Studies on the Conversion of Glucose into Lactic Acid in the Ehrlich Ascites Tumour

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The most widely substantiated biochemical characteristic of neoplastic tissue is the high rate of conversion of glucose into lactic acid in the presence of oxygen, first described by Warburg (1930). The significance of this high aerobic glycolysis remains controversial (Warburg, 1956; Weinhouse, 1956; Burk, 1956). Another characteristic of neoplastic tissue is the depression of the respiration rate on adding glucose, described by Crabtree (1929). This Crabtree effect has recently been confirmed and studied by many others (Brin & McKee, 1956; Chance, 1953; Chance & Hess, 1956; Gatt, Krinsky & Racker, 1956; Ibsen, McCarty & McKee, 1956; Kun, Talalay & Williams-Ashman, 1951; McKee, Lonberg-Holm & Jehl, 1953; Bloch-Frankenthal & Weinhouse, 1957; Emmelot, 1958).

The present study was initiated from the point of view of radiotherapy. Oxygen is known to be an important factor in the sensitivity of biological material to ionizing radiations (Crabtree & Cramer, 1934). It has been suggested that the oxygen tension in some neoplastic tissue may be sufficiently low to affect adversely the benefit available from radiotherapy (Thomlinson & Gray, 1955). This has, in fact, been shown in model systems (Scott, 1957), and more recent clinical trials of oxygen administered under pressure during irradiation have given promising results (Churchill-Davidson, Sanger & Thomlinson, 1957).

The inhibition of respiration of neoplastic cells by glucose seemed to be an attractive method of decreasing tissue-oxygen gradients, and thereby preferentially increasing the oxygen tension inside a tumour, while having little or no effect on the blood supply or on the oxygen tension of neighbouring healthy tissue. Before studying the oxygen at different concentrations of blood glucose *in vivo*, more information was required on the nature of the effect of glucose and of the conditions for maximum inhibition of respiration *in vitro*. Ehrlich ascites tumour cells were used for these studies, since their rate of respiration is very dependent on glucose concentration.

The glucose and lactic acid biochemistry of Ehrlich ascites tumour cells in mice has already been studied in considerable detail. Glucose is obtained from the blood supply of the host via the peritoneal fluid at sufficient rate to maintain the cells in maximum glycolysis, the lactic acid produced being returned to the blood (Kemp & Mendel, 1957). *In vitro* the cells respire rapidly for many hours without added glucose. Their respiratory quotient indicates that they are not metabolizing glucose (Dickens & Simer, 1930), but probably some lipid substance (Slechts, Jakubovic & Sorm, 1955). When glucose is added the rate of respiration falls, and the glucose is rapidly converted into L-lactic acid (Brin, 1953). The carbon dioxide produced comes partly from the residual respiration and partly from oxidation of some of the glucose (Bloch-Frankenthal & Weinhouse, 1957). No single theory to explain the effect of glucose on respiration appears to have received general acceptance, and more experimental evidence seemed desirable.

MATERIALS AND METHODS

The Ehrlich ascites tumour cells used in this study were peritoneal transplants of 5–8 days in mice of a heterogeneous strain. The mice were killed and the fluid was withdrawn immediately before each experiment. The fluid was pooled from the minimum number of mice required to provide sufficient material. In order to approximate as nearly as possible to the conditions *in vivo*, the ascitic fluid was used as such without further treatment, and always contained some blood. The Ehrlich ascites tumour was the tetraploid strain of Klein. Ascitic fluid from a tumour-bearing mouse contained a remarkably constant number of cells, $1.309 \times 10^8 \pm 0.043 \times 10^8/\text{cm}^3$, with an average cell volume of $1903 \mu^3$ and variation coefficient of 15% (Klein & Revesz, 1953). Each experiment consisted of a comparison between control and experimental portions taken from the same pooled fluid. Cell counts were not always made; thus no comparison should be made between the absolute values obtained in different experiments.

Measurements of respiration were made with the conventional Warburg technique. The main compartment of the manometer flasks contained 1.0 ml. of buffer, and either 1.0 ml. of ascitic fluid or 0.5 ml. of ascitic fluid, and 0.5 ml. of 0.154M-NaCl soln. Glucose in 0.5 ml.

