Effects of Insulin-Induced Hypoglycaemia on the Fate of Glucose Carbon Atoms in the Mouse

By R. VRBA

Medical Research Council Neuropsychiatric Research Unit, Carshalton, Surrey

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1. [U- ^{14}C]Glucose was injected into mice and the distribution of ^{14}C in various chemical fractions of the whole body was determined at times from 15 min. to 8 hr. after injection. 2. At 1 hr. after injection 31.8% of the recovered ^{14}C was found in the expired air and 26.7% was found in the isolated glycogen, lipids, proteins, nucleic acids and in other acid-insoluble carbon compounds ('residual ^{14}C '). The rest (41.5%) was combined in acid-soluble substances. 3. When insulin was injected 5 min. or 1 hr. before injection of [U- ^{14}C]glucose, and the mouse was killed 1 hr. later, the ^{14}C content of expired air, glycogen, protein and 'residual ^{14}C ' was not significantly affected; but the incorporation of ^{14}C into lipids was increased two- to three-fold. 4. Chromatography of the lipids on silicic acid columns and by thin-layer chromatography showed that the main effect of insulin injection was to increase the incorporation of ^{14}C into fatty acids. 5. A significant increase of ^{14}C after insulin injection was also found in a glyceride in which the ^{14}C was combined in glycerol.

Injection of insulin into an experimental animal causes hypoglycaemia: the concentration of glucose in the blood and in the organs is decreased, and most of the free glucose is utilized. The numerous experiments that have been performed to account for the glucose which has disappeared have been reviewed by Stadie (1954), Chain, Beloff-Chain & Pocchiari (1956), Fisher (1960) and Young (1962). It has been suggested that the main effect of insulin on glucose metabolism is to accelerate the conversion of glucose into carbon dioxide, glucose 6-phosphate, fatty acids, triglycerides, lipids, glycogen or proteins. In some experiments, after the injection of insulin, a rise in liver glycogen has been observed, in others a fall in glycogen and in others no change (cf. Fisher, 1960). Several authors investigated the effect of insulin on particular organs, under different experimental conditions, and studied the changes in particular metabolites. From the vast amount of accumulated data, which are sometimes contradictory, it is at present difficult to assess the net effect of insulin injection (insulin-induced hypoglycaemia) on glucose metabolism in the whole intact body. There is also relatively little information about the fate of injected glucose in the organism as a whole.

The objects of the investigations described below were: (1) to obtain data on the quantitative relationship between the conversion of glucose into carbon dioxide and the incorporation of glucose

carbon into the acid-insoluble fraction (acid-insoluble glycogen, lipids, proteins, nucleic acids and other acid-insoluble substances); (2) to obtain information on the effect of insulin-induced hypoglycaemia on the fate of glucose carbon atoms in the intact body as a whole.

A part of this work has been published in preliminary form (Vrba, 1964, 1965*a,b*).

METHODS

Animals. Grey male mice of the Schofield strain were used in this study. At the time the mice were killed they weighed 25–35 g. All mice were fed *ad libitum* on the M.R.C. diet (B-41) until injection with [U- ^{14}C]glucose or insulin. In the period of time between the injection and the killing of the animal (a period ranging from 15 min. to 8 hr. but mostly 1 hr.) food and water were withdrawn.

Chemicals. [U- ^{14}C]Glucose was obtained from The Radiochemical Centre (Amersham, Bucks.). The radiochemical purity was determined by the suppliers by dilution with glucose and conversion into penta-acetate. Crystalline insulin was used as supplied by Allen and Hanburys Ltd. (London, W. 1) and the British Drug Houses Ltd. (Poole, Dorset) (Batch no. 2287).

Experiments without injection of insulin. Male mice (25–30 g.) were first treated with antibiotics to decrease the intestinal flora (neomycin, 250 mg./kg., in 0.2 ml. of water by mouth, 24 hr. before glucose injection). They were then injected intraperitoneally with 15 μC of [U- ^{14}C]glucose and killed by being dropped into liquid N_2 either immediately after the injection (control animals) or at times ranging from 15 min. to 8 hr. after injection. During this

Scheme 1. $\rightarrow^{14}\text{C}$ indicates that the fraction was made up to volume and that suitable samples were then plated on planchets and the radioactivity was counted. For further details see 'Chemical fractionation procedure 1' in the Methods section.

period each mouse was kept in a desiccator, to which three gas-absorption flasks (containing 15 ml. of 2N-NaOH each) were attached in series. Air was constantly sucked from the desiccator and passed together with the expired CO_2 through the three gas-absorption bottles, where all expired $^{14}\text{CO}_2$ was trapped. The contents of the absorption bottles were then combined and together with washings from the bottles made up to 100 ml. and, after suitable further dilution, directly plated for counting. The frozen carcass was subjected to chemical fractionation procedure 1 (see below).

