The Production of Pyruvic Acid, Oxaloacetic Acid and α -Oxoglutaric Acid from Glucose by Tissue in Culture

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Glycolysis and respiration of cancer tissue *in vitro* have been the objects of many studies (see Warburg, 1956a, b; Weinhouse, 1955, 1956). Work with tissue in culture offers the general advantages that the cells are not only physiologically active, since in principle they proliferate indefinitely, but that they are also uniform. Therefore, the characteristic metabolism of cancer tissue is not 'diluted' by the metabolism of healthy tissue or modified by it as it is in intact animals. Moreover, the composition of the medium and the gas phase as well as the temperature can be varied at will within certain limits. However, the mass of tissue in culture is small (in many cases, 0.1–1 mg. dry weight/roller tube), and therefore sensitive methods are needed to determine the metabolites.

In these Laboratories, the production, from radioactive substrates, of certain metabolites by normal mesenchyma tissue and by HeLa tumour tissue has been determined in various experimental conditions (see Broda, Suschny & Kellner, 1958). Special attention was given to the mechanisms of energy production. The ratio of the amounts of radiocarbon in lactic acid (end-product of fermentation) and in carbon dioxide (end-product of respiration) after incubation of the tissue with radioglucose for a few hours or days was called the '*q* value' (Suschny, Kellner, Broda, Figdor & Rücker, 1958). The term 'fermentation' rather than 'glycolysis' is used here in view of the possibility that lactic acid arises through other pathways than that of Embden-Meyerhof-Parnas, e.g. through the pentose phosphate cycle.

Both with mesenchyma and HeLa tissue, the *q* values tend to increase when the tissues are exposed to certain damaging influences, e.g. treatment with trypsin, but they also increase with an improvement in the supply of glucose (Suschny *et al.* 1958). A shift from respiration to aerobic glycolysis with increasing supply of glucose is, of course, known from manometric experiments as the 'Crabtree effect' or—more frequently though misleadingly—as the 'inverse Pasteur effect'.

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Significant differences between mesenchyma and HeLa tissue were found only in extreme conditions: when the supply of glucose was reduced to a very low level, and the *q* values consequently were brought down radically, only the normal tissue survived. With HeLa, no conditions were found where *q* was less than 1, i.e. where the utilization of glucose by respiration predominated over that by fermentation. On the other hand, the suppression of respiration by cyanide was, within a rather wide range of concentrations, tolerated by HeLa, but not by mesenchyma. Both results point to a shift in relative importance from respiration to aerobic glycolysis on the transition from a normal cell to a cancer cell.

In following up this line of work, it was important to determine the stationary concentrations of key intermediates of glycolysis and respiration in the same two kinds of tissue. Pyruvic acid, oxaloacetic acid and α -oxoglutaric acid were chosen. The concentrations of oxaloacetic acid and α -oxoglutaric acid relative to those of pyruvic acid should be depressed in tumour tissue if the operation of the citric acid cycle is partly inhibited. Again, the assay was done by radioactivity so that the amounts of the keto acids derived from (radioactive) glucose may be obtained. These amounts are exceedingly small.

In these experiments only about 0.1 μ C of radiocarbon/ml. was applied. The resulting radiation dose, approx. 0.2 rep/day (equivalent to a γ -ray dose of 0.2 r./day), is not expected to damage the tissue to any noticeable extent, and in fact no damage has been observed. Damage has, however, been noted (in other experiments with tissue in culture) from doses of the order of 10 reps/day, as delivered by 1 μ C of radiophosphorus/ml. (Kellner, Suschny, Broda & Rücker, 1959).

EXPERIMENTAL

The mesenchyma tissue ('fibroblasts') was obtained from legs of chick embryos 10 days old and grown in roller tubes at 37° as described previously (Kellner & Stockinger, 1957). The nutrient medium (1 ml./tube) consisted of 4% of

