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Amino Acid Utilizations and Protein Synthesis at Various Proliferation Rates, Population Densities, and Protein Contents of Perfused Animal Cell and Tissue Cultures*

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ABSTRACT: Amino acid utilization and production by animal cells have been described previously in suspension cultures or other cell culture type systems but not for cultures having tissue-like cell densities. In this work, tissue-like Jensen sarcoma cultures were produced and maintained by a perfusion system which controlled pH, maintained adequate nutrient concentrations, and eliminated metabolic waste products. Rates of amino acid utilization and production were determined during periods of 0.8-, 1.0-, 3.2-, and 3.6-day population doubling times and for cell densities ranging from the equivalent of 0.24 to 8.4 monolayers (preconfluent *vs.* postconfluent). Nutritionally essential amino acid rates of utilization per hour per microgram of cell protein were quite constant (*e.g.*, 3.3 ± 0.2 pmoles of histidine), indicating a continuous rate of protein synthesis despite the progressively slower proliferation and higher population density. Subsequently, it was shown that a progressively increasing amount of synthesized protein appeared in the culturing medium as the rate of proliferation decreased and multiple-layered tissue-like cultures were produced. The phenomena of cell crowding and contact did not appear, therefore, to be a controlling factor in protein synthesis in the perfused tumor cell system.

Parallels were noted between utilization of most of the nutritionally essential amino acids and protein amino acid composition, but relative rates of utilization of valine, isoleucine, and leucine were variable depending upon their relative concentrations in the culture fluid. Based on relative amino acid composition of total cell protein, the most extensively utilized amino acids were serine, glutamine, cysteine, and methionine.

Amino acid utilization and production by animal cells in suspension cultures or other cell culture[1] type systems have been described in some detail (*e.g.*, Westfall *et al.*, 1955; Eagle, 1959; Kagawa *et al.*, 1960; Pasieka *et al.*, 1960; Lucy, 1960; McCarty, 1962; Mohberg and Johnson, 1963; Kuchler, 1964; Tritsch and Moore, 1964). Such information is particularly applicable to (a) improvements in culture media, (b) an understanding of the capacities of dispersed cells to grow and multiply, and (c) applications of the cell culture technique, such as in virus propagation.

Similar studies in cultures of tissue-like[1] cell densities have not been made heretofore primarily because some means of automated feeding and elimination is required for production, maintenance, and establishment of homeostasis in this type of culture as described recently (Kruse and Miedema, 1965a). In the present work a perfusion system was used to develop multiple-layered, tissue-like cultures, and the rates of amino acid utilization and production were determined for

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[1] In this discussion the term "cell culture" denotes cells not organized in tissues, such as in suspension or preconfluent dispersions on glass or plastic, while the terms "tissue-like" and "tissue culture" denote confluent, multiple-layered, sometimes interwoven (with intercellular substance) masses of cells; these connotations are in keeping with recent recommendations of the Committee on Terminology, Tissue Culture Association, Inc.

