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Kinetic Analysis of Insulin Action on Amino Acid Uptake by Isolated Chick Embryo Heart Cells

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1. Isolated chick embryo heart cells were used to investigate the mode of action of insulin on the transport of three naturally occurring amino acids: L-proline, L-serine and glycine. Initial velocities of uptake were measured over a period of 5 min with an 80-fold range of amino acid concentration. Corrections for amino acid diffusion, incorporation into protein and conversion into carbon dioxide were introduced. 2. The uptake processes approximated Michaelis–Menten kinetics within definite ranges of amino acid concentrations. A single transport system for proline and at least two transport systems for serine and glycine were detected. 3. The kinetic effects of insulin on transport systems for the amino acids tested were consistent with an acceleration of the maximal velocity of the process, without substantial changes in substrate concentration for half-maximal transport velocity. 4. These hormonal effects were not essentially altered by the corrections for amino acid incorporation into protein and conversion into carbon dioxide.

Insulin is known to enhance the uptake of amino acids by muscle tissue (Manchester & Wool, 1963; Wool, Castles & Moyer, 1965). Previous attempts to analyse the mechanism of this action of the hormone were performed with non-utilizable amino acid analogues (Akedo & Christensen, 1962; Elsas, Albrecht & Rosenberg, 1968; Guidotti *et al.* 1968*a*; Guidotti, 1971). Kinetic studies with naturally occurring amino acids have been limited hitherto by the fact that substantial fractions of these compounds are metabolized intracellularly when they are added *in vitro* to muscle preparations at the concentrations required for such experiments (Guidotti *et al.* 1968*b*; Guidotti, Lüneburg & Borghetti, 1969; Manchester, 1970). In a previous paper (Guidotti *et al.* 1969) we described the preparation of cell suspensions from chick embryo hearts. We hoped that this insulin-sensitive single-cell system might be useful in investigations of initial rate kinetics of transport of natural amino acids (and effects of insulin thereon); the rapid equilibration of the entire cell population with the tracer present in the medium was likely to allow studies over short times of incubation, minimizing the fraction of amino acid taken up by the cells that is metabolized.

It is the purpose of the present paper to define the kinetics of transport of three naturally occurring amino acids (glycine, L-proline and L-serine) in isolated chick embryo heart cells and to analyse the nature of the action of insulin on the entry process of these molecules. Glycine, proline and serine were selected for this study because they are actively accumulated against a concentration difference by the cardiac cell, are metabolized at a relatively slow rate (as compared with other natural amino acids), and respond to insulin with a remarkable acceleration of the rate of their penetration (Guidotti *et al.* 1969). Some metabolic conversions of these natural substrates during uptake were investigated in addition to the simple accumulation of labelled compound.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Glycine, L-[U-^{14}C]proline, L-[U-^{14}C]-serine and [*methoxy*-^{3}H]inulin were obtained from New England Nuclear Corp. (Boston, Mass., U.S.A.); unlabelled amino acids and glucose were from British Drug Houses Ltd. (Poole, Dorset, U.K.). Collagenase (batch CLS 9FC, 137 units/mg) was purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.); bovine insulin (Lilly, batch no. PJ4609, 23.8 units/mg) was a gift from Dr Mary Root (Eli Lilly Research Laboratories, Indianapolis, Ind., U.S.A.).

Preparation of isolated heart cells and preincubation. The procedures for heart dissection from 7-day-old chick embryos and isolation of cells from hearts were as described by Guidotti *et al.* (1969). The isolated cells, suspended in Krebs–Ringer bicarbonate buffer, pH 7.4, (Cohen, 1959) containing 8 mM-glucose (1×10^{7}–2.5×10^{7} cells/ml), were placed in silicone-treated glass vessels and preincubated for 60 min at 37.5°C under continuous mild stirring in the absence and in the presence of insulin (0.5 unit/ml). Samples were withdrawn for protein determination by the method of Lowry, Rosebrough,

Farr & Randall (1951) and by a biuret procedure (Layne, 1957), with crystallized bovine plasma albumin for protein standards.

Incubation. Incubations were carried out in a Dubnoff metabolic shaker oscillating at a rate of 75 cycles/min at 37.5°C. Samples (1 ml) of cell suspension were transferred into silicone-treated conical flasks containing 1 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, supplemented with 8 mM-glucose and the labelled amino acid under study at concentrations in the range 0.25–20 mM (0.125–10 mM after addition of the cells); the specific radioactivity of labelled amino acids was varied to maintain the radioactivity of the medium between 1 and 2 μCi/ml. The gas phase was O_2+CO_2 (95:5). The flasks were incubated for 5 min in the absence and in the presence of insulin (0.5 unit/ml). In additional experiments the amino acid concentration in the incubation medium ranged from 40 to 100 mM; in these experiments incubation lasted 10 min.

