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STIMULATION OF ALANINE TRANSPORT AND METABOLISM BY DIBUTYRYL CYCLIC AMP IN THE HEPATOCYTES FROM FED RATS

ASSESSMENT OF TRANSPORT AS A POTENTIAL RATE-LIMITING STEP FOR ALANINE METABOLISM

JOHN D. McGIVAN *, JANE C. RAMSELL and JEAN H. LACEY

Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, (U.K.)

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(1) Cyclic AMP stimulated alanine transport in isolated hepatocytes by approx. 30%, in the range 0.2–5 mM alanine. (2) Alanine utilisation was also stimulated by cyclic AMP. The rates of transport and metabolism were comparable, both in the presence and absence of cyclic AMP. (3) At concentrations of alanine above 1 mM, addition of ouabain, or the reduction of the Na^+ concentration, could partially inhibit transport without affecting the rate of metabolism. (4) At these alanine concentrations, stimulation of metabolism by cyclic AMP was associated with a decrease in the intracellular to extracellular alanine concentration ratio. (5) At alanine concentrations below 0.5 mM, or at higher concentrations when transport was inhibited by reducing the Na^+ concentration, cyclic AMP caused an increase in the alanine concentration ratio. (6) It is concluded that at concentrations of alanine above 1 mM, alanine transport is not rate-limiting for alanine metabolism in hepatocytes from fed rats, and cyclic AMP stimulates alanine metabolism primarily by an effect on an intracellular reaction. At physiological concentrations of alanine, however, alanine transport appears to be rate-limiting in agreement with a previous report.

Introduction

One of the major functions of the liver is the conversion of alanine, an end-product of nitrogen metabolism in muscle, to glucose and urea. Gluconeogenesis from alanine is stimulated by glucagon in perfused liver [1,2] and in isolated hepatocytes [3,4]. The action of glucagon on gluconeogenesis is considered to be mediated via an increase in the intracellular concentration of cyclic AMP, but the actual step which is stimulated by cyclic AMP has not been identified.

The first step in the metabolism of alanine involves its transport across the plasma membrane. In isolated hepatocytes, alanine transport is mediated mainly by a Na^+-dependent carrier system having properties similar to the 'A' system in Ehrlich ascites cells [5,6]. The kinetic properties of alanine transport in hepatocytes from both fed and starved rats have been established [7,8].

Studies of the effect of hormones on amino acid transport have been performed mainly using the non-metabolised analogue, aminoisobutyrate, as substrate. Aminoisobutyrate transport has been shown to be stimulated by glucagon and cyclic AMP in liver slices [9], isolated perfused liver [10], cultured liver cells [11] and liver cells in suspension [12]. In hepatocytes, aminoisobutyrate is not a specific substrate for the 'A' system [13] and direct measurements of the effects of hormones on alanine transport are required. Edmonson and Lumeng [14] have recently reported that alanine transport in hepatocytes is stimulated by glucagon.

* To whom correspondence should be addressed.

In a recent paper, Sips et al. [15] have measured incracellular and extracellular alanine concentrations in suspensions of hepatocytes perifused with alanine, and have concluded that alanine transport limits the rate of alanine catabolism at alanine concentrations in the physiological range. In the present paper measurements of intracellular and extracellular alanine concentrations in the presence and absence of cyclic AMP are used to assess under what conditions the increase in the rate of alanine metabolism caused by cyclic AMP can be attributed to a primary effect on alanine transport. A preliminary report of some of this work has been published [16].

Methods

Hepatocytes were isolated from the livers of normally fed male Wistar rats by the method of Berry and Friend [17], as modified by Krebs et al. [18]. The cells were washed and incubated in Krebs-Henseleit bicarbonate-buffered saline, pH 7.4 [19], containing 2% (w/v) bovine albumin (fraction V). The albumin had been previously dialysed but not defatted. In incubations where cyclic AMP was used, the cells were preincubated with 0.1 mM dibutyryl cyclic AMP for 10 min before the initiation of the reaction to be studied. For the measurement of alanine metabolism, the cell suspension was incubated under a gas phase of 95% O_2 + 5% CO_2. Alanine was added to the incubations and the reaction terminated after 15 min by the addition of $HClO_4$ (3.5% (w/v) final concentration). After centrifugation at 10 000 × *g* for 1 min, the supernatant was neutralised with 3 M KOH and assayed for alanine.

For the measurement of alanine transport in the absence of metabolism, cells were preincubated with 1 mM amino-oxyacetate, which completely inhibits the metabolism of alanine [7,6], and were then incubated with ^{14}C-labelled alanine, together with [^3H]inulin as a marker of the extracellular space. Transport was terminated by centrifugation through silicone oil into $HClO_4$, and uptake of alanine was taken as the amount of [^{14}C]alanine appearing in the inulin-impermeable space. The amount of alanine transported was linear with time over the first 2 min, and full details of this method have been published previously [7,8].

