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Amino Acid Uptake in the Developing Chick Embryo Heart

THE EFFECT OF INSULIN ON α-AMINOISOBUTYRIC ACID ACCUMULATION

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1. The uptake of ^{14}C-labelled α-aminoisobutyric acid by 5-day-old chick embryo hearts was investigated *in vitro*, together with the effect of insulin thereon. 2. At equilibrium the distribution ratio of this amino acid analogue between intracellular and extracellular water attained values greater than unity. Insulin enhanced the rate of α-aminoisobutyric acid accumulation and increased the value of its final concentration in the cell water. 3. The rate of α-aminoisobutyric acid accumulation and the effect of insulin on it were independent of the presence of glucose in the incubation medium. Bovine and chicken insulin were equally effective, and the action of the hormone was specifically prevented by an anti-insulin serum but not by puromycin. 4. A linear relationship was observed between the intracellular accumulation of the analogue and the logarithm of the insulin concentration in the range 50 μunits–100 m-units/ml. of incubation medium. 5. Evidence was obtained for the occurrence of two different transport processes for α-aminoisobutyric acid in the chick embryo heart: one subject to saturation and one that was not saturated by reasonable concentrations of the analogue. Insulin increased the effectiveness of the saturable component, increasing the maximal velocity of transport without altering the concentration for half-maximal velocity of transport, and decreased the contribution of the non-saturable component.

The stimulatory effect of insulin on the transport of amino acids across the cell membrane has received increasing attention in recent years. In muscle tissue, the detailed studies by Manchester & Wool (1963), Scharff & Wool (1965*b*) and Wool, Castles & Moyer (1965) established the experimental conditions under which the hormonal effect can be clearly demonstrated with most of the natural amino acids. The model amino acid α-aminoisobutyric acid, which is not metabolized because of the absence of α-hydrogen, was used by Akedo & Christensen (1962) to investigate the mechanism by which insulin affects the transport process in rat diaphragm.

As part of a study of the regulation by insulin of carbohydrate, lipid and protein metabolism in the chick embryo heart during ontogeny (Guidotti & Foà, 1961; Guidotti, Kanameishi & Foà, 1961; Foà, Melli, Berger, Billinger & Guidotti, 1965; Loreti, Gaja, Ragnotti, Borghetti & Guidotti, 1965; Guidotti, Loreti, Gaja & Foà, 1966), this and the accompanying paper (Guidotti *et al.* 1968) are devoted to a description of the effects of the hormone on the transport and accumulation of amino acids in this biological preparation. It is the purpose of the present paper to define the conditions under which insulin accelerates the uptake of α-aminoisobutyric acid by the chick embryo heart during the early stages of development as well as to explore, in some detail, the nature of the hormonal control of the movement of this model amino acid across the cardiac cell membrane.

MATERIALS AND METHODS

Chemicals. α-Amino[1-^{14}C]isobutyric acid was obtained from The Radiochemical Centre (Amersham, Bucks.); unlabelled α-aminoisobutyric acid and inulin were purchased from Mann Research Laboratories (New York, N.Y., U.S.A.); gelatin was from Difco Laboratories (Detroit, Mich., U.S.A.); puromycin dihydrochloride was purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.); 2,5-diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were obtained from Packard Instrument Co. Inc. (Downers Grove, Ill., U.S.A.); reagent-grade toluene and sulphosalicylic acid were from E. Merck A.-G. (Darmstadt, Germany); reagent-grade ethylene glycol monomethyl ether (methylCellosolve) was from C. Erba (Milan, Italy). Bovine insulin (Lilly, batch no.

466368, 26 units/mg.) was a gift from Dr Mary Root (Eli Lilly Research Laboratories, Indianapolis, Ind., U.S.A.); chicken insulin, assayed at 23 units/mg., was a gift from Dr I. Arthur Mirsky (University of Pittsburgh, Pa., U.S.A.).

Tissue preparation. Fertile chicken eggs (White Leghorn; Incubatoio Milanese, Milan, Italy) were incubated for 5 days in a commercial farm incubator. At appropriate intervals the eggs were opened, the embryos transferred to a large watch-glass containing Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit, 1932) and the hearts dissected out by means of thin blunt glass rods. All dissections were carried out on a warming plate keeping the temperature of the medium at 37·5°; a thin stream of O_2+CO_2 (95:5) was allowed to bubble through the buffer. With practice, each dissection took less than 20 sec. per heart, and the entire dissection period for a group of hearts never exceeded 10 min.

Preincubation. After two preliminary rinsings in glucose-free Krebs–Henseleit bicarbonate buffer the hearts were allowed to beat for 15 min. in the same medium supplemented with 8 mM-glucose, except in those experiments concerned with the effect of the absence of glucose. Other additions made during this phase are specified in the Tables. The preincubation was sufficient to empty the heart of all residual blood (Guidotti *et al.* 1961). The hearts were then blotted gently with acid-hardened Whatman filter paper (no. 50) and transferred to the incubation medium.

