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Effect of Cell Concentration on the Uptake of Amino Acids by Rat Liver Parenchymal Cells in Suspension

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Summary. The accumulation of several amino acids in the acid-soluble fraction and their incorporation into protein in rat liver parenchymal cell suspensions, has been shown to depend on the concentration of cells in the incubation medium; the uptake, both in the acid-soluble and the acid-insoluble fractions, decreased as the cell concentration increased from 0.03×10^6 cells/ml upwards, reaching a plateau at high cell concentrations ($3–5 \times 10^6$ cells/ml). The uptake values at high cell concentrations were the same as those obtained in liver slices in which a similar effect was not observed. Evidence is presented which suggests that this phenomenon is mediated by a material released from the cells in suspension, which is inhibitory to enhancement of the uptake of amino acids by these cells over and above the value obtained in normal, adult liver slices.

Several investigators have shown that in mammalian cell suspensions (e.g., spermatozoa, reticulocytes, liver cells in suspension, and FLS ascitic tumor cells), incorporation of exogenous labeled amino acids into intracellular protein decreases as the concentration of cells in the incubation medium increases (Bhargava, Bishop & Work, 1959; Bhargava & Bhargava, 1962; Friedman & Epstein, 1967; Jezyk & Liberti, 1969; Blade, Blat & Harel, 1968; Blade & Harel, 1968). A similar cell concentration effect has been demonstrated in regard to the uptake of ^{32}P-phosphate and its incorporation into phospholipids in BHK and FLS ascitic tumor cells (Blade & Harel, 1965; Blade, Harel & Hanania, 1966); uptake of amino acids by the FLS ascitic tumor and by polyoma-transformed BHK 21 cells (Blade *et al.*, 1968; Blade & Harel, 1968; Bellanger & Harel, 1969); uptake of glucose by strain L mouse cells (Whitfield & Rixon, 1961); uptake of puromycin by pig kidney cell suspensions (Cass, 1972); and uptake of α-aminoisobutyric acid (Dickson, 1970) by rat liver cells in suspension.

In this paper we show that an increase in cell concentration in suspensions of liver cells leads to a progressive reduction—to a limiting value—in the uptake of amino acids into both the acid-soluble and the acid-insoluble fractions of the cell. This control of uptake is shown to be exercised

Table 1. Effect of conditioned media on the total uptake of labeled amino acids by liver cell suspensions at "low" and "high" cell concentrations [a]

Con-tainer (s)	Medium of incubation	Total uptake of the amino acids (dpm/mg protein)					
		Expt. 1		Expt. 2		Expt. 3	
		0.5×10^6 cells/ml	5.0×10^6 cells/ml	0.5×10^6 cells/ml	5.0×10^6 cells/ml	0.5×10^6 cells/ml	5.0×10^6 cells/ml
Separate	KRP buffer/MEM	28,500	7,610	37,880 (41,020)	13,470 (12,780)	26,020	7,910
Separate	Conditioned medium from 0.5×10^6 cells/ml	24,130	6,570	23,510	13,810	13,060	7,820
Separate	Conditioned medium from 5.0×10^6 cells/ml	15,930	6,680	9,190	13,240	7,900	6,760
Separate	Pronase-treated conditioned medium from 5.0×10^6 cells/ml	—	—	20,830 (20,350)	—	—	—
Separate	Heat-treated conditioned medium from 5.0×10^6 cells/ml	—	—	—	—	14,970	—
Two-compart-ment chamber	KRP buffer	—	—	15,790	13,950	—	—

