Accumulation of Amino Acids in Muscle of Perfused Rat Heart

EFFECT OF INSULIN

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1. Rat heart perfused with Krebs–Henseleit bicarbonate buffer released material containing ninhydrin-positive nitrogen, but the amount was less than that reported to be released by diaphragm; glucose, but not insulin, decreased the release of ninhydrin-positive nitrogen and increased the concentration of the same material in the intracellular water of heart. 2. When heart was perfused with a mixture of amino acids and glucose, there was actually a net uptake, and an increase in intracellular concentration, of ninhydrin-positive nitrogen. Changes in the concentration of ninhydrin-positive nitrogen did not accurately reflect changes in concentration of amino acids. 3. The effect of insulin on the actual concentration of individual amino acids in heart muscle was examined by perfusing the heart with a mixture of amino acids and other ninhydrin-positive substances in the same concentration as they are found in plasma. 4. The effect of insulin on the concentrations of amino acids in the medium and in the intracellular water of the heart was determined after perfusion for different periods of time. No clear or meaningful effect of insulin was observed, despite the fact that insulin significantly increased the accumulation, in each of the same hearts, of radioactivity from amino[^{14}C]isobutyric acid.

In circumstances similar to that in which it increases the incorporation of labelled amino acids into protein of muscle, insulin also enhances the accumulation of radioactivity from six of the natural utilized amino acids (glycine, proline, hydroxyproline, serine, methionine and threonine) but not from 13 others (for references see Wool, 1964). However, in the presence of sufficient puromycin to suppress protein synthesis, the accumulation of radioactivity from a number of the amino acids, not previously responsive to insulin, is increased by the hormone (Castles & Wool, 1964). However, with but rare exceptions (Guroff & Udenfriend, 1961), what has been examined is the ratio of radioactivity in the tissue water to that in the medium after incubating diaphragm, or perfusing heart, with a single radioactive amino acid. In reality, then, what has been studied is the accumulation of radioactivity rather than amino acid, and there is no assurance that the ratio so determined accurately reflects the concentration ratio for the unlabelled amino acid. What is required for a proper study of the regulation by insulin of amino acid penetration into muscle is a chemical determination of the exact amount of each amino acid in intracellular water in circumstances reflecting, as faithfully as possible, those found physiologically. It is the purpose of the present paper to report the results of a study of this kind. We have perfused rat heart, a preparation whose advantages for the study of amino acid transport have been described by Manchester & Wool (1963), with medium containing a mixture of amino acids in the same concentration as they occur in plasma, and examined the effect of insulin on the ability of the heart to concentrate individual amino acids; in the accompanying paper (Scharff & Wool, 1965) the effect of insulin in the presence of puromycin is described.

METHODS

Chemicals. The amino acids and other ninhydrin-positive substances and the sodium pyruvate were obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.); ninhydrin, hydrindantin, thiodiglycol and BRIJ 35 were from Pierce Chemical Co. (Rockford, Ill., U.S.A.); methylCellosolve was from Union Carbide Chemicals Corp. (Chicago, Ill., U.S.A.). The albumin used was either bovine albumin powder, fraction V (Armour Pharmaceutical Co., Chicago, Ill., U.S.A.), freed of fat and insulin according to the method of Garland, Newsholme & Randle (1962), or untreated crystalline albumin (human) purchased from Nutritional Biochemicals Corp. The [^{14}C]inulin (0·23μc/mg.) was obtained from Volk Radiochemical Co. (Skokie, Ill., U.S.A.); the α-amino[1-^{14}C]isobutyric acid (98μc/mg.) was from New England Nuclear Corp. (Boston, Mass.,

U.S.A.). The insulin (ox zinc–insulin crystals), assayed at 25·2 units/mg. and containing less than 0·1% of glucagon, was a gift from Dr O. Behrens of Eli Lilly and Co. (Indianapolis, Ind., U.S.A.); it was dissolved in 3·3 mN-HCl to form a stock solution of 20 units/ml.

Preparation and perfusion of hearts. Male rats of the Sprague–Dawley strain, weighing 200–300 g., were maintained under standard conditions (Wool & Krahl, 1959) and allowed free access to food and water at all times. The technique and apparatus used for the removal of hearts and their perfusion with a small volume of recirculating perfusion medium was essentially that described by Morgan, Henderson, Regen & Park (1961). Animals were anaesthetized by the intraperitoneal administration of 0·2 ml. of a mixture of pentobarbitol (15 mg./ml.) and heparin (750 units/ml.)/100 g. body wt. About 15 min. later the rats were killed by decapitation; each heart was rapidly excised and placed in ice-cold 0·9% NaCl. After the heart stopped beating it was tied to the perfusion cannula and washed through with 25–30 ml. of medium (see below) from a separate wash-out chamber, the wash-out fluid being collected in a graduated cylinder; the perfusion was then switched by means of a three-way stopcock to the main chamber, which contained 12 ml. of medium. To keep the heart submerged in fluid during perfusion, an inner chamber (1·6 cm. × 2·5 cm.) supported by marbles resting on the sintered-glass filter, was added to the apparatus described by Morgan *et al.* (1961). A head of pressure of about 5 cm. Hg was maintained during the perfusion; fluid emerging from the heart entered the inner chamber, overflowed into the main chamber where it equilibrated with O_2+CO_2 (95:5), passed through the sintered-glass filter and was returned by a peristaltic pump to a bubble trap from which the fluid again entered the heart. Perfusion was at 37°. Regular cardiac contractions were usually maintained during the entire perfusion period. Hearts that did not contract satisfactorily were discarded.

Wash-out and perfusion media. The basic medium was Krebs–Henseleit bicarbonate buffer (Krebs & Henseleit, 1932) equilibrated with O_2+CO_2 (95:5), pH 7·4, but containing one-half the usual concentrations of Mg^{2+} and Ca^{2+} (Zachariah, 1961). The basic medium was used to wash blood from the hearts that were not further perfused. The wash-out medium in all other cases was the same as the final perfusion medium except for the omission of the radioactive substrate, insulin and glucose.

Table 1 gives the composition of the supplemented perfusion media used in several of the experiments. The supplemented media were prepared immediately before use by diluting a stock solution of the ninhydrin-positive substances (100 times the final concentration, except for tyrosine and cystine) with the basic medium and by adding the other substances needed (the concentrations are given in Table 1); the pH was then adjusted to 7·4.

Extracellular space. The extracellular space was taken to be equal to the volume of distribution of [^{14}C]inulin; in muscle inulin is confined to an extracellular location and occupies a space equal to the chloride space if sufficient time is allowed for equilibration (Cotlove, 1954; White & Rolf, 1956).

For determination of the extracellular space *in vitro* [^{14}C]inulin was added to the perfusion medium at a concentration of 0·02 μc/ml. After perfusion the heart was removed from the cannula and placed briefly in ice-cold 0·9% NaCl; a portion of the apex was cut off, blotted on hard filter paper, weighed and placed in 3 ml. of water in a boiling-water bath for 5 min. The perfusion medium was diluted tenfold. Samples (1 ml.) of heart extract and of diluted medium were pipetted on to stainless-steel planchets, each with concentric rings, and dried under an infrared lamp, and the radioactivity was determined in a Geiger–Müller gas-flow counter (model D–47; Nuclear–Chicago Corp., Des Plaines, Ill., U.S.A.) having an efficiency of about 40%. Corrections were made for background and self-absorption.

For determination of the extracellular space *in vivo* the [^{14}C]inulin was injected intravenously (10 μc/kg. body wt.) immediately after the animals had been nephrectomized under ether anaesthesia. The animals were allowed free access to water, but were not fed; 15 hr. after the inulin injection the animals were killed by decapitation and the hearts removed, and a 100–300 mg. portion from the apex was blotted, weighed and put in 3 ml. of water in a boiling-water bath for 5 min. Blood was collected in beakers

Table 1. *Composition of the supplemented perfusion media*

The basic medium was Krebs–Henseleit bicarbonate buffer with half the usual concentrations of Ca^{2+} and Mg^{2+}, to which was added a stock solution of the ninhydrin-positive substances as described in the text.

	Final concn. (μmoles/100 ml.)	
	Medium *A*	Medium *B*
Amino acids		
Aspartic acid	5·1	3·44
Threonine	28·5	25·0
Glutamine	38·0	—
Asparagine	39·4	—
Serine	36·1	55·1
Glutamic acid	25·8	11·5
Proline	22·2	32·6
Citrulline	—	8·52
Glycine	54·8	27·0
Alanine	65·4	40·9
Valine	18·4	18·9
Cystine (half)	43·2	6·66
Methionine	59·9	4·50
Isoleucine	10·2	9·01
Leucine	19·5	15·0
Tyrosine	10·3	7·67
Phenylalanine	10·4	7·51
Ornithine	—	6·44
Lysine	62·7	35·0
Tryptophan	—	6·99
Histidine	6·8	7·49
Arginine	12·6	41·2
Non-amino acids		
Taurine	—	35·0
Ammonia	—	100
Pyruvic acid	—	200
Albumin	—	500 mg./100 ml.

containing heparin at the time the animals were killed and the plasma separated by centrifugation and diluted tenfold with water. Samples (1ml.) of diluted plasma and heart extract were plated and the radioactivity was determined as described above.