Measurement of amino acid accumulation. Incubation was terminated by adding to the flask 8 ml of ice-cold Krebs–Ringer bicarbonate buffer, pH 7.4, containing [^{3}H]inulin (0.4 μCi/ml). The flasks were swirled to provide rapid mixing and the content was filtered off as reported by Guidotti *et al.* (1969), except that micro fibre-glass filters (Millipore, type AP20) replaced the cellulose plastic filters used previously. The whole procedure (from addition of cold buffer to the end of filtration) took 20 $\pm$ 2 s. Cell-loaded filters were extracted by boiling in 3% (w/v) sulphosalicylic acid (Guidotti *et al.* 1969). Samples of extraction fluids were added to a scintillation mixture composed of toluene–methyl-Cellosolve (7:3), 2,5-diphenyloxazole (0.4%, w/v) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.01%, w/v), and their radioactivity was counted in a Packard Tri-Carb spectrometer with efficiencies of 50–60% for ^{14}C and of 10–15% for ^{3}H. Radioactivities of samples of ^{14}C-labelled amino acid-containing medium, [^{3}H]inulin-containing buffer and suitable mixtures of the two were counted likewise. Portions of extraction fluids were used for chemical identification of tissue radioactivity by electrophoretic analysis on Schleicher and Schuell paper (type no. 2317) with acetic acid–formic acid–water (2:3:15, by vol.), pH 1.8, for 2 h in a refrigerated Electrophorator (10 V/cm). Labelled amino acid spots were located with the aid of marker amino acids run concurrently, sprayed with ninhydrin (0.2%, w/v, in water-saturated butan-1-ol), and by means of radioactive tracing with a radiochromatogram scanner (model 7201, Packard Instrument Co. Inc.). Zones containing radioactivity were cut out, placed in counting vials and their radioactivities counted in a Packard Tri-Carb spectrometer after addition of the scintillation mixture (composition as mentioned above).

Amino acid incorporation into protein and conversion into $^{14}CO_2$. The procedures for measuring the incorporation of [^{14}C]glycine, [^{14}C]proline and [^{14}C]serine into protein and their conversion into CO_2 were essentially identical with those described by Guidotti *et al.* (1968*b*).

Calculations. The intracellular radioactivity (d.p.m./ml of cell water) of the ^{14}C-labelled amino acid under study was calculated as follows: the radioactivity (d.p.m. of ^{14}C) accumulated by the cells, obtained by the difference between the total radioactivity (d.p.m. of ^{14}C) of the extraction fluid from the cell-loaded filter and the radioactivity (d.p.m. of ^{14}C) present extracellularly [as estimated by the radioactivity of [^{3}H]inulin, on the assumption that this molecule was restricted to the extracellular compartment, and by the actual (d.p.m. of ^{14}C)/(d.p.m. of ^{3}H) ratio directly measured in standard samples], was divided by the volume of intracellular water (in ml) in the actual experiment (calculated from data on protein content as described by Guidotti *et al.* 1969).

The intracellular concentration of the amino acid, expressed in μmol/ml of cell water, was obtained by dividing the intracellular radioactivity (d.p.m./ml of cell water) by the radioactivity of the medium at the beginning of the incubation (d.p.m./ml) and multiplying the result by the concentration of the amino acid in μmol/ml of medium.

Experiments with high concentrations of amino acid in the medium (40–100 mM) were used to determine the rate constants for diffusion by extrapolation to infinite concentration (Akedo & Christensen, 1962). The formulation of Christensen & Liang (1966) was utilized to correct net uptake velocity for the diffusion component. The results relating substrate concentration to velocity were analysed by graphical transformations (Dowd & Riggs, 1965) and by computer methods. Separation of serine and glycine transport into component systems was achieved by programming a digital computer (Univac 1108) to read out the total transport velocity from the summation of independent Michaelis–Menten expressions, on the assumption that single-transport components contributed independently to the observed saturatable uptake of the amino acid. The Fortran V programme for this analysis (providing the best estimates of V and K_m values for each transport system) has been developed by Dr V. Stampanoni Bassi (Centro di Calcolo, Università di Milano) from a general automatic procedure for function optimization (Buzzi Ferraris, 1968).

