BBA 75666

EVALUATION OF KINETIC PARAMETERS FOR UPTAKE OF AMINO ACIDS BY CELLS: EFFECT OF INSULIN ON THE ACCUMULATION OF AMINOISOBUTYRATE AND CYCLOLEUCINE BY ISOLATED RAT DIAPHRAGM MUSCLE AND CHICK EMBRYO HEARTS

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(Received December 23rd, 1970)

SUMMARY

An attempt has been made to evaluate some of the kinetic parameters governing the uptake of amino acids by muscle by measuring the steady-state concentration ratios established on incubation of the tissue *in vitro*. The data are analysed in terms of the steady state resulting from a balance between saturable uptake and efflux processes in conjuction with diffusion. For aminoisobutyrate and cycloleucine the data were compatible with efflux being solely by diffusion, but it was also possible to fit the data to conditions such that efflux by a saturable process and by diffusion were both occurring. Measurement of initial entry rates after preloading hearts with aminoisobutyrate gave results consistent with the first possibility only. Such measurements also suggested that the parameters governing a saturable uptake process changed during the course of uptake, but that the rate constant for diffusion remained the same. The rate of approach to equilibrium was found to be faster the higher the external concentration of amino acid. Both these observations would be consistent with accumulated amino acid progressively interfering with further operation of a carrier.

For aminoisobutyrate the enhancement of accumulation in the presence of insulin appeared to result from an increase in the maximal velocity of the transport rate rather than from any change in apparent K_m. For cycloleucine there was some diminution in the K_m with insulin but again a rise in the transport rate.

INTRODUCTION

Insulin enhances the uptake of amino acids by muscle[1,2]. This action of the hormone is most clearly demonstrated in its effects on the accumulation by various isolated tissue preparations of certain model amino acids — aminoisobutyrate and cyclo-

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leucine being two particularly responsive[3,4]. Uptake of amino acids by cells may be partly by diffusion, but active transport demonstrating saturation and Michaelis–Menten like kinetics also plays an important part. Stimulation of uptake by insulin could result from an increase in the maximal velocity of the uptake process (V), a change in apparent affinity constant (K_s) of the solute (S) for the putative carrier, a change in the value of the diffusion rate constant (K_D) for first order diffusion or by changes in any two or all three.

If uptake of an amino acid not already contained in a cell consists of diffusion operative in either direction across the membrane together with an active process functional in only one direction, the time course of uptake is given by

$$c = S\left(1 + \frac{V}{(K_s + S)K_D}\right)(1 - e^{-K_D t}) \qquad (1)$$

where c is the intracellular concentration present at time t with an external solute concentration of S. From measurement of initial rates of entry with different values of S, AKEDO AND CHRISTENSEN[4] concluded that insulin enhanced the uptake of amino-isobutyrate by intact rat diaphragm by lowering K_s from about 16 to 1.6 mM, without appreciably changing V. Others have confirmed this[5]. On the other hand, some of us[6], using the same technique but working with the chicken embryo heart, found that insulin raised V by 45 % and lowered K_D (from 0.37 to 0.25 h^{-1}) without affecting K_s (2.35 mM). The reason for this divergence of results is not immediately obvious.

Isolated diaphragm or heart does not constitute the ideal biological system with which to measure kinetic parameters, *inter alia* both because of the inherent variability between successive pieces of tissue and because uptake of amino acid into intracellular water involves the passage of the amino acid from the bathing solution through the interstitial fluid. In for example efflux experiments it is possible that the apparent rate of efflux could be materially influenced by immediate reaccumulation of the liberated amino acid whilst still in the interstitial fluid before its escape into the suspending medium. The same problem arises in uptake experiments. Measurement of kinetic parameters of the uptake of amino acids by muscle is of interest because of response to hormones which the ascites tumour cell for example does not show. We have therefore investigated the usefulness of an alternative procedure for estimating kinetic constants which tends to minimise the problems likely to arise in systems with interstitial fluid.

As t increases, Eqn. 1 simplifies to

$$(c - S)K_D = \frac{V \cdot S}{(K_s + S)} \qquad (2)$$

which indicates that equilibrium is eventually attained when the rate of efflux by diffusion equals the saturable uptake process. Eqn. 2 rearranges to

$$\Delta = \frac{V}{K_D} - K_s \cdot \frac{\Delta}{S} \qquad (3)$$

where $\Delta = c - S$. Thus by plotting Δ against Δ/S for the steady-state concentrations of amino acid attained between tissue and medium at different values of S it should prove possible to make estimates of K_s and V/K_D. We do not need to assume that the rate of exchange of the extracellular compartment with the medium is substan-

tially faster than with that of the intracellular pool and the problems of immediate reabsorption following efflux do not arise. Moreover determination of K_s from Eqn. 2 does not require exact knowledge of the intracellular space since the same term appears on both sides of the equation.

The correctness of this procedure depends, however, on the validity of Eqns. 1–3. The goodness of fit obtained might be regarded as support for their use, but there is clearly a possibility that as the value of c increases a saturable efflux process may play an appreciable role in addition to diffusion. Such might be implied from the time course published in the earlier experiments of some of us[6], which, when compared with the theoretical curves derived by substituting the values of V, K_s and K_D obtained from initial velocity experiments in Eqn. 1, shows (Fig. 1) that the experimental curve plateaus off sooner than expected. The existence of a saturable efflux process will of course vitiate the validity of determinations of K_D estimated from efflux experiments at temperatures compatible with a functional mediation.

