

# Observations on the Metabolic Behavior of a Clone of Mouse Liver Cells Grown in Agitated Fluid Suspension

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The strain of mouse cells designated NCTC clone 1469 was developed several years ago (5) from a single cell isolated from a culture of the parenchymal liver epithelium of a young mouse of the C3H-HeN line (4). The strain grows well in T-60 flasks in a stock medium of 40 per cent horse serum, 20 per cent chick embryo extract, and 40 per cent balanced saline (v/v).<sup>1</sup> Under these circumstances it stores glycogen rapidly in the presence of suitable precursors.<sup>2</sup> Several years ago its parent cell strain NCTC 721 was also found to store glycogen in considerable quantity (12). The study reported here was one phase of an attempt to grow the strain in agitated fluid suspension with continuous gassing (1). In part of the study, counts were made at each fluid change and samples of the pertinent fluids taken for analysis. At the end of the experimental period the cells were harvested for study. The adjustment to growth in the shaker flasks was accompanied by certain peculiarities in the growth and metabolism of the strain that are brought out in the text.

## MATERIALS AND METHODS

The inoculum to start the experiment was the pooled cells from 10 T-60 flasks. They had grown up to this point in the stock fluid mentioned earlier. The ten flasks yielded approximately 300 million cells. The inoculum was planted in 200 ml. of nutrient fluid that differed from the standard stock in that the embryo extract was replaced by 20 per cent (v/v) of an ultrafiltrate of the balanced saline extract (1:1.25 v/v) of the freshly laid hen's egg exclusive of the shell and membrane (8). The nutrient fluid was replaced on a 48-48-72-hour schedule weekly. An effort was made to adjust the fluid volume to the cell population at each fluid change. This involved, at each fluid change, measurement of the population by nuclei count (10). At the end of 30 days the cells were taken for analysis.

<sup>1</sup> Balanced saline means the high bicarbonate buffered saline with glucose used in this laboratory (2).

<sup>2</sup> B. B. Westfall, V. J. Evans, E. V. Peppers, N. M. Hawkins, H. A. Kerr, W. T. McQuilkin, and W. R. Earle. The Glycogen Content of Cell Suspensions Prepared from Massive Tissue Culture. II. Comparison of 15 Cell Strains after Long Cultivation (in preparation).

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The pertinent cell-free fluids were also taken for analysis. These were:

1. The used medium from the cells.
2. The control, incubated, gassed, and shaken medium with no cells.
3. An aliquot of the medium kept at 37° C. with no cells, no shaking.
4. An aliquot of the freshly made medium at each fluid change.
5. An aliquot kept at 5° C. for the stated interval between fluid change.

The fluids were used for the estimation of the glucose used by the cells and the lactic acid produced (11). Alpha-keto acids and total free amino acids, amines, and amides were also deter-

TABLE 1  
NUMBER OF CELLS, TOTAL FLUID VOLUME, AND AMOUNT OF GLUCOSE IN THE MEDIUM FOR CULTURE OF THE 1469 STRAIN LIVER CELLS

TIME (days)	VOLUME OF FLUID (ML.)		NUMBER OF NUCLEI		GLUCOSE SUPPLIED TO CELLS (MG/100 ML)
	Old	New	Million total	Millions/ml	
0		200	299	1.49	260
2	200	175	169	0.85	260
4	175	125	97	0.56	260
7	125	75	49	0.39	260
9	75	75	55	0.74	260
11	75	150	144	1.93	320
14	150	400	360	2.53	260
16	400	420	626	1.57	320
18	420	420	493	1.17	320
21	420	250	259	0.62	320
23	250	300	250	1.00	320
25	300	350	308	1.03	260
28	350	400	943	2.69	380
30	400		1114	2.80	290

mined (13-15). The cells were taken for estimation of wet and dry weights, total protein, total intracellular free amino acids, and glycogen. The procedures used are detailed in the references listed. Throughout the paper the terms cell number and cell nuclei are used as synonymous.

## RESULTS

The data are summarized in Tables 1, 2, and 3 and in Chart 1. Table 1 contains the volumes of medium used, the total populations of the cultures, the number of nuclei/ml of nutrient medium, and the concentration of the glucose furnished to the cells. The glucose concentration was increased in

all instances over the usual 1 mg/ml nutrient fluid in an effort to maintain a continuously available supply of the carbohydrate. This was done because it was known that in the T-60 unshaken flasks the glucose supplied to the cells at the 1 mg/ml level was exhausted in the presence of 1.5 million cells/ml by 24 hours after fluid renewal (12). The figure 1.5 million is that usually attained when good growth takes place.

