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EFFECTS OF GLUCOSE AND OXYGEN ON THE METABOLISM OF GLYCINE BY EHRLICH ASCITES TUMOR CELLS *IN VITRO*

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SUMMARY

Ehrlich ascites tumor cells were incubated for 30 min in a salts medium containing 2 mM [2-^{14}C]glycine under 95 % oxygen, air, or nitrogen. The portion of total recovered radioactivity not present as free glycine was 4.1–4.2 % without added sugar and 7.2–7.6 % with 5.5 mM glucose. Considerable incorporation into free amino acids, especially serine and taurine, occurred.

Glucose stimulated the intracellular concentration of glycine; it increased the total radioactivity in serine, taurine, alanine, glutamic acid, acid-soluble purines, and nucleic acid purines, but decreased that in aspartic acid and protein. The concentrations of some amino acids differed after incubation in the presence or absence of glucose, whether or not glycine was added. Composition of the gas phase had much less effect on incorporation into free amino acids; however, increased O_2 tension enhanced incorporation into protein and nucleic acid purines.

INTRODUCTION

Many studies of the incorporation of glycine into nucleic acids by ascites tumor cells *in vitro* have involved low concentrations of this amino acid, *e.g.*, 0.17 mM (see refs. 1–5), which may be limiting for protein synthesis. The incubation medium has usually included glucose, and the gas phase has contained 95 % nitrogen of air[1–5]. On the other hand, investigations of glycine uptake have utilized relatively high levels of glycine, usually in a medium without added glucose and under an atmosphere of 95 % oxygen[6–10]; it has been reported by one investigator that under these conditions no significant incorporation of glycine occurs[11].

Glucose has been found to stimulate the incorporation of glycine and formate into purines[2,12,13], whereas high oxygen tension has been reported to inhibit nucleic acid synthesis and cell growth in tissue culture[14]. Recent publications by the Montreal group[15–18] on glycine uptake by ascites cells incubated with moderately high levels of glycine have included observations on incorporation into protein and the effect of glucose. However, no measurements were made of incorporation into acid-soluble compounds or nucleic acids. We, therefore, thought it of interest to subject ascites cells

Abbreviation: KRB, Krebs–Ringer–bicarbonate buffer.

to a relatively short incubation with a moderately high level (2 mM) of glycine, in order to test the effects of glucose and oxygen tension on both the uptake of glycine and its incorporation into acid-soluble as well as macromolecular products.

METHODS

Ascites cell suspension

The Ehrlich EF strain (hypotetraploid) ascites tumor, obtained from Dr. C. HEIDELBERGER, was carried in female white Swiss mice and used seven days after inoculation. Cells were centrifuged for 5 min at 1470 × *g*, washed twice with 3 times their volume of isotonic saline, centrifuged for 7 min, and suspended in KRB. These procedures were carried out at room temperature.

KRB[19] had been used in many of the investigations of glycine uptake[7–10]. The composition is quite similar to that of ROBINSON's salts mixture[20] which, fortified with 5.5 mM glucose, was the medium in the incorporation studies[1–5].

Incubations

To the main compartment of 20-ml Warburg flasks were added 2.2 ml KRB and 1.0 ml cell suspension containing the equivalent of approx. 40 mg dry cell mass. To the side arm was added 8.0 μmoles [2-^{14}C]glycine, $4 \cdot 10^6$ counts/min/μmole, in 0.8 ml KRB. The 2.2 ml KRB in some flasks contained enough glucose to make the concentration 5.5 mM in the final volume of 4.0 ml. The flasks were filled with the appropriate gas mixture O_2–CO_2 (95:5), air–CO_2 (95:5) or N_2–CO_2 (95:5) by evacuating 3 times with a vacuum pump and allowing the flasks to fill after each evacuation. The flasks were placed in a water bath at 37.5° for 5 min, glycine was tipped into the main compartment, and the incubation was continued for 30 min with shaking. The flasks were then removed to an ice bath and subsequent operations were carried out at 3°.

Separation of acid-soluble and acid-insoluble fractions

The contents of each flask were centrifuged for 10 min at 20000 × *g*. Using the values of HEINZ[8,11], it was calculated that under these conditions not more than 1 % of the medium was trapped by the packed cells. The supernatant media from duplicate flasks were pooled, and perchloric acid was added to a final concentration of 0.2 M. The cells were extracted 3 times with 2.0-ml portions of 0.2 M perchloric acid, and the extracts from duplicate flasks were combined to give the acid-soluble fraction of the cells.