To take account of this possibility Eqn. 2 can be modified to

$$\frac{V \cdot S}{(K_s + S)} = (c - S)K_D + \frac{V \cdot c}{(K_c + c)} \tag{4}$$

where K_c is the affinity constant for the efflux process and V is assumed to reach the same maximal velocity in either direction. Though not amenable to graphical manipulation we have sought to evaluate its significance to the present data by computer fitting to a rearranged form:

$$\frac{V}{K_D} \cdot \frac{S}{(K_s + S)} = (c - S) + \frac{V}{K_D} \cdot \frac{c}{(K_c + c)} \tag{5}$$

since V and K_D cannot be independently determined.

From the previously reported figures for the chicken embryo heart[6] equilibrium

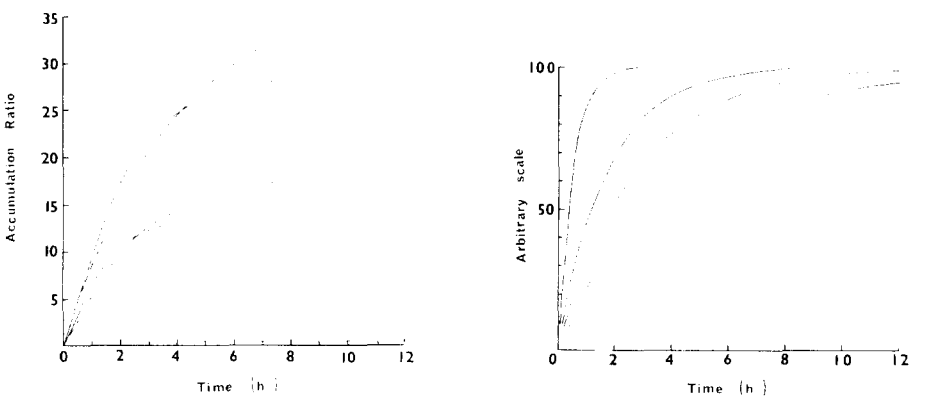


Fig. 1. Experimental and theoretical time courses for the uptake of aminoisobutyrate by chicken embryo hearts. ———, results shown in Fig. 1 of ref. 6; · · · · · , calculated time courses obtained by inserting in Eqn. 1 the constants they derived. $S = 2$ mM, the upper lines are data in the presence of insulin; the lower ones in its absence.

Fig. 2. Plots of the function $(1 - e^{-K_D t})$ for different values of K_D. The values of K_D used for the different curves successively from left to right are 1.98, 0.58, 0.37 and 0.25 h^{-1} (taken from refs. 6 and 10).

appeared to be attained in 6–8 h. According to Eqn. 1 the rate of approach to equilibrium depends on K_D, being faster the greater its value. LONDON AND SEGAL[7] found values of 0.58 and 1.98 h^{-1} for K_D for efflux of aminoisobutyrate and cycloleucine from isolated diaphragm. The function $(1-e^{-K_D t})$ for these values of K_D and for 0.25 and 0.37 h^{-1} found previously for aminoisobutyrate in heart[6] are shown in Fig. 2 to give some idea of the likely periods of incubation required to reach reasonable steady-state conditions. Whether the prediction of Eqn. 1 and Fig. 2 about the rate of approach to equilibrium is correct is considered further in the RESULTS section.

MATERIALS AND METHODS

Aminoiso[1-^{14}C]butyrate was obtained from the Radiochemical Centre, Amersham, Bucks, England; [^{14}C]cycloleucine (1-aminocyclopentane carboxylic acid) came from New England Nuclear Chemicals, Dreieichenhain, West Germany. Each was diluted as appropriate with unlabelled material.

Hemidiaphragms were taken from non-fasting albino rats about 100 g weight and incubated at 37° with shaking in Krebs–Ringer bicarbonate gassed with O_2–CO_2 (95:5, v/v). No glucose was added to the incubation medium. Insulin when present had a concn. of 0.1 unit/ml. To minimise inter-animal variation the hemidiaphragms were systematically distributed between incubation flasks with different concentrations of amino acid so that no pair of hemidiaphragms from any one rat were contained in any single flask. In the experiments with aminoisobutyrate incubation was for 8 h; by the end of this period the tissue still looked healthy but was more fragile to touch. Reference to Fig. 2 suggests that by 8-h equilibrium will be 85 % complete even if K_D is as low as 0.25 h^{-1} and 99 % complete if K_D is 0.58 h^{-1} (ref. 7). With cycloleucine incubation was for 6 h.

At the end of the incubation the tissue was removed, gently blotted and weighed and the accumulated amino acid extracted by boiling with water[8]. Samples of tissue extract and medium were counted in a liquid scintillation system consisting of 1 ml of aqueous extract *plus* 10 ml of scintillator (0.4 % 2,5-bis-2-(5-tert.-butylbenzoxazolyl) thiophene in toluene–Triton X-100 (2:1, v/v)). This system counts ^{14}C with about 70 % efficiency and shows little quenching. Entry of amino acid into the tissue led to lowering of its concentration in the medium. The concentration of amino acid remaining in the medium at the end of the incubation was calculated from the radioactivity present.

The concentration of amino acid in the intracellular water of the tissue was calculated from the radioactivity found in a known weight of tissue and the concentration of amino acid in the final medium by correcting for the dry weight of the tissue (0.23 g/g wet wt.) and assuming that the amount of solute in the extracellular fluid (0.27 ml/g) was the same as that of the medium. The precise volume of the various fluid compartments is somewhat variable[9]. Slight variations should have little influence on estimates of K_s, they will affect the absolute but not the relative values of V/K_D.