From the population figures of Table 1 it will be noted that there was very heavy cell mortality in the first week of the experiment. The transition to the shaker flask evidently requires drastic adjustment on the part of the cells, since eight out of

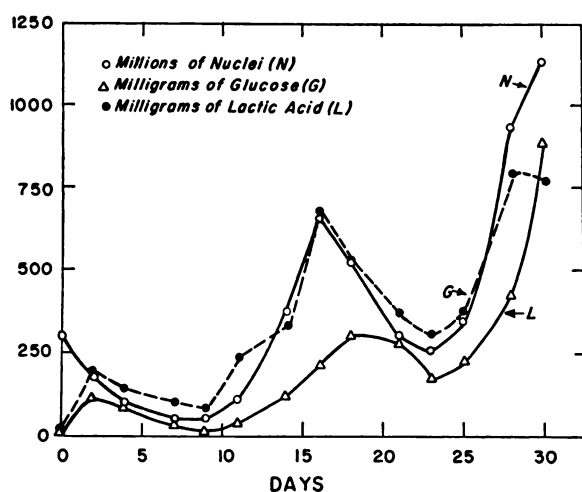


CHART 1.—Time course of the cell population, glucose usage, and lactic acid production by the NCTC clone 1469 liver cells.

ten were destroyed by the 7th day. By the 16th day there was then an increase of 13 $\times$  from the nadir on the 7th. A subsequent low was reached on the 23d day, with a second sharp growth spurt to 4 $\times$  the new low, at which time the cells were harvested, on the 30th day of the experiment. Chart 1 is a graph of the total cell population and total glucose utilization at the sampling intervals. There is good parallelism, except for the final point where the glucose used was outstripped by the increase in cell nuclei. Chart 1 also contains the similar graph of lactic acid produced and remaining in the cultures in excess of that supplied by the medium. The horse serum used in the medium contained 30 mg/100 ml lactic acid, so that the medium samples had 12.5 mg/100 ml from this source when it was added to the cultures. The lactic acid figures have had this amount subtracted from them. Table 2 lists the glucose used, lactic,  $\alpha$ -amino, and  $\alpha$ -keto acids produced—calculated to the 100 mil-

lion cell basis. The medium supplied to the cells contained 0.8 mg/100 ml total  $\alpha$ -keto acids expressed as pyruvic acid and 3.5 mg. N as  $\alpha$ -amino acids, amides, and amines; the analyses have taken these into account.

When the shaken control flask was centrifuged at each fluid change, it was noted that there was accumulating in the flask a mass of apparently precipitating protein. Since this centrifuged out of the fluid it continued to accumulate for the entire period in the flask so that all was present in the final fluid taken for analysis at the end of the experimental period. Six aliquots of 30 ml. each of the shaken, control medium were centrifuged at 80  $\times$  *g*, and the precipitate was weighed. The wet weight per aliquot was 53 mg. or 1.76 mg protein precipitate/ml medium. The precipitate dried down to give 1.56 mg dry weight/100 mg wet precipitate, which indicated that the precipitate con-

TABLE 2  
GLUCOSE, LACTIC, AMINO, AND ALPHA-KETO ACIDS OF  
THE MEDIUM DURING CULTURE OF THE  
STRAIN 1469 LIVER CELLS

Day of sampling	Total cells (millions)	Glucose used (mg.)	Lactic acid, excess (mg.)	Alpha-keto acids, excess (mg.)	Alpha-amino acids, excess (mg.)
1	235	93	60	8	11
3	133	134	92	0	*
5.5	73	160	65	12	18
8	52	155	52	15	15
10	99	118	44	9	3
12.5	262	315	51	3	4
15	503	120	49	0.4	1
17	550	88	46	1	7
19.5	376	85	68	1.5	8
22	255	108	64	3.5	*
24	270	125	58	1.5	*
26.5	622	151	28	1.2	0.5
29	1044	71	73	0.9	0.5

Cell populations in this table have been adjusted to the middle of the sampling period. Results are calculated to the 100 million cell basis.

\* No analysis for amino acids made at these periods.

By "excess" in columns 4, 5, and 6 is meant the quantity found present over that supplied to the cells by the fresh medium.

tained a great amount of water. In order to calculate wet weight, dry weight, and total protein on the cell mass, it had to be assumed that the separated protein occurs in the same quantity in the medium containing the cells and carried with it identical amounts of dry matter and water. It was not possible to separate the cells from the similar protein of the medium in which they grew.