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of 0.154M-NaCl soln. was added by tipping in from the side bulb; 0.5 ml. of 0.154M-NaCl soln. containing no glucose was always tipped into control flasks at the same time. Carbon dioxide was absorbed on filter paper saturated with 0.15 ml. of 5N-KOH soln. in the centre well. Measurements were made at 37° in an atmosphere of air. The buffer at pH 7.4 was made by mixing 4.3 vol. of 0.154M- Na_2HPO_4 to 1 vol. of 0.154M- NaH_2PO_4 . The buffer at pH 6.0 was made by mixing 1 vol. of 0.154M- Na_2HPO_4 with 7.3 vol. of 0.154M- NaH_2PO_4 . Potassium salts were avoided after the inhibitory action of high concentrations of K^+ ions (McKee *et al.* 1953) had been confirmed in this Laboratory. The buffer concentration had to be kept as high as was possible without upsetting the osmotic pressure of the system, to avoid gross changes in pH during the experiment, from the large quantities of lactic acid produced. Even so, the pH at the end of an experiment was frequently as much as 0.2 pH unit lower than at the beginning.

Glucose measurements were made by the method of King (1951). A sample (0.1 ml.) of the fluid was added to 1.8 ml. of isotonic CuSO_4 reagent (made by adding 6.06 g. of anhydrous Na_2SO_4 and 3.0 g. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ to 1 l. of water). Aqueous 10% (w/v) $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ soln. (0.1 ml.) was added and the mixture shaken and centrifuged at 540 g for 5 min. Glucose was estimated in a portion of the supernatant fluid. Lactic acid was measured by the method of Barker & Summerson (1941); 0.5 or 1.0 ml. of 10% (w/v) trichloroacetic acid in water was added to the 0.5 or 1.0 ml. sample to be estimated respectively. The mixture was diluted with water to 25 ml. and centrifuged at 970 g for 5 min., and lactic acid determined on portions of the supernatant.

Fig. 1. Respiration of Ehrlich ascites tumour cells at 37° in air in Warburg manometer; CO_2 was removed by KOH. Δ , 1 ml. of cells + 1.5 ml. of 0.154M-NaCl soln.; $\circ$, 1 ml. of cells + 1.5 ml. of 0.154M-NaCl and glucose soln. to a final concentration of 400 mg./100 ml.

The volume of the liquid trapped between centrifuged cells was measured by suspending about 10 ml. of cells in 100 ml. of sucrose-NaCl soln. (containing 5 g. of sucrose and 8.15 g. of NaCl/l.), centrifuging for 10 min. at 940 g and resuspending in fresh sucrose-NaCl soln. A portion was then centrifuged under standard conditions (970 g at the tip of the centrifuge tube for 5 min.) in a graduated tube. The total volume of the packed cell mass was noted. The supernatant was poured off and replaced with a volume of NaCl soln. exactly equal to the volume of the cell mass and mixed by vigorous shaking. A sample (1 ml.) was diluted to 50 ml. with 0.154M-NaCl soln., the cells were removed by centrifuging for 10 min. at 940 g and sucrose was determined in a sample of the supernatant by the anthrone method of Seifter, Dayton, Novic & Muntwyler (1950). The mean of eight determinations showed that 1 ml. of cells centrifuged under these standard conditions contained 0.44 ± 0.04 ml. of fluid between them.

RESULTS

Fig. 1 is a typical curve obtained by plotting the rate of respiration with and without added glucose against time. In this particular experiment no buffer has been added. Even so, the respiration continues at a high rate for many hours although falling slightly with time. The rate of respiration is significantly less in the presence of glucose.

Fig. 2. Effect of pH on the magnitude of inhibition of respiration rate of Ehrlich ascites tumour cells on adding glucose. Cell suspension (1.0 ml.) and 1.0 ml. of 0.154M-phosphate buffer at the appropriate pH were added to each Warburg flask. The figures are the fall in respiration rate on tipping in glucose in 0.154M-NaCl soln. to a final concentration of 1000 mg./100 ml., as percentages of the rate in the control flask in which 0.154M-NaCl soln. only was tipped.