Hearts (15 ± 1 mg wet wt.), dissected from 5-day-old chick embryos[6], were incubated in 4 ml of Krebs–Ringer bicarbonate buffer supplemented with 8 mM glucose. Incubation was at 37.5° in an atmosphere of O_2–CO_2 (95:5, v/v) for the proper period of time (8 h with aminoisobutyrate and 6 h with cycloleucine in equilib-

ration experiments; 1–12 h in time course experiments; 20–80 min after 6–10 h preincubation in experiments of flux rate at steady state). When present, insulin was added at a concentration of 0.2 unit/ml of medium. At the end of the incubation the hearts were blotted, weighed and the accumulated amino acid extracted by boiling in 3 % (w/v) sulphosalicylic acid[6]. Samples of tissue extract and of initial and final medium were added to a scintillation mixture and counted in a Tri-Carb spectrometer. The means for calculating the intracellular and extracellular concentrations of the amino acid in these experiments were as described by GUIDOTTI *et al.*[6].

Cardiac cell suspensions, obtained by collagenase treatment of 7-day-old chick embryo hearts[10], were incubated for 1–5 h, at 37.5° in Krebs–Ringer bicarbonate supplemented with 8 mM glucose and ^{14}C-cycloleucine in an atmosphere of O_2–CO_2 (95:5, v/v). Isolated cell concentration was 0.6 ± 0.1 mg protein per ml. The procedures for extraction of amino acids, measurement of radioactivity and calculation of the intracellular concentration of amino acid were as previously described[10].

Fitting of values of the constants in Eqn. 5 by computer involved scanning various appropriate ranges to find minimum variances on substitution in the equation.

TABLE I

THE UPTAKE OF ^{14}C-AMINOISOBUTYRATE BY ISOLATED RAT DIAPHRAGM AND CHICKEN EMBRYO HEARTS AT VARIOUS EXTERNAL SOLUTE CONCENTRATIONS

Incubation was for 8 h. All concentrations are mM. For diaphragm each figure is the mean ± S.E. of four observations and the calculated concentrations are derived from Eqn. 2 for values of K_s and V/K_D of 1.29 mM and 6.9 μmoles/ml in the absence of insulin and 1.29 mM and 22.8 μmoles/ml in its presence. For the embryo hearts each figure is the mean ± S.E. of three observations and the calculated concentrations are derived from Eqn. 2 for values of K_s and V/K_D of 0.78 mM and 33.8 μmoles/ml in the absence of insulin and 0.76 mM and 76.0 μmoles/ml in its presence.

Diaphragm muscle			*Embryo hearts*		
Concn. in medium at end of incubation	*Concn. in intracellular water*	*Calc. concn. in intracellular water*	*Concn. in medium at end of incubation*	*Concn. in intracellular water*	*Calc. concn. intracellular water*
No insulin added					
0.093	0.54 ± 0.019	0.56	0.28	9.3 ± 0.93	9.2
0.19	1.16 ± 0.073	1.05	0.58	15.3 ± 1.5	15.0
0.47	2.11 ± 0.036	2.33	0.98	19.7 ± 1.2	19.8
0.95	3.62 ± 0.11	3.89	1.99	25.3 ± 0.91	26.3
2.95	8.09 ± 0.16	7.75	5.00	32.5 ± 1.4	34.3
10.0	16.3 ± 0.29	16.1	9.84	41.7 ± 1.3	41.2
			19.7	54.9 ± 1.1	52.2
Insulin added					
0.078	1.56 ± 0.06	1.38	0.27	19.4 ± 0.96	20.1
0.16	2.42 ± 0.04	2.68	0.56	33.6 ± 1.13	32.7
0.43	5.57 ± 0.20	6.13	0.95	44.5 ± 3.0	43.1
0.88	9.5 ± 0.34	10.1	1.93	56.6 ± 3.6	56.4
2.81	20.8 ± 1.44	18.4	4.88	69.4 ± 1.2	70.6
10.0	30.2 ± 1.17	30.2	9.87	79.3 ± 0.56	80.4
			19.5	93.4 ± 1.2	92.6

RESULTS

Apparent values of kinetic parameters under steady-state conditions

In the first experiments hemidiaphragms and embryo hearts were incubated with various concentrations of aminoiosobutyrate for 8 h — a period which seemed on the basis of recorded values of K_D likely to lead to near equilibrium conditions. Both tissues continue to respire linearly throughout this period (ref. 11 and unpublished observations). Table I shows the intracellular concentration of aminoisobutyrate attained at various external solute concentrations and in Fig. 3 (a–b) is shown a plot of Δ *versus* Δ/S for these values. For the figures from diaphragm, fitting of the data by least squares to Eqn. 3 gives a value (± S.E.) for K_s of 1.29 ± 0.24 and 1.29 ± 0.19 mM in the absence and presence of insulin and V/K_D of 6.9 ± 0.76 and 22.8 ± 3.2 μmoles/ml. K_s in these experiments is similar to that found by AKEDO AND CHRISTENSEN[4] in the presence of insulin. With the embryo hearts values of 0.78

