Amino Acid Uptake in Isolated Chick Embryo Heart Cells

EFFECT OF INSULIN

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1. The preparation of cell suspensions by treatment of chick embryo hearts with collagenase at various stages of development is described. 2. Measurements of oxygen consumption, incorporation of labelled leucine into protein and accumulation of labelled α -aminoisobutyric acid against a concentration gradient indicated a long-lasting viability of the isolated heart cells *in vitro*; a satisfactory preservation of subcellular structures, including plasma membrane, was assessed by electron-microscopic examination. 3. The rate of α -aminoisobutyric acid accumulation by cardiac cells isolated from hearts at different stages of embryological development decreased with aging; insulin stimulated the intracellular accumulation of this amino acid analogue. 4. Insulin increased the uptake by isolated heart cells of several ^{14}C -labelled naturally occurring amino acids; however, the fraction of amino acid taken up by the cells that was recovered free intracellularly, and therefore the concentration ratio (between intracellular water and medium), was enhanced by the hormone only with glycine, proline, serine, threonine, histidine and methionine. When isolated heart cells were incubated in the presence of a mixture of labelled amino acids, the addition of insulin increased the disappearance of radioactivity from the medium. 5. The general pattern of amino acid transport (in the absence and in the presence of insulin) in isolated cardiac cells was similar to that found in intact hearts, suggesting that the biological preparation described in this paper might be useful for studies of cell permeability and insulin action.

Insulin stimulates the transport of amino acids across the cell membrane in muscle tissues (Manchester & Wool, 1963; Scharff & Wool, 1965b; Wool, Castles & Moyer, 1965). Previous attempts to elucidate the mechanism of this stimulation made use of complex biological preparations, including rat diaphragm (Akedo & Christensen, 1962; Elsas Albrecht & Rosenberg, 1968) and chick embryo heart (Guidotti *et al.* 1968a). Both these experimental models consist of three compartments: incubation medium, extracellular space and intracellular space. This fact renders the kinetic treatment of transport processes somewhat more complicated than in single-cell (two-compartment) systems (Robertson, 1957).

In an effort to develop a single-cell system for transport studies in insulin-sensitive muscle tissues, it was found that, if chick embryo hearts were treated with collagenase, cell suspensions were readily obtained that met the requirements for these studies. It is the purpose of the present paper to define the conditions for the preparation of cell suspensions from chick embryo hearts at various

stages of development, to investigate the viability of the isolated cells *in vitro* and to verify the adequacy of the biological preparation for studies of cell permeability. The effects of insulin on uptake and intracellular accumulation of amino acids in the isolated heart cells is also reported.

MATERIALS AND METHODS

Chemicals. α -Amino[1- ^{14}C]isobutyric acid, [1- ^{14}C]glycine, L-[ring-2- ^{14}C]histidine, L-[1- ^{14}C]leucine, L-[U- ^{14}C]phenylalanine, L-[U- ^{14}C]proline, L-[U- ^{14}C]valine and U- ^{14}C -labelled L-amino acid mixtures were obtained from The Radiochemical Centre (Amersham, Bucks.); L-[U- ^{14}C]lysine, L-[Me- ^{14}C]methionine, L-[U- ^{14}C]serine, L-[U- ^{14}C]threonine and [carboxyl- ^{14}C]inulin were purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.); unlabelled amino acids and glucose were from British Drug Houses Ltd. (Poole, Dorset); Proanalysis Grade acetic acid, aq. ammonia, butan-1-ol, ethylene glycol monomethyl ether (methyl-Cellosolve), propan-1-ol, sulphosalicylic acid, sodium deoxycholate and toluene were purchased from E. Merck A.-G. (Darmstadt, Germany); Hyamine hydroxide 10X, 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.); collagenase prepared from *Clostridium histolyticum* was obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.); Bactotrypsin (1:250) was from Difco Laboratories (Detroit, Mich., U.S.A.); testicular hyaluronidase (300 U.S.P. units/mg.) was a gift from Professor Angelo Bairati (Department of Anatomy, University of Milan, Italy); crystallized bovine plasma albumin was purchased from Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex); 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. Fertile fowl eggs (White Leghorn, Incubatoio Schaganski, Milan, Italy) were incubated for 5–19 days in a commercial farm incubator. At appropriate intervals the eggs were opened, the embryos removed and the hearts dissected out by means of thin blunt glass rods. The hearts were transferred to a large watch-glass containing Krebs–Ringer bicarbonate buffer, pH 7.4 (Cohen, 1959), supplemented with 8 mM-glucose, and allowed to beat until all residual blood had been released. These operations were carried out on a warming plate; the temperature of the medium was maintained constant at 37.5° and a thin stream of O₂ + CO₂ (95:5) was allowed to bubble through the buffer.