Total tissue water. A small portion (50–100mg.) of heart muscle was placed in a tared beaker, weighed, dried to constant weight in an oven at 100° and the tissue water calculated from the loss in weight of the tissue.

Accumulation of amino[^{14}C]isobutyric acid. The accumulation by perfused heart of amino[^{14}C]isobutyric acid was measured in the manner described by Manchester & Wool (1963).

Preparation of samples for analysis of ninhydrin-positive substances. (*a*) Heart muscle. For the preparation of protein-free extracts for chromatographic analysis and determination of the concentration of amino acids and other ninhydrin-positive substances, use was made of sulphosalicylic acid to deproteinize the samples (Hamilton, 1962), and advantage taken of the observation by Manchester & Young (1960) that when muscle is heated in water at 100° for 5min. all the free amino acids are released. Heart ventricle muscle (500–800mg.) was boiled for 5min. either in 5ml. of 3% (w/v) sulphosalicylic acid, or in 3ml. of water followed by the addition of 1ml. of 20% (w/v) sulphosalicylic acid. The two methods gave similar values for the concentration of ninhydrin-positive substances, but the latter was found to precipitate protein more efficiently and for that reason it was generally used. In either case the solution was clarified by filtration through Whatman no. 50 filter paper and the sample frozen until the analysis could be carried out; before analysis the sample was thawed and the pH adjusted to 2·2–2·5 with 2N-NaOH. A 1–2ml. sample was used for chromatographic analysis.

To test the efficiency of extraction, hearts were perfused for 15min. with 0·01μc of amino[^{14}C]isobutyric acid/ml. and the extraction was then carried out in the usual manner. The residue remaining after precipitation of the protein was washed several times; the radioactivity in the several washes, and in the residue as well, was determined. Of the total amino[^{14}C]isobutyric acid contained in the heart, 95% was recovered in the original supernatant and an additional 3·5% in the first wash. Only insignificant amounts of radioactivity were present in the subsequent washes or in the heart residue. Similar results were obtained when the amino[^{14}C]isobutyric acid was added after the heart had been heated in sulphosalicylic acid, indicating that the radioactivity not contained in the original supernatant was most likely accounted for by liquid remaining with the residue and that the extraction of amino acid was complete.

The efficiency of the extraction procedure was tested in one other way. Portions of three hearts that had been perfused with amino[^{14}C]isobutyric acid were dissolved in 88% formic acid; the remaining tissue from the same three hearts was boiled in 3% sulphosalicylic acid. The radioactivity in the dissolved heart muscle and that in the heart muscle extract was determined. The concentration of radioactivity in the dissolved heart was 12800$\pm$1970counts/min./g., and in the heart extract 15900$\pm$1500counts/min./g.; the difference was not significant for the six observations and the results support the conclusion that the method of extraction is efficient.

(*b*) Plasma. Blood was collected in beakers containing heparin at the time the animals were killed, and the plasma separated by centrifugation. The protein was precipitated by addition of an equal volume of 10% (w/v) sulphosalicylic acid. The gelatinous precipitate was removed after the addition of acid-washed 'filtering aid' by filtering through Whatman no. 3 paper. The subsequent treatment of the sample was the same as with heart muscle extracts; a 1ml. sample was used for the chromatographic analysis.

Perfusion medium. The perfusion medium was prepared for analysis in a manner similar to that described for plasma except that the protein was precipitated with one-third volume of 20% (w/v) sulphosalicylic acid; 1ml. was analysed.

Analysis of ninhydrin-positive substances. Samples were analysed with a Technicon Amino Acid Autoanalyzer, a one-column chromatographic system adapted from the methods of Spackman, Stein & Moore (1958) and Piez & Morris (1960). Two different procedures were used for the separation and determination of ninhydrin-positive substances in extracts of muscle, plasma and perfusion medium. The first required 21·5hr. for a complete analysis, gave good separation of most of the substances usually found in the extracts (the exceptions being serine, glutamine and asparagine), and the reproducibility was better than $\pm$3%. The second procedure required only 6·5hr. for a complete analysis and is similar to that described by Thomson & Miles (1964); by programming three separate column systems in series, it was possible to carry out three analyses in a day. Resolution was not as good as with the longer procedure but separation below the half-height was obtained for most substances; reproducibility was $\pm$3–5%. Resolution of proline and citrulline was not possible with the second procedure, but their concentration could be calculated from the difference in extinction at 440 and 570mμ (see below). In the 6·5hr. procedure glutamine and asparagine were eluted with threonine. Neither procedure consistently separated hydroxyproline from glutathione; the concentration of hydroxyproline was therefore not always determined.

Table 2 gives the buffers and gradients for the two procedures. A much steeper gradient (pH2·57–10·00; 0·2–2·4M-Na^+) and a faster flow rate (1·5ml./min.) were used to resolve the ninhydrin-positive substances in 6·5hr. In the 21·5hr. chromatogram the flow rate was 0·5ml./min.; proline was eluted between glutamic acid and citrulline by keeping the column temperature at 45° for the first 2·5hr., and at 60° for the remainder of the analysis; for the 6·5hr. chromatograms the column temperature was maintained at 60° throughout. In either case the column was washed free of substances not completely eluted during analysis with Na_3PO_4 (0·2M with respect to Na^+). It was then regenerated with pH2·88 buffer (Table 2) for the longer analysis, and with pH3·20 buffer (same composition as pH2·88 buffer, Table 2) for the shorter one.

The ninhydrin reaction for analysis of the column effluent was carried out according to the method described by Moore & Stein (1954). An external standard of 0·05mM-L-leucine was used as a routine to test the accuracy of the analytical system; an internal standard of 0·25μmole of DL-norleucine (Walsh & Brown, 1962) was added to the column with each sample. The concentrations of the individual ninhydrin-positive substances was calculated from the area of the peak by using the 'height times width procedure' of Spackman *et al.* (1958); when necessary, a correction was made

Table 2. *Buffers and gradients for column chromatography*

The buffers were all 0·05 M with respect to citrate and contained in each litre: 5 ml. of thiodiglycol and 10 ml. of BRIJ 35 solution (prepared by adding 50 g. of BRIJ 35 to 100 ml. of water). The pH 2·57 buffer had in addition 110 ml. of methanol in each litre. The times for completion of the chromatograms (6·5 and 21·5 hr.) are shown.

	Volume (ml.)									
	pH 2·57 (0·2 M-Na^+)		pH 2·88 (0·2 M-Na^+)		pH 3·80 (0·2 M-Na^+)		pH 5·00 (0·80 M-Na^+)		pH 10·00 (2·4 M-Na^+)	
Chamber	21·5 hr.	6·5 hr.	21·5 hr.	6·5 hr.	21·5 hr.	6·5 hr.	21·5 hr.	6·5 hr.	21·5 hr.	6·5 hr.
1	—	75	75	—	—	—	—	—	—	—
2	—	25	75	50	—	—	—	—	—	—
3	—	—	75	75	—	—	—	—	—	—
4	—	—	75	50	—	25	—	—	—	—
5	—	—	—	—	70	75	5	—	—	—
6	—	—	6	—	9	20	60	55	—	—
7	—	—	—	—	—	—	75	35	—	40
8	—	—	—	—	—	—	75	—	—	75
9	—	—	—	—	—	—	75	—	—	75

in accordance with the variations in the internal standard. Glutathione was not oxidized and converted into the *S*-sulphonate form as described by Moore, Spackman & Stein (1958), since the reduced form was consistently eluted as a sharp peak before, and well separated from, aspartic acid. Glutathione could not, however, be separated from methionine oxidation products and the values for glutathione may reflect that failure.

To estimate the concentration of threonine, a sample of the heart extract was made 1 N with respect to HCl and placed in a boiling-water bath for 2 hr. Under those conditions the amide nitrogen of asparagine and glutamine is hydrolysed (Hamilton, 1945; Bergmeyer, 1963). Chromatography of the sample now allowed estimation of threonine (or serine in the 21·5 hr. analysis) without interference by glutamine and asparagine. The hydrolysed sample was used only for the determination of the concentration of threonine or serine; the concentrations of other amino acids were determined on a sample that had not been hydrolysed. A control experiment carried out with a standard solution of threonine, serine, glutamine and asparagine revealed that complete hydrolysis of the two amides did occur and that the concentrations of threonine and serine could be determined accurately.