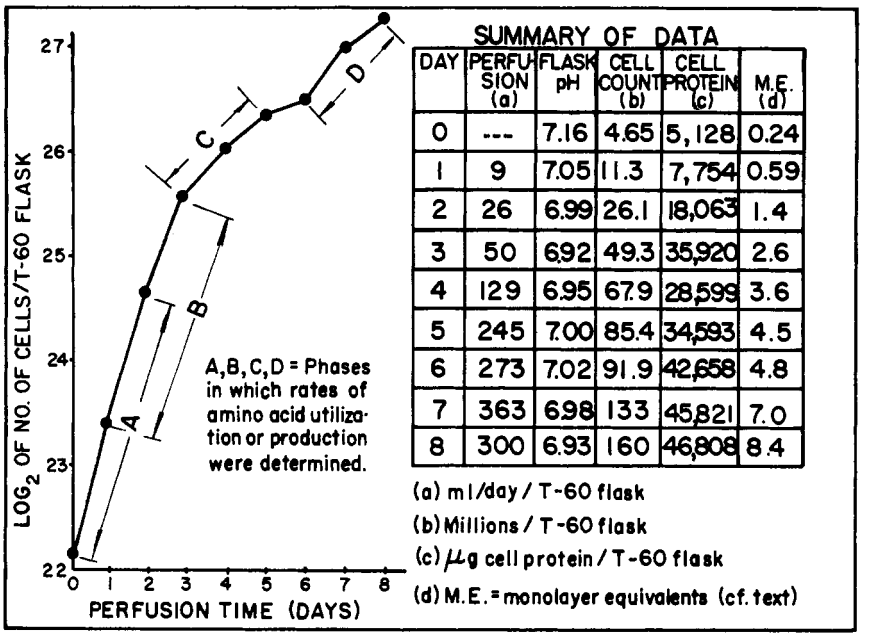

FIGURE 1: Proliferation curve of perfused Jensen sarcoma cell and tissue cultures, employed in determination of rates of amino acid utilization and production (*cf.* Table II) at various proliferation rates, population densities, and protein contents. Population doubling times ($\log_2$) for each of the four periods, A, B, C, and D, were equivalent to 0.8, 1.0, 3.2, and 3.6 days, respectively. Rates of perfusion (milliliters of medium 7a plus 10% whole calf serum per day per T-60 flask), culture pH, cell counts, protein contents, and monolayer equivalents (ME = 1.0 = a confluent sheet one-cell thick) are given in the inset.

these as well as for the dispersed cell cultures from which they emanated. Thus, the principal objective was concerned not so much with amino acid dynamics as they pertain to considerations a–c above, but primarily with respect to their variation with (d) the effects of population density on cell function *vs.* proliferation, and (e) the control of protein synthesis by cell crowding and contact (*e.g.*, Green and Goldberg, 1963; Levine *et al.*, 1965; Stoker, 1967). One example of variation related to considerations d and e has been cited recently (Kruse and Miedema, 1965a) wherein marked changes in utilization or production of glutamic acid, proline, and glycine in cell *vs.* tissue cultures of WI-38 human diploid fibroblasts were related to the onset of specific protein secretion.

In addition to the considerations above, some comparisons of amino acid utilization with protein amino acid composition were made. Also, since it was found that cell protein content decreased markedly in the tissue-like cultures, amino acid utilization and production were examined on the basis of changes in cellular protein content as well as on the basis of changes in cell numbers. This aspect of amino acid economics in animal cell and tissue culture has not been examined previously.

Methods

Jensen sarcoma cell cultures were initiated in stoppered T-60 flasks from freshly excised tumors carried intramuscularly in female Holtzman rats. After 28 hr, eight of them were transferred to a perfusion system for replicate cultures described previously (Kruse *et al.*, 1963; Kruse and Miedema, 1965a). Medium 7a (*cf.* White *et al.*, 1963), containing 21 amino acids (L isomers) and 10% whole calf sera, was employed throughout. At daily intervals effluents from each of the cultures were collected and frozen, and one culture was terminated for cell counts and protein assay. The latter was measured by the Lowry method as adapted for cell culture (Oyama and Eagle, 1956). Cells were enumerated by hemocytometer counts and nuclei determinations (Sanford *et al.*, 1951). Periodic tests for the presence of bacteria or mycoplasma were negative.

Amino acids in the culture fluids were determined with a Technicon Corp. amino acid analyzer; prior to analysis, aliquots of each culture fluid sample were deproteinized with picric acid (Stein and Moore, 1954; McCarty, 1962; Block *et al.*, 1966). Since Jensen sarcoma cells require asparagine in the medium (McCoy *et al.*, 1959), an overlap with threonine, serine, and glutamine occurred in the column eluent. This was resolved by a second analysis of each sample, in which aspartic and glutamic acids were first adsorbed on Dowex 1-X8, the eluent was deproteinized, and the amides were deamidated by refluxing in 2 N HCl for 1 hr (Patterson *et al.*, 1963). Subsequent analysis gave concentrations of asparagine (as aspartic acid), threonine, serine, and glutamine (as glutamic acid). These

