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INFLUENCE OF SERUM AND AMINO ACIDS ON THE ACCUMULATION OF AMINOISOBUTYRATE BY RAT HEPATOMA CELLS

A DEDIFFERENTIATION OF TRANSPORT ROUTES?

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Rat hepatoma cells accumulate considerably less 2-aminoisobutyrate after cultivating in the absence of serum – the change in rate of aminoisobutyrate uptake takes place within 1 h of serum starvation. Starvation of amino acids by contrast raises aminoisobutyrate uptake in the presence or absence of serum, but the cells are much less responsive to amino acid supply than to availability of serum. Phosphate (10 mM) reduced aminoisobutyrate uptake by cells grown in serum to that exhibited by serum-starved cells. Aminoisobutyrate uptake by cells grown in serum was reduced by glycine, proline, alanine, serine, glutamine, methylaminoisobutyrate and 2-aminonorbornane-2-carboxylate, the effects of methylaminoisobutyrate and 2-aminonorbornane-2-carboxylate being additive. However, similar inhibition phenomena were not seen for cells deprived of serum where aminoisobutyrate uptake tended to a relatively constant level insensitive to inhibitory influences, yet substantially greater than that arising by simple diffusion. The comparative insensitivity of our hepatoma line when starved of serum to competition and repression phenomena is in contrast to findings of others. Our results also suggest a lack of clear delineation of specificities for the A and L transport systems as usually defined.

Introduction

In an earlier communication from this laboratory [1] it was found that rat hepatoma cells cultured in the presence of serum accumulated more 2-aminoisobutyrate than when cultured in medium lacking serum. In the latter case, though uptake had the kinetic characteristics of diffusion, it was nonetheless much greater than that observed at 4°C. Moreover, uptake by cells deprived of serum was still subject to inhibition in the presence of alanine at 37°C, though not at 4°C. Uptake by cells cultured without serum likewise remained responsive to insulin. However, the ability of prior incubation with alanine to depress subsequent aminoisobutyrate uptake (i.e., repression) was only seen when the preincubation medium contained serum.

In these earlier experiments, minimal essential medium was used throughout. This medium contains defined amounts of free amino acids and it seems probable that optimal aminoisobutyrate uptakes and responses were not achieved. In the present experiments, we have sought to investigate more fully the influence of a number of amino acids, including methylaminoisobutyrate and 2-aminonorbornane-2-carboxylate (BCH), reputedly specific for Systems A and L, respectively [2], on aminoisobutyrate uptake by hepatoma cells cultured in the presence and absence of serum.

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Abbreviation: BCH, 2-aminonorbornane-2-carboxylate.

Materials and Methods

Incubation media. Incubation media used were as follows. Modified Hank's balanced salt solution (medium I) containing (mmol/l) Na^+, 146; K^+, 5.8; Mg^{2+}, 0.4; Ca^{2+}, 1.26; Cl^-, 144; phosphate, 0.61; SO_4^{2-}, 0.4; HCO_3^-, 8.9; plus 5.5 mM glucose. Earle's balanced salt solution containing (mmol/l) Na^+, 143; K^+, 5.4; Mg^{2+}, 0.41; Ca^{2+}, 1.8; Cl^-, 124; phosphate, 0.8; SO_4^{2-}, 0.41; HCO_3^-, 26; plus 5.5 mM glucose. Phosphate-buffered saline containing (mmol/l) Na^+, 165; Cl^-, 150; phosphate, 10; pH adjusted to 7.4. Minimal essential medium (medium II) containing the same ions and concentration of glucose as medium I. In addition medium II contained the following L-amino acids (mM): arginine, 0.60; histidine, 0.27; lysine, 0.40; leucine, 0.38; isoleucine, 0.38; valine, 0.37; phenylalanine, 0.19; tryptophan, 0.05; tyrosine, 0.29; glycine, 0.10; serine, 0.10; threonine, 0.40; glutamine, 2.0; cysteine, 0.26 and methionine, 0.10. Vitamin C and 50 units of both penicillin G and streptomycin were also present together with 5% foetal calf serum as indicated.