Acid-soluble and acid-insoluble fractions from medium

The perchloric acid-treated medium was centrifuged for 10 min at 1470 × *g*. The precipitate (0.05–0.1 ml) was suspended in 0.2 ml 0.2 M perchloric acid and recentrifuged. The supernates from the two centrifugations were combined to give the acid-soluble fraction of the medium. The precipitate was washed twice more with 2-ml portions of 0.2 M perchloric acid; supernates from these washings were discarded. The final precipitate was dissolved in 1.0 ml concentrated formic acid for radioactivity measurements.

A 0.1-ml sample of the acid-soluble fraction of the medium was removed and

diluted to 10.0 ml for radioactivity measurements. The remainder was treated with KOH and centrifuged to remove the perchloric acid (as $KClO_4$). The precipitate was washed with 0.5 ml 0.1 N KOH and recentrifuged. The two supernates were combined, adjusted to pH 2 with HCl, and used for amino acid analysis.

Acid-soluble fraction of cells

The acid-soluble fraction of the cells was divided into two portions; 7.2 ml was taken for amino acid analysis and 4.8 ml, for nucleotide analysis. The former portion was treated with KOH and centrifuged to remove the $KClO_4$. The supernate was adjusted to pH 2 with HCl and used for amino acid analysis. A 0.1-ml aliquot was removed from the portion for nucleotide analysis and diluted to 10.0 ml for radioactivity measurements. The remainder was hydrolyzed for 45 min at 115° under pressure of 15 lbs./in^2. Adenine and guanine were isolated from the hydrolysate for radioactivity measurements as described by WILLIAMS AND SCHILLING[21].

Acid-insoluble fraction of cells

The acid-insoluble precipitate was heated at 100° for 30 min in 4.0 ml 0.4 M perchloric acid to hydrolyze nucleic acids[1]. The protein residue after centrifugation (10 min at 1470 × *g*) was washed twice with 1.0-ml portions of 0.4 M cold perchloric acid, and these washes were added to the supernate to give the nucleic acid extract. A 0.1-ml aliquot was removed and diluted to 10.0 ml for radioactivity measurements. The remainder was placed on a Dowex-50 column (0.8 × 1.5 cm), and nucleic acid adenine and guanine were isolated for radioactivity measurements as described by WILLIAMS AND SCHILLING[21]. The protein residue was dissolved in 5.0 ml concentrated formic acid for radioactivity measurements.

Isolation of amino acids

Amino acids in acid-soluble fractions (of medium and of cells) were separated by on-exchange chromatography essentially as described by MOORE AND STEIN[22], but with Dowex-50 X5 in place of Dowex-50 X4. After collection of 1-ml fractions, 0.1 ml was removed from each and the remainder was analyzed by the ninhydrin method of ROSEN[23] with minor modifications*. After the individual amino acids had been located by ninhydrin analyses, the 0.1-ml aliquots corresponding to each peak were pooled for radioactivity measurements.

It was found from complete amino acid analyses of two cellular acid-soluble extracts that none of the amino acids eluted after leucine exhibited significant radioactivity. Therefore, in subsequent analyses the columns were developed through leucine and then stripped with 1 N NaOH. The chromatographic fractions obtained from the acid-soluble extracts of medium were generally pooled for radioactivity determinations into "pre-glycine", glycine, and "post-glycine", but in certain instances complete analyses (through leucine) were done.

Measurement of radioactivity and presentation of data

Suitable aliquots were plated on aluminum planchets and counted in a flow counter. Corrections were made for self absorption where necessary. Total radio-

* The pH and concentration of the acetate buffer were raised slightly (450 g sodium acetate: $3H_2O$ + 55 ml glacial acetic acid/l (pH 5.0–5.7)) and the sodium cyanide concentration was reduced by half.

activity in the Tables has been calculated for the entire volume of each acid-soluble fraction pooled from duplicate incubation flasks. As described above, aliquots were removed for radioactivity measurements before preparation of samples for amino acid chromatography. Radioactivity and concentration values for individual amino acids were adjusted by correcting for activity lost (less than 10 %) during preparation and analysis.