Recovery of amino acids and other ninhydrin-positive substances. To test whether loss of amino acids or other ninhydrin-positive substances occurred during the preparation of the sample or its subsequent chromatography, the efficiency of recovery of a standard mixture of amino acids, glutathione and ammonia added to a heart extract was determined (Table 3). Muscle from two separate hearts was divided into a control and experimental sample, boiled in sulphosalicylic acid and 0·5 μmole of each of 22 ninhydrin-positive substances added to the experimental sample. The samples were analysed (21·5 hr. procedure) and the difference in concentration (after correction for the amount of material originally present in the sample) was determined.

The recovery of most of the added amino acids was good (Table 3). Exceptions were: glutamic acid, which can be ascribed to a technical difficulty peculiar to the glutamic

Table 3. *Recovery of ninhydrin-positive substances added to the extraction medium*

Paired halves from two hearts that had been washed free of blood were combined to form two samples. The extraction of ninhydrin-positive substances was, in one case (*A*), carried out with sulphosalicylic acid containing 0·5 μmole of each of 22 different ninhydrin-positive substances, and in the other (*B*) with sulphosalicylic acid alone. The samples were analysed and the recoveries calculated from the difference. The percentage recovered is given by: (increase in ninhydrin-positive substance/amount added) × 100.

	Increase in concn. ($A-B$) (μmoles)	Recovery (%)
Aspartic acid	0·910	182
Threonine	0·491	98
Serine	0·450	90
Glutamic acid	0·360	72
Citrulline	0·482	97
Glycine	0·511	102
Alanine	0·480	96
Valine	0·479	96
Cystine (half)	0·517	103
Methionine	0·487	97
Isoleucine	0·486	97
Leucine	0·479	96
Tyrosine	0·546	109
Phenylalanine	0·551	110
Ornithine	0·507	101
Lysine	0·534	107
Tryptophan	0·452	90
Histidine	0·486	97
Arginine	0·504	101
Glutathione	0·820	164
Ammonia	2·440	488

acid peak in the control chromatogram; aspartic acid, which, for reasons unknown, has often given erratic results when heart extracts are analysed (one possibility is that heart extracts have an unidentified substance that elutes with aspartic acid); glutathione, which also cannot be reliably measured, partly because of contamination with methionine oxidation products (see above) and partly because some hydrolysis may occur in the acid conditions used in the preparation of the samples for chromatography (perhaps also because it reacts poorly with ninhydrin; the extinction at 570mμ for glutathione is only 12% of that for leucine); ammonia, which most probably was due to contamination of the acid solution of the added standard (to prevent a similar occurrence with unknown samples, the extracts were covered and frozen immediately after preparation and thawed just before analysis).

Release of ninhydrin-positive nitrogen. A sample was removed from the perfusion medium at stated intervals with a capillary pipette; heart-muscle ninhydrin-positive nitrogen was extracted at the end of the perfusion period by heating the muscle at 100° for 5min. in 10ml. of water. The sample was deproteinized by the addition of 3% sulphosalicylic acid (Hamilton, 1962) and the ninhydrin-positive nitrogen determined by a method adapted from that of Moore & Stein (1948). The ninhydrin reagent contained the following: 75ml. of methylCellosolve; 25ml. of 4M-sodium acetate buffer, pH5·5; 15ml. of 10% (w/v) ninhydrin in methylCellosolve; 4ml. of 4% (w/v) $SnCl_2,2H_2O$ in methylCellosolve. The reagent was prepared immediately before it was used. To a 1ml. portion of the protein-free sample was added 1·8ml. of ninhydrin reagent and the sample was heated in a boiling-water bath (in a marble-capped tube) for 20min. The sample was then cooled rapidly, diluted to 10ml. with 95% (v/v) ethanol and agitated with a stream of air or O_2 to remove the colour due to excess of hydrindantin. The extinction at 570mμ was determined with a Unicam model SP.600 spectrophotometer. The unknown samples were analysed in triplicate and compared with a standard (L-leucine) determined at the same time. Precautions were taken to prevent contamination of the reagents with ammonia. In determining the total ninhydrin-positive nitrogen released at each time-interval, a correction was made for the volume and amount removed for the prior analyses. The results are expressed as μmoles of ninhydrin-positive nitrogen released/100ml. of cell water; for the purpose of calculation tissue water was assumed to be 812μl./g. wet wt. of tissue and the extracellular water 320μl./g. (cf. Tables 4 and 5).

Calculations. The inulin space, the volume of tissue water necessary to contain the inulin at its concentration in medium or plasma, was calculated according to the following formula:

$$\text{Inulin space in } \mu\text{l./g. wet wt. of heart} = \frac{\text{counts/min./g. wet wt. of heart}}{\text{counts/min./ml. of medium or plasma}} \times 1000$$

To calculate the intracellular concentrations of the ninhydrin-positive substances in muscle it was assumed that the concentration in extracellular fluid was the same as that in the perfusion medium. For hearts merely washed through with Krebs–Henseleit bicarbonate buffer containing no additions, the extracellular concentration of ninhydrin-positive substances was assumed to be nil (see below). The following formula was used:

$$\mu\text{moles/100ml. of cell water} = \frac{\text{T.W.} - {}^{f}\text{E.C.F.} \times \text{E.C.F.}}{{}^{f}\text{I.C.}}$$

where T.W. is the concentration in tissue water in μmoles/100ml., E.C.F. is the concentration in extracellular fluid in μmoles/100ml., ^{f}E.C.F. is the fraction of the tissue water that is extracellular and ^{f}I.C. is the fraction of the tissue water that is intracellular. For heart ^{f}E.C.F. was 0·40 and ^{f}I.C. was 0·60 (see below).

To calculate the concentrations of citrulline and proline when they could not be separated by chromatography, advantage was taken of the fact that the product of citrulline's reaction with ninhydrin absorbs strongly at 570mμ and only weakly at 440mμ, whereas the reverse is true for proline. With an appropriate standard solution the relative extinctions of the two substances can be determined at the two wavelengths; since extinctions and hence peak areas are additive, two simultaneous equations can be formulated and the area contributed by each substance determined. The total area at each wavelength (in parentheses) is given by the following:

$$(570)^{c+p} = (570)^{c} + (570)^{p} \quad (1)$$

$$(440)^{c+p} = (440)^{c} + (440)^{p} \quad (2)$$

where $(\ )^{c+p}$ is the total area measured on the chromatogram and $(\ )^{c}$ and $(\ )^{p}$ refer to the contribution made at each wavelength by citrulline and proline respectively. If A is a constant representing the relative extinction of proline at 570mμ to that at 440mμ, and B the relative extinction of citrulline at 440mμ to that at 570mμ, then:

$$(570)^{p} = A \times (440)^{p} \quad (3)$$

$$(440)^{c} = B \times (570)^{c} \quad (4)$$

Substituting eqns. (3) and (4) in eqns. (1) and (2) respectively gives the two simultaneous equations:

$$(570)^{c+p} = (570)^{c} + A \times (440)^{p} \quad (5)$$

$$(440)^{c+p} = B \times (570)^{c} + (440)^{p} \quad (6)$$

Solving for the area of each substance at its wavelength of maximal absorption gives the final equations:

$$(440)^{p} = \frac{B \times (570)^{c+p} - (440)^{c+p}}{AB - 1} \quad (7)$$

$$(570)^{c} = (570)^{c+p} - A \times (440)^{p} \quad (8)$$

which can be used to calculate proline and citrulline concentrations from the area of the combined peaks. A and B were found to have values of 0·15 and 0·18 respectively.

RESULTS

Tissue water and inulin space of rat heart muscle. The total tissue water of rat heart muscle was found to average 812μl./g. wet wt. and to remain relatively constant during 80min. of perfusion (Table 4). The value accords with that reported by Bleehan & Fisher (1954) and Morgan *et al.* (1961).

Table 4. *Total water content of rat heart muscle*

The hearts were perfused with bicarbonate buffer unless otherwise indicated. The concentration of glucose, when present, was 11·1 mM, that of insulin was 0·1unit/ml. and that of albumin was 5mg./ml. The values are the means ± S.E.M. of the numbers of observations in parentheses. The composition of medium *B* is given in Table 1.

Expt. no.	Addition to the medium	Water content (μl./g.) After perfusion for (min.)……0	5	10	20	60	80
1	None	812±1 (12)		810±2 (5)		817±13 (11)	810±5 (4)
	Glucose			811±1 (4)		826±14 (4)	818±4 (4)
2	None		830±3 (3)		841±7 (3)		835±6 (3)
	Insulin		835±7 (3)		841±1 (3)		837±9 (3)
3	None		816±7 (3)		819±8 (3)		821±9 (3)
	Albumin		814±1 (3)		812±3 (3)		822±12 (3)
4	Medium *B*					812±1 (5)	
	Medium *B* + insulin					807±6 (4)	
5	–	785±2 (15)*					

* Hearts were not washed out or perfused.

