THE METABOLISM OF HUMAN EMBRYONIC AND MALIGNANT CELLS AND THEIR RESPONSE TO INSULIN

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Knowledge of the metabolic differences between normal and malignant cells is based mainly on measurements of their oxygen consumption and their ability to convert glucose to lactic and pyruvic acids (*i.e* glycolysis.) In WARBURG's view[1,2] the fundamental distinguishing feature is the relatively high glycolytic activity of the cancer cell. Experiments with isotopically-labelled glucose have yielded more detailed information on its role in supplying energy and materials for synthesis in the cell (*cf.*[3]), but they have not resolvet the conflicting views on the basic metabolic differences between normal and malignant cells[4].

We have approached the same problem —the study of the metabolic properties of cancer cells—by applying the tissue culture technique to the measurement of metabolic rates in actively growing embryonic and malignant cells. On theoretical grounds, it seems appropriate to compare malignant cells with embryonic cells rather than with the corresponding adult cells, since neoplasia involves a return to the primitive features of undifferentiated cells. The crucial distinction is that embryonic cells differentiate and become integrated in the body, whereas the malignant cells lack this ability and are autonomous and destructive. As hormones undoubtedly have some control over cellular differentiation, they could possibly provide a way of revealing differences between embryonic and malignant cells. We have begun a systematic investigation of such differences by studying the response of the two cell types to insulin.

A standard procedure for studying the growth of cell strains and their glucose utilization has been developed and our results show why previous investigators[5,6,7] found no consistent relationship between these two measurements in tissue cultures. It is also evident from our results that metabolic quotients expressed in the conventional terms of fresh or dry weight provide an inadequate basis for distinguishing between embryonic and malignant cells and are to some extent responsible for the conflicting views mentioned above.

METHODS

Origin of the cell strains

We obtained human embryonic tissues (from two 20-week embryos at surgical termination of pregnancy) in a sterile condition from a nearby hospital, and cultured from these a number of cell strains, which multiplied at approximately the same rates as the human carcinoma cells. The carcinoma strains came from three patients: the HeLa strain is an epidermoid carcinoma of the cervix, cultured originally by GEY, COFFMAN AND KUBICEK[8]; HEP 1 is of similar origin and HEP 2 is an epidermoid carcinoma of the larynx of a male patient, both having been originally

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maintained as heterologous transplants by TOOLAN[9] and subsequently grown in tissue culture at the Sloan-Kettering Institute for Cancer Research, New York. Only one cell type could be seen in the lung and skin cultures and in the three strains of carcinoma cells, but there were some fibroblastic cells amongst the epithelial sheets of kidney cells. Stocks of the cells were cultivated in Earle T-flasks or in medicinal flats: the malignant strains may be kept indefinitely, the embryonic cells, on the other hand, deteriorated and ceased to grow after 10–12 weeks of rapid multiplication and frequent subculturing.

Procedure for studying cell metabolism

The cells on test were grown in special roller tubes[10] in which the homogenization and extraction of cell constituents can subsequently be carried through to the final step of oxidizing the deoxyribonucleic acid (DNA) precipitate obtained in the SCHMIDT AND THANNHAUSER procedure[11]. As we have determined the constant value for the DNA phosphorus (DNAP) content per average cell for most of the strains used, we can calculate from the total DNAP content per tube, the number of cells present and the amount per average cell of any other constituent which we measure[12].

Sixteen to twenty-four replicate tubes are set up by subculturing from stock flasks, using a 1% trypsin (Difco 1:250) solution in saline to obtain a suitable cell suspension. By keeping the pH of the media between 6.6 and 7.0, the cells can be deposited evenly over the surface area during the rotation of the tubes on a roller-drum at 37°. These are previously coated with a film consisting of equal volumes of fowl plasma and 25% chick embryo extract. The film is very thin, as excess of the plasma mixture is removed by centrifuging lightly, and it is made to clot before the tubes are inoculated by reducing the pH (originally 9–9.5) with a mixture of 20% CO_2 in air and incubating for 30 minutes at 37°. In this way replicate tubes can be prepared in which there are no cell clumps, so avoiding any local necrosis or nutritional deficiencies. The cells are grown for a few days before they are put on test and any defective tubes are eliminated.

The preparation and handling of media

Two kinds of media have been used: one, the growth-promoting medium (GPM) consists of 5% chick embryo extract and 20% human serum in a modified Connaught medium 858[13], the other, the maintenance medium (MM) contains only 20% human serum in the same chemically-defined solution. As used by us, this contains the eight water-soluble vitamins found essential by EAGLE[14] for Earle's L strain and the HeLa cell strain, but is without uridine triphosphate and the group of coenzymes, which HEALY, FISHER AND PARKER[15] included in medium 858 to replace some of the B-vitamin group. Following the observations of FELL AND MELLANBY[16] and LASNITZKI[16] of the stimulating effect of high concentrations of vitamin A on the multiplication of soft-tissue cells, vitamin A acetate was present in a concentration of 1 mg per litre and the media were made up freshly at frequent intervals to avoid loss of activity. In all the present tests, the initial glucose concentration was about 2 mg per ml (*i.e.* 11.1 μmoles per ml).

The pH of the medium was always brought to about 7.4 at the time of feeding the cultures, by gassing the tubes with air mixtures containing appropriate concentrations of carbon dioxide. The percentage of CO_2, which varied between 3–8%, could be judged from the colour of the phenol red indicator incorporated in the medium, and the required gas mixtures were obtained by controlling the rates of flow of nitrogen, oxygen and carbon dioxide cylinders by means of a Rotameter flow unit. The gas mixture was washed by allowing it to stream through a 1% $CuSO_4$ solution, and spray or bacterial contamination was removed by passage through a sterile cotton-wool filter.