Fig. 2 shows the relationship between the extent of the inhibition of respiration (Crabtree effect) in Ehrlich ascites tumour cells and the pH of the buffer added. The figures for the percentage inhibition are the respiration rates in the absence of glucose, minus the respiration rates in the presence

Fig. 3. Rate of removal of glucose from Ehrlich ascites tumour cells shaken in air at 37°. The system contained cell suspension (2 ml.) and 2 ml. of 0.154 M-NaCl soln. and 4 ml. of buffer, pH 6.0 (Δ) or pH 7.4 (○), and glucose to a final concentration of 100 mg./100 ml.

Fig. 4. Conversion of glucose into lactic acid by Ehrlich ascites tumour cells when the concentration of glucose is high (600 mg./100 ml.). Cell suspension (5 ml.) and 5 ml. of buffer, pH 7.4, and glucose were shaken at 37° in air. ○, Glucose; Δ, lactic acid.

of glucose, as a percentage of the respiration rates in the absence of glucose, the rate of respiration being determined from the initial slope of the line obtained by plotting uptake against time. The Crabtree effect virtually disappears at pH 6.0. Fig. 3 shows that this is not due to an inability of the cells to utilize glucose at this pH. Although the rate of consumption of glucose is less at pH 6.0 than at pH 7.4, it is still far from negligible.

Since the rate of utilization of glucose by Ehrlich ascites tumour cells is very high, care is required to ensure that sufficient glucose remains during the entire experiment to avoid spurious results. Figs. 4 and 5 show the effect of adding high and low concentrations of glucose to these cells. The result of using adequately high initial concentrations of glucose (above 300 mg./100 ml.; Fig. 4) is that it is not completely consumed during the experiment, and the concentration of the lactic acid produced continues to rise. In Fig. 5 the smaller amount of glucose added is quickly consumed, and the lactic acid concentration ceases to rise and eventually starts to fall.

In Fig. 6 the initial change in respiration rate on adding glucose is plotted against the glucose concentration. The Crabtree effect is nearly independent of the glucose concentration above 100 mg./100 ml., but begins to fall off more rapidly below this level. Thus it was necessary to ensure that the glucose concentration remained above 100 mg./100 ml. throughout the experiment. For this

Fig. 5. Conversion of glucose into lactic acid by Ehrlich ascites tumour cells when the concentration of glucose is low (100 mg./100 ml.). Cell suspension (5 ml.) and 5 ml. of buffer, pH 7.4, containing glucose were shaken at 37° in air. ○, Glucose; Δ, lactic acid.

Table 1. *Distribution of lactic acid between Ehrlich ascites tumour cells and the peritoneal fluid*

Control cells were centrifuged immediately after withdrawal. Glucose in 0.154M-NaCl soln. was added to treated cells to a final concentration of 90.9 mg./100 ml. and shaken in air at 37° for 10 min., then cooled in ice and centrifuged. All readings are averages of duplicate samples.

	Lactic acid (mg./ml. of sample)		
	Centrifuged supernatant	Centrifuged cell fraction	Cells corrected for trapped supernatant
Control (no glucose)	0.78	0.31	0.00
Glucose added	1.06	0.86	0.70

Fig. 6. Effect of glucose concentration on the inhibition of the respiration rate of Ehrlich ascites tumour cells. The system contained cell suspension (1 ml.) and 1 ml. of 0.154M-buffer, pH 7.4, and the requisite glucose in 0.5 ml. of 0.154M-NaCl soln.

reason, 1000 mg. of glucose/100 ml. was used as the starting concentration in Fig. 2.

To test whether the resultant lactic acid was in part responsible for the Crabtree effect, an analysis for lactic acid was carried out on the cells and on the fluid surrounding them. In a typical experiment, a sample (6.9 ml.) of ascitic fluid containing Ehrlich ascites tumour cells was immediately cooled in ice-water and, within 10 min. of collection, was centrifuged for 5 min. at 970 g. The supernatant was carefully removed by pouring into a separate tube and drying the wet glass surface with filter paper, leaving 3.05 ml. of hard-packed cells. 0.154M-Sodium chloride soln. (3.05 ml.) was added and thoroughly mixed. The dilution was necessary to make the cell suspension sufficiently fluid to be accurately pipetted. Tri-

Table 2. *Distribution of lactic acid between washed Ehrlich ascites tumour cells and the peritoneal fluid*

Cells were first washed almost free of lactic acid by centrifuging and resuspending in 0.154M-NaCl soln. Glucose in 0.154M-NaCl soln. was added to a final concentration of 90.9 mg./100 ml. Cells were shaken in air at 37° and samples withdrawn at intervals. All readings are averages of duplicate samples.