Table 5. *Inulin space of rat heart muscle*

The hearts were perfused with bicarbonate buffer unless otherwise indicated. The concentration of [^{14}C]-inulin in the perfusion medium was 0·01 μc/ml., that of glucose, when present, was 10 mM, that of insulin was 0·1unit/ml. and that of albumin was 4·5mg./ml. In the experiment *in vivo* 10 μc of [^{14}C]inulin/kg. body wt. was given 15hr. before the animal was killed. The values are the means ± S.E.M. of the numbers of observations in parentheses. The composition of medium *B* is given in Table 1.

Expt. no.	Addition to the medium	After perfusion for (min.)…0	Inulin space (μl./g.) 5	10	20	30	60	80
1	None		320±22 (6)	356±24 (8)	346±10 (11)	346±13 (9)	340±13 (8)	
	Glucose			322±15 (8)			364±12 (8)	
2	None				334±7 (25)		329±16 (13)	
	Insulin				315±10 (24)		316±13 (14)	
3	None		313±22 (3)		346±27 (3)			449±21 (3)†
	Albumin		285±17 (3)		340±13 (3)			414±23 (3)†
4	Medium *B*						324±14 (4)	
	Medium *B* + insulin						320±9 (4)	
5	–	249±6 (10)*						

* The inulin space was determined *in vivo*.
† Difference that is significant ($P < 0·05$).

The amount of water in heart muscle was not appreciably altered by the addition to the medium of glucose, of insulin, of albumin or of a number of amino acids and like substances (i.e., the use of medium *B*), nor of both the last-named and insulin.

In hearts that were not washed out or perfused the tissue water tended to be lower, 785 μl./g. wet wt., a difference of 27 μl./g. The reason for the small difference is not known.

The inulin space of rat heart muscle was 320 μl./g. wet wt. after perfusion for 5min. and remained relatively constant during 60min. of perfusion (Table 5). There was, however, a significant increase in the inulin space between 60 and 80min. and for that reason no subsequent experiment was continued beyond 60min. The size of the extracellular space was not altered by addition to the media of any of a number of substrates (Table 5).

In hearts that had not been perfused the inulin space was, on the average, 249 μl./g. wet wt., 66 μl./g. less than in hearts that had been perfused for as short a time as 5min. (Table 5). The extracellular space, as measured herein with inulin, accords with similar measurements made with sorbitol (Morgan *et al.* 1961; Fisher & Young, 1961), raffinose, or by compression (Fisher & Young, 1961), and this was true of the heart muscle of the intact animals and for heart perfused *in vitro*.

The initial increase in the size of the extracellular compartment during perfusion is not easily accounted for. Morgan *et al.* (1961) suggested that it was due to an equivalent increase in total tissue water; however, the change in tissue water (27 μl./g.) in the present experiments was not sufficient to account for the entire increase.

For the purpose of calculation, in any experiment the value for tissue water and the extracellular space as determined under exactly the same circumstances was used. In general, 40% of the tissue water was taken to be extracellular (320/812), and 60% intracellular. In many instances tissue water was determined on a portion of the same heart muscle as was the concentration of ninhydrin-positive substances.

Concentration of ninhydrin-positive substances in the wash-out fluid from perfused hearts. To calculate the intracellular concentration of amino acids and other ninhydrin-positive substances from the concentration in tissue water, the concentration in the extracellular fluid must be known. For hearts that were perfused the extracellular concentration was assumed to be the concentration in the perfusion medium; for hearts washed free of blood with Krebs–Henseleit bicarbonate buffer, but not further perfused, the extracellular concentration was assumed to be nil. To test the latter assumption, the heart was washed free of blood with 25ml. of buffer, and an additional 10ml. of medium was perfused through the heart, collected and the amount of ninhydrin-positive substances determined. The amount of ninhydrin-positive substances was 1·32 μmoles for the 10ml. of medium collected, of which only 0·22 μmole was amino acid. On that basis it can be calculated that the concentration of amino acid in the extracellular fluid is here less than 3% of that in the intracellular water and for that reason may be disregarded in the calculation of the latter.

Not all of the ninhydrin-positive material ordinarily present in the wash-out medium is derived from plasma contained in the extracellular space. That that is so was established in the following way. A heart was washed through with 50ml. of buffer, the perfusion effluent being collected in ten 5ml. fractions. Each fraction was deproteinized and the total ninhydrin-positive nitrogen determined. Plasma from the same animals was analysed in a like manner. From the concentration of ninhydrin-positive nitrogen in the plasma (6·55 μmoles/100ml.) and the weight of the heart (913mg.) it was calculated that 1·9 μmoles of ninhydrin-positive nitrogen were present in the extracellular space. Almost exactly that amount (1·97 μmoles) was recovered in the first 10ml. of wash-out fluid; the subsequent 5ml. fractions (i.e., fractions 3–10) had relatively constant amounts of ninhydrin-positive nitrogen (the values in μmoles were: 0·39; 0·50; 0·46; 0·39; 0·38; 0·39; 0·34). The results indicate that plasma is removed from the extracellular space by the first 10ml. of perfusion medium and that thereafter the concentration of ninhydrin-positive material in the extracellular space is sufficiently low so that it may safely be ignored. Moreover, it appears that during the wash-out of the heart with buffer there is slow leakage of ninhydrin-positive material from the intracellular compartment. Though the total amount of material in the wash-out medium is so small as to preclude reliable estimation of the amount of individual amino acids, the general pattern was found on analysis to reflect the concentration observed in heart muscle (results not shown).

Release of ninhydrin-positive nitrogen by perfused rat heart. During incubation of isolated rat diaphragm ninhydrin-positive material is released into the medium (Kline, 1949; Manchester, 1961) and the release appears to be a regulated process (Kline, 1949). Manchester (1961) reported that insulin decreased the release of ninhydrin-positive nitrogen by isolated diaphragm and, in addition, decreased the amount remaining in the diaphragm at the end of the incubation period. However, the effect of the hormone was small; glucose added to the medium was without effect.

The results with perfused rat heart (Table 6) were not the same as obtained by Manchester (1961) with diaphragm. Hearts perfused with Krebs–Henseleit bicarbonate buffer did release ninhydrin-positive nitrogen, but the amount was considerably less than that reported by Manchester (1961) for diaphragm. The values were: at 30min., heart 65 μg./g., diaphragm 186 μg./g.; at 60min., heart 110 μg./g., diaphragm 251 μg./g. (The values for release of ninhydrin-positive nitrogen by diaphragm were taken from Manchester, 1961.) The difference in the initial release of ninhydrin-positive nitrogen can most probably be ascribed to the leakage of material from diaphragm muscle that has many cut fibres, whereas all the fibres of perfused heart are intact. The interpretation is supported by the observation that the release of ninhydrin-positive nitrogen by diaphragm is greater than that by heart during the first 30min., but the difference is not nearly as great during the second 30min. period, i.e., in the period 30–60min. 65 μg./g. was released by diaphragm and 54 μg./g. by heart.

Insulin by itself did not significantly alter the release of ninhydrin-positive nitrogen (Table 6), and that was so whether the heart was perfused with Krebs–Henseleit bicarbonate buffer or a medium supplemented with amino acids (medium *A*). Insulin did, however, increase the concentration

Table 6. *Release of ninhydrin-positive nitrogen by perfused rat heart*

The hearts were perfused with Krebs–Henseleit bicarbonate buffer unless otherwise stated; the concentration of insulin, when present, was 0·1unit/ml. and that of glucose was 11mM. The values are the means ±S.E.M. of the numbers of observations in parentheses. The composition of medium *A* is given in Table 1. A negative value for material released represents a net uptake of ninhydrin-positive nitrogen. N.S., Not significant.

Addition to the medium	Total ninhydrin-positive nitrogen released (μmoles/100ml. of cell water) After perfusion for (min.)..... 10	30	60	Final concn. in heart (μmoles/100ml. of cell water)
(1) None	397±25·5 (12)	955±87·3 (12)	1750±64·3 (16)	6950±290 (16)
(2) Insulin	470±62·2 (12)	1020±101 (12)	1880±129 (12)	8010±340 (12)
P value versus (1)	N.S.	N.S.	N.S.	<0·05
(3) Glucose	440±133 (4)	772±166 (4)	868±137 (8)	9250±683 (8)
P value versus (1)	N.S.	N.S.	<0·001	<0·001
(4) Glucose and insulin	367±65·0 (4)	823±39·0 (4)	881±167 (8)	8180±304 (8)
P value versus (1)	N.S.	N.S.	<0·001	<0·01
(5) Glucose and medium *A*	−560±105 (6)	−485±223 (6)	37±178 (6)	11770±703 (6)
P value versus (1)	<0·001	<0·001	<0·001	<0·001
P value versus (3)	<0·001	<0·001	<0·01	<0·05
(6) Glucose, medium A and insulin	−366±203 (6)	−220±192 (6)	131±226 (6)	12000±1130 (6)
P value versus (5)	N.S.	N.S.	N.S.	N.S.

of ninhydrin-positive nitrogen in the intracellular water of hearts perfused with Krebs–Henseleit bicarbonate medium but not of hearts perfused with medium *A*; in the former case the increase in intracellular concentration cannot be accounted for by a concomitant change in medium concentration.