Experiments with injection of insulin. Male mice (fed *ad libitum*) weighing 25–35 g. were used. A batch of 12 mice was divided into three groups of four mice each (groups A, B and C). Each mouse received a subcutaneous injection of [^{14}C]glucose over the left flank (16.7 μC of [^{14}C]glucose in the presence of 0.023 mg. of carrier glucose, dissolved in 0.5 ml. of 0.9% NaCl solution). Group A received only [^{14}C]glucose, whereas groups B and C also received insulin. Insulin was injected subcutaneously over the right flank. Crystalline insulin dissolved in 0.9% NaCl was used, the volume of injection varying between 0.1 and 0.4 ml. Groups B and C received the insulin 5 min. and 1 hr. respectively before the injection of [^{14}C]glucose. The doses of insulin were 0.05, 0.10, 0.2 and 0.4 unit for the respective animals of each group of four. The mice were killed 1 hr. after the injection of [^{14}C]glucose by dropping them into liquid air. In all instances a marked hypoglycaemia was observed and the effect of insulin on the fate of glucose carbon atoms was basically similar (Table 1). The geometrical increase in the dose of insulin and its injection either almost simultaneously with or 1 hr. before the injection of [^{14}C]glucose was intended to ensure that the results obtained are not related to a particular stage of insulin hypoglycaemia but are valid for various stages of insulin hypoglycaemia in general. This assumption has been justified by the experimental results (Table 1 and Fig. 4).

Expired $^{14}\text{CO}_2$ was collected as described in experiments without injection of insulin and with the frozen carcass subjected to chemical fractionation procedure 2 (see below).

Chemical fractionation procedure 1 (cf. Scheme 1). (a) Acid-soluble fraction. The frozen carcass was powdered in a precooled brass mortar and the homogeneous powder obtained was transferred quantitatively into 5 vol. of 5% (w/v) HClO_4 , cooled to 0°. The tissue was homogenized for 3 min. in the high-speed Ultra-Turrax homogenizer (Janke und Kunkel K.G., Staufen/Br., Germany), with blades rotating at 24000 rev./min. The homogenate was centrifuged at 1500–2000g for 30 min. in an MSE Major centrifuge (0°), and the sediment rehomogenized in another 5 vol. of 5% HClO_4 . (All other homogenizations and centrifugations mentioned below were performed in a similar way.) This procedure was repeated three times, and the combined supernatants were made up to 500 ml. A 40 ml. portion of the acid-soluble fraction was neutralized with 60% (w/v) KOH at 0°, the pH was adjusted with 0.1 N-HCl and 0.1 N-KOH by using indicator paper, and the precipitated KClO_4 was separated by sedimentation overnight in a cold room (4°) and centrifugation. The precipitate was resuspended in 40 ml. of water and removed by sedimentation and centrifugation. The combined supernatants (fraction A.S.) were made up to volume and plated for counting [cf. below under (g)].

(b) Crude lipids. The acid-insoluble residue from the above procedure was homogenized in 5 vol. of Bloor's mixture (ethanol-diethyl ether, 3:1, v/v) and after standing for a week at room temperature the suspension was centrifuged. The combined supernatants were made up to 500 ml. with methanol and suitable portions were plated for counting (fraction L.1).

(c) Purified lipids. The foregoing 'lipid fraction' contains impurities, and was purified in the following way: 10 ml. was neutralized with 60% (w/v) KOH followed by 0.1 N-HCl to the point of decolorization of phenolphthalein and centrifuged to eliminate KClO_4 , the supernatant was evaporated to dryness *in vacuo* in a boiling-water bath and the dry residue kept at 100° for 30 min. This treatment decomposes the protein-lipid complexes. The dry residue was resuspended in 10 ml. of chloroform-methanol (2:1, v/v) and extracted by mechanical rotation for 6 hr.; the solution was centrifuged, the sediment was re-extracted with another 5 ml. of chloroform-methanol and suitable portions of the combined supernatant ('purified lipids') were plated for counting (fraction L.2).