After two rinsings in fresh buffer and two additional rinsings in a Ca²⁺- and Mg²⁺-free Krebs–Ringer bicarbonate buffer, the hearts were blotted, minced (when dissected from 10-day-old or older hearts) and transferred (up to 1 g. of wet tissue) to a 25 ml. silicone-treated glass vessel containing 6 ml. of a solution of collagenase (2.5 mg./ml.) in Ca²⁺- and Mg²⁺-free Krebs–Ringer bicarbonate buffer equilibrated with O₂ + CO₂ (95:5). The vessel, equipped with a plastic-coated magnetic bar, was the inner part of a glass apparatus whose outer chamber was connected to a constant-temperature water circulator adjusted at 37.5°; the apparatus was set on the plate of a magnetic stirrer.

Complete dispersion of the tissue was obtained in 30 min. of incubation under continuous gentle stirring (60 rev./min.) and occasional sucking in and out by a silicone-treated blunt-bored pipette. The resulting cell suspension was passed through a sintered-glass filter funnel (pore size 40–60 µm.) to remove stromal and vascular material, and centrifuged for 5 min. at 250g. The supernatant was discarded and the cells were washed by suspending them in 8 ml. of Krebs–Ringer bicarbonate buffer supplemented with 8 mM-glucose and centrifuging for 5 min. at 250g. This procedure was repeated twice. All these steps were run in an atmosphere of O₂ + CO₂ (95:5). In some experiments the buffer mixtures used in the preparation of the cells contained bovine albumin (1%, w/v); in other experiments trypsin (0.5 mg./ml.), hyaluronidase (5 mg./ml.) or both substituted for collagenase as dissociating agents.

The isolated heart cells were finally suspended in an appropriate buffer volume. The suspension was swirled to ensure uniform distribution and the number of cells/ml. of suspension was determined by counts in a standard blood-counting chamber. Suspension samples were withdrawn for microscopy and protein determination by the method of Lowry, Rosebrough, Farr & Randall (1951) and by a biuret procedure (Layne, 1957).

Microscopy. Cell suspensions were examined as a routine by phase-contrast microscopy and by light-microscopy after

fixation with formaldehyde (10%, v/v), smearing and staining of the smears with haematoxylin and eosin.

In some experiments suspensions of heart cells, before and after incubation in glucose-supplemented Krebs–Ringer buffer, were centrifuged, fixed in glutaraldehyde, post-fixed in osmium tetroxide and embedded in Epon for electron-microscopic observation.

Protein content and intracellular water. Cells isolated from 5–19-day-old chick embryo hearts were suspended in Krebs–Ringer bicarbonate buffer containing [carboxyl-¹⁴C]inulin (0.4 µC/ml.; specific radioactivity 3.3 mC/g.). After repeated mixing the suspension was centrifuged for 20 min. at 20000g in the cold. The supernatant was collected for radioactivity measurement. Three large samples of wet sediment were rapidly weighed on a Cahn electrobalance and used for the following determinations: (i) total water, measured from the loss in weight of the wet sample after drying to constant weight in an oven at 100°; (ii) water trapped extracellularly, measured from the volume of distribution of labelled inulin in the sediment. Sediment samples were placed in 3 ml. of water in a boiling-water bath and extracted for 5 min. The distribution volume of inulin was calculated as the ratio between the radioactivity (d.p.m.) extracted from 1 g. of wet sediment and the radioactivity (d.p.m.)/ml. of supernatant; (iii) protein content, assayed by the method of Lowry *et al.* (1951) after treatment of the sample with sodium deoxycholate.

Intracellular water volumes (calculated by the difference between total water and water trapped extracellularly), wet weights (obtained by correcting the wet weight of the sediment for the weight of water trapped extracellularly) and dry weights (obtained by correcting the dry weight of the sediment for the weight of salts trapped extracellularly) of the isolated heart cells as a function of cell protein content at various stages of embryological development were then determined and used for subsequent calculations.

Oxygen consumption and incorporation of ¹⁴C-labelled leucine into protein. Oxygen consumption by isolated heart cells, suspended in Krebs–Ringer phosphate buffer (Cohen, 1959) containing 8 mM-glucose and supplemented with streptomycin (50 µg./ml.) to avoid bacterial contamination, was measured by standard manometric Warburg procedures (Umbreit, 1959).

For the study of the incorporation of labelled leucine into protein, isolated cardiac cells were incubated in Krebs–Ringer bicarbonate containing 8 mM-glucose and 0.3 mM-L-[1-¹⁴C]leucine (specific radioactivity 1.3 mC/m-mole) either in the presence or in the absence of albumin (1%, w/v). Incubation was at 37.5° in an atmosphere of O₂ + CO₂ (95:5). The procedures for protein purification and radioactivity measurements were substantially similar with those described previously (Guidotti *et al.* 1968b).

