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THE EFFECTS OF BIOREGULATORS UPON AMINO ACID TRANSPORT AND PROTEIN SYNTHESIS IN ISOLATED RAT HEPATOCYTES

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Summary

Isolated rat hepatocytes prepared by an enzyme perfusion technique possess a functional amino acid transport system and retain the capacity to synthesize protein. Amino acid transport was studied using the non-metabolizable amino acid analog α-aminoisobutyric acid. The transport process was time, temperature and concentration dependent. Similarly, leucine incorporation into protein was time and temperature dependent being optimal at 37°C. Amino acids, fetal calf serum, growth hormone and glucose all produced small, reproducible increases in protein synthesis rates. Bovine serum albumin diminished the uptake of α-aminoisobutyric acid and leucine incorporation into protein. The amino acid content on either side of the cell membrane was found to affect transport into or out of the cellular compartment (transconcentration effects). High cell concentrations decreased transport and protein synthesis as a result of isotopic dilution of labelled amino acids with those released by the hepatocytes. This was consistent with the capacity of naturally occurring amino acids to compete with α-aminoisobutyric acid for uptake into the hepatocyte. In order to define more precisely the effects of bioregulators on transport and protein synthesis it will be necessary to define and subfractionate cellular compartments and proteins which are the specific targets of cellular regulation.

Introduction

The isolated hepatocyte has become a highly studied cell [1]. Enzymatic dispersion of liver results in the production of high yields of morphologically

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intact hepatic parenchymal cells [2]. Such cell preparations retain the capacity to synthesize lipids [3,4] glycerolipids [5], phospholipids [6] and protein [7,8] through apparently normal metabolic pathways. Isolated hepatocytes respond to hormones and cyclic nucleotides with changes in the rates of gluconeogenesis [9,10] and glycogenolysis [11,12]. The synthesis and secretion of fibrinogen [13], albumin [13], and bile salts [14], characteristics of liver function are retained in isolated hepatocytes. However, cellular isolation does result in the loss of specialized membrane regions such as the bile canaliculi on the cell surface [1]. Further, isolated liver cells have been reported to be freely permeable to amino acids and lose much of their amino acid content during preparation [7]. More recently reports of functional α-aminoisobutyric acid accumulation by hepatocytes have appeared [15,16].

Despite reports of hormonal effects on amino acid transport and protein synthesis in liver tissue [17—22], only relatively small, although reproducible responses to human growth hormone [15], insulin [23] and glucagon [24] on amino acid transport in isolated hepatocytes have been observed. In view of these small hormonal responses in hepatocytes apparently functional in other regards, it seemed profitable to characterize more completely the effects of bioregulators upon amino acid transport and protein synthesis in isolated liver cells. The non-metabolizable amino acid analog α-aminosiobutyric acid was used to study transport as the properties of this compound have been extensively documented in several cell and tissue systems [16,24—27]. α-Aminoisobutyric acid is not incorporated into protein and, as a consequence, amino acid transport into liver cells was distinguishable from normally occurring subsequent metabolic events such as protein synthesis.

Experimental Procedure

Materials. Male, Sprague-Dawley rats (150—180 g), both normal and hypophysectomized, were purchased from Charles River Laboratories. Hypophysectomized animals were not used for at least 2 weeks after operation to insure complete removal of the pituitary (based on lack of growth). [1-^{14}C]Leucine and α-aminoiso[1-^{14}C]butyric acid were purchased from New England Nuclear and were used without further purification. Non-radioactive materials utilized were: α-aminoisobutyric acid, all naturally occurring amino acids, ouabain, sodium pyruvate (Sigma); trichloroacetic acid (J.T. Baker); bovine serum albumin, fraction V, crystallized, and fatty acid poor (Miles Laboratories, Inc.); crude collagenase, Type I, from *Clostridium histolyticum* (125—200 units/mg, Worthington Biochemical Co.); fetal calf serum and Hanks balanced salt solutions (Grand Island Biological Co.). The scintillation fluid used consisted of 3.79 l of toluene (Mallinckrodt), 19 g PPO (Nuclear Associates, Inc.), 1.43 g dimethyl-POPOP (Packard) and 150 ml of BBS-3 solubilizer (Beckman). All compounds used were of the highest grade commercially available.

Hepatocytes were prepared by a modification of the method of Berry and Friend [2]. Using this procedure, $2 \cdot 10^6$—$20 \cdot 10^6$ cells were isolated per liver. Cellular viability, as assayed by the ability of the cells to exclude the vital dye trypan blue, ranged from 80 to 95% when initially isolated. Viability generally did not diminish by more than 10—20% over the course of experiments. Most

of the cells isolated by this technique appeared microscopically to be free intact hepatic parenchymal cells.

α-Aminoisobutyric acid uptake and leucine incorporation into protein. Uptake of α-aminoisobutyric acid, by isolated hepatocytes, was measured by a modification of the method of Gardner and Levy [28]. Cells ($2 \cdot 10^6$—$20 \cdot 10^6$ per ml) were incubated in Hanks balanced salt solution containing calcium (1.0 mM) and magnesium (0.8 mM) and 1% fatty acid-free albumin at 37°C for 10 min. Aliquots of cell suspension were pipetted into incubation flasks containing varying amounts of medium, α-aminoisobutyric acid and α-aminoiso-[1-^{14}C]butyric acid, all equilibrated to 37°C. The flasks were shaken during the course of the experiment in order to insure interaction between the cells and the incubation mixture. The moment of cell addition was considered to be time zero.