(d) Nucleic acid fraction. The carcass residue, after lipid extraction, was homogenized in 50 ml. of 10% (w/v) NaCl solution and kept at 100° for 1 hr. in a boiling-water bath. The tissue was sedimented by centrifugation. This procedure, recommended for nucleic acid extraction (Davidson & Smellie, 1952), was repeated three times. The combined NaCl extracts were treated with 2 vol. of 95% (v/v) ethanol to precipitate nucleic acids, the precipitate was washed with ethanol, the supernatants were combined and suitable portions were plated for counting. This supernatant (N.A.1) contained radioactive acid-insoluble impurities that are not nucleic acids. The precipitate was dissolved in 5 ml. of 0.3 N-KOH and kept overnight at 38° to decompose nucleic acids into soluble nucleotides (Davidson & Smellie, 1952). KOH was eliminated by neutralization with diluted 5% (w/v) HClO_4 , the precipitated KClO_4 was washed with 5 ml. of water in the centrifuge tube and the combined supernatants (N.A.2) were made up to 25 ml. with water. Portions of this supernatant were plated for counting. A further portion was mixed with 5 vol. of ethanol to precipitate acid-insoluble glycogen and other compounds that may become soluble during boiling with 10% NaCl but are not hydrolysed during the treatment with KOH. The precipitated glycogen was dissolved in 5 ml. of water and reprecipitated with 5 vol. of ethanol. (The radioactivity of this sediment is included in fraction N.A.2 and included in the final calculation into 'residual ^{14}C '.) The combined supernatants, which represent a 'purified nucleic acid fraction' (N.A.3), were made up to volume and plated for counting.

(e) Crude protein. A part (500 mg.) of the dried residue from the pellet remaining after extraction of the nucleic acid fraction was homogenized and dissolved in 100 ml. of 90% (v/v) formic acid, and portions of this fraction (P.1) were plated for counting.

(f) Purified amino acids from proteins. Another portion of the crude protein fraction was homogenized in 15 ml. of 5% (w/v) trichloroacetic acid and kept in a water bath at 90° for 15 min. with constant stirring. This eliminates nucleic acids, acid-insoluble glycogen and free amino acids retained by absorption (Schneider, 1945; Kits van Heijningen & Kemp, 1955). The suspension was centrifuged, the supernatant discarded and the residue homogenized in 10 ml. of 6 N-HCl and hydrolysed in a sealed Pyrex test

tube for 18 hr. at 110°. The hydrolysate was evaporated to dryness *in vacuo* and dissolved in 10 ml. of water, neutralized with 0.1 N-NaOH, and passed through a column (15 cm. $\times$ 1 cm.) of Zeo-Karb 225 (H⁺ form) cation-exchange resin. The water effluent (100 ml.) was discarded and the amino acids were eluted with 200 ml. of N-NH₃; the eluate was evaporated to dryness *in vacuo*, the residue was dissolved in water, filtered and made up to volume, and suitable portions of this fraction (P.2, 'purified amino acids from proteins') were plated for counting.

(g) Determination of radioactivity. All samples were pipetted on to plastic planchets (diam. 2.5 cm.), three different portions being used for calculation of self-absorption, and then dried in desiccators over conc. H₂SO₄ and NaOH pellets. From each sample 1000 counts were taken (in triplicate) in a Nuclear-Chicago automatic gas-flow counter fitted with a Micromil window. The samples were corrected as necessary for self-absorption and background.

(h) Calculation of results and of the 'residual ¹⁴C'. The results are expressed as percentages of the total ¹⁴C recovered. The total ¹⁴C per mouse is obtained from the sum of the following fractions: CO₂ + A.S. + L.1 + N.A.1 + N.A.2 + P.1. From the three mice killed at zero time, a mean of $(1552 \pm 62) \times 10^3$ counts/min. per mouse was obtained. From five mice killed at 15 min.-8 hr. after injection of [U-¹⁴C]glucose a mean of $(1560 \pm 42) \times 10^3$ counts/min. per mouse was obtained.

The acid-insoluble fraction (Fig. 1) represents the sum of L.1 + N.A.1 + N.A.2 + P.1, whereas 'residual acid-insoluble ¹⁴C' (Fig. 2) represents (L.1 + N.A.1 + N.A.2 + P.1) - (L.2 + N.A.3 + P.2), i.e. the sum of the unpurified fractions minus the sum of the purified fractions (cf. Scheme 1). The fraction of 'residual acid-insoluble ¹⁴C' includes acid-insoluble glycogen, 'lipid-bound' proteins and 'protein-bound' lipids. These were eliminated during the purification procedures applied for obtaining 'purified lipids' and 'purified amino acids from proteins'. The ¹⁴C lost during the purification procedure is also included in this fraction.

Chemical fractionation procedure 2 (cf. Scheme 2 and Tables 1 and 2). (a) Acid-soluble substances and acid-soluble glycogen. At 1 hr. after injection of [U-¹⁴C]glucose the mouse was removed from the desiccator and dropped into liquid air. The frozen carcass was powdered in a precooled brass mortar and the homogeneous powder obtained was transferred quantitatively into 5 vol. of 5% (v/v) HClO₄, cooled to 0°. The tissue was homogenized for 3 min. as in fractionation procedure 1, the sediment was rehomogenized five times with fresh portions of HClO₄ and the combined supernatants were made up to 1000 ml. A 100 ml. portion of this acid-soluble fraction was neutralized with 60% (w/v) KOH at 0° until decolorization of phenolphthalein occurred, the pH was adjusted to neutral reaction with 0.1 N-HCl and 0.1 N-KOH by using indicator paper, and the precipitated KClO₄ was separated by sedimentation overnight in a cold room (0°) and centrifugation. The precipitate was resuspended in 40 ml. of water and removed by sedimentation and centrifugation. The combined supernatants were evaporated to dryness in a rotary evaporator, and transferred with four 5 ml. portions of water into a volumetric flask through a funnel with filter paper, which eliminated a considerable amount of KClO₄. The filtrate was made up to 25 ml. Of this, 5 ml. was mixed with 25 ml. of ethanol, left overnight in the cold room and