Glucose decreased the loss of ninhydrin-positive nitrogen by hearts perfused with Krebs–Henseleit bicarbonate buffer and increased the intracellular concentration; the addition of insulin did not produce a greater effect than that of glucose alone (Table 6). When hearts were perfused with medium *A*, glucose actually led to a net uptake of ninhydrin-positive nitrogen during the first 30min. of perfusion and restricted the loss during the subsequent 30min. to a relatively small amount; glucose also increased the intracellular concentration of ninhydrin-positive nitrogen when hearts were perfused with medium *A*. The effect of glucose on the release of ninhydrin-positive nitrogen is not apparent until after 30min. of perfusion and not statistically significant until 60min., whereas the addition of an amino acid mixture (medium *A*) produces a prompt decrease in the release of ninhydrin-positive nitrogen.

Table 7 records both the concentrations of amino acids and other substances released by hearts that had been perfused for 60min. with Krebs–Henseleit bicarbonate buffer or medium *B* and the concentrations of the same materials in the intracellular water of the hearts at the completion of the perfusion. (For comparison Table 7 has the concentrations of the same substances in the intracellular water of hearts washed free of blood with 25ml. of medium *B* and not further perfused.) It is apparent that changes in the concentration of ninhydrin-positive nitrogen do not accurately reflect changes in amino acid concentration. Of a total of 4410μmoles of ninhydrin-positive material released to the medium from each 100ml. of intracellular water by hearts perfused with Krebs–Henseleit buffer, only 1500μmoles, or 34%, is amino (or imino) acid. An even smaller proportion of the ninhydrin-positive nitrogen in intracellular water is amino acid; only 1700μmoles of a total of 8660μmoles/100ml. (or 20%) in hearts perfused with Krebs–Henseleit bicarbonate buffer, and 2422μmoles of a total of 8512μmoles (28%) for hearts perfused with medium *B*.

Perfusion of the hearts with a supplemented medium (medium *B*) almost completely prevents the release of ninhydrin-positive substances that occurs when hearts are perfused under similar conditions with Krebs–Henseleit buffer (Table 7). The concentration in the medium of most of the individual amino acids was lower at the end of the perfusion with medium *B* than at the start, indicating a net uptake of amino acids by the heart.

The initial intracellular concentration of most ninhydrin-positive substances was maintained relatively constant during 60min. of perfusion with medium *B*; only aspartic acid and taurine were significantly decreased in concentration, and the concentrations of isoleucine, leucine, phenylalanine and arginine were significantly increased (Table 7). However, perfusion with an amino acid-free medium (Krebs–Henseleit bicarbonate buffer) led to a decrease in the intracellular concentrations of

Table 7. *Changes in the concentrations of ninhydrin-positive substances in rat heart and medium after perfusion for 60 min. with Krebs–Henseleit bicarbonate buffer or medium* B

The hearts were perfused for 60 min. with either Krebs–Henseleit bicarbonate buffer or medium *B*. In the control experiment the hearts were washed free of blood with 25 ml. of medium *B* but not further perfused. The release of ninhydrin-positive substances for the experiment with medium *B* was the difference between the amount originally present in the medium and that present at the end of the experiment; an intracellular volume of 0·50 ml. was assumed for the heart in calculating the release per 100 ml. of cell water. A negative value for release indicates a decrease in the medium concentration of the substance during the perfusion. Most values are the means ± S.E.M. of three observations.

	Concn. of ninhydrin-positive substances in heart (μmoles/100 ml. of cell water)			Ninhydrin-positive substances released into medium (μmoles/100 ml. of cell water)	
	Control	Medium *B*	Bicarbonate buffer	Medium *B*	Bicarbonate buffer
Amino acids					
Hydroxyproline	—	29·0 ± 0·70	38·5 ± 5·80	—	—
Aspartic acid	604 ± 69·1**	102 ± 22·4	138 ± 7·02	47·0	81·9
Threonine	183	86·2 ± 33·7	79·9 ± 5·30	−19·2	314
Serine†	337	297 ± 42·4	73·7 ± 15·1**	−158	58·5
Glutamic acid	847 ± 63·6	753 ± 12·0	692 ± 79·7	45·6	175
Proline	47·1 ± 11·7	75·4 ± 14·7	16·0 ± 3·44*	−86·4	45·5
Citrulline	29·5 ± 1·85	25·9 ± 2·63	32·1 ± 1·52	−4·08	13·4
Glycine	136 ± 6·39	167 ± 15·6	95·6 ± 8·94*	67·2	136
Alanine	464 ± 69·4	380 ± 39·6	357 ± 93·4	144	348
Valine	21·2 ± 3·85	33·8 ± 4·00	12·0 ± 0·68**	−38·4	34·4
Cystine (half)	—	3·10 ± 0·10	9·38 ± 0·82**	−26·8	1·34
Methionine	12·7 ± 2·32	18·9 ± 2·32	8·94 ± 0·67	−4·08	15·0
Isoleucine	11·3 ± 1·56*	23·7 ± 3·71	7·49 ± 0·48*	−31·2	13·4
Leucine	23·8 ± 2·40*	37·1 ± 0·50	12·4 ± 1·52***	−33·6	34·4
Tyrosine	15·2 ± 2·87	18·0 ± 2·72	9·59 ± 0·72*	−6·48	20·0
Phenylalanine	10·8 ± 2·08*	20·8 ± 2·83	7·66 ± 0·73*	7·20	24·0
Ornithine	9·21 ± 1·92	10·9 ± 0·42	4·69 ± 1·36*	0·96	61·6
Lysine	121 ± 23·7	109 ± 5·60	72·0 ± 4·31**	4·80	79·5
Histidine	21·8 ± 2·44	33·0 ± 3·85	11·5 ± 2·34**	−10·1	31·8
Tryptophan	3·78 ± 0·83	6·77 ± 2·79	—	−20·6	—
Arginine	53·9 ± 4·50**	118 ± 6·34	42·8 ± 1·79***	−118	32·7
Non-amino acids					
Taurine	6070 ± 1040*	3090 ± 457	3800 ± 186	442	1270
Glutathione	822 ± 180	867 ± 166	1010 ± 632	—	—
Ammonia	2500 ± 473	2100 ± 321	2160 ± 425	192	1600
Total					
Amino acids	2730 ± 110	2422 ± 173	1713 ± 52·5*	−240	1500
Non-amino acids	9390 ± 1600	6060 ± 478	6970 ± 776	634	2870
Ninhydrin-positive substances	12120 ± 1700	8580 ± 59·8	8680 ± 82·1	394	4370

* $P < 0·05$ versus medium *B*; ** $P < 0·01$ versus medium *B*; *** $P < 0·001$ versus medium *B*.
† Includes glutamine and asparagine calculated as serine.

most of the amino acids. The release into the medium of specific ninhydrin-positive substances by heart perfused with Krebs–Henseleit buffer could not be correlated with the change in concentration of that substance in the intracellular water of the heart. The amount of material accumulating in the perfusion medium in general exceeded the change in concentration in the heart by a factor 2 or more, indicating that the amino acids lost from the heart were renewed by protein breakdown.

Concentration of ninhydrin-positive substances in rat heart muscle and plasma. The intracellular concentration of amino acids and like material in heart muscle and in plasma of rats is recorded in Table 8. In general, the values accord with those reported before for plasma and for other types of muscle (Schurr, Thompson, Henderson, Williams & Elvehjem, 1950; Kaplan & Nagareda Shimizu, 1963; Ryan & Carver, 1963); however, the values for heart muscle are different from those reported by Manchester & Wool (1963) and the reason for the difference is not known.

Table 8. *Concentrations of ninhydrin-positive substances in rat heart muscle and plasma*

Each heart was perfused with 25ml. of medium *B*. The values for plasma are from Scharff & Wool (1964). Most, but not all, values are the means ± S.E.M. of three observations.