Preparation of chick embryo extract (EE)

This is made by a modification of the method of BRYANT, EARLE AND PEPPERS[17] who employed hyaluronidase to reduce the viscosity of embryo extract in order to prevent the clogging of sterilizing filters. In our procedure, 11–12 day embryos are crushed in pairs in 25 ml screw-cap bottles, and to each is added 15 ml of saline, based on EARLE's formula, but lacking sodium bicarbonate and glucose, and incorporating penicillin and streptomycin at concentrations of 100 μg per ml. The embryos are kept overnight at —15° before centrifuging at 2000 r.p.m. for 20 minutes to remove solid material. In this way, an approximately 25% crude EE is obtained.

It is first incubated at 37° with hyaluronidase (5 mg of a Benger's preparation per 100 ml medium) for 2 hours. However, as we found that filtration through Fords Sterimats (type GS/PH) was still too slow after this treatment, we tried the effect of reducing viscosity further with trypsin. A greatly improved filtration rate and an equally active extract could be obtained by following the hyaluronidase treatment with the addition of 1 ml of 1% trypsin per 100 ml extract and incubating at 37° for 4 hours. A clear filtrate is obtained, to which is added the sterile solution of sodium bicarbonate omitted in the preparation of EARLE's saline. This preparation of EE retains its growth-promoting activity when stored at —15° for 2–3 months. Any residual trypsin activity is eliminated by the presence of soya bean trypsin inhibitor in medium 858 at a concentration of 1 mg per 100 ml.

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Test procedure

At the start of each test, four replicate tubes were removed and the tissue kept for determination of initial cell number (based on DNAP) and nucleic acid phosphorus (NAP) content. Each remaining tube received 2 ml of GPM or MM and this was removed and replaced by fresh medium at 24 hour intervals, the used medium being collected and stored at —15° until required for analysis. The replicate tubes were put on test when the amount of tissue present was judged to require less than 70 % of the available glucose, taking into account a possible 2- to 3-fold growth in GPM over 72 hours, the standard time for each test. The medium from the daily collections of each tube was combined, the analysis being carried out on a total volume of 6 ml. At the end of the test, the tissue was washed with cold saline and stored at —15° until the determinations of lipid phosphorus (LP), ribonucleic acid phosphorus (RNAP), DNAP, and in some cases, protein nitrogen (PN) could be carried out. All tests were carried out under aerobic conditions.

In all the tests on insulin action, the hormone was present in the medium in the concentration of 0.1 unit per ml. Stock solutions of 2 units per ml were made up in acidified saline from crystalline pork insulin (free from glucagon) prepared by the Novo-Terapeutisk Laboratorium, Copenhagen.

Chemical determinations

The analysis of the cells for LP, RNAP and DNAP was carried out by a modified SCHMIDT AND THANNHAUSER[18] procedure and the phosphorus was determined by the method of FONTAINE[19]. Suitable modifications of three standard methods were applied to the analysis of the medium. After precipitation of interfering substances with barium hydroxide and zinc sulphate solution, glucose was determined by the anthrone reagent[20]. For lactic acid determination by a modification[21] of the Barker and Summerson procedure, another portion of the medium was treated with 20 % copper sulphate and solid calcium hydroxide, and a third portion was deproteinized with sodium tungstate for the measurement of total keto acids with the phenylhydrazine reagent[22]. Chromatography of the derived phenylhydrazones on paper strips by the method EL HAWARY AND THOMPSON[23], showed that for each cell strain 60–75 % of the total keto acid was pyruvate, with α-ketoglutaric acid as the chief remaining constituent. The medium and tissue from each tube were analyzed separately, duplicate determinations being made where ever possible. Tests were repeated to confirm a particular response, but the results quoted for each cell strain are based on the mean figures from a typical test.

Determination of the DNAP content per nucleus

The cultures from a number of tubes or flasks were pooled and the cells brought into suspension by 1 % trypsin in saline. After washing with saline, they were resuspended in ice-cold 0.1 *M* citric acid and the cytoplasmic material disintegrated by vigorous agitation with a platinum spatula attached to an electric motor. The nuclear suspension was centrifuged to remove cytoplasmic debris, and this process was repeated until the nuclei were reasonably clean. They were finally suspended in 0.1 *M* citric acid containing 0.1 % crystal violet[24], and the number of nuclei in a known volume were estimated by haemocytometer counts. Six counts were made on each suspension and replicate determinations of DNAP were obtained on suitable samples, each containing at least 2 million nuclei per ml.

Method of expressing metabolic quotients

In order to make possible comparison with the results of other investigators using tissue culture or manometric methods, we have expressed our results for metabolites in the following terms: (1) μmoles per mg NAP per hour. (2) μmoles per 10^9 cells per hour. (3) μmoles per mg PN per hour. (4) μl (mm^3) per mg dry weight per hour (22.4 μl are equivalent to 1 μmole).

Glucose consumption, lactic acid and keto acid production are determined, as described above, by analysis of the used and unused medium, and the corresponding number of cells or amounts of cell constituents (in this case the total nucleic acid phosphorus or NAP) are obtained by taking the mean of the values from tubes sampled at the start and the end of the growth period. This provides a common basis of reference which takes into account the different extent of cell multiplication in GPM and MM. The metabolic quotients expressed in terms of PN or dry weight are calculated from the quotients based on cell number, using the mean values determined for the protein N or dry weight per million cells with each cell type.

RESULTS

Composition of the cell strains

The most important difference between the embryonic and the malignant cell lies in their content of DNAP per nucleus (Table I). It was not possible to measure this in all the embryonic strains, but the value obtained for the skin fibroblasts

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(0.69 picograms per nucleus or 0.69 μg per million cell nuclei) is very close to previous estimates of the average amount in the human diploid nucleus[25,26], and there is no reason to suppose that the other embryonic cells would be different in this respect. On the other hand, all three human carcinoma cells had values close to the tetraploid value (approximately 1.4 picograms per nucleus) for human cell nuclei.