chick embryo extract, 40% of human ascitic fluid and 56% of Gey's buffered salt solution (8.0 g. of NaCl, 0.37 g. of KCl, 0.23 g. of NaHCO_3 , 0.17 g. of CaCl_2 , 0.21 g. of MgCl_2 , 0.15 g. of Na_2HPO_4 , 0.03 g. of KH_2PO_4 , 0.07 g. of MgSO_4 and 1 g. of glucose/l.). (The glucose content of this mixture was very nearly 1 mg./ml.) About 4–5 days after explantation, the tissue in each tube was treated for 20 min. at pH 8.4 with 1 ml. of pancreatin solution with an activity equivalent to 0.11 mg. of crystallized trypsin to produce a cell suspension, the contents of the tubes were combined, the trypsin was removed by repeated washing with the buffered salt solution and the cells were resuspended in embryo extract and counted in a haemocytometer. A number of plasma-coated roller tubes were charged with 0.1 ml. of the suspension each, and incubated. Within a few hours, the cells settled on the wall as a monolayer. Now 1 ml. of nutrient medium was added and the incubation continued. At any time the cells could be counted again, in some of the tubes, after resuspending with trypsin. It was found that the cell counts decreased during the first day after the monolayer was formed; thereafter these counts increased. Monolayers of HeLa cells were made in an analogous manner from the HeLa tissue grown in these Laboratories for several years. The average dry weight of one HeLa cell is 7.15×10^{-10} g. (Leslie, Fulton & Sinclair, 1957), and the average weight of one of our chicken mesenchyma cells is about two-thirds of that value.

Randomly labelled radioglucose was obtained from Amersham and purified here by paper chromatography (Sverak, Suschny & Broda, 1953). Three days after the formation of the monolayer, the radioglucose was added along with 1 ml. of new nutrient medium, and incubation continued for 24 hr. Thereafter the metabolites were isolated, subjected to combustion by a wet method (Van Slyke & Folch, 1940) after addition of a known amount of inactive carrier, the radioactivity of the CO_2 measured with the gas Geiger counter (with almost 100% yield) for 16 min. and the activity referred to the total quantity of the metabolite; the measuring methods were the same as described before (Broda, 1955; Suschny *et al.* 1958). Whenever the original activity of the glucose was different from $0.1 \mu\text{C}/\text{tube}$, the activities of the metabolites were calculated to this standard value.

In the work to be reported here, lactic acid and CO_2 , as well as the three keto acids, were determined. The assay of the lactic acid and the CO_2 (Suschny *et al.* 1958) was always done on parallel but identical roller tubes; the detailed results will be given elsewhere (O. Suschny, G. A. Abdel-Tawab, G. Kellner, E. Broda & W. Rücker, in preparation), but some of the results will be used below. The numbers of cells per tube, at the end of each experiment, were also measured in parallel tubes. All determinations of metabolites or cell counts were done on three separate samples and the averages of these three independent results listed in the tables.

The principle of the isolation of the keto acids is as follows (Abdel-Tawab, Broda & Kellner, 1959); the tubes are cooled by ice immediately after incubation, the contents are homogenized and deproteinized and all carbonyl compounds are converted into the yellow 2:4-dinitrophenylhydrazones; these are extracted with ether, and the dinitrophenylhydrazones of the keto acids separated from other dinitrophenylhydrazones through the solubility of the former in alkali. Finally, the individual keto acid

dinitrophenylhydrazones are isolated by descending chromatography on paper impregnated with 0.1 M-phosphate buffer, pH 7.4 (composed of 81.8 ml. of 0.1 M- Na_2HPO_4 and 18.2 ml. of 0.1 M- KH_2PO_4), with a solvent system consisting of butanol saturated with aq. 3% (w/v) NH_3 . The dinitrophenylhydrazone of each keto acid is eluted from its spot and determined by colorimetry or by gas Geiger counting. Special colorimetric tests with known quantities of keto acids showed the yields to be at least 80%. As the radioactivities of the keto acids were rather low, the contents of several (as a rule, 4) parallel tubes were combined for isolation; however, the values in Table 1 are referred to single tubes.

It was necessary to find out whether the medium by itself, i.e. in absence of tissue, metabolized radioglucose. Therefore some blank tubes were incubated along with the tubes containing tissue, and the metabolites were isolated and their radioactivities measured. However, for CO_2 and the keto acids the blank values were always negligible (Suschny *et al.* 1958; Abdel-Tawab *et al.* 1959). Some small amounts of a radioactive acid behaving like lactic acid were found in the absence of tissue, and their radioactivity was deducted from that found in the presence of tissue; however, this correction was small.