To measure the alanine distribution ratios during alanine metabolism, cells (12–14 mg protein/ml) were incubated with alanine, 1 μC/ml ^3H_2O and 0.1 μC/ml [*carboxy*-^{14}C]inulin. After the appropriate time interval, 0.8 ml aliquots of the suspension were spun through silicone oil into $HClO_4$, as previously described [4], using an Eppendorf centrifuge (model 3200). A sample of the supernatant was immediately acidified with 3.5% (w/v) $HClO_4$. Alanine was assayed in both pellet and supernatant fractions after neutralisation, and ^{14}C and ^3H were estimated in both fractions, as described previously [7]. The amount of alanine measured in the pellet was corrected for the alanine carried down in the extracellular water, as determined by the inulin space.

Alanine was estimated by the method of Williamson et al. [20], using alanine dehydrogenase. Reagent blanks consisted of incubation medium which had been acidified and neutralised as above. Cell protein was measured by a biuret method [21], with bovine serum albumin as standard.

Results

Effect of cyclic AMP on alanine transport and metabolism

It is known that gluconeogenesis from alanine in isolated hepatocytes is stimulated by glucagon, the effect probably being mediated by cyclic AMP. To determine whether alanine transport into the cell is a possible site of regulation, the effect of cyclic AMP on alanine transport was determined under conditions where alanine metabolism was completely inhibited by aminooxyacetate.

Fig. 1. shows the initial rate of alanine transport at various external alanine concentrations, and the effect of cyclic AMP. Transport was stimulated by cyclic AMP by about 30% at concentrations of alanine up to 4 mM. These results are the means ± S.E. for four determinations on a single batch of cells, and similar results were obtained with five separate cell preparations (Table I).

Half maximal stimulation of alanine transport was observed at 40 μM cyclic AMP, and this stimulation of alanine transport was unaffected by preincubation of the cells with either 0.1 or 1 mM cycloheximide (results not shown), in agreement with previous findings [14].

Alanine metabolism was measured by incubating

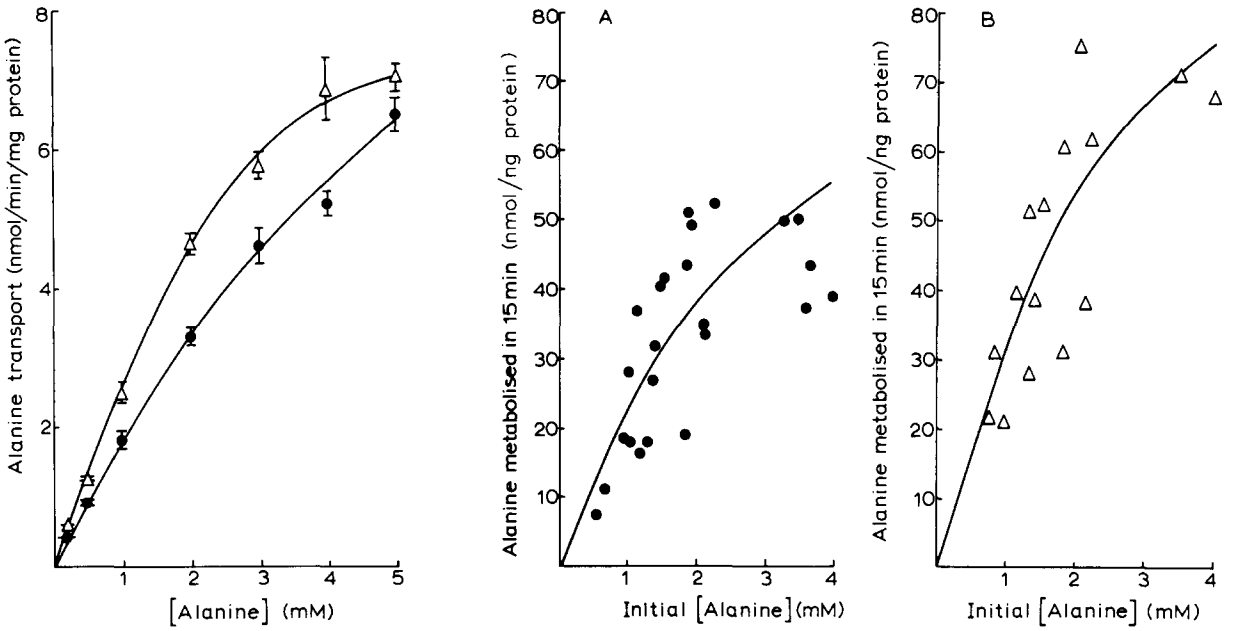

Fig. 1. Effect of cyclic AMP on the initial rate of alanine transport. Hepatocytes (10–12 mg protein/ml) were preincubated for 10 min with 1 mM aminooxyacetate in the presence or absence of 0.1 mM dibutyryl cyclic AMP. The initial rate of alanine transport was determined from the uptake of [^{14}C]alanine after 2 min. Each point represents the mean ± S.E. for four determinations on a single batch of cells. ●———●, controls; △———△, + cyclic AMP.