TABLE I

THE DEOXYRIBONUCLEIC ACID (DNA) CONTENT AND CELL COMPOSITION OF HUMAN EMBRYONIC AND MALIGNANT CELLS

Cell strain	*Average amounts in µg per million cells***				
	DNAP	*RNAP*	*LP*	*PN*	*Dry weight*
Embryonic skin fibroblasts	0.69 ± 0.03*	1.77	1.33	—	510
Embryonic kidney cells	—	1.20	1.65	31.6	513
Embryonic lung fibroblasts	—	0.84	0.97	15.2	252
HeLa carcinoma cells	1.36 ± 0.03	3.31	2.87	67.1	715
HEP 1 carcinoma cells	1.38 ± 0.01	1.80	1.56	34.5	470
HEP 2 carcinoma cells	1.41 ± 0.02	2.49	1.99	58.8	639

* Figures for DNA phosphorus are means ± standard error.
** Abbreviations: LP = lipid phosphorus; RNAP = ribonucleic acid phosphorus; PN = protein nitrogen.

From microscopic examination it could be seen that the smallest cells were HEP 1 and the largest of the three strains were the HeLa cells, although both originated as cervical carcinoma. The chemical composition of the cells, expressed as the average amounts of constituents per million cells and derived from a number of tests with each strain, is given in Table I. Again, in keeping with the relative sizes of the malignant series, the smallest values for LP, RNAP, PN and dry weight are obtained with HEP 1 and the largest values with the HeLa cells. When the carcinoma cells are kept on MM, their contents may be as much as 25% below the values given in Table I, and when growing rapidly in GPM they can be 25% higher than these values. The embryonic cells are also variable in composition, but from our results it appears they are not necessarily larger in GPM than in MM as with the malignant cells.

Glucose utilization and the size of the cultures

Table II summarizes the results of medium and tissue analysis for the three embryonic and the three malignant cell strains, and it shows how far the extent of cell multiplication depends on the type of medium (GPM or MM). The values for the average cell number and the average NAP per tube take into account these differences in growth and the different initial numbers of cells per tube. If no increase in cell number has occurred, as with the lung fibroblasts, then the glucose consumption and total acid production is associated solely with tissue maintenance. In all the other tests, there is an increase in cell number, and glucose has had to supply energy for both growth and maintenance.

In earlier experiments[27] with cultures of HeLa cells, in which measurements were made of cell number and of changes in the medium day by day, it was found that the average cell number for each 24 hour period was directly proportional to the

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part of the utilized glucose which was not accounted for in the production of lactic acid and keto acids. As a matter of convenience, we shall describe this portion of the glucose utilized as the glucose "oxidized" (see DISCUSSION, p. 374). It is derived by subtracting half the value (in μmoles) for the total acid produced from that for glucose utilized.

TABLE II

CHANGES IN TISSUE AND MEDIUM OVER TEST PERIODS OF 72 HOURS

Cell strain	*Medium* (No. of tubes)*	*Cell multiplication***	*Average cell no.*** (millions)*	*Average NAP μg*	*Total glucose utilized μmoles*	*Total§ acid produced μmoles*	*Glucose§§ 'oxidised' μmoles*
Embryonic skin fibroblasts	GPM (3)	4.56	4.38	7.98	45.0	73.1	8.43
	MM (3)	1.14	5.47	11.38	50.3	81.6	9.58
Embryonic kidney cells	GPM (2)	2.45	2.03	3.77	20.2	32.7	3.83
	GPM (2)	1.80	4.16	7.55	34.4	53.9	7.43
Embryonic lung fibroblasts	MM (3)	1.00	4.36	6.69	36.0	57.3	7.34
HeLa carcinoma cells	GPM (4)	2.56	7.66	30.78	44.7	34.2	27.6
	MM (4)	1.40	6.00	22.71	29.6	25.7	16.5
HEP 1 carcinoma cells	GPM (4)	2.11	12.34	40.41	52.7	39.3	33.0
	MM (4)	1.61	10.95	33.64	49.2	48.8	24.8
HEP 2 carcinoma cells	GPM (3)	2.80	3.60	16.83	23.8	25.2	11.2

* GPM is growth-promoting medium, MM is maintenance medium (see METHODS).

** Cell multiplication is the ratio of the final cell number to the initial cell number per tube (calculated from DNAP determinations).

*** Average cell number and average NAP (RNAP + DNAP) are obtained by calculating the means of initial and final values.

§ Total acid production is obtained by estimating separately the lactic acid and total keto acid content of the medium from each tube.

§§ The glucose "oxidised" denotes the portion of glucose utilized which does not appear as lactic acid and keto acids in the medium (see text).

A direct linear relationship between the average NAP or cell number per tube and the glucose "oxidized" has now been found to hold for all the cell types investigated, whether they are embryonic or malignant, as can be seen by reference to Table II. When correlation coefficients for each series are calculated from the results for individual tubes, the highest are those for NAP and the glucose "oxidized". For embryonic cells the correlation is + 0.944, and for malignant cells, + 0.962. With the embryonic cells, the relationship is simply $y = x$, when y is glucose "oxidized" in μmoles and x is NAP as μg phosphorus; for the malignant cells, the equation is $y = 0.815x$. The glucose "oxidized" also parallels the average cell number (or DNAP), since the coefficients for the embryonic and malignant series are + 0.983 and + 0.877 respectively. No other pairs of measurements show such close dependence on one another for both cell types. Although the correlation between NAP and total glucose utilization is + 0.923 for the embryonic cells, it is only + 0.809 for the malignant series.