Measurement of uptake and accumulation of labelled amino acids. Cells isolated from 5–19-day-old chick embryo hearts were suspended in Krebs–Ringer bicarbonate buffer (1 × 10⁷–3 × 10⁷ cells/ml.) containing 8 mM-glucose. Samples (1 ml.) of cell suspension were transferred into silicone-treated conical flasks containing 1 ml. of Krebs–Ringer bicarbonate buffer supplemented with 8 mM-glucose and the labelled amino acid (or amino acid mixture) under study at concentrations between 0.2 and 4 mM; the specific radioactivity of labelled compounds was varied to maintain the radioactivity of the medium between 0.1 and 2 µC/ml. The gas phase was O₂ + CO₂ (95:5). The flasks were incubated

at 37.5° in a Dubnoff metabolic shaker oscillating at 75 cyc./min. When added, insulin was present at a concentration of 0.2 unit/ml. of medium. Incubation was terminated by adding to the flasks 8 ml. of ice-cold Krebs-Ringer bicarbonate. The flasks were swirled to provide rapid mixing and the diluted cell suspensions were quickly poured into precooled stainless-steel filter holders (cylindrical shape, capacity 10 ml.) equipped with cellulose plastic filters (type SM 11302, pore size 3 μ m.; Sartorius-Membranfilter G.m.b.H., Göttingen, Germany) and connected to a vacuum pump by means of a suction flask containing a centrifuge tube placed directly under the stem of the filter holder. Filtration took 5–40 sec., depending on the absolute number of cells to be filtered. Filters with the cells retained on their surfaces were transferred into open long-necked glass ampoules containing 2 ml. of 3% (w/v) sulphosalicylic acid (when α -aminoisobutyric acid was studied) or 1.5 ml. of aqueous 1.5 mM solution of the unlabelled amino acid (or amino acid mixture) under study (when natural amino acids were investigated). The ampoules were placed in a boiling-water bath and refluxed for 10 min. Diluted incubation media collected in the centrifuge tubes were refiltered in the same manner and the filters so obtained, contaminated with medium but no cells, were extracted as described for the cell-loaded partners. Samples of the filtrates were diluted tenfold with 3% (w/v) sulphosalicylic acid (when α -aminoisobutyric acid was studied) and fivefold with aqueous 1.5 mM solution of the unlabelled amino acid (or amino acid mixture) under study (when natural amino acids were investigated). In each experiment samples of initial medium were diluted likewise. After centrifugation, three 0.2 ml. portions of each extraction fluid and diluted medium were added to 10 ml. volumes of a scintillation mixture composed of toluene-methyl Cellosolve (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 with an efficiency approaching 70% (as assessed by the use of internal standards) and a counting error less than 1%. When natural amino acids had been investigated, the remaining portions of extraction fluids and media were used for chromatographic analysis on silica-gel-coated polyethylene terephthalate Eastman chromatogram sheets (type 6061). Procedures of chromatographic development with water-saturated butan-1-ol-acetic acid (4:1, v/v), amino acid location and radioactivity measurements with a Tri-Carb spectrometer were essentially identical with those described by Guidotti *et al.* (1968b), except in experiments concerned with the uptake of 14 C-labelled amino acid mixtures where thin-layer and paper chromatograms were developed with propan-1-ol-aq. ammonia (sp.gr. 0.88) (33:17, v/v) and explored with a radiochromatogram scanner (model 7201, Packard Instrument Co. Inc.).

Calculations. The intracellular radioactivity (d.p.m./ml. of cell water) attributable to the labelled amino acid under study was calculated as follows: the radioactivity (d.p.m.) accumulated by the cells (obtained by the difference between the radioactivity of the extraction fluid from the cell-loaded filter and the radioactivity of the extraction fluid from the cell-free filter) was multiplied by a coefficient defining the fraction of radioactivity due to the amino acid in the extraction fluid of the cell-loaded filter (as measured by the ratio between the radioactivity of the amino acid after chromatographic development of extraction fluid samples and the radioactivity of equivalent-volume samples of undeveloped

extraction fluid spotted on the chromatographic sheet) and the result was divided by the volume of intracellular water (in ml.) in the actual experiment (as calculated from data on protein content). Owing to the presence of a small quantity of incubation medium trapped in the filter, the use of the fluid extracted from the cell-loaded filter to evaluate the fraction of radioactivity referable to the amino acid accumulated intracellularly might be questionable; however, the error introduced by this procedure becomes negligible when the radioactivity contaminating the filter is much smaller than the radioactivity accumulated by the cells or when the value of the fraction of radioactivity due to the amino acid in the extraction fluid from the cell-loaded filter approaches the value of the same fraction in the medium or when both occur. These conditions were satisfied in our experiments. Studies with labelled inulin showed also that the cell layer retained on the filter surface did not hold measurable amounts of incubation medium.