During the packing of the cells in the centrifuge tube it was noted that the cell-containing mass packed better and stayed in place in the bottom of the centrifuge tube better than did the protein

from the acellular medium. An experiment wherein a weighed mass of strain 1469 cells from the T-60 flasks was packed down, washed, resuspended, weighed, and then added to a weighed mass of the protein precipitate, centrifuged, and once more weighed indicated that there was more efficient packing of the protein in the presence of the cells; e.g., 100 mg. of the wet cells mixed with 106 mg. of the wet protein gave 184 mg. total combined wet weights. The total mass of the wet cells in the flask on the 30th day of the experiment was then either 1.2 or 1.34 gm., depending on whether one assumed that packing would be better in the joint presence of the protein and cells or not. The total mass of the cells plus precipitated protein was 1.9 gm. If one assumes that this is all cells and that the protein precipitate from the medium did not occur in the presence of the cells, the wet weight of a million cells would be 1.7 mg., i.e., 1.9/1,114 million; the wet weight after correcting for possible lower volume, i.e., lower weight of the protein in the presence of the cells, would be 1.2 mg./million cells and, neglecting shrinkage, would be 1.06 mg./million cells. All these are decidedly less than the wet weight found for the parent 721 strain (2.75 mg./million) when grown in T-60 flasks (12). When the cells from the fluid suspension culture were taken for dry weight, four aliquots gave  $29.7 \pm 0.7$  per cent dry weight. This value is close to that for the livers of the parent strain of mice (12), as shown in Table 3, and is 40 per cent greater than found in cells of this same strain grown in T-60 static flask cultures. It appears possible that the increased dry weight should be ascribed to a rounding-up with cell volume reduction during growth in an agitated suspension. However, this might also result from rapid proliferation with reduction in nuclear size. From the one viewpoint of per cent dry weight it would appear that growth is more nearly like that in the intact animal's tissue than is growth in the T-60 static cultures.

The cells at harvesting had 29 mg glycogen/100 gm wet weight.

The amino acids from the protein-free ultrafiltrates of the used supernatant fluid at 5.5 days (Table 2) were put on paper for 2-dimensional chromatographic examination (11). All appeared to be increased 2 or 3 times over those of the medium with no exposure to the cells. The similar experiment for the 29th day gave evidence of decreases in several of the acids such as the leucines, valine, phenylalanine, and threonine, but a tripling of the concentration of the alanine. The dinitrophenylhydrazones of the  $\alpha$ -keto acids of the 1 and 5.5 periods of Table 2 were subjected to paper chromatographic separation (13). In milli-

grams/100 ml of used medium the fluid of the 1-day period of Table 2 had in it the highest concentration of  $\alpha$ -keto acids encountered to date in any culture—namely, 18 mg. Presumably, this reflects a severe disturbance in the normal pathway of glucose metabolism, since about 85 per cent of the figure was found to be pyruvic acid, with  $\alpha$ -ketoglutaric acid as 5 per cent. In the solvent system used there was a fast moving spot beyond the more rapidly moving spots attributed to the pyruvic acid hydrazone (14). Hydrolytic reduction (7, 9) of the dinitrophenylhydrazones eluted from this spot with subsequent paper chromatography to separate the formed amino acids (7) indicated that much of the fraction was to be ascribed to the hydrazones of  $\alpha$ -ketoisovaleric acid and  $\alpha$ -ketoisocaproic acid (6, 7, 9).

It was not possible to devise a satisfactory solvent system for the separation from each other of the dinitrophenylhydrazone of  $\alpha$ -ketoisocaproic and  $\alpha$ -ketoisovaleric acids on the paper.

The fast moving hydrazones accounted for most of the remaining 10 per cent of the total. The recovered amino acids from the hydrolytic reduction accounted for less than half as much. There were, however, two unidentified ninhydrin-positive spots on the amino acids chromatogram after the hydrolytic reduction.

## DISCUSSION

It has been the custom in this laboratory to refrain from making calculations with respect to the metabolism of the cells during periods of easily demonstrable extensive cell death, since it was thought that trying to analyze dying cells in any way could be very misleading. Due caution should be exercised in drawing conclusions from periods in the experiment presented here for such times as the first 2-day interval when the keto acid concentration reached unprecedented values and the amino acid concentration likewise was higher than that in the medium supplied to the cells for their subsistence. It is the general experience in the still cultures, when there is good growth, that the total free amino acid concentration declines and the  $\alpha$ -keto acids reach perhaps 10 per cent of the value recorded during this period of great cell destruction.