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We have not measured the amount of metabolites actually present in the cells at the end of the tests, but even with the largest numbers of cells per tube, the volume of the cells is calculated from estimates of fresh weight to be less than one-sixtieth of the 2 ml of medium per tube. Therefore, in assessing the results, the portion of intra-cellular metabolites may be neglected.

TABLE III

METABOLIC QUOTIENTS CALCULATED IN TERMS OF TOTAL NUCLEIC ACID PHOSPHORUS (NAP) FOR HUMAN EMBRYONIC AND MALIGNANT CELLS

Cell strain	*Medium (No. of tubes)*	*Lactic acid concn.* µmoles per ml*	*Quotients in µmoles per mg NAP per hour***		
			Q glucose utilized	*Q total acid produced*	*Q glucose 'oxidised'*
Embryonic skin fibroblasts	GPM (3)	13.2	78.3	127.2	14.5
	MM (3)	14.7	61.6	99.8	11.7
Embryonic kidney cells	GPM (2)	11.5	74.5	122	13.5
	GPM (2)	9.6	63.0	97.6	13.3
Embryonic lung fibroblasts	MM (3)	10.5	74.5	118.6	15.2
Mean values for embryonic cells (± S.E.)		11.9 ± 0.92	70.4 ± 3.4	114 ± 5.7	13.6 ± 0.59
HeLa carcinoma cells	GPM (4)	10.3	20.1	16.5	11.8
	MM (4)	6.4	18.1	15.5	10.3
HEP 1 carcinoma cells	GPM (4)	7.5	18.1	13.5	11.4
	MM (4)	8.6	20.3	20.2	10.2
HEP 2 carcinoma cells	GPM (3)	5.6	19.7	20.8	9.3
Mean values for malignant cells (± S.E.)		7.7 ± 0.83	19.3 ± 0.48	17.3 ± 1.38	10.6 ± 0.46

* Figures give the average concentrations of lactic acid in 6 ml of medium collected over 72 hour tests.

** Definitions as in footnotes to Table II.

Metabolic differences between embryonic and malignant cells

Tables III–V give the metabolic quotients for glucose utilization and acid production for embryonic and malignant cells in terms of four aspects of the cell population in the tubes. Irrespective of the standard of reference (total nucleic acid, cell number, protein nitrogen or dry weight), the obvious distinction is that the average rates for glucose consumption and total acid production are always much higher for the embryonic cells than for the tumour cells.

From the column in Table III giving the concentrations of lactic acid in the total volume of medium collected in each 72 hour period, it can be seen that these are on the average 55% higher for the embryonic cells than for the carcinoma cells. The higher quotients for glucose utilization and total acid production of the embryonic cells are understandable if we envisage that, under tissue culture conditions, lactic acid and keto acids leak out of the cells into the medium until their normal equilibrium concentration within the cells is reached.

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We have been able to test this hypothesis with cultures of carcinoma cells (HEP 2) but not so far with embryonic cells. If it is correct, there should be higher rates of acid production in the first few hours of growth on fresh medium. This, in fact, is the

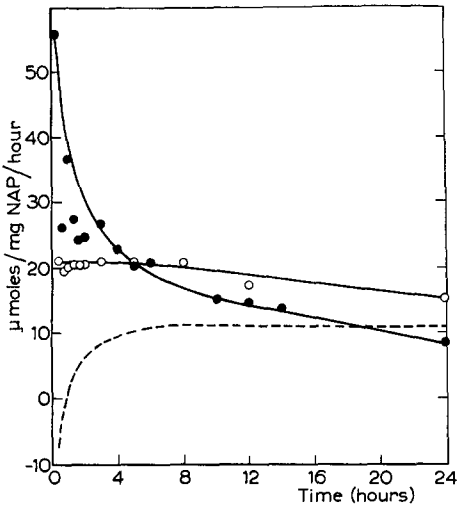

Fig. 1. Average rates of glucose utilization (O—O) and lactic acid production (●—●) over increasing periods of time during maintenance of HEP 2 in MM. The cultures were transferred to 2 ml volumes of fresh medium at zero time. The amounts of medium available were insufficient for the measurement of keto acids, and consequently the glucose 'oxidized'" (-----) was estimated by subtracting the glucose equivalent of lactic acid from the glucose utilized. (Total keto acids in other tests with similar cell populations accounted for about 10% of total acid production.) NAP was determined by measuring RNAP and DNAP in each roller tube.

case as can be seen in Fig. 1, in which the metabolic quotients were calculated from measurements made at 20–60 minute intervals over a 12 hour period and subsequently at 14 and 24 hours under conditions in which there could be little change in cell number. In the first hour the average rate of lactic acid production was 37 µmoles

TABLE IV

METABOLIC QUOTIENTS CALCULATED IN TERMS OF CELL NUMBER FOR HUMAN EMBRYONIC AND MALIGNANT CELLS

Cell strain	*Medium*	*Quotients in µmoles per 10^6 cells per hour*		
		Q glucose utilized	*Q total acid produced*	*Q glucose 'oxidized'*
Embryonic skin fibroblasts	GPM	140	229	26.0
	MM	127	207	23.4
Embryonic kidney cells	GPM	136	218	27.0
	GPM	114	178	25.0
Embryonic lung fibroblasts	MM	106	169	22.9
Mean values for embryonic cells (± S.E.)		125 ± 6.4	200 ± 11.5	24.9 ± 0.77
HeLa carcinoma cells	GPM	94.2	77.7	55.4
	MM	72.3	61.5	43.2
HEP 1 carcinoma cells	GPM	59.2	44.3	37.0
	MM	63.4	62.8	31.9
HEP 2 carcinoma cells	GPM	91.6	97.5	42.8
Mean values for malignant cells (± S.E.)		76.1 ± 7.16	68.8 ± 4.14	42.1 ± 3.24