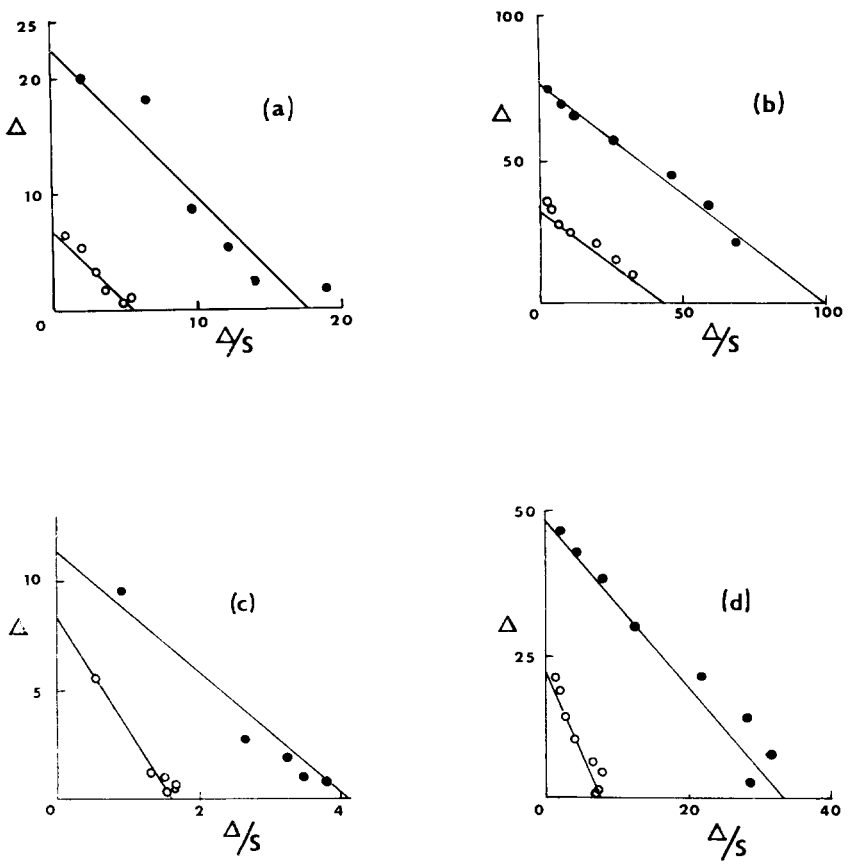


Fig. 3. Steady-state concentration of aminoisobutyrate (a, b) and cycloleucine (c, d) in isolated rat diaphragm muscle (a, c) and chicken embryo hearts (b, d), plotted as the concentration difference (Δ) between intracellular fluid and medium against Δ/medium concentration. ○, in the absence of insulin; ●, in its presence. The slope (K_s) is unchanged by insulin for aminoisobutyrate but decreases for cycloleucine.

TABLE II

THE UPTAKE OF ^{14}C-CYCLOLEUCINE BY ISOLATED RAT DIAPHRAGM AND CHICKEN EMBRYO HEARTS AT VARIOUS EXTERNAL SOLUTE CONCENTRATIONS

Incubation was for 6 h. All concentrations are mM. For diaphragm each figure is the mean ± S.E. of four observations and the calculated concentrations are derived from Eqn. 2 for values of K_s and V/K_D of 5.2 mM and 8.2 μmoles/ml in the absence of insulin and 2.75 mM and 11.2 μmoles ml in its presence. For the embryo each figure is the mean ± S.E. of three observations and the calculated concentrations are derived from Eqn. 2 for values of K_s and V/K_D of 2.8 mM and 22.5 μmoles/ml in the absence of insulin and 1.4 mM and 48.8 μmoles/ml in its presence.

Diaphragm muscle			*Embryo hearts*		
Concn. in medium at end of incubation	*Concn. in intracellular water*	*Calc. concn. in intracellular water*	*Concn. in medium at end of incubation*	*Concn. in intracellular water*	*Calc. concn. intracellular water*
No insulin added					
0.11	0.27 ± 0.01	0.28	0.098	0.77	0.87
0.15	0.40 ± 0.02	0.38	0.245	2.06	2.08
0.26	0.68 ± 0.02	0.65	0.49	4.36	3.88
0.51	1.28 ± 0.07	1.24	0.98	6.97	6.88
1.01	2.31 ± 0.13	2.34	2.47	12.3	13.1
10.0	15.6 ± 0.19	15.4	4.95	18.6	19.4
			9.93	28.5	27.5
			19.9	40.7	39.7
Insulin added					
0.11	0.53 ± 0.05	0.54	0.093	2.73	3.13
0.15	0.78 ± 0.02	0.73	0.23	7.47	7.12
0.26	1.18 ± 0.05	1.23	0.47	13.7	12.7
0.51	2.17 ± 0.11	2.26	0.95	21.5	20.7
1.01	3.68 ± 0.20	4.02	2.42	32.1	33.3
10.0	19.4 ± 0.36	18.8	4.90	42.9	42.9
			9.85	52.7	52.6
			19.9	66.0	65.5

and 0.76 mM for K_s in the absence and presence of insulin are found and 33.8 and 76.0 μmoles/ml respectively for V/K_D.

Table II and Fig. 3(c–d) show the results of similar experiments with cycloleucine. Incubation was for 6 h. With diaphragm there was some lowering of K_s on addition of insulin, from 5.21 ± 0.61 to 2.75 ± 0.37 mM and a rise in V/K_D from 8.2 ± 0.54 to 11.2 ± 1.0 μmoles/ml. For the embryo hearts K_s also declined with insulin from 2.9 to 1.4 mM whilst V/K_D more than doubled from 23 to 49 μmoles/ml. It was noticed that the point for the lowest concentration in both the presence and absence of insulin did not fit the line drawn through the other data. The reason for this is possibly explicable later.

Alternative values of the kinetic parameters

That the data of Tables I and II for two different tissues in two different conditions are consistent with Eqn. 3 as indicated graphically in Fig. 3, suggests that the formulation provides a possible description of events. It was thought, however, desirable to see whether the data could also fit Eqn. 5 and what would then be the value of K_s, K_c and V/K_D. The first attempts at computer evaluation gave values

TABLE III

COMPUTER DERIVED VALUE OF V/K_D, K_s AND K_e THAT ARE COMPATIBLE WITH THE DATA OF TABLES I AND II AND Eqn. 5

The variance is $\frac{\sum\left(\frac{\text{actual } c - \text{calculated } c}{c}\right)^2}{n}$ for each particular set of observations and the proposed values of the constants.