Culture of cells and measurement of aminoisobutyrate accumulation. Rat hepatoma cells, originally derived by treatment of rats with 3-methyl-4-dimethylaminoazobenzene [3], were grown in medium II containing 5% foetal calf serum, as previously described [1]. Cells were cultured in flat glass bottles with a surface area of 194 cm^2. After one week, the cells were trypsinized and equally distributed in 125-ml Falcon flasks, with a surface area of 43 cm^2, and having a cell count of about $5 \cdot 10^6$. After four days of culture, the number of cells had doubled. Conditions were standardised in this manner to avoid variations of cell number and degree of confluency which can affect amino acid transport [4]. After four days, the culture medium was replaced by either medium II plus serum as hitherto, or medium II containing no serum, or as otherwise indicated in the text and tables. After about 20 h, the medium was replaced by, unless otherwise indicated, medium I containing no added serum or amino acids except 1 mM amino-[^{14}C]isobutyrate. Incubation was continued for 10 min at 37°C. Cells were quickly washed twice with ice-cold phosphate-buffered saline. They were then scraped off the culture flasks and dispersed in 5 ml phosphate-buffered saline. Duplicate 2-ml samples were mixed with 0.1 ml Triton X-100 and boiled for 1 h. The entire sample was mixed with 10 ml of dioxan-based scintillation fluid and the ^{14}C content was measured. Cells were counted with a Coulter counter. Their viability was estimated by trypan blue exclusion to be 90–92%. Cell size was not changed by preincubation for various periods in the absence of serum and cell diameter was about 13 μm. The intracellular concentration of aminoisobutyrate was calculated as nmol/10^6 cells.

Results

Effects of various media

The finding [1] that the uptake of aminoisobutyrate by cells continuously cultured in medium II containing serum is greater than when serum is withdrawn from the culture medium 20 h previously has been confirmed in the present work (Table I). In the experiments in Table I, line 1, serum was present during the 10-min period of aminoisobutyrate uptake, but the presence or absence of serum at this stage is without effect (results not shown).

Use of medium II, which contains amino acids, as the medium from which to measure aminoisobutyrate

TABLE I

UPTAKE OF AMINOISOBUTYRATE BY HEPATOMA CELLS IN VARIOUS MEDIA

Cells were cultured in medium II with or without serum for the previous 20 h. Aminoisobutyrate transport was measured for 10 min in the medium indicated. Values are nmol/10^6 cells per 10 min and are the mean ± S.E. of mean of the number of observations shown in parentheses.

Medium during aminoisobutyrate accumulation	Aminoisobutyrate accumulated	
	− serum	+ serum
Minimal essential medium (medium II)	5.3 ± 0.4 (9)	10.0 ± 0.5 (9)
Hank's buffered salt solution (medium I)	12.0 ± 1.1 (14)	30.8 ± 1.8 (18)
Earle's buffered salt solution	16.5 ± 0.4 (4)	34.2 ± 2.1 (12)
Phosphate-buffered saline	17.9 ± 1.9 (7)	12.4 ± 0.6 (12)
Medium I + 10 mM phosphate		15.6 ± 0.4 (4)

uptake does not measure the full potential for amino acid accumulation, since much larger uptakes were observed when the amino acids were removed at this stage, namely by use of medium I in place of medium II (Table I). Earle's solution gave similar results to medium I. The greater uptake of aminoisobutyrate in medium I as opposed to medium II was observable with cells cultured in the presence of serum or when previously starved of serum for 20 h.

That the ionic composition of medium I and of Earle's solution are important for support of aminoisobutyrate transport can be seen when phosphate-buffered saline is used as the incubation medium. Under these conditions, aminoisobutyrate transport by cells, cultured in the presence of serum, was severely reduced when phosphate-buffered saline replaced medium I. However, replacement of medium I by phosphate-buffered saline did not diminish aminoisobutyrate uptake by cells cultured in the absence of serum. Addition of inorganic phosphate, the buffering component of phosphate-buffered saline, to medium I at its concentration in phosphate-buffered saline markedly reduced aminoisobutyrate uptake by cells cultured in serum (Table I). The reason for this is not known.

