BBA 3896

GLUTAMATE OXIDATION IN THE DIFFERENTIATING SLIME MOLD

II. STUDIES *IN VITRO*

MARGARETE BRÜHMÜLLER AND BARBARA E. WRIGHT

The John Collins Warren Laboratories of the
Huntington Memorial Hospital of Harvard University at Massachusetts General Hospital,
Boston, Mass. (U.S.A.)

(Received August 20th, 1962)

SUMMARY

The mechanism by which CO_2 is formed from glutamate in extracts of *Dictyostelium discoideum* does not involve a direct non-oxidative decarboxylation, since the reaction is inhibited anaerobically both *in vivo* and *in vitro*. α-Ketoglutarate is implicated as an intermediate in the reaction, since (a) it is decarboxylated at the same rate as glutamate, (b) it competitively dilutes out $^{14}CO_2$ from [1-^{14}C]glutamate. All the data are compatible with the conclusion that the rate of glutamate oxidation *in vivo* is substrate-controlled throughout differentiation.

INTRODUCTION

The companion paper demonstrates that the rate of $^{14}CO_2$ evolution from [1-^{14}C]-glutamate *in vivo* increases as differentiation proceeds. It has been postulated that the enhanced activity of this reaction is caused by progressive increases in the intracellular glutamate concentration. This conclusion is based upon a comparison of the molar concentration of glutamate in the cells to the K_m of partially purified glutamic acid dehydrogenase, as assayed by DPNH formation. The primary purpose of the present study is, therefore, to determine whether the $^{14}CO_2$ assay also involves glutamic acid dehydrogenase in the conversion *in vivo* of glutamate to CO_2. Furthermore, *in vitro* evidence is presented relative to the role of glutamate as the rate-limiting substrate in its conversion to CO_2 via α-ketoglutarate.

MATERIALS AND METHODS

DL-[1-^{14}C]Glutamic acid was purchased from Volk Radiochemical Company with the specific radioactivity of 3.0 mC/mmole. The α-[1,2-$^{14}C_2$]ketoglutaric acid was obtained from Nichem Inc. and had a specific radioactivity of 0.98 mC/mmole equally labeled in both C atoms. Enzyme extracts from *D. discoideum* were prepared as follows: the amoebae were grown with *Escherichia coli* as the bacterial associate on complex media and starved on 2% agar plates[1]. The cells were harvested at the desired stage of differentiation in 0.01 M Tris (pH 7.0). Cell extracts were obtained by using an Aminco-

French pressure cell. In order to get maximum protein yields at the fruit stage, the spore suspension was put through twice.

After centrifugation for 10 min at 3000 × *g*, protein was estimated by the micromethod of SAKAGUCHI modified by ZAMENHOF AND CHARGAFF[2]. Bovine albumin was used as standard protein solution for the calibration curve, and the measurements were carried out in a Zeiss Spectrophotometer, Model PMQ II.

$^{14}CO_2$ Determination

To estimate the $^{14}CO_2$ liberated from the labeled glutamate or α-ketoglutarate, incubations were carried out in stoppered Warburg vessels of 7 ml capacity at room temperature. An average incubation mixture of 1.0 ml total volume was composed as follows: the main vessel contained 50 μmoles potassium phosphate buffer (pH 6.5); 0.5 μmole DPN; 0.1 μmole DL-[1-^{14}C]glutamate or 0.25 μmole α-[1,2-$^{14}C_2$]ketoglutarate; the sidearm was supplied with 0.2 ml 5 N H_2SO_4 which was tipped into the incubation mixture to terminate the reaction. The simultaneously released $^{14}CO_2$ was absorbed by 0.1 ml 14% NaOH in the center well. 5 minutes after tipping the acid in each vessel, 0.05 ml of the NaOH was transferred into the scintillation vial containing 0.15 ml H_2O and 10 ml scintillation gel[3,4] and assayed for ^{14}C in the Packard Tri-Carb Liquid Scintillation Spectrometer model 314-DC. Each vial was counted 3 times and an average value was taken. Unless specified otherwise in a table, the lowest counts used in the data to be presented were at least 5 times above background.

