NUTRITIONAL REQUIREMENTS FOR GROWTH OF A MOUSE LYMPHOMA IN CELL CULTURE

L. A. HERZENBERG[1] and R. A. ROOSA[2,3]

Laboratory of Cell Biology, National Institute of Allergy and Infectious Diseases and Laboratory of Biology, National Cancer Institute, National Institutes of Health, Bethesda, Md., U.S.A.

Received November 30, 1959

For comparative *in vivo* and *in vitro* biological studies on mammalian cells it is necessary to have a cell which can be grown in culture or in an animal. The mouse lymphoma P-388 appeared to be such a cell. It was established in culture in 1956 and on many occasions since this time cultures of these cells have been injected into the peritoneal cavities of compatible inbred strains of mice and there have given rise to transplantable ascites tumors. These tumor cells have then been able to grow when reintroduced into culture [1].

For quantitative biochemical and genetic studies of these cells in culture it was desirable to develop a chemically defined medium. This paper describes the development of such a medium which serves well for the long-term propagation of these mouse lymphoma cells from large inocula as well as from isolated cells.

MATERIALS AND METHODS

Medium: Eagle's basal medium [2] supplemented as noted was used throughout this work.

Serum.—Calf serum was purchased from Microbiological Associates, Bethesda, Maryland. Dialysis was carried out for 24 hours against running tap water at 5–10°C, then against 4 changes of 20 volumes of 0.85 per cent NaCl for 24 hours each at 4°C. The water dialysis alone was not sufficient to remove growth-promoting activity of the serum when added to the basal medium.

Cell line.—The cells used in this work were derived from a mouse lymphoma (P-388) induced by methylcholanthrene in a female DBA/2 mouse and after 49 passages in CDBA/2 F_1 mice, was introduced into culture in a complex medium. To avoid confusion due to possible differences of the cells as we have maintained them from those maintained in the original medium, we have designated our cell line ML (mouse lymphoma) 388.

[1] Present address: Dept. of Genetics, Stanford University, Stanford, California.
[2] Post-Doctoral Research Fellow, National Cancer Institute.
[3] Present address: Wistar Institute, Philadelphia, Pennsylvania, U.S.A.

Maintenance of cultures.—These cells attach to glass, and were maintained in flat-bottomed, stoppered prescription bottles or in T flasks in Eagle's medium supplemented with 5 per cent calf serum at 37°C. In later work, 10^{-3} *M* pyruvate was routinely included in the medium used for cell maintenance. Cultures were fed as needed (judged by acidity of the medium) and were subdivided when the glass surface became crowded (about once a week). When the population density was adjudged too low to maintain a proper pH, the flasks were gassed with a small volume of 10 per cent CO_2–90 per cent air.

At the beginning of this work, several aliquots of the culture were slow-frozen [12] in ampoules and stored in a dry-ice chest. Cultures were revived from these ampoules after the majority of this work was complete, and the nutritional studies were repeated to insure that these properties of the cell line had not changed while the work was in progress.

Growth measurement.—Approximately 10^5 cells were inoculated into a series of T-15 culture flasks in 2.5 ml of maintenance medium. After 24 hours incubation at 37°C (to allow attachment of the cells to the glass), the medium was removed, the cells were washed and then fed with experimental medium (2.5 ml per flask). This was designated zero time. Additional feedings were made on days 2, 4 and 5.

At zero time and at suitable intervals thereafter, flasks were harvested by removing the medium and washing twice with 0.85 per cent NaCl (room temperature). Protein was determined by a modification of the Lowry method [9] and nuclei counts were made in a hemocytometer using Sanford's diluent [11]. Determinations were always done on at least two flasks. Agreement between flasks was usually within 10 per cent for protein determinations and 20 per cent for cell counts.

Growth from isolated cells (colony formation).—Monodisperse cell suspensions (>95 per cent single cells) were prepared by removing recently fed cell layers from the glass with rubber policemen and pipetting up and down several times. Dispersed cells were inoculated in 5 ml of medium per 60 mm petri dish. Incubation was in a vibration-free 37°C incubator with a water-saturated atmosphere of 5 per cent CO_2–95 per cent air.