To introduce corrections for the fractions of amino acid incorporated into protein and metabolized to CO_2 after penetration into the cell, the data of the pertinent experiments were expressed as μmol of amino acid (incorporated into protein or converted into CO_2)/ml of cell water and the fraction of them that entered the cell by the saturatable component of transport was calculated (incorporation into protein and conversion into CO_2 were considered as irreversible entry processes; see Christensen & Liang, 1966). The degradation of proline and serine to CO_2 was probably overestimated because the process has been considered as a simple decarboxylation of the two uniformly labelled amino acids; however, the error introduced in the subsequent corrections was negligible, since the contribution of this component to the saturatable transport of proline and serine is very small (less than 1%) in our experimental conditions.

RESULTS

The experimental conditions were such that the rate of intracellular accumulation of the labelled amino acids was linear over the entire incubation period at all the external concentrations used.

The extrapolation to infinite concentration (Akedo & Christensen, 1962) of the data from experiments with high amino acid concentrations in the medium (40–100 mM) provided values of

apparent rate constants for diffusion of 0.051, 0.006 and 0.019 min^{-1} for proline, serine and glycine respectively. Insulin did not alter these values.

Initial uptake velocities of glycine, proline and serine, determined over an 80-fold range of amino acid concentration in the medium (0.125–10 mM), were corrected for the non-saturatable component (so designated with the usual reservations about the meaning of this term: Christensen & Liang, 1966; Guidotti *et al.* 1968*a*) and plotted by either the double-reciprocal method ($1/v$ versus $1/[S]$) or by the method in which velocity is plotted against velocity over concentration (v versus $v/[S]$). In both cases straight lines were obtained with proline over the entire range of amino acid concentration in the medium, suggesting that, under these conditions, the uptake process approximated Michaelis–Menten kinetics. Data for serine and glycine could be plotted to lines with two different slopes.

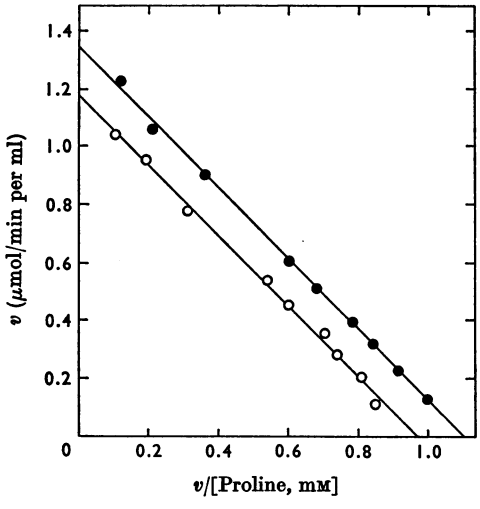

Fig. 1. Initial velocities (v) of proline uptake (saturatable process) by isolated chick embryo heart cells plotted against the ratio of velocity to medium concentration. The data points are means of four experiments in the absence (○) and in the presence (●) of insulin. Curves are derived by the method of the least squares. The experimental details are given in the text.

Fig. 1 shows the plot of v versus $v/[S]$ for proline. The presence of insulin enhanced V (maximal initial velocity of transport) by approx. 15% (Table 1) without altering the apparent K_m (substrate concentration for half-maximal transport velocity). The correction for the fraction of amino acid incorporated into protein and converted into CO_2 that entered the cell irreversibly by the saturatable process did not change substantially the values of the kinetic constants or the extent of insulin stimulation of V (Table 1). Indeed, the increment of v introduced by this correction was rather small, ranging between 9% (at the lowest amino acid concentration in the medium) and 3–4% (at the highest concentration); most of it was attributable to incorporation into protein.

With serine (Fig. 2) the initial velocity of uptake by the saturatable process tended to increase more rapidly than was expected at external amino acid

Table 1. *Effect of insulin on the kinetic constants derived from studies of transport of glycine, proline and serine in chick embryo heart cells*

Values of the kinetic constants of amino acid transport by the saturatable process were calculated from the curves presented in Figs. 1, 2 and 3 by using computer methods (see text). Values in the two columns at the right were calculated after correction of the data of initial uptake velocity (by the saturatable process) for the fractions of amino acid incorporated into protein and converted into CO_2 that entered the cell irreversibly by the saturatable process. The two components of serine and glycine uptake have arbitrarily been numbered I (low-concentration system) and II (high-concentration system) for convenient reference.