The intracellular accumulation of labelled amino acids in isolated heart cells, expressed as concentration ratio between intracellular water and medium, was calculated by dividing the intracellular radioactivity attributable to the amino acid (d.p.m./ml. of cell water) by the radioactivity (d.p.m./ml.) due to the amino acid in the medium at the end of the incubation (as evaluated by multiplying the radioactivity of the medium by a coefficient defining the fraction of it referable to the amino acid; the coefficient was measured by the ratio between the radioactivity of the amino acid after chromatographic separation of medium samples and the radioactivity of equivalent-volume samples of undeveloped medium spotted on the chromatographic sheet).

Coefficients defining the fraction of radioactivity due to the amino acid in extraction fluids and final media are equal to unity with non-utilizable amino acid analogues; hence they were omitted when the accumulation of α -aminoisobutyric acid was calculated.

The uptake of labelled amino acids by isolated heart cells was measured by the disappearance from the medium and expressed as μ moles/10 mg. wet cell wt. The amount of amino acid in the medium at the end of the incubation was obtained by dividing the radioactivity (d.p.m.) due to the amino acid in the final medium by the specific radioactivity of the initial medium.

The fraction of labelled amino acid taken up by the heart cells that was recovered free intracellularly was evaluated by dividing the intracellular radioactivity attributable to the free amino acid by the radioactivity referable to the amino acid that disappeared from the medium in the actual experiment, and expressed as a percentage.

The uptake of radioactivity from 14 C-labelled amino acid mixtures by isolated cardiac cells was calculated by the difference between the radioactivity (d.p.m.) of the initial medium and the radioactivity (d.p.m.) essentially referable to unchanged amino acids in the final medium, and expressed as d.p.m./mg. wet cell wt. The amino acid radioactivity in the final medium was evaluated by subtracting the radioactivity of non-migrating compounds after chromatographic development of medium samples from the radioactivity of equivalent-volume samples of undeveloped medium spotted on the chromatographic sheet. (The rationale for this procedure arose from the results of radiochromatographic analyses of final media showing that detectable peaks of radioactivity were associated only with zones corresponding to the amino acids present in the initial mixture and with a

zone at the application point; the peak at the origin did not coincide with any amino acid and represented mainly peptide radioactivity as shown by its sharp decrease in experiments performed in the presence of puromycin.)

RESULTS

General characteristics of the preparation. Phase-contrast- and light-microscopic observations showed that the treatment of chick embryo hearts with collagenase resulted in a complete cell dispersion (Plate 1) with a fairly high proportion of pulsating elements and no clumping of the cells. Decidedly inferior results were obtained with trypsin and hyaluronidase as dissociating agents. These findings confirm those of Cavanaugh, Berndt & Smith (1963). The yield of the procedure of cell isolation by collagenase averaged 2×10^8 – 3×10^8 cells/g. of wet cardiac tissue. Freshly prepared suspensions were composed of cells from all parts of the heart and included contractile elements, stromal cells and few blood cells. Myocardial elements predominated largely in cell suspensions obtained from young embryonic hearts (5 and 7 days); the number of stromal cells increased in cell suspensions from older hearts. The contribution of broken cells to the total population was negligible.

The electron-microscopic examination of isolated myocardial cells, before and after a 2 hr. incubation in glucose-supplemented Krebs–Ringer bicarbonate buffer, demonstrated a satisfactory preservation of the subcellular structures including plasma membrane (Plate 1).

The intracellular water of isolated heart cells did not vary appreciably during the embryological development: between 0.813 ml./g. of cells in preparations obtained from 5-day-old hearts and 0.805 ml./g. of cells in preparations from 19-day-old hearts. The protein content increased with the development: between 131 mg./g. of cells in preparations obtained from 5-day-old hearts and 165 mg./g. of cells in preparations from 19-day-old hearts.

Viability of isolated heart cells. Oxygen consumption, incorporation of labelled leucine into protein and intracellular accumulation of α -aminoisobutyric acid against a chemical gradient were considered adequate criteria to investigate cell viability *in vitro*.

The uptake of oxygen by isolated cell suspensions obtained from 7-day-old hearts (cell concentration: 4×10^6 cells/ml. of Krebs–Ringer phosphate; incubation at 37.5° in atmosphere of oxygen) was constant for 3 hr. with a Q_{O_2} (μ l. of oxygen/mg. of dry wt./hr.) of -14.2 and decreased slightly during the next 4 hr. down to values of -11 (Fig. 1). Q_{O_2} values of -15.1 and -16.5 have been reported for intact hearts and heart slices of 7-day-old chick

Fig. 1. Oxygen consumption of isolated cardiac cells, measured by standard manometric Warburg procedures (Umbreit, 1959). Cell suspensions (4×10^6 cells/ml.), prepared from 7-day-old chick embryo hearts, were incubated at 37.5° in Krebs–Ringer phosphate buffer supplemented with 8 mM-glucose and streptomycin (50μ g./ml.). The gas phase was O_2 .

embryos (Warburg & Kubowitz, 1927; Bhattacharya & MacDougall, 1959).