RESULTS

To establish the linearity of $^{14}CO_2$ evolution from [1-^{14}C]glutamate and α-[1,2-$^{14}C_2$]-ketoglutarate *in vitro*, four identical incubations were stopped by acid addition at intervals of 15 min and a linear dependence of $^{14}CO_2$ formation with time resulted. Thus, the data on rate of $^{14}CO_2$ evolution from [^{14}C]glutamate/h were obtained from four such individual values at three stages of development (Fig. 1). Similar results were obtained for α-[1,2-$^{14}C_2$]ketoglutarate.

Preliminary experiments using the Thunberg technique with methylene blue showed that glutamate as well as α-ketoglutarate were oxidized in the presence of crude extracts. In order to pursue this observation quantitatively, the dependence of $^{14}CO_2$ evolution from [1-^{14}C]glutamate on aerobiosis was determined at 3 different stages of development with intact cells and with extracts. Table I indicates that the $^{14}CO_2$ formation from [1-^{14}C]glutamate is completely dependent on an oxidative reaction at all three stages *in vivo* and *in vitro*. In order to insure complete anaerobiosis, incubations were carried out after treatment with $Na_2S_2O_4$ in the presence of safranin. After adding a few crystals of $Na_2S_2O_4$ to two vessels, one flask was gassed with pure N_2 for 30 min, whereas the control flask was left open and shaken gently until complete aerobiosis was again indicated by the dye.

The results presented in Table I exclude a direct non-oxidative decarboxylation of glutamate and, alternatively, indirectly implicate glutamic acid dehydrogenase and hence α-ketoglutarate as an intermediate in CO_2 formation from glutamate. In Table II is seen the DPN dependence of CO_2 formation from glutamate as well as from α-ketoglutarate in an aged and dialyzed cell extract at preculmination. For aging the intact pseudoplasmodia were incubated on plain agar at 5° for about three days,

and the extract, prepared in the usual way, was dialyzed against 0.01 M Tris (pH 7.0) overnight. The lack of a greater dependence on DPN is probably due to the fact that it is very difficult to resolve for catalytic amounts. Charcoal treatment of extracts and $(NH_4)_2SO_4$ fractionation did not give a greater dependence.

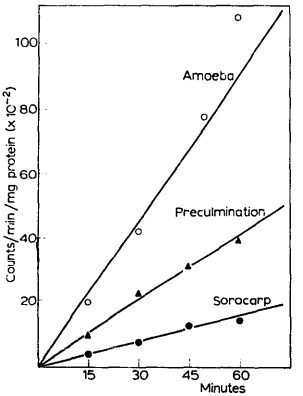

Fig. 1. The rate of CO_2 evolution *in vitro* as a function of time at three developmental stages. See METHODS for experimental details.

To provide more direct evidence for the participation of α-ketoglutarate as an intermediate in the conversion of glutamate to CO_2, experiments were carried out to determine the dilution in radioactivity of $^{14}CO_2$ from [1-^{14}C]glutamate by adding unlabeled α-ketoglutarate to the incubation mixture (Table III). The experiment

TABLE I

DEPENDENCE OF $^{14}CO_2$ EVOLUTION FROM [1-^{14}C]GLUTAMATE ON AEROBIOSIS *in vivo* AND *in vitro*

The concentration of [1-^{14}C]glutamate present was $2 \cdot 10^{-4}$ M. Comparable cell aliquots *in vivo* were used at each developmental stage. The reaction was started by tipping the substrate into the incubation mixture after closing the vessel (see text).

Stage	Atmosphere	*In vivo*		*In vitro*	
		Counts/min/ cell aliquot	*Dependence (%)*	*μmoles CO_2/h/mg protein ($\times 10^3$)*	*Dependence (%)*
Amoeba	Air	1622		9.2	
	N_2	140	91	0.3	97
Preculm.	Air	5046		4.14	
	N_2	96	98	0.2	95
Sorocarp	Air	702		2.84	
	N_2	36	95	0.3	90

was performed using a glutamate concentration of $1 \cdot 10^{-4}$ M, similar to that found *in vivo*, and an incubation time of 15 min in the presence of a crude pseudoplasmodium extract. The concentration of unlabeled α-ketoglutarate used was that at which three other Krebs-cycle dicarboxylic acids showed no effect in inhibition or dilution of $^{14}CO_2$ liberation from [1-^{14}C]glutamate ($6 \cdot 10^{-3}$ M). The data obtained strongly suggest that α-ketoglutarate is the only intermediate in the pathway of CO_2 formation from glutamate.