At appropriate intervals after the initiation of influx, 100-μl triplicate aliquots of cell suspension were removed from each incubation vessel with an Eppendorf pipette. The suspension was layered over 250 μl of a 5% sucrose solution in a Beckman microfuge tube and centrifuged at 10 000 × *g* in a Beckman 152 microfuge. The supernatant fluid was aspirated from above the resulting cell pellet which was subsequently washed twice with 250 μl of ice-cold sucrose. After each wash, the tube was recentrifuged to insure that the cell pellet remained intact. Trichloroacetic acid (10%, 100 μl) was added to the washed cell pellet which was then disrupted with a fine wire rod in order to disperse the cells and release accumulated α-aminoisobutyric acid. The top half of the microfuge tube, which was free of solution, was removed with a pair of scissors and the remainder of the tube was inverted and placed in a glass scintillation vial. The scintillation vial was vigorously agitated in order to release all of the solution and disrupted cellular material within the microfuge tube into the counting vial. Scintillation fluid (10 ml) was then added to the vial.

Assay of the radioactivity was performed in a Beckman liquid scintillation spectrometer, Model 230. Internal standards demonstrated that quenching was negligible using the assay procedure described above. No variation in quenching was observed from one sample to another when evaluated by an automatic external standard. Aliquots of the incubation medium, to which 100 μl of a 10% trichloroacetic acid solution were added, were also assayed. The molar concentration of α-aminoisobutyric acid in the incubation medium was then calculated. The number of mol of labelled α-aminoisobutyric acid incorporated into cell suspensions was calculated and this value was then normalized to the number of mol of α-aminoiso[1-^{14}C]butyric acid accumulated per cell. The net increase or decrease in α-aminoisobutyric acid is reported as the number of mol of α-aminoisobutyric acid incorporated per cell per unit time. Control experiments, in which the cell pellets were extensively washed with 10% trichloroacetic acid demonstrated that none of the α-aminoisobutyric acid accumulated by hepatocytes was incorporated into protein.

In assaying for leucine incorporation into protein, after the initial centrifugation described above, trichloroacetic acid (10%, 250 μl) was added to the cell pellet which was disrupted with a fine wire rod in order to break the cells. The microfuge tube was centrifuged for 1 min and the supernatant was aspirated

from above the trichloroacetic acid precipitate. The washing procedure was repeated one additional time to insure complete removal of all free amino acids. In control experiments in which the non-metabolizable amino acid analog α-aminoiso[1-^{14}C]butyric acid was used in place of [1-^{14}C]leucine, radioactivity in the supernatant was reduced to background (no α-aminoisobutyric acid being incorporated into trichloroacetic acid-precipitable material) by this second wash. Water (100 μl) was placed above the precipitate which was disrupted with a fine wire rod. The procedure was then as described above for α-aminoisobutyric acid uptake.

Efflux experiments. Hepatocytes were loaded with α-aminoisobutyric acid by incubation with this amino acid for 2 h at 37°C in Hanks balanced salt solution containing 1% albumin. The cells were isolated by low speed centrifugation (400 rev./min, 50 × *g*, 2 min) and washed twice with ice-cold buffer. Finally, the cells were redistributed in buffer which was pre-equilibrated to the appropriate temperature. Triplicate 100-μl aliquots were withdrawn from the incubation flasks at stated time intervals for microfuge assay, as already described. The α-aminoiso[1-^{14}C]butyric acid activity of the dispersed cell pellets was expressed as a percent of the amount measured at the moment at which incubation was initiated, which was considered to be time zero.

Balanced amino acid mixture. In those experiments to which an amino acid mixture was added, as described in the text, a balanced amino acid solution was prepared as described by Minemura et al. [29]. This amino acid mixture was proportioned to approximate the amino acid content of rat plasma [29].

Results

The accumulation of α-aminoisobutyric acid by isolated hepatocytes was time, temperature and concentration dependent. Initially, there was rapid uptake of α-aminoisobutyric acid by the liver cells. Subsequently, the rate at which α-aminoisobutyric acid was accumulated diminished as steady-state conditions were approached. Addition of sodium pyruvate, an energy source, to the incubation solution did not alter the initial rate of uptake or the final concentration of α-aminoisobutyric acid accumulated (not shown).

Naturally occurring amino acids were found to compete with varying efficacies, with α-aminoisobutyric acid, for transport into liver cells (Table I). Alanine and methionine were most efficient in their capacity to compete with α-aminoisobutyric acid for uptake suggesting that these amino acids shared a common carrier-mediated transport system. The relative capacity of added amino acids to inhibit α-aminoisobutyric acid accumulation did not vary with time as similar results were obtained at both 30 and 60 min of incubation.

Ouabain inhibited the accumulation of α-aminoisobutyric acid in isolated hepatocytes. After 90 min of incubation α-aminoisobutyric acid (2.1 mM) accumulation was 71.7 and 40.5% of control in the presence of 10^{-4} and 10^{-3} M ouabain, respectively. The uptake of α-aminoisobutyric acid into hepatocytes was dependent upon the concentration of Na^+ in the medium. Uptake increased as a function of Na^+ concentration up to about 80 mM Na^+, above which concentration it remained essentially constant. A significant proportion of the α-aminoisobutyric acid transport did not, however, require the presence of sodium.