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per mg NAP per hour as compared with quotients of 14.7 and 8.2 for the average rates per hour over 12 and 24 hour periods respectively. If the rates of lactic acid production over 20 minutes periods are derived from the curve in Fig. 1, negative values are obtained for periods after 18 hours and it is probable that lactic acid

TABLE V

METABOLIC QUOTIENTS IN TERMS OF PROTEIN NITROGEN (PN) AND DRY WEIGHT FOR EMBRYONIC AND MALIGNANT CELLS

Cell types and number investigated		*Q glucose utilized*	*Q total acid produced*	*Q glucose 'oxidised'*
		*μmoles per mg PN per hour**		
Human embryonic cells (2)	Range	3.6–7.0	5.6–11.1	0.79–1.51
	Mean values	5.0	7.5	1.05
Human carcinoma cells (3)	Range	1.1–2.0	0.9–2.6	0.64–1.19
	Mean values	1.60	1.44	0.88
		*μmoles per mg dry weight per hour**		
Human embryonic cells (3)	Range	5.0–9.4	7.4–15.0	1.03–2.04
	Mean values	6.4	9.9	1.30
Human carcinoma cells (3)	Range	2.3–3.2	1.9–3.4	1.35–1.76
	Mean values	2.9	2.6	1.57

* The protein nitrogen and dry weight could not be determined in each test, and the values are derived from the quotients per 10^9 cells per hour in Table IV, using the average amounts for PN and dry weight per million cells (Table I).

utilization from the medium was taking place at this stage. This certainly does occur in our roller tube cultures when the cell population is large and glucose concentrations are reduced to 40% or less of the original values over a 24 hour test period[27]. Throughout the 24 hour test, it can be seen (Fig. 1) that there was a gradual decline in the rate of glucose utilization, while the quotient for glucose "oxidized" remains constant after the first 3–4 hours.

The quotients for glucose "oxidized" in Tables III–V show how much the interpretation of cell characteristics depends on the terms of reference. On a "per cell" basis, the malignant cells are obviously using this portion of the total glucose uptake at a higher rate than the embryonic cells (Table IV). Relative to the total nucleic acid in the cells, it is the embryonic cells which have the greater requirement for glucose (Table III), and the same is true when the rate is expressed in terms of protein nitrogen (Table V). On a dry weight basis, there is no clear distinction in the rates of glucose "oxidized" for the two cell types (Table V).

Response of embryonic and malignant cells to insulin

Both embryonic and malignant cells respond to the presence of insulin by increased synthesis of cell constituents. In Table VI it can be seen that there were greater amounts of RNA formed in insulin-treated cultures, and we have found this to be true also of phospholipid and protein synthesis. The lower amounts of DNA in most of the culture tubes treated with insulin indicates that cell division has been inhibited. This deduction is supported by our previous observation that the average amount of DNA per nucleus in the HeLa cells is unaltered by the action of insulin[28].

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TABLE VI

INFLUENCE OF CRYSTALLINE INSULIN (0.1 unit per ml) ON GROWTH AND METABOLISM OF HUMAN EMBRYONIC AND MALIGNANT CELLS

Cell strain and medium	*Test*	*Increase* in RNAP* %	*Increase* in DNAP* %	*Ratio** RNAP / DNAP*	*Glucose utilized µmoles*	*Total acid produced µmoles*
Embryonic skin fibroblasts (MM)	Control	12	14	2.06	50.3	81.4
	Insulin	61	32	2.51	56.0	83.0
Embryonic skin fibroblasts (GPM)	Control	470	357	1.67	45.0	73.1
	Insulin	542	334	1.98	45.6	68.7
HeLa carcinoma cells (MM)	Control	55	49	1.97	29.6	25.7
	Insulin	109	31	3.19	45.8	40.4
HEP 1 carcinoma cells (MM)	Control	50	61	1.22	49.2	48.8
	Insulin	69	50	1.48	67.9	74.6
HEP 2 carcinoma cells (MM)	Control	46	51	1.56	62.7	69.9
	Insulin	58	21	2.10	71.5	84.9

* Percentage increases of final amounts of RNAP and DNAP per roller tube over the corresponding initial amounts.
** These are the ratios of the total amounts per tube of RNAP and DNAP.

The striking feature about the response of the cells to insulin (0.1 unit per ml of medium) was the greatly increased glycolysis of the malignant cells (Table VI). By comparison, the effects on the glucose metabolism of the embryonic cells were small, although there was a lower production of acids relative to the amount of glucose consumed. From Table VII it is evident that insulin significantly reduced the rate of

TABLE VII

INFLUENCE OF CRYSTALLINE INSULIN (0.1 unit per ml) ON THE RATES OF GLUCOSE UTILIZATION AND METABOLITE PRODUCTION

Cell strain and medium	*Test*	*Lactic acid concn. µmoles per ml*	*Ratio LA* / KA*	*Quotients in µmoles per mg NAP per hour*		
				Q glucose utilized	*Q total acid produced*	*Q glucose "oxidized"*
Embryonic skin fibroblasts (MM)	Control	14.7	12.3	61.8	100	11.7
	Insulin	14.7	11.7	57.8	86	14.7
P		< 0.2	< 0.4	< 0.2	< 0.01	< 0.2
Embryonic skin fibroblasts (GPM)	Control	13.2	9.6	78.3	127	14.5
	Insulin	12.5	8.7	80.5	124	18.5
P		< 0.01	< 0.001	0.05	0.2	< 0.01
HeLa carcinoma cells (MM)	Control	6.4	15.0	18.1	15.7	10.1
	Insulin	9.7	19.7	24.7	21.8	13.8
P		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
HEP 1 carcinoma cells (MM)	Control	8.6	7.3	20.3	20.2	10.2
	Insulin	12.7	11.3	27.4	30.1	12.1
P		< 0.01	—**	< 0.01	< 0.01	< 0.01
HEP 2 carcinoma cells (MM)	Control	12.2	6.9	23.7	26.1	10.6
	Insulin	14.7	11.6	28.5	33.8	11.6
P		—**	—**	< 0.02	< 0.05	0.20

* LA = lactic acid; KA = total keto acids (mainly pyruvic and α-ketoglutaric).
** No values because means were based on triplicate determinations on pooled samples.

