Glutamine Metabolism in Ehrlich Ascites-Carcinoma Cells

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It is well known that an exogenous supply of glutamine is required by the Ehrlich ascites-carcinoma cell for optimum incorporation *in vitro* of ^{14}C -labelled amino acids into the tissue proteins (Rabinovitz, Olson & Greenberg, 1956; Fraser, 1960). Moreover, several investigators have shown that little, if any, free glutamine is found in these cells (Rabinovitz *et al.* 1956, 1957, 1959; Kvamme & Svenneby, 1961). Roberts & Frankel (1949) demonstrated that there is a lower glutamine content in tumours than in the corresponding normal tissues. Furthermore, the concentration of free glutamine in the C1498 leukaemia increases as the tumour regresses; there is no detectable free glutamine in the rapidly developing leukaemia (Roberts & Borges, 1955).

Glutamine is rapidly destroyed by the Ehrlich ascites cell (Rabinovitz *et al.* 1956, 1957, 1959; Kvamme & Svenneby, 1961), but little is known about the rate of glutamine oxidation and its conversion into compounds other than glutamate. The present study is devoted to an examination of the metabolism of glutamine by the Ehrlich ascites-carcinoma cells.

EXPERIMENTAL

Chemicals. [^{14}C]Glycine, DL-[^{14}C]leucine and L-[^{14}C]glutamine (^{14}C designates uniformly labelled with ^{14}C) were purchased from Merck and Co. Ltd., Montreal. Methionine sulfoximine was obtained from the California Corp. for Biochemical Research. Pyruvic acid, phenylpyruvic acid (as sodium salts), α -oxoglutaric acid and oxaloacetic acid were products of Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Cell preparations. Ehrlich ascites-carcinoma cells were grown for 6–8 days in Swiss white mice. The animals were killed by cervical dislocation and the ascites fluid was removed with a Pasteur pipette. The cells were washed several times by suspending in ice-cold, calcium-free Krebs–Ringer phosphate medium (Umbreit, Burris & Stauffer, 1951) and centrifuging for 20 sec. in the six-place head of the International (clinical model) centrifuge at 800g. The supernatant, containing most of the blood elements, was decanted and the procedure repeated twice. The packed-cell volume was determined by centrifuging the loosely packed cells for 2 min. at 800g and finally the cells

were resuspended in sufficient calcium-free Krebs–Ringer phosphate medium to give a 1:10 dilution. A portion (1 ml.) of this suspension was used in each incubation vessel. The dry weight of such a cell preparation was 9–10 mg.

Incubation methods. The final volume of each incubation flask was 3 ml. unless stated otherwise. Ehrlich ascites cells were incubated at 37°, either in conventional Warburg manometric vessels or in Erlenmeyer flasks which were shaken in a temperature-controlled water bath. The medium used was Krebs–Ringer phosphate, without calcium but sometimes with the further addition of sodium hydrogen carbonate, together with supplements as indicated in the individual experiments. Radioactive amino acids were diluted with the corresponding non-radioactive compounds. In manometric experiments, the radioactive amino acid (0.1 ml.) was placed in the side arm of the vessel, and was tipped in after thermal equilibration at 37° for 10 min. Subsequent incubation was normally for 1 hr.

Measurement of uptake of amino acids. The measurement of uptake of radioactive amino acids into Ehrlich ascites cells was carried out as follows. The cell suspension in the manometric vessel was transferred to an ice-cold centrifuge tube containing 7 ml. of Krebs–Ringer phosphate medium. The suspension was centrifuged for 45 sec. in the International clinical model centrifuge, the supernatant decanted and the cells were packed by further centrifuging for 1 min. The remaining supernatant fluid was removed with a syringe. To the washed cells was added 3 ml. of 95% ethanol, and a minimum period of 30 min. was allowed for extraction of the ethanol-soluble materials. After centrifuging, a sample of the supernatant was plated on an aluminium disk and counted for radioactivity. The above method is essentially that described by Johnstone & Scholefield (1959). The concentration factors given refer to the ratio of total ethanol-soluble radioactivity found per unit volume of cells compared with the radioactivity found per unit volume of medium. In the calculation of the concentration of radioactivity in the medium, allowance was made for the decrease in concentration of radioactivity in the medium during incubation due to utilization by the cells.

Measurement of incorporation of amino acids into proteins. The precipitate remaining after extraction with 95% ethanol was washed with 5% trichloroacetic acid and centrifuged again. In experiments where the uptake of amino acids was not measured, the reaction was stopped by the direct addition of an equal volume of 10% trichloroacetic acid to the incubation medium. Subsequent operations were performed according to the method of Simkin & Work (1957).