the glycogen was dissolved in 5 ml. of water and reprecipitated three times with ethanol as described below (c). The combined supernatants were evaporated to dryness, dissolved in 5 ml. of water and 0.15, 0.20 and 0.25 ml. portions were plated on plastic planchets for counting. Glucose was estimated in 0.2 ml. portions of this glycogen-free acid-soluble fraction by using glucose oxidase by the method of Guidotti, Colombo & Foa (1961).

The precipitated and purified glycogen was dissolved in water, made up to 5 ml. and 0.2, 0.4 and 0.6 ml. portions were plated for counting. In suitable portions glycogen was estimated by using the anthrone reagent method of Seifter, Dayton, Novic & Muntwyler (1950).

(b) Lipids. The acid-insoluble residue from the above procedure was homogenized in 200 ml. of ethanol-acetone (1:1, v/v), leached overnight at room temperature and centrifuged. The sedimented pellet was homogenized with 200 ml. of chloroform-methanol (2:1, v/v), containing 1 vol. of conc. HCl in 300 vol. of the organic solvent. The homogenate was leached overnight at room temperature and centrifuged. The combined supernatants were made up to 500 ml. with methanol and 0.2, 0.3 and 0.4 ml. portions were plated for counting. Then 25 ml. or 50 ml. portions were used for silicic acid chromatography as described below.

(c) Acid-insoluble glycogen. The tissue residue remaining after extraction with HClO₄ and with organic solvents was spread on Petri dishes (15 cm. diam.) and air-dried in a fume cupboard under a constant current of air. The pellet was thoroughly powdered in a mortar and the yield weighed to the nearest milligram.

A 400 mg. sample of the acid-insoluble residue was suspended in 10 ml. of 30% (w/v) KOH and kept in a boiling-water bath for 3 hr. After cooling, 3 vol. of ethanol was added to the mixture, which was kept overnight in a cold room. The glycogen was separated by centrifugation, the supernatant carefully decanted and discarded, and the pellet dissolved in 6 ml. of water and centrifuged again. The water-insoluble sediment was discarded, and the combined supernatants (including washings of the water-insoluble sediment) were mixed with 30 ml. of ethanol, left in the cold room overnight and centrifuged. The supernatant was decanted and the test tube drained for 1 min. The pellet, consisting mainly of glycogen, was dissolved in water, made up to 10 ml. and 0.2, 0.3 and 0.4 ml. portions were plated for counting. In suitable portions glycogen was estimated by using the anthrone reagent method of Seifter *et al.* (1950).

(d) Purified amino acids from proteins. Of the dry acid-insoluble defatted residue, 250 mg. was suspended in 30 ml. of 5% (w/v) trichloroacetic acid and kept in a water bath for 30 min. at 90° under constant stirring. This procedure eliminates nucleic acids (Schneider, 1945), traces of absorbed free amino acids and acid-insoluble glycogen (Kits van Heijningen & Kemp, 1955). The undissolved residue was separated by centrifugation and the supernatant was discarded. The extraction with hot trichloroacetic acid was repeated. The residue was homogenized in 15 ml. of 6 N-HCl with the Ultra-Turrax homogenizer and hydrolysed in a sealed Pyrex test tube for 18 hr. at 110°. Thereafter the protein-bound amino acids were purified and isolated, and their radioactivity was measured as described in chemical fractionation procedure 1 (Scheme 1).

(e) 'Residual ¹⁴C'. A 250 mg. portion of the dried residue

Scheme 2. $\rightarrow^{14}\text{C}$ indicates that the fraction was made up to volume and that suitable samples were then plated on planchets and the radioactivity was counted. For further details see 'Chemical fractionation procedure 2' in the Methods section.

from the pellet remaining after extraction of the lipids was homogenized and dissolved in 50 ml. of 90% (w/v) formic acid, and 0.2, 0.4 and 0.6 ml. portions of this fraction were plated for counting.