TABLE I

THE EFFECT OF ADDED AMINO ACIDS ON α-AMINOISOBUTYRIC ACID ACCUMULATION

Isolated hepatocytes were equilibrated for 10 min at 37°C in Hanks balanced salt solution containing 1% albumin. Aliquots of cell suspension were added to pre-equilibrated incubation flasks containing α-amino-iso-[1-^{14}C]butyric acid, α-aminosiobutyric acid, 20 mM of the unlabelled amino acid and buffer. The final α-aminoisobutyric acid concentration present was 2.1 mM. The flasks were shaken and aliquots were taken from each flask after 30 or 60 min of incubation for assay of α-aminoisobutyric acid accumulation. Each point represents the mean of triplicate determinations.

Amino acid present	Percent accumulation relative to control with α-aminoisobutyric acid alone	
	30 min incubation	60 min incubation
Leu	86	81
Phe	80	89
Val	82	84
Gly	59	59
Pro	53	46
Thr	46	52
Ser	39	42
Ala	29	33
Met	26	29

α-Aminoisobutyric acid uptake varied with the extracellular concentration of α-aminoisobutyric present in the medium. The uptake of α-aminoisobutyric acid increased hyperbolically up to a concentration of 5 mM in the medium. At concentrations greater than 5 mM, accumulation increased in an apparently linear fashion with respect to the applied α-aminoisobutyric acid concentration.

The effect of time and the extracellular α-aminoisobutyric acid concentration on α-aminoisobutyric acid influx are shown in Fig. 1. At each concentration of α-aminoisobutyric acid studied, linear uptake was observed over the time period investigated. The deviation of the slope of the observed lines from a zero intercept on the *y*-axis increased with the applied α-aminoisobutyric acid concentration in the external medium. The lack of a zero intercept upon the *y*-axis suggested the rapid establishment of either significant α-aminoisobutyric acid efflux or a transconcentration effect (the capacity of intracellular amino acid to affect the influx rate of extracellular amino acids).

Hepatocytes were preloaded with increasing concentrations of α-aminoisobutyric acid for 2 h prior to initiating influx with α-aminoiso[1-^{14}C]butyric acid. There was a progressively greater decrease in the initial rate of uptake as the preincubation concentration of α-aminoisobutyric acid was increased (Fig. 2). At a preloaded α-aminoisobutyric acid concentration of 10 mM, after 20 min, uptake was reduced by 40% relative to cells not previously preloaded.

The efflux of α-aminoisobutyric acid from hepatocytes was temperature dependent (Fig. 3). At 4°C, the α-aminoisobutyric acid content of the cells decreased to 55% after 75 min. In contrast at 37°C, 85% of the α-aminoisobutyric acid content of these cells was lost within the same period of time. The addition of unlabelled α-aminoisobutyric acid to the efflux medium resulted in an increased rate of α-aminoiso[1-^{14}C]butyric acid loss from preloaded cells. The effect of extracellular α-aminoisobutyric acid was observed only after 40 min.

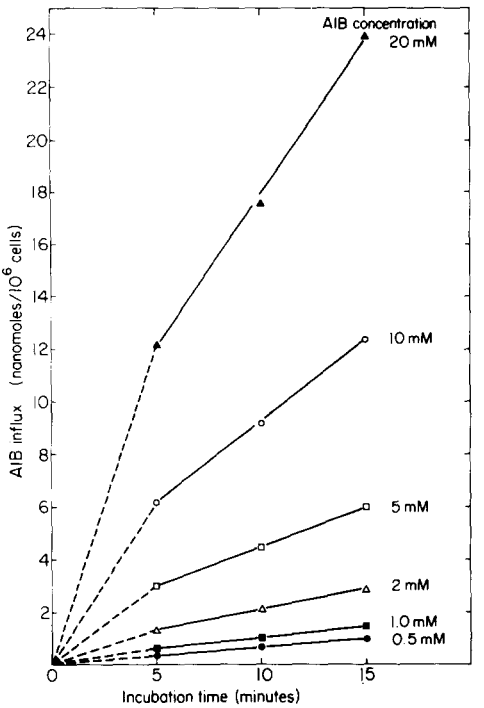

Fig. 1. Uptake as a function of extracellular α-aminoisobutyric acid (AIB) concentration and time. Cells were equilibrated in Hanks balanced salt solution that was 1% in albumin for 10 min at 37°C. Aliquots of cell suspension were added to pre-equilibrated incubation vessels containing α-aminoiso[1-^{14}C]butyric acid and varying concentrations of α-aminoisobutyric acid. The flasks were shaken and aliquots were removed after 5, 10 and 15 min for assay of α-aminoisobutyric acid uptake. Each experimentally determined point represents the average of three determinations.

The observation of a monocomponent course of efflux suggested that α-aminoisobutyric acid released from the hepatocytes did not accumulate in sufficient concentration to affect the efflux rate.

Leucine incorporation into protein was both time and temperature dependent. The concentration of leucine incorporated per hepatocyte increased with temperature. After 4 h of incubation at 23°C, for example, approx. 3 nmol of leucine were incorporated per 10^7 cells; at 37°C, approx. 12 nmol of leucine were incorporated per 10^7 cells. Leucine uptake increased rapidly during the first 2 h of study. The rate of uptake diminished thereafter.

The addition of an amino acid mixture which simulated the composition of that found in rat plasma enhanced leucine incorporation into protein (Table II). Prior to 90 min added amino acids did not produce a significant effect on leucine incorporation. Subsequent to 150 min the presence of amino acids resulted in reproducible increases in leucine uptake. For example, after 300 min, added amino acids produced a 21% increase in leucine incorporation relative to controls.