Fig. 2. Effect of cyclic AMP on alanine metabolism in hepatocytes. Hepatocytes (10–12 mg protein/ml) were preincubated in the presence or absence of cyclic AMP for 10 min. Alanine was added, and a sample of the incubation was immediately removed and protein-depleted. Alanine metabolism in the remainder of the incubation was stopped by protein depletion after 15 min. Alanine metabolism in 15 min was taken as the difference between the alanine content of the incubation at zero time and at 15 min. The values shown were obtained from 14 separate cell preparations. The curve drawn through these points was the best fit as determined by the method of least squares. ●———●, controls; △———△, + cyclic AMP.

the hepatocytes for 15 min with various concentrations of alanine in the absence of aminooxyacetate. In this system, alanine metabolism is best measured as alanine disappearance rather than the formation of products. Although there is little endogenous alanine, glycogen and lactate are present in cells from fed rats, and effects of cyclic AMP on the metabolism of these compounds may interfere with the overall production of glucose and CO_2, with alanine as substrate.

Fig. 2 shows the net disappearance of alanine as a function of the initial alanine concentration, in experiments using 14 different batches of cells. 0.1 mM cyclic AMP stimulated alanine metabolism at all alanine concentrations used. The rates of transport and metabolism of alanine in this system should not be limited by the availibility of fatty acids since both rates were found to be unaffected by the addition of 1 mM oleate (results not shown).

The rates of alanine metabolism observed varied considerably between different batches of cells. Nevertheless, comparison of the results in Figs. 1 and 2 indicates that the initial rates of transport and metabolism in the range 0.5–5 mM alanine are the same to within a factor of less than 2, and both processes are stimulated by cyclic AMP to a similar extent. It is therefore possible that the stimulation of alanine metabolism by cyclic AMP is primarily due to a stimulation of the alanine transport system under these conditions. However, this would apply only if transport were the rate-determining step in alanine metabolism.

TABLE I

STIMULATION OF ALANINE TRANSPORT BY CYCLIC AMP

Alanine transport was measured as described in Fig. 1. Experiments were performed using five batches of cells and taking 2–4 replicates for several concentrations. The values given are the overall mean ± S.E. for the total number of determinations shown in parentheses. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control without cyclic AMP, using a Student's t-test

[Alanine] (mM)	Initial rate of transport (nmol/min/mg protein)	
	Control	+cyclic AMP
0.2	0.56 ± 0.06 (8)	0.69 ± 0.08 (8)
0.5	1.00 ± 0.07 (12)	1.30 ± 0.09 (12) ***
1.0	1.95 ± 0.13 (12)	2.83 ± 0.15 (12) ***
2.0	3.81 ± 0.25 (10)	5.07 ± 0.16 (12) ***
3.0	4.59 ± 0.22 (4)	5.75 ± 0.21 (4) **
4.0	4.98 ± 0.31 (8)	6.32 ± 0.43 (8) *
5.0	7.73 ± 0.59 (8)	8.01 ± 0.51 (8)

Effect of inhibition of alanine transport on the rate of alanine metabolism

The transport of alanine in isolated hepatocytes is strongly dependent on the Na^+ concentration, and is sensitive to inhibition by ouabain [7]. If alanine transport were rate-limiting for alanine metabolism, both processes should be inhibited by ouabain to the same extent and with the same ouabain concentration dependence. Fig. 3 shows that a significant inhibition of transport of 2 mM alanine is obtained by preincubation of the cells with 30 μM ouabain. At low concentrations of ouabain, a small but reproducible stimulation of alanine metabolism was observed, and alanine metabolism was progressively inhibited at higher concentrations. However, the ouabain concentration dependences of the inhibition of transport and metabolism were not identical. In particular, the addition of 0.15 mM ouabain inhibited transport by 17.0 ± 1.5% (mean ± S.E. for three preparations) without significantly affecting metabolism. Essentially similar results were obtained with 1 mM alanine.