[a] The conditioned media were prepared by incubating suspensions of rat liver parenchymal cells at two different concentrations (0.5×10^6 and 5.0×10^6 cells/ml) in KRP buffer (Expts. 1 and 2) or MEM (Expt. 3), at 37 °C for 1.5 hr. The cells were removed by centrifugation; the supernatant was dialyzed at 4 °C for 8 hr against 5 changes (2 liters each) of the above buffer in Expts. 1 and 2 only. In Expts. 2 and 3, portions of the conditioned medium were treated with pronase or subjected to heat treatment as in the text. A fresh batch of cells in suspension was then prepared in KRP buffer (Expts. 1 and 2) or MEM (Expt. 3); an appropriate volume containing the required number of cells was dispensed in a series of centrifuge tubes and sedimented at $200 \times g$. The supernatant was decanted and the cells suspended in the required medium (KRP buffer, MEM, conditioned medium from 0.5 or 5.0×10^6 cells/ml, or pronase or heat-treated conditioned medium). The cell suspension was then transferred quantitatively to 25 or 50 ml Erlenmeyer flasks (separate but same-size containers were used for the two cell concentrations), or to a two-compartment cell (*see text;* one compartment contained 0.5×10^6 cells/ml and the other 5.0×10^6 cells/ml). A mixture of ^{3}H-labeled amino acids (phenylalanine, histidine and lysine) in 0.2 ml of KRP buffer (or water) was then added and the cells incubated for 1.5 hr at 37 °C. The total incubation volume was, for separate containers, 10 ml in Expts. 1 and 3, and 5 ml in Expt. 2, and for the two-compartment cell, 15 ml in each compartment. The final concentrations of the

by a nondialyzable material released from the cells into the medium; the concentration of this material apparently regulates the increase in the rate of uptake of amino acids by the liver cells over and above the rate obtained in normal, adult liver slices.

Materials and Methods

Animals

These were the same as described in an accompanying communication (Kumar & Bhargava, 1975).

Radioactive Chemicals

^{14}C-labeled histidine, methionine, phenylalanine and leucine were obtained from Radiochemical Centre, Amersham, Bucks., U.K.; ^{14}C-labeled *Chlorella* protein hydrolysate from Bhabha Atomic Research Centre (BARC), Bombay, or from N.V. Philips Duphar, Holland; and the ^{3}H-amino acids from BARC, Bombay.

Enzymes

Enzite-pronase (insolubilized pronase) was purchased from Miles Lab Inc., Kankakee, Ill.

Liver Parenchymal Cell Suspensions

The cell suspensions were prepared by the method of Jacob and Bhargava (1962) except that in some experiments, Ca^{2+}-free Locke's solution was used instead of sucrose for the dispersion of the cells. For experiments using conditioned medium (Table 1) the perfusion was done at 37 °C instead of in the cold, and the cells were dispersed at 37 °C in Ca^{2+}-free Locke's solution. Wherever necessary, the cell suspensions prepared from several animals were pooled. Cell concentration was determined using a hemocytometer.

Slices

These were cut free-hand from liver perfused with citrated Ca^{2+}-free Locke's solution (Jacob & Bhargava, 1962). In experiments requiring a comparison to be made of the cell suspensions and the slices, both tissue preparations were obtained from the same animal.

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labeled amino acids in Expts. 1 and 2 were: phenylalanine, 1.15 pmoles (7.4×10^6 dpm)/ml; histidine, 1.01 pmoles (7.4×10^6 dpm)/ml; and lysine, 2.22 pmoles (7.4×10^6 dpm)/ml. In Expt. 3, the final concentrations were: phenylalanine, 0.2 μmoles (7.4×10^6 dpm)/ml; histidine, 0.2 μmoles (7.4×10^6 dpm)/ml; and lysine, 0.4 μmoles (7.4×10^6 dpm)/ml. After incubation, the cells were processed for determination of the total uptake as in the text. Values in parenthesis are for duplicates.

Incubation Media

One of the following three media was used for incubation: (a) Ca^{2+}-free Krebs original Ringer's phosphate (KORP) buffer (Dawson, Elliott, Elliott & Jones, 1959) containing glucose (15 mM) and penicillin (100 IU/ml); (b) Ca^{2+}-free Krebs-Ringer's phosphate (KRP) buffer (Umbreit, Burris & Stauffer, 1949) containing glucose and penicillin as above; or (c) Eagle's minimal essential medium in Hanks balanced salt solution, containing penicillin as above (MEM).