This radioactivity represents the total ^{14}C in the remainder of the tissue after extraction with organic solvents. From this amount the radioactivity of the acid-insoluble glycogen and that of the purified amino acids of protein were subtracted. The resulting 'residual ^{14}C ' includes nucleic acids, 'tissue-bound' lipids and the ^{14}C lost during the purification of the amino acids from proteins and of the glycogen. It represents 3.8–7.2% of the total ^{14}C recovered from the mouse and its amount was not affected by the injection of insulin.

(f) Determination of radioactivity. The procedure was the same as in chemical fractionation procedure 1.

Silicic acid chromatography of lipids: procedure A. The chromatographic separation of lipids was carried out by using silicic acid columns as described by Fillerup & Mead (1953) and Dvorkin, Chetverikov & Shmelev (1963), with minor modifications. The modified procedures are described below.

A column (15 cm. $\times$ 1 cm.) of silicic acid (Mallinckrodt analytical reagent, 100 mesh) was prepared and 'activated' by washing with successive portions (100 ml. each) of methanol, acetone, diethyl ether and light petroleum (b.p. 60–80°).

Of the lipid fraction 25 ml. was neutralized with a few drops of 30% (w/v) KOH to eliminate traces of free HClO_4 , and the surplus alkali was carefully eliminated with 0.1 N-HCl to the point of decolorization of phenolphthalein. The lipid solution was concentrated on a rotary vacuum evaporator to a volume of about 10 ml. and transferred drop by drop with a Pasteur pipette from the evaporation flask to a piece of thoroughly defatted cotton wool. (Before use, the cotton wool was soaked in a mixture of chloroform-methanol and the organic solvents were squeezed off. This was repeated three times. The defatted cotton wool was air-dried before use.) The flask in which the neutralized lipid mixture was concentrated was rinsed with three portions (5 ml. each) of chloroform-methanol (2:1, v/v) and these washings were transferred to the same piece of cotton wool. A cold stream of air from a hair-drier was used to accelerate the evaporation. The air-dried cotton wool containing the lipid sample was cut into small pieces with scissors, and these pieces were transferred to the top of the silicic acid column and covered with a sufficient amount of light petroleum to prevent drying of the column. Thereafter 200 ml. portions of the following solvent were passed through the column: 1% (v/v) diethyl ether in light petroleum (b.p. 60–80°); 50% (v/v) diethyl ether in light petroleum; 25% (v/v) methanol in diethyl ether.

Of the effluent 25 ml. fractions were collected and evaporated to dryness, and the residue was dissolved in 3 ml. of chloroform-methanol (2:1, v/v). Then 0.2, 0.3 and 0.4 ml. portions were plated, and the ^{14}C was counted and corrected for self-absorption and background as described above.

Silicic acid chromatography of lipids: procedure B. A silicic acid column (30 cm. $\times$ 3 cm.) was used, and, after the column had been activated (as described in procedure A above), a sample of total lipids was put on the column on a piece of cotton wool as described above and the lipids were eluted with the following solvents: 1% (v/v) diethyl ether in light petroleum (1000 ml.); 50% (v/v) diethyl ether in

light petroleum (1000 ml.); chloroform (1000 ml.); chloroform-methanol (9:1, v/v) (500 ml.); chloroform-methanol (4:1, v/v) (500 ml.); chloroform-methanol (3:2, v/v) (500 ml.); chloroform-methanol (1:4, v/v) (500 ml.); methanol (500 ml.). Fractions (50 ml.) were collected from the column and the radioactivity was measured by plating 1 and 2 ml. portions. In 10 ml. portions of each fraction phosphorus was estimated by the method of Martin & Doty (1949).

Hydrolysis of lipids. To localize the ^{14}C in the radioactive lipid samples, the lipids were hydrolysed and the non-saponifiable, saponifiable and residual water-soluble components were separated. A sample of crude lipid (one-tenth of the total lipid extract of the mouse) was separated on a silicic acid column (procedure A) into three fractions. The fractions were evaporated to dryness on a vacuum rotary evaporator. To the residue, 2 ml. of 30% (w/v) KOH and 15 ml. of ethanol were added, and the mixture was incubated at 70° for 3 hr. in a water bath.

(a) Non-saponifiable fraction. After hydrolysis, 15 ml. of water was added to the hydrolysate, and the mixture extracted three times with 50 ml. portions of light petroleum (b.p. 60–80°) in a separating funnel. The combined light-petroleum extracts were washed with 50 ml. of 0.3% (w/v) KOH followed by 50 ml. of water. The upper phase was evaporated to dryness and dissolved in 10 ml. of neutralized ethanol.

(b) Saponifiable fraction. The washings from the non-saponifiable fraction were combined with the saponified fraction, acidified with N-H₂SO₄ (methyl orange) and extracted four times with 50 ml. portions of light petroleum (b.p. 60–80°). The combined light-petroleum extracts were washed twice with 50 ml. of water. The upper phase was evaporated to dryness and the residue dissolved in 10 ml. of neutralized ethanol. The lower phase represents the water-soluble fraction.