In most experiments, the keto acids were determined separately within the tissue and within the medium. In this case the nutrient medium from each tube was decanted, the tissue quickly rinsed twice with 1 ml. of ice-cold saline (0.9% NaCl) solution, and the washings were combined with the decanted medium. Thereafter homogenization of the tissue, separate deproteinization of both tissue and supernatant medium, and separate treatment with 2:4-dinitrophenylhydrazine were carried out as described. It should be remembered, however, that with this procedure the keto acids within the plasma coating of the tubes are measured together with those actually within the tissue.

RESULTS

In Tables 1 and 2, the experiments with either mesenchyma or HeLa tissue are arranged in the order of decreasing numbers of cells per tube, i.e. of increasing supply of glucose per cell.

The radioactivities (Table 1) are given in 'disintegrations/min.' in the whole of the sample. The values listed were obtained, of course, by calculating, from the activities actually measured, the total amount of carbon in the sample (by multiplication) and that in a single tube (by division) (see Suschny *et al.* 1958). The lowest activities of the keto acids, accepted as significant, were 6 min.^{-1} (per 4 combined tubes) above a background of 40 min.^{-1} . As all values given in the table are means of three independent determinations on parallel samples (4 tubes each), the standard deviation corresponding to this minimum activity is $\pm 1.0 \text{ min.}^{-1}$. Measured activities of 2 min.^{-1} or less (per 4 tubes), again with an s.d. of $\pm 1.0 \text{ min.}^{-1}$, are considered as not significant, and entered in the table as zero. In fact, not a single measurement yielded an apparent activity of more than 2 but less than 6 min.^{-1} .

Table 1. *Radioactivities (per tube) of metabolites*

For details of incubation and extraction of cells, see Experimental section. For definition of *q*, see introduction. The cell counts refer to the end of the incubation, except for Expts. F1 and F3, in which they refer to the beginning of the incubation. The final cell counts are not known in Expts. F1 and F3; therefore the keto acids cannot be related to the cell counts.

Expt.	10 ⁻⁵ × cell count	Lactic acid		CO ₂ (disintegrations/min.)	q	Pyruvic acid (disintegrations/min.)			Oxaloacetic acid (disintegrations/min.)			α-Oxoglutaric acid (disintegrations/min.)		
		(disintegrations/min.)				Tissue	Medium	Total	Tissue	Medium	Total	Tissue	Medium	Total
						(a) Fibroblasts								
F1	15	11 900		9700	1.2	—	—	628	—	—	24	—	—	57
F2	9, 54	29 100		5070	5.7	38	291	329	17	0	17	19	0	19
F3	2, 5	39 200		2470	15.8	—	—	385	—	—	25	—	—	20
F4	3, 81	35 500		2030	17.5	30	790	820	20	0	20	20	0	20
						(b) HeLa								
H1	10	31 630		8830	3.6	15	162	177	9	15	24	6	44	50
H3	4, 5	11 330		1170	9.6	27	325	352	20	30	50	18	38	56
H2	2	15 130		1480	10.2	12	105	117	7	11	18	8	38	46
H4	0, 7	3 460		180	19.2	33	288	321	23	49	72	20	34	54

In Table 2, the quantities of the keto acids are expressed in $\mu\text{g.}/10^7$ cells of the glucose from which they were derived ('equivalent glucose'). For the purpose, the specific activity of the carbon of the keto acids at the time of isolation (i.e. after incubation for 24 hr.) is assumed to be the same as that of the carbon in the glucose. This assumption is safe, as much more radiocarbon was found in the two end-products of metabolism (lactic acid and CO₂) than in the keto acids; therefore, the mean life of the keto acids must have been comparatively small. For example, from a radioactivity of 3000 disintegrations/min. in the CO₂ accumulated by mesenchyma after 24 hr. and an activity of 20 disintegrations/min. in the stationary α-oxoglutaric acid, an average life of α-oxoglutaric acid of the order of a few minutes is obtained. Wherever a keto acid is found in two different pools, i.e. inside and outside the cells (e.g. for pyruvic acid), and is, no doubt, metabolized at different speeds in the two pools, a similar semiquantitative estimate is more difficult. It is clear, however, from the orders of magnitude involved that, in these cases as well, the specific activities of the carbon atoms in the keto acids must closely approach those of the glucose at the time of isolation.