	Concn. in heart (μmoles/100ml. of cell water)	Concn. in plasma (μmoles/ 100ml.)	Heart/plasma concentration ratio
Amino acids			
Aspartic acid	604±69·1	3·76±0·31	160
Threonine	183	25·2±0·84	7·3
Serine	110±5·89	53·2±2·30	2·1
Glutamic acid	847±63·6	11·3±1·10	75
Proline	47·1±11·7	32·5±0·07	1·4
Citrulline	29·5±1·85	8·70±0·36	3·4
Glycine	136±6·39	27·8±1·20	4·9
Alanine	464±69·4	40·9±0·63	11·3
Valine	21·2±3·85	18·8±1·10	1·1
Cystine (half)	—	6·14±0·84	—
Methionine	12·7±2·32	4·46±1·05	2·8
Isoleucine	11·3±1·56	8·78±0·31	1·3
Leucine	23·8±2·40	15·2±0·50	1·6
Tyrosine	15·2±2·87	7·28±0·69	2·1
Phenylalanine	10·8±2·08	7·64±0·57	1·4
Ornithine	9·21±1·92	6·53±0·44	1·4
Lysine	121±23·7	34·6±1·41	3·5
Histidine	21·8±2·44	7·31±0·23	3·0
Tryptophan	3·80±0·83	6·89±2·21	0·55
Arginine	53·9±4·50	17·5±0·21	3·1
Non-amino acids			
Taurine	7070±1040	35·6±4·40	200
Glutathione	822±180	—	—
Ammonia	2500±473	37·7±5·49	66
Total			
Amino acids	2730±111	354±26·2	7·7
Non-amino acids	10390±1600	73·3±7·22	140
Ninhydrin-positive substances	13120±1730	427±32·5	31

Three amino acids, glutamic acid, alanine and aspartic acid, account for 53% of the total concentration of amino acids in heart; only four other amino acids (threonine, serine, glycine and lysine) are present in concentrations in excess of 100 μmoles/100ml. of cell water. The concentration ratio (heart/plasma) for most amino acids is between 1 and 5; however, a few have a ratio greater than 10 (aspartic acid, glutamic acid and alanine). A large proportion of the ninhydrin-positive material in heart muscle is not amino acids but taurine, glutathione and other substances. The concentration ratio for the non-amino acids is exceedingly high.

Effect of insulin on the concentration of ninhydrin-positive substances in perfused rat heart. Insulin added *in vitro* increases the accumulation by muscle (isolated diaphragm or perfused heart) of radioactivity from amino[^{14}C]isobutyric acid and of radioactivity from several, but not all, ^{14}C-labelled utilized amino acids (Kipnis & Noall, 1958; Manchester & Young, 1960; Akedo & Christensen, 1962; Guroff & Udenfriend, 1961; Manchester & Wool, 1963; Wool, 1964). An experiment was undertaken to determine whether insulin affected the actual concentration of individual amino acids in heart muscle. For that purpose rat heart was perfused with a mixture (medium *B*) of ninhydrin-positive substances in the same concentrations as they are found in plasma (Table 8), and containing also pyruvate (an energy substrate whose utilization is not influenced by insulin; Villee, White & Hastings, 1952) and albumin, and the effect of insulin on the concentrations of amino acids and like material in the medium and in the intracellular water of the heart was determined after perfusion for different periods of time. To authenticate, before undertaking the laborious analysis, the occurrence of a physiological effect of insulin on the heart, amino[^{14}C]-isobutyric acid was added to the medium and the effect of the hormone on its concentration ratio determined in samples of the same muscle and medium that were subsequently analysed for their concentration of individual ninhydrin-positive substances. Insulin has been shown to increase

Table 9. *Accumulation of amino*[^{14}C]*isobutyric acid by perfused rat heart*

Hearts were perfused with medium *B* containing 0·05 μc of amino[^{14}C]isobutyric acid/ml.; the concentration of insulin, when present, was 0·1unit/ml. The hearts are those analysed in the experiment recorded in Table 10; the effect of insulin to increase accumulation of radioactivity from amino[^{14}C]isobutyric acid served to authenticate the occurrence of an insulin effect. The values are the means ± S.E.M. of three observations.

	Accumulation $\left(\frac{\text{counts/min./ml. of cell water}}{\text{counts/min./ml. of medium}}\right)$			
Insulin	After perfusion for (min.).....5	10	30	60
−	0·00	0·14 ± 0·08	0·99 ± 0·13	1·72 ± 0·27
+	0·17 ± 0·08	0·49 ± 0·00	1·35 ± 0·02	3·03 ± 0·69

the accumulation of radioactivity from amino[^{14}C]-isobutyric acid in heart muscle (Manchester & Wool, 1963), an observation that was confirmed (Table 9); in the presence of insulin the ratio of radioactivity in intracellular water to that in the medium was, for each time-period studied, greater than in the absence of the hormone.

The concentrations of amino acids in the medium remained constant during 60min. of perfusion of heart in control experiments (Table 10), and were not sensibly changed by the addition of insulin. (The apparent increase in the concentrations of aspartic acid and taurine after 60min. of perfuson was not observed in a subsequent experiment.)

The concentrations of amino acids and like material in the intracellular water of perfused heart evinced some variability (Table 10). The total amino acid concentration tended to decrease as a function of time; the decrease could be accounted for by a relatively large fall in the concentrations of aspartic acid, glutamic acid and alanine (amino acids that are readily transaminated in muscle); the concentrations of most of the other amino acids, especially leucine, tyrosine, phenylalanine, ornithine, lysine, histidine, tryptophan and arginine, however, increased during the perfusion. The concentrations in heart muscle of taurine, glutathione and ammonia decreased during the perfusion. The addition of insulin to the perfusion medium did not produce a significant change in the total concentration of amino acids in heart muscle; the concentration was slightly decreased after 10 or 60min. of perfusion (i.e., 6 and 15% respectively), but that was not the case after 5 or 30min. of perfusion. Examination of the concentrations of individual amino acids in heart muscle gave no clearer, or more meaningful, pattern. The concentrations of serine, proline and threonine tended to be increased, although not significantly so, in the presence of insulin; the accumulation in muscle of radioactivity from those three amino acids is increased by insulin (Akedo & Christensen, 1961; Wool, 1964); the same is true of radioactivity from glycine and methionine, but their concentrations in heart muscle were not consistently affected by the hormone. The concentrations in heart muscle of a number of amino acids (alanine, isoleucine, leucine, lysine, histidine and arginine) were actually lower when insulin was present. Insulin also decreased the intracellular concentration of taurine. Inasmuch as the concentrations of amino acids in the medium remained relatively constant, the concentration ratio (heart/medium) followed the changes in intracellular concentration.

Most frequently, then, insulin was without effect on the concentrations of amino acids in either the medium or the intracellular water of perfused heart; such changes as were induced by the hormone were quantitatively small, both positive and negative, and, finally, provided no clearly meaningful pattern. This was true despite the fact that insulin significantly increased the accumulation, in each of the very same hearts, of radioactivity from amino[^{14}C]isobutyric acid.

DISCUSSION

The existence of an intracellular pool of free amino acids, maintained at a concentration greater than in plasma, was first recognized by Van Slyke & Meyer (1913–14). The general characteristics of the process whereby the amino acids are concentrated has since been studied for a variety of tissues (for reviews see Wilbrandt & Rosenberg, 1961; Christensen, 1955; Holden, 1962) without the exact nature, or the character of its regulation by hormones (or other agents), having been revealed. Certainly, the actual intracellular concentration of individual amino acids is the resultant of a number of vectors: of those tending to increase the concentration by the entry of amino acids from the extracellular compartment, by the addition of amino acids from protein hydrolysis, or by the synthesis of new amino acids; and of those tending to decrease

Table 10. *Effect of insulin on the concentration of ninhydrin-positive*

Hearts were perfused with medium *B* and with or without insulin (0·1unit/ml.). The values are the means ±S.E.M. of three and are included with threonine for the hearts perfused for 5min., and with serine for the other hearts.

