AMINO ACID AND GLUCOSE UPTAKE IN RELATION TO PROTEIN SYNTHESIS IN CELLS GROWING IN TISSUE CULTURE

R. SINCLAIR* AND I. LESLIE

Department of Biochemistry, The Queen's University, Belfast (N. Ireland)

(Received July 4th, 1958)

SUMMARY

1. Replicate roller tube cultures of short-term human foetal lung and kidney cells, of a transformed human liver cell strain (HLM) and of long-term human malignant strains (HEP 1 and HEP 2) have been used to investigate amino acid uptake, protein synthesis and carbohydrate metabolism during cell multiplication in growth-promoting medium. The uptake of 13 individual amino acids has been followed by quantitative paper chromatography.

2. The glucose "oxidised" (*i.e.* the portion of the glucose consumed which cannot be accounted for by the production of lactic and keto acids) is related to the total amino acid uptake in an approximately one to one molar ratio in cultures of HEP 1, HEP 2 and HLM cells.

3. The total free amino acid nitrogen utilised was sufficient to account for the new protein formed during the growth of HEP 1 and HEP 2 cells, but was less than the requirement for protein synthesised in HLM and foetal cell cultures.

4. Glutamine was extensively or entirely removed from the medium during the growth of all the cell types and the glutamic-aspartic acid pool was increased or unchanged during growth.

5. The non-essential amino acids, proline, serine and glycine were utilised by all the cells, but only the foetal cells appeared to make use of alanine.

6. All the cells utilised leucine, isoleucine, phenylalanine, threonine and valine, and most of the cultures appeared to use cystine for growth.

7. There was no evidence of leakage of non-essential amino acids from HEP 1 cells maintained on medium lacking these metabolites.

INTRODUCTION

Much has been learned about nutritional requirements of animal cells in tissue culture by growing them in chemically-defined medium supplemented with a small amount of serum. By comparing the growth response of cells growing on such media with that of cells growing on medium depleted of a single component (*e.g.* an amino acid), EAGLE[1], HAFF AND SWIM[2], MCCOY, MAXWELL AND NEUMAN[3], and MORGAN AND

* Present address: Roscoe B. Jackson Laboratory, Bar Harbour, Maine, U.S.A.

References p. 57.

MORTON[4] have been able to specify which of the possible constituents are essential for growth.

For tissue culture studies on the metabolism and response of cells to hormones, another aspect of nutrition is important, namely, the extent of utilisation of amino acids, carbohydrates and other metabolites under specified experimental conditions. In this work, we have employed a chemically-defined medium (CDM), Connaught 858 which was found by HEALY, FISHER AND PARKER[5] to be adequate to support the growth of certain clones of Earle L strain cells. In our experience, human cells from foetal lung, human malignant strains, HEP 1* and HEP 2*, and a transformed strain, HLM (see METHODS), multiply relatively rapidly when medium 858 is supplemented with human serum and 5 % chick embryo extract. The purpose of this investigation has been to find out how amino acid and glucose utilisation are related to protein synthesis under these conditions.

METHODS

Origin of the cell strains

Human embryonic lung tissue was cultured from a 20-week foetus at the surgical termination of pregnancy. Cells from this tissue grew for 8–10 weeks before the growth potential was lost. The malignant strains HEP 1 and HEP 2 have been maintained under tissue culture conditions in this laboratory since January, 1956. HLM is a transformed strain of human foetal liver cells first cultured from a 20-week male foetus in March, 1956. It has been shown that these transformed cells have biochemical properties similar to those of malignant cells[6].

The preparation of medium

Two types of medium have been used. The growth-promoting medium (GPM) consisted of (a) Connaught 858, a chemically-defined medium modified by omitting the co-enzymes and including all the corresponding B vitamins at a concentration of 1 μg/l, (b) 20 % of whole human serum and (c) 20 % of chick embryo extract prepared as previously described[7]. The maintenance medium contained 90 % of modified Connaught 858 and 10 % dialysed human serum. The serum was dialysed against 25 vol. of Earle's saline for 4 h at 4°, as described by SATO, FISHER AND PUCK[8].

Test procedure

Replicate cultures were set up in roller tubes from cells brought into suspension by a 1 % trypsin solution in saline or by a mixture of 0.1 % trypsin and 0.002 *M* versene in Ca- and Mg-free saline. The use of versene in sub-culturing operations has been discontinued because alterations in the metabolic characteristics of stock cells have since occurred during the period in which it was used[9]. Before each test, the cells were allowed to become established for two days in GPM and a sample group of four tubes was removed for initial estimations. Each remaining tube received 2 ml of GPM or test medium at the beginning of the test period of 72 h. At 24-h intervals the

* Dr. A. E. MOORE, Sloan-Kettering Institute of Cancer Research, New York, kindly supplied the cultures of these cells from which our stock was grown. Both strains are of human origin, HEP 1 was derived from an epidermoid carcinoma of the cervix, and HEP 2 from an epidermoid carcinoma of the larynx of a male patient. The cells were first maintained as heterologous explants by TOOLAN[51].