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total acid production into the medium by embryonic cells, when they were maintained in MM under aerobic conditions, whereas, under the same conditions it increased the rate of total acid production of malignant cells. The presence of embryo extracts in the medium, as in GPM, is known to reduce insulin activity[28,29], and although the tests on embryonic fibroblasts with this medium show the same trend as before, the differences are not significant (Table VII).

While lactic acid concentrations in the spent medium were little affected by insulin in fibroblast cultures, they were always much greater with the insulin-treated carcinoma cells. A consistent qualitative difference between the two cell types lies in the behaviour of the ratio LA/KA: its value is reduced by insulin with embryonic cells and always increased with carcinoma cells. This was confirmed for cultures of embryonic kidney cells, where the ratios averaged from tests on flask cultures were 11.7 for the controls and 9.6 for insulin-treated cultures.

DISCUSSION

Aerobic glycolysis of embryonic and malignant cells

In discussions of his view that high aerobic glycolytic activities are associated only with malignant cells, WARBURG[1,2,4] has repeatedly emphasized that any acid production by embryonic cells under aerobic conditions is solely due to damage inflicted on their respiratory systems. If rat embryo or chorion is handled carefully and immersed in amniotic fluid of serum, he finds that a relatively high respiration is accompanied by the complete absence of acid production. In WARBURG's opinion, respiratory damage in cells leads to their death, or, if it is not lethal, then to their transformation to malignant forms.

Our present studies show that glycolysis, in the sense of the production of lactic and pyruvic acids into the medium is clearly a feature of both vigorously growing embryonic and malignant cells under tissue culture conditions. The high rates of acid production with the embryonic cells were found throughout the 10–12 weeks when they were multiplying without noticeable change in morphology and at mean generation times varying from 30–70 hours.

On what grounds could these contradictory observations be reconciled? It is possible that respiratory damage occurred in the period of 4–6 hours which elapsed between the death of the embryo and the inoculation of culture flasks with cell suspensions. If this is the case, it appears that defective embryonic cells can continue to multiply rapidly for at least 10 weeks before morphological changes become obvious and their growth potential is lost. The general experience of tissue culturists that many lines of embryonic cells die off after 8–12 passages in growth flasks could then be the result of initial damage to the cellular respiratory system instead of being due to nutritional deficiency or chronic toxicity of media as suggested by SWIM AND PARKER[30].

On the other hand, there is evidence that lactic acid production into the medium is associated with the growth of embryonic cells in circumstances where respiratory damage is less likely to have occurred. Using fresh explants of chick embryonic tissues, WILLMER[5], WILSON, JACKSON AND BRUES[6], PAUL AND PEARSON[31] and the present authors[32] have found appreciable lactic acid production, and, when they can be calculated, the ratios of lactic acid production: glucose utilization are of the same

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order (*i.e.* > 1 on a molar basis) as found with human embryonic cells. Only JONES AND BONTING[33] report one instance of the complete absence of acid production in cultures of chick heart cells but this seems to be the exception among all their other aerobic tests, and was found when 80% of the glucose (present initially at 1 mg per ml) had been utilized. With malignant cell cultures, much lower amounts of lactic acid are obtained when 70% or more (of the original 2 mg per ml) of the glucose disappears from the medium[27], but the utilization of the acid has not proceeded in our experiments to the extent of removing the entire amount from the medium.

In support of his view, WARBURG[4] also cites the fact that no increase in lactic acid can be detected in the efferent vessels from embryonic tissues. There remains, however, the evidence of Finnish investigators[34] that the concentrations of respiratory enzymes of human foetal cells increases with age (a feature also observed in animal embryonic tissues[35]) and that the blood of premature infants contains higher concentrations of pyruvic acid than that of full-term infants. It is relevant too that WARBURG's[1]* own values for the lactic acid concentrations in the blood passing between foetus and placenta are in the region of 9 μmoles per ml, which is close to our average of 10.7 μmoles per ml (Table III), and 3–4 times higher than the concentration in the maternal blood vessels. It does still seem possible that the normal environment of embryonic cells contains higher concentrations of lactic and pyruvic acids than those present in the adult body fluids and that these metabolites are not all oxidized but may provide material for fat synthesis, as suggested by RÄIHÄ[34].

The conclusion that aerobic glycolysis is relatively high for embryonic tissues and is not peculiar to malignant cells has been put forward on various occasions, and, in particular, by O'CONNOR[36] and WEINHOUSE[3,4]. VILLEE[37] in his extensive studies of the metabolism of human foetal tissues by manometric methods, also finds considerable production of lactic and pyruvic acids and his value of 2.56 μl per mg dry weight per hour for the rate of glucose consumption by embryonic kidney slices is of the same order as ours.

Glucose utilization and the size of the cell population

If glucose is the main source of energy for growth and maintenance, it should be possible to relate the amounts of tissue in replicate cultures to the amounts of glucose disappearing from the medium. However, previous investigators who have carried out studies of this nature all report that they found no direct and consistent relationship between the size of cell populations and glucose utilization[5,6,7].