The rate of incorporation of L-[1- 14 C]leucine into protein of isolated cardiac cells (prepared from 10-day-old chick embryo hearts) was linear over 4 hr. of incubation and independent of the presence of albumin in the medium. If the incorporation of labelled leucine into protein of isolated heart cells was calculated in quantitative terms (from absolute radioactivities of protein and specific radioactivity of the tracer) values of 6.8μ moles/g. of cell protein/hr. were obtained, which are slightly higher than those reported previously for intact hearts (from 10-day-old chick embryos) (Loreti, Gaja, Ragnotti, Borghetti & Guidotti, 1965) and compare well with values of 6.5μ moles/g. of protein/hr. observed with heart slices under similar experimental conditions (L. Loreti, A. F. Borghetti & G. G. Guidotti, unpublished work).

Cell suspensions prepared from 5–19-day-old chick embryo hearts accumulated α -amino[1- 14 C]-isobutyric acid in the intracellular water against a concentration gradient (Fig. 2). The distribution ratios of this model amino acid between intracellular water and medium increased linearly for 2 hr. in cells obtained from 5- and 7-day-old hearts and almost linearly in cells from older hearts. The

EXPLANATION OF PLATE I

(a) and (b) Light-microscope photographs of isolated cardiac cells prepared from collagenase-treated 7-day-old chick embryo hearts. Cells were fixed with formaldehyde, smeared and stained with haematoxylin and eosin. Magnification: $\times 60$ (a) and $\times 350$ (b). (c) Electron micrograph of isolated myocardial cells (obtained from collagenase-treated 7-day-old hearts) incubated for 2 hr. in glucose-supplemented Krebs-Ringer bicarbonate buffer. Note the satisfactory preservation of the subcellular structures: N, nucleus; Mt, mitochondrion; R, ribosomes; Mf, myofibrils; G, glycogen. The centrifugation of the cell suspension before fixation and embedding accounts for the close contact of the cells in the photograph. Magnification: $\times 25000$.

Fig. 2. Time-course of α -amino[1- 14 C]isobutyric acid accumulation by isolated cardiac cells at various stages of embryological development. Cell suspensions, prepared from 5-day-old (○), 7-day-old (Δ), 10-day-old (□), 14-day-old (●) and 19-day-old (▲) chick embryo hearts, were incubated in Krebs-Ringer bicarbonate buffer containing 8 mM-glucose and 0.5 mM- α -amino[1- 14 C]isobutyric acid (specific radioactivity 0.2 mc/m-mole). Final cell concentration was 5×10^6 cells/ml. Incubation was at 37.5° in an atmosphere of $O_2 + CO_2$ (95:5). The procedures for amino acid extraction and radioactivity measurements are described in the text. The experimental points are means of three different experiments.

Fig. 3. Effect of insulin on α -amino[1- 14 C]isobutyric acid accumulation by isolated cardiac cells. Cell suspensions, prepared from 7-day-old chick embryo hearts, were incubated in Krebs-Ringer bicarbonate buffer containing 8 mM-glucose and 0.5 mM- α -amino [1- 14 C]isobutyric acid (specific radioactivity 0.2 mc/m-mole) in the absence (○) and in the presence (●) of insulin (0.2 unit/ml.). Final cell concentration was 5×10^6 cells/ml. Incubation was at 37.5° in an atmosphere of $O_2 + CO_2$ (95:5). The procedures for amino acid extraction and radioactivity measurements are described in the text. The experimental points are means of three different experiments.

values attained after 2 hr. of incubation were inversely related to the age of the heart; a pronounced decrement in the concentrative capacity of the cardiac cells was observed between the seventh and the tenth days of embryological development. Expressed as distribution ratios or recalculated in terms of intracellular concentrations, the values of α -aminoisobutyric acid accumulation by cells isolated from 5- and 10-day-old hearts agree with those reported previously for intact hearts at the same stage of development (Loreti *et al.* 1965; Guidotti *et al.* 1968a). In some experiments bovine serum albumin (1%, w/v) was added to the incubation medium; this addition did not change the rate of intracellular accumulation of α -aminoisobutyric acid.

Effect of insulin on the uptake and accumulation of amino acids. Insulin stimulated the intracellular accumulation of α -amino[1- 14 C]isobutyric acid by cell suspensions obtained from 7-day-old chick embryo hearts (Fig. 3). The effect of the hormone on the concentration ratio between intracellular water and medium of this amino acid analogue in isolated cells was of the same order of magnitude as that observed in intact hearts (Loreti *et al.* 1965; Guidotti *et al.* 1968a) and was independent of the

presence of albumin in the incubation medium. The addition to the medium of 1 mM-dithiothreitol, a reagent known to reduce disulphides quantitatively (Cleland, 1964), abolished the stimulation by insulin of α -aminoisobutyric acid accumulation even in experiments with incubation periods as short as 15 min. and decreased markedly the basal accumulation of this analogue in longer-lasting experiments.