Incubation. Incubations were carried out in a Dubnoff metabolic shaker oscillating at a rate of 75 cyc./min. at 37·5°. In most experiments the hearts (five to eight) were placed in conical flasks or in beakers containing 2–4 ml. of Krebs–Henseleit bicarbonate buffer, pH 7·4, equilibrated with O_2+CO_2 (95:5) and incubated for 1–10 hr. When the incubation period exceeded 60 min. the flasks were sealed after gassing to prevent evaporation. The basic medium contained 8 mM-glucose and α-amino[1-^{14}C]isobutyric acid at concentrations ranging between 0·1 and 150 mM. The specific activity of the latter was varied to maintain the radioactivity of the medium between 0·1 and 0·5 μc/ml. The osmolarity of the medium was always adjusted to 0·30–0·33 by varying the total salt concentration. Insulin was dissolved in 3 mN-HCl at a concentration of 40 units/ml. When required the hormone was added to the incubation medium at a concentration of 0·2 unit/ml. except in those experiments where the effect of its concentration was investigated; in the latter experiments gelatin (1 mg./ml.) was added to the incubation medium to prevent insulin loss on glass surfaces and to ensure the even distribution of the hormone. When present puromycin was added at a concentration of 100 μg./ml. of medium. Other experimental conditions varied as described below.

Measurement of the accumulation of α-amino[1-^{14}C]*isobutyric acid.* At the end of the incubation period the hearts were quickly blotted on acid-hardened Whatman filter paper (no. 50), weighed on a Cahn electrobalance (sensitivity less than 2 μg.) and transferred into open long-necked glass ampoules containing 2 ml. of 3% (w/v) sulphosalicylic acid (Scharff & Wool, 1965*a*). The ampoules were placed in a boiling-water bath and refluxed for 10 min. After cooling and centrifugation, five 0·2 ml. portions of each extraction fluid were added to 10 ml. volumes of a scintillation mixture composed of toluene–methylCellosolve (7:3, v/v), 2,5-diphenyloxazole (0·4%, w/v) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0·01%, w/v) and counted in a Tri-Carb spectrometer (model 3365; Packard Instrument Co. Inc.) with an efficiency approaching 70% (as assessed by the use of internal standards) and a counting error of less than 1%. Samples of the initial and final medium were diluted tenfold with 3% (w/v) sulphosalicylic acid and counted in the same manner.

Extracellular space and total tissue water. The extracellular space of chick embryo hearts was taken to be equal to the volume of distribution of inulin. For the determination of this volume inulin was added to the medium at a concentration of 0·5 mg./ml. (Guidotti *et al.* 1966). After the selected period of incubation ten to 15 hearts were removed, blotted on acid-hardened filter paper, weighed and extracted for inulin (Del Monte, 1961). Inulin was assayed by the method of Roe, Epstein & Goldstein (1949). Total water was determined from the loss in weight of the blotted wet tissue after drying to constant weight in an oven at 100°.

Preparation of insulin antiserum and incubation in the presence of antiserum. Insulin antigen was prepared by mixing 1 part of bovine insulin solution (5 mg./ml.) with a mixture of 3 parts of mineral oil and 1 part of Falba wax gently heated to the melting point. Next 0·3 ml. of this antigen, containing approx. 8 units of insulin, was injected intradermally into guinea pigs every 2 weeks for 6 months. Hypoglycaemia was prevented by means of subcutaneous injections of glucose (1 g./kg. body wt., in a sterile 10% solution). Ten days after the last injection of antigen the blood of guinea pigs was collected by cardiac puncture, allowed to clot and centrifuged. The pooled antisera had an insulin-binding capacity of about 0·8–1 unit/ml.

A 0·2 ml. sample of either normal guinea-pig serum or antiserum was added to 0·8 ml. of Krebs–Henseleit bicarbonate buffer containing 8 mM-glucose with or without 40 m-units of insulin. This mixture was incubated for 110 min. at 37·5° in an atmosphere of O_2+CO_2 (95:5), after which time 1 ml. of Krebs–Henseleit bicarbonate buffer containing 8 mM-glucose and 0·4 mM-α-amino[1-^{14}C]isobutyric acid was added. Then 10 min. later five hearts, which had been incubated for 15 min. in bicarbonate buffer in another flask, were added and the incubation was allowed to continue for an additional 90 min. After the incubation period the hearts were blotted, weighed and extracted for radioactivity measurements as described above.

Wash-out experiments. In these experiments the hearts were incubated for 5 hr. in Krebs–Henseleit bicarbonate buffer containing 8 mM-glucose and 2 mM-α-amino[1-^{14}C]-isobutyric acid (specific activity 0·75 mc/m-mole) with or without insulin (0·2 unit/ml.). A suitable number of hearts were blotted, weighed and extracted for radioactivity measurements as described above; additional hearts were blotted, weighed, transferred to a large volume of α-amino-isobutyric acid-free medium (extracellular/intracellular fluid volume ratio 500:1) with or without insulin (0·2 unit/ml.) and incubated for 3 hr., during which time the rate of α-amino[1-^{14}C]isobutyric acid transfer out of the tissue was followed by measurements of radioactivity on samples of medium. At the end, the hearts were blotted, reweighed and extracted for radioactivity measurements as described above.

Calculations. The intracellular radioactivity R_i (counts/min./ml. of cell water) was calculated according to the formula:

$$R_i = \frac{R_t - R_e}{W_t - S_e}$$

where R_t is total radioactivity (counts/min.)/g. wet wt. of tissue as determined from direct measurement of the extraction fluid, R_e is radioactivity (counts/min.) contained in the extracellular space, calculated by multiplying the radioactivity (counts/min.)/ml. of the final medium by the volume of the extracellular space in ml./g. wet wt. of tissue (for this calculation it was assumed that the concentration of α-aminoisobutyric acid in the extracellular space at the end of the incubation was the same as its concentration in the final medium), and S_e and W_t are extracellular space and total water respectively, expressed in ml./g. wet wt. of tissue as determined by direct measurement (see above).