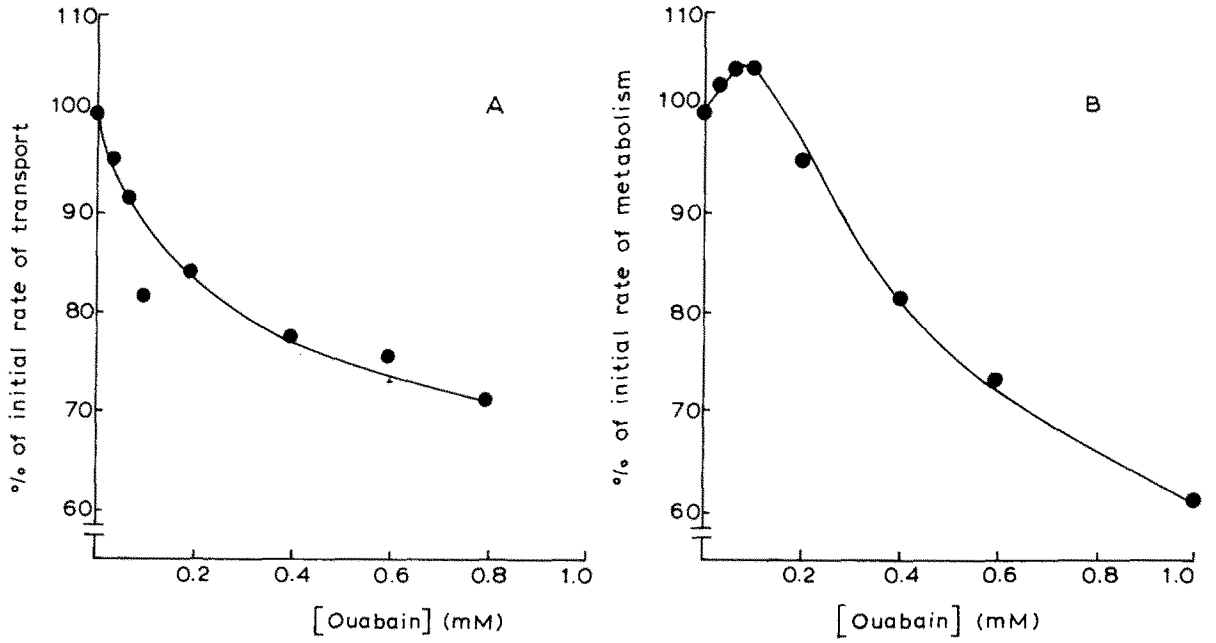

Fig.3. Effect of ouabain on the transport and metabolism of alanine. Cells were preincubated for 10 min with various concentrations of ouabain. 2 mM alanine was added, and the initial rate of alanine transport was measured after 2 min, as described in Methods. Alanine utilisation was measured after 15 min in parallel incubations. The values shown are the means from three separate cell preparations. A, Transport; B, metabolism.

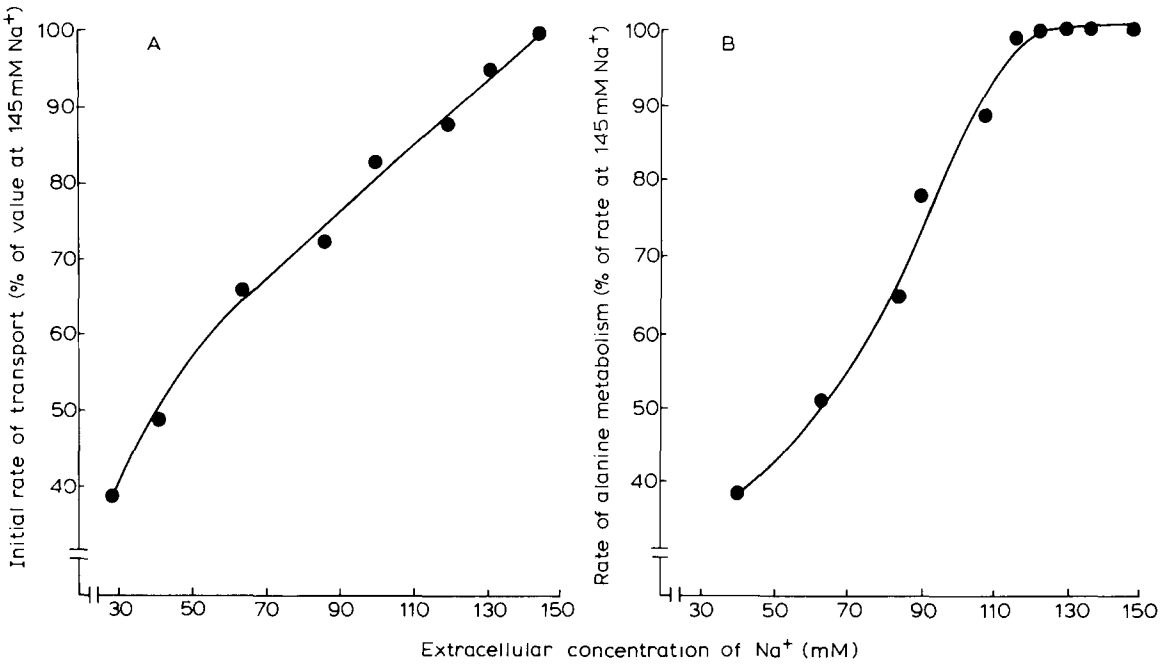

Fig. 4. Effect of varying the Na^+ concentrations of the medium on the transport and metabolism of alanine. Cells were preincubated in either Krebs-Henseleit buffer, or in a modification of this medium where part of the NaCl component was replaced by an equal concentration of choline chloride to give the final Na^+ concentrations shown. Alanine was added at a concentration of 2 mM. Alanine transport and metabolism were measured as described in Methods. The results shown are the means from three separate cell preparations. A, Transport; B, metabolism.