References p. 68.

spent medium was collected into 2 % tungstic acid precipitant, and replaced by fresh medium. The spent medium and the tissue present at the end of the test were stored at —20° until analysed.

Chemical determinations

The tissue was separated into lipid (L), ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and protein fractions by suitable modification of the SCHMIDT AND THANNHAUSER procedure[10]. Phosphorus (P) was determined by the method of FONTAINE[11] and protein nitrogen (PN) by micro-Kjeldahl method. In the medium analysis, glucose was measured by the anthrone method and lactic acid by the *p*-hydroxydiphenyl procedure devised by BARKER AND SUMMERSON[12]. The α-keto acids were determined using the phenylhydrazine reagent[13] and total α-amino acids by the ninhydrin method of COCKING AND YEMM[14]. Ammonia was estimated by the CONWAY microdiffusion technique[15]. As the reaction between ammonia and ninhydrin gives extinction values approximately one-third of those obtained with comparable amounts of amino acids, the total amino acid uptake was adjusted to allow for the corresponding ammonia production.

Determinations on twelve individual amino acids were made by paper chromatographic separation of their dinitrophenyl derivatives[16]. The pH of 5-ml samples of the tungstate filtrates of the used and unused reagent was brought to 8 by the dropwise addition of 0.1 *N* sodium hydroxide. 2 % dinitrofluorobenzene (DNFB) in ethanol was added to make the solution 0.2 % with respect to DNFB and the pH was maintained near 8 by adding drops of 0.1 *N* sodium hydroxide. The condensation reaction was allowed to proceed overnight at 37° and the unused reagent extracted by washing 3 times with an equal volume of ether. An equal volume of 2 *N* hydrochloric acid was added to acidify the solution and the DNP-amino acids were extracted by washing 5 times with ether. A suitable fraction of the ether extract was concentrated by evaporation and spotted at one corner of 10″ × 10″ Whatman No. 1 chromatographic paper.

After overnight equilibration with 0.8 *N* ammonia, the first dimension separation was made ascendingly with "toluene" solvent consisting of 10 vol. toluene, 6 vol. chloroethanol, 3 vol. pyridine and 6 vol. of 0.8 *N* ammonia. The paper was dried at 40° and the second dimension separation was made with 1.5 *M* phosphate solution (1.0 *M* sodium dihydrogen phosphate, 0.5 *M* disodium hydrogen phosphate) by descending chromatography, thus allowing the phosphate buffer to drip off the serrated lower edge of the paper. The yellow spots were outlined under ultra-violet light filtered through Wood's glass, excised, and eluted in 4 ml water by warming at 60° for 15 min. Their extinction values were measured at 360 mμ in a Unicam SP 500 spectrophotometer.

RESULTS

The three components of GPM are Connaught medium 858, chick embryo extract and human serum. Representative values for the total α-amino groups, as determined by the ninhydrin procedure, are given at the foot of Table I, and it can be seen that approximately 50 % of the amino acid pool is supplied by medium 858, 40 % by the embryo extract and the remainder by the human serum.

References p. 68.

Table I also shows the approximate amount of 12 of the amino acids in the components of GPM. It should be noted that it is not possible to separate glutamic acid and aspartic acid, nor leucine and isoleucine by the LEVY chromatographic procedure[16], but for convenience the values determined for these pots will be credited to glutamic acid and leucine respectively. The method does not provide information on the changes in arginine, cysteine, histidine, methionine, tryptophan and tyrosine, and is not altogether reliable for cystine. However, from the assessment (see footnote, Table I) of the total content of the other 12 amino acids in GPM, it can be seen that these represent nearly 4600 μmoles in a total of 7400 μmoles/l of medium, or 62 % of the whole amino acid pool (column 5). This was confirmed by the analysis of batches of GPM, using glycine as a standard for calculating the concentrations both of the total amino acids by the ninhydrin procedure and of the individual amino acids measured chromatographically. For eight such determinations, 50–80 % of the total pool was supplied by the 12 amino acids in approximately the same proportions as indicated in column 5, Table I.