Measurement of radioactivity of respiratory carbon dioxide. To measure the radioactivity of the respiratory CO_2 , the alkali-impregnated filter paper from the centre well of the Warburg vessel was transferred to a centrifuge

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tube. The centre well was washed three to four times with water and the washings were added to the centrifuge tube. The contents of the tube were then made to approximately 5 ml. with water and mixed, and the tube was stoppered and left overnight for the potassium carbonate to be extracted from the paper. The following day the paper was removed and rinsed with water to bring the total volume of eluate to approximately 7 ml. To this was added 0.1 ml. of 2M-NH₄Cl and 2 ml. of a saturated solution of BaCl₂. A few drops of a dilute Na₂CO₃ solution were also added so that the final amount of BaCO₃ precipitate was 8–10 mg. After standing for 30 min., the BaCO₃ precipitate was centrifuged, washed twice with water, twice with acetone and plated from a small volume of acetone on a weighed aluminium planchet for counting. All the BaCO₃ precipitate was used.

Counting of radioactivity. The radioactivity was estimated with a thin mica-window Geiger-Müller tube and automatic Tracerlab scaler and timer. The efficiency of the counter was 6%. The counted data were corrected for background. No corrections for self-absorption were found to be necessary when measurements were made of uptake of amino acids or of incorporation into proteins (less than 5 mg. of protein/plate). The radioactivity of the BaCO₃ samples was corrected for self-absorption, a self-absorption curve for BaCO₃ constructed from the data of Calvin, Heidelberger, Reid, Tolbert & Yankwich (1949) being used.

Chromatography and radioautography. Ascending chromatography in two dimensions on 10 in. squares of Whatman no. 1 paper was always used, in various solvent systems. For the chromatography of amino acids, the solvents used were 2-methylpropan-2-ol-ethyl methyl ketone-aq. NH₃ (sp.gr. 0.88)-water (50:50:15:25, by vol.) (Wyatt, Loughheed & Wyatt, 1956) for the first phase, and for the second phase butan-2-ol-90% formic acid-water (100:15:25, by vol.). The radioactive spots were localized by radioautography and the radioactivities of the spots measured directly on the paper with a Tracerlab probe with a mica window of 28 mm. diam., and a Tracerlab scaler. The amino acids on the chromatograms were located after counting by dipping the papers into a solution of ninhydrin (1%, v/v, of a saturated aq. solution in acetone) and drying in air at room temperature. For the chromatography of organic acids, the solvents used were 2-ethoxyethanol-eucalyptol-90% formic acid (50:50:20, by vol.) for the first phase and 2-ethoxyethanol-aq. NH₃

(sp.gr. 0.88)-water (80:5:15, by vol.) for the second phase (Cheftel, Munier & Macheboeuf, 1953). The individual acids were located on the chromatograms by spraying with a neutral ethanolic solution (0.1%) of bromocresol green.

RESULTS

When Ehrlich ascites-carcinoma cells are incubated in Krebs-Ringer phosphate medium with [U-¹⁴C]glutamine, the isotope is readily concentrated within the cells, both anaerobically in the presence of glucose and aerobically. Aerobically, in the absence of further additions, the concentration factor for radioactivity between cells and medium is 6.1 after incubation for 1 hr. In the presence of glucose aerobically, the concentration factor is 4.9. These data agree with those of Kvamme & Svenneby (1961) that glucose, under aerobic conditions, inhibits the uptake of glutamine. Under anaerobic conditions in the presence of glucose, the concentration factor reaches 8.5. However, paper-chromatographic examination of the ethanol extracts of the cells shows that most of the radioactivity is present in compounds other than glutamine, chiefly glutamic acid and aspartic acid (Table 1). Anaerobically, an additional compound, probably α -oxoglutaric acid from its chromatographic properties, was detected. This compound reached its highest concentration in the presence of pyruvate, accounting for a total of 2300 counts/min. in the experiment reported in Table 1. The results in Table 1 also show that various compounds have a profound effect on the total concentration of radioactivity in the cells and on the distribution of radioactivity between the three major radioactive substances within the cells. Unless specifically stated otherwise, over 90% of the total radioactivity was distributed between glutamine, glutamic acid and aspartic acid.