TABLE I: Illustrating Maintenance of Adequate Amino Acid Levels in Culture Fluid during 8-Day Perfusion Experiment.[a]

Amino Acids (amides)	Perfusion Time (hours)						
	0 Influent Medium (mM)[b]	21	45	67	117	141	189
		Effluent Media (mM)					
Asparagine	0.29	0.17	0.14	0.15	0.17	0.24	0.22
Glutamine	1.30	0.65	0.60	0.64	0.83	1.03	0.97
Valine	0.18	0.15	0.12	0.13	0.16	0.17	0.17
Isoleucine	0.32	0.27	0.23	0.24	0.27	0.29	0.29
Leucine	0.33	0.29	0.23	0.24	0.29	0.31	0.31
Lysine	0.61	0.56	0.50	0.52	0.56	0.56	0.57

[a] Jensen sarcoma cells were cultured for 8 days in a perfusion system (see text and Figure 1); cell numbers increased from 4.65 to 160 $\times$ 10^6 cells/culture (equivalent to 0.24–8.4 confluent layers) and rates of perfusion varied from 9 to 363 ml/day. For illustrative purposes, concentrations of only the two amides and four amino acids are given; similar data were employed for subsequent calculations of rate data given in Table II. [b] Concentrations (by analysis) of other amino acids in influent medium 7a plus 10% whole calf sera (see text) were: alanine, 0.23; aspartic acid, 0.17; glutamic acid, 0.27; proline, 0.16; ornithine, 0.02; citrulline, 0.01; serine, 0.20; glycine, 0.19; threonine, 0.15; cystine ($^1/_2$) 0.10; methionine, 0.10; tyrosine, 0.09; phenylalanine, 0.11; histidine, 0.20; and arginine, 0.36. L-Hydroxyproline and L-tryptophan were included in medium 7a at 0.015 and 0.030 mM, respectively, but were not determined by amino acid analysis.

latter columns also served as duplicate analyses for the other amino acids (except tyrosine, which was adsorbed partially on Dowex 1-X8 resin), *e.g.*, the basic amino acids and two (ornithine and citrulline) present in very low concentrations. Standard mixtures of amino acids were analyzed periodically for calculation of integration constants and all determinations were corrected for recoveries of three amino acids (L-homoserine, DL-norleucine, and L-α-amino-β-guanidinopropionic acid) added to each sample. Concentrations of hydroxyproline and tryptophan, present in fresh medium 7a at 0.015 and 0.030 mM, respectively, were not determined.

Amino acid analyses were made also on several samples of deproteinized fresh and 37° incubated medium 7a to obtain an average amino acid composition in the influent medium for each of the periods of investigation throughout the 8-day perfusion experiment (*cf.* Figure 1). Fresh medium was added to the influent reservoirs at approximately 48-hr intervals.

Subsequently, rates of amino acid utilization and production per cell and per microgram of cellular protein were calculated knowing (a) amino acid concentrations in the influent and effluent media over each time interval, (b) volumes of perfusate, (c) cell numbers, and (d) protein contents, by equations used for similar rate calculations (Rueckert and Mueller, 1960; McCarty, 1962).

The extent of production of extracellular protein by the sarcoma cells was evaluated by a perfusion experiment with the influent medium containing 0.04 μc/ml of uniformly labeled L-[^{14}C]lysine; the initial inoculum of cells was prepared from Jensen cultures which had undergone multiple generations in the labeled medium for maximal labeling of the cellular protein. The [^{14}C]lysine was purified prior to use by a method reported previously (Kruse *et al.*, 1962) for purification of labeled amino acids. The protein from 2.0-ml aliquots of effluent media at daily intervals was obtained by precipitation with 0.5 ml of 50% TCA[2] followed by two washings each of cold 10% TCA, ethanol, ether, and water containing 6 mM unlabeled L-lysine. Each of the residual protein samples was solubilized in NCS reagent (Hansen and Bush, 1966), made up to 5.0 ml with XDC diluent (Bruno and Christian, 1961), and assayed with a Nuclear-Chicago Corp. Mark I scintillation counter. Rates of production of protein into the surrounding medium were made on a per cell basis by calculations referred to above.