	Diaphragm				*Embryo hearts*			
	V/K_D (μmoles/ml)	K_s (mM)	K_e (mM)	*Variance*	V/K_D (μmoles/ml)	K_s (mM)	K_e (mM)	*Variance*
Aminoisobutyrate								
— Insulin	7.2	1.38	∞	0.0043	34	0.78	∞	0.0010
	8.5	1.7	133	0.0061	49	0.96	127	0.0042
					67	0.90	55	0.0072
+ Insulin	22.9	1.33	∞	0.0087	76	0.76	∞	0.0007
	852	0.074	0.98	0.0217	195	0.52	56	0.0012
					384	0.27	21.2	0.0022
Cycloleucine								
— Insulin	8.6	5.4	∞	0.0006	23.0	2.90	∞	0.0047
	16.1	6.7	47	0.0010	27.1	3.63	446	0.0077
	6280	0.017	0.041	0.0026				
+ Insulin	12.4	3.2	∞	0.0024	49	1.40	∞	0.0040
	19.7	4.1	64	0.0069	61	1.45	267	0.0038
	5124	0.018	0.070	0.0186				

of K_c in excess of 10^6 M. Thus effectively the data were found to fit preferentially to Eqn. 3 as is consistent with the graphical fitting. Further evaluations, however, in which the upper range of values for K_c was restricted, showed that additional solutions can be found in which K_c has finite and potentially realistic values. These in the case of V/K_D range over several orders of magnitude (Table III). A variety of values for the constants of Eqn. 5 will thus adequately describe the experimental observations so far. The theoretical curves derived from the different solutions do not superimpose precisely: the lower the values of K_s and K_c and the higher V/K_D the steeper the slope at low values of S but discrepancies are too small for it to be possible to discriminate between the solutions, even if many more points were obtained, given the normal standard deviation of the data. Extension of the theoretical curves to cover four orders of magnitude (from 0.01 to 100 mM) shows no greater divergence. It must be concluded therefore that consideration of data at steady-state conditions will not allow unequivocal determination of the kinetic parameters.

Two procedures were considered which might provide information differentiating between the various sets of values recorded for the kinetic parameters in Table III: (a) determinations of initial velocity of entry calculated from uptake of labelled amino acid added to the system after equilibrium had been attained with unlabelled amino acid, and (b) time courses of the approach to equilibrium.

Uptake of labelled amino acid under steady-state conditions

Fig. 4 compares the uptake of labelled aminoisobutyrate by hearts pre-incubated to equilibrium with unlabelled amino acid as against uptake by hearts pre-incubated without amino acid. The presence of unlabelled amino acid diminishes the rate of uptake of label and provides no indication for the existence of exchange diffusion. (Others[12,13] also have found with aminoisobutyrate no evidence of exchange diffusion.) In Table IV are the rates of uptake of amino acid observed for different concentrations of aminoisobutyrate under steady-state conditions. Evaluation of

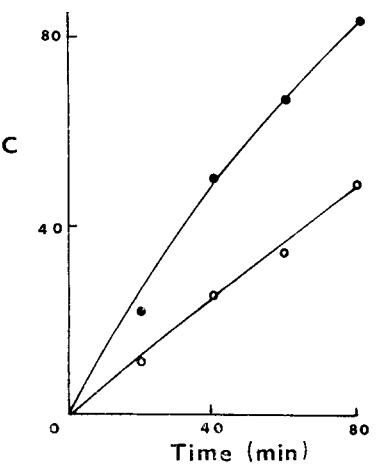


Fig. 4. Comparison of rate of uptake by chicken embryo hearts of labelled aminoisobutyrate (○) when cells have not been preincubated with unlabelled amino acid and (●) when cells have been incubated for 6 h with unlabelled amino acid. In both experiments medium concentration of aminoisobutyrate when present was 2 mM.

TABLE IV

RATE OF ENTRY OF LABELLED AMINOISOBUTYRATE INTO CHICKEN EMBRYO HEARTS PREVIOUSLY LOADED WITH UNLABELLED AMINOISOBUTYRATE

The values of K_s, V and K_D were derived by fitting them to the equation: initial rate of entry (which was linear for the first hour) $= V \cdot S/(K_s + S) + K_D \cdot S$. Figures in parentheses are calculated values using derived constants.

Concn. in the medium (mM)	*Rate of aminoiso[^{14}C]butyrate uptake (μmoles/ml intracellular water per h)*	
	No insulin added	*Insulin added*
0.5	5.4 (5.4)	8.0 (8.0)
1	7.8 (7.9)	11.4 (11.6)
2	10.2 (10.4)	14.8 (15.0)
5	13.7 (13.6)	19.0 (18.7)
10	16.4 (16.4)	21.2 (21.3)
K_s (mM)	0.78	0.78
V (μmoles/ml per h)	13.3	20.2
K_D (h^{-1})	0.41	0.26

K_s, K_D and V gives the values indicated. The diffusion rate constant (K_D) does not change depending on whether initial rates of entry are made in the presence or absence of preloading, but the values of K_s and V for the operation of a putative carrier system are lower in the preloaded state than those found from initial entry rates[6] and the values for V/K_D (and for K_s) are virtually identical to those found under steady-state conditions to be consistent with Eqn. 2. These results must indicate first that efflux is largely by diffusion, with saturable efflux of little significance, and secondly that the presence of intracellular amino acid in some manner lowers the values of the parameters governing uptake. It is possible that the presence of intracellular amino acid interferes with further uptake through a sequestering of the carrier on the inner side of the cell membrane. Further evidence interpretable in this sense is discussed next.