TABLE I

THE RELATIVE CONTRIBUTIONS OF CONNAUGHT MEDIUM 858, EMBRYO EXTRACT (EDF) AND HUMAN SERUM TO THE AMINO ACID CONCENTRATIONS IN THE GROWTH-PROMOTING MEDIUM (GPM)

Columns 1	2	3	4	5
Amino acids determined by paper chromatography	*Concentrations expressed as μmoles/l GPM**			
	Medium 858	*EDF*	*Human serum*	*Total in GPM*
Alanine	170	120	80	370
Glutamic and aspartic acids	440	420	10	870
Glutamine	410	120	110	640
Glycine	400	250	40	690
Leucine and isoleucine	370	200	40	610
Phenylalanine	90	60	10	160
Proline	210	140	40	390
Serine	140	90	20	250
Threonine	150	160	25	335
Valine	130	90	50	270
Totals for above amino acids	2510	1650	425	4585
Total amino acid as determined by ninhydrin method	3820	3190	470	7400

* It should be noted that most of the values given are approximations, since they are based on the extinctions obtained with standard amounts of the dinitrophenyl derivative of glycine and apply to representative samples of EDF and human serum.

In determining the extent of glucose utilisation in growing cultures, it is necessary to distinguish between the glucose transformed to lactic and pyruvic acids and the glucose which cannot be accounted for by the production of these metabolites. The latter portion we have called the glucose "oxidised' without intending to imply that it is entirely converted to carbon dioxide.

When the values for the constituents utilised from the medium are compared with those for protein formed in the cells, two correlations become evident. There is an approximately "one to one" molar relationship between the glucose "oxidised" (Table II, column 5) and the uptake of α-amino acids (column 6) for most of the cells

References p. 68.

in these tests. In 46 individual pairs of measurements of glucose "oxidised" and amino acid utilisation from these and other unpublished tests, the significant correlation coefficient of +0.76 was obtained. Secondly, it can be seen in Table II that the total amino acid nitrogen incorporated (column 8) corresponds to the net protein increments (column 9) of HEP 1 and HEP 2 cells but not for the foetal lung fibroblasts nor for the transformed HLM cells.

TABLE II

GLUCOSE AND AMINO ACID UTILISATION IN RELATION TO PROTEIN SYNTHESIS IN CULTURES OF HUMAN FOETAL, MALIGNANT AND TRANSFORMED CELLS GROWN FOR 72 HOURS

Columns 1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Type of cells with No. of tubes in brackets	*MGT* h*	*Glucose utilised µmoles*	*Total acid** produced µmoles*	*Glucose*** "oxidised" µmoles*	*α-Amino§ groups utilised µmoles*	*Ammonia produced µmoles*	*AA-N§§ utilised µg atoms*	*Protein-N formed µg atoms*
Lung (3)	27	40.8	63.2	9.2	8.9	4.5	11.4	15.3
Lung (2)	52	47.8	93.0	1.3	4.5	5.7	5.8	—
HEP 1 (5)	48	34.8	45.9	11.8	11.9	1.9	15.2	14.5
(s.e.)		(1.1)	(1.1)	(0.4)	(0.3)	(0.2)		(1.1)
HEP 2 (5)	60	31.0	27.6	17.2	14.6	1.0	18.7	16.4
(s.e.)		(0.2)	(0.8)	(0.4)	(0.3)	(0.2)		(1.1)
HLM (5)	45	29.4	40.0	9.4	9.1	2.6	11.7	14.7
(s.e.)		(1.6)	(2.9)	(0.7)	(0.5)	(0.3)		(0.8)
Initial amounts in medium		67.0	8.0	—	38.4	2.3	—	—

* MGT = mean generation time.
** Total acid = lactic acid + α-keto acids.
*** Glucose "oxidised" = glucose not accounted for by total acid production.
§ α-Amino groups = amino acids determined by ninhydrin method.
§§ AA-N = total amino acid nitrogen.
s.e. = standard error.

The glucose "oxidised" is calculated on the assumption that the lactic and keto acid carbon atoms originate entirely from the metabolism of glucose. However, if certain of the non-essential amino acids are deaminated to an appreciable extent, carbon from this source will find its way into the lactic and keto acid pool. That such deamination is relatively small in cultures of malignant and transformed cells can be judged from the production of free ammonium ions in the medium during growth, where it represents only from 7–14 % of the total amino acid uptake. By contrast, the free ammonia produced by cultures of lung fibroblasts may be 50 % or more of the amino uptake on a molar basis (Table II, column 7).