TABLE II

DPN DEPENDENCE OF $^{14}CO_2$ EVOLUTION FROM [1-^{14}C]GLUTAMATE AND α-[1,2-$^{14}C_2$]KETOGLUTARATE

An aged, dialyzed extract of preculminating cells was used (see text for details).

Substrate	*Molar concentration* ($\times 10^3$)	*μmoles CO_2/h* ($\times 10^3$)		*DPN dependence* (%)
		Without DPN	*With DPN*	
[1-^{14}C]Glutamate	0.36	19.0	37.0	48
α-[1,2-$^{14}C_2$]Ketoglutarate	1.6	16.7	22.0	24

TABLE III

DILUTION OF $^{14}CO_2$ FROM [1-^{14}C]GLUTAMATE BY VARIOUS KREBS-CYCLE INTERMEDIATES

All non-radioactive compounds were present at a concentration of $6 \cdot 10^{-3}$ M.

Unlabeled compound	*Counts/min/h*	*Dilution* (%)
None	1152	0
α-Ketoglutarate	56	95
Fumarate	1402	0
Malate	1226	0
Oxalacetate	1275	0

TABLE IV

STIMULATION OF $^{14}CO_2$ EVOLUTION FROM [1-^{14}C]GLUTAMATE BY DPN AND PYRUVATE

5 μmoles DPN were added where indicated. A dialyzed extract of pseudoplasmodia was used in this experiment (see text).

Substrate	*Concentration* ($\times 10^4$ M)	*μmoles CO_2/h* ($\times 10^3$)		*DPN dependence* (%)
		Without DPN	*With DPN*	
[^{14}C]Glutamate	1	0.34	0.70	51
[^{14}C]Glutamate + Pyruvate	1 1	1.30	1.63	20
[^{14}C]Glutamate + Pyruvate	1 10	1.46	1.90	23

Evidence for the possible participation of a transaminase (as well as the dehydrogenase) in α-ketoglutarate formation from glutamate is shown in Table IV. $^{14}CO_2$ evolution from labeled glutamate is stimulated by pyruvate and DPN in the presence of a dialyzed extract of pseudoplasmodia which had been kept as intact cells at 5° for a few days for a better depletion of endogenous substances. The striking effect on the stimulation of CO_2 evolution even at very low pyruvate levels suggests that this transaminase reaction may play a role in CO_2 formation from glutamate in the intact cell. However, in the absence of knowledge concerning the presence of various keto-acids *in vivo*, we cannot yet assess the significance of this pathway. It is of incidental interest to note the lesser DPN dependence in the presence of pyruvate. These results are compatible with the data of Table II, in which CO_2 evolution starting from α-ketoglutarate was less dependent on DPN than CO_2 evolution from glutamate.

Previous data *in vitro* have indicated a decrease in glutamic acid dehydrogenase activity assayed by DPNH formation and no change in glutamate–pyruvate transaminase during development[5]. In order to confirm these data on glutamic acid dehydrogenase by an independent assay and also to compare the activity of this enzyme with α-ketoglutarate dehydrogenase as a function of developmental stage, CO_2 evolution from glutamate as well as from α-ketoglutarate was determined. Table V demonstrates that at the low concentration, comparable to the endogenous glutamate level, the rates of CO_2 liberation from glutamate and α-ketoglutarate are very similar, being greatest at the amoebae stage and about equal in the two later stages. These data provide supporting evidence for the intermediary role of α-ketoglutarate, which would require that the rate of CO_2 liberation from α-ketoglutarate should be at least as high as from glutamate.