Time courses of amino acid uptake

Determination of time courses of rate of approach to equilibrium should offer a means of distinguishing between the different solutions listed in Table III on the grounds that the time taken to reach a steady state if described by Eqn. 1 is independent of the value of S, whereas this is not so when K_c has a realistic value. The time course for uptake of solute which when $t = \infty$ will become Eqn. 5 is

$$(c_1 + c)K_D \cdot t = (c_\infty + K_c) \cdot \ln \left(\frac{1}{1 - \frac{c}{c_\infty}} \right) - (c_1 - K_c) \cdot \ln \left(1 + \frac{c}{c_1} \right) \qquad (6)$$

where c is equal to the intracellular concentration at time t, c_∞ is c when $t = \infty$ and c_1 is the second root of the quadratic which arises in determination of c_∞ on substitution of values in Eqn. 5. Plots of t and c show that a steady state is more rapidly reached when S is low and conversely takes longer to achieve as S rises. The differences in the speed of attainment of steady state according to Eqn. 6 for varying concentrations of S when K_s and K_c are very small is dramatic but less so when K_c is round 50 mM.

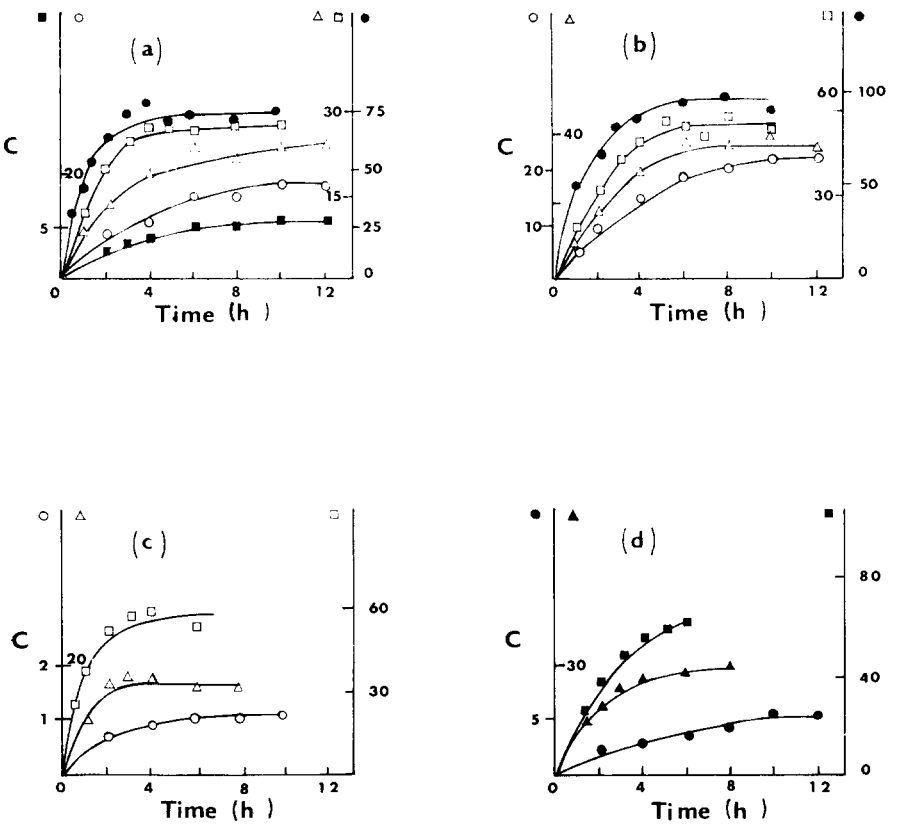


Fig. 5. Time courses of uptake by chicken embryo hearts of aminoisobutyrate (a, b) and cycloleucine (c, d) in the absence (a, c) and presence of insulin (b, d) at different medium concentrations of amino acid. c is in μmoles/ml of cell water. Note that the scale for c is different for different values of S. (a) S (mM): ■, 0.1; ○, 0.3; △, 0.6; □, 2; ●, 20. (b) S (mM): ○, 0.3; △, 0.6; □, 2; ●, 20. (c) S (mM): ○, 0.1; △, 2; □, 20. (d) S (mM): ●, 0.1; ▲, 2; ■, 20.

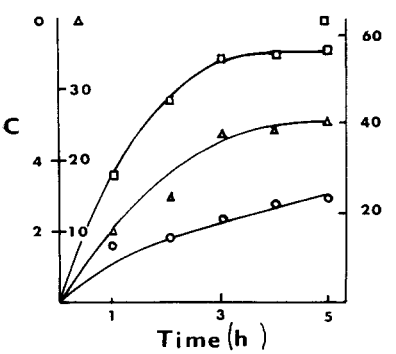


Fig. 6. Time course of uptake of cycloleucine by isolated heart cells. c is in μmoles/ml of cell water. The scale for c is different for different values of S (mM): ○, 0.1; △, 1; □, 20.

The time course of uptake of aminoisobutyrate and cycloleucine at different external solute concentrations by intact embryo hearts and isolated cardiac cells are shown in Figs. 5 and 6. The speed of approach to equilibrium is found to be greater for higher values of S. This is the converse of the predictions of Eqns. 5 and 6, but is consistent with the idea that increasing intracellular concentration of amino acid diminishes the capacity of the machinery to accumulate further material. As the size of the kinetic parameters declines so the existing degree of accumulation will more rapidly approach the steady-state concentrations applicable to that situation. It will be noticed (Fig. 5) that for similar values of S the time taken to reach equilibrium in the presence of insulin is longer (and absolute value of c higher) than in its absence.