Possible sources of the ammonium ion production can be found by examination of the changes in individual amino acids during growth. Fig. 1 shows the extinction values for thirteen amino acids separated by paper chromatography from the initial and spent medium in tests on foetal and malignant cells. The most significant feature of amino acid uptake by the malignant and transformed cells is the extensive utilisation of glutamine. In the tests with HEP 1 and HEP 2 strains, the glutamine was completely exhausted, about 50 % of the glucose was utilised, and the cells multiplied 2 to 3-fold. The HLM cells used about 75 % of the glutamine. In every case there was

References p. 68.

an increase in aspartic and glutamic acid in the medium. As there is approximately 5 μmoles of these amino acids initially per 6 ml of medium and they increase by 25–50 % during growth, the deamination of glutamine could well account for the increase of 1.0 to 2.6 μmoles ammonia recorded in Table II.

As noted above, cultures of foetal cells cause the production of more free ammonia than is found with the long-term strains. Fig. 1 also shows the pattern of amino acid

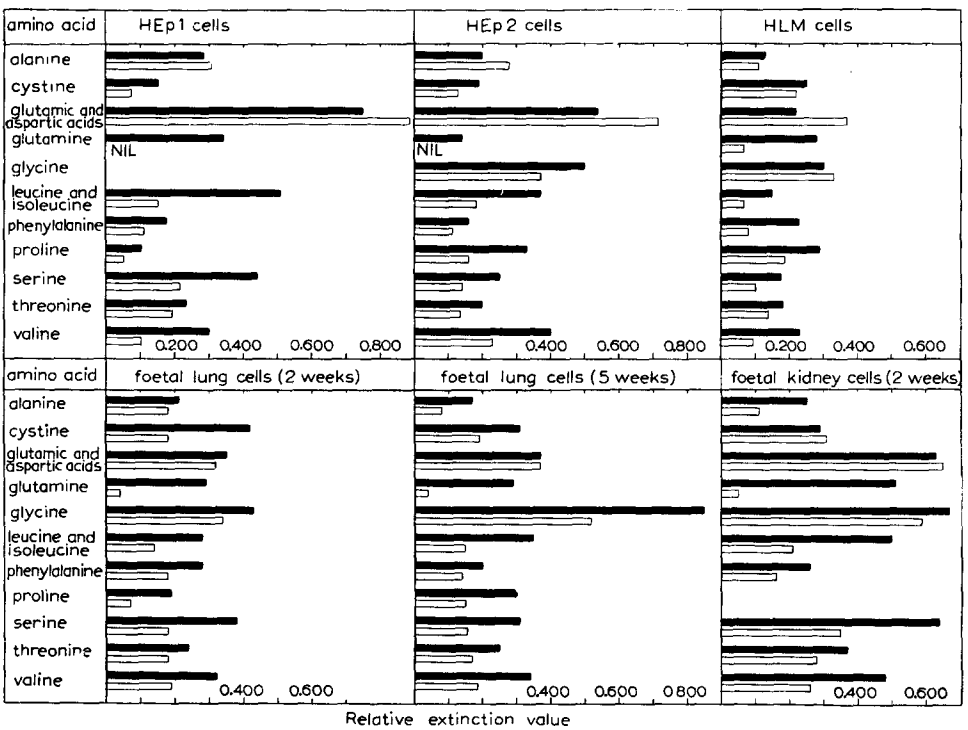

Fig. 1. The relative amounts of individual amino acids in the initial medium and in the used medium of tests with human foetal and malignant cells. Black columns: extinction values for DNP-amino acids in initial, control medium; open columns: extinction values for DNP-amino acids in used medium collected over the 72-h tests.

uptake of human lung cells; glutamine is again very extensively used, but there is not a corresponding increase in the glutamic-aspartic acid spots, while alanine and glycine are both reduced during growth.

There are features of amino acid utilisation which foetal and malignant cells share in common. Proline and serine, two non-essential amino acids for human cells, are both utilised to an appreciable extent. This also applies to the remaining amino acids (*i.e.* leucine, isoleucine, phenylalanine, threonine and valine), all of which are considered to be essential in tissue cultures of human cells. Finally, as Fig. 1 makes clear, the uptake of essential amino acids represents a range of 30–70 % of the amounts in the initial medium, while, with the exception of serine and proline, the non-essential amino acids are not utilised or are even produced during growth. Against this complex pattern of utilisation, the average uptake of 30 % of the amino acid pool, as determined by the ninhydrin procedure, would appear to be a reasonable estimate.

References p. 68.