	Insulin	After perfusion for (min.).....5	Concn. in heart (μmoles/100ml. of cell water) 10	30	60
Amino acids					
Aspartic acid	−	531±93·5	359±57·9	102±18·3	416±134
	+	518±88·6	366±3·54	177±13·3	313±218
Threonine	−	386±10·0	77·8±1·52	75·9±3·43	85·6±5·49
	+	489±45·4	72·1±0·30	93·4±2·89	94·2±11·0
Serine	−	89·8±11·2	301±54·3	305±25·6	386±43·3
	+	125±8·18	332±12·5	398±36·7	403±31·8
Glutamic acid	−	919±62·2	700±22·2	746±239	561±115
	+	792±57·4	676±30·0	861±67·4	539±134
Proline	−	59·1±8·32	37·9±2·98	59·0±2·29	71·4±10·9
	+	62·6±6·41	39·0±6·90	75·5±5·87	92·5±9·57
Citrulline	−	26·3±1·48	27·9±0·32	32·5±4·44	42·2±4·68
	+	29·2±2·63	29·4±3·60	31·9±8·23	42·2±6·32
Glycine	−	133±5·85	140±14·7	119±8·29	145±14·9
	+	138±9·54	119±4·00	144±1·75	146±13·4
Alanine	−	509±16·8	512±14·4	385±39·7	282±114
	+	560±25·7	398±26·9	402±23·0	237±93·1
Valine	−	26·0±1·99	21·6±1·74	22·9±1·33	29·6±3·97
	+	24·5±0·38	19·6±3·05	27·6±0·52	26·0±3·66
Cystine (half)	−	—	—	—	—
	+	—	—	—	—
Methionine	−	15·9±1·68	12·0±0·42	12·9±1·08	13·3±0·92
	+	14·2±0·96	11·4±1·10	14·6±0·84	13·1±1·06
Isoleucine	—	15·2±2·42	14·3±0·82	14·6±1·04	17·7±1·48
	+	12·3±1·02	10·5±1·12	16·2±0·64	14·6±2·94
Leucine	−	26·2±2·00	29·7±2·11	32·7±1·39	39·1±4·10
	+	23·2±0·79	24·8±0·95	31·8±0·24	30·7±4·98
Tyrosine	−	13·8±2·72	15·4±1·22	14·8±0·06	19·8±1·90
	+	14·1±0·10	13·7±0·10	15·5±1·12	15·3±0·46
Phenylalanine	−	13·1±1·17	17·7±1·28	17·7±0·56	21·6±2·89
	+	11·5±1·73	16·1±0·81	17·9±0·90	17·5±0·24
Ornithine	−	9·82±0·98	10·4±0·55	12·7±0·81	11·8±1·28
	+	10·1±0·39	9·77±1·03	11·4±1·00	10·6±1·84
Lysine	−	87·8±4·04	98·0±7·37	94·6±7·94	117±12·0
	+	94·6±7·67	92·1±4·70	81·7±0·84	112±3·51
Histidine	−	21·0±1·41	28·1±2·63	29·8±2·85	32·4±1·14
	+	22·0±0·57	23·9±6·60	25·0±0·54	25·3±1·73
Tryptophan	−	5·71±0·52	9·55±0·24	7·99±0·84	9·15±1·75
	+	6·33±0·41	8·97±1·33	9·72±1·66	8·47±1·84
Arginine	−	64·5±3·06	83·8±8·99	100±1·51	131±11·3
	+	67·7±3·78	82·6±1·80	91·2±6·42	115±3·78
Non-amino acids					
Taurine	−	5450±886	4960±87·6	4740±157	4700±194
	+	6017±624	4762±399	4290±282	3690±107
Glutathione	−	993±68·5	408±24·8	367±25·6	423±7·99
	+	985±198	401±11·4	432±36·3	333±13·3
Ammonia	−	2620±159	1680±78·9	1590±66·5	1780±193
	+	2233±222	1511±15·0	1590±187	1389±68·2
Total					
Amino acids	−	2950±196	2500±59·9	2182±111	2430±86·4
	+	3010±158	2350±89·6	2510±114	2290±94·1
Non-amino acids	−	9060±869	7050±131	6690±138	6940±224
	+	9340±573	6680±402	6420±450	5410±163
Ninhydrin-positive substances	−	12010±967	9550±191	8873±225	9370±241
	+	12350±520	9030±492	8930±556	7700±236

the concentration by the exit of amino acids from the cell, by the utilization of amino acids for protein synthesis, or by their metabolic transformation. The interplay of that array of factors is reflected in the wide range of concentrations and concentration gradients for individual amino acids in muscle (Table 8). In rat heart muscle the average concentration ratio is 7·71, yet only three amino acids (aspartic acid, glutamic acid and alanine) exceed that value; for most the value is far less. The actual concentrations of aspartic acid, glutamic acid and alanine are also higher than those of the other amino acids, probably a reflection of the crucial role those three have in reactions that link carbohydrate and amino acid metabolism (Meister, 1955). Alanine, aspartic acid and glutamic acid are also present in muscle protein in high concentrations (Szent-Györgyi, 1960). The exceedingly high concentration of taurine in muscle, especially heart muscle, has been noted before (Awapara,

substances in perfused rat hearts and in the perfusion medium

observations with the exception of hearts perfused for 10 min. (two observations). Glutamine and asparagine were not hydrolysed

Concn. in medium (μmoles/100 ml.)				Heart/medium concentration ratio			
5	10	30	60	5	10	30	60
4·46±0·42	4·57±0·36	4·79±0·70	4·56±0·22	124±30·0	64·0±5·71	23·2±6·26	92·0±28·9
5·08±0·88	5·03±0·08	4·84±0·36	7·00±0·15	116±39·4	72·8±1·63	37·2±6·55	43·7±27·0
24·1±0·24	23·2±0·57	23·0±0·41	24·2±0·12	16·0±1·41	3·34±0·14	3·29±0·13	3·54±0·22
24·7±0·74	23·4±0·80	23·6±0·52	23·5±0·71	19·7±1·44	3·08±0·10	3·97±1·49	4·05±0·58
53·4±1·13	55·3±0·53	54·1±0·85	57·1±0·68	1·69±0·75	5·42±0·92	5·64±0·52	6·75±0·24
52·8±0·99	53·2±1·40	53·4±2·05	58·3±2·44	2·37±0·15	6·26±0·40	7·27±0·65	6·98±0·79
10·2±0·00	11·1±0·35	11·6±0·55	11·7±0·69	90·0±5·99	63·3±0·37	64·6±5·52	42·2±5·99
10·0±0·24	10·4±0·10	11·8±0·30	13·7±0·67	79·1±7·31	65·0±3·49	72·8±4·10	39·1±9·67
34·3±1·64	32·4±0·20	32·1±0·82	31·7±0·69	1·75±0·32	1·17±0·29	1·83±0·03	2·09±0·24
35·8±2·33	33·0±1·40	31·7±1·27	29·8±0·90	1·77±0·26	1·19±0·26	2·37±0·09	3·09±0·24
6·73±0·32	7·62±0·41	7·94±0·23	8·25±0·20	3·94±0·38	3·68±0·16	4·08±0·51	5·40±0·58
6·82±0·12	7·66±0·20	8·23±0·21	8·05±0·18	4·28±0·38	3·85±0·57	3·86±0·98	5·23±0·76
26·6±0·33	28·0±0·56	27·3±0·29	28·3±0·38	5·00±0·10	5·01±0·61	4·37±0·32	5·33±0·50
26·0±0·35	26·4±1·60	27·9±0·25	28·2±0·78	5·32±0·42	4·58±0·43	5·17±0·10	5·20±0·56
40·8±0·77	44·2±1·57	41·5±1·00	43·9±1·35	12·5±0·61	11·6±0·69	9·28±0·93	5·79±0·64
42·1±1·39	40·8±0·85	42·8±1·07	41·8±2·89	13·4±1·01	9·74±0·66	9·39±0·56	6·48±0·68
17·7±0·73	17·9±0·31	17·7±0·32	17·8±0·26	1·48±0·15	1·21±0·12	1·30±0·08	1·66±0·21
18·2±0·29	17·6±0·30	17·9±0·35	17·5±0·29	1·35±0·04	1·11±0·16	1·55±0·06	1·48±0·19
2·18±0·22	1·82±0·19	1·81±0·16	1·73±0·16	—	—	—	—
1·69±0·08	1·66±0·15	1·82±0·07	1·36±0·11	—	—	—	—
4·59±0·25	4·23±0·16	4·04±0·04	4·27±0·74	3·52±0·54	2·86±0·19	3·20±0·25	3·12±0·18
4·65±0·36	4·21±0·00	4·19±0·06	3·88±0·18	3·09±0·40	2·71±0·26	3·49±0·15	3·37±0·29
8·58±0·19	8·52±0·04	8·09±0·15	8·19±0·21	1·79±0·32	1·68±0·10	1·80±0·10	2·16±0·13
8·52±0·16	8·64±0·30	8·13±0·19	7·80±0·98	1·44±0·10	1·22±0·13	1·99±0·06	1·85±0·34
15·0±0·12	15·0±0·09	14·0±0·15	14·5±0·49	1·79±0·18	1·98±0·13	2·33±0·10	2·72±0·38
14·3±0·18	14·0±0·16	14·1±0·23	13·6±0·67	1·63±0·04	1·78±0·08	2·26±0·04	2·24±0·29
6·75±0·11	7·59±0·16	7·08±0·15	7·48±0·10	2·07±0·42	2·03±0·13	2·09±0·04	2·65±0·29
6·79±0·08	6·96±0·09	7·80±0·43	7·49±0·17	2·08±0·02	1·97±0·01	2·00±0·19	2·05±0·12
6·94±0·06	7·49±0·16	7·05±0·18	7·34±0·05	1·89±0·19	2·36±0·12	2·51±0·14	2·94±0·40
7·03±0·09	6·98±0·14	7·83±0·48	7·43±0·09	1·63±0·24	2·31±0·01	2·31±0·25	2·35±0·01
6·31±0·41	7·02±0·09	6·32±0·14	6·15±0·10	1·56±0·14	1·49±0·07	2·01±0·16	1·93±0·22
6·55±0·26	6·10±0·28	6·57±0·04	6·20±0·08	1·55±0·09	1·62±0·30	1·74±0·16	1·71±0·30
35·2±0·49	36·6±0·61	35·0±0·55	35·8±0·64	2·50±0·15	2·67±0·18	2·70±0·20	3·26±0·29
34·6±0·72	34·6±0·55	36·2±0·58	35·4±0·49	2·73±0·17	2·67±0·18	2·26±0·06	3·16±0·11
7·45±0·32	7·49±0·18	6·96±0·03	7·34±0·03	2·84±0·29	1·72±0·20	4·20±0·43	4·41±0·14
6·61±0·18	7·08±0·52	7·27±0·30	7·49±0·14	3·33±0·06	1·26±0·10	3·45±0·14	3·38±0·29
4·58±0·54	5·62±0·37	5·94±0·24	6·30±0·10	1·31±0·26	3.73±0·26	1·36±0·18	1·48±0·28
4·73±0·46	5·16±0·68	6·06±0·24	6·41±0·18	1·38±0·24	3·46±1·18	1·63±0·34	1·33±0·23
36·2±0·40	37·5±1·67	37·4±0·30	38·4±0·81	1·78±0·09	2·26±0·35	2·69±0·22	3·39±0·22
37·5±0·18	36·8±2·65	38·4±1·06	37·7±0·85	1·80±0·09	2·26±0·22	2·39±0·22	3·02±0·06
42·8±6·44	51·9±2·67	61·1±4·98	64·6±2·15	129±10·9	96·0±4·60	76·3±7·68	72·6±1·85
58·2±9·30	56·4±2·45	57·8±3·15	81·0±1·59	107±17·4	84·2±3·40	76·0±0·60	45·7±2·23
—	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—
107±7·06	102±3·24	98·3±1·36	111±1·22	24·8±3·08	16·5±0·95	16·1±0·81	16·0±0·20
121±8·65	98·4±0·54	106±1·78	112±2·34	19·7±2·89	15·4±0·10	15·1±2·00	12·5±0·86
352±3·05	363±2·92	354±4·42	363±4·42	8·40±0·62	6·87±0·19	6·16±0·34	6·68±0·16
354±5·24	350±10·5	360±7·97	356±11·4	8·61±0·64	6·72±0·46	6·98±0·32	6·45±0·45
150±5·55	154±5·50	161±4·58	175±1·78	60·4±4·67	45·9±1·47	41·6±1·94	39·6±0·91
179±16·6	155±1·73	163±2·74	193±5·00	53·0±0·54	31·1±10·2	39·3±2·43	28·1±1·41
502±8·38	517±7·97	515±7·45	539±3·85	23·9±1·84	18·5±0·65	17·2±0·66	17·4±0·38
533±21·7	504±7·52	524±5·55	549±14·0	23·2±1·99	17·9±1·20	17·1±1·07	14·1±0·78