		Uptake by saturatable transport		Values corrected for amino acid utilization	
Amino acid	Insulin	Apparent K_m (mM)	V (μmol/min per ml)	Apparent K_m (mM)	V (μmol/min per ml)
Proline	−	1.22	1.18	1.17	1.20
	+	1.23	1.35	1.17	1.37
Serine I	−	0.59	0.75	0.40	0.59
	+	0.59	0.89	0.38	0.67
Serine II	−	8.83	2.88	7.09	2.89
	+	9.27	3.51	6.88	3.43
Glycine I	−	0.14	0.04	0.16	0.05
	+	0.15	0.06	0.18	0.07
Glycine II	−	7.94	2.61	8.25	2.79
	+	8.72	3.08	9.47	3.37

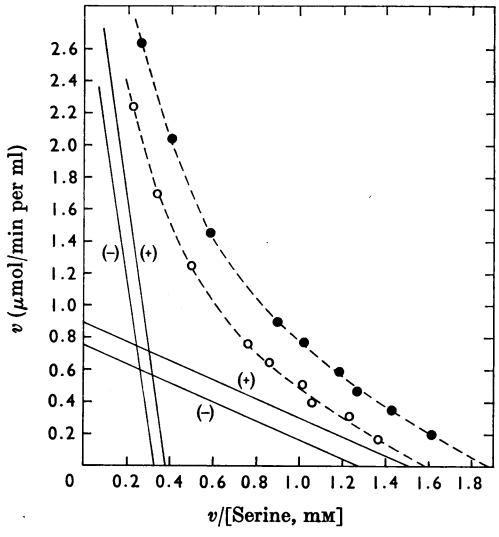

Fig. 2. Initial velocities (v) of serine uptake (saturatable process) by isolated chick embryo heart cells plotted against the ratio of velocity to medium concentration. The data points are means of three experiments in the absence (○) and in the presence (●) of insulin. Broken lines (total serine transport) are drawn according to the fitting of the data obtained by the digital-computer method given in the text. Continuous lines are single-transport systems in the absence (−) and in the presence (+) of insulin as derived from values of V and K_m (see Table 1) of two overlapping components separated by computer analysis.

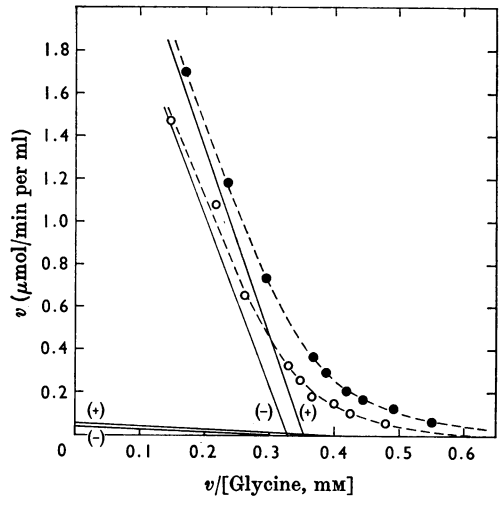

Fig. 3. Initial velocities (v) of glycine uptake (saturatable process) by isolated chick embryo heart cells plotted against the ratio of velocity to medium concentration. The data points are means of four experiments in the absence (○) and in the presence (●) of insulin. Broken lines (total glycine transport) are drawn according to the fitting of the data obtained by the digital-computer method given in the text. Continuous lines are single-transport systems in the absence (−) and in the presence (+) of insulin as derived from values of V and K_m (see Table 1) of two overlapping components separated by computer analysis.

concentrations higher than 1 mM, suggesting the presence of more than one transport system for this amino acid. Two independent overlapping components, consistent with Michaelis–Menten kinetics, were separated by computer analysis. As indicated in Table 1, they were defined by different values of V and K_m (and subsequently called system I and II according to their primary operational range at low and high substrate concentration respectively). Insulin enhanced V (by approx. 20%) of both systems of mediation and did not alter (or increased slightly) their apparent K_m. The intracellular conversion of serine into glycine did not affect these results substantially. As estimated by electrophoretic procedures, this conversion ranged between 8% and 12% at all the external concentrations used in the presence and in the absence of insulin. Correction of the experimental initial velocities shown in Fig. 2 with these conversion data did not alter the pattern of the uptake curves for serine and lowered the values of V by approx. 10% without affecting insulin stimulation. This correction was not made in subsequent calculations. The correction for the fraction of amino acid incorporated into protein and converted into CO_2 decreased the values of K_m (more for the system I than for the system II) and of V for the system I; it did not alter appreciably the values of V for the system II of mediation (Table 1). With serine, as with proline, amino acid incorporation into protein was the major source of these changes. No substantial alterations in the effects of the hormone were brought about by this correction.