One possible explanation for this anomaly emerges when lactic acid and keto acid production into the medium are measured simultaneously with glucose uptake. It seems probable from our results that these metabolites leak out of the cells until the external concentrations approach those normally existing inside the cells *in vivo*. If the amount of glucose required to provide for this leakage is subtracted from the total utilized, we find a very high correlation between the remainder (which we have provisionally called the glucose "oxidized") and the average amount of tissue per tube, whatever the type of cell.

The description of this portion of utilized glucose as the glucose "oxidized" is not intended to imply that it is entirely converted to carbon dioxide. As it happens,

* p. 303 of reference.

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there is unlikely to be much glycogen formation in the cultures, particularly in the malignant cells[7], and the amount of ribose incorporated in RNA and derived from glucose would involve only 2–2.5% of the glucose "oxidized". If, however, the assumption is made that the entire portion is oxidized as far as carbon dioxide, the volume of oxygen required will be 0.75 μl per 100,000 cells per hour for HeLa cells growing in GPM. GIFFORD, ROBERTSON AND SYVERTON[38] find that oxygen uptake is directly proportional to the cell number in HeLa cell cultures, which utilize approximately 1 μl per 100,000 cells per hour[39]. The agreement between the values is reasonably good and it would suggest that the major part of the energy required for growth is likely to be provided by the glucose "oxidized", although some other metabolites in culture media will also be subject to oxidation.

The energy produced by the breakdown of glucose for the lactic and pyruvic acid leaking into the medium will, of course, be available for synthetic processes. On the assumption that the conversion of glucose to these metabolites provides 2 or 8 moles of energy-rich phosphate compounds per mole of glucose, while approximately 38 moles are produced for each mole of glucose oxidized to carbon dioxide, it can be estimated that with the malignant cell cultures about 10% of the energy comes from excess glycolysis, while the proportion is about 20% for the embryonic cells.

The interpretation of metabolic quotients

The metabolic quotients for glucose utilization and acid production in tissue culture (and manometric) vessels are abnormally high, because they reflect a response to an abnormal environment. Under tissue culture conditions, as we have shown, lactic acid production diminishes in intensity from the highest rates achieved in the first hour in fresh medium, until the stage is reached when glucose concentration may be limiting and there is actual utilization of the lactic acid by the cells. But while the metabolic quotients for glucose utilization and acid production vary according to the changing concentrations in the medium, the quotients for glucose "oxidized" remain remarkably constant, and are more likely to represent the normal rates of glucose utilization.

MCILWAIN AND RODNIGHT[40,41] have drawn attention to the consequences of studying metabolism in manometric vessels when brain slices are suspended in 50 or more times their own volume of medium. In such circumstances, a high percentage (62%) of the glucose utilized is converted to lactic acid and the escape of metabolites from the cells into fresh medium may reduce respiratory rates by 20–45%. By maintaining the slices in a moist atmosphere and in minimal amounts of saline, they were able to reduce the conversion to lactic acid to 15% and to obtain higher levels of respiration. Similarly, in their studies on tissue explants in culture media, PAUL AND PEARSON[31] observed that respiration is depressed by renewal of medium. The important distinction between manometric and tissue culture studies of metabolism is that the manometric measurements are made over the period (2–3 hours) during which leakage of metabolites into the medium is highest, while tissue culture measurements cover 24 hour periods in which initial acid production can give way to the utilization of the metabolites at later stages.

The consequence of these differences may be judged by comparing metabolic rates for malignant strains as measured by the two methods. From recent studies

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carried out by BURK and his collaborators on the highly malignant cell line 1742 originating from mouse adipose tissue[42], WARBURG[2] reports an aerobic glycolysis quotient of 30, obtained in conditions in which the cells may have been suspended in as much as 300 times their own volume of culture medium (BURK AND SCHADE[4]) On expressing our results with human cells on the same dry weight basis (Table V) we obtain average quotients for aerobic glycolysis of 2.6 for human carcinoma cells. The differences can be partially explained by the fact that over the first three hours (the period in which manometric measurements are made) the acid production by our carcinoma strain was about three times faster than the average rate over 24 hours. BURK AND SCHADE[4] have suggested that adjustments should be made for species differences in basal metabolic rates, and if that were done, using relative values of 160 and 25 for mice and man, the gap would be more than closed.

TABLE VIII

METABOLIC QUOTIENTS FOR NORMAL AND LEUKAEMIC HUMAN LEUCOCYTES
(From data of BECK AND VALENTINE[49]) *

Cells	*Quotients as μmoles per 10^9 cells per hour*	
	Q glucose utilized	*Q lactic acid produced***
Normal leucocytes	140 ± 23	301 ± 51
Chronic myelocytic leukaemia	52 ± 9	127 ± 17
Chronic lymphocytic leukaemia	31 ± 3	48 ± 7

* Values given are means ± S.E.
** Our comparable values are the quotients for total acid production in Table IV. Of this total acid, 85–95% was lactic acid.

It would be preferable to compare our results with similar measurements made on other human cells, and this is possible in the case of studies on the metabolism of human leucocytes. BECK AND VALENTINE[43] separated these from normal and leukaemic blood and by suspending the cells in a buffered medium, they were able to measure the glucose consumption and lactic acid production and to express their results on a "per cell" basis. Their quotients are shown in Table VIII and our comparable values are in Table IV. The quotients are of the same order, and there is a striking similarity in the relationship between normal and leukaemic leucocytes and that between embryonic and carcinoma cells. In each set of results the rates of glucose utilization and lactic acid production are higher for the normal (or embryonic) cells than for the malignant cells.

The choice of reference standards

In most manometric studies of the metabolism of normal and malignant cells, quotients are expressed as μl (or mm^3) of metabolite per mg dry (or fresh) weight per hour. In one instance, in the studies of BERENBLUM, CHAIN AND HEATLEY[44] on the metabolism of normal and neoplastic skin epithelium, total NAP was used as a standard, and the authors came to the unorthodox conclusion that relatively high aerobic glycolysis (coupled with a low R.Q.) is a normal physiological event and not peculiar to malignant cells.