Radioactive tracer experiment.—Cells in logarithmic growth [10] were allowed to increase 10-fold in media containing the labeled precursor compound. The medium from the last day was saved for isolation and determination of pyruvate as its dinitrophenyl hydrazone. Cell layers were washed twice with 0.85 per cent NaCl in the cold and harvested with cold 5 per cent trichloracetic acid. The precipitate was extracted at 90°C with 5 per cent trichloracetic acid and then hydrolyzed in 6 *N* HCl in a sealed tube at 115°C for 16 hours. Glycine, serine and alanine were isolated from the hydrolysate by chromatography on DOWEX-50 [7]. Radioactivity determinations were made in a thin-end-window, gas-flow counter.

RESULTS

ML 388 grows continuously in cell culture in Eagle's medium supplemented with 5 per cent calf serum with a minimum doubling time of approximately 20–24 hours. Growth is not sustained, however, when 5 per cent

dialyzed calf serum replaces the whole serum (Fig. 1). Rather than attempt to fractionate the material removed on dialysis of the serum to identify in this way the missing growth factor(s), we examined a large number of likely compounds for possible growth-promoting activity when added to the dialyzed-serum-containing medium (DSM).

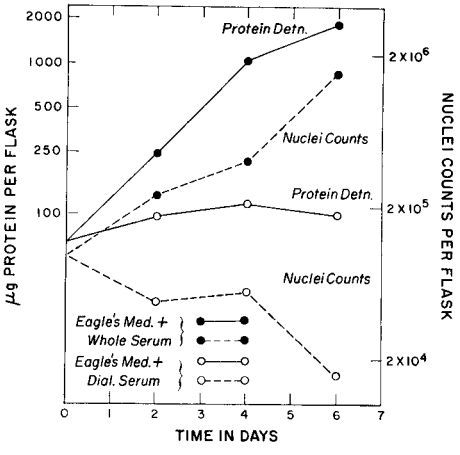

Fig. 1.

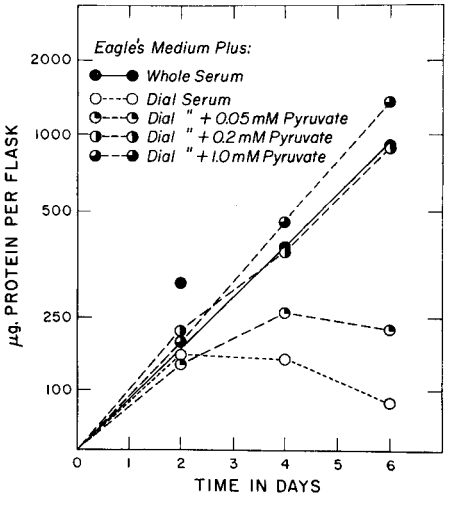

Fig. 2.

Fig. 1. Growth kinetics of ML 388 in mass culture with dialyzed or whole serum supplements.
Fig. 2.—Growth kinetics of ML 388 as a function of pyruvate concentration in the medium.

Data presented in Table I and Fig. 2 show that glycine, serine, pyruvate and to a lesser extent alanine all support growth of 388 in mass culture when added to the DSM. Pyruvate gives maximal growth at concentrations greater than 2×10^{-4} *M*, glycine and serine are maximally effective at 5×10^{-5} *M* and higher while alanine even at 10^{-3} *M*, does not allow the maximum growth rate to be achieved. The other natural amino acids not found essential for the growth of a number of other mammalian cell lines and not included in Eagle's medium (aspartic acid, asparagine, proline, hydroxyproline and glutamic acid) do not support growth of 388 when added either singly or in combination.

Having established that pyruvate, serine, glycine and, less effectively, alanine act as alternative growth factors for these mouse lymphoma cells in culture, it became of interest to determine how these compounds could serve interchangeably. We, therefore, undertook to determine the metabolic relationships between these compounds in 388 by isotopic tracer experiments.