Fetal calf serum increased leucine incorporation into protein (Table III). An 11% increase, relative to controls, was observed within 10 min of the initiation

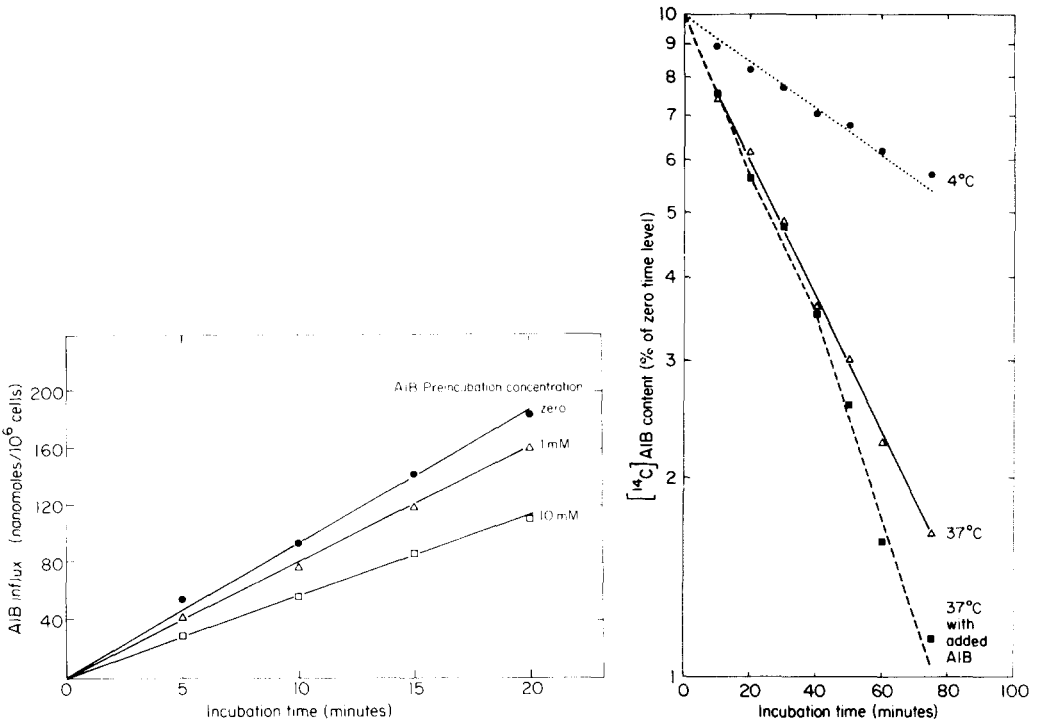

Fig. 2. The effect of preliminary incubation with α-aminoisobutyric acid on uptake. Hepatocytes were pre-equilibrated in Hanks balanced salt solution which was 1% in albumin, for 10 min at 37°C. Aliquots of cell suspension were added to incubation flasks containing buffer alone or buffer and α-aminoisobutyric acid of varied concentration. These were shaken at 37°C for 2 h. At the end of this time the contents of each vessel were added to 10 ml of ice-cold Hanks balanced salt solution containing 1% albumin and centrifuged (50 × *g*, 2 min). The supernatant was aspirated and the resulting cell pellet was resuspended in 3.9 ml of buffer containing α-aminoiso[1-^{14}C]butyric acid (0.2 mM) at 37°C. These incubation mixtures were shaken and aliquots were taken after timed intervals. Each point is the mean of triplicate determinations.

Fig. 3. Time course of α-aminoiso[1-^{14}C]butyric acid (AIB) efflux. Hepatocytes were equilibrated for 10 min at 37°C in Hanks balanced salt solution containing 1% albumin. Aliquots of this suspension were added to incubation flasks containing α-aminoiso[1-^{14}C]butyric acid, α-aminoisobutyric acid and Hank's balanced salt solution/1% albumin. The final α-aminoisobutyric acid concentration achieved was 2.1 mM. These were shaken for 1 h to load the cells with α-aminoisobutyric acid. The suspensions were centrifuged (4°C, 2 min, 50 × *g*) and the resulting cell pellet was washed once with ice-cold buffer. The cell pellets were finally resuspended in 5 ml of buffer equilibrated to the appropriate temperature. In one instance, sufficient α-aminoisobutyric acid was added to the external medium to produce a final α-aminoisobutyric acid concentration of 1.65 mM. Samples were withdrawn immediately after resuspension and at stated time intervals. The α-aminoisobutyric acid content of the cells was assayed as described in Experimental Procedure and expressed as a percent of the zero time value. Each point represents the mean triplicate determinations.

of incubation; a maximal effect of 20% was obtained after 20 min. Serum-mediated response was sustained for at least 90 min. Rat and newborn calf serum (not shown) produced similar increases in leucine incorporation.

Glucose increased the incorporation of leucine into protein (Table IV). Effects were obtained within 30 min and were maintained for at least 3 h. In the absence of albumin, glucose did not affect leucine incorporation. Crystallized, fraction V and fatty acid-poor albumin were all equally efficacious

TABLE II

THE EFFECT OF ADDED AMINO ACIDS ON PROTEIN SYNTHESIS IN ISOLATED RAT HEPATOCYTES

Hepatocytes were equilibrated in Hanks balanced salt solution/1% fatty acid-poor albumin solution for 10 min at 37°C. Aliquots of cell suspension were added to temperature-equilibrated incubation flasks containing [1-^{14}C]leucine, leucine (0.23 mM) and either amino acid solution (40 μl) or medium. The final incubation volume was 4090 μl. The assay was then as described in the section entitled Experimental Procedure. Each point represents the mean of triplicate determinations.