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Our results illustrate how much the order of values for metabolic quotients can vary according to the standards of reference. If we compare the embryonic and malignant cells on a "cell for cell" basis, then the quotients for glucose "oxidized" based on cell number are always larger for malignant than for embryonic cells (Table IV). By using NAP as a reference standard, the order is reversed, and the quotients for embryonic cells are now slightly greater than those for malignant cells (Table III). This situation arises because of the higher amounts of NAP per cell of the malignant series, and it would seem to indicate that glucose utilization depends to a large extent upon the nucleic acid content of the cells.

When the same quotients are expressed on the basis of unit dry weight, differences no longer exist between these for embryonic and malignant cells (Table V) The explanation lies in the different number of cells per unit dry weight of each cell type. Because the lung fibroblasts are (at least) half the size of the other cells (Table I), their unit dry weight contains twice or more the number of cells as the same unit of any other strain. This difference causes the range of the quotients for embryonic cells to overlap the range for malignant cells.

Expression of quotients on the basis of dry weight has been criticized by BACH[45] on the grounds that errors are inherent in the method as applied to the tissue slices used in manometric studies. From our results it is clear that quotients expressed on a unit weight basis (whether dry or fresh) will be misleading if, for example, the malignant cells are much smaller than the cells of origin, as is the case in comparison of hepatoma and normal liver cells (*cf.*[46]). High values for the quotients for hepatoma[47] could be the consequence of greater cellularity and a lower proportion of extracellular material.

It would seem more logical to put results on a "per cell" basis, since discussions are always made on the assumption that one cell type is being compared with another (*i.e.* on a "cell for cell" basis). However, quotients based on NAP are useful in showing how metabolic activity may vary from one cell type to another, particularly as the nucleic acids are almost entirely confined to the cell and are involved in the protein synthesis of cell maintenance and growth.

Response of embryonic and malignant cells to insulin

Very little information is available on the influence of insulin on tumour cell metabolism. By using manometric methods, WOODS, WIGHT, HUNTER AND BURK[48] were able to show that anaerobic glycolysis of mouse melanoma slices was increased by 40% in the presence of insulin, whereas mouse brain remained unaffected under the same conditions. From their later studies[49] came evidence that there is an insulin: anti-insulin mechanism operating in certain melanotic tumours, but absent from the more-anaplastic strains and the Krebs-2 ascites tumour.

From our results it is clear that insulin can stimulate RNA (and phospholipid and protein[32]) synthesis in cultures both embryonic and malignant cells. The two cell types are quite distinctive, however, as regards the effect of insulin on glucose metabolism. With the embryonic cells a slightly lower proportion of the glucose appeared as lactic acid and keto acids in the medium, while with the carcinoma cells the production of these metabolites (and of glucose consumption) was greatly increased. This difference, together with the distinctive values of DNA per nucleus (Table I) and of glycolytic rates (Table III), provide a means of distinguishing

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between embryonic cells and any suspected malignant strains which may originate in cultures of normal cells[50].

The increased glycolysis of the malignant cells apparently overtakes the oxidative capacity of the cells, and the increases in the LA:KA ratio (Table VII) suggests that reduced cozymase (DPNH) is produced faster than it can be removed by mitochondrial respiratory systems. On the other hand, the reduction of the LA:KA ratio with insulin-treated embryonic cells suggests that in this case they contain a higher proportion of the oxidized cozymase as compared with the controls. However, it would be unwise to draw any definite conclusions on the action of insulin on the DPN^+:DPNH ratio within the cells, since direct measurements of the pyridine nucleotides by different investigators have produced conflicting reports of its effect on the proportions of the oxidized and reduced forms in animal[51, 52, 53].

As a working hypothesis, it seems reasonable to take the view that the characteristics which distinguish malignant cells from other cells lie in the adjustment of their metabolism to hormone action. PETERS[54] has recently proposed that hormone action must take place at specified localities on the "cytoskeleton", and our results might be explained if it is assumed that insulin produces a different response in the malignant cells because of the altered nature of its mitochondria and, possibly, of its nucleus. It is significant that ^{131}I-labelled insulin has been found to penetrate rapidly into liver cells and to become firmly attached to the mitochondrial and nuclear membranes[55].

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SUMMARY

1. A tissue culture procedure has been developed for measuring rates of glucose utilization and lactic acid and keto acid production in cultures of human embryonic and malignant cells. Tetraploid values for the DNA content per nucleus were found in the three carcinoma strains (HeLa, HEP 1, HEP 2), and normal diploid values were obtained for cultures of the skin fibroblasts.

2. Very high correlations ($r > +0.94$) were obtained between the average amount of NAP in the roller tubes over 72 hours and the portion of the utilized glucose which did not appear in the medium in the form of lactic acid and keto acids. This portion has been termed the glucose "oxidized". Total glucose utilization was partly dependent on the extent of leakage of lactic acid and keto acids into the medium.

3. The metabolic quotients for glucose utilization and total acid production were higher for embryonic cells than for malignant cells, irrespective of the standard of reference (NAP, cell number, protein nitrogen or dry weight).

4. The relative order of the quotients for glucose "oxidized" varied according to the standard of reference and indicated that quotients based on dry weight were unsuitable for distinguishing between embryonic and malignant cells.

5. The response of skin fibroblasts and kidney cells to insulin was qualitatively different from the response of the carcinoma cells. With embryonic cells there were decreases in acid production relative to glucose utilization and in the ratio of lactic acid:keto acid in the medium, as compared with the controls. With carcinoma cells, increases in these ratios accompanied a much

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more intensive glycolysis than found in control cultures. Insulin significantly increased RNA formation in both cell types.

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