In some experiments, a "conditioned medium" obtained following incubation of liver cells in suspension at specified cell concentrations in KRP buffer or in MEM for 1.5 hr, was used; it was processed before use as described in the legend to Table 1. A portion of it was incubated with Enzite-pronase (2 mg/ml) at 37 °C for 1 hr; the enzyme was removed by centrifugation and the supernatant was dialyzed. In some experiments, a portion of the conditioned medium was heated at 90 °C for 30 min, cooled and centrifuged to remove heat-precipitable material.

Incubation for the Uptake Studies

The cells or slices were incubated in the stated medium, with the labeled amino acid(s) added in 0.1–0.2 ml of 0.9 NaCl, in 25–50 ml Erlenmayer flasks (one for each time point) at 37 °C in a constant temperature water-bath with shaking at 50 oscillations/min. In some experiments using conditioned media, incubations were made in a two-compartment chamber consisting of two identical cylindrical cells of Perspex, $1^1/_2''$ in diameter and $2^1/_4''$ deep, separated by a 50-mm diameter Millipore membrane filter (pore size 1.0 μ) and held together by nylon bolts and nuts. Every possible care was taken to prevent bacterial contamination; all the containers and solutions were sterilized before use. In many experiments, 0.1–0.2 ml of the incubation mixture was plated on blood-agar; no contamination was detected.

Estimation of the Uptake of Labeled Amino Acids

Following incubation with labeled amino acid(s) for the desired period, the cell preparation was processed for estimation of one of the following: (a) radioactivity in the whole cells or slices; (b) radioactivity in the total cellular or tissue protein; (c) radioactivity in the total free amino acid pool; or (d) radioactivity in the intracellular free pool of the amino acid which was used for the study of uptake. In several experiments, (b) was estimated along with (c) and/or (d) in the same set of samples.

For estimation of (a), the washed cell preparation *(see below)* was dissolved in 2–4 ml of 0.6 N NaOH, and radioactivity and protein were estimated. In some experiments, the washed cells were homogenized in distilled water and radioactivity estimated in the homogenate.

For estimation of (b), an excess of the unlabeled precursor was added to the incubation mixture and the cells or slices sedimented in the cold at $100 \times g$. They were washed rapidly 6–8 times with KORP buffer, each time with the same volumes as used for incubation, until the washings were virtually free of radioactivity. Trichloroacetic acid (TCA: 5%; 2–5 ml) was added, the precipitate washed twice with cold 5% TCA, treated with 5% TCA for 30 min at 90 °C, and washed again twice with 5% TCA. Lipids were removed from the residue and the specific radioactivity of the protein fraction determined in the usual way (Bhargava & Bhargava, 1962). In experiments in which the incubation was made in MEM, the cold TCA-washed precipitate was treated with ether to remove TCA and dissolved in 2–4 ml of 0.6 N NaOH as for (a).

For estimation of (c), the combined cold TCA supernatants obtained following precipitation with 5% TCA were used. TCA was removed with ether and the resultant aqueous fraction passed through a 150–200 mesh IR-120 ion exchange column (12 × 0.9 cm). The free amino acids were eluted with 0.5 or 2.0 N ammonia. The amino acid eluate, comprised of the first 10 ml of the eluant after ammonia started eluting, was concentrated by freeze drying and its radioactivity and total amino acid content estimated. In experiments in which the incubations were done in MEM, the radioactivity was estimated in the combined TCA supernatants, without prior removal of TCA and chromatography.

For estimation of (d), the total amino acid fraction was chromatographed in two dimensions on Whatman no. 1 paper, the amino acids visualized with ninhydrin and the spot corresponding to the labeled amino acid used eluted as usual (Lederer & Lederer, 1954). Radioactivity and amino acids were estimated in the eluate, the latter colorimetrically (Moore & Stein, 1948).

Protein estimations were done by the method of Lowry, Rosebrough, Farr and Randall (1951).

Estimation of the Specific Activity of the Medium Amino Acids

At the end of incubation, the cells were sedimented by centrifugation at $100 \times g$. The supernatant was centrifuged for 1 hr at $105{,}000 \times g$ in a preparative ultracentrifuge and treated with an equal volume of 10% TCA or 6% perchloric acid. TCA was removed from the supernatant as above and PCA by precipitation as $KClO_4$ in the cold. The total amino acid fraction was then isolated by chromatography on an IR-120 column and the specific activity of the desired amino acid determined by chromatography on paper as above.