Since it was evident that certain of the non-essential amino acids were not utilized appreciably in growing cultures, or were, in fact, in greater concentration in the medium after growth had occurred, the effect of growing cells in medium deficient in these amino acids was investigated. The control medium for these tests consisted of Connaught medium 858 supplemented with 10% dialysed human serum (medium A), and in the deficient medium, alanine, glutamic and aspartic acids, glycine, proline and hydroxyproline, and serine were omitted, and the concentration of cysteine was reduced from 1.6 to 0.3 μmoles/ml (medium B). Glutamine was maintained in both media at its normal concentration of 100 mg/l.

In test carried out with HEP 1 cells, growth, as estimated on this occasion by the increase in protein in the tubes, was only of the order of 10 % above the initial amounts over 72 h in the deficient medium. Even in the control medium it did not exceed 30 %. Fig. 2 shows the changes in some of the free amino acids under these conditions. The feature common to both types of media is the near-exhaustion of the glutamine pool,

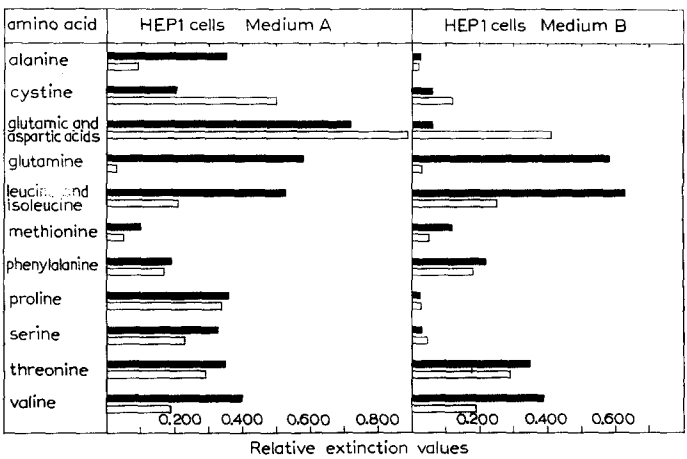

Fig. 2. The relative amounts of individual amino acids in the initial and used media A and B (see text). Black columns: extinction values for DNP-amino acids in the initial, control media; open columns: extinction values of DNP-amino acids in media collected during 72 h test with cultures of HEP 1 cells.

TABLE III

GLUCOSE AND AMINO ACID UTILISATION AND PROTEIN FORMATION IN CULTURES OF HEP 1 CELLS GROWING FOR 72 HOURS ON MEDIA A AND B*

Medium, with No. of tubes in brackets	*Glucose utilised*	*Total acid produced*	*Glucose** "oxidised"*	*α-Amino** groups utilised*	*Protein-N formed*
	μmoles or μg atoms per roller tube				
Medium A (4)	35.2	59.8	5.3	4.7	2.8
(s.e.)	(0.6)	(2.0)	(0.8)	(0.2)	(0.4)
Medium B (4)	29.0	53.4	2.3	3.4	1.0
(s.e.)	(1.2)	(1.4)	(0.6)	(0.3)	(0.3)

* Medium A consisted of Connaught 858 with 10% dialysed human serum; medium B was similar except that the non-essential amino acids were omitted (see text).

** See abbreviations for Table II.

References p. 68.

and the significantly large increases in the glutamic-aspartic acid pool. It is also obvious that there was no leakage into the medium of alanine, proline and serine, when these compounds were omitted (medium B).

Although only a small amount of growth was apparent in these cultures, the cells remained healthy in appearance and from Table III it can be seen that they were metabolically active. There was considerable production of lactic acid and keto-acids (shown as total acid in Table III), and the utilisation of α-amino groups exceeded the requirements for the amounts of protein formed in the cultures.

DISCUSSION

Glucose metabolism and amino acid uptake

The glucose "oxidised" represents the portion of glucose carbon which is transformed by cellular metabolism to the carbon of the pentoses, purines, pyrimidines, lipids, amino acids and carbon dioxide. It probably supplies a large part of the energy and material for synthesis, since it is found to be highly correlated with the average cell population and thus with the extent of growth of the cultures[7]. The present studies have shown that in a medium containing a complete and adequate supply of free amino acids, HEP 1, HEP 2 and HLM cells utilise approximately one mole of amino acids for each mole of glucose "oxidised". This relationship does not apply, however, to certain cultures of human embryonic cells, in which glucose "oxidised" is very small or cannot be measured by the microchemical methods employed in these investigations.