R_i was used to evaluate the intracellular concentration of α-amino[1-^{14}C]isobutyric acid in hearts, expressing it either as distribution ratio between intracellular and extracellular water [by dividing R_i by the radioactivity (counts/min.)/ml. of the medium at the end of the incubation] or directly as μmoles/ml. of intracellular water [by dividing R_i by the radioactivity (counts/min.)/ml. of the medium at the beginning of the incubation and multiplying the result by the concentration of α-aminoisobutyric acid in μmoles/ml. of the medium].

In order to analyse the kinetics of α-aminoisobutyric acid influx and efflux the data from accumulation and wash-out experiments were expressed as μmoles/g. wet wt. of tissue; for this calculation the radioactivity (counts/min.)/g. wet wt. accumulating or remaining in tissue (as determined by direct measurement or by subtraction from released radioactivity; see above) was divided by the radioactivity (counts/min.)/ml. of the initial medium and the result multiplied by the concentration of α-aminoisobutyric acid in μmoles/ml.

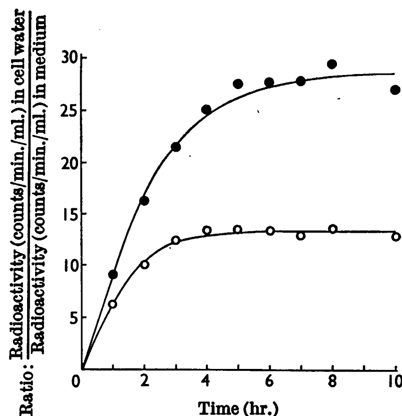


Fig. 1. Time-course of α-amino[1-^{14}C]isobutyric acid accumulation by 5-day-old chick embryo hearts in the absence (○) and in the presence (●) of insulin. Five hearts were preincubated for 15min. in Krebs–Henseleit bicarbonate buffer containing 8mM-glucose and subsequently incubated in 4ml. of the same medium supplemented with 2mM-α-amino[1-^{14}C]isobutyric acid (specific activity 75μc/m-mole). Bovine insulin was 0·2unit/ml. Incubation was at 37·5° in an atmosphere of O_2+CO_2 (95:5). Under these experimental conditions the concentration of the amino acid in the medium did not change substantially during the incubation period. The experimental points are means of three different experiments. Curves were drawn according to the fitting of the data obtained by using the digital-computer method of Berman, Shahn & Weiss (1962a) and Berman, Weiss & Shahn (1962b).

RESULTS

General characteristics of the preparation

The total tissue water of 5-day-old chick embryo hearts was found to average 0·886ml./g. wet wt. of tissue after 60min. of incubation and to remain constant during the following 9hr. This value was not appreciably altered by the addition to the medium of α-aminoisobutyric acid, insulin, gelatin or puromycin (for the last-named substance the incubation period did not exceed 2hr.). The inulin space of 5-day-old chick embryo hearts was 0·350ml./g. wet wt. after 60min. of incubation, increased to 0·377ml./g. wet wt. after 90–120min. and remained relatively constant during the next 8hr. These values are in accord with those obtained in previous measurements as well as those calculated from kinetic data of [^{14}C]glucose wash-out (Guidotti *et al.* 1966). The addition of α-aminoisobutyric acid, insulin, gelatin or puromycin (the latter substance in 2hr. incubations) did not alter substantially the extracellular space volume of the cardiac tissue. The reason for the initial increase in the inulin space is not known.

Uptake of α-aminoisobutyric acid by chick embryo hearts

Effects of insulin. The time-course of the accumulation of α-amino[1-^{14}C]isobutyric acid by 5-day-old chick embryo hearts incubated in the absence and in the presence of insulin is illustrated in Fig. 1. In both cases the model amino acid, added to the medium at an initial concentration of 2mM, was actively accumulated intracellularly against a chemical gradient. Its distribution ratio between intracellular and extracellular water increased almost linearly over the first 2hr. of incubation and approached a constant value after 3–4hr. in the absence and after 5–6hr. in the presence of insulin. The hormone enhanced the rate of accumulation of the amino acid and doubled the value of its concentration in the cell water at equilibrium.

The rate of α-aminoisobutyric acid accumulation and its acceleration by insulin were found to be independent of the presence of glucose in the incubation medium (Table 1), a result that accords with the observations of Manchester & Young (1960) on rat diaphragm. Bovine and chicken insulin produced a comparable stimulation of α-aminoisobutyric acid accumulation (Table 1); results indicating that bovine and chicken insulin were equally effective in stimulating the incorporation of labelled amino acids into protein of the chick

Table 1. *Effects of bovine insulin, chicken insulin, glucose, and puromycin on the accumulation of α-amino*[1-^{14}C]*isobutyric acid by 5-day-old chick embryo hearts*

Five hearts were preincubated for 15min. in Krebs–Henseleit bicarbonate buffer (with or without the additions described below) and subsequently incubated for 90min. in 2ml. of the same medium supplemented with 0·2mM-α-amino[1-^{14}C]isobutyric acid (specific activity 0·5mc/m-mole), in the absence or in the presence of the following additions: bovine and chicken insulin, 0·2unit/ml.; glucose, 8μmoles/ml.; puromycin, 100μg./ml. Incubation was at 37·5° in an atmosphere of O_2+CO_2 (95:5). Values are means±S.E.M. with numbers of experiments in parentheses.

	Ratio: Radioactivity (counts/min./ml.) in cell water / Radioactivity (counts/min./ml.) in medium		
Additions	No insulin	Bovine insulin	Chicken insulin
No glucose	22·7±1·6 (4)	33·3±2·2* (4)	—
Glucose	20·7±0·7‡ (9)	29·7±0·4*‡ (9)	29·6±1·1*§ (5)
Puromycin (+glucose)	13·1±0·7 (7)	15·5±0·4† (7)	—

* $P<0.01$ as compared with 'no insulin'.
† $P<0.02$ as compared with 'no insulin'.
‡ $P>0.05$ as compared with 'no glucose'.
§ $P>0.05$ as compared with 'bovine insulin'.