Influence of amino acids during aminoisobutyrate uptake

Which amino acids in medium II are responsible for reducing the ability of cells growing in serum to accumulate aminoisobutyrate is shown in Table II. The basic and hydrophobic amino acids have relatively small influence by comparison with amino acids such as glycine, serine, threonine, glutamine, cysteine and methionine, which either singly or together reduced aminoisobutyrate uptake to the level of cells cultured without serum and incubated without amino acids. Conversely, the basic amino acids, the hydrophobic amino acids and glycine, serine and threonine seemed to increase aminoisobutyrate uptake by cells cultured in the absence of serum. Heaton and Gelehrter [5] found that a mixture of glutamine, histidine and methionine at 2, 0.2 and 0.1 mM, respectively, was as effective as a mixture very similar to that normally present in medium II. The importance of glutamine as a noncompetetive inhibitor of System A has been recently noted [6]. The potency of methionine (and/or in our case cysteine) at the low concentrations at which they were added is also of interest.

TABLE II

EFFECT OF AMINO ACIDS PRESENT IN MEDIUM II ON THE UPTAKE OF AMINOISOBUTYRATE BY HEPATOMA CELLS

Amino acids were added to medium I containing no serum at the concentrations they are present in medium II. Cells had been cultured in medium II with or without addition of serum for the previous 20 h. Values are nmol/10^6 cells per 10 min and each is the mean of the number of observations shown in parenthesis.

Amino acids added	Aminoisobutyrate uptake	
	– serum	+ serum
None	12.0 (14)	30.8 (18)
Arg, His, Lys	17.4 (2)	24.0 (2)
Leu, Ile, Val, Phe, Trp, Tyr	18.4 (2)	27.7 (2)
Gly, Ser, Thr, Gln, Cys, Met	7.3 (4)	9.7 (4)
Gly, Ser	16.7 (2)	12.7 (2)
Thr	22.0 (2)	14.1 (2)
Gln	10.3 (2)	8.2 (2)
Cys, Met	8.7 (2)	8.4 (2)

The effects of individual amino acids added at the same concentrations as the amino[^{14}C]isobutyrate are studied further in Table III. As was to be expected, alanine, serine and proline, amino acids using System A mediation preferred by aminoisobutyrate, and glutamine were inhibitory towards aminoisobutyrate uptake by cells cultured in serum. Glycine was also inhibitory, but apparently less so than the other amino acids. Of the naturally occurring amino acids, leucine was unexpectedly inhibitory towards cells cultured in serum. As expected, the aminoisobutyrate analogue, methylaminoisobutyrate, was inhibitory, but again, unexpectedly, BCH proved almost as effective an inhibitor as methylaminoisobutyrate. Neither leucine, glutamine, methylaminoisobutyrate nor BCH had much effect on cells cultured in the absence of serum.

Effect of pH of incubation medium

Table IV shows the uptake of aminoisobutyrate by cells when the pH of the medium containing the amino[^{14}C]isobutyrate was varied. Increase of pH above 7.4 decreased somewhat accumulation by cells

TABLE III

EFFECT OF VARIOUS AMINO ACIDS ON THE UPTAKE OF AMINOISOBUTYRATE BY HEPATOMA CELLS

Amino acids were added to medium I containing no serum at a concentration of 1 mM. Cells had been cultured in medium II with or without addition of serum for the previous 20 h. Values are nmol/10^6 cells per 10 min and each is the mean ± S.E. of the mean of the number of observations shown in parentheses.

Amino acids added	Aminoisobutyrate uptake	
	– serum	+ serum
None	12.0 ± 1.1 (14)	30.8 ± 1.8 (18)
Glycine		22.7 ± 0.9 (8)
Proline		14.0 ± 1.4 (3)
Alanine		14.0 ± 2.3 (4)
Serine		13.1 ± 0.5 (4)
Leucine	16.4 ± 0.7 (4)	12.6 ± 1.2 (4)
Glutamine	10.3 (2)	10.8 ± 1.5 (10)
Methylaminoisobutyrate	13.9 ± 1.1 (8)	10.1 ± 0.9 (8)
BCH	14.8 ± 1.5 (4)	13.1 ± 1.8 (4)

cultured in the presence of serum. Lowering the pH to 6.0 and 3.4 resulted in marked decrease in aminoisobutyrate uptake by cells cultured either in the presence or the absence of serum.