DISCUSSION

The fate of the radioglucose in the tissue is identical with that of the total exogenous glucose. Moreover, for the following reason it must be almost identical with that of the total carbohydrate. The amount of carbohydrate in tissue is small (Šlechtá, Jakubovič & Šorm, 1955; Willmer, 1954). Yet a fair fraction of the exogenous glucose is utilized during the experiment (usually 5–15%, according to Table 1). This is possible only if the exogenous glucose rapidly penetrates the tissue and floods the endogenous carbohydrate.

As regards the keto acids, the following features emerge from the tables. Wherever the concentrations of oxaloacetic acid and α-oxoglutaric acid change, they do so in parallel. Apparently these changes reflect the role of the citric acid cycle as a whole. Thus there are no grounds to assume a specific block within the cycle which would cause an increase in the amount of some members at the expense of other members of the cycle.

The content of pyruvic acid per cell, in the mesenchyma or HeLa tissue, increases with a rise of the glucose supply. Some of the pyruvic acid diffuses into the medium. The total quantity of pyruvic acid in the medium is always higher than that within the tissue. However, as the volume of tissue plus plasma coating is so much less than that of the medium, the concentration of the pyruvic acid must always be less in the medium than in the tissue.

Table 2. *Stationary contents of keto acids*

Expt.	Pyruvic acid (PA) ($\mu\text{g./10}^7$ cells)			Oxaloacetic acid (OAA) ($\mu\text{g./10}^7$ cells)			α -Oxoglutaric acid (OGA) ($\mu\text{g./10}^7$ cells)			PA/OAA	PA/OGA
	Tissue	Medium	Total	Tissue	Medium	Total	Tissue	Medium	Total	in	in
										tissue	tissue
(a) Fibroblasts											
F2	1.7	12.7	14.4	0.74	0	0.74	0.83	0	0.83	2.3	2.0
F4	3.3	86.7	90.0	2.2	0	2.2	2.2	0	2.2	1.5	1.5
(b) HeLa											
H1	0.63	6.8	7.4	0.38	0.63	1.0	0.25	1.8	2.0	1.7	2.5
H3	2.5	30.1	32.6	1.9	2.8	4.7	1.7	3.5	5.2	1.3	1.5
H2	2.5	21.9	24.4	1.5	2.3	3.8	1.7	8	9.7	1.7	1.5
H4	19.5	169	189	13.5	29	42.5	12	20	32	1.4	1.6

In Table 2, the ratios between the amounts of pyruvic acid (PA), on the one hand, and of oxaloacetic acid (OAA) or α -oxoglutaric acid (OGA) on the other hand (PA/OAA or PA/OGA), are found to be 1.6 ± 0.3 and 1.8 ± 0.4 respectively. From the lack of an increase of this ratio with a rise in the glucose supply per cell, it can be concluded that in the given range the rate per molecule of the oxidative decarboxylation of pyruvic acid, followed by condensation with oxaloacetic acid and the reactions of the citric acid cycle, does not depend much on the concentration of glucose or pyruvic acid. In other words, the enzymes of the citric acid cycle (located in the mitochondria) do not lag appreciably behind the glycolytic enzymes (located in the soluble parts of the cytoplasm).

The pyruvic acid in the medium as well as in the tissue is acted upon by lactic dehydrogenase. True, the smallness of the blank values of lactic acid (after incubation of medium alone with radioglucose) indicates only low glycolytic activity of the medium. However, in contact with growing tissue the medium may take up glycolytic enzymes from dying cells, and reduced diphosphopyridine nucleotide by diffusion from intact cells as well. Thus the rise in pyruvic acid in the medium, caused by an increase in the supply of glucose per cell, may contribute a great deal to the increase in q , which is observed to be a consequence of this improved supply of glucose, and is the radiometric analogue of the manometric 'inverse Pasteur effect'.

A numerical comparison of the amounts, per cell, of lactic acid or CO_2 accumulated, or of the amounts, per cell, of the keto acids present in the stationary state, between mesenchyma and HeLa is not useful, as the two kinds of tissue were derived from different species. However, the ratios between the various metabolites, in the two kinds of tissue, can be compared.

The ratios PA/OAA and PA/OGA are not significantly different in mesenchyma and HeLa tissue. But there is a striking qualitative difference

in that tumour tissue, in contrast with mesenchyma tissue, loses part of the two keto acids of the citric acid cycle into the medium. This can hardly be attributed to differences in permeability, although this remote possibility will be tested experimentally. It seems more likely that the two keto acids diffuse out of the tissue if they are not dealt with rapidly enough by the enzymes of the citric acid cycle, i.e. if the efficiency of the mitochondria is impaired.