Fig. 4 shows the dependence of alanine transport and metabolism on the Na^+ concentration of the medium. Reduction of $[Na^+]$ from 150 mM to 110 mM inhibited the rate of transport of 2 mM alanine by 20% without affecting the rate of metabolism; at lower Na^+ concentrations, both transport and metabolism were progressively inhibited. These results indicate that at the alanine concentration used transport is not rate-limiting for metabolism, but a partial inhibition of transport by ouabain or a

TABLE II

EFFECT OF DIBUTYRYL CYCLIC AMP ON CELLS METABOLISING ALANINE

Hepatocytes (14.3 mg protein/ml) were incubated at 37°C with 5 mM alanine, and 0.1 mM dibutyryl cyclic AMP was added to one portion of the cell suspension after 15 min. Results are given as the mean ± S.E. of five observations from one experiment: similar results were obtained with four separate cell preparations, using protein concentrations in the range 10–20 mg/ml, and initial alanine concentrations of 3 and 5 mM. * $P < 0.001$ vs. control at 30 min.

Time at which the sample was taken	Intracellular [alanine] (mM)	[Alanine] in medium (mM)	Concentration ratio (in/out)
15 min, just before addition of cyclic AMP	6.53 ± 0.23	3.74 ± 0.09	1.75 ± 0.09
30 min: control	7.40 ± 0.16	2.93 ± 0.01	2.53 ± 0.05
30 min: + cyclic AMP	5.65 ± 0.086 *	2.64 ± 0.020 *	2.14 ± 0.020 *

Alanine disappearance between 15 and 30 min: –control, 56.6 nmol/mg; +cyclic AMP, 76.9 nmol/mg.

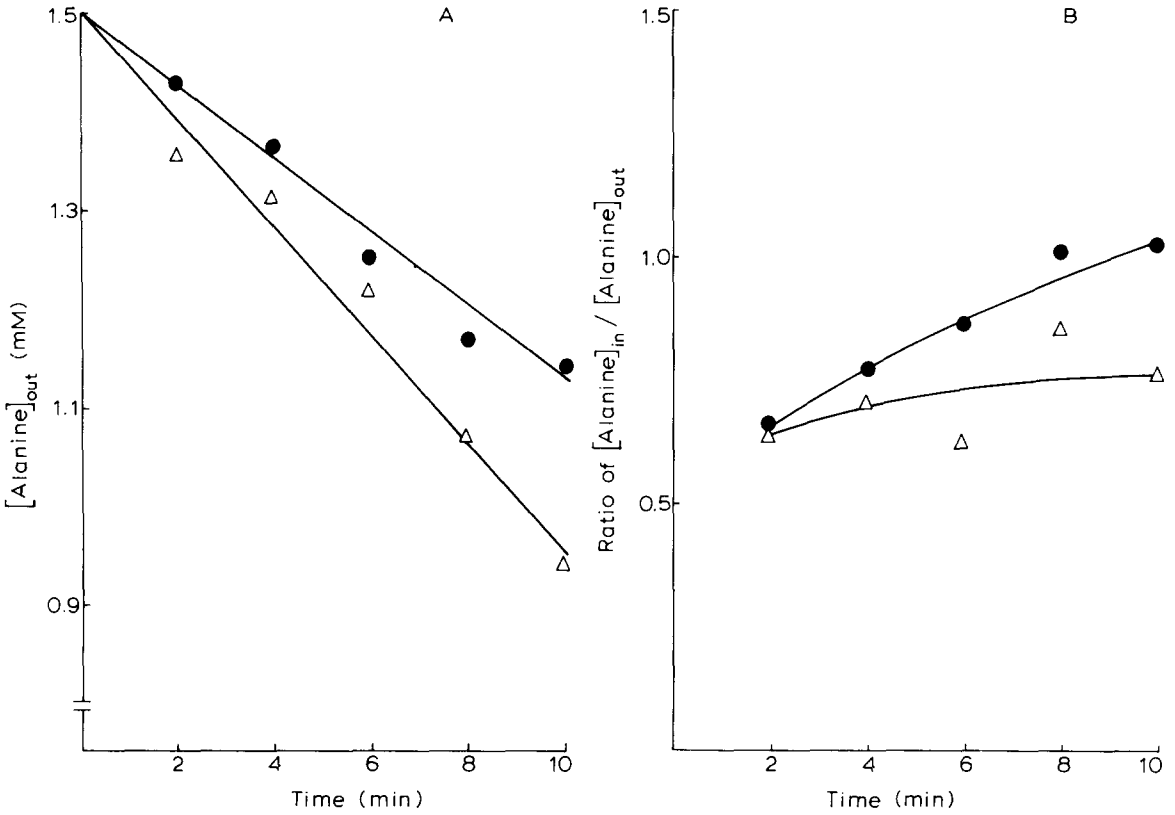

Fig. 5. Effect of cyclic AMP on alanine metabolism and the alanine distribution ratio in hepatocytes incubated in Krebs-Henseleit buffer (145 mM Na^+). Hepatocytes were preincubated for 10 min in the presence or absence of 0.1 mM dibutyryl cyclic AMP. 1.5 mM alanine was added and samples were taken at intervals for the determination of the intracellular and extracellular concentrations of alanine, as described in Methods. A, Effect of cyclic AMP on alanine utilisation; B, effect of cyclic AMP on the alanine distribution ratio. ●——●, controls; △——△, + cyclic AMP.

decrease in Na^+ concentration can cause the transport process to become rate-limiting.

