE[22], yielding pyruvate, H_2S and cystine. This interpretation also suggests the role of a "cofactor", *i.e.*, the boiled extract of liver acetone powder. Thiopyruvate is known to arise by the transamination of α-ketoglutarate and cysteine as described by CAMMARATA AND COHEN[23], yielding glutamate as one of the end products. It is also known that thiopyruvate reacts enzymically with glutamate (and glutamine) to yield S, which can then be reduced to H_2S in the presence of a H donor (cysteine or thioethanol[22]). The overall reactions may be summarized in the following equations:

1. cysteine + pyruvate ⟶ alanine + β-thiopyruvate[22,23]
2. β-thiopyruvate + 2 cysteine ⟶ H_2S + cystine[22] + pyruvate (to CO_2)
3. 1/2 HDP + cystine ⟶ glycerophosphate + 2 cysteine
   ↓
   (to pyruvate ⟶ CO_2)

Both CO_2 and H_2S formation are determined by factors influencing all three reaction sequences. It also follows that the CO_2/H_2S ratio may vary accordingly (see Tables III and IV), and approaches 2 μM CO_2: 1 μM H_2S.

The deproteinized extract of rat liver probably contains α-ketoglutarate and glutamine, and thus activates CO_2 and H_2S production in lysed cells, where these components are diluted. It should be mentioned that chromatographic analyses revealed an SH containing keto acid in extracts of *E. histolytica*, which observation supports the proposed hypothesis. Since *E. histolytica* is not a suitable microorganism for enzyme isolation studies, we are in the process of studying these reactions in other cells which may enable us to obtain a clearer picture of the interrelationship between carbohydrate and sulfur metabolism.

SUMMARY

1. Suspensions of *Endamoeba histolytica* form CO_2 and H_2S under anaerobic conditions from sugars in the presence of cysteine (or cystine).

2. Evidence was obtained which indicated that glucose is phosphorylated and HDP is fermented to pyruvate which is then decarboxylated.

References p. 159.

3. It was shown that during the fermentation of glucose (or HDP in broken cells) triose phosphate is oxidized by cystine (or another ortho substituted aromatic disulfide). The oxidation of the resulting thiols occurs by way of a "labile S compound" (β-thiopyruvate or a derivative of it) which is enzymically desulfurated and the S reduced to H_2S.

RÉSUMÉ

1. Des suspensions d'*Endamoeba histolytica* produisent, en anaérobiose, CO_2 à partir des hydrates de carbone et H_2S à partir de la cystéine.

2. Il apparait que les hexoses sont transformés, via le fructosediphosphate et le triose-phosphate en acide pyruvique; ce dernier est ensuite décarboxylé.

3. Au cours de la fermentation anaérobie, l'oxydation du triosephosphate est liée à une réduction enzymatique de la cystine; celle-ci peut être remplacée par un disulfure aromatique synthétique, substitué en position "ortho". L'oxydation ultérieure des thiols formés est due à un composé labile, probablement l'acide β-thiopyruvique ou un corps voisin, lequel est désulfuré en engendrant H_2S.

ZUSAMMENFASSUNG

1. Suspensionen von *Endamoeba histolytica* entwickeln unter anaeroben Umständen CO_2 und H_2S in Gegenwart von Glukose und Cystein.

2. Es wurde bestätigt dass in diesem Protozoon eine phosphorolytische Glykolyse stattfindet. Fruktosediphosphat wird via Triosephosphorsäure zu Brenztraubensäure überführt, die dann dekarboxyliert wird.

3. Während des anaeroben Zuckerabbaus oxidiert das Cystin Glycerinaldehydphosphorsäure; Cystin kann auch durch ein synthetisches orthosubstituiertes aromatisches Disulfid ersetzt werden. In einer zweiten Phase reagieren die in der ersten Reaktion entstandenen Thiole mit S, das aus einer labilen Schwefelverbindung (wahrscheinlich β-Thiobrenztraubensäure oder dessen Derivat) durch Desulfurierung entsteht. Dabei wird das Disulfid regeneriert und das S zu H_2S reduziert.

REFERENCES

1 E. KUN AND J. L. BRADIN Jr., *Biochim. Biophys. Acta*, 11 (1953) 312.
2 E. C. FAUST, E. H. SADUN, M. G. EVERRITT, J. L. BRADIN Jr. AND R. A. LEWIS, *J. Parasitol.*, 36(4) (1950) 336.
3 W. BALAMUTH, *Am. J. Clin. Pathol.*, 16 (1946) 380.
4 E. H. SADUN, I. M. KRUPP AND M. G. EVERRITT, *Proc. Soc. Exptl. Biol. Med.*, 80 (1952) 272.
5 J. L. BRADIN Jr. AND E. KUN, *J. Parasitol.*, 38 (1952) (suppl.) 12; 39 (1953) (suppl.) 12; *Proc. Soc. Protozool.*, 4 (1953) 8.
6 D. E. WYKOFF, *Thesis*, Tulane Univ. 1951.
7 L. H. ALMY, *J. Am. Chem. Soc.*, 47 (1925) 1381.
8 S. B. BARKER AND W. H. SUMMERSON, *J. Biol. Chem.*, 138 (1941) 535.
9 T. E. FRIEDEMANN AND G. E. HAUGEN, *J. Biol. Chem.*, 147 (1943) 415.
10 M. L. ANSON, *J. Gen. Physiol.*, 24 (1941) 399.
11 R. M. MCCREADY AND W. Z. HASSID, *J. Am. Chem. Soc.*, 66 (1944) 561.
12 A. H. BLATT, *Org. Syntheses Coll.*, Vol. II, John Wiley and Sons, Inc., New York, 1943, p. 580.
13 S. SMILES AND J. STEWART, *J. Chem. Soc.*, 119 (1921) 1792.
14 H. GILMAN, *Org. Syntheses Coll.*, Vol. I. New York, 1941, 504.
15 L. M. CLARK, *J. Chem. Soc.*, Part II (1928) 2320.
16 J. A. SIBLEY AND A. L. LEHNINGER, *J. Biol. Chem.*, 177 (1949) 859.
17 P. CHAIX AND C. FROMAGEOT, *Enzymologia*, 6 (1939) 33.
18 P. DESNUELLE, E. WOOKEY AND C. FROMAGEOT, *Enzymologia*, 8 (1940) 225.
19 P. DESNUELLE AND C. FROMAGEOT, *Compt. rend.*, 216 (1943) 359.
20 C. FROMAGEOT, P. CHAIX AND J. THIBAUT, *Bull. soc. chim. France*, 13 (1946) 202.
21 C. FROMAGEOT, E. WOOKEY AND P. CHAIX, *Enzymologia*, 9 (1940) 198.
22 A. MEISTER, P. E. FRAZER AND S. V. TICE, *J. Biol. Chem.*, 206 (1954) 561.
23 P. S. CAMMARATA AND P. P. COHEN, *J. Biol. Chem.*, 187 (1950) 439.

Received June 13th, 1955