At first glance, this result seems to be in contradiction with the approximate equality of the PA/OAA and PA/OGA ratios, or with that of the q values, between the two kinds of tissue. However, the loss of the keto acids from the tissue need not be reflected in an appreciable reduction of respiration. While the mean life of the keto acids is short within the tissue, diffused-out oxaloacetic acid and α -oxoglutaric acid can be metabolized only after re-entry into the tissue, i.e. after a relatively long time. From the similarity of the fates of the oxaloacetic acid and the (more stable) α -oxoglutaric acid within the medium it must also be concluded that little oxaloacetic acid only is lost by non-catalytic decarboxylation. Therefore even a small fractional loss of keto acids from the tissue will lead to a comparatively high stationary concentration within the medium.

SUMMARY

1. Monolayers of chick-embryo mesenchyma tissue and of human tumour tissue (HeLa) were incubated for 24 hr. with radioactive glucose. The metabolites lactic acid, carbon dioxide, pyruvic acid, oxaloacetic acid and α -oxoglutaric acid were isolated and their radioactivities measured with the gas Geiger counter.

2. When the supply of glucose per cell is raised, the ratio of radioactive lactic acid to radioactive carbon dioxide accumulated (' q value') increases sharply, indicating a shift from respiration to fermentation ('inverse Pasteur effect').

3. In stationary conditions, the quantities of the three keto acids can be calculated from their radioactivities. These quantities are small and correspond to the lactic acid or carbon dioxide generated during a few minutes only.

4. The average amount of each keto acid within a cell increases in parallel with the supply of glucose, but the ratio of the three keto acids does not change appreciably. Thus the enzymes of the citric acid cycle can, to a large extent, cope with an increase in the supply of pyruvic acid.

5. Pyruvic acid diffuses, from both mesenchyma and tumour tissue, into the medium where it is acted upon by lactic dehydrogenase. In the given conditions, the inverse Pasteur effect can partly be explained as being due to this reaction.

6. Oxaloacetic acid and α -oxoglutaric acid diffuse into the medium from tumour, but not from mesenchyma tissue. This loss is attributed to some malfunction of the mitochondria in tumour tissue. But it is shown that this malfunction need not express itself in any great increase of the ratio of pyruvic acid to oxaloacetic acid or α -oxoglutaric acid within the cells or any great decrease in the rate of respiration.

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Carbon Assimilation by *Pseudomonas oxalaticus* (OX 1)

1. FORMATE AND CARBON DIOXIDE UTILIZATION DURING GROWTH ON FORMATE

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Some non-photosynthetic micro-organisms can derive all their carbon and energy needs from oxalate or formate. It has been suggested that the organisms might obtain energy by oxidizing these compounds to carbon dioxide, and then synthesize cellular constituents from carbon dioxide (Bhat & Barker, 1948; Schatz, Isenberg & Trelawney, 1953; van Niel, 1954; Jayasuriya, 1955; Jakoby & Bhat, 1958). The isolation by Khambata & Bhat (1953) of *Pseudomonas oxalaticus* (OX 1), capable of growing on either formate or oxalate as the sole carbon and energy source, provided a suitable organism to investigate the reactions leading from these substrates to cell constituents. Results presented here and in the next paper are concerned with the carbon assimilation by *P. oxalaticus*

(OX 1) grown on formate. A preliminary account of part of this work has been published (Quayle & Keech, 1958).

METHODS

Maintenance and growth of organism. Cultures of *Pseudomonas oxalaticus* (OX 1) (Khambata & Bhat, 1953) were obtained from the National Collection of Industrial Bacteria (Culture no. 8642) and maintained on slopes consisting of: 5 mm-potassium oxalate, 3 mm-sodium phosphate-potassium phosphate buffer, pH 7.0, essential salts [50 mg. of $(\text{NH}_4)_2\text{SO}_4$, 10 mg. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg. of CaSO_4 /100 ml. of medium], 0.1% of yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.) and 1% (w/v) of agar-agar (Davis, New Zealand). Stock cultures were grown at 30°, stored at 2° and subcultured every 4 weeks.