Log-phase cells were inoculated into the basal medium containing either carbon-labeled glucose, pyruvate or serine. After a 10-fold increase in protein had occurred, the specific acitivities of the serine, glycine and alanine in the cell protein and of pyruvate in the medium were determined. Table II, column 1, shows that, starting with medium containing uniformly labeled ^{14}C-glucose and unlabeled pyruvate, the three amino acids, and pyruvate as well, derive a large portion of their carbon from glucose. Although this experiment does not allow us to establish a precursor arrangement among

TABLE I. *Serine, glycine and alanine as supplements for growth in mass culture of ML 388. The indicated additions were made to DSM.*

Supplement		Protein after 6 days referred to inoculum as 1
None		2.6
glycine	5×10^{-5} *M*	9.3
,,	2×10^{-4} *M*	16
,,	1×10^{-3} *M*	16
1-serine	5×10^{-5} *M*	13
,,	2×10^{-4} *M*	17
,,	1×10^{-3} *M*	18
1-alanine	5×10^{-5} *M*	3.5
,,	2×10^{-4} *M*	4.1
,,	1×10^{-3} *M*	6.3
5 % whole calf serum (replacing dialyzed)		16

TABLE II. *Contribution of carbon by various precursors to amino acids in cell protein and to pyruvate in the medium. Cell protein increased 10-fold.*

	C^{14}-Labeled precursor compound		
	Glucose (5×10^{-3} *M*)	Pyruvate (10^{-3} *M*)	Serine (10^{-4} *M*)
Observed compound	Average specific activity per C atom precursor = 100		
Serine	66	1	35
Glycine	74	1.2	47
Alanine	57	21	0.0
Pyruvate	63	12	0.0

the four alternative growth factors, as the originally unlabeled pyruvate in the medium rapidly becomes radioactive, it does make clear that ML 388 is able to synthesize all of these compounds from glucose. When labeled pyruvate and unlabeled glucose are in the starting medium (Table II, column 2), only alanine and pyruvate become labeled. On the other hand, when labeled serine is used in conjunction with unlabeled glucose (Table II, column 3) both protein-serine and protein-glycine become labeled, whereas alanine and pyruvate do not have any detectable radioactivity. Thus these cells cannot appreciably interconvert the carbon of serine and glycine with pyruvate and alanine although each of these sets of compounds are synthe-

TABLE III. *Essentiality of pyruvate and 1-serine (or glycine) for colony formation by ML 388. Approximately 150 cells were plated in 60 mm petri dishes in 5 ml of Eagle's medium plus 5 % dialyzed calf serum with the indicated additions.*

Supplement	Colonies per dish (average of 4 dishes)
0	0
10^{-3} *M* pyruvate	3
10^{-4} *M* 1-serine	0
10^{-3} *M* pyruvate + 10^{-4} *M* 1-serine	68
10^{-3} *M* pyruvate + 10^{-4} *M* glycine	48
10^{-3} *M* 1-alanine + 10^{-4} *M* 1-serine	0
10^{-3} *M* pyruvate + 10^{-3} *M* 1-alanine	0

TABLE IV. *The effect of various concentrations of pyruvate and 1-serine on colony formation by ML 388. Approximately 200 cells were plated in 60 mm petri dishes in 5 ml of Eagle's medium plus 5 % dialyzed calf serum with the indicated additions.*

Concentration of Na pyruvate (*M*)	Colonies per dish (average of 4–6 dishes) Concentration of 1-serine (*M*)				
	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}
0	0	0	0	0	0
5×10^{-5}	0	0	0	4	0
2×10^{-4}	1	2	8	55	15
10^{-3}	26	25	36	71	49
10^{-2}	44	44	36	70	44

sized from glucose and members of either set support maximal growth of ML 388 in mass culture.

Nutritional needs for growth from large inocula of many cell lines in culture are often somewhat less demanding than when isolated cells are planted in relatively large volumes of media [6]. ML 388 proved to follow this general trend, but in a somewhat unusual manner. Although isolated cells form visible colonies in 7–10 days in Eagle's medium supplemented with 5 per cent whole calf serum they do not divide in 5 per cent DSM. When the compounds which act as growth factors in mass culture of 388 are used one at a time to supplement the DSM, only pyruvate allows some small colonies to form. However, when both serine and pyruvate are added, full growth into colonies is obtained (see Table III). As under mass culture conditions, glycine replaces serine on an equimolar basis. In contrast, how-

TABLE V. *α-Keto acids as supplements for mass culture and isolated cell growth of ML 388. The indicated additions were made to DSM for mass culture growth measurements and to DSM plus 10^{-4} M l-serine for colony formation determinations.*