Results

Figure 1 depicts the proliferation curve and summarizes the experimental data for perfusion rates, pH, cell counts, protein, and calculation of monolayer equivalents (*cf.* Kruse and Miedema, 1965a); four time periods of determination of amino acid utilization are also designated. Over the entire 8-day period about five doublings ($\log_2$) of the population occurred in progressing from an initial number of 4.65 $\times$ 10^6 cells (24% of the 60-cm^2 glass surface covered with

[2] Abbreviations used: TCA, trichloroacetic acid; PPLO, mycoplasma.

TABLE II: Rates of Amino Acid Utilization and Production (+) Based on Cell Numbers and on Cell Protein Content (in parentheses) in Perfused Jensen Sarcoma Cell and Tissue Cultures.[a]

Amino Acid	Proliferation Phase			
	A	B	C	D
Alanine	+4.3 (+3.3)	+4.2 (+6.0)	+2.0 (+3.8)	+2.5 (+6.9)
Aspartic acid	+2.9 (+3.3)	+1.4 (+2.0)	+2.9 (+5.5)	0.6 (1.6)
Glutamic acid	+12.1 (+9.3)	+7.8 (+11.2)	+21.2 (+39.5)	+15.2 (+41.7)
Proline	3.0 (2.3)	2.5 (3.6)	+0.5 (+0.9)	+0.5 (+1.5)
Ornithine	+1.9 (+1.5)	+1.0 (+1.4)	+1.2 (+2.2)	+4.8(+13.1)
Citrulline	+0.3 (+0.2)	0.1 (0.2)	0.1 (0.2)	0.2 (0.6)
Serine	13.9 (10.7)	7.7 (11.0)	5.1 (9.5)	3.8 (10.5)
Glycine	2.3 (1.8)	+0.2 (+0.3)	+0.9 (+1.7)	+0.8 (+2.2)
Asparagine	23.6 (18.1)	10.9 (15.6)	14.2 (26.5)	8.2 (22 4)
Glutamine	101 (77.6)	44.1 (63.0)	55.1 (103)	37.6 (103)
Threonine	5.9 (4.5)	2.9 (4.1)	1.3 (2.4)	+0.5 (+0.5)
Cystine (1/2)	3.1 (2.3)	1.8 (2.5)	1.4 (2.6)	0.4 (1.2)
Methionine	5.1 (3.9)	2.5 (3.6)	1.7 (3.1)	1.1 (2.9)
Valine	7.3 (5.6)	4.1 (5.8)	3.0 (5.6)	1.3 (3.4)
Isoleucine	10.6 (8.1)	6.6 (9.4)	5.5 (10.3)	4.0 (11.0)
Leucine	12.7 (9.8)	7.7 (11.0)	5.0 (9.3)	3.2 (8.8)
Tyrosine	2.6 (2.0)	1.3 (1.9)	1.1 (2.1)	+1.1 (+1.0)
Phenylalanine	4.2 (3.2)	3.2 (4.5)	1.8 (3.3)	0.6 (1.6)
Histidine	4.2 (3.2)	2.2 (3.1)	1.8 (3.4)	1.3 (3.5)
Lysine	14.1 (10.8)	7.1 (10.1)	5.6 (10.4)	3.8 (10.5)
Arginine	4.9 (3.7)	1.8 (2.6)	2.8 (5.2)	1.4 (3.9)
Doubling time (days)	0.8	1.0	3.2	3.6
ME range[b]	0.24–1.4	0.59–2.6	2.6–4.5	4.8–8.4

[a] Calculated as explained in text from amino acid analyses of influent and effluent media (illustrated in Table I) and expressed as 10^{-9} μmole of amino acid/hr per cell and (in parentheses) as picomoles of amino acid per hour per microgram of cell protein. For graphic representation of proliferation phases A–D, see Figure 1. [b] ME = monolayer equivalents, an expression signifying the extent to which the glass surface was covered with cells (Kruse and Miedema, 1965a); ME = 1.0 = a confluent sheet one-cell thick.