The addition of sodium hydrogen carbonate to the incubation medium increases the respiratory

Table 1. *Concentration of [U-¹⁴C]glutamine and distribution of radioactivity among intracellular amino acids in Ehrlich ascites-carcinoma cells incubated aerobically and anaerobically*

Concentration of [U-¹⁴C]glutamine was 2 mM, initial activity 6.7×10^4 counts/min./vessel. Concentration of α -oxo acids and NaHCO₃ was 3 mM under aerobic conditions, and that of glucose 10 mM. Incubation was carried out in 3 ml. of Krebs-Ringer phosphate medium for 1 hr. at 37°, aerobically in air and anaerobically in N₂ + CO₂ (93:7). Anaerobically, 10 mM-glucose and 28 mM-NaHCO₃ were also present.

Additions	Conditions	Q _{O₂}	Total uptake of ¹⁴ C (counts/min.)	Total counts/min.		
				Glutamine	Glutamic acid	Aspartic acid
Nil	Aerobic	7.4	9 400	3 530	3 400	2 470
NaHCO ₃	Aerobic	12.4	14 800	3 750	5 200	6 050
Glucose	Aerobic	3.7	7 000	2 360	4 350	470
Q_{CO₂}						
Nil	Anaerobic	44	14 800	6 750	7 900	Nil
Pyruvate	Anaerobic	41	13 600	6 150	5 650	Nil
Phenylpyruvate	Anaerobic	41	14 000	6 250	7 080	Nil

rate from $Q_{0.1}$ 7.4 to $Q_{0.2}$ 12.4 and the amount of glutamine taken into the cell by nearly 60%. However, the concentration of free glutamine within the cell is only slightly raised above that of the control, but marked increases are seen in the concentrations of glutamic acid and especially of aspartic acid. Aerobically, the addition of glucose to the incubation medium almost completely eliminates the accumulation of aspartic acid. Anaerobically, in the presence of glucose, the concentration of radioactivity from $[U-^{14}C]$ glutamine within ascites cells reaches the same value as aerobically in the presence of sodium hydrogen carbonate, but no formation of aspartic acid from glutamine can be detected.

Effects of sodium hydrogen carbonate and oxaloacetate on the distribution of radioactivity from $[U-^{14}C]$ glutamine. The effects of sodium hydrogen carbonate on the distribution of radioactivity from $[U-^{14}C]$ glutamine in Ehrlich ascites cells as a function of time are presented in Fig. 1 (a and b). All the glutamine is utilized within 1 hr., and the rate of glutamine utilization is increased in the presence of sodium hydrogen carbonate. The rate of formation and the accumulation of glutamic acid is increased in the early stages of incubation (0–30 min.) in the presence of sodium hydrogen carbonate. Subsequently, the rate of glutamic acid utilization and the rate of aspartic acid formation are increased.

The effects of oxaloacetic acid are similar to those observed with sodium hydrogen carbonate, in that the rate of utilization of glutamine is increased (Fig. 1c), but little isotope accumulates as aspartic acid inside the cell. Moreover, the addition of oxaloacetic acid not only lowers the total incorporation of radioactivity into aspartic acid, but the total amount of aspartic acid itself (as revealed by ninhydrin) is greatly reduced.

Effects of glucose, pyruvate, lactate and phenylpyruvate on the distribution of radioactivity from $[U-^{14}C]$ glutamine. Glucose, pyruvate and lactate all influence the distribution of radioactivity from $[U-^{14}C]$ glutamine (Table 2). Glucose, pyruvate and lactate markedly decrease the concentration of $[^{14}C]$ aspartic acid, and increase the concentration of $[^{14}C]$ glutamic acid. Glucose, unlike pyruvate and lactate, decreases the total uptake of $[^{14}C]$ glutamine as well as the concentration of $[^{14}C]$ glutamine in the cells. Pyruvate and lactate stimulate the total uptake of radioactivity as well as the concentration of $[^{14}C]$ glutamine in the cells.

Phenylpyruvate has little effect on the total uptake of radioactivity, or on the concentration of $[^{14}C]$ glutamine in the cells. However, unlike pyruvate and lactate, phenylpyruvate decreases the concentration of $[^{14}C]$ glutamate in the cells.