The effects of insulin on uptake and intracellular accumulation of naturally occurring amino acids by cell suspensions prepared from 7-day-old chick embryo hearts are presented in Table 1. In these experiments the initial concentration of the labelled amino acids in the incubation medium was between 0.1 and 2 mM; the lower concentration value for each amino acid tended to approximate to its concentration in chick blood (Charkey, Wilgus, Patton & Gassner, 1950) and egg yolk between 6 and 9 days of development (Williams, DaCosta, Newman & Marshall, 1954).

After 2 hr. of incubation the uptake (measured as disappearance from the medium) of labelled amino acids by isolated heart cells varied between 2% (with 2 mM-phenylalanine) and 33% (with 0.3 mM-proline) of the amount present in the initial medium. Insulin increased markedly the uptake of glycine, serine and valine at 2 mM concentration and slightly

Table 1. *Effects of insulin on uptake and accumulation of labelled amino acids by isolated heart cells*

Cell suspensions obtained from 7-day-old chick embryo hearts were incubated for 10 and 120 min. in Krebs-Ringer bicarbonate buffer containing 8 mM-glucose and the labelled amino acid, in the absence and in the presence of insulin (0.2 unit/ml.). Final cell concentration was 1.5×10^7 cells/ml. (23 ± 1 mg. wet wt.). Incubation was at 37.5°C in an atmosphere of O₂ + CO₂ (95:5). The procedures for amino acid purification, radioactivity measurements and calculations are described in the text. The values are means of two or three experiments.

Amino acid added	Initial concn. (mM)	Uptake of amino acid (μ mole/10 mg. wet wt.)		Intracellular free labelled amino acid (% of uptake)		Ratio	Concn. of free labelled amino acid in cell water	
		No insulin	Insulin	No insulin Incubation for 120 min.	Insulin		Concn. of free labelled amino acid in medium	
							No insulin	Insulin
[1- ¹⁴ C]Glycine	0.25	0.064	0.070	19	25		9.18	14.1
[1- ¹⁴ C]Glycine	2.0	0.239	0.305	25	34		4.36	8.00
L-[U- ¹⁴ C]Proline	0.3	0.082	0.085	18	21		8.78	10.3
L-[U- ¹⁴ C]Proline	2.0	0.400	0.405	9	14		2.76	4.34
L-[U- ¹⁴ C]Serine	0.5	0.117	0.121	31	36		13.3	16.1
L-[U- ¹⁴ C]Serine	2.0	0.278	0.347	39	42		8.23	9.18
L-[U- ¹⁴ C]Threonine	0.25	0.039	0.040	14	18		3.44	4.35
L-[U- ¹⁴ C]Threonine	2.0	0.193	0.196	15	17		1.97	2.18
L-[<i>ring</i> -2- ¹⁴ C]Histidine	0.1	0.012	0.015	8	8		2.72	2.88
L-[<i>ring</i> -2- ¹⁴ C]Histidine	2.0	0.203	0.203	16	26		2.56	3.26
L-[<i>Me</i> - ¹⁴ C]Methionine	0.1	0.027	0.023	5	8		2.16	3.25
L-[<i>Me</i> - ¹⁴ C]Methionine	2.0	0.447	0.336	4	6		0.93	1.43
L-[1- ¹⁴ C]Leucine	0.2	0.033	0.040	5	3		1.22	0.97
L-[1- ¹⁴ C]Leucine	2.0	0.160	0.163	8	8		0.83	0.81
L-[U- ¹⁴ C]Valine	0.2	0.031	0.033	6	4		1.46	1.03
L-[U- ¹⁴ C]Valine	2.0	0.046	0.091	23	21		0.68	0.73
L-[U- ¹⁴ C]Lysine	0.3	0.042	0.045	6	6		1.29	1.17
L-[U- ¹⁴ C]Lysine	2.0	0.242	0.253	6	5		0.71	0.79
L-[U- ¹⁴ C]Phenylalanine	0.13	0.008	0.009	13	12		1.03	1.08
L-[U- ¹⁴ C]Phenylalanine	2.0	0.031	0.032	31	28		0.65	0.57
Incubation for 10 min.								
[1- ¹⁴ C]Glycine	0.25	—	—	—	—		0.57	1.05
L-[U- ¹⁴ C]Proline	0.3	—	—	—	—		0.82	1.23
L-[U- ¹⁴ C]Serine	0.5	—	—	—	—		0.88	1.10

increased the uptake of most of the other amino acids tested; the uptake of 2 mM-histidine was unaffected and the uptake of 0.1 mM- and 2 mM-methionine decreased in the presence of the hormone.