cells) to a final population of 160 × 10^6 cells/T-60 (sufficient cells to cover the 60-cm^2 surface 8.4 times). During the periods selected for amino acid analysis (Figure 1A–D), doubling times were equivalent to 0.8, 1.0, 3.2, and 3.6 days, respectively. Since 35 ml of medium was in each flask, the rate of 363 ml/day per T-60 on the 7th day was equivalent to *ca.* ten medium changes in 24 hr. The perfusion rate on the last day was inadvertently allowed to decrease somewhat instead of increase. Culture pH for days 1–8 was held at 6.98 ± 0.07, similar to the 6.95–7.19 pH range of interstitial fluid reported (Gullino *et al.*, 1965) in five types of rat tumors *in vivo*. Total cell protein increased through day 3, then changed very little even though cell numbers tripled during days 3–8. Protein content per cell (column c/b, Figure 1) remained constant during days 1–3, then dropped markedly in the multiple-layered populations; for periods A–D (Figure 1), mean protein contents per 10^6 cells were 827, 702, 518, and 367 μg, respectively.

Table I gives concentrations of some of the amino acids present in influent and effluent media to illustrate that perfusion conditions maintained adequate levels of these nutrients throughout the 8-day period. For the sake of brevity only the two amides and four amino acids are listed; similar data were obtained for the others. Other illustrations of the environmental control obtained in the perfusion system have been given recently (Miedema and Kruse, 1965; Kruse and Miedema, 1965a,b).

Rates of amino acid utilization and production are given in Table II for the two periods, A and B, of rapid proliferation and the two periods, C and D, of slow proliferation. Serine, asparagine, glutamine, isoleucine, leucine, and lysine were consumed at high rates. All of these except serine are nutritionally essential components of the culture medium for proliferation of the Jensen sarcoma *in vitro*. In general, the utilization of essential amino acids on a *per cell basis* decreased with decreasing rate of proliferation. Several amino acids were produced into the medium in appreciable amounts, notably alanine, aspartic

acid, and glutamic acid. Production of the last was increased in the tissue culture populations, periods C and D, whereas it dropped sharply in a similar experiment with WI-38 human diploid cells (Kruse and Miedema, 1965a) derived from normal tissue. Proline was utilized during rapid but not slow proliferation, and glycine showed a progressive change from utilization to production. Both of these trends are the reverse, also, of previous findings with WI-38 cells (Kruse and Miedema, 1965a).

Profiles of arginine utilization and ornithine and citrulline production supported the negative tests cited above for the presence of mycoplasma (PPLO), since most strains of the latter have been found to utilize arginine extensively. The excessive arginine utilizations frequently cited in the literature of animal cell culture are now known to have been due to the then unrecognized presence of PPLO contaminants. In the present study ornithine production increased significantly in the slowest proliferative, highest cell density period (D). A similar result was recorded by Tritsch and Moore (1964); whether this was due to an induction of appreciable arginase activity was not investigated.

Considerably different results were obtained when the rate calculations were made on a *cell protein content basis* (Table II, data in parentheses), rather than on cell numbers. Here the rate of utilization of essential amino acids, except the two amides, was remarkably constant regardless of the fact that throughout the course of the experiment cell numbers increased and protein contents decreased at significantly different rates. These results indicate that the tumor cells continued to synthesize protein despite the decreased proliferation rate and increased population density.

This finding was confirmed in another perfusion experiment with ^{14}C-labeled lysine in the influent medium; the experiment was limited to 6 days due to depletion of the labeled medium. Table III shows an increased production of protein into the surrounding medium as proliferation decreased and population density increased. Cell counts (Table III) during the 6-day period were quite similar to those for the first 6 days in the previous perfusion experiment (Figure 1). The total counts per minute in extracellular protein increased more than fivefold. The increment in protein production each day (Table III, column 4) was obtained from the sum of activities in culture flask and effluent medium less that in the flask terminated on the previous day. From these increments the rate of protein production per hour per cell was calculated; as shown, it first declined during the period of rapid proliferation and then increased to become greatest during the last day when proliferation was minimal.