In view of these findings it is probable that the points for the lowest concentrations of S in Tables I and II represent incomplete equilibration. Additional experiments in which incubation proceeded for longer, however, gave only marginally different results for the kinetic constants.

DISCUSSION

As far as the authors are aware the present procedure has been little used hitherto for determination of kinetic constants of solute accumulation by tissues[14,15]. It has the advantage that it minimises the need for speed and accuracy of timing in manipulating and transferring tissue from various bathing solutions. It also avoids the problem of the influence of rate of transfer of solute between medium and extracellular fluid which arises when rate of uptake to or efflux from the intracellular compartment are being measured. On the other hand steady-state measurements alone do not allow deduction of unequivocal figures. What the present results emphasise is that the values of the kinetic parameters of the uptake process (saturable component) change with the intracellular solute concentration, a phenomenon similar to the "trans-inhibition" described by other authors[6]. Values derived under steady-state conditions thus represent lower limiting values whereas data of initial entry rates provide the upper limits. Impressive is the fact that though both K_s and V decline as the value of c rises, K_D appears to remain unchanged. As mentioned in the introduction it seems possible that in a three-compartment system an apparent decrease in K_D in the presence of insulin might be a consequence of an increase in V making for more efficient reabsorption of material leaking from the cell into the interstitial fluid. That the determined values of K_D from efflux[6] are consistent with the data obtained in steady-state conditions, which minimise aberrations resulting from the use of a three-compartment system, suggests that the values of K_D are in fact correct. Several implications follow from this: (a) that insulin as well as influencing the activity of the active, presumably carrier mediated, process of uptake can also affect non-active diffusion; (b) that the rate of diffusion, unlike other parameters, does not change as the intracellular concentration of solute changes; (c) that the more rapid attainment of steady state as S increases is indicative of some alteration in parameters due to c (or S) since from K_D attainment of equilibrium would not be expected to be so rapid, and (d) that the rate of interchange of material between medium and interstitial fluid in the embryo hearts is sufficiently rapid such as not to prejudice direct measurement of K_D from efflux.

An alternative explanation to that suggested above for difference in time taken to reach equilibrium for different values of S can be based on the question of the extent to which diffusion through the interstitial space might influence the rate of cellular uptake. Thus if entry into the interstitial fluid is solely by diffusion, the rate of solute movement will be proportional to the concentration gradient, which will of necessity be minimal at the lowest media concentrations. A low rate of entry of amino acid into the interstitial fluid, particularly in proportion to the rate of active uptake into the cell, might be expected to slow down the approach to equilibrium, which is what is observed here. The rate of diffusion of amino acid from medium to extracellular fluid is not known. Unfortunately the cell water of embryo hearts at five days is penetrated by sorbitol[17], but measurements with mature rat heart suggest a value of K_D (subsequently called K_D' to distinguish from K_D for intracellular movement) for entry of sorbitol into extracellular fluid of 150 h^{-1} (ref. 18).

One can attempt to assess the extent to which diffusion through the extracellular space might limit cellular uptake on the following basis. The rate of net uptake will be greatest when intracellular concentration is low and will approximate to

$$\frac{V \cdot S}{(K_s + S)} + K_D \cdot S \tag{7}$$

What concentration gradient from medium to extracellular space is necessary to ensure an inflow equal to cellular uptake, or to what extent is the concentration in the extracellular fluid (S') less than S? A steady state will arise when

$$K_D'(S - S') = \frac{V \cdot S'}{(K_s + S')} + K_D \cdot S' \tag{8}$$

If we assume for the embryo hearts in the presence of insulin that $K_D' = 100\ h^{-1}$, $V = 41$ μmoles/ml per h (ref. 6) and $K_s = 0.76$ mM (the term including K_D is insignificant if K_D is less than 1 h^{-1}), then when $S = 0.1$ mM, S' is around 0.06 mM. Thus under these circumstances entry of amino acid into the extracellular fluid will delay cellular uptake. However, the values chosen are likely to represent the extreme situation, that is with K_D' at its lowest likely value, with K_s as derived from the steady-state data and in the presence of insulin, and taking V at its maximum. The calculation is also based on the lowest value of S employed and is calculated before efflux from the cell contributes to the extracellular pool. The results with isolated cardiac cells, showing a faster attainment of steady state as S increases even in a two-compartment system, support the argument that the extracellular transfer of the amino acid in the intact tissue cannot be regarded as an important rate-limiting step for uptake, other than in extreme conditions.

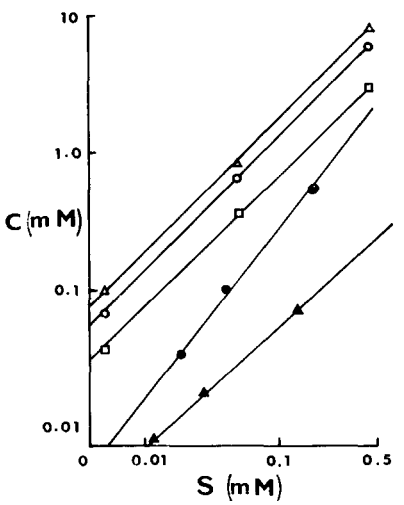
In the calculations investigating the existence of a saturable efflux process the value of V was assumed to be equal both inwards and outwards[19]. It is possible that this assumption is unjustified. Competition by amino acids in the cell with aminoisobutyrate or cycloleucine for attachment to the carrier will slow down efflux, but this should show as an increase in K_c rather than lower V. A V outwards lower than inwards would render the process less effective, and it is not clear what circumstances would raise it. It is of course implicitly assumed that the intracellular amino acids are free in solution[20]. For what purpose the cells so avidly accumulate the amino acids is

not clear, a concentration of aminoisobutyrate of nearly 100 mM being attainable by the embryo hearts (Fig. 5).