(c) Water-soluble fraction. The washings from the saponifiable fraction were added to the residual phase of the hydrolysate and made up to 200 ml. with water.

Suitable portions of all three fractions were used for measurements of radioactivity.

RESULTS

At 1 hr. after the intraperitoneal injection of [^{14}C]glucose about 40% of the ^{14}C had been eliminated from the organism as $^{14}\text{CO}_2$, whereas about 20% was incorporated into the acid-insoluble fraction (Fig. 1). This acid-insoluble fraction consists of proteins, lipids, nucleic acids and 'residual' carbon (Fig. 2). Lipids and proteins isolated after extensive purification contained at least 3.7 and 3.1% respectively of the total ^{14}C (Fig. 2). At 8 hr. after the intraperitoneal injection of [^{14}C]glucose, 82.5% of the labelled carbon had been expired as $^{14}\text{CO}_2$. Only 2.9% of the ^{14}C remained in the acid-soluble fraction (Fig. 1).

The anabolism of glucose, i.e. its utilization for the synthesis of substances of higher molecular weight than glucose, reached a maximum about 1 hr. after its injection (Figs. 1 and 2). Therefore in experiments in which insulin-induced hypo-

Fig. 1. Distribution of ^{14}C in $^{14}\text{CO}_2$ (O), acid-soluble substances (●) and acid-insoluble substances (▲) in mice after the intraperitoneal injection of $[\text{U-}^{14}\text{C}]$ glucose. Male mice (25–30 g.) were injected with $15\mu\text{C}$ of $[\text{U-}^{14}\text{C}]$ glucose in 1 ml. of water in the presence of 0.038 mg. of carrier glucose and killed (at intervals as indicated in graph) by being dropped into liquid N_2 . For further experimental details see 'Chemical fractionation procedure 1' in the Methods section and Scheme 1.

Fig. 2. Incorporation of ^{14}C into acid-insoluble substances in mice after the intraperitoneal injection of $[\text{U-}^{14}\text{C}]$ glucose. ●, Purified protein-bound amino acids; ▲, purified lipids; ○, nucleic acids; ■, residual acid-insoluble ^{14}C . For experimental conditions see Fig. 1 and 'Chemical fractionation procedure 1' in the Methods section, and also Scheme 1.

glycaemia was produced the period of 1 hr. was selected for a study of the effect of insulin injection on the fate of glucose carbon atoms.

The experimental animals were divided into three groups: a control group that received only $[\text{U-}^{14}\text{C}]$ glucose, and two hypoglycaemic groups, one receiving insulin injection 5 min. before $[\text{U-}^{14}\text{C}]$ glucose and the other receiving insulin injection 1 hr. before $[\text{U-}^{14}\text{C}]$ glucose.

The only significant increase of ^{14}C as a result of insulin injection was in the lipids (Table 1). In the control group, a mean of 6.2% of the recovered ^{14}C was found in the lipids. Injection of insulin increased the amount of ^{14}C in the lipids two- to three-fold. This increase is statistically significant. No increase of ^{14}C was observed in the expired air, or in glycogen, proteins or any other fraction, suggesting that the main effect of insulin-induced hypoglycaemia on glucose metabolism consists in an increased conversion of glucose into lipids. Changes of glycogen are recorded in Table 2.

In further experiments various classes of lipids were separated to see if insulin-induced hypoglycaemia increased the incorporation of glucose carbon atoms into lipids generally, or if particular lipid constituents were affected. Separation of lipids from control animals and insulin-injected

animals was carried out on silicic acid columns. The injection of insulin caused a marked increase in the incorporation of glucose carbon into the non-polar lipid fraction (eluted with solvent 1), and a smaller increase in the polar lipid fraction (eluted with solvent 3) (Fig. 3). In the control as well as in the insulin-injected animals the incorporation of glucose carbon into the fractions of medium polarity was negligible. These results are statistically significant and are valid for all doses of insulin used in the experiments (Fig. 4).

After hydrolysis of the non-polar fraction (eluted with 1% diethyl ether in light petroleum), the radioactivity of this fraction was completely recovered in the fatty acids. Almost all of the radioactivity of the polar lipids (eluted with 25% methanol in diethyl ether) was found in the water-soluble constituents of the hydrolysate (Table 3).

These experiments showed that insulin injection caused an increased incorporation of ^{14}C into two constituents of the lipids. This conclusion was confirmed with samples of crude lipids by using a combined and more elaborate chromatographic procedure (Fig. 5).