Relative rates of utilization of a few of the amino acids from medium 7a appeared to be disproportionate to their contents in cell protein. For example, the percentages of isoleucine and leucine in Jensen sarcoma cell protein were found to be 4.35 and 8.84, respectively (unpublished data); yet these two amino acids were utilized at approximately equal rates (Table II). This implied either a need by the tumor cells for isoleucine other than for protein synthesis or it reflected an amino acid imbalance in the culture medium. This uncertainty was resolved by short tests in stoppered cultures, using two media identical except for the relative concentrations of valine, isoleucine, and leucine. As shown in Table IV the "excessive" utilization of isoleucine disappeared when the culture medium contained these three amino acids in nearly the same relative concentrations as they are present in Jensen sarcoma cell protein. This result was not unexpected, since similar alterations in relative utilizations of valine, isoleucine, and leucine have been observed both *in vitro* and *in vivo* (*e.g.*, Mohberg and Johnson, 1963; Paul, 1959; Tannous *et al.*, 1966). In addition, Table IV presents a comparison of utilization with protein amino acid composition for 14 of the amino acids utilized, by ratios with lysine being 1.00. Asparagine and glutamine contents in protein were estimated from data reported previously from this laboratory (Patterson *et al.*, 1963). Glutamine was consumed from both media at rates tenfold in excess of requirements for protein synthesis. Similarly, excessive quantities of serine, cysteine, methionine, and isoleucine (from 7a only) were utilized. Reasons for the apparent insufficient consumption of tyrosine and phenylalanine, and of threonine from 7a VIL, were not investigated. In general, profiles of utilization of most of these 14 amino acids resembled rather closely their relative proportions in the cell protein, as shown in Table IV (also Table II). Such relationships have generally not been observed in previous studies of amino acid utilization (Kagawa

TABLE III: Production of Extracellular Protein by Perfused Cultures of Jensen Sarcoma Cells.[a]

Day Cell Count	($\times 10^{-6}$)	Effluent Protein: Total Cpm ($\times 10^{-3}$)	Effluent Protein: ΔCPM/Day ($\times 10^{-3}$)	Effluent Protein: Cpm/hr Cell ($\times 10^{3}$)
0	6.21	—	—	—
1	12.7	10.6	3.1	1.43
2	26.8	11.0	1.2	0.28
3	43.7	24.0	16.3	1.92
4	60.2	34.5	23.7	1.92
5	84.2	40.7	27.6	1.61
6	84.4	54.6	46.2	2.28

[a] Jensen sarcoma cells were cultured for 6 days in a perfusion system (see text) with the influent medium containing 0.04 μc/ml of uniformly labeled L-[^{14}C]-lysine; prior to perfusion the inoculum of cells was derived from cells carried through several generations in the labeled medium. Extractions of protein, radioassays, and rate calculations were made as described in the text.

TABLE IV: Comparison of Rates of Amino Acid Utilization with Composition of Jensen Sarcoma Cell Protein.[a]

Amino Acid	Protein	Utilization	
		Medium 7a	Medium 7a VIL
Serine	0.85	1.43	1.34
Asparagine	0.40	0.60	0.47
Glutamine	0.53	5.41	5.65
Threonine	0.80	0.84	0.50
Cystine (1/2)	0.15	0.23	0.24
Methionine	0.25	0.36	0.39
Valine	0.77	0.72	0.83
Isoleucine	0.55	0.92	0.63
Leucine	1.12	1.09	1.04
Tyrosine	0.35	0.24	0.27
Phenylalanine	0.43	0.28	0.35
Lysine	*1.00*	*1.00*	*1.00*
Histidine	0.26	0.22	0.26
Arginine	0.77	0.76	0.74

[a] Cells were cultured for 50 hr in stoppered T-60 flasks in mediums 7a and 7a VIL (valine, isoleucine, and leucine content of 0.18, 0.32, and 0.33, and 0.25, 0.17, and 0.31 mM, respectively). Amino acid composition of protein was calculated from four analyses of acid hydrolysates (and enzymatic digests for the two amides, *cf.* Patterson *et al.*, 1963) and rates of utilization were determined as described in text. *For purposes of comparing amino acid composition with rates of amino acid utilization, all values are related to lysine as unity.*

et al., 1960; McCarty, 1962; Mohberg and Johnson, 1963; Chung *et al.*, 1966) in animal cell cultures.