1956; Garvin, 1960; Scharff & Wool, 1964). The functional significance of taurine remains a mystery.

Perfusion of the rat heart with a medium free of amino acids resulted in the release of ninhydrin-positive substances into the medium (Tables 6 and 7). The losses from the tissue were partially compensated for, presumably by protein breakdown, so that after 60 min. of perfusion the intracellular concentration of amino acids was about 60% of that originally present and some 40 times that of the medium. Guroff & Udenfriend (1960) reported that the tyrosine concentration was unchanged in diaphragms incubated for 90 min. in an amino acid-free medium. They concluded from that observation, and from the failure to obtain specific activity equilibrium between extracellular and intracellular tyrosine, that the endogenous tyrosine in muscle is not in rapid diffusion equilibrium with the external medium, but rather that the endogenous tyrosine is in a compartment separate

from the tyrosine accumulated in response to an elevated external concentration. No evidence has been obtained in the present study of 'compartmentation' of tyrosine in perfused rat heart; the intracellular tyrosine was decreased by perfusion with amino acid-free medium to about the same extent as was the total amino acid pool.

The presence in the perfusion medium of amino acids in the concentrations that they occur in plasma prevented the loss of amino acids into the medium. This is consonant with the finding of Christensen & Streicher (1949) that rat hemidiaphragms incubated *in vitro* will not release glycine into the medium if the medium contains a concentration of glycine equal to that of the plasma. At lower concentrations glycine release did occur, and at greater concentrations there was uptake of the amino acid.

The failure to observe an effect of insulin on the release of ninhydrin-positive nitrogen by perfused rat heart does not accord with the small but consistent decrease the hormone produces in isolated rat diaphragm (Manchester, 1961). The difference might have been attributed to a peculiarity of one or the other of the two preparations had not Akedo & Christensen (1962) found no effect of insulin on the exodus of preaccumulated radioactive glycine from diaphragm, a finding that does accord with our results with heart muscle. We are, however, still unable to explain the increase by insulin of the concentration of ninhydrin-positive nitrogen in hearts that had been perfused for 60min. with an amino acid-free medium.

Again, in contrast with the findings with isolated diaphragm (Manchester, 1961), the addition of glucose to the perfusion medium did significantly decrease the release of ninhydrin-positive nitrogen by the perfused rat heart. It is possible that in the heart, in contrast with isolated diaphragm, amino acid transport is limited in the absence of added oxidizable substrate by the availability of energy. The heart is contracting, and it is reasonable to suppose that its energy requirements are greater than those of isolated diaphragm. That the supply of energy can in some circumstances be limiting for amino acid transport has been shown by Newey & Smyth (1964). They found that glycine transport into everted intestinal sacs was increased by the addition of glucose to the medium. An analogous observation has been made with respect to protein synthesis in perfused rat heart. Glucose increases the incorporation of radioactivity from [^{14}C]glycine into protein of perfused rat heart (Wool & Manchester, 1962), but not into the protein of isolated rat diaphragm (Wool & Krahl, 1959).

One additional point need be made. Because the proportion of non-amino acids is so great the use of total ninhydrin-positive material, without fractionation, as a measure of amino acid metabolism in muscle, as has sometimes been done (Kline, 1949; Manchester, 1961), seems inappropriate. The ninhydrin reaction is not specific for α-amino acids (Harding & MacLean, 1916), nor is the intensity of the colour developed with the reagent constant for equimolar amounts of the individual substances that react with ninhydrin (Moore & Stein, 1948; Spackman *et al.* 1958). Many of the non-amino acid ninhydrin-positive substances give a less intense colour reaction; for example, the intensity of the colour developed with ninhydrin and measured at 570mμ is, for taurine and glutathione, only 12% of that obtained with a like amount of leucine, and for ammonia only 70%. For all of these reasons alterations in the concentration of the total of ninhydrin-positive substances is not a reliable guide to changes in the concentration of amino acids.

It was the purpose of the present study to determine the effect of insulin on the concentration, in the intracellular water of perfused heart, of each of the individual amino acids in circumstances reflecting as faithfully as possible those found physiologically. To do so required a method for the efficient extraction and accurate analysis of amino acids as well as conditions of perfusion of heart that allowed for reasonable maintenance of the intracellular amino acids at a physiological concentration and for sufficiently long a time so as to be able to measure hormone effects if they occurred. Those several objectives were achieved. The extraction of amino acids from muscle with sulphosalicylic acid at 100° proved efficient and simple; recovery and reproducibility were excellent. The determination of the concentration of each of the individual amino acids in the extracts was accurate; the single column used to chromatograph the amino acids gave good separation; the automatic analytical system provided reproducible results.

Several of the advantages of the perfused heart for the study of transport of substrates have been described before (Manchester & Wool, 1963). We now find, in addition, that the intracellular concentrations of amino acids in perfused heart can be maintained constant for as long as 60min., provided that the perfusion medium is supplemented with a mixture of amino acids, a source of energy and albumin; moreover, the total tissue water and the size of the extracellular space remain constant.

Despite the fact that the methods appeared in each respect to be adequate, no clear or meaningful effect of insulin on the concentrations of amino acids in the medium or in the intracellular water of heart was observed. The results, taken alone, can hardly be reckoned to support the conclusion that insulin increases the accumulation of amino acids in muscle. However, it is possible that in the

circumstance of the experiment an influence of insulin to promote amino acid entry into heart muscle was masked by an effect of the hormone to increase the utilization of amino acids for protein synthesis. The possibility is real, for insulin does accelerate the synthesis of protein in heart muscle (Wool & Manchester, 1962; Manchester & Wool, 1963), and, as Castles & Wool (1964) have shown, when protein synthesis is inhibited with puromycin, and only then, the hormone will increase the accumulation in muscle of radioactivity from several amino acids. For that reason we have tested the effect of insulin on the concentrations of amino acids in hearts perfused with sufficient puromycin so as to suppress protein synthesis all but completely. The observation that in the presence of the antibiotic insulin does increase the accumulation of amino acids in heart muscle is described in detail in the accompanying paper (Scharff & Wool, 1965).

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