Supplement	Protein after 6 days, referred to inoculum as 1		Visible colonies/dish after 9 days (average of 4 dishes)	
none	Exp. 1	1.0	Exp. 3	0
pyruvate 1×10^{-3} *M*		7.0		45
glyoxylate 1×10^{-4} *M*		5.0		0
,, 2×10^{-4} *M*		10		—
,, 1×10^{-3} *M*		2.9		0
α-Keto *n*-butyrate 1×10^{-4} *M*		2.5		0
,, 1×10^{-3} *M*		13		41
,, 2×10^{-3} *M*		12		—
α-Keto isovalerate 1×10^{-4} *M*		1.1		0
,, 1×10^{-3} *M*		1.1		0
α-Keto isocaproate 1×10^{-4} *M*		0.9		0
,, 1×10^{-3} *M*		0.9		0
α-Keto *n*-caproate 1×10^{-4} *M*		1.1		0
,, 2×10^{-4} *M*		3.9		—
,, 2×10^{-3} *M*		5.8		0
none	Exp. 2	2.8		
pyruvate 1×10^{-3} *M*		14.7		
α-Keto *n*-valerate 1×10^{-4} *M*		2.4		0
,, 1×10^{-3} *M*		2.4		3

ever, alanine does not substitute for pyruvate or serine (or glycine) for colony formation.

The results of a typical experiment showing the pyruvate and serine concentrations needed for maximal colony formation are shown in Table IV. Serine has an optimal concentration range around 10^{-4} *M* while pyruvate is equally effective at all concentrations greater than 2×10^{-4} *M*.

A number of α-keto acids were tried as possible substitutes for pyruvate. Of these, α-keto-*n*-butyrate replaced pyruvate on an equimolar basis both in mass culture and for growth of isolated cells. Glyoxylate and α-keto-*n*-caproate are growth factors in mass culture, but are not effective as either pyruvate or serine (or glycine) replacements for colony formation. In the presence of α-keto-*n*-valerate a few small colonies developed, but no growth-promoting activity in mass cultures was demonstrated. All the other α-keto acids tried including α-keto α-methyl valerate, α-keto isovalerate, α-keto isocaproate, α-keto glutarate and α-keto adipate were completely ineffective in both growth situations.

A large number of other compounds, many of which bad been reported to be growth-supporting or stimulating in various tissue culture situations, were inactive for 388. These included Kreb's cycle intermediates, fats, fatty acids, purines, pyrimidines, nucleosides, vitamins and cholesterol.

DISCUSSION

The requirement for serine exhibited by ML 388 under cloning conditions is essentially the same as that found by Lockart and Eagle for a number of other mammalian cell lines [6]. These authors ascribe this requirement to a low serine pool and the inability of the cells to retain this pool when suspended at a very low cell density. In contrst to the cells these workers investigated, with 388 cells glycine replaces serine on an equimolar basis.

The alternate requirement for serine or pyruvate under mass culture conditions and the absolute requirement for both these compounds for growth from single cells invites explanation. Serine (or glycine) is required in relatively high concentrations to support growth from large inocula, whereas much lower concentrations suffice for isolated cell growth. However, the same high concentration of pyruvate is required either for mass culture or for colony formation, and this concentration is far in excess of the amount metabilized or removed by the cells—particularly under cloning conditions. The demonstration, using labeled materials, that 388 actually synthesizes the compounds shown to be required for growth indicates that these materials

may not be needed as metabolic precursors or end products in the same way as an "essential" amino acid or vitamin, but suggests that they serve in some other manner.

Ascites cells in general and 388 in particular have very limited respiratory capacities and metabolize glucose almost exclusively via glycolysis. For a glycolytic cycle to continue indefinitely in the absence of sufficient respiration, one of the products of glycolysis must be reduced in order to reoxidize the pyridine nucleotide coenzymes which have become reduced in the glycolytic process. With 388 growing in Eagle's medium, the reduction of pyruvate to lactate must serve this essential function since approximately 95 per cent of the glucose disappearing from the medium is accounted for as lactate produced [4]. If, however, a relatively small amount of the pyruvate formed in glycolysis is lost from the cells before it can be reduced, and if the cells are unable to reconcentrate this pyruvate from the medium, the pyridine nucleotides of the cells would become completely reduced and glycolysis, and consequently growth, would have to cease. In the absence of any other means of oxidizing the reduced coenzymes, loss of only 0.1 μmole of pyruvate per gram of cells is sufficient to reduce the cell layer's entire complement of pyridine nucleotides, assuming 100 μg of pyridine nucleotide per gram wet weight of cells. This amount of pyruvate will give a concentration of pyruvate in the medium of only 4×10^{-7} *M*. These cells do have a limited oxidative capacity, however, and traces of oxygen are essential for growth, though not for survival. Thus, these cells can perhaps tolerate loss of slightly more pyruvate than estimated before suffering complete reduction of the pyridine nucleotides. On the other hand, the estimate may be too low as growth may cease when only a slight change in the normal ratio of oxidized to reduced pyridine coenzymes has been produced.