Results and Discussion

Figs. 1 and 2 show that the incorporation of ^{14}C-histidine into protein in rat liver parenchymal cells in suspensions at 3 hr was dependent on the concentration of the cells in the incubation medium in the concentration range 0.03–2.1 $\times 10^6$ cells/ml; the incorporation decreased as the cell concentration increased, and the cell concentration effect became progressively less marked with increasing cell concentration. Fig. 2 shows that the above cell concentration effect was also observed in regard to the accumulation of the radioactive amino acid in the total free amino acid pool or in the histidine pool. Similar observations were made for the uptake of ^{14}C-histidine, both in the acid-soluble and the acid-insoluble fractions, at various time points below 3 hr (*cf.* Bhargava & Bhargava, 1962). These observations show that under the experimental conditions used, the amount of histidine transported per cell at any time point decreased with increasing cell concentration within a wide range of cell concentrations. Figs. 3–5 show that this phenomenon was not cinfined to histidine; it was observed with all the amino acids tried (methionine, phenylalanine, leucine and lysine, used singly or in mixture) within a

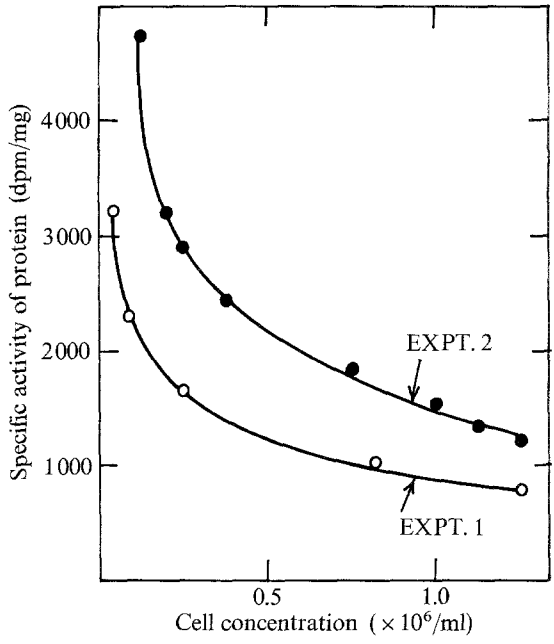

Fig. 1. Effect of cell concentration on the incorporation of ^{14}C-histidine into protein in liver parenchymal cell suspensions. The cells were incubated in 6.0 ml of KORP buffer for 3 hr with ^{14}C-histidine [5.6 nmoles (4.4×10^5 dpm)/ml in Expt. 1, and 6.7 nmoles (5.2×10^5 dpm)/ml in Expt. 2]. The cells were washed and the incorporation into protein estimated as described in the text

wide range of external amino acid concentrations and in two types of incubation media [KORP (or KRP) buffer and MEM]. No similar effect was observed with liver slices (Fig. 6); when slices and cell suspensions obtained from the same liver were used, the uptake of amino acids in the slices was about the same as the limiting value observed at high cell concentrations (generally $3–5 \times 10^6$ cells/ml) in the case of cell suspensions.

It seemed to us that a possible, trivial explanation of the phenomenon could be the dilution of the exogenous labeled amino acids with unlabeled amino acids leaking out of the cells; the extent of this dilution would increase with increasing concentration of the cells in the incubation medium. Although we did find some dilution of the labeled precursor during incubation, it was far too small to explain the magnitude of the cell concentration effect observed by us (Fig. 4). Further, a similar concentration effect was observed when the cells were incubated in a tissue culture medium (Fig. 5 and Table 1) in which case any leakage of amino acids from the cells during incubation could not significantly alter the specific activity of the amino acid(s) in the incubation medium.