Formation of $^{14}CO_2$ from $[^{14}C]$ glutamine by Ehrlich

Fig. 1. Distribution of radioactivity in intracellular amino acids of Ehrlich ascites-carcinoma cells incubated with L- $[U-^{14}C]$ glutamine. Each incubation mixture contained, in 6 ml. of Krebs-Ringer phosphate medium: 6 μ moles of L- $[U-^{14}C]$ glutamine (6.7×10^4 counts/min.) and 45 mg. dry wt. of washed cells. In (a) no further additions were made; in (b) 3 mM- $NaHCO_3$ was present; in (c) 3 mM-sodium oxaloacetate was present. Incubation was at 37° in air; samples (1 ml.) were removed at intervals for analysis of glutamine (●), glutamic acid (▲) and aspartic acid (○).

ascites-tumour cells. Typical results presented in Table 2 show the respiration and the production of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]$ glutamine by Ehrlich ascites cells and the influence of glucose, lactate and α -oxo acids. With the exception of glucose and lactate, the compounds tested increase the production of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]$ glutamine. Sodium hydrogen carbonate and oxaloacetate also increase the $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]$ glutamine. Increases in respiratory rate are noted in the presence of sodium hydrogen carbonate, oxaloacetate and pyruvate. However, the increase in the production of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]$ glutamine is usually greater than the increase in the respiratory rate. The largest difference is seen with oxaloacetate, which increases the production of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]$ glutamine by 93 %, but the increase in the respiratory rate is only 34 %. Whereas respiration is inhibited by glucose (Crabtree effect) by nearly 40 %, the production of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]$ glutamine is inhibited much less (Table 2). This means that the expired CO_2 actually has a higher specific activity in the presence of glucose than in its absence. Since glucose is oxidized by Ehrlich ascites cells in addition to being glycolysed (Bloch-Frankenthal & Weinhouse, 1957), it might have been expected that the specific activity of the $^{14}\text{CO}_2$ would be decreased, because of isotopic dilution.

Effects of malonate on the distribution of radioactivity from $[\text{U-}^{14}\text{C}]$ glutamine on production of $^{14}\text{CO}_2$. Although the presence of 30 mM-malonate causes a 44 % inhibition of respiration (Table 2), there is little significant change in the total uptake of radioactivity. However, the distribution of the radioactivity is appreciably altered. The concentration of ^{14}C aspartic acid is decreased whereas that of ^{14}C glutamic acid is increased. The decrease in ^{14}C from $[\text{U-}^{14}\text{C}]$ glutamine in the presence of malonate is equivalent to the decrease in the respiratory activity (Table 2). A small amount of

^{14}C succinate also accumulates in the presence of malonate.

Effects of glutamine and α -oxo acids on incorporation of $[\text{1-}^{14}\text{C}]$ glycine into proteins. It is well known that glutamine influences the rate of amino acid incorporation into proteins of Ehrlich ascites cells (Rabinovitz *et al.* 1956; Fraser, 1960; Quastel, 1961). The present data have shown that various compounds alter the metabolism of $[\text{U-}^{14}\text{C}]$ glutamine by these cells, and the effects of some of the substances on the incorporation of $[\text{1-}^{14}\text{C}]$ glycine into proteins was therefore examined. Under aerobic conditions, pyruvate, oxaloacetate, glutamine and glucose stimulate the incorporation (Table 3). The addition of methionine sulfoximine abolishes the effects of pyruvate, oxaloacetate and glucose, but not of glutamine. Similar results are obtained with $[\text{1-}^{14}\text{C}]$ leucine used in place of $[\text{1-}^{14}\text{C}]$ glycine.

Phenylpyruvate inhibits the incorporation of both $[\text{1-}^{14}\text{C}]$ leucine and $[\text{1-}^{14}\text{C}]$ glycine into the proteins of Ehrlich ascites cells. It is apparent that the incorporation is more extensively inhibited by phenylpyruvate than is the respiration. There is considerable variation in the extent of inhibition that may be caused by a given concentration of phenylpyruvate with different preparations of cells; e.g. variations in the specific activity of the proteins from 40 to 110 counts/min./mg. have been obtained with no inhibitor present. With 3 mM-phenylpyruvate, inhibitions of leucine and glycine incorporation ranging from 30 to 70 % have been observed. Phenylalanine and phenyl-lactate are much less inhibitory than is phenylpyruvate, and phenylacetate is without inhibitory effect. α -Oxoglutarate has little effect on the incorporation of amino acids into proteins and on the respiratory activity.

The inhibition caused by phenylpyruvate may be reversed under aerobic conditions by any of the compounds which stimulate incorporation, namely pyruvate, oxaloacetate, glucose and glutamine.