As recorded in Table 2, there is nothing particularly noteworthy in the glucose consumption or lactic acid production during the interim. During the period of greatest cell destruction, i.e., the day 1 of Table 2, the  $\alpha$ -keto acids were high in concentration as previously noticed. At the times of lowest total cells, i.e., the 7th and 22d days, they were about 8 times the value supplied to the cells by the

medium. The large increase in ninhydrin-positive materials in the ultrafiltrate from the used medium recorded as  $\alpha$ -amino acids in Table 2 is probably attributable to the proteolytic activity of the materials released by the cells as destroyed as well as to the transaminases both from the cells and from the medium. The transaminase activity is probably responsible for the increase in alanine on the 29th day when many of the other amino acids were decreased through usage by the cells. The free amino acids that could have been released from inside the cells by the cellular destruction are very much less than enough to account for the increase in free amino acids of the medium. Markert (8), using labeled glucose has found that the alanine of the medium is labeled after use of the medium by growing liver cells.

TABLE 3

DRY WEIGHT AND PROTEIN CONTENT OF THE LIVER OF THE MOUSE OF ORIGIN, OF THE CULTURED CELLS, AND OF THE DERIVED CULTURES

Tissue cell strain or tissue source and conditions	Dry weight of tissue (gm/100 gm)	Protein content (gm/100 gm)
Liver, C3H/HeN mouse <i>in vivo</i>	34.7	21.6
NCTC 721 liver epithelium, T-60 flasks, sm.	17.5	11.5
NCTC 1469 liver epithelium, T-60 flasks, sm.	17.7	11.5
NCTC 1469 liver epithelium, Shaker flasks. WUF instead of EE.	29.7	21.0

sm = stock medium: horse serum, chick embryo extract plus saline.

EE = 1:1 balanced saline extract of the 9.5-day-old chick embryo.

WUF = 1:1.25 extract of the unincubated newly laid hen's egg used as the protein-free ultrafiltrate.

The large mass of protein separating out in the shaken medium is a disturbing factor in all work with cells under these circumstances. The precipitate contained a considerable proportion of the cholesterol and other lipides of the medium but behaved like a partially denatured protein when attempts were made to examine it electrophoretically. Correcting as best one can for the precipitated protein, the data of Table 3 indicate that the cells are higher in solid constituents than when grown in T-60 flasks. Data are not yet available on such key materials as deoxyribonucleic acid, but such materials would probably be increased as much as 40 per cent in the agitated cultures compared with the cultures in T-60 flasks. This statement would apply only to the wet weight basis for comparison and is not meant to imply that the total per cell would be increased.

The strain 1469 cells under study derived from one cell of a culture of cells which were in turn

derived from an original explant of what was presumed to be mouse liver epithelium. Due caution must be exercised in considering them as liver epithelial cells at the present stage. The facts that they have a certain glycogen content and evoke relatively large increases in keto acids of the medium are suggestive, but not definitive.

The experimental work presented here has been considered a continuous experiment running 31 days. Actually, since complete fluid change and cell counts were made at short intervals, i.e., 2 and 3 days, the work may also be viewed as thirteen separate experiments with varying population levels. Looked at in this manner, the variations in glucose utilization shown in Table 2 are very large.

## SUMMARY

The mouse liver epithelial cells from clone NCTC 1469 were grown in agitated suspensions in a medium of horse serum, whole fresh hen's egg extract ultrafiltrate, and balanced saline (40-20-40 v/v) for 30 days. Glucose consumption and lactic acid production paralleled the cell quantity rather closely. During periods of massive cell death the free amino acids detectable in the medium were greatly increased. At times when there was a declining population, the  $\alpha$ -keto acids in the medium were greatly increased following use of it by the cells. When there was good cell growth there was a decrease in certain of the free amino acids of the medium regarded as needed for cell growth. Alanine was, however, increased in concentration under the latter circumstances. During the initial 48 hours of cultivation of the cells in agitated culture the  $\alpha$ -keto acids in the used medium reached the highest concentration, 18 mg/100 ml, so far encountered in any cultures; 15 mg/100 ml of this was pyruvic acid, indicating a serious disturbance in glucose metabolism. At the time the cells were harvested they had 140 mg glycogen/100 gm cell protein.

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