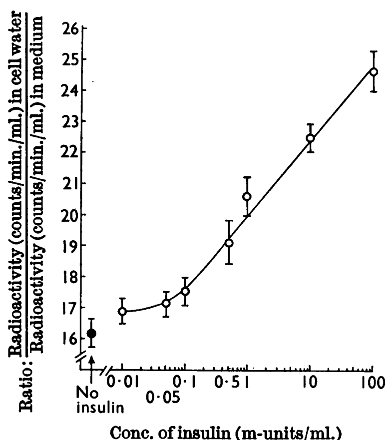


Fig. 2. Accumulation of α-amino[1-^{14}C]isobutyric acid in 5-day-old chick embryo hearts incubated in the presence of different concentrations of insulin (note logarithmic scale). Values are means±S.E.M. of ten experiments. The arrow points to the control value (●) obtained when the hearts were incubated in the absence of the hormone. Five hearts were preincubated for 15min. in Krebs–Henseleit bicarbonate buffer containing 8mM-glucose and subsequently incubated for 90min. in 4ml. of the same medium supplemented with 0·25mM-α-amino[1-^{14}C]isobutyric acid (specific activity 0·5mc/m-mole), gelatin (1mg./ml.) and bovine insulin. Incubation was at 37·5° in an atmosphere of O_2+CO_2 (95:5). In these experiments the concentration of the amino acid in the medium remained substantially constant during the incubation.

embryo heart at late stages of development have been reported (Loreti *et al.* 1965). Puromycin, added to the medium at a concentration that suppressed protein synthesis almost completely (as shown by a 95% inhibition of [^{14}C]leucine incorporation into protein of 5-day-old chick embryo hearts after 60min. of incubation), substantially decreased the intracellular accumulation of α-aminoisobutyric acid, but did not prevent its stimulation by insulin (Table 1). Persistence of the insulin effect on the accumulation of α-aminoisobutyric acid under conditions of puromycin-suppressed protein synthesis has been reported by Fritz & Knobil (1963) in rat diaphragm and by Scharff & Wool (1965*b*) in the perfused rat heart.

All experiments described above were carried out with high concentrations of insulin in the incubation medium. Examination of the response of the 5-day-old chick embryo heart to lower concentrations of the hormone (Fig. 2) revealed an approximately linear relationship between the intracellular accumulation of α-aminoisobutyric acid and the logarithm of the insulin concentration in the range 50μunits–100m-units/ml. The minimum effective concentration was 10μunits/ml.; however, a statistically significant effect ($P<0.02$) was first obtained with concentrations of 50μunits/ml. It should be pointed out that, under favourable conditions, the metabolism of the rat heart (Bleehen & Fisher, 1954) and rat diaphragm (Vallance-Owen & Hurlock, 1954) is affected by similarly low concentrations of insulin and that a linear relationship between the initial velocity of hexose penetration and the logarithm of insulin concentration in the range 20–1000μunits/ml. has been reported for frog sartorius muscle (Wohltmann & Narahara, 1966).

The specificity of the insulin effect was demonstrated in experiments where the acceleration of the

intracellular accumulation of α-aminoisobutyric acid by the hormone was abolished by an insulin antiserum prepared from guinea pigs (see the Materials and Methods section). When normal guinea-pig serum was added to the incubation medium the distribution ratio of α-aminoisobutyric acid between intracellular and extracellular water was $22\cdot1\pm0\cdot6$ in the absence of insulin and increased significantly ($P<0\cdot05$) to $26\cdot4\pm1\cdot3$ on addition of the hormone. Under the same experimental conditions, but with antiserum in place of normal serum, the distribution ratios of the amino acid analogue were $21\cdot5\pm0\cdot9$ in the absence and $21\cdot8\pm0\cdot4$ in the presence of insulin. (Values are means of four experiments ± S.E.M.)

Evidence for saturable and non-saturable transfer systems. When 5-day-old chick embryo hearts were incubated with increasing concentrations of α-aminoisobutyric acid (Table 2) in the absence of insulin, the intracellular concentration of the amino acid analogue increased rapidly up to extracellular concentrations of 2–5mM, then more slowly and almost linearly, but without tendency to reach a definite plateau even at the highest extracellular concentrations used. Under these conditions the distribution ratios of α-aminoisobutyric acid between intracellular and extracellular water were much greater than unity when its external concentrations were low, but decreased below unity at the highest concentrations, the decrement being more pronounced for extracellular concentrations increasing from 1 to 10mM. In the presence of insulin the rate of intracellular accumulation of α-aminoisobutyric acid was accelerated at low external concentrations, but approximated to the rate of amino acid accumulation achieved in the absence of insulin at extracellular concentrations higher than 20mM (Table 2). Also, in this case the distribution ratios of α-aminoisobutyric acid between intracellular and extracellular water largely exceeded unity at low external amino acid concentrations (being greater than the corresponding ratios found in the absence of the hormone) and decreased below unity at the highest external concentrations (approximating to the corresponding ratios found in the absence of insulin).