Table 2. *Respiration, uptake and distribution of radioactivity from $[\text{U-}^{14}\text{C}]$ glutamine in intracellular amino acids of Ehrlich ascites-carcinoma cells*

Concentration of L- $[\text{U-}^{14}\text{C}]$ glutamine was 2 mM, initial activity 6.7×10^4 counts/min./vessel. Concentration of α -oxo acids was 3 mM, DL-lactate 6 mM, glucose 10 mM and malonate 30 mM. Incubation was in 3 ml. of Krebs-Ringer phosphate medium for 1 hr. at 37° in air.

Expt. no.	Additions	Q_{O_2}	%	Total $^{14}\text{CO}_2$ (counts/min.)	%	Total uptake (counts/min.)	Total counts/min.		
							Glutamine	Glutamic acid	Aspartic acid
1	Nil	9.0	100	7 250	100	10 100	3 000	3 000	3 920
	Glucose	5.6	60	5 540	77	8 600	1 930	6 070	870
	Pyruvate	11.1	122	9 250	128	11 300	3 420	5 460	1 010
	Lactate	11.1	122	7 480	103	11 400	3 420	6 500	1 180
2	Nil	8.8	100	6 900	100	11 400	3 280	3 050	5 040
	Phenylpyruvate	9.3	106	8 900	129	10 900	3 250	2 330	5 400
3	Nil	8.5	100	7 630	100	11 700	3 720	4 430	4 760
	Malonate	3.7	44	3 000	39	11 300	3 890	6 570	750

Table 3. *Influence of α -oxo acids, glutamine, glucose and methionine sulfoximine on incorporation of [1- 14 C]glycine into the proteins of Ehrlich ascites-carcinoma cells under aerobic conditions*

Concentration of [1- 14 C]glycine was 2 mM, initial activity 10^5 counts/min./vessel. Concentration of glutamine and α -oxo acids was 3 mM, glucose 10 mM and that of methionine sulfoximine 2 mM. Incubation was for 1 hr. at 37° in Krebs-Ringer phosphate medium and the gas phase was air. In incubations with glucose, 28 mM- NaHCO_3 was also present and the gas phase was $\text{O}_2 + \text{CO}_2$ (93:7). A preincubation period of 30 min. with methionine sulfoximine was given before the addition of other substrates.

Additions	Expt. 1		Expt. 2		Expt. 3	
	Q_{O_2}	Incorporation (counts/min./mg. of protein)	Q_{O_2}	Incorporation (counts/min./mg. of protein)	Q_{O_2}	Incorporation (counts/min./mg. of protein)
Nil	7.6	52	8.0	71	7.7	52
Phenylpyruvate	4.9	21	6.5	39	—	—
Pyruvate	9.0	86	10.3	116	10.0	100
Oxaloacetate	10.7	96	11.5	126	11.7	105
α -Oxoglutarate	8.0	57	9.5	75	—	—
Glutamine	8.5	80	9.5	104	9.2	87
Glucose	—	75	—	—	—	85
Phenylpyruvate + pyruvate	8.7	82	—	—	—	—
Phenylpyruvate + oxaloacetate	9.9	90	—	—	—	—
Phenylpyruvate + α -oxoglutarate	5.8	20	—	—	—	—
Phenylpyruvate + glutamine	9.0	81	10.3	100	—	—
Phenylpyruvate + glucose	—	76	—	—	—	—
Glutamine + pyruvate	—	—	11.5	126	—	—
Glutamine + oxaloacetate	—	—	13.3	167	—	—
Glutamine + α -oxoglutarate	—	—	10.3	129	—	—
Methionine sulfoximine	—	—	—	—	7.7	44
Glutamine + methionine sulfoximine	—	—	—	—	9.2	87
Pyruvate + methionine sulfoximine	—	—	—	—	9.2	55
Oxaloacetate + methionine sulfoximine	—	—	—	—	10.0	50
Glucose + methionine sulfoximine	—	—	—	—	—	50

No reversal of the inhibition caused by phenylpyruvate can be obtained with a number of other amino acids tested, including glutamic acid, aspartic acid, asparagine, leucine, valine, phenylalanine and α -aminobutyric acid at equimolar concentrations. High concentrations of glutamic acid (20 mM), however, are as effective as 3 mM-glutamine in reversing the inhibition caused by phenylpyruvate under aerobic conditions. In addition, glutamine is the only amino acid tested which stimulates the incorporation of [1- 14 C]glycine into proteins.