It is possible to make some approximate calculations relating the extent of amino acid incorporation to the amount of energy available for all cell activities by assuming that in both cell types energy is derived entirely from glucose catabolism. It is assumed that for each mole of lactic acid produced, 2 moles of ATP are obtained, for each mole of pyruvic acid, the gain is 8 moles of ATP, and when each mole of glucose "oxidised" is completely converted to carbon dioxide, approximately 38 moles of ATP are formed. From the extent of glycolysis and "oxidation" in malignant and transformed cell cultures, it can be calculated that 1 peptide bond is formed for every 52 molecules of ATP produced, and in embryonic cells, that there is a new peptide bond for every 57 ATP molecules. These are certainly overestimations, since only part of the glucose "oxidised" will be converted to carbon dioxide. It will be necessary in future studies to try to assess how ATP production is distributed between the synthetic pathways of protein, nucleic acid, lipid and other cells constituents in growing embryonic and malignant cells.

Protein formation in tissue cultures

Discussion of the energy production which accompanies peptide bond synthesis in intact cells presupposes that, in tissue culture, proteins are formed *de novo* from free amino acids in the medium, or from amino acids released from ingested protein within the cells. The amino acids in the medium appear to be the predominant source of the newly-formed protein in cultures of HEP 1 and HEP 2 cells, but this may not apply to short-term cultures of foetal tissue nor to the transformed HLM cells, in which amino acid uptake was considerably less than the incorporation indicated by protein production.

References p. 68.

The demonstration by HOAGLAND, ZAMECNIK AND STEPHENSON[18] of soluble enzymes in liver cell cytoplasm which "activate" amino acids and prepare their incorporation on RNA templates provides a basic mechanism for the utilisation of free amino acids for protein synthesis. There is, moreover, growing evidence in favour of the amino acid pool rather than peptides or serum proteins as the source of protein formation in animal tissues *in vivo*[19,20]. By contrast, tumour cells appear able to incorporate plasma proteins directly without any release of the component amino acids[21-23]. These reports, however, leave unexplained the lack of peptide intermediates in the tissue fluid and imply the existence of a mechanism of extensive degradation and rearrangement which would be required to transform one protein into another.

The evidence from tissue culture investigations is contradictory. WINNICK AND WINNICK[25] and FRANCIS AND WINNICK[26] showed the incorporation of isotopically-labelled protein from embryo extract by chick cells, and there have been a number of subsequent reports of protein incorporation from media by both normal and malignant cell cultures[27-30]. When embryo extract is used in the medium, it is possible that protein becomes absorbed on the cells rather than forming part of the cell protein, especially as it tends to precipitate out when acidic metabolites accumulate in the medium[31]. The work of CORNWELL AND LUCK[32] and of EAGLE, PIEZ AND FLEISCHMAN[33] shows that caution is also necessary in interpreting isotopic incorporation unless it is demonstrated that net protein synthesis is occurring.

Whatever the beneficial role of protein in the cultivation of cells, it is clear that tissue culture strains are not always able to utilise it and that they can, in fact, become adapted to grow in the absence of protein. Studies such as those of EAGLE and his colleagues[34] illustrate the necessity of including 13 free amino acids in the medium. Even when serum protein is present to provide a source of these amino acids, the omission of one of them will stop growth and cause the cells to deteriorate. It is also established that certain long-term cell strains can become adapted to grow on a completely chemically-defined medium in the absence of protein[35,26]. The beneficial effect of protein, which is particularly evident with freshly-explanted cells, may to some extent depend on absorbed components, such as fatty acids and cholesterol[8]. The stimulating effect of certain peptide fractions on the growth of cells kept in chemically-defined medium, as reported by WAYMOUTH AND MATTHEWS[37], may indicate that components with a specific catalytic activity are necessary for optimal cell metabolism.

Amino acid uptake by tissue cultures

In long-term strains of human cells (HEP 1, HEP 2 and HLM) and in the short-term lines obtained from foetal tissues, certain consistent features in the pattern of amino acid uptake are found in rapidly growing cultures. In every test, glutamine was completely or extensively removed from the medium, and in the long-term strains this was accompanied by an increase in the glutamic-aspartic acid pool.

The role of glutamine is known to be of key importance in the nutrition of mammalian cells. Glutamine forms one-fourth of the total amino acid in the blood and is involved in such diverse reactions as amide exchange[38], transamination[39,40], purine biosynthesis[41,42] and is, of course, directly incorporated into protein. Its extensive utilisation in growing cultures is, therefore, not surprising and its complete removal from the medium could clearly impose a serious limit to the extent of cell multiplica-

References p. 68.

tion. The presence of glutamic acid in the medium may not meet the situation, since EAGLE *et al.*[43] found that glutamic acid could substitute for glutamine only irregularly and at best incompletely, although it was incorporated into the protein of both malignant and embryonic cells whether glutamine was present or not.