The low concentration of glutamate used in the experiment described in Table V was expected to be rate-limiting. In fact, by increasing the glutamate concentration by a factor of 100, and hence approaching substrate saturation, it was possible to raise the rate of CO_2 formation 10-fold. These results are compatible with previous data *in vitro* on the K_m of glutamic acid dehydrogenase and with the conclusion that the amount of glutamate inside the cell is below substrate saturation and rate-limiting. In order to determine whether the endogenous level of glutamate is also rate-limiting if the transaminase is active (*i.e.*, if pyruvate is present), incubations were carried out in the

TABLE V

$^{14}CO_2$ EVOLUTION FROM [1-^{14}C]GLUTAMATE AND α[1,2-$^{14}C_2$]KETOGLUTARATE AT DIFFERENT STAGES OF DEVELOPMENT *in vitro*

A, amoeba; P, preculmination; S, sorocarp. The corrected values for the sorocarp stage starting with glutamate as substrate take into account the lower specific activity of glutamic acid dehydrogenase at this stage[5].

Substrate	*Molarity* (× 10^4)	*Specific activity* (counts/min/μmole)	*μmoles $^{14}CO_2$/h/mg protein* (× 10^2)			
			A	*P*	*S*	*S(corr.)*
α-[1,2-$^{14}C_2$]-Ketoglutarate	2.5	$5 \cdot 10^5$	4.5	2.2	1.0	
[1-^{14}C]Glutamate	1.0	$1.5 \cdot 10^6$	6.5	2.6	1.0	1.6
[1-^{14}C]Glutamate	110.0	$1.5 \cdot 10^4$	85.0	22.0	15.0	20.0

presence of 0.002 M pyruvate with increasing amounts of glutamate. As can be seen in Table VI, an increase in glutamate concentration causes an increased rate of CO_2 formation also under conditions where a transaminase reaction could occur.

TABLE VI

CO_2 EVOLUTION AS A FUNCTION OF GLUTAMATE CONCENTRATION IN THE PRESENCE OF PYRUVATE
Pyruvate was present at a concentration of 0.002 M.

Glutamate molarity ($\times 10^4$)	*μmoles CO_2/h/mg protein* ($\times 10^2$)
1.0	5.4
6.0	10.2
12.0	12.7
52.0	28.0

To establish the degree to which α-ketoglutarate participates in the reaction, dilution experiments were carried out at three different stages of development. As can be seen in Table VII, the percentage dilution at the two later stages is about 90 %, whereas it is somewhat lower at the earlier stage of development. The amount of non-radioactive α-ketoglutarate added (4 μmoles) and the amount of labeled α-ketoglutarate present, assuming it is the only precursor of $^{14}CO_2$ (0.006 μmoles at preculmination), could result in a dilution of about 1000:1. Thus, if α-ketoglutarate were

TABLE VII

DILUTION OF $^{14}CO_2$ EVOLUTION FROM [1-^{14}C]GLUTAMATE BY UNLABELED α-KETOGLUTARATE AT THREE STAGES OF DEVELOPMENT

Radioactive glutamate was present at a concentration of $1 \cdot 10^{-4}$ M and non-radioactive α-ketoglutarate at $4 \cdot 10^{-3}$ M. Incubations were carried out for 15 min in the presence of an extract of pseudoplasmodia (see text).

Stage	*α-Ketoglutarate*	*μmoles CO_2/15 min/mg protein* ($\times 10^4$)	*Dilution* (%)
Amoebae	—	14.5	
Amoebae	+	4.4	70
Preculm.	—	6.0	
Preculm.	+	0.54	90
Sorocarp	—	3.42	
Sorocarp	+	0.26	92

the only intermediate, virtually all of the radioactive α-ketoglutarate should be diluted out, instead of 90 %. A possible explanation for these results is to invoke the existence of a partially enzyme-bound α-ketoglutarate, not in complete equilibrium with added, non-radioactive α-ketoglutarate. If this situation existed, it might be predicted that (a) at higher glutamate concentrations, relatively more enzyme-bound α-ketoglutarate would exist, hence giving less dilution by non-radioactive α-ketoglutarate, (b) the percent dilution by α-ketoglutarate as a function of time should be constant. Table VIII shows that the degree of dilution by α-ketoglutarate is constant with time and is in fact lessened at higher concentrations.