Effects of α -oxo acids on amino acid incorporation under anaerobic conditions. The effects of the α -oxo acids on amino acid incorporation were determined under conditions where oxidation and conversion into glutamine cannot take place. Ehrlich ascites cells are capable of incorporating amino acids into proteins under anaerobic conditions in the presence of glucose (Rabinovitz, Olson & Greenberg, 1955; Quastel & Bickis, 1959). Under these conditions, significant inhibitions of incorporation of [1- 14 C]glycine into proteins are obtained with pyruvic acid, phenylpyruvic acid and oxaloacetic acid (Table 4). α -Oxoglutarate is without effect. The inhibitions may be reversed by the addition of glutamine to the incubation medium, but no reversal can be obtained with a number of other amino acids. The extent of inhibition by phenylpyruvate is less under anaerobic conditions than

Table 4. *Influence of α -oxo acids and glutamine on incorporation of [1- 14 C]glycine into proteins of Ehrlich ascites-carcinoma cells under anaerobic conditions*

Concentration of [1- 14 C]glycine was 2 mM, initial activity 10^5 counts/min./vessel. Concentration of glutamine and α -oxo acids was 3 mM. Incubation was for 1 hr. at 37° in Krebs-Ringer NaHCO_3 medium with 10 mM-glucose in an atmosphere of $\text{N}_2 + \text{CO}_2$ (93:7). The acids were present as sodium salts.

Additions	Incorporation (counts/min./mg. of protein)
Nil	94
Phenylpyruvate	76
Pyruvate	64
Oxaloacetate	62
α -Oxoglutarate	95
Glutamine	125
Glutamine + phenylpyruvate	124
Glutamine + pyruvate	125
Glutamine + oxaloacetate	120
Glutamine + α -oxoglutarate	119

under aerobic and is usually of the order 15–20%. It therefore appears that the stimulation of incorporation by pyruvate and oxaloacetate depends on the oxidation of these substances and their conversion into glutamine.

Glutamine as a source of energy. These studies showed that the effect of glutamine on the incorporation of amino acids into proteins is not merely

Table 5. *Influence of glutamine, oxaloacetate, glucose and sodium hydrogen carbonate on the incorporation of [1-¹⁴C]glycine into the proteins of Ehrlich ascites-carcinoma cells*

Concentration of [1-¹⁴C]glycine was 2 mM, initial activity 10⁵ counts/min./vessel. Concentration of glutamine and sodium oxaloacetate was 3 mM, glucose 10 mM and NaHCO₃ 28 mM. Incubation was for 1 hr. at 37° in 3 ml. of Krebs-Ringer phosphate medium.

Gas phase	Additions	Incorporation (counts/min./mg. of protein)	
		Expt. 1	Expt. 2
O ₂	Nil	78	85
	Glutamine	95	105
	Oxaloacetate	—	123
	Glutamine + oxaloacetate	—	164
O ₂ + CO ₂ (93:7)	NaHCO ₃	58	77
	NaHCO ₃ + glutamine	114	170
	NaHCO ₃ + oxaloacetate	—	118
	NaHCO ₃ + glutamine + oxaloacetate	—	158
	NaHCO ₃ + glucose	87	—
	NaHCO ₃ + glucose + glutamine	113	—
N ₂ + CO ₂ (93:7)	NaHCO ₃ + glucose	88	—
	NaHCO ₃ + glucose + glutamine	114	—

due to glutamine acting as a constituent amino acid of the newly formed proteins as suggested by Rabinovitz *et al.* (1956). In most preparations of cells with Q₀ less than 10, glutamine increases Q₀. The increase in Q₀, however, is less than the increase in amino acid incorporation (Table 3). Optimum incorporation and respiratory activity are obtained when the cells are incubated with glutamine and oxaloacetic acid, although glutamine in association with other α -oxo acids stimulates the amount of incorporation and respiratory activity to a greater degree than glutamine alone (Table 3).

The same incorporation of amino acids into proteins occurs in the presence of glutamine and oxaloacetate and with glutamine alone if the incubation is carried out in a medium containing a phosphate-bicarbonate buffer, pH 7.4, rather than phosphate alone at the same pH. No further enhancement of incorporation is obtained upon the addition of oxaloacetate or glucose (Table 5).

Identical stimulations of incorporation of glycine into proteins by glutamine in the presence of 28 mM-sodium hydrogen carbonate (gas phase: O₂ + CO₂, 93:7), can be obtained with 3 mM-sodium hydrogen carbonate in an atmosphere of air, when measurements of respiration can also be carried out. The increases in the respiratory rate of Ehrlich ascites cells observed in the presence of glutamine upon the addition of either sodium hydrogen carbonate or of oxaloacetate to the incubation medium occurs concomitantly with increased rates of incorporation of amino acids into proteins.