RESULTS

Tables I and II show the distribution of total radioactivity and the specific activities of certain cellular components. Only those amino acids which had marked total radioactivity are listed. Considerable incorporation into free amino acids, proteins, and purines occurred during a 30-min incubation. Overall recovery of radioactivity in the 5 groups was 89 ± 5 %. Glucose increased the total radioactivities of serine, taurine, alanine, and glutamic acid, but decreased that of aspartic acid. In the presence of glucose both total radioactivities and specific activities of serine, taurine, and glutamic acid were highest under anaerobic conditions; those of alanine and aspartic acid were

TABLE I

DISTRIBUTION OF TOTAL RADIOACTIVITY AFTER 30-min INCUBATION WITH [2-^{14}C]GLYCINE

	$O_2 + CO_2$		$Air + CO_2$		$N_2 + CO_2$ + Glucose
	Control	+ Glucose	Control	+ Glucose	
	Counts/min 80 mg dry wt. cells*				
Acid-soluble					
Total	$20.3 \cdot 10^6$	$27.0 \cdot 10^6$	$22.9 \cdot 10^6$	$24.7 \cdot 10^6$	$24.3 \cdot 10^6$
Glycine	$19.7 \cdot 10^6$	$25.2 \cdot 10^6$	$22.2 \cdot 10^6$	$22.9 \cdot 10^6$	$22.5 \cdot 10^6$
Serine	244 000	$1.29 \cdot 10^6$	391 000	$1.28 \cdot 10^6$	$1.41 \cdot 10^6$
Taurine	14 200	91 100	12 400	87 400	115 000
Alanine	8 890	42 700	15 100	27 000	29 200
Aspartic acid	11 100	8 470	6 270	4 410	4 950
Glutamic acid	4 370	12 400	7 040	9 180	17 800
Adenine**	5 650	30 700	2 700	14 900	22 200
Guanine**	4 120	5 740	5 210	7 600	4 970
Carbohydrates***	14 000	60 000	4 180	126 000	60 900
Acid-insoluble					
Total	867 000	829 000	774 000	671 000	648 000
Protein	618 000	580 000	549 000	436 000	403 000
Nucleic acid					
Adenine	693	2 900	360	1 630	1 450
Guanine	928	4 960	651	3 220	3 280
	Counts/min/7.5 ml medium*				
Acid-soluble					
Total	$32.9 \cdot 10^6$	$32.5 \cdot 10^6$	$34.6 \cdot 10^6$	$32.5 \cdot 10^6$	$30.7 \cdot 10^6$
Glycine	$32.1 \cdot 10^6$	$30.8 \cdot 10^6$	$33.7 \cdot 10^6$	$30.7 \cdot 10^6$	$29.0 \cdot 10^6$
Acid-insoluble	14 900	15 100	32 700	37 600	21 700

* Combined results from duplicate incubation flasks. Acid-soluble fractions from duplicate flasks were pooled for analysis. Acid-insoluble fractions were analyzed separately.

** Includes purines from nucleotides and nucleosides.

*** This fraction would include carbohydrates and any other compounds not absorbed on Dowex-50 in the sodium form.

highest under 95 % oxygen. Glucose exhibited an apparent stimulation of the conversion of glycine to carbohydrates. Evidently glucose and its derivatives served to "trap" labeled carbohydrate intermediates.

As found by others[2,12], glucose greatly enhanced incorporation of glycine into acid-soluble adenine and nucleic acid adenine and guanine. A less marked stimulation of incorporation into acid-soluble guanine occurred. Although labeling of nucleic acid purines in the presence of glucose was similar under air and nitrogen (as was noted by LEPAGE[1]), it was higher under 95 % oxygen than under air, both with and without glucose. Labeling of cellular protein was also highest under 95 % oxygen; however, glucose depressed incorporation of label into this fraction.

TABLE II

SPECIFIC ACTIVITIES OF CELLULAR COMPONENTS AFTER 30-min INCUBATION WITH [2-^{14}C]GLYCINE

	$O_2 + CO_2$		$Air + CO_2$		$N_2 + CO_2$ + Glucose
	Control	+ Glucose	Control	+ Glucose	
	counts/min/mg				
Protein	1 1600 ± 100*	9 660 ± 120	9 770 ± 300	7 580 ± 100	7 700 ± 400
	Counts/min/μmole				
Nucleic acid adenine	172 ± 8	614 ± 4	74 ± 4	32[illegible] ± 9	309 ± 15
Nucleic acid guanine	225 ± 51	1 108 ± 64	169 ± 10	723 ± 37	802 ± 50
Acid soluble	5 120 ± 120	22 400 ± 200	2 250 ± 80	14 500 ± 400	15 600 ± 500
Acid soluble guanine	32 400**	45 500	36 400	48 800	39 300
Glycine	$3.30 \cdot 10^6$	$3.28 \cdot 10^6$	$3.43 \cdot 10^6$	$3.40 \cdot 10^6$	$3.31 \cdot 10^6$
Serine	$1.74 \cdot 10^6$	$2.11 \cdot 10^6$	$2.61 \cdot 10^6$	$2.46 \cdot 10^6$	$2.93 \cdot 10^6$
Taurine	3 600	22 000	2 940	22 100	33 300
Alanine	23 100	35 000	3[illegible] 000	26 500	25 200
Aspartic acid	14 600	20 200	7 940	13 800	14 100
Glutamic acid	6 150	16 800	8 000	14 800	27 000

* Difference from the mean of duplicate incubation flasks.
** Acid-soluble guanine from duplicate flasks was pooled for determination.