While it is well established that both fresh explants and longterm lines from mammalian tissues require glutamine as an essential growth factor[3,43–45], freshly-explanted avian cells appear to produce glutamine rather than utilise it, when they are maintained without growth on a chemically-defined medium[46]. Under the same conditions, monkey kidney cells and human malignant cells utilise glutamine[47]. It is not yet clear whether these differences are a reflection of basic metabolic differencess between the cells or whether they indicate cell autolysis in tissue explants when total protein is diminishing. It may be relevant to this situation that glutamine is rapidly utilised by tumour cells *in vivo* (ROBERTS AND TANAKA[48]) but that it is released from regressing tumours into the surrounding fluid[49].

Besides glutamine, 12 of the amino acids have been found to be essential for the growth of human cell cultures[1]. 6 of these (*i.e.* threonine, leucine, isoleucine, phenylalanine, valine and cystine) were routinely measured by the LEVY paper-chromatographic method and all were consistently utilised during growth. Of the so-called "non-essential" amino acids, proline and serine were invariably removed to a marked extent (30–60 %) and glycine was usually taken up, indicating that the rate of synthesis of these amino acids did not keep pace with their requirement by the cells.

These results agree in general with the few published observations on the changes in medium amino acids during the course of cell growth. Serine is reported to be essential for the growth of rabbit fibroblasts[2] and the absence of either serine or alanine from a medium supporting the growth of cultures of Walker carcinoma 256 resulted in a smaller final cell population[3]. With HeLa cells, however, WESTFALL, PEPPERS AND EARLE[27] found no demonstrable utilisation of glycine, alanine, proline or serine from a medium of high protein content and with a small amino acid pool. Glycine is reported to stimulate the growth of first passage cultures of monkey kidney cells[50].

When the malignant HEP 1 cells were presented with a medium thoroughly depleted of the "non-essential" amino acids (*i.e.* alanine, glutamic and aspartic acids, proline, hydroxyproline, serine, glycine) and in which cysteine was greatly reduced, the cultures grew only to a small extent. However, they metabolised actively (Table III) and although appreciable amounts of lactic acid and some keto-acid leaked out of the cells, there was no corresponding release of "non-essential" amino acids, with the sole exception of glutamate. This almost certainly came from the glutamine in the medium, since it was again extensively utilised even though growth was negligible.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. D. C. HARRISON for helpful discussions and Miss MARGARET YARNELL, B.Sc. for her assistance with the tissue culture work. They are glad to acknowledge the generosity of Messrs. Benger Laboratories Limited in supplying the hyaluronidase used in the preparation of chick embryo extract. They are grateful to a number of colleagues and to the staff of Belfast Hospitals and the Blood Transfusion Service for their co-operation. Finally, they wish to thank Mr. L. DICKSON for his valuable and skilled technical assistance.

References p. 68.

The work has been supported by grants from the Medical Research Council and the British Empire Cancer Campaign.