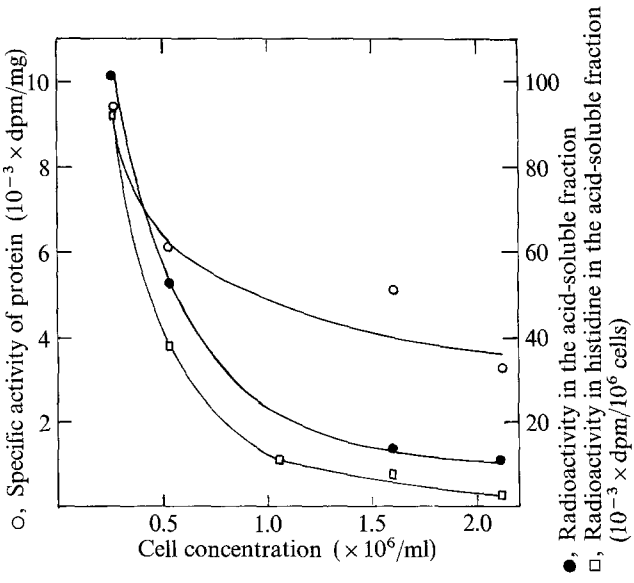

Fig. 2. Comparison of the effect of cell concentration on the accumulation of ^{14}C-histidine in the acid-soluble pool with that on its incorporation into cellular protein in liver parenchymal cell suspensions. The cells were incubated with ^{14}C-histidine [5.6 nmoles (4.4×10^5 dpm)/ml] and the radioactivity incorporated into cellular protein estimated as in Fig. 1. Radioactivity in the ether-extracted acid-soluble fraction of the cells and in the histidine fraction obtained after chromatography, was estimated as described in the text

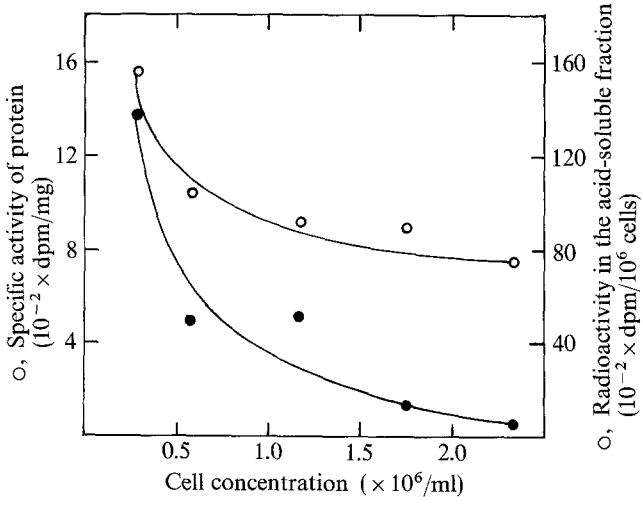

Fig. 3. Effect of cell concentration on the accumulation of ^{14}C-methionine in the acid-soluble pool and on its incorporation into cellular protein in liver parenchymal cell suspensions. The cells were incubated with ^{14}C-methionine [8.5 nmoles (5.5×10^5 dpm)/ml] and its accumulation in the acid-soluble pool and incorporation into cellular protein estimated as in Fig. 2