In the case of two extracts of medium (air + glucose and nitrogen + glucose), radioactivities of individual amino acids were determined instead of those of pre- and post-glycine fractions. For the group under air and with glucose, total radioactivities (in counts/min) in the medium were as follows: serine, 862000; taurine, 370000; alanine, 31000; aspartic acid, 21000; glutamic acid, 6080. Total radioactivities of these amino acids for the group under nitrogen and with glucose were quite similar.

Specific activities of protein, purines, and taurine and glutamic acid roughly paralleled the total counts/min (Tables I and II). However, specific activities of serine, alanine and aspartic acid underwent smaller changes with experimental conditions than did their total activities, and the changes were not always in the same direction. The cellular levels of these free amino acids are shown in Table III. Glucose caused a considerable increase in the concentration of serine and alanine, but a decrease in that of aspartic acid.

To see if these changes in amino acid concentrations occurred without added glycine, ascites cells were incubated in KRB medium with and without glucose, under air + CO_2. Triplicate flasks, each containing approx. 40 mg dry wt. cells in 4.0 ml total volume, were pooled for analysis; thus a larger amount was available for amino acid determinations than in the experiments with labeled glycine. However, results in Table IV have been calculated for 80 mg dry wt. cells to allow direct

TABLE III

CELLULAR CONTENTS OF VARIOUS AMINO ACIDS AFTER 30-min INCUBATION WITH [2-^{14}C]GLYCINE

	$O_2 + CO_2$		$Air + CO_2$		$N_2 + CO_2$ + Glucose
	Control	+ Glucose	Control	+ Glucose	
	μmoles/80 mg dry wt. cells				
Glycine	5.97	7.69	6.47	6.73	6.80
Serine	0.14	0.61	0.15	0.52	0.48
Taurine	4.00	4.14	4.22	3.96	3.45
Alanine	0.38	1.22	0.42	1.02	1.16
Aspartic acid	0.76	0.42	0.79	0.32	0.35
Glutamic acid	0.71	0.74	0.88	0.62	0.66

TABLE IV

CONTENTS OF VARIOUS AMINO ACIDS IN CELLS AND MEDIUM AFTER 30-min INCUBATION WITHOUT ADDED GLYCINE UNDER AIR + CO_2

	Cells		Medium	
	μmoles/80 mg dry wt. cells		μmoles/7.5 ml medium	
	Control	+ Glucose	Control	+ Glucose
Glycine	2.22	2.77	1.22	1.41
Serine	0.06	0.60	0.03	0.30
Taurine	7.64	6.69	0.20	0.50
Alanine	0.38	1.05	0.26	0.54
Aspartic acid	1.03	0.34	0.08	0.06
Glutamic acid	0.41	0.43	0.15	0.18
Threonine	0.44	0.30	0.48	0.32
Glycerophosphoethanolamine*	0.88	0.80	0.04	0.04
Phosphoethanolamine	0.96	0.76	0.03	0.04

* Expressed as microequivalents of leucine.

comparisons of Tables III and IV. Columns were developed through leucine, and all amino acids present in significant amounts were determined. Glucose caused striking increases in cellular and medium levels of serine and alanine, and a decrease in cellular aspartic acid. Moreover, the distribution ratios of aspartic acid and taurine were noticeably depressed. Thus, in the experiments with added (labeled) glycine, two interrelated effects of glucose must be distinguished: increased conversion of glycine to other free amino acids, as shown by a higher total radioactivity in these compounds, and changes in the concentrations of various amino acids, whether or not

excess glycine is added. Specific activities will reflect the combination of these two effects.

Glucose appeared to enhance the intracellular concentration of glycine by the cells, especially in the presence of 95 % O_2 (Table I). This small but consistent effect was demonstrated more clearly by computing distribution ratios (Table V). For the groups in which labeled glycine was added, the distribution was calculated from both total radioactivity and μmoles of glycine; the values agreed closely. As reported by others[10,11,24], the intracellular/extracellular ratio was lowered by increasing the total amount of glycine in the system.

TABLE V

PARTITIONING OF FREE GLYCINE BETWEEN CELLS AND MEDIUM*

		*Distribution ratio***
$O_2 + CO_2$	Control	11.2
	+ Glucose	15.1
Air + CO_2	Control	11.8
	+ Glucose	13.2
$N_2 + CO_2$	+ Glucose	14.0
Air + CO_2 (No added glycine)	Control	38.6
	+ Glucose	41.8

* It was assumed that the cells contained 84 % water and that this value remained constant.