Fig. 5 shows that the lipid extract obtained from

Table 1. *Effect of insulin injection on the fate of [U-¹⁴C]glucose carbon atoms in the mouse*

Male mice (25–35 g.) were injected subcutaneously with 16.7 μ C of [U-¹⁴C]glucose in 0.5 ml. of water in the presence of 0.023 mg. of carrier glucose and killed by being dropped into liquid air 1 hr. later. Mice in groups B and C received insulin subcutaneously in doses indicated in the Table. For further details see 'Chemical fractionation procedure 2' in the Methods section and Scheme 2. Results are expressed as percentages $\pm$ M.E.M. of the recovered ¹⁴C. (M.E.M., mean error of the mean.)

Distribution of ¹⁴ C (%)											
Acid-soluble substances				Acid-insoluble substances							
Injected insulin (unit)	Time of injection of insulin before [U- ¹⁴ C]glucose	Acid-soluble substances		Expired CO ₂	Acid-soluble (not including glycogen)	Acid-soluble glycogen	Tissue-bound glycogen	Lipids	Purified protein-bound amino acids	Residual ¹⁴ C	Total glucose in mouse (mg.)
		substances	glycogen								
Group A	—	37.9	38.2	37.9	6.9	5.0	4.6	3.6	4.4	3.8	21.5
		21.8	45.9	31.8	6.8	7.5	6.4	4.7	4.4	6.8	20.5
		26.9	46.6	±4.5	5.9	4.0	±0.9	4.6	±0.3	5.5	43.5
		40.6	35.3	±2.7	3.1	3.3	7.4	4.8	5.6	19.5	
Group B	5 min.	26.0	32.9	37.9	3.7	5.9	20.0	3.8	3.7	7.6	6.9
		37.9	23.9	33.5	1.2	7.2	6.3	17.5	4.4	7.9	4.1
		27.2	±4.1	±2.5	2.5	8.0	±0.8	16.6	±2.4**	6.8	±0.5
		42.7	32.7	3.2	3.2	4.1	9.0	2.7	5.7	3.4	
Group C	1 hr.	18.2	52.3	5.2	5.2	4.6	8.1	4.5	7.2	7.2	10.2
		32.1	31.7	39.0	2.9	4.1	4.6	18.3	3.9	7.0	6.6
		37.7	±4.1	±4.6	2.2	5.1	±0.2	10.9	±2.1*	6.6	±0.4
		30.0	38.4	5.5	5.5	4.6	12.7	3.4	5.5	4.6	±1.2**

* $P < 0.05$; ** $P < 0.01$.

Table 2. *Effect of insulin on acid-soluble and acid-insoluble glycogen in the mouse*

Results are expressed as mg. of glucose equivalents/100g. fresh wt. of whole animal $\pm$ M.E.M. All other details are as given in the legend to Table 1.

	Insulin injected (unit)	Time of injection of insulin before [U- ¹⁴ C]glucose	Glycogen content (mg. of glucose equivalents/100 g. body wt.)	
			Acid-soluble glycogen	Acid-insoluble glycogen
Group A	0	—	123.3	49.6
	0	—	290.0	67.1
	0		193.3	55.2
	0		146.2	56.9
			188.2 ± 36.9	57.2 ± 3.6
Group B	0.05	5 min.	53.2	91.8
	0.10		38.8	68.7
	0.20		33.3	42.3
	0.40		23.2	59.2
			37.1 ± 6.2*	65.5 ± 10.3
Group C	0.05	1 hr.	26.0	20.7
	0.10		11.4	65.2
	0.20		7.3	40.3
	0.40		67.2	50.3
			28.0 ± 13.7*	44.1 ± 9.3

* $P < 0.01$.

Fig. 3. Effect of insulin injection on the distribution of [U-¹⁴C]glucose carbon atoms in lipids of the mouse. Silicic acid column chromatography (procedure A) was used. Arrows indicate solvents used for elution: 1, 1% diethyl ether in light petroleum; 2, 50% diethyl ether in light petroleum; 3, 25% methanol in diethyl ether. $\circ$, Controls, no insulin injected; $\blacktriangle$, insulin injected (a, 1.8 units/kg.; b, 3.1 units/kg.).

Fig. 4. Effect of insulin injection on the distribution of [U-¹⁴C]glucose carbon atoms in lipids of the mouse. Male mice (23–35g.) were injected subcutaneously with 16.7 μ C of [U-¹⁴C]glucose in 0.5ml. of water in the presence of 0.023mg. of carrier glucose and killed by immersion in liquid air 1 hr. later. One-tenth of the total lipid extract was separated on silicic acid columns. Group A, controls, no insulin injected; group B, insulin injected 5 min. before [U-¹⁴C]glucose; group C, insulin injected 1 hr. before [U-¹⁴C]glucose. In groups B and C the points from left to right represent animals that received 0.05, 0.10, 0.20, 0.40 unit of insulin respectively. (Increasing the dose of insulin does not further increase the incorporation of ¹⁴C into the fraction eluted with solvent 1.) Solvent 1, 1% diethyl ether in light petroleum; solvent 2, 50% diethyl ether in light petroleum; solvent 3, 25% methanol in diethyl ether. Vertical lines represent M.E.M.