Time	Leucine incorporated (nmol/10^7 cells)		P
	Without added amino acids	With added amino acids	
15	2.80 ± 0.18	3.03 ± 0.11	n.s.
30	6.00 ± 0.19	6.33 ± 0.33	n.s.
45	9.58 ± 0.14	9.90 ± 0.26	n.s.
60	12.78 ± 0.33	12.93 ± 0.31	n.s.
90	17.45 ± 0.39	18.65 ± 0.48	<0.05
120	21.94 ± 1.07	23.13 ± 0.56	n.s.
150	24.96 ± 0.76	26.23 ± 0.33	<0.05
180	27.96 ± 0.54	29.23 ± 0.31	<0.02
240	34.61 ± 0.52	38.73 ± 1.11	<0.01
300	45.33 ± 3.34	54.68 ± 0.40	<0.01

n.s., not significant.

in mediating the effect of glucose. The largest effects of glucose were obtained after longer incubation times; a 29% increase in incorporation, relative to controls, was observed after 150 and 180 min. α-Aminoisobutyric acid accumulation was not affected by glucose suggesting that the effect of glucose was not upon the amino acid transport system.

TABLE III

THE EFFECT OF SERUM ON PROTEIN SYNTHESIS IN ISOLATED RAT HEPATOCYTES

Hepatocytes from a hypophysectomized rat were equilibrated in Hanks balanced salt solution/1% fatty acid-poor albumin solution for 10 min at 37°C. Aliquots of cell suspension were added to temperature-equilibrated incubation flasks which contained [1-^{14}C]leucine (0.23 mM), standard amino acid solution (40 μl), somatotropin (10^{-7} M) and either fetal calf serum (50 μl) or medium. The final incubation volume was 4090 μl. The assay procedure was then as described in the section entitled Experimental Procedure. Each point represents the mean of triplicate determinations.

Time (min)	Leucine incorporated (nmol/10^7 cells)		P
	Without added serum	With added serum	
10	1.41 ± 0.10	1.56 ± 0.04	<0.05
20	3.38 ± 0.14	4.05 ± 0.17	<0.01
30	5.43 ± 0.19	6.23 ± 0.20	<0.01
35	6.01 ± 0.45	7.18 ± 0.26	<0.02
40	7.27 ± 0.39	8.13 ± 0.31	<0.05
50	8.56 ± 0.42	9.80 ± 0.15	<0.01
60	10.25 ± 0.30	11.33 ± 0.33	<0.01
75	12.05 ± 0.54	13.28 ± 0.56	<0.05
90	13.39 ± 0.20	14.69 ± 0.61	<0.02
120	16.01 ± 0.60	16.84 ± 0.61	n.s.

n.s., not significant.

TABLE IV

THE EFFECT OF GLUCOSE ON LEUCINE INCORPORATION INTO ISOLATED HEPATOCYTES

Hanks balanced salt solution was prepared with and without the presence of glucose. Hepatocytes were equilibrated in each medium which contained 1% fatty acid-poor albumin for 10 min at 37°C. Aliquots of cell suspension were added to temperature-equilibrated incubation flasks which contained [1-^{14}C]-leucine, and unlabelled leucine (0.23 mM). The assay procedure was then as described in the section entitled Experimental Procedure (Fig. 2). Each point represents the mean of triplicate determinations.

Time (min)	Leucine incorporated (nmol/10^7 cells)		P
	Without added glucose	With added glucose	
15	0.41 ± 0.12	0.45 ± 0.01	n.s.
30	0.70 ± 0.03	0.79 ± 0.03	<0.05
45	1.09 ± 0.03	1.31 ± 0.03	<0.001
60	1.54 ± 0.05	1.89 ± 0.07	<0.01
90	2.43 ± 0.09	3.08 ± 0.001	<0.001
120	3.28 ± 0.04	4.00 ± 0.05	<0.05
150	3.95 ± 0.14	5.10 ± 0.30	<0.01
180	4.98 ± 0.05	6.42 ± 0.01	<0.001

n.s., not significant.

Human somatotropin produced no effect on amino acid transport in hepatocytes obtained from hypophysectomized rats when buffer or buffer supplemented either with fetal calf serum or an amino acid mixture was used in the incubation (data not shown). However, a small but reproducible cellular response to somatotropin was elicited when both fetal calf serum and the amino acid mixture were added to the incubation medium (Table V). A similar response was not observed in studies conducted using hepatocytes obtained from non-hypophysectomized rats. Effects of somatotropin were observed within 10 min of the initiation of incubation. A maximal increase of α-aminoisobutyric acid uptake of 14% was observed after 50 min of incubation. Thereafter the differential between cells incubated in the presence and absence of somatotropin remained essentially constant.

Somatotropin elicited small, reproducible alterations in protein synthesis (Table VI). Cells obtained from both hypophysectomized and normal rats (not shown) were affected similarly. Increased incorporation occurred within 5 min of the initiation of incubation; effects were observable for at least 5 h thereafter. A dose vs. response relationship was not obtained in these and similar experiments in which the somatotropin concentration was varied from 10^{-14} to 10^{-6} M. A maximal effect of somatotropin (21%) was achieved at a hormone concentration of 10^{-7} M after 30 min. At 10^{-9} M somatotropin, responses were, for the most part, not significant as evaluated by a standard t-test.