The relatively high pyruvate concentration needed for growth (greater than 2×10^{-4} *M*) may indicate that pyruvate enters the cells only with difficulty, and offers an explanation of why metabolically produced pyruvate lost from the cells cannot be reutilized for growth.

The ability of α-keto-*n*-butyrate and not of the other α-keto acids to substitute for pyruvate is consistent with the interpretation we have placed on the pyruvate requirement. α-keto-*n*-butyrate is by far the next best substrate for lactic dehydrogenese after pyruvate itself and can thus be used for the oxidation of reduced pyridine nucleotide.

The ability of serine (or glycine) to substitute for pyruvate in mass cultures, but not with isolated cells, is not understood. These amino acids not only replace pyruvate as a growth factor, but also have the same stimulatory

effect on glycolysis. The tracer experiments showed that there is no appreciable conversion of serine (or glycine) to pyruvate. The common effects may be mediated through an as yet undefined feedback mechanism, perhaps at the level of 3-phosphoglyceraldehyde, a common intermediate in the synthesis of both serine and pyruvate from glucose. This compound either can be reduced to α-glycerophosphate with a concomitant oxidation of DPNH, or can be metabolized by the pathway described by Ichihara and Greenberg [5] to yield serine. This latter results in a net formation of two molecules of reduced pyridine nucleotide while the former oxidizes one moleculeof DPNH per molecule of 3-phosphoglyceraldehyde metabolized. A shortage of DPN would be helped by favoring the reduction to α-glycerophosphate.

The requirement of pyruvate for the growth of cells in culture is not unique with 388. Eagle [3] has recently found that mouse embryo cells need pyruvate for growth and Neumann and McCoy [8] have reported that the Walker-Carcinoma 256 requires pyruvate. However, unlike 388, pyruvate can be replaced by α-keto glutarate in the Walker-Carcinoma 256.

SUMMARY

An established cell culture of a mouse lymphoma (P-388) was found to require pyruvate and L-serine or glycine for growth from isolated cells in culture. For sustained growth in mass culture, only one of these compounds is essential although all of them are synthesized from glucose under this condition and the cells cannot synthesize pyruvate from serine or glycine or *vice versa*. Pyruvate can be replaced by α-keto-*n*-butyrate both for growth in mass culture and from isolated cells.

REFERENCES

1. Dawe, C. J. and Potter, M., *Am. J. Pathol.* **33**, 603 (1957).
2. Eagle, H., *Science* **122**, 501 (1955).
3. —— *Ibid.* **130**, 432 (1959).
4. Herzenberg, L., unpublished results.
5. Ichihara, A. and Greenberg, D. M., *Proc. Natl. Acad. Sci.* **41**, 605 (1955).
6. Lockart, R. Z., Jr. and Eagle, H., *Science* **129**, 252 (1959).
7. Moore, S. and Stein, W. H., *J. Biol. Chem.* **192**, 663 (1951).
8. Neuman, R. E. and McCoy, T. A., *Proc. Soc. Exptl. Biol. Med.* **98**, 303 (1958).
9. Oyama, V. I. and Eagle, H., *Proc. Soc. Exptl. Biol. Med.* **91**, 305 (1956).
10. Salzman, N. P., *Biochim. et Biophys. Acta* **31**, 158 (1959).
11. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K. and Shannon, J. E., *J. Natl. Cancer Inst.* **11**, 773 (1951).
12. Stulberg, C. S., Soule, H. D. and Berman, L., *Proc. Soc. Exptl. Biol. Med.* **98**, 428 (1958).