DISCUSSION

The inhibitions of amino acid incorporation into proteins by α -oxo acids under anaerobic conditions, and the specific reversal of these inhibitions by

glutamine, indicate that the inhibitions are due to a depletion of intracellular glutamine. This interpretation is supported by the following facts. (a) A number of α -oxo acids have been shown to accelerate the deamidation of glutamine (Meister & Tice, 1950; Meister, 1953, 1954). This reaction is associated with a transamination reaction between glutamine and an α -oxo acid, resulting in the formation of ammonia, α -oxoglutarate and the corresponding amino acid. (b) It is well known that glutamine is a limiting factor in amino acid incorporation into proteins in Ehrlich ascites cells (Rabinovitz *et al.* 1956) and that (c) tumour cells contain low concentrations of glutamine (Roberts & Frankel, 1949). Therefore compounds which decrease the amount of glutamine in the cells would be expected to inhibit amino acid incorporation into the proteins. In addition, methionine sulfoximine, which inhibits glutamine synthesis, inhibits amino acid incorporation (Rabinovitz *et al.* 1956).

The failure to obtain inhibition of amino acid incorporation under anaerobic conditions, with α -oxoglutarate, in contrast with the effects of pyruvate, oxaloacetate and phenylpyruvate, is probably due to the limited permeability of α -oxoglutarate.

Attempts were made to determine the depletion of intracellular glutamine that may have been brought about by α -oxo acids under anaerobic conditions. However, the endogenous concentration of glutamine in these cells is below the level of detection with the present techniques. This observation is consistent with those of previous investigators.

In agreement with data previously presented by Fraser (1960) we have observed that aerobic incubation of Ehrlich ascites cells in a medium con-

taining sodium hydrogen carbonate without further supplements inhibits incorporation of amino acids into proteins, whereas, in the presence of glutamine, marked stimulations are observed. Such an effect of sodium hydrogen carbonate can now be attributed to its accelerating the oxidation of glutamine. Thus in the absence of exogenous glutamine, sodium hydrogen carbonate causes a more rapid depletion of intracellular glutamine. However, with added glutamine, the increased respiratory activity in the presence of sodium hydrogen carbonate leads to an increased incorporation of amino acids into proteins since extra energy and glutamine are both available.

Phenylpyruvate is the only keto acid to inhibit amino acid incorporation into protein aerobically. This is no doubt due to the fact that aerobically pyruvate and oxaloacetate are rapidly oxidized. Increases in the respiratory activity between 20 and 40% are obtained with pyruvate and oxaloacetate. There is no indication that phenylpyruvate is oxidized. In fact, it usually inhibits respiration at concentrations above 3 mM. It is already known that phenylpyruvate inhibits the incorporation of L-[^{14}C]tyrosine into liver proteins (Bickis, Kennedy & Quastel, 1957).

The results indicate that the oxidation of pyruvate, oxaloacetate and glucose leads to the synthesis of glutamine. The inhibitory effects of phenylpyruvate on the incorporation of amino acids are reversed aerobically by pyruvate, oxaloacetate, glucose and glutamine, whereas anaerobically, in the absence of α -oxo acid oxidations, only glutamine reverses the inhibitions.

An examination of the metabolism of [U- ^{14}C]glutamine by Ehrlich ascites-carcinoma cells has revealed that the major end products of glutamine metabolism are carbon dioxide, glutamic acid and aspartic acid. Kvamme & Svenneby (1961) reported that 50–70% of the glutamine utilized is converted into glutamic acid. The present results indicate that the amount of aspartic acid formed from glutamine may exceed the amount of glutamic acid, but that the time-factor must be considered in assessing the extent of conversion of glutamine into other compounds. Recent studies (Kini & Quastel, 1959; Krebs & Bellamy, 1960; Borst & Slater, 1960) have shown the interconversion of glutamic acid and aspartic acid in several tissues. This clearly arises from transamination of oxaloacetate by glutamate and subsequent oxidation of α -oxoglutarate along the citric acid cycle. Krebs & Bellamy (1960) and Borst & Slater (1960) have suggested that glutamic dehydrogenase plays a limited role in glutamate oxidation, transamination being the predominant reaction for removal of the α -amino group. Transamination may also occur directly between glutamine and oxalo-

acetic acid, according to the following scheme (Meister, 1953, 1954):

(1) Glutamine + oxaloacetic acid $\rightarrow$ α -oxoglutaramate + aspartic acid.