Effect of stimulation of alanine metabolism by cyclic AMP on the internal to external alanine concentration ratio

Information about the rate-limiting step in alanine metabolism can be obtained by observing the intracellular to extracellular alanine concentration ratio during the metabolism of alanine by liver cells. If transport of alanine across the cell membrane were rate-limiting and cyclic AMP stimulates transport alone, then on addition of cyclic AMP, an increase in the concentration ratio should occur, together with an increase in the rate of metabolism. If transport were not rate-limiting, then cyclic AMP must be assumed to activate an intracellular process in addition to activation of transport. In this case, addition of cyclic AMP should cause a stimulation of metabolism associated with a decrease in the intracellular to extracellular alanine concentration ratio.

Table II shows the result of a representative experiment designed to investigate this point. In this experiment, net accumulation of alanine occurred, and alanine metabolism between 15 and 30 min was stimulated by 36% and the intracellular to extracellular ratio decreased. Similar results (not shown) were obtained in several experiments using initial alanine concentrations of 3 and 5 mM, and cell concentrations in the range 10–20 mg protein/ml. Therefore, it appears that at these alanine concentrations, transport was not rate-limiting and that the stimulation of

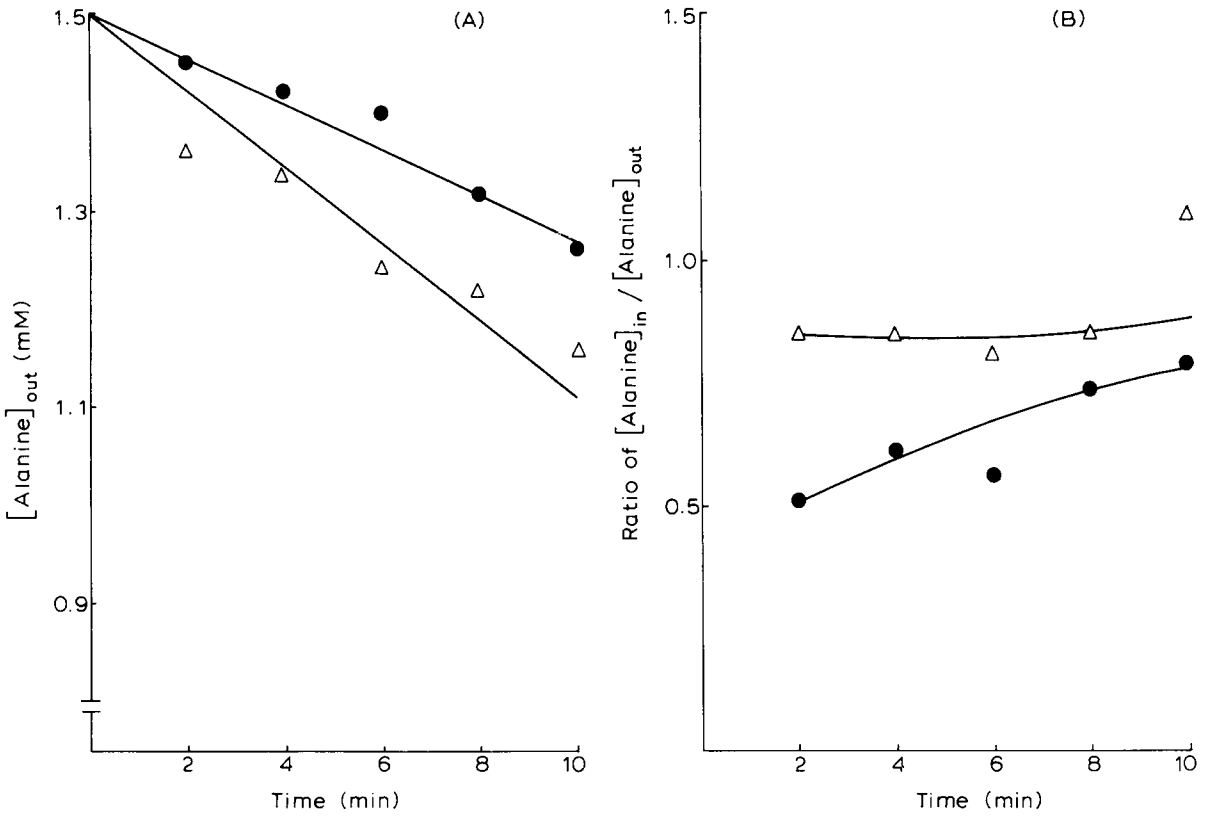

Fig. 6. Effect of cyclic AMP on alanine metabolism and alanine distribution ratio in hepatocytes incubated in a low Na^+ medium. Hepatocytes were incubated in a modified Krebs-Henseleit buffer containing 75 mM Na^+, as described in Table III. Details of the experiment are the same as in the legend to Fig. 5. A, Effect of cyclic AMP on alanine metabolism; B, effect of cyclic AMP on the alanine distribution ratio. ●——●, controls; △——△, + cyclic AMP.

metabolism by cyclic AMP was primarily due to the stimulation of an intracellular event.