Discussion

The profiles of amino acid utilization and production in each of the four phases of proliferation were quite similar, the most marked difference being increased utilization of glutamine and production of glutamic acid in the tissue culture periods (C and D, Figure 1). Thus, the summation of anabolic and catabolic pathways of the tumor cells with regard to amino acids was a relatively constant property whether or not they were dispersed as isolated cells or closely packed in tissue-like densities. This property is one which might be predicted for tumors such as the Jensen sarcoma, since it has been transplanted for many years and in all probability consists of cells reduced to the single capacity of growth and multiplication. In contrast, cells in tissue-like densities derived from normal tissue may behave quite differently. We reported recently (Kruse and Miedema, 1965a) that increased population density and decreased proliferation rate of WI-38 human diploid cells was accompanied by marked changes in production or utilization of glutamic acid, proline, and glycine. In fact, these changes were just the reverse of those found in the present study with tumor cells. Whereas cells of diverse origins may exhibit similar nutritional and metabolic behavior *in vitro* when induced to proliferate in cell culture systems, the above comparison shows clearly that they may elicit markedly different responses when produced and maintained in tissue-like densities *in vitro*. We hypothesize that other differences among animal cell types of diverse origins will be more readily ascertained in "tissue" rather than in "cell" culture.[1] Thus, in addition to the control of environmental factors such as pH and nutrient supply, one of the advantages of most potential in a perfusion type culture system, such as that used herein, is that tissue cultures can be produced and maintained readily from a variety of cell types.

The high rate of serine utilization was of interest because most cells *in vitro*, including the Jensen sarcoma, have an appreciable capacity for its synthesis. The influent medium contained 0.20 mM L-serine and this may have caused an appreciable lowering of serine synthetic pathways, as reported for KB cells (Pizer, 1964), such that the exogenously supplied serine was utilized preferentially.

An unexpected finding was that rates of utilization of amino acids were constant with respect to cell protein content despite marked changes in rates of proliferation. That is, the Jensen tumor cells synthesized protein at a constant rate throughout the course of the experiment, implying that the new protein was used primarily for cell multiplication purposes during rapid proliferation of the cell culture phases (A and B, Figure 1) and mostly expressed into the surrounding medium during the tissue culture phases (C and D, Figure 1) when cell multiplication was slowed markedly. The observed changes in cell protein content are consistent with such an interpretation, and recently Eidam and Merchant (1965) have shown protein accumulation in extracellular fluid of plateau phase cell cultures. Further, the [^{14}C]lysine experiment in the present study demonstrated conclusively that the rate of production of extracellular protein increased as proliferation decreased and tissue-like cultures formed. From these considerations we conclude that cell crowding and contact had no controlling effect on total protein synthesis in the malignant cells employed in the present perfusion system experiments. In contrast, there is evidence that nonperfused cultures of cells of apparent nonmalignant character are contact and population density sensitive in this regard, *i.e.*, protein synthesis (Levine *et al.*, 1965; Todaro *et al.*, 1965; Loewenstein and Kanno, 1966). It should be of interest to explore further this suggested difference *in vitro* between cells derived from normal and malignant tissues. It is possible, though, that the apparent variation in response to crowding and contact may be a reflection of the different *in vitro* environments, *i.e.*, perfusion *vs.* nonperfusion systems. For example, serum has been shown to be a requirement for protein synthesis in primary cultures of chick embryo cells

(Amos and Moore, 1963) and the factor(s) responsible are thought to be of limited stability (Amos, 1966). If cell–serum interaction is necessary for expression of the factor(s), perfusion culture conditions may maintain effective levels of them while nonperfused cultures, with but daily renewals of medium, would not.

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