(2) α -Oxoglutaramate $\rightarrow$ α -oxoglutaric acid + ammonia.

(3) Sum: glutamine + oxaloacetic acid $\rightarrow$ aspartic acid + α -oxoglutarate + ammonia.

In accordance with this scheme the addition of any α -oxo acid which transaminates with glutamine or glutamic acid, resulting in the formation of α -oxoglutarate and subsequently oxaloacetate, would be expected to increase the production of $^{14}\text{CO}_2$ from [U- ^{14}C]glutamine. The results have shown that pyruvate and phenylpyruvate cause an increased production of $^{14}\text{CO}_2$ from [U- ^{14}C]glutamine. The addition of glutamine alone to Ehrlich ascites cells does not result in optimum respiratory activity, although there is a rapid conversion of glutamine into glutamic acid. It appears that the oxidation of glutamine–glutamic acid is limited by the rate of oxidation of glutamic acid by glutamic acid dehydrogenase. In the absence of added keto acids, it would be expected that inhibitions of oxaloacetate production would decrease the extent of $^{14}\text{CO}_2$ and [^{14}C]aspartate production from [U- ^{14}C]glutamine. Thus the addition of malonate, which inhibits succinic acid dehydrogenase, causes an extensive inhibition of $^{14}\text{CO}_2$ and [^{14}C]aspartate production whereas the [^{14}C]glutamate accumulation is greatly increased. The accumulation of only small amounts of succinate in the presence of malonate also supports the conclusion that the oxidation of glutamate requires the presence of oxaloacetate under the present experimental conditions. The present studies also indicate that the accumulation of aspartate from glutamine or glutamic acid is quantitatively important only in those cells which have an insufficient supply of acetyl-CoA. Thus, the addition of compounds such as glucose, lactate and pyruvate, but not of phenylpyruvate, the oxidation of which leads directly to the formation of acetyl-CoA, brings about substantial decreases in the accumulation of [^{14}C]aspartate, whereas the [^{14}C]glutamate concentration is greatly increased. Kini & Quastel (1959), in their studies on carbohydrate–amino acid interrelations, also concluded that the accumulation of [^{14}C]aspartic acid from [^{14}C]carbohydrate sources is increased if the supply of acetyl-CoA is limited.

SUMMARY

1. The oxidation of glutamine in Ehrlich ascites-carcinoma cells proceeds through α -oxoglutarate and other intermediates of the tricarboxylic acid cycle. In cells lacking sufficient suitable precursors of acetyl-coenzyme A, the

oxidation of glutamine results in the formation of aspartic acid.

2. In the presence of substances giving rise to acetyl-coenzyme A, the rate of oxidation of glutamine is decreased, but the extent of oxidation is increased.

3. The rate of glutamine oxidation is increased by sodium hydrogen carbonate and α -oxo acids.

4. The incorporation of amino acids into proteins is inhibited under anaerobic conditions by pyruvate, oxaloacetate and phenylpyruvate. This inhibition is reversed specifically by glutamine.

5. The incorporation of amino acids into proteins is inhibited under aerobic conditions by phenylpyruvate, and this inhibition is reversed by glutamine, pyruvate, oxaloacetate and glucose.

6. Glutamine may serve as a source of energy under certain circumstances for the incorporation of amino acids into proteins by these cells.

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Biochemical Changes in the Tissues of Animals Injected with Iron

3. LIPID PEROXIDATION*

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The presence in tissues of acid-fast polymers, composed of condensation products of peroxidized lipid with protein, is characteristic of a number of experimental and clinical conditions, of which old age is by far the most common. Lipofuscin polymers of apparently similar type are readily produced in animals injected with massive doses of

iron-dextran (Golberg, Smith & Martin, 1957), and constitute part of the evidence for a relative lack of antioxidant capacity accompanying tissue siderosis. In the rat this effect is manifested as vitamin E deficiency, accentuated by an inadequate supply of dietary tocopherol or by an excessive proportion of unsaturated fat, but not by lack of vitamin A (Golberg & Smith, 1958, 1960).

The biosynthesis of acid-fast polymers depends on the initial formation of auto-oxidized unsaturated lipids (Casselman, 1951). We have pre-

* Part 2: Golberg, Martin & Smith (1960b).

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