According to Christensen & Liang (1966*a*) these results can be interpreted to indicate that the total uptake of α-aminoisobutyric acid by the heart cell is dependent on two different processes, one subject to saturation and one failing to exhibit saturation within the range of concentrations used and hence following Fick's Law over this range. The non-saturable component was isolated and measured as described by Akedo & Christensen (1962), by plotting the cell-water/medium distribution ratios

Table 2. *Effect of insulin on the accumulation of α-aminoisobutyric acid by 5-day-old chick embryo hearts incubated with increasing concentrations of amino acid*

Five to eight hearts were preincubated for 15min. in Krebs–Henseleit bicarbonate buffer containing 8mM-glucose (with or without insulin) and subsequently incubated for 60min. in 4ml. of the same medium supplemented with α-amino[1-^{14}C]isobutyric acid at concentrations ranging between 0·1 and 150mM (radioactivity adjusted to 0·1–0·5μc/ml. of medium). Bovine insulin was 0·2unit/ml. Incubation was at 37·5° in an atmosphere of O_2+CO_2 (95:5). Under all experimental conditions the concentration of the amino acid in the medium remained substantially constant during the incubation. Values for total uptake are means ± S.E.M. of three experiments. Values in the two columns at the right were obtained by correcting the total uptake data for the contribution of the non-saturable component (Akedo & Christensen, 1962), considered as an irreversible entry process, whose kinetic constants were 0·368hr.$^{-1}$ in the absence and 0·246hr.$^{-1}$ in the presence of insulin.

Concn. of α-aminoisobutyric acid in medium (mM)	Intracellular concn. of α-aminoisobutyric acid (μmoles/ml. of cell water)			
	Total uptake		Uptake by saturable transport	
	No insulin	Insulin	No insulin	Insulin
0·1	1·19±0·03	1·64±0·09	1·16	1·62
0·2	2·28±0·03	3·18±0·12	2·21	3·13
0·5	5·11±0·22	7·59±0·07	4·93	7·47
1·0	9·29±0·59	12·52±0·40	8·93	12·28
2·0	14·40±1·25	19·85±1·13	13·67	19·36
5·0	21·84±0·75	29·75±0·74	20·00	28·52
10·0	26·71±1·45	34·80±0·40	23·03	32·34
20·0	33·13±0·95	41·40±1·37	25·77	36·48
50·0	46·23±1·26	49·68±1·15	27·83	37·38
75·0	56·93±1·13	59·55±2·41	29·33	41·10
100·0	63·96±1·40	64·98±2·11	27·16	40·38
150·0	81·90±2·82	77·03±0·23	26·70	40·13

for α-aminoisobutyric acid against the reciprocal of its extracellular concentration and then extrapolating to infinite concentration. By this method apparent rate constants of 0·37 hr.$^{-1}$ in the absence and 0·25 hr.$^{-1}$ in the presence of insulin were calculated, suggesting that the hormone tended to decrease the efficiency of the non-saturable transfer process. Total uptake values were then corrected for this component (considered as an irreversible entry process) and the resulting data (Table 2) were analysed for Michaelis–Menten kinetics on the assumption that they were initial velocities (i.e. independent of time and the intracellular concentration of the amino acid). The validity of this assumption was supported by 30 min. experiments (not shown), which furnished values for the saturable process that were about one-half of those obtained in the 1 hr. experiments presented in Table 2. The applicability of the Michaelis–Menten equation was demonstrated by graphical transformations that yielded straight lines in plots of $1/v$ versus $1/[S]$, $[S]/v$ versus $[S]$ and v versus $v/[S]$ (where v represents α-aminoisobutyric acid uptake by the saturable transport and $[S]$ is the concentration of the amino acid in the medium). The plot of v versus $v/[S]$ (Fig. 3) was used to estimate the kinetic constants (Dowd & Riggs, 1965): K_m (substrate concentration for half-maximal transport velocity), $2{\cdot}35\times10^{-3}$ M either with or without insulin; V (maximal initial velocity of transport), 28 μmoles/ml. of cell water/hr. in the absence and 41 μmoles/ml. of cell water/hr. in the presence of insulin. Allowing for some uncertainties in the slopes, these results suggested that, in the 5-day-old chick embryo heart, insulin did not affect the apparent affinity of the saturable transport site for α-aminoisobutyric acid and tended to raise the maximal velocity of amino acid transport.

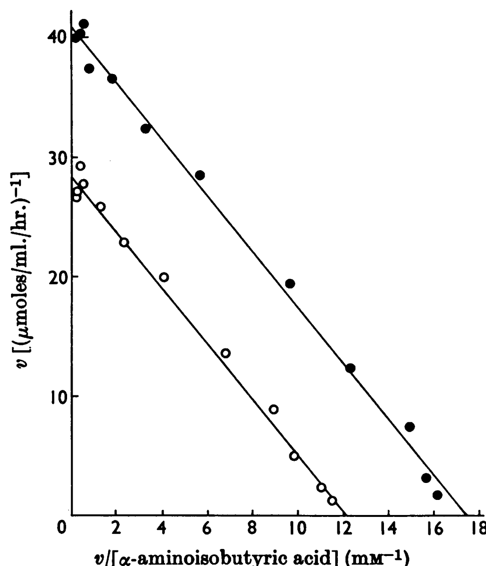


Fig. 3. Michaelis–Menten analysis of α-aminoisobutyric acid uptake (saturable component) by 5-day-old chick embryo hearts. The data points are plotted from the values of α-aminoisobutyric acid uptake by saturable transport, v, against $v/[S]$ (where [S] represents the extracellular concentration of amino acid given in Table 2). Curves, derived by the method of the least squares, yielded the following kinetic constants: in the absence of insulin (○), K_m $2{\cdot}35\times10^{-3}$ M and V 28·2 μmoles/ml./hr.; in the presence of insulin (●), K_m $2{\cdot}35\times10^{-3}$ M and V 40·9 μmoles/ml./hr. The experimental details are the same as given in Table 2.