Bovine serum albumin diminished α-aminoisobutyric acid transport and leucine incorporation into protein in hepatocytes (Fig. 4). The effect of albumin was time dependent being obtained within 10—15 min of the initiation of incubation; the largest responses were observable after longer periods of incubation. After 3 h, α-aminoisobutyric acid uptake relative to control was 87% in the presence of fatty acid-poor albumin; similarly leucine incorporation

TABLE V

EFFECT OF SOMATOTROPIN ON α-AMINOISOBUTYRIC ACID ACCUMULATION

Cells were equilibrated for 10 min in Hanks balanced salt solution containing 1% albumin at 37°C. Aliquots of cells were added to temperature-equilibrated reaction vessels containing α-aminoiso[1-^{14}C]-butyric acid (0.20 mM final concentration), fetal calf serum (final concentration, 1%), a sufficient concentration of amino acid mixture to simulate the concentration in rat plasma [22] and somatotropin. This mixture was shaken and aliquots were taken for assay at appropriate timed intervals. Each point represents the mean of triplicate determinations.

Time (min)	nmol α-aminoisobutyric acid accumulated/10^6 cells		Uptake with somatotropin X 100	P
	Control	Somatotropin added	Uptake without somatotropin	
10	44.0 ± 1.8	48.3 ± 3.5	109.9	<0.02
15	64.6 ± 2.1	70.1 ± 1.0	108.5	<0.02
20	87.7 ± 4.7	89.7 ± 5.9	102.3	n.s.
25	104.4 ± 3.0	114.9 ± 5.4	110.0	<0.01
30	117.9 ± 4.7	133.8 ± 3.9	113.5	<0.01
35	141.7 ± 3.4	159.5 ± 2.6	112.6	<0.001
40	161.3 ± 3.3	171.6 ± 2.0	106.8	<0.01
45	177.7 ± 5.3	195.0 ± 3.1	109.7	<0.01
50	186.7 ± 9.8	213.0 ± 6.9	114.0	<0.02
60	246.5 ± 16.7	276.5 ± 4.6	112.2	<0.05
90	281.2 ± 11.4	308.7 ± 12.0	109.8	<0.02
120	326.3 ± 4.7	369.5 ± 11.3	113.2	<0.001

n.s., not significant.

was 85% of control. Responses to crystallized and fraction V albumin were similar. After 3 h, α-aminoisobutyric acid uptake was 60% of control in the presence of these forms of albumin. Leucine incorporation was 66 and 56% control in the presence of fraction V and crystallized albumin, respectively.

TABLE V

EFFECT OF SOMATOTROPIN ON LEUCINE INCORPORATION IN ISOLATED HEPATOCYTES

Hepatocytes from a hypophysectomized rat were equilibrated in Hanks balanced salt solution/1% fatty acid-poor albumin solution for 10 min at 37°C. Aliquots of cell suspension were added to temperature-equilibrated incubation flasks which contained [1-^{14}C]leucine, leucine (0.23 mM), standard amino acid solution (40 μl), fetal calf serum (40 μl) and somatotropin solution (10^{-9}, 10^{-8}, 10^{-7} M)or medium. The final incubation volume was 4080 μl. The assay procedure was then as described in the section entitled Experimental Procedure. Each point represents the mean of triplicate determinations. The *P* values reported were obtained by comparison of each experiment conducted with somatotropin with the control study in which no hormone was present.

Time (min)	Leucine incorporation (nmol/10^7 cells)						
	No somatotropin	10^{-9} M somatotropin	*P*	10^{-8} M somatotropin	*P*	10^{-7} M somatotropin	*P*
30	7.51 ± 0.70	8.24 ± 0.13	n.s.	8.63 ± 0.22	<0.05	9.07 ± 0.08	<0.02
60	18.40 ± 0.17	18.95 ± 0.10	<0.01	20.07 ± 0.07	<0.001	19.98 ± 0.84	<0.05
90	26.84 ± 0.46	27.26 ± 0.43	n.s.	28.69 ± 0.18	<0.01	28.40 ± 0.37	<0.01
120	32.26 ± 0.41	33.13 ± 0.42	n.s.	34.48 ± 0.18	<0.01	33.81 ± 0.14	<0.01
150	35.82 ± 0.37	38.20 ± 1.66	n.s.	39.59 ± 1.22	<0.01	38.05 ± 1.94	n.s.
180	39.02 ± 0.21	39.63 ± 0.25	<0.02	41.63 ± 0.49	<0.01	40.98 ± 0.33	<0.01

n.s., not significant.