** Distribution ratio = $\frac{\text{glycine/ml intracellular water}}{\text{glycine/ml extracellular water}}$

TABLE VI

SUMMARY OF GLYCINE INCORPORATION

		Total recovered counts not counts not present as free glycine (%)
$O_2 + CO_2$	Control	4.2
	+ Glucose	7.2
Air + CO_2	Control	4.1
	+ Glucose	7.4
$N_2 + CO_2$	+ Glucose	7.6

The percentages of total recovered counts which were not present as free glycine are summarized in Table VI. While the values were relatively low (4.1–7.6 %), the stimulating effect of glucose on the overall incorporation of glycine was consistent and striking. Although composition of the gas phase significantly affected incorporation into certain cellular components, its effect on total incorporation was not pronounced.

DISCUSSION

The finding that glycine was metabolized to various products in both cells and medium during a 30-min incubation is contrary to previous observations under similar experi-

mental conditions that no significant incorporation occurred[11]. The amount of incorporation into the acid-insoluble fraction is in good agreement with the values of ELLIS AND SCHOLEFIELD[17,18]. In addition, the present studies have shown that the cellular acid-soluble fraction and the medium contain significant amounts of radioactivity in compounds other than glycine. Nevertheless, when 2 mM glycine was added under our various incubation conditions, 92–96 % of the recovered radioactivity (and 80–87 % of the total initial radioactivity) was in the form of free glycine. It therefore seems that the assumption made by HEINZ AND PATLAK[25] in deriving equations for glycine transport (*viz.*, that under the present experimental conditions the radioactivity of the cells and medium is due entirely to free glycine) is justified, since the precision of the constants in these equations is considered to be only ± 10–20 % (see ref. 26). In experiments involving lower concentrations of glycine in the medium, the fraction undergoing metabolic interconversions might become large; however, as noted earlier, most studies of amino acid transport have involved relatively high levels of glycine.

The enhancement of glycine uptake by glucose is in agreement with results of ELLIS AND SCHOLEFIELD[17], who found a slight stimulation by 10 mM glucose under similar incubation conditions with an air atmosphere. (These workers equated the entire alcohol-soluble fraction to the glycine pool.) However, our results for the effect of glucose on incorporation of label into the insoluble fraction differed. We observed a moderate depression with 5.5 mM glucose, whereas ELLIS AND SCHOLEFIELD found a slight increase with 10 mM glucose during incubations of 45 min. QUASTEL AND BICKIS[15], on the other hand, reported that 10 mM glucose had no effect on the aerobic incorporation of 2 mM glycine into protein.

The stimulation of incorporation into protein and nucleic acids during a short incubation is in contrast to results in tissue culture, where high oxygen tensions had a powerful growth inhibitory effect, including a generalized reduction in biosynthesis of DNA, RNA, and protein[14]. However, these inhibitions occurred gradually and achieved maximal effectiveness after 24–48 h of exposure. An interesting contrast arises between the nucleic acid precursors glycine and adenine. Adenine apparently enters the Ehrlich ascites cell by free diffusion[27] but finds one or more acceptors readily available[28,29], whereas glycine enters via an active mechanism[25,30] and can be maintained there in the free state. Thus, the net effect achieved by the tumor cell in terms of accessibility of these two precursors appears to be similar.

The marked changes in concentrations of some free amino acids when glucose was added with or without excess glycine point out the importance of experimental conditions in studies of amino acid uptake. Moreover, glucose significantly affected the conversion of glycine to free amino acids and other cellular components. The observation that serine was the major acid-soluble product formed from glycine during the 30-min incubation is of particular importance, since these two amino acids compete with each other for concentrative uptake[31]. Under a given set of conditions, uptake could be influenced not only by the efficiency of competition but by that of conversion.

ELLIS AND SCHOLEFIELD[32] have shown an increase in the acid-soluble nucleotide content of Ehrlich ascites cells incubated aerobically in the presence of adenine and glucose. Results suggested that ATP was the predominant nucleotide synthesized. Maintenance of ATP levels could help explain the stimulatory effect of glucose on glycine uptake by an energy-dependent active transport system, and on various

metabolic interconversions. The increase in alanine and the corresponding decrease in aspartic acid might be the result of transaminase activity. Further investigation would be necessary to clarify the mechanisms involved in these changes brought about by glucose. However, it is clear that the glucose level must be considered in studies of amino acid uptake and incorporation.

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