This experiment was repeated under conditions where transport may be assumed to be rate-limiting for metabolism, i.e., by incubation of the cells in a medium containing 75 mM Na^+ (low Na^+ medium), as shown by the results of Fig. 4. Under these conditions, the initial rate of alanine transport was stimulated by cyclic AMP to the same extent as in normal Krebs-Henseleit medium (Table III). The effect of the addition of cyclic AMP on the intracellular to extracellular concentration ratio of alanine is shown as a function of time in Figs. 5 and 6. In the normal medium (Fig. 5), cyclic AMP caused a decrease in the concentration ratio and an increase in the rate of metabolism. In the low Na^+ medium (Fig. 6), of cyclic AMP again caused an increase in the rate of metabolism, but this was accompanied by an increase in the concentration ratio of alanine.

The elevated alanine concentration ratio observed in Fig. 6 confirms that alanine transport is rate-limiting in low Na^+ medium. In addition, the increase in this ratio implies that cyclic AMP has not stimulated the intracellular utilisation of alanine to the same extent as in the normal medium. Lactate, after its conversion to pyruvate, is metabolised by the same pathway as alanine. Attempts to study the effects of cyclic AMP and low Na^+ medium on lactate utilisation were complicated by the differential breakdown of endogenous glycogen, and yielded inconclusive results.

In the experiments shown in Figs. 5 and 6, the external concentration of alanine varied in the range 1.5–0.9 mM during the course of the incubation. It

TABLE III

STIMULATION OF ALANINE TRANSPORT BY CYCLIC AMP IN NORMAL KREBS-HENSELEIT MEDIUM AND IN A MEDIUM CONTAINING 75 mM Na^+

Cells (10–12 mg protein/ml) were suspended in either Krebs-Henseleit medium or in a modification of this medium in which 70 mM of the total NaCl component was replaced with 70 mM choline chloride, giving a final concentration of 75 mM Na^+. Alanine transport was measured in the presence and absence of cyclic AMP, as described in Methods. The data in the second column are taken from Table I; the third and fourth columns show data from experiments with two separate batches of cells where each value was determined in triplicate. Values are given as means ± S.E. of the ratio of the values in the presence and absence of cyclic AMP

[Alanine] (mM)	Ratio of initial rates of transport in the presence and absence of cyclic AMP		
	Krebs-Henseleit medium	75 mM Na^+ medium	
		Expt. 1	Expt. 2
0.2	1.23 ± 0.15	1.16 ± 0.11	1.25 ± 0.02
0.5	1.30 ± 0.10	1.25 ± 0.08	1.25 ± 0.05
1.0	1.45 ± 0.09	1.28 ± 0.09	1.36 ± 0.03
2.0	1.33 ± 0.07	1.23 ± 0.07	1.68 ± 0.09
3.0	1.25 ± 0.06	1.36 ± 0.10	1.29 ± 0.05
4.0	1.27 ± 0.09	1.16 ± 0.08	1.22 ± 0.06

is concluded that in this concentration range alanine transport is rate-limiting only in low Na^+ medium, and that in normal medium the stimulation of alanine metabolism by cyclic AMP is not primarily due to a stimulation of the transport process.

Fig. 7 shows the effect of adding cyclic AMP to hepatocytes incubated with low concentrations of alanine. At external alanine concentrations greater than 0.5 mM, cyclic AMP caused a decrease in the intracellular alanine concentration, in accordance with the results presented above. However, at external alanine concentrated below 0.5 mM, an increase in the intracellular alanine concentration was observed on addition of cyclic AMP. It therefore appears that in the concentration range 0–0.5 mM, alanine transport is rate-limiting for alanine metabolism, and stimulation of alanine metabolism may be primarily due to stimulation of the transport process. In the system used, the accurate measurement of alanine at low extracellular alanine concentrations is difficult. Sips et al. [15] have used a perifusion technique to overcome this problem.

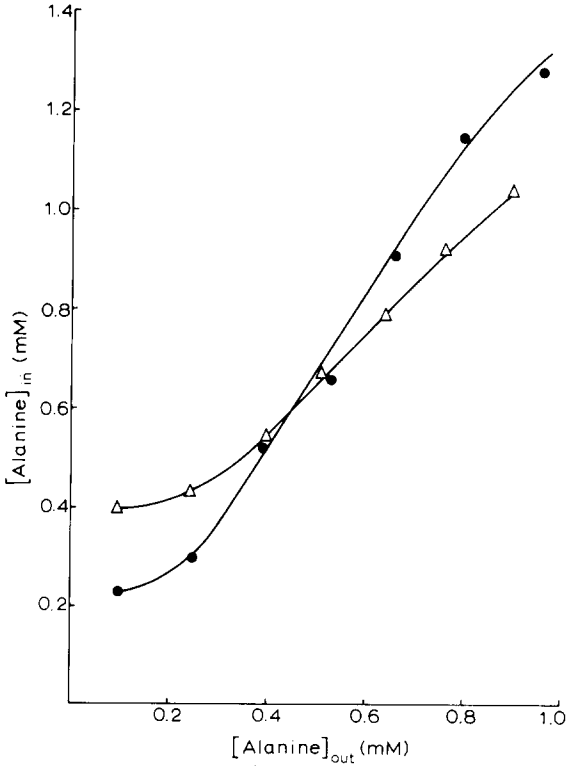

Fig. 7. Effect of cyclic AMP on intracellular alanine levels at various concentrations of alanine. Cells were preincubated with or without 0.1 mM dibutyryl cyclic AMP and alanine was added at various concentrations between 0 and 1.2 mM. After a further 5 min incubation, the intracellular and extracellular alanine concentrations were determined, as described in Methods. ●——●, controls; △——△, + cyclic AMP.