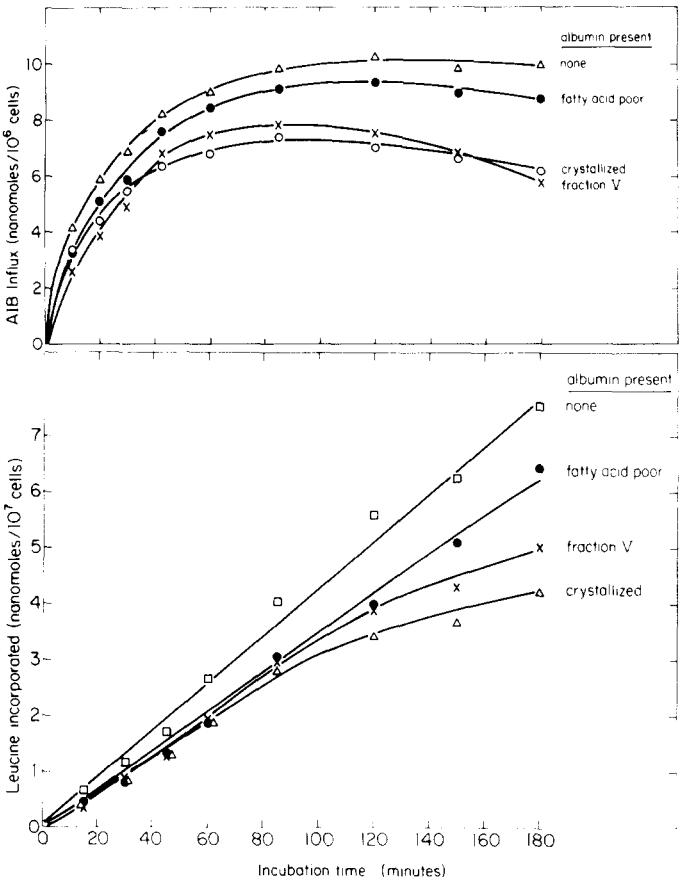

Fig. 4. The effects of albumin on α-aminoisobutyric acid (AIB) uptake and leucine incorporation into protein. 1% Hanks balanced salt solution/albumin solutions were prepared using crystallized, fraction V and fatty acid-poor albumin. Hepatocytes were equilibrated in each medium for 10 min at 37°C. Aliquots of cell suspension were then added to incubation flasks containing α-aminoiso[1-^{14}C]butyric acid (0.2 mM) or [1-^{14}C]leucine and leucine (0.23 mM). The assay procedure is as described in the section entitled Epxerimental Procedure. Each point represents the mean of triplicate determinations.

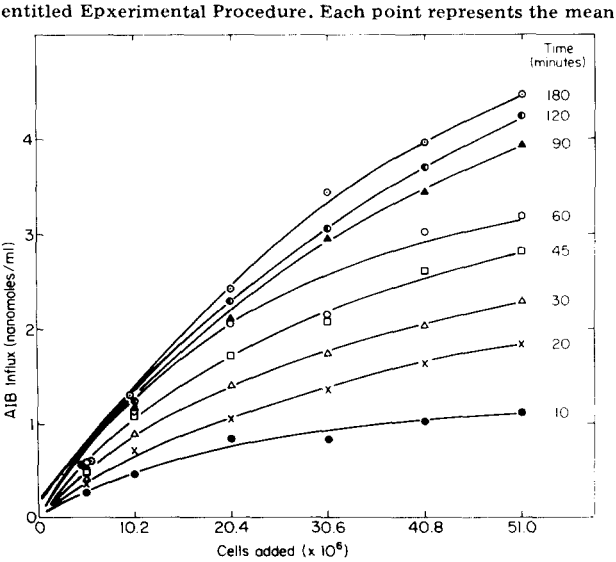

Fig. 5. The effect of cell concentration on amino acid (AIB) uptake. Hepatocytes were equilibrated in Hanks balanced salt solution/1% fatty acid-poor albumin for 30 min at 37°C. Increasing volumes of cell suspension were then added to the incubation flasks containing α-aminoiso[1-^{14}C]butyric acid (0.2 mM). The assay was then as described in the section entitled Experimental Procedure. Each point represents the mean of triplicate determinations.

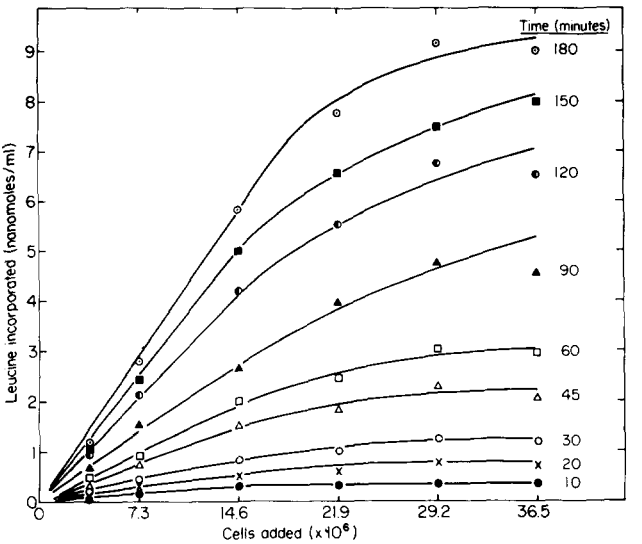

Fig. 6. The effect of cell concentration on leucine incorporation. Hepatocytes were equilibrated in Hanks balanced salt solution/1% fatty acid-poor albumin for 30 min at 37°C. Increasing volumes of cell suspension were then added to incubation flasks containing [1-^{14}C]leucine, and leucine (total concentration: 0.2 mM). The assay was then as described in the section entitled Experimental Procedure. Each point represents the mean of triplicate determinations.

The uptake of α-aminoisobutyric acid and leucine incorporation into protein in hepatocytes did not increase linearly with cell concentration (Figs. 5 and 6). At cell concentrations up to $10 \cdot 10^6$—$15 \cdot 10^6$ cells per ml an apparently linear uptake was observed. At higher cell concentrations, a progressively greater deviation from linearity was observed. The effects of high cell concentration were obtainable within 10 min of the initiation of incubation and maintained for at least 180 min.

Discussion

Preparations of isolated rat hepatocytes accumulate the non-metabolizable amino acid analog, α-aminoisobutyric acid. The properties of α-aminoisobutyric acid transport in liver cells were strikingly similar to those previously described for the isolated fat cell and fat cell ghost [30,31]. Uptake was time, temperature and concentration dependent. Part of the transport mechanism was dependent upon sodium although a significant fraction did not seem to require this ion. This suggested that amino acid transport was linked to the active sodium-potassium transport process. Ouabain was, therefore, effective in inhibiting α-aminoisobutyric acid accumulation presumably by inhibition of the ($Na^+ + K^+$)-ATPase system. Neither sodium pyruvate nor glucose, when added to media free of these energy sources, produced any effect upon the rate or final concentration of α-aminoisobutyric acid accumulated. As isolated, these cells possessed sufficient energy reserves to support a functional amino acid transport system for some period of time.