Influx and efflux of α-aminoisobutyric acid. Table 3 provides data on the net accumulation (influx minus efflux) of α-aminoisobutyric acid by chick embryo hearts and on the efflux of α-aminoisobutyric acid from hearts preloaded with the amino acid (in the absence and in the presence of insulin). These data were fitted to a model, proposed on the basis of the experimental values and requirements, consisting of two compartments, medium and tissue, with transfer constants (or turnover rates) between them; solution required specification of the initial conditions and a model arrangement for rendering constant the concentration of the tracer in the first compartment (medium) as imposed by the experimental results. The second compartment (tissue) was not resolved for intracellular and extracellular space since the transfer of α-aminoisobutyric acid through the latter space was too rapid to be evaluated with sufficient accuracy. Least-squares fitting of the data to the model was obtained by using digital computer techniques with an IBM 7094 and the SAAM programme (Berman *et al.* 1962*a*,*b*), which yielded values for the transfer constants and estimates for their standard deviations. This procedure provided satisfactory solutions of the model both in the absence and in the presence of insulin. Table 4 shows the influx and efflux rate constants for the α-aminoisobutyric acid movements between medium and tissue. In the presence of insulin a substantial increase in the influx rate and a marked decrease in the efflux rate of the amino acid were observed. The values of the efflux rate constants in the absence and in the presence of the hormone are comparable with the values of the corresponding rate constants of the non-saturable transfer process (Table 2) that were obtained under experimental conditions in which the concentration ratios of α-aminoisobutyric acid between intracellular and extracellular water were lower than unity. These results are reflected in the data of Fig. 1, which revealed a definite enhancement of the initial concentration ratio of the analogue

Table 3. *Accumulation and wash-out of α-aminoisobutyric acid in 5-day-old chick embryo hearts: effect of insulin*

In accumulation experiments the hearts were preincubated for 15 min. in Krebs–Henseleit bicarbonate buffer containing 8 mM-glucose and subsequently incubated in the same medium supplemented with 2 mM-α-amino[1-^{14}C]-isobutyric acid (specific activity 75 μc/m-mole). Insulin (bovine) was 0·2 unit/ml. Incubation was at 37·5° in an atmosphere of O_2+CO_2 (95:5). Incubation conditions were such that the concentration of the amino acid analogue in the medium did not change appreciably during the experiments. At the intervals indicated below hearts were removed, blotted, weighed and extracted for radioactivity measurements. Values are means ± S.E.M. of three experiments. In wash-out experiments the hearts were incubated for 5 hr. as above in the absence and in the presence of insulin. At the end of this preloading period, during which equilibrium took place, a suitable number of hearts were removed, blotted, weighed and extracted for measuring the concentration of the labelled analogue in the tissue; additional hearts were blotted, weighed, transferred into 10 ml. of α-aminoisobutyric acid-free medium (extracellular/intracellular fluid volume ratio 500:1) either with or without insulin (0·2 unit/ml.) and incubated for 3 hr. At the intervals indicated below 0·1 ml. samples of the incubation media were withdrawn and assayed for radioactivity. Incubations were carried out at 37·5° in an atmosphere of O_2+CO_2 (95:5), and care was taken to maintain this atmosphere constant and to avoid medium evaporation during sample removal. At the end the hearts were blotted, reweighed and extracted for radioactivity measurements. The cumulative release and the concentration of α-aminoisobutyric acid remaining in the tissue during the second incubation period were calculated from the α-aminoisobutyric acid concentration in hearts extracted after the preloading incubation and from the radioactivity in the media to which the hearts had been transferred for wash-out. The α-aminoisobutyric acid concentration in the tissue at the end of the wash-out period as determined by direct measurement coincided with the value calculated by difference from released radioactivity within the limits of experimental error. Values are means ± S.E.M. of four experiments.

	α-Aminoisobutyric acid accumulation in hearts (net flux inward) (μmoles/g. wet wt. of tissue)			α-Aminoisobutyric acid remaining in hearts (efflux) (μmoles/g. wet wt. of tissue)	
Time (min.)	No insulin	Insulin	Time (min.)	No insulin	Insulin
60	7·37 ± 0·19	10·29 ± 0·33	0	13·08 ± 0·41	25·66 ± 0·10
120	11·02 ± 0·72	17·24 ± 0·60	2	12·30 ± 0·38	24·94 ± 0·05
180	13·43 ± 0·66	22·66 ± 1·53	5	11·98 ± 0·36	24·56 ± 0·07
240	14·42 ± 0·55	26·28 ± 0·55	10	11·52 ± 0·35	24·03 ± 0·08
300	14·46 ± 0·73	28·70 ± 0·71	15	11·20 ± 0·35	23·64 ± 0·09
360	14·31 ± 0·61	28·82 ± 1·77	20	10·81 ± 0·32	23·23 ± 0·10
420	13·85 ± 0·66	28·96 ± 0·58	25	10·58 ± 0·33	22·82 ± 0·11
480	14·48 ± 0·09	30·64 ± 1·17	30	10·21 ± 0·36	22·45 ± 0·09
600	13·77 ± 1·27	27·99 ± 2·26	40	9·62 ± 0·38	21·71 ± 0·10
			50	9·01 ± 0·36	20·95 ± 0·10
			60	8·45 ± 0·36	20·20 ± 0·10
			75	7·70 ± 0·34	19·18 ± 0·15
			90	6·97 ± 0·33	18·13 ± 0·15
			120	5·71 ± 0·30	16·35 ± 0·14
			180	3·78 ± 0·23	13·48 ± 0·13

between intracellular and extracellular water in the presence of the hormone as well as a pronounced increase of the eventual equilibrium attained.