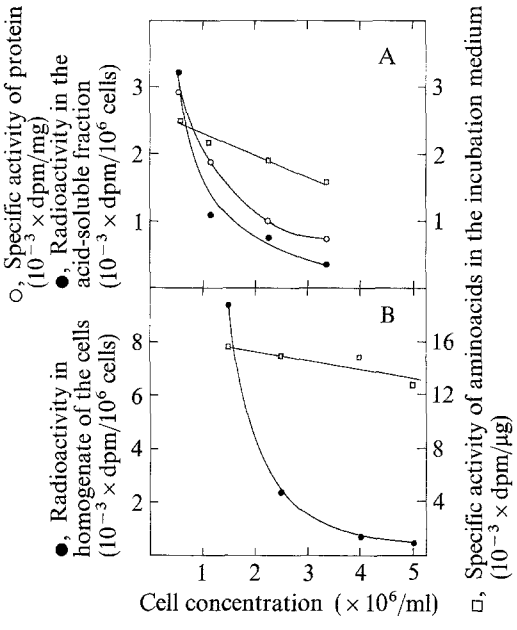

Fig. 4. Effect of cell concentration on the transport of ^{14}C-phenylalanine and ^{14}C-leucine in liver parenchymal cell suspensions and on the specific radioactivity of the amino acid in the medium after incubation. The cells were incubated (*A*) in 4.0 ml of KORP buffer for 3 hr with ^{14}C-phenylalanine [0.303 μmoles (11.1×10^5 dpm)/ml]; or (*B*) in 5.0 ml of Ca^{2+}-free KRP buffer for 2 hr with ^{14}C-leucine [0.382 μmoles (11.1×10^5 dpm)/ml]. The incubation mixture was separated into the cell and the medium fractions as described in the text. In *A*, the accumulation of the amino acid in the free amino acid pool of the cells and its incorporation into protein were separately estimated as in Fig. 2; in *B*, the total transport was estimated by measurement of radioactivity in the homogenate of washed cells. Specific radioactivity of the amino acid in the medium was estimated in the acid-soluble fraction as in the text

The cell concentration effect cannot also be explained by the selective absorption at low cell concentrations of radioactive contaminants present in limiting amounts in the amino acid preparations used, as (a) chromatographic separation showed that all the radioactivity in the amino acid pool was due to the amino acid used as precursor (e.g. Fig. 2), and (b) the effect was not abolished or reduced when the labeled precursor was pretreated, before use, with low-concentration cells. The effect was not due to depletion of the substrate from the medium by high-concentration cells as in no case was more than 10% of the substrate taken up by the cells.

Two types of explanation of this phenomenon seemed possible: (A) the effect was a consequence of *direct* cell contact, the extent of which contact would increase as the concentration of cells increased; or (B)

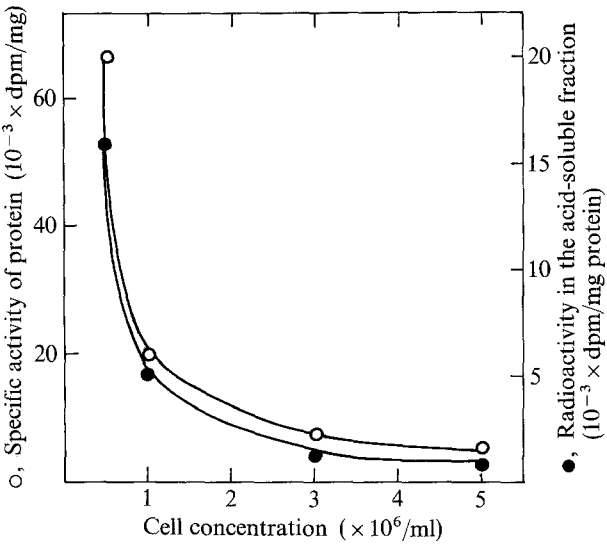

Fig. 5. Effect of cell concentration on the accumulation of a mixture of ^{3}H-labeled amino acids in the acid-soluble pool and on their incorporation into cellular protein in liver parenchymal cells in suspension. The cells were incubated in 10 ml of MEM for 1.5 hr with a mixture of ^{3}H-phenylalanine [0.2 μmoles (7.4×10^6 dpm)/ml], ^{3}H-histidine [0.2 μmoles (7.4×10^6 dpm)/ml] and ^{3}H-lysine [0.4 μmoles (7.4×10^6 dpm)/ml]. The accumulation of amino acids in the acid-soluble pool and their incorporation into cellular protein, was estimated by measurement of radioactivity in the TCA supernatant and in the TCA-precipitate dissolved in 0.6 N NaOH, respectively. For details, *see text*