Transconcentration effects were observed in studies of α-aminoisobutyric

acid uptake and efflux. Variations from simple accumulation were observable within 5 min at even very low concentrations of α-aminoisobutyric acid (0.5 mM). For this reason no attempt has been made to fit the observed data to any particular model in a quantitative manner. The observation of such subtle effects gave added assurance, however, that the cellular isolation process did not produce significant damage to the plasma membrane transport system.

Naturally occurring amino acids competed with α-aminoisobutyric acid for uptake suggesting the presence of a carrier-mediated transport mechanism. The α-aminoisobutyric acid transport system appeared to be used preferentially by amino acids belonging to the A series described by Oxender and Christensen [32]. Amino acids accumulated by hepatocytes were rapidly incorporated into protein. Digestion of the trichloroacetic acid-precipitable fraction obtained from [1-^{14}C]leucine-hepatocyte incubates with pronase resulted in the recovery of free leucine and other radioactive ninhydrin-positive material (not shown). Pronase specifically cleaves peptide units with high efficiency [33]. This would imply that the leucine present in the trichloroacetic acid precipitates was incorporated into protein through peptide bonds.

Increased intracellular levels of free amino acids results in enhanced protein synthesis in mammalian cells [34—36]. The addition of an amino acid mixture to hepatocytes resulted in increased incorporation of leucine into protein (Table II). A lag time of at least 1 h was observed prior to the observation of a significant effect. The addition of other amino acids decreased α-aminoisobutyric acid accumulation by competition for carrier-mediated transport systems. The interval required for an added amino acid mixture to produce an effect upon protein synthesis may have reflected the time necessary for a sufficient concentration of amino acids to be transported into the cell. Hepatocyte suspensions possessed sufficient pools of intracellular amino acids to permit completely active protein synthesis for a significant period of time without the presence of more substrate.

Under normal circumstances the intracellular pool of free amino acids and the energy reserves available to the cell will regulate protein synthesis. Glucose, for example, supported amino acid incorporation into protein in Ehrlich ascites tumor cells [37]. Similarly, the addition of glucose to hepatocyte incubates free of this energy source resulted in increased incorporation of [1-^{14}C]leucine into protein (Table IV). Glucose had no effect on α-aminoisobutyric acid transport into hepatocytes thereby differentiating amino acid transport and protein synthesis by their response to amino acids and glucose.

Serum increased the incorporation of [1-^{14}C]leucine into protein (Table III). The factors present in serum which affect cellular metabolism may be either macromolecular [36,39] or of low molecular weight [40]. Among the low molecular weight substrates are glucose and free amino acids. Polypeptide hormones are among the macromolecular bioregulators present within serum. Somatotropin (Table V) and insulin (not shown) each produced small increases in α-aminoisobutyric acid uptake and protein synthesis in hepatocytes. The effects of somatotropin were observable in cells obtained from both normal and hypophysectomized rats.

Bovine serum albumin decreased the concentration of leucine incorporated into protein in isolated hepatocytes (Fig. 4). Crystallized albumin was more

effective than fraction V albumin which in turn was more effective than fatty acid-poor albumin in this inhibition. Albumin produced a similar effect upon α-aminoisobutyric acid transport into the hepatocyte (Fig. 4). It may be that the effects of albumin were related to the content of free fatty acid bound to this macromolecular carrier. Rodbell [41] found that isolated fat cells released protein to the medium after albumin became saturated with fatty acids. The accumulation of intracellular fatty acids and protein release were inhibited by providing either excess albumin or glucose to the medium which accelerated the re-esterification of intracellular fatty acids [41]. These studies suggest that similar mechanisms might be operative in the hepatocyte cell system as the effect of glucose was solely on protein synthesis and not amino acid transport.

The uptake of α-aminoisobutyric acid (Fig. 5) and incorporation of leucine into protein (Fig. 6) did not increase linearly with the cell concentration. Incorporation of labelled amino acids into protein decreased with cell concentration in spermatozoa [42], ascites tumor cells [43] and liver cells isolated by a number of different methods [44—46]. Seglen [47] found that hepatocytes may release amino acids to the medium in sufficient concentration to isotopically dilute extracellular labelled amino acid pools. The diminished uptake of α-aminoisobutyric acid observed at elevated cell concentrations becomes rational when viewed as potentially resulting from release of intracellular amino acids from the cell. The transconcentration effects of α-aminoisobutyric acid pools, both intracellular and extracellular, represent a direct confirmation that amino acid transport from either side of the cell membrane is directly affected by the pool of substrate into which it is to be transported. The efficacy of a number of naturally occurring amino acids in competing with α-aminoisobutyric acid for uptake into the hepatocyte suggests that the relationship between intra- and extracellular pools of substrate may largely determine cellular response to changes in amino acid concentrations. Although in the present study specific intracellular amino acid pools were not subcompartmentalized, ultimately this will need to be done. The small observed effects of bioregulators upon α-aminoisobutyric acid transport and protein synthesis represent observations conducted upon the total intracellular compartment which was not subfractionated. It may well be that bioregulators may exert much larger effects upon specific protein or amino acid pools. This study has, therefore, pointed to the need to define specific cellular compartments for more elaborate study when investigating the effects of hormones or other bioregulators.

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