Time (min.)	Lactic acid (mg./ml. of sample)		
	Centrifuged supernatant	Centrifuged cell fraction	Cells corrected for trapped supernatant
10	0.57	0.67	0.75
210	0.64	0.58	0.53
315	0.52	0.42	0.34

chloroacetic acid was added to duplicate 0.5 ml. samples of supernatant and duplicate 1.0 ml. samples of the diluted cell suspension. All samples were made up to 25 ml. with water for lactic acid analysis.

The results given in Table 1 show that when a correction was made for the supernatant fluid mechanically trapped between the centrifuged cells (see Methods) there was virtually no lactic acid inside the cell. This represents the state of ascites cells *in vivo* where the glucose concentration is known to be extremely low (Kemp & Mendel, 1957). In a similar experiment made in the presence of glucose, 0.5 ml. of 0.154M-sodium chloride solution containing 5 mg. of glucose was added to 5 ml. of freshly drawn tumour bearing ascitic fluid. The mixture was shaken in a conical flask in an adapted Warburg water tank at 37° in air for 10 min. and then cooled in ice-water. The cells were centrifuged and lactic acid was estimated as before. The lactic acid concentration in the cells, although still less than in the supernatant, had risen to a relatively high value (Table 1). The concentration in the supernatant is greater than in the previous experiment, being the sum of the lactic acid originally present and that amount released by the cells fermenting glucose.

In the next experiment (Table 2) the cells in 19 ml. of tumour-bearing ascitic fluid were first washed almost free of lactic acid by centrifuging at 970 g for 5 min. and resuspending them in 0.154M-sodium chloride solution to a final volume of 19 ml. 0.154M-Sodium chloride solution (1.9 ml.) containing 19 mg. of glucose was added and the mixture was shaken in a stoppered conical flask containing air in the water bath at 37°. Samples (5 ml.) were withdrawn at intervals, cooled in ice-water, centrifuged and lactic acid determinations made on cells and supernatant as before. In this experiment, after 10 min., the intracellular lactic acid concentration was higher than in the external medium. Under the conditions used the glucose added would be consumed within the first 30 min. of the experiment. After 3.5 hr. the intracellular concentration of lactic acid was less, and fell faster during the next 1.75 hr., than the concentration outside the cell.

DISCUSSION

Previous investigators of the Crabtree effect have suggested various mechanisms for its explanation. Brin & McKee (1956) have proposed that it is related to the concentration of inorganic phosphate. Racker (1956), Chance & Hess (1956) and Shacter (1955), suggest that the effect is not due solely to a pH change. The evidence against a simple pH change is that methylene blue and dinitrophenol abolish the effect without altering the overall pH change, and iodoacetic acid prevents the pH change without altering the Crabtree effect. Racker (1956) and Wu & Racker (1957) suggested an alternative mechanism involving the cytoplasmic shuttling of an adenine nucleotide. Bloch-Frankenthal & Weinhouse (1957) have pointed out that the point of inhibition must be such as to act on the oxidation of endogenous substrate as well as on the oxidation of glucose, without affecting glycolysis.

Although we have no proof that intracellular pH is responsible for the Crabtree effect, we have shown that the intracellular lactic acid concentration is increased after addition of glucose and, under special conditions, can be higher than in the external medium. We therefore cannot exclude the possibility that an intracellular increase in hydrogen-ion concentration may have some part in the reduction of respiration rate. The fact that the intracellular lactic acid concentration can be very different from the concentration in the external fluid shows that the combined ability of the cell to remove lactic acid by oxidation and to transport it to the external fluid is not large in comparison with the rate of conversion of glucose into lactic acid. Further studies on the removal of lactic acid from the cell are in progress.