Influence of amino acids during culture (adaptive regulation)

Table V studies the effect of culturing for various periods in the absence of amino acids (amino acid starvation) by replacing medium II with medium I. The table also compares the effect of serum deprivation for periods shorter than the usual 20 h. The difference in capacity for aminoisobutyrate uptake is clearly visible after serum deprivation for as little as 1 h and is proportionally fairly similar whether culture medium did or did not contain added amino acids. Withholding amino acids during culture increased the capacity for aminoisobutyrate uptake irrespective of whether serum was present or not, but the magnitude of the serum effect was much greater than that of the amino acids. Though in the cultures with serum the serum itself adds some amino acids to the medium, there is no source in the cultures without serum. The amino acids contained in medium II do not constitute the full range of naturally occurring amino acids and many are present at comparatively

TABLE IV

EFFECT OF pH ON THE UPTAKE OF AMINOISOBUTYRATE BY HEPATOMA CELLS

Cells were cultured in medium II with or without serum for the previous 20 h. Aminoisobutyrate uptake was measured after 10 min of incubation in medium I with the pH adjusted to the values indicated. Values are nmol/10^6 cells per min and, except for those at pH 7.4, each is the mean of two observations.

pH	Aminoisobutyrate uptake	
	– serum	+ serum
3.4	2.6	2.1
6.0	5.2	4.1
7.4	12.0 (14)	30.8 (18)
7.7	13.2	22.1
9.0	15.6	22.1

low concentrations. This may explain their comparatively small influence. For whatever reason, the increase in aminoisobutyrate uptake following amino acid starvation, either due to release of transinhibition or due to derepression, was not nearly as marked as that observed with hepatomas by Heaton and Gelehrter [5] and Kelley and Potter [7,8].

If cells are preincubated with alanine, glycine or glutamine for 1 h in the presence of serum, the capac-

TABLE V

EFFECT OF VARYING PREINCUBATION CONDITIONS ON THE CAPACITY OF HEPATOMA CELLS TO ACCUMULATE AMINOISOBUTYRATE

Prior to measurement of aminoisobutyrate uptake for 10 min from medium I containing no serum, cells were cultured for the stated periods of time in medium I or II with or without addition of serum as indicated. Values are nmol/10^6 cells per min and each is the mean of two observations.

Period of incubation (h)	Aminoisobutyrate uptake			
	Medium II		Medium I	
	– serum	+ serum	– serum	+ serum
1	8.1	38.7	13.2	46.6
2	8.4	22.4	15.0	36.6
5	8.8	33.2	14.5	56.3
10	9.1	21.1	12.7	42.6
20	8.7	30.1	14.8	32.1

TABLE VI

EFFECT OF VARYING PREINCUBATION CONDITIONS ON THE CAPACITY OF HEPATOMA CELLS TO ACCUMULATE AMINOISOBUTYRATE

Prior to measurement of aminoisobutyrate uptake for 10 min from medium I containing no serum, cells were incubated for 1 h in medium I with or without serum, with the stated concentrations of the amino acids. Values are nmol/10^6 cells per 10 min and, except as indicated, each is the mean of two observations.

Amino acid added	Aminoisobutyrate	
	– serum	+ serum
None	13.2	46.6
Alanine (5 mM)	10.5 (4)	13.8
Glycine (1 mM)	13.8 (5)	11.9
Glutamine (2 mM)	7.7	10.1

ity for subsequent aminoisobutyrate uptake is much reduced (Table VI), as observed earlier using cells cultured in medium II [1]. As also seen in Table V, preincubation for only 1 h in the absence of serum results in a marked decrease in subsequent uptake of aminoisobutyrate (Table VI). Effects of the amino acids when added to cells in the absence of serum were less marked than in its presence. In this regard, our results contrast markedly with those of Heaton and Gelehrter [5] and Kelley and Potter [7,8] whose hepatoma cells were treated similarly to our serum-starved cells, yet showed responses more like our cells cultured in serum. The reason for this difference is not clear. Although their units of aminoisobutyrate uptake are expressed in terms of mg of protein and ours in terms of 10^6 cells, it appears that our basal rate of aminoisobutyrate uptake is 2–3-fold higher than theirs. The magnitude of the effect of serum in our experiments is similar to that described by Oxender et al. [4].