However, these results might also be obtained if, at higher glutamate concen-

trations, a significant amount of glutamate is decarboxylated directly, without involving α-ketoglutarate as an intermediate. Therefore, a study was carried out on the effect of a high glutamate concentration on the degree of inhibition by anaerobiosis of CO_2 formation from glutamate. An experiment similar to that summarized in Table I demonstrated that even at a glutamate concentration of 10^{-2} M, CO_2 evolution is completely inhibited under anaerobic conditions. Thus, the most reasonable explanation for the observed results is the existence of a partially enzyme-bound α-ketoglutarate as an intermediate in the conversion of glutamate to CO_2.

TABLE VIII

PERCENT DILUTION OF $^{14}CO_2$ FROM [1-^{14}C]GLUTAMATE BY α-KETOGLUTARATE AS A FUNCTION OF GLUTAMATE CONCENTRATION AND AS A FUNCTION OF TIME

The concentration of unlabeled α-ketoglutarate present was $4 \cdot 10^{-2}$ M.

Molarity [1-^{14}C]glutamate ($\times 10^4$)	*Incubation time (min)*	*μmoles CO_2/min/mg protein* ($\times 10^4$)		*Dilution (%)*
		Without α-ketoglutarate	*With α-ketoglutarate*	
1.0	10	1.0	0.228	78
	20	3.6	0.43	88
	30	6.2	0.6	90
	40	7.5	0.9	88
110.0	10	11.5	4.25	63
	20	20.0	8.15	59
	30	25.0	10.7	57
	40	43.0	16.7	61

Although no such reaction has been reported to our knowledge, glutamate might be oxidized by a DPN-linked dehydrogenase not involving α-ketoglutarate. This possibility was excluded by demonstrating that α-ketoglutarate competitively inhibits $^{14}CO_2$ evolution from glutamate. The data presented in Fig. 2 strongly implicate α-ketoglutarate as the only intermediate in the conversion of glutamate to CO_2.

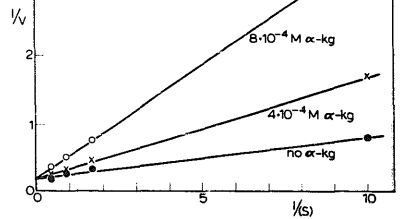

Fig. 2. A plot of $1/V$ against $1/(S)$ according to LINEWEAVER AND BURK. The reaction is $^{14}CO_2$ evolution from [1-^{14}C]glutamate, and the inhibitor is unlabeled α-ketoglutarate. V, μmoles CO_2/mg protein/h ($\times 10^3$) and (S), μmoles glutamate/ml.

DISCUSSION

Glutamate oxidation is undoubtedly one of the main pathways responsible for the conversion of amino acids into citric acid cycle intermediates during the course of slime mold differentiation. It has been shown that the activity of this reaction (but not the concentration of the enzymes involved) increases during development, in response to intracellular glutamate accumulation. The actual source of this glutamate is unknown; it could arise through the degradation of glutamate-rich proteins, or through conversion from other amino acids. These studies emphasize the dangers of equating enzymic concentration *in vitro* with enzymic acitvity *in vivo* and point out the importance of possible alterations during differentiation in the physico-chemical environment of an enzyme. There are a number of other differentiating systems in which an enzymic potential has been shown to be in great excess of the availability of endogenous substrates[6].

ACKNOWLEDGEMENT

This investigation was supported by a PHS grant (RG-8958) from the Division of Medical Sciences, Public Health Service.

This is publication No. 1096 of the Cancer Commission of Harvard University.

REFERENCES

[1] G. U. LIDDEL AND B. E. WRIGHT, *Develop. Biol.*, 3 (1961) 265.
[2] S. ZAMENHOF AND E. CHARGAFF, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 702.
[3] C. F. GORDON AND A. L. WOLFE, *Anal. Chem.*, 32 (1960) 574.
[4] F. E. KINARD, *Rev. Sci. Instr.*, 28 (1957) 293.
[5] B. E. WRIGHT, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 798.
[6] B. E. WRIGHT, in M. FLORKIN AND H. MASON, *Comparative Biochemistry*, Vol. V, A (1962).