The amount of the different amino acids recovered free intracellularly after 2 hr. of incubation represented a rather small fraction of that which entered the cell. Expressed as percentage of uptake, this fraction was between 3% (with 0.2 mM-leucine) and 42% (with 2 mM-serine), being remarkably low with methionine, leucine and lysine. It tended to increase when the initial concentration of the amino acid in the medium was raised, except in experiments with proline, threonine, methionine and lysine. Insulin definitely enhanced this fraction in experiments with glycine, proline, serine, threonine, methionine and histidine (for the last-named amino acid only at 2 mM concentration) and tended to depress it with leucine, valine, lysine and phenylalanine.

The intracellular accumulation of labelled amino acids, expressed as concentration ratio between intracellular water and medium, was definitely greater than unity and was enhanced by the presence of insulin for at least six amino acids. These were glycine, proline, serine, threonine, histidine and methionine. There was no evidence of substantial intracellular accumulation of leucine, valine, lysine and phenylalanine, and insulin caused only minor changes in the distribution ratios of these amino acids. Preliminary experiments (not shown) with other naturally occurring amino acids indicated that the hormone did not affect the cell water/medium concentration ratios of arginine, aspartic acid and glutamic acid. Attempts to investigate the effect of insulin on the intracellular accumulation of L-cysteine were unsuccessful because of the difficulty of maintaining the amino acid in the reduced form. The use of dithiothreitol for this purpose, as suggested by Crawhall & Segal (1966), was unsatisfactory since, in the presence of

this reagent, the ability of the heart cells to respond to the hormone was rapidly destroyed (see above).

Table 1 finally shows that the stimulatory effect of insulin on the intracellular accumulation of the natural amino acids glycine, proline and serine by isolated cardiac cells can be detected after experimental periods as short as 10 min., when the amino acid concentration in cell water is still lower than that in medium in the absence of the hormone (i.e. under conditions in which inward transport should predominate).

It is recognized, of course, that the limited number of observations with each individual amino acid renders the results reported above and their interpretation somewhat uncertain. Further investigations will be required to assess the reliability of some unusual effects of insulin as the marked stimulation of the uptake of 2 mM-valine (not reflected by an increase of its intracellular concentration), the enhancement of the intracellular accumulation of 2 mM-histidine (without changes in its uptake) and the decreased uptake of methionine (under conditions in which its intracellular accumulation is raised). In particular, a complete balance accounting for all the amino acid taken up by the cells, including values of oxidation and incorporation into protein, is needed to obtain more meaningful information when the intracellular utilization is larger than accumulation.

In view of these difficulties, it is encouraging that results substantially similar to those presented in Table 1 have been obtained with isolated rat diaphragm (Wool, 1964; Wool *et al.* 1965) and perfused rat heart (Manchester & Wool, 1963; Scharff & Wool, 1965b). Moreover, the results of glycine and leucine accumulation and of leucine uptake and utilization by the isolated heart cells (and the effects of insulin on them) agree well with those reported for intact chick embryo hearts (Guidotti *et al.* 1968b).

Experiments with cell suspensions (1.5×10^7 cells/ml.) prepared from 7-day-old chick embryo hearts and incubated for 2 hr. in Krebs-Ringer bicarbonate containing 8 mM-glucose and $2 \mu\text{C}$ of a mixture of 14 U- ^{14}C -labelled L-amino acids (specific radioactivity 54 mc/mg. atom of carbon/ml.) showed that insulin caused a small but significant increase of the uptake of radioactivity by the cardiac cells. In the absence of the hormone the uptake was 164405 ± 1785 d.p.m./mg. wet cell wt. and increased to 175884 ± 2978 d.p.m./mg. wet cell wt. when insulin was present in the incubation medium. (Values are means of four experiments $\pm$ S.E.M.) This increment is statistically significant ($P < 0.01$ using a *t* test for paired variates). Experiments with rat hearts perfused with amino acid mixtures yielded inconclusive results for the effect of insulin on amino acid uptake (Scharff & Wool, 1965a).

However, when protein synthesis was inhibited by adding puromycin to the system, insulin increased the concentration of total amino acids in the tissue and decreased it in the perfusion medium (Scharff & Wool, 1965b).

DISCUSSION

Suitability of the biological model. The results presented in this paper indicate that the cardiac cells isolated by collagenase treatment from chick embryo hearts at various stages of development maintain the intrinsic metabolic properties of the intact tissue. In particular, the similarity in the patterns of amino acid transport in the absence and in the presence of insulin by both isolated cardiac cells and intact hearts demonstrates that the major characteristics of permeability of the cell membrane, including insulin sensitivity, have been preserved in isolated cells.