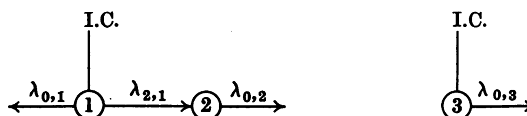
DISCUSSION

Suitability of the biological model: justification of experimental conditions and assumptions. The properties of the 5-day-old chick embryo heart that make it a desirable model for studies of tissue permeability *in vitro* have been discussed previously (Guidotti *et al.* 1961). Among them are: (i) the minimal variability in heart size and weight; (ii) the probability that the tissue had never been exposed to insulin, since the pancreatic islets of the chick embryo do not appear to be functional on the fifth day of development (Thommes, 1960); (iii) the remarkable viability of the heart *in vitro*, demonstrated by the fact that the heart beat regularly, consumed oxygen at a constant rate, maintained a gradient of amino acid concentration between tissue and medium, and preserved a normal electron-microscopic appearance for more than 15 hr. when suspended in a Krebs–Henseleit buffer supplemented with glucose (Guidotti, 1967).

Of the various experimental variables considered in the present study, the concentration of α-aminoisobutyric acid in the incubation mixture deserves brief discussion. It will be noted that, except in saturation experiments, the concentration of the

Table 4. *Influx and efflux transfer constants for α-aminoisobutyric acid movements between medium and tissue in 5-day-old chick embryo hearts: effects of insulin*

For experimental details see Table 3. The data for net flux inward and efflux of α-aminoisobutyric acid (as given in Table 3) were fitted simultaneously to the model with the following computing procedure:



where $\lambda_{2,1}$ is the transfer constant for movement into tissue from medium, $\lambda_{0,3}$ ($=\lambda_{0,2}$) is the transfer constant for movement into medium from tissue, $\lambda_{0,1}$ ($=-\lambda_{2,1}$) is the condition imposed to maintain constant the concentration in compartment 1, compartment 1 indicates the external medium, and compartments 2 and 3 indicate the tissue. The system formed by compartments 1 and 2 was used to fit the data of net flux inward; the system of compartment 3 was used for efflux data. I.C. are the initial conditions as follows: 2 μmoles/ml. of medium for compartment 1 either in the absence or in the presence of insulin; 13·08 μmoles/g. wet wt. of tissue in the absence and 25·66 μmoles/g. wet wt. of tissue in the presence of insulin for compartment 3 (see Table 3). The transfer constants are expressed as turnover rates ± S.D.

Experimental conditions	Transfer constants (hr.$^{-1}$)		Difference from control (%)	
	$\lambda_{2,1}$	$\lambda_{0,3}$	$\lambda_{2,1}$	$\lambda_{0,3}$
Control	3·63 ± 0·16	0·45 ± 0·02	—	—
Insulin	5·02 ± 0·24	0·27 ± 0·02	+38	−40

amino acid analogue in the medium was either 0·2–0·25 mM or 2 mM. The justification for this choice is as follows: the lower concentrations were used on the basis of preliminary kinetic experiments (Loreti *et al.* 1965) when linear rates of α-aminoisobutyric acid accumulation in the cell water were desired; the 2 mM concentration was selected when the experimental design required that both the saturable and non-saturable components be involved in the uptake process [at this extracellular concentration the contribution of the non-saturable component to the entry rate of the model amino acid did not exceed 5% after 1 hr. of incubation (Table 2), but the high intracellular concentration that resulted from the establishment of a gradient was likely to have rendered the non-saturable process quantitatively very significant in determining the exit rate of the amino acid analogue].

The major assumption on which the preceding and the following speculations on the concentrative aspects of the uptake process are based is that all transported amino acid exists free in solution within the cell, none of it being associated with intracellular binding sites. Though the question of the physical state of intracellular amino acids is still largely unresolved (Holden, 1962), direct evidence indicating that the concentrative nature of the uptake process for amino acids is not due to binding on to intracellular macromolecules has appeared (Udenfriend, Zaltzman-Nirenberg & Guroff, 1966). Accuracy in calculating the intracellular concentration of α-aminoisobutyric acid requires the additional assumption that all intracellular water is available for its distribution; if this were not the case, as has been reported for sugars (Morgan, Henderson, Regen & Park, 1961; Guidotti *et al.* 1966), intracellular concentrations of the analogue and distribution ratios have been underestimated. Finally, a correct estimate of the apparent diffusion constants for non-saturable transfer, obtained at very high extracellular concentrations of α-aminoisobutyric acid, requires that the partial replacement of salts in the incubation medium by the amino acid analogue does not impair energy metabolism or other cellular functions.