Discussion

The stimulation of alanine transport by cyclic AMP reported in this paper does not involve the synthesis of new protein since it occurs rapidly, and it is not prevented by preincubation of the cells with cycloheximide. These results are similar to the reported effects of glucagon and cyclic AMP on aminoisobutyrate transport in hepatocytes [12]: long-term effects involving de novo synthesis of protein have also been reported [9,11,22]. In the case of alanine transport, stimulation by glucagon is biphasic [14], the initial phase being independent of protein synthesis.

The similarity between the rates of transport and metabolism of alanine in hepatocytes, together with the parallel effects of cyclic AMP on both processes,

indicates that alanine transport is a potential rate-limiting step for gluconeogenesis. However, at concentrations of alanine above 1 mM, alanine transport does not appear to be rate-limiting. This is concluded from the experiments involving inhibition of transport and also the measurement of steady state levels of intracellular alanine. Cyclic AMP must therefore, in addition to its direct effect on transport, activate a subsequent step in the pathway of alanine metabolism. Mallette et al. [2] measured steady-state levels of gluconeogenic intermediates after perfusion of the liver with alanine. Their results were interpreted as showing that glucagon stimulated a rate-limiting reaction between pyruvate and phospho*enol*pyruvate. Pyruvate carboxylation in isolated mitochondria is stimulated after treatment of rats with glucagon [23]. Glucagon and cyclic AMP cause the phosphorylation and subsequent inactivation of pyruvate kinase [24]. It has also been suggested that alanine aminotransferase may limit the rate of gluconeogenesis from alanine under certain conditions [2].

Ths physiological concentration of alanine in plasma is in the range 0.2–0.5 mM [25,26]. The data in Fig. 7 show that the intracellular alanine concentration is relatively higher on adding cyclic AMP to cells at external alanine concentrations in this range. However, at higher external alanine concentrations the internal alanine concentration becomes relatively lower in the presence of cyclic AMP. Under conditions of low external [Na^+], where alanine transport is known to be rate-limiting (Fig. 6), addition of cyclic AMP caused an increase in the alanine concentration ratio. The results of Fig. 7 may therefore be interpreted as showing that alanine transport must be rate-limiting at low, physiological concentrations of external alanine.

Previous studies on the transport of alanine in hepatocytes from starved rats [7] showed that 0.5 mM ouabain inhibited alanine transport by 50% without affecting the rate of metabolism of alanine (in contrast to the results shown for fed rats in Fig. 3). On the basis of this and similar experiments it was concluded that alanine transport was not limiting for alanine metabolism in cells from starved rats at alanine concentrations between 0.5 and 10 mM. No experiments were performed at lower concentrations of alanine. It should be noted that alanine transport at 0.5 mM alanine is much faster in hepatocytes from starved rats [7] than in hepatocytes from fed rats. Similar results have been obtained for the transport of α-aminoisobutyrate [27]. In cells from starved rats, the rate of transport of 0.5 mM alanine is nearly double the rate of metabolism [7] while in cells from fed rats both rates were similar. These results may suggest that in starved rats transport is less likely to become limiting at low alanine concentrations. However, we have not systematically studied this problem in starved rats.

In the present paper, alterations in the rate of alanine metabolism and intracellular alanine concentrations on addition of activators and inhibitiors of alanine transport have been used to assess the role of alanine transport in the regulation of alanine metabolism. While this paper was in preparation, Sips et al. [15] published a study in which a different approach to the same problem was employed. Cells from both fed and starved rats were perifused with alanine and the intracellular alanine concentrations at various concentrations of alanine in the perifusate were measured. It was found that the intracellular alanine concentration varied little over a wide range of external alanine concentrations although the rate of metabolism was strongly dependent on the external concentration. These results were interpreted to show that at physiological alanine concentrations the transport of alanine across the plasma membrane is limiting for its subsequent catabolism. The data in Fig. 7 of the present paper confirm this conclusion. Under the conditions in our experiments, hepatocytes from fed rats contained a low intracellular concentration of alanine when suspended in an alanine-free medium. In contrast, Sips et al. [15] observed intracellular alanine concentrations of 1 mM in cells perifused with alanine-free medium. The reason for this discrepancy is not clear at present.

Sips et al. [15] did not specifically indicate at what concentration of alanine, if any, transport ceases to be rate-limiting. Our results indicate that at alanine concentrations in excess of 1 mM in fed rats, alanine transport is no longer limiting for metabolism.

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