SUMMARY

1. Below pH 6.0 the Crabtree effect in Ehrlich ascites tumour cells (i.e. inhibition of respiration caused by addition of glucose) is very small or non-existent. The rate of removal of glucose from solution by the cells is slower at pH 6.0 than at pH 7.4, but is still considerable.

2. As taken from the mouse, Ehrlich ascites tumour cells have little or no lactic acid in them. The lactic acid is essentially all in the ascitic fluid.

3. When excess of glucose is added *in vitro*, the lactic acid content of the cells becomes greater than that of the fluid. Some time after all the added glucose has been used the situation is reversed again and there is a progressive decrease in intracellular lactic acid concentration.

4. The increased intracellular concentration of lactic acid after addition of glucose shows that a relationship between the concurrent increased inhibition of respiration observed and a lowered intracellular pH cannot be excluded.

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The Distribution of Bound Acetylcholine and Choline Acetylase in Rat and Pigeon Brain

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Mann, Tennenbaum & Quastel (1938) presented evidence that about three-quarters of the acetylcholine in guinea-pig brain was bound to some protein constituent of the cell, and later the same authors (1939) suggested that this was a complex of acetylcholine and its synthesizing enzyme. It has since been pointed out (Braganca & Quastel, 1952) that bound acetylcholine could be acetylcholine held within the mitochondria, and from indirect evidence del Castillo & Katz (1956) conclude that intracellular granules containing acetylcholine may function in synaptic transmission.

Hebb & Whittaker (1958) have located bound acetylcholine and choline acetylase in particulate fractions from guinea-pig and rabbit brain. Bound acetylcholine and choline acetylase have also been found in mitochondrial preparations from locust-head tissue (Bellamy, 1958).

This paper records the distribution of bound acetylcholine and choline acetylase in rat- and pigeon-brain homogenates fractionated by differential centrifuging and provides evidence that both the enzyme and its stored product occur in the same particulate fraction.

METHODS

Preparation and fractionation of the homogenates. Brains from rat or pigeon were forced through a chilled stainless-steel Fisher mincer (Jouan, Paris) and homogenized with 4 vol. of 0.25M-sucrose soln. at 0° in a stainless-steel homogenizer of the Potter-Elvehjem type (working volume 40 ml.). The clearance of the pestle in the cylinder was 0.005 in. and it was moved up and down eight times, revolving at a speed of 2000 rev./min.

The homogenate was diluted with a further 2 vol. of 0.25M-sucrose soln. and fractionated at the gravitational forces and times described later. Each fraction was

examined by phase-contrast microscopy and it was found that the nuclear and microsomal fractions were not entirely free from mitochondria. After sedimentation of each fraction it was washed three times with 0.25M-sucrose soln. (one-third of the original volume of the tissue mince) and the washings were added to the supernatant fluid from that fraction. The last centrifuging of the fractions was carried out at 25 000 g for 15 min. and the tightly packed residue resuspended in 9 vol. of 0.25M-sucrose soln. The microsomal fraction was not washed but resuspended immediately in 9 vol. of 0.25M-sucrose soln. All the operations were carried out as close to 0° as possible.

Preparation of electron micrographs. Small samples of the mitochondrial and microsomal pellets were fixed in 1% osmic acid adjusted to 0.25 osmolar with sucrose, then dehydrated and embedded in butylmethacrylate as described by Bellamy (1958). Sections were cut and examined under the electron microscope by Dr G. Meek of the Department of Anatomy, Oxford.

Extraction and estimation of acetylcholine. Acetylcholine in the tissue preparations was extracted with 1% trichloroacetic acid soln. after brief heat treatment as described previously (Bellamy, 1958), except that the ion-exchange resin was not used. The extracted acetylcholine was measured with the frog rectus abdominis-muscle preparation described by Chang & Gaddum (1933), with the controls suggested by Feldberg (1942-43).

Determination of dry weights. The dry weights of tissue samples were determined as acid-insoluble dry matter according to the method of Werkheiser & Bartley (1957).

RESULTS

Distribution of bound acetylcholine in brain tissue. No inhibitor of acetylcholine esterase was added during the preparation of the homogenate or during the fractionation procedure. This ensured that only acetylcholine bound to tissue components was present (Mann *et al.* 1939). A sample of pigeon-brain homogenate contained 13.6 µg. of bound