REFERENCES

1 H. EAGLE, *Science*, 122 (1955) 501.
2 R. F. HAFF AND H. E. SWIM, *J. Gen. Physiol.*, 41 (1957) 91.
3 T. A. MCCOY, M. MAXWELL AND R. E. NEUMAN, *Cancer Research*, 16 (1956) 979.
4 J. F. MORGAN AND H. J. MORTON, *J. Biophys. Biochem. Cytol.*, 3 (1957) 141.
5 G. M. HEALY, D. C. FISHER AND R. C. PARKER, *Proc. Soc. Exptl. Biol. Med.*, 89 (1955) 71.
6 I. LESLIE, W. C. FULTON AND R. SINCLAIR, *Nature*, 178 (1956) 1179.
7 I. LESLIE, W. C. FULTON AND R. SINCLAIR, *Biochim. Biophys. Acta*, 24 (1957) 365.
8 G. SATO, H. W. FISCHER AND T. T. PUCK, *Science*, 126 (1957) 961.
9 Unpublished results.
10 G. SCHMIDT AND S. J. THANNHAUSER, *J. Biol. Chem.*, 161 (1945) 83.
11 T. D. FONTAINE, *Ind. Eng. Chem., Anal. Ed.*, 14 (1942) 77.
12 S. B. BARKER AND W. H. SUMMERSON, *J. Biol. Chem.*, 138 (1941) 535.
13 T. E. FRIEDEMANN AND G. E. HAUGEN, *J. Biol. Chem.*, 147 (1943) 415.
14 E. C. COCKING AND E. W. YEMM, *Biochem. J.*, 58 (1954) xii.
15 E. J. CONWAY, *Microdiffusion Analysis and Volumetric Error* (4th Ed.), Lockwood and Son Ltd., London, 1957.
16 A. L. LEVY, in D. GLICK, *Methods of Biochemical Analysis*, Vol. 2, Interscience Publ. Inc., New York, 1955, p. 360.
18 M. B. HOAGLAND, P. C. ZAMECNIK AND M. L. STEPHENSON, *Biochim. Biophys. Acta*, 24 (1957) 215.
19 N. W. PENN, S. MANDELES AND H. S. ANKER, *Biochim. Biophys. Acta*, 26 (1957) 349.
20 J. L. SIMKIN AND T. S. WORK, *Nature*, 179 (1957) 1214.
21 C. L. YUILE, B. G. LAMSON, L. L. MILLER AND G. WHIPPLE, *J. Exptl. Med.*, 93 (1951) 539.
22 A. L. BABSON AND T. WINNICK, *Cancer Research*, 14 (1954) 606.
23 H. BUSCH, S. SIMBONIS, D. C. ANDERSON AND H. S. N. GREENE, *Yale J. Biol. Med.*, 29 (1956) 105.
24 D. M. GREENBERG, *Cancer Research*, 15 (1955) 421.
25 R. E. WINNICK AND T. WINNICK, *J. Natl. Cancer Inst.*, 14 (1953) 519.
26 M. D. FRANCIS AND T. WINNICK, *J. Biol. Chem.*, 202 (1953) 273.
27 B. B. WESTFALL, E. V. PEPPERS AND W. R. EARLE, *Am. J. Hyg.*, 61 (1955) 326.
28 J. G. BACHTOLD AND L. P. GEBHARDT, *Exptl. Cell Research*, 13 (1957) 432.
29 H. N. KENT AND G. O. GEY, *Proc. Soc. Exptl. Biol. Med.*, 94 (1957) 205.
30 S. GRAFF AND K. S. MCCARTY, *Exptl. Cell Research*, 13 (1957) 348.
31 J. N. DAVIDSON AND I. LESLIE, *Exptl. Cell Research*, 2 (1951) 366.
32 D. G. CORNWELL AND J. M. LUCK, *Arch. Biochem. Biophys.*, 73 (1958) 391.
33 H. EAGLE, K. A. PIEZ AND R. FLEISCHMAN, *J. Biol. Chem.*, 228 (1957) 847.
34 H. EAGLE, V. I. OYAMA AND LEVY, *Arch. Biochem. Biophys.*, 67 (1957) 432.
35 W. T. MCQUILKIN, V. J. EVANS AND W. R. EARLE, *J. Natl. Cancer Inst.*, 19 (1957) 885.
36 C. WAYMOUTH, personal communication.
37 C. WAYMOUTH AND J. MATTHEWS, *Abstr. 9th Ann. Meeting Tissue Culture Assoc.*, (1958) 32.
38 H. WAELSCH, *Advances in Enzymol.*, 13 (1952) 237.
39 A. E. BRAUNSTEIN, *Advances in Protein Chem.*, 3 (1947) 1.
40 A. MEISTER, *Physiol. Revs.*, 36 (1956) 103.
41 D. A. GOLDTHWAIT, R. A. PEABODY AND G. R. GREENBERG, *J. Am. Chem. Soc.*, 76 (1954) 5258.
42 S. C. HARTMAN, B. LEVENBERG AND J. M. BUCHANAN, *J. Am. Chem. Soc.*, 77 (1955) 501.
43 H. EAGLE, V. I. OYAMA, M. LEVY, C. L. HORTON AND R. FLEISCHMAN, *J. Biol. Chem.*, 218 (1956) 607.
44 A. FISCHER, *J. Natl. Cancer Inst.*, 13 (1953) 1399.
45 B. B. WESTFALL, V. J. EVANS, E. V. PEPPERS, N. M. HAWKINS AND W. R. EARLE, *J. Natl. Cancer Inst.*, 17 (1956) 131.
46 A. E. PASIEKA, H. J. MORTON AND J. F. MORGAN, *J. Natl. Cancer Inst.*, 16 (1956) 995.
47 A. E. PASIEKA, H. J. MORTON AND J. F. MORGAN, *Canad. J. Biochem. Physiol.*, 36 (1958) 171.
48 E. ROBERTS AND T. TANAKA, *Cancer Research*, 16 (1956) 204.
49 E. ROBERTS AND P. R. F. BORGES, *Cancer Research*, 15 (1955) 697.
50 H. EAGLE, A. E. FREEMAN AND M. LEVY, *J. Exptl. Med.*, 107 (1958) 643.
51 H. W. TOOLAN, *Cancer Research*, 14 (1954) 660.