At steady state the fitting of data (Table III) is consistent with efflux being either by diffusion alone, or by a saturable process together with diffusion. The data are not compatible, except possibly for cycloleucine movements in diaphragm, with efflux being solely by a saturable process — reflecting itself in very high V/K_D values. Plots of log c against log S (shown for diaphragm in ref. 1) do not produce the straight line which is to be expected if diffusion plays a negligible role in efflux since Eqn. 4 rearranges to

$$\log c = \log S + \log (K_D/K_s) \tag{9}$$

This is of particular interest in that analysis of data for uptake of amino acids by brain slices and the small intestine[21] have been shown to do just the reverse, namely to fit Eqn. 9 but not Eqn. 2. CHARALAMPOUS[22] has recently found from non-steady-state kinetics a K_s for aminoisobutyrate uptake by KB cells of about 0.4 mM and a K_c for efflux of 6.5 mM. Following the example of COHEN[21] the figures provided by LEMBACH AND CHARALAMPOUS[23] for steady-state concentrations of aminoisobutyrate and serine in KB cells can be tested for fit to Eqn. 9 or 2. Although there are only three points for each experiment they fall in a remarkably straight line on the log/log plots (Fig. 7) from which it may be concluded that diffusion plays a relatively small part in amino acid movements in these cells; this despite their finding and that of SCHOLEFIELD[15] that efflux was first order.



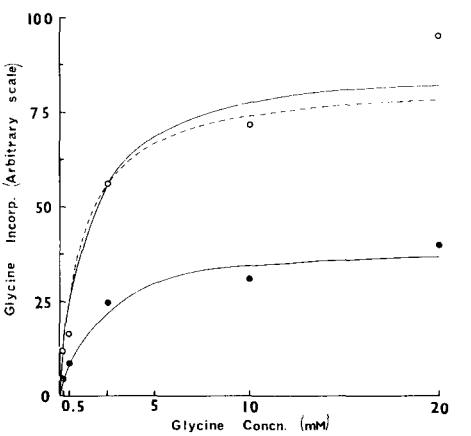


Fig. 7. Plot of log c *versus* log S for the steady-state distribution of aminoisobutyrate in normal (△, ●) and inositol-deficient (○, □, ▲) KB cells. From data (Tables 7 and 8) of LEMBACH AND CHARALAMPOUS[23].

Fig. 8. The rate of incorporation of [^{14}C]glycine into the protein of isolated rat diaphragm with various glycine concentrations. The circles represent the data of HECHTER AND HALKERSTON[24] in the presence (○) and abscence (●) of insulin. The solid lines are extrapolated Michaelis–Menten curves with values of $K_m = 1.43$ mM, $V = 88.4$ and $K_m = 1.73$, $V = 40$. The broken curve is from $K_m = 1.24$, $V = 83.4$. For origin of values, see text.

A final important question is the significance of apparent K_m 's for transport processes. As others have pointed out curves tending to an upper limiting value lend themselves all too readily to analysis in this way. With the uptake of unutilised amino acids it is possible to regard the uptake process as essentially one step and the above analysis therefore rational. However, given a certain latitude of experimental error, it is readily possible to fit a Michaelis–Menten curve to the data shown in Fig. 8 for the incorporation of [^{14}C]glycine into protein of diaphragm. These figures which come from HECHTER AND HALKERSTON[24] were not intended for analysis in this way, but values of 1.73 ± 0.17 mM for the K_m for incorporation of glycine in the absence of insulin and 1.24 ± 0.26 in its presence are readily obtainable by normal graphical means. By the procedure of BARBER *et al.*[25], which minimises the influence of a variable V in different pieces of tissue, the data show an apparent K_m of 1.73 ± 0.4 mM in the absence of insulin and 1.43 ± 0.2 in its presence. Protein synthesis clearly is a more complex process than merely uptake of amino acid. However, the values of K_m are remarkably close to what is obtained by substituting figures provided by CHRISTENSEN AND RIGGS (Fig. 1 of ref. 26 for uptake of glycine into ascites cells), namely 1.54 mM. In a different context it is interesting to note that HARRIS AND MANGER[27] found a K_m for succinate oxidation by intact mitochondria substantially larger than when they used mitochondrial fragments, from which they deduced that the larger K_m reflected the transport step. Similar arguments are implicitly assumed when kinetic parameters for glucose uptake are measured from rates of $^{14}CO_2$ production[28,29]. However, the effects of insulin on K_s and V for glucose uptake by responsive cells are confused[30–38]. It is of interest that both testosterone and estradiol promote uptake of amino acids by responsive tissues and in these instances the enhancement appears to result from a rise in V rather than change in K_s (refs. 39,40). Unfortunately, the significance of kinetic measurements is minimised by the absence of clear information as to the chemical nature of the carrier.

ACKNOWLEDGMENTS

We are grateful to Professor A. F. Huxley for advice and for derivation of Eqn. 6 and to Mr. A. Dunlop for help with computing. The skilled technical assistance of Dr. G. C. Gazzola with some of the experiments is appreciated. The work was assisted by grants from the National Institutes of Health (AM-05290), U.S. Public Health Service, Bethesda, Md., U.S.A. (to G.G.G.) and from the Medical Research Council of Great Britain (to K.L.M.).

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