Kinetic parameters of aminoisobutyrate uptake

In earlier studies, the apparent K_m of aminoisobutyrate uptake from medium II was found to be about 8 mM for cells cultured in serum and approached diffusion kinetics for cells deprived of serum [1]. Redetermination of the apparent K_m in medium I for cells cultured in serum gave a value of approx. 11 mM and a V value about 3-fold larger than previously observed. This result was unexpected. It was anticipated that measurement of apparent K_m in the absence of the amino acids present in medium II would result in a lower value without there being a marked change in V. In fact, the precise opposite was seen. Moreover, in partial contradiction of earlier results [1], cells cultured in the absence of serum did not show a very striking increase in apparent K_m (19 mM) by comparison with cells cultured in serum.

On the grounds that glutamine is the amino acid in medium II present in the largest concentration, the influence of glutamine was studied further. Its addition at 2 mM to the medium during aminoisobutyrate uptake had negligible effect on K_m (approx. 12 mM), but V was approximately halved. Kilberg et al. [6] have also recently observed noncompetitive inhibition of System A by glutamine.

The difficulty of obtaining meaningful kinetics of amino acid uptake where multiple routes of uptake are involved is well known and these results might be explicable if it were possible to differentiate between the different transport mechanisms used. However, as noted above (Table III), it was unexpected to find, on the basis of experiments of others showing the specificity in inhibition of BCH and methylaminoiso-

TABLE VII

EFFECTS OF METHYLAMINOISOBUTYRATE AND BCH, SINGLY AND TOGETHER, ON THE UPTAKE OF AMINO-[^{14}C]ISOBUTYRATE BY HEPATOMA CELLS

Cells were cultured in medium II with serum for the previous 20 h. Aminoisobutyrate uptake was measured from a number of different concentrations after 10 min incubation in medium I in the presence of 1 mM concentrations of methylaminoisobutyrate and/or BCH as indicated. Values are nmol/10^6 cells per 10 min and each value is the mean of four observations.

Amino acids added to medium	Aminoisobutyrate uptake with concn. of aminoisobutyrate (mM) in medium being:			
	1	2.5	5	10
No addition	23.1	50.7	79.5	122
Methylaminoisobutyrate	8.2	15.6	35.7	64
BCH	10.9	27.0	34.5	73
Methylaminoisobutyrate + BCH	2.1	6.5	11.6	23

butyrate, that both these compounds inhibited aminoisobutyrate uptake in a similar fashion. Table VII extends these observations, showing inhibition of aminoisobutyrate uptake by methylaminoisobutyrate and BCH singly and together at various aminoisobutyrate concentrations. Table VII shows that methylaminoisobutyrate and BCH both inhibit aminoisobutyrate uptake to a roughly similar extent. The inhibition produced by each compound persists over a 10-fold range of aminoisobutyrate concentrations. Furthermore, the effects of the two compounds were additive and the extent of aminoisobutyrate uptake observed when the two amino acids were present was roughly that observed previously [1] for simple diffusion. It is not clear from these results whether both methylaminoisobutyrate and BCH produce a toxicity towards transport not hitherto observed, which seems unlikely, or whether in our cells transport of aminoisobutyrate occurs through both A and L routes which are extremely sensitive to inhibition by the two inhibitors.

Discussion

It is frequently concluded that uptake of aminoisobutyrate is by sodium-dependent transport systems. Both Kelley and Potter [7] and Edmonson et al. [9] found System L and diffusion to account for less than 10% of its uptake in hepatocytes. Kelley and Potter [7] concluded that System ASC accounted for only 5% of uptake in hepatocytes, whereas Le Cam and Freychet [10] found up to 30% entering via this transport mode. It is not clear to what extent System ASC functions in our experiments. Aminoisobutyrate uptake is much lowered by lowering the pH (Table IV) to which System A is especially sensitive. Though uptake by serum-cultured cells is very sensitive to inhibition by both serine and alanine (Table III), if these amino acids largely use System A in hepatoma cells as well as in hepatocytes [9], this result is to be expected. It therefore seems reasonable to conclude from this observation that most aminoisobutyrate uptake in our cells, particularly those continuously cultured in serum, is via System A.