The results obtained with glycine were similar to those reported for serine (Fig. 3) except that the point of inflexion of the curves occurred at a lower external amino acid concentration either in the presence or in the absence of insulin. Two independent overlapping transport systems were resolved (subsequently called system I and II according to their primary operational range at low and high substrate concentration respectively) and their kinetic constants defined (Table 1). Again, insulin enhanced the value of V for both systems of mediation and increased slightly the value of apparent K_m. As estimated by electrophoretic separation, the conversion of glycine into serine in tissue water was lower than 3% at all the external concentrations used. Correction of initial entry rates shown in Fig. 3 with these conversion data did not alter the pattern

of the uptake curves for glycine and it was omitted in subsequent calculations. When the data of initial velocity were corrected for the fraction of amino acid incorporated into protein and converted into CO_2 the values of apparent K_m and V were slightly increased (more for the system I than for the system II of mediation); insulin stimulation of V was substantially unchanged.

DISCUSSION

Isolated chick embryo heart cells represent a suitable biological preparation for the study of initial rate kinetics in muscle because of the lack of intervening tissue between the cell and the bathing medium. This structural property avoids the problem of diffusion of labelled molecules throughout the extracellular space; moreover, the fast and complete contact of the entire cell population with the compounds present in or added to the medium allows very short incubation times, minimizing intracellular metabolic effects.

The fact that heart cells are sensitive to insulin (Guidotti *et al.* 1969) offered the opportunity to investigate the effects of the hormone on the kinetics of transport of naturally occurring amino acids in a muscle tissue, extending to these molecules studies previously carried out with non-utilizable amino acid analogues (Akedo & Christensen, 1962; Elsas *et al.* 1968; Guidotti *et al.* 1968*a*; Guidotti, 1971). From measurements of initial velocities of uptake of α-aminoisobutyric acid by the rat diaphragm, Akedo & Christensen (1962) concluded that insulin acted by increasing the affinity of the transport site for the model amino acid without affecting the maximal velocity of the process. Elsas *et al.* (1968) have confirmed this. In contrast, our previous experiments (Guidotti *et al.* 1968*a*) with the same methodology applied to the chick embryo heart indicated that the hormone enhanced the maximal velocity without altering transport affinity. The result was confirmed when the kinetic constants of α-aminoisobutyric acid transport were evaluated by procedures based on measurements of steady-state values of distribution resulting from a balance between saturatable uptake and efflux processes connected with diffusion (Guidotti, 1971). It is noteworthy that the latter mathematical treatment provided evidence for a stimulation by insulin of maximal transport velocity also in rat diaphragm (Manchester, 1968).

The results of the present experiments with three naturally occurring amino acids, glycine, proline and serine indicate that insulin promotes the transfer of these molecules from the extracellular to the intracellular compartment by increasing the effectiveness of saturatable systems assumed to obey Michaelis–Menten kinetics. The pattern of the curves obtained with glycine and serine suggests the occurrence of at least two transport systems with differing affinities and capacities for both the amino acids. Similar findings for glycine (interpreted to indicate multiple transport systems) have been reported for rabbit reticulocytes (Winter & Christensen, 1965) and isolated renal tubules (Hillman, Albrecht & Rosenberg, 1968). Multiple transport systems have also been identified for proline in mammalian kidney (Hillman & Rosenberg, 1969; Mohyuddin & Scriver, 1970); our results indicate that this is not the case for isolated chick embryo heart cells (at least in a range of proline concentrations compatible with physiological values). In the present study, a detailed characterization of the various transport systems for glycine and serine (by competitive inhibition, metabolic inhibitors, ionic replacement etc.) was not attempted. However, as far as the action of insulin is concerned, the different systems of mediation for serine and glycine proved to respond similarly to the single transport system detected for proline. The kinetic effects of insulin on these systems are consistent with an acceleration of the maximal velocity of transport, without substantial changes in substrate concentration for half-maximal transport velocity. As reported above, the same result has been obtained with α-aminoisobutyric acid (Guidotti *et al.* 1968*a*).

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