Mode of action of insulin on α-aminoisobutyric acid movements. The results presented in this paper indicate that α-aminoisobutyric acid, a non-utilizable amino acid analogue that seems to be transported across the cell membrane in a manner similar to that of some natural amino acids (Noall, Riggs, Walker & Christensen, 1957), undergoes concentrative transfer in 5-day-old chick embryo hearts. Insulin affects the movements of this analogue at concentrations that extend to physiological levels by a specific mechanism that is independent of the presence of glucose. Akedo & Christensen (1962) demonstrated that the entry of α-aminoisobutyric acid into rat diaphragm occurred by two different processes, one subject to saturation

(mediated transport) and one that failed to be saturated by any reasonable concentration of the analogue (either free diffusion, or transport mediated by reaction sites that are abundant or of very low affinity for the amino acid, or both). Our results suggest that the same is true for the chick embryo heart, since the uptake process could be resolved in two components, one consistent with Michaelis–Menten kinetics and one formally indistinguishable from free diffusion. It should be noted, however, that in the absence of insulin the contribution of the non-saturable component [as calculated as described by Christensen & Liang (1966*a*) assuming $V/K_m \simeq 12$ and a rate constant for the non-saturable component $K_D = 0{\cdot}37\,hr.^{-1}$] at low substrate concentrations accounted for only approx. 3% of the total uptake in chick embryo heart, whereas it represented approx. 40% of the total uptake in rat diaphragm (Akedo & Christensen, 1962). In the presence of insulin further discrepancies became manifest; the hormone, which did not affect the rate of the component not subject to saturation in rat diaphragm, decreased the rate of this process in chick embryo heart. In the latter tissue the contribution of the non-saturable component, as calculated from $V/K_m \simeq 17$ and $K_D = 0{\cdot}25\,hr.^{-1}$, represented 1·5% of the total uptake at low substrate concentration in the presence of insulin; under similar conditions, this contribution was approx. 4·5% of the total uptake in rat diaphragm (Akedo & Christensen, 1962). Since the apparent diffusion constants were of the same order of magnitude in the two tissues, and since the contribution of the non-saturable component to the total uptake at low amino acid concentrations was 13-fold greater in diaphragm than in heart in the absence of insulin and only threefold greater in the presence of the hormone, one may infer that the saturable component of the transport systems for α-aminoisobutyric acid is more efficient in the chick embryo heart than in rat diaphragm in the absence of insulin but less sensitive to the action of the hormone. Indeed, insulin appeared to act differently on the saturable component of transport: by increasing the maximal velocity without changing the affinity of the transport site for α-aminoisobutyric acid in chick embryo heart and by increasing the affinity of the transport site for the analogue without altering the maximal velocity of the process in rat diaphragm. We have no convincing explanation for this discrepancy. In our treatment, the calculation of the kinetic constants for the saturable component of transport involved the correction of total uptake data for the contribution of the non-saturable component, considered as an irreversible entry process (Table 2). If this component is assumed to approach a thermodynamic equilibrium of distribution with the ratio between intracellular and extracellular water being unity (Christensen & Liang, 1966*b*), its contribution to the total uptake would be negative when the mean distribution ratio (between intracellular and extracellular water) exceeds unity. Recalculation of the kinetic constants of the saturable component after the correction of total uptake data according to this condition (Christensen & Liang, 1966*b*) still indicated an effect of insulin to increase maximal velocity of transport and revealed a slight effect of the hormone on transport affinity, which appeared to increase by approx. 10–20%. However, this result is uncertain since the plot of v versus $v/[S]$ used to estimate the kinetic constants yielded a line slightly concave upwards for points obtained at high extracellular substrate concentrations, so that the applicability of the Michaelis–Menten equation was questionable. This difficulty was avoided when efflux transfer constants determined in wash-out experiments (Tables 3 and 4) were used as rate constants of the non-saturable process (considered to approach a thermodynamic equilibrium of distribution) for correcting total uptake data. Under these circumstances the plot of v versus $v/[S]$ was compatible with linearity, but again the calculation of the kinetic constants for the saturable component showed that insulin increased maximal velocity without changing the affinity of the transport site for α-aminoisobutyric acid. It is pertinent to note that the saturable transport system for glucose that appears in the chick embryo heart after the seventh day of development (Guidotti & Foà, 1961) is sensitive to insulin, which tends to lower the concentration for half-maximal transport velocity without altering the transport maximum (Guidotti *et al.* 1966).

The fact that in chick embryo heart insulin was found to restrain the non-saturable component of α-aminoisobutyric acid transport supports the work by Christensen & Liang (1966*a*) showing that this component is not identical with free diffusion and disproves the view that insulin acts by simply removing non-specific barriers to diffusion. This action of the hormone would be expected to affect α-aminoisobutyric acid efflux more strongly than its influx, since the concentration of the amino acid analogue is much higher in the intracellular than in the extracellular compartment except during the first few minutes of incubation. The experimental verification of an insulin-mediated decrease in α-aminoisobutyric acid efflux from the cardiac tissue (Tables 3 and 4) and the correspondence among the values of the efflux rate constants and rate constants of the non-saturable transfer process in the absence and in the presence of insulin (Tables 2 and 4) appear to confirm this prediction, since it is unlikely that the hormone would substantially alter the rate of a saturable outward process at

high intracellular substrate concentrations, which should tend to saturate it; however, no attempts were made to establish the existence of a saturable component participating in α-aminoisobutyric acid efflux.

The results described above lead to the conclusion that insulin enhances the intracellular accumulation of α-aminoisobutyric acid in the 5-day-old chick embryo heart, not only by increasing the effectiveness of the saturable component in achieving apparent uphill transport, but also by decreasing the contribution of the non-saturable component, which tends to lower the intracellular concentration of the amino acid analogue when its concentration ratio between intracellular and extracellular water exceeds unity. Presumably, as the rate of the inward transport of α-aminoisobutyric acid by the saturable component increases and the rate of its outward transport by the non-saturable component decreases, a substantial increment in the overall concentration gradient results. At the present time we do not know whether natural amino acids will respond to insulin in a similar manner; a preliminary description of the effects of insulin on the accumulation of glycine and L-leucine by 5-day-old chick embryo hearts is the subject of the accompanying paper (Guidotti *et al.* 1968).

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