This conclusion is supported by many of the results in Tables II and III with the exception of the unexpected observation of inhibition of uptake in the serum-grown cells by leucine and BCH. Christensen and colleagues [11,12] have emphasised the errors that may arise from not recognising overlapping specificities. For example, methylaminoisobutyrate can inhibit leucine accumulation by the Ehrlich cell [11] and leucine can inhibit alanine uptake [9,13]. That aminoisobutyrate uptake should be inhibited by its methyl analogue is consistent with its uptake via System A, but inhibition by BCH (Table III) has not to the best of our knowledge been previously reported, though aminoisobutyrate can inhibit accumulation of BCH [14]. However, severe inhibition of aminoisobutyrate uptake by phenylalanine and by relatively high concentrations of leucine has been found by others [15–17].

The observations of Tables III and VII suggest either that both leucine and BCH can interact with System A or that a substantial portion of aminoisobutyrate uptake in our experiments is via System L, if it is correct to assume, which some authors question [14], that this is the route used mainly by leucine and BCH. That methylaminoisobutyrate can depress leucine accumulation [11] makes it not surprising that leucine can interfere with aminoisobutyrate accumulation, but this observation does not tell us whether the mutual competition is via System A or L. In the experiments reported by Christensen [11], methylaminoisobutyrate and BCH together had an additive effect on leucine accumulation as we have observed for aminoisobutyrate (Table VII).

It is possible to speculate that under the growth conditions of hepatoma cells, a dedifferentiation of transport systems occurs, which allows a broader range of transport specificities than usually found. Kelley and Potter [8] noted that different hepatoma cell lines show considerable variability in the extent of aminoisobutyrate uptake via the L system and with cell line H-35 80% of uptake was via this route. The sensitivity, therefore, of our cells to both methylaminoisobutyrate and BCH and their additive effects is consistent with appreciable uptake via either route, and reinforces the view of McClellan and Schafer [14] that a distinction between A and L systems is not always easily discerned.

The results with cells deprived of serum prior to measurement of aminoisobutyrate uptake are not easy to explain. That competition phenomena exist is readily seen from the results of Table I using medium I and Earle's solution versus medium II, but in Table

II it is only cysteine and methionine or mixtures containing them that show inhibitory effects. Conversely, addition of many of the other amino acids tends to enhance uptake. In contrast to cells cultured in serum where preincubation with alanine, glycine or glutamine leads to marked diminution in subsequent aminoisobutyrate uptake, preincubation of the serum-deprived cells with these amino acids has little effect. In other words, adaptive regulation of aminoisobutyrate uptake on amino acid starvation (increase) or after preloading cells with amino acids (decrease) was only observed when serum was present. It is curious that the uptake by the control serum-deprived cells is usually close to the value attained with serum-cultured cells either when preincubated with certain amino acids or when uptake is in their presence – yet this value (approx. 10–12 nmol/10^6 cells/10 min) is clearly greater than that observed either when cells are incubated for long periods at 4°C [1] or when both methylaminoisobutyrate and BCH are present together (Table VII).

Oxender et al. [4] noted with Balb/3T3 cells that removal of serum led to decreased System A activity and increased System L activity. There was also an increase in intracellular free amino acids. They did not study the effect of these changes on aminoisobutyrate uptake. However, it is clear that the uptake characteristics that we have observed in serum-deprived cells would be generally consistent with aminoisobutyrate uptake by an L type mediation (operation of System A being inhibited by intracellular amino acids) where exchange diffusion of other exogenous amino acids entering the cell more rapidly than aminoisobutyrate then leaving could facilitate uptake of the latter. Aminoisobutyrate is not normally regarded as participating actively in exchange reactions, but it appears that it can to some extent.

The inhibition by inorganic phosphate noted in Table I, if the foregoing arguments are correct, would be specifically of System A, a result that we have not seen previously reported.

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