The properties that make the chick embryo heart a desirable model for studies of tissue permeability *in vitro* have been examined previously (Guidotti, Kanameishi & Foà, 1961; Guidotti *et al.* 1968a). A biological preparation composed of cells isolated from the same source presents additional advantages over the intact tissue. Among them are: (i) much faster and more complete contact of the entire cell population under study with the compounds (substrates, hormones, inhibitors) present in or added to the incubation medium (this condition allows decreased reaction times and tends to enlarge metabolic responses); (ii) lower complexity of the biological model, represented by a two-compartment system (this attribute simplifies the analysis of the experimental observations and the mathematical treatment involved); (iii) more uniform composition of the biological model, consisting of randomly mixed cell populations (this property minimizes the biological variability within the experiment).

The suitability of the isolated heart cells for studies of cell permeability is further stressed by their remarkable viability *in vitro*, even in the absence of membrane-protective agents (e.g. albumin) in the incubation medium. In particular, a constant rate of incorporation of labelled amino acids into protein over 4 hr. of incubation, as found with cells isolated from chick embryo hearts, has never been observed by us with other preparations from adult mammalian tissues including rat liver and kidney slices, mouse and rat isolated diaphragm, Ehrlich ascites-carcinoma cells and Yoshida ascites-hepatoma cells. Perhaps the long-lasting integrity *in vitro* of complex intracellular mechanisms in isolated (chick embryo) heart cells depends on the superior adaptability of the embryonic cells to simpler environments and on the specific property of the avian embryo to develop as an isolated system

without a continuous supply of nutrients from a maternal circulation.

It therefore appears that the use of collagenase as a dissociating agent and of chick embryo hearts as biological source provides a favourable insulin-sensitive single-cell system from a muscle tissue. An insulin-sensitive single-cell system from adipose tissue, obtained by collagenase treatment, has been described by Rodbell (1964).

Developmental aspects of amino acid-transport systems. The general pattern of the values for the intracellular accumulation of 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) at different stages of embryological development indicates a pronounced decrease in the concentrative capacity of isolated cell suspensions with aging. Whether this decrease reflects a fall in the efficiency of the amino acid-transport systems inherent in the individual cells or depends on changes in the composition of the cell population during the ontogeny of the heart remains to be elucidated. Histological examinations, showing an increase of the number of stromal cells with the age of the heart, would support the second possibility if the assumption is made of a less-efficient amino acid accumulation by these cells; however, a change in the concentrative capacity of the myocardial cell as the development of the heart proceeds cannot be excluded on this simple ground and radioautographic studies at the cellular level (with techniques preventing loss or displacement of water-soluble compounds) will be needed to solve this problem.

Uptake and accumulation of natural amino acids. Insulin was found to increase the uptake by isolated heart cells of most of the amino acids tested. In contrast, a stimulatory effect of insulin on the intracellular concentration has been observed only with amino acids (glycine, proline, serine, threonine, histidine and methionine) that were accumulated intracellularly against a concentration gradient in the absence of the hormone. The failure of insulin to enhance the intracellular concentration of amino acids (leucine, valine, lysine and phenylalanine) whose ratios of distribution between cell water and medium approached unity may have been connected with a simultaneous acceleration by the hormone of their intracellular utilization. Indeed, the fraction of these amino acids that was recovered free intracellularly decreased, in the presence of insulin, in spite of the stimulation of their total uptake (Table 1). These findings suggest that the observation of a definite stimulatory effect of the hormone on the intracellular accumulation of amino acids in isolated heart cells requires rates of uptake higher than the corresponding rates of intracellular utilization and

a lack of insulin effect on the latter process. Whether or not this interpretation is correct the results presented, indicating that insulin increases the uptake of the amino acids tested (the sole exception being methionine) and stimulates the intracellular accumulation of six (including methionine) out of ten amino acids, support the conclusion that the hormone promotes the transfer of most, if not all, natural amino acids across the cell membranes of isolated chick embryo heart cells.

Previous investigations (Guidotti *et al.* 1968a) showed that α -aminoisobutyric acid was accumulated in chick embryo hearts by two different transport systems and that insulin enhanced the intracellular accumulation of this amino acid analogue by affecting both these processes. Although these studies provide information on the nature of the hormonal control of the movement of this model amino acid across the cardiac cell membrane, a conclusive interpretation of the mechanism of insulin action on amino acid transport into muscle tissues requires kinetic studies with naturally occurring molecules. Unfortunately, a reliable analysis of the kinetics of transport is difficult with natural amino acids, because substantial fractions of these compounds are metabolized when they are supplied at the concentrations required for such experiments. The single-cell system described in this paper, which is capable of a prompt response (within minutes) to insulin, might be useful to surmount this difficulty. In fact, a drastic decrease of incubation times, minimizing the fraction of amino acid taken up by the cells that is metabolized, should make it possible to analyse with sufficient accuracy the transport kinetics of natural amino acids in muscle and perhaps to clarify the mechanism of action of insulin thereon.

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