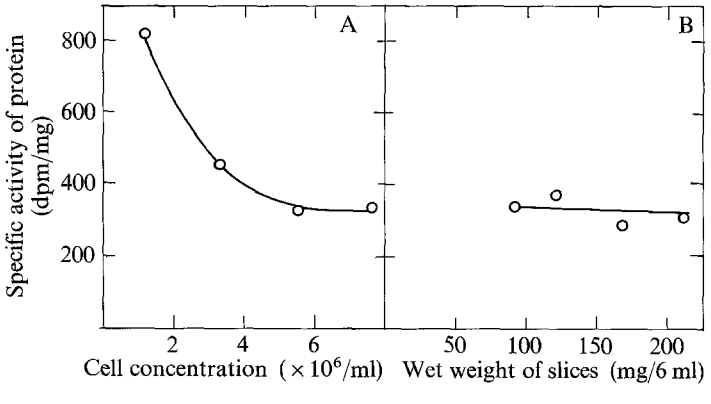

Fig. 6. Effect of cell/tissue concentration on the incorporation of ^{14}C-histidine into protein in (*A*) liver parenchymal cell suspensions, and (*B*) perfused liver slices. Incubations were made with ^{14}C-histidine [5.6 nmoles (4.4×10^5 dpm)/ml] and radioactivity incorporated into cell/tissue protein estimated as in Fig. 1. Rat liver slices contain, on an average, 81×10^6 parenchymal cells per gram wet wt of liver (Iype, Bhargava & Tasker, 1965)

the effect was mediated by a factor (or factors) released from the cells into the medium, the concentration of which factor would increase as cell concentration increased. We, therefore, did a series of experiments in which we used "conditioned media" obtained by incubation of liver parenchymal cells at "low" (0.5×10^6 cells/ml) and at "high" (5×10^6 cells/ml) cell concentrations. If possibility A were true, conditioned medium derived from the high-concentration cells should have no effect on the uptake of amino acids by the low-concentration cells; if possibility B held true, the uptake in the low-concentration cells in the presence of the above conditioned medium, should be reduced to the same level as obtained in the high-concentration cells. Table 1 shows that conditioned medium obtained following incubation of liver cells at the high cell concentration, had a strong inhibitory effect on the uptake of amino acids by cells incubated at the low cell concentration. These experiments strongly support possibility B: that the cell concentration effect observed by us is mediated through a factor(s) released from the cells. This view was further supported by experiments in which an incubation chamber with two compartments separated by a membrane filter which would allow macromolecules but not cells to pass through, was used; one compartment contained cells, at the low cell concentration and the other at the high cell concentration (Table 1). While the uptake by the high-concentration cells in the two-compartment chamber was the same as was obtained when the cells were incubated at the high cell concentration separately, the uptake by the low-concentration cells in this chamber was reduced to the level obtained in the high-concentration cells, from a threefold higher value obtained when the low-concentration cells were incubated separately.

As would be expected, the extent of inhibition of amino acid uptake by the low-concentration cells appeared to depend on the concentration of the inhibitory material released into the incubation medium by the cells. The inhibitory effect of conditioned medium from the *low*-concentration cells on amino acid uptake by fresh cells incubated at the low cell concentration, was lower than that obtained with conditioned medium from the high-concentration cells.

The uptake by the high-concentration cells was not further reduced by the conditioned medium derived from the high-concentration cells. As the normal uptake in the high-concentration cells represented a limiting value (Fig. 6), the inhibitory effect on amino acid uptake of the factor released from liver cells during incubation would appear to be confined to the increase in the rate of uptake *over and above* this limiting value.

As the conditioned media were dialyzed before use, the transport inhibitory material in them should be a macromolecule. In a series of experiments (some of which are given in Table 1), pronase or heat treatment of the conditioned medium from cells incubated at the high cell-concentration, resulted in a substantial (60–75%) reduction in the ability of the conditioned medium to inhibit transport over and above the limiting value obtained in the high-concentration cells; this suggests that the inhibitory material is at least partly protein.

While it would be premature to speculate on the precise biological role of the above transport inhibitory material for which we have obtained evidence, it is tempting to suggest that such inhibitors of transport may have a role in regulation of growth. Recent work in several cell systems (e.g., Bhargava, Allin & Montagnier, *unpublished observations*) has shown that induction of resting mammalian cells into division is accompanied by a rapid rise in the rate of uptake of nutrients. Such transport inhibitors may aid in the maintenance of the "resting state" by preventing a rise in the rates of transport of essential nutrients *over and above* the level required for cell maintenance.

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