a hypoglycaemic animal gave two well-defined peaks of radioactivity. Fractions 3 and 74 (Fig. 5), which contain the peaks of radioactivity, were subjected to thin-layer chromatographic separation (Figs. 6 and 7). In each case only one radioactive

Table 3. *Distribution of [U - ^{14}C]glucose carbon atoms in lipids of the mouse after insulin injection*

The mouse was killed 1 hr. after the injection of [U - ^{14}C]glucose. Insulin (1.8 units/kg.) was injected 5 min. before [U - ^{14}C]glucose. The lipids were fractionated by chromatography on silicic acid columns by procedure A. Results are expressed as percentages of ^{14}C in total lipids. For further details see 'Hydrolysis of lipids' in the Methods section.

Fraction	Eluent	Distribution of ^{14}C (%)		
		1% Diethyl ether in light petroleum	50% Diethyl ether in light petroleum	25% Methanol in diethyl ether
Non-saponifiable		1	1	1
Saponifiable		78	2	2
Water-soluble		0	2	13

Fig. 5. Distribution of [U - ^{14}C]glucose carbon atoms in lipids of the mouse. Insulin (0.40 unit) was injected subcutaneously into a 29 g. male mouse 1 hr. before [U - ^{14}C]glucose and the mouse was killed 1 hr. after the latter. The lipids were chromatographed on a silicic acid column (see 'Silicic acid column chromatography: procedure B' in the Methods section). Arrows indicate solvents used for elution: 1, 1% diethyl ether in light petroleum; 2, 50% diethyl ether in light petroleum; 3, chloroform; 4, chloroform-methanol (9:1, v/v); 5, chloroform-methanol (4:1, v/v); 6, chloroform-methanol (3:2, v/v); 7, chloroform-methanol (1:4, v/v); 8, methanol. ●, ^{14}C ; ○, P after digestion with $HClO_4$, estimated by the method of Martin & Doty (1949).

spot was obtained. The radioactivity of the non-polar fraction (eluted from silicic acid columns with 1% diethyl ether in light petroleum) was concentrated on thin-layer plates in one spot corresponding to methyl esters of fatty acids. Sterol esters are the only naturally occurring lipids that can be eluted from silicic acid columns with light petroleum containing only 1% of diethyl ether (Fillerup & Mead, 1953; Hirsch & Ahrens, 1958; Lis, Tinoco & Okey, 1961). This led me to believe that this fraction consisted mainly of sterol esters (Vrba, 1965a,b). Further investigation showed that this conclusion was wrong, as under my experimental conditions the main part of this fraction consisted of methyl esters of fatty acids, which were ap-

parently formed by transesterification during the process of extraction of lipids with mixtures of organic solvents containing ethanol, methanol and traces of perchloric acid.

The fractions containing phospholipids and other polar radioactive lipids (eluted from silicic acid columns with 25% methanol in diethyl ether) were pooled, evaporated to dryness and hydrolysed in 10% (w/v) hydrochloric acid for 30 min. at 100° in a boiling-water bath according to the method of Klenk (1940). This procedure should liberate carbohydrate from lipids. The acid hydrolysate was extracted four times with equal volumes of light petroleum and the extract discarded. The water-soluble part of the lipid hydrolysate was then

evaporated to dryness *in vacuo* to eliminate hydrochloric acid, the residue was dissolved in a small amount of water and portions of this solution were subjected to descending chromatography on Whatman no. 1 paper, with four different solvent systems for separation. After chromatography the filter-

paper strips were air-dried and scanned for radioactivity in a Baird and Tatlock Ltd. radioactive chromatogram counter. The results of this experiment are recorded in Fig. 8. With four different solvent systems in each case only one radioactive spot was recorded on each strip, this giving further

Fig. 6. Thin-layer chromatography of the non-polar lipid fraction 3 (Fig. 5) obtained after chromatography of the total lipids of the mouse on silicic acid columns. Ascending chromatography was used, with the solvent system 5% (v/v) diethyl ether in light petroleum (b.p. 40–60°) (Rowe, 1964). 1, Cholesteryl oleate (marker); 2, palmitic acid (marker); 3, methyl palmitate (marker); 4, sample of fraction 3 (Fig. 5). Zones (as indicated) of this sample were removed from the chromatographic plate, mounted into a small column, eluted with chloroform-methanol (2:1, v/v), plated and counted. Spots were located with iodine vapours.

Fig. 7. Thin-layer chromatography on silicic acid of the polar lipid fraction no. 74 (Fig. 5). A portion of this fraction was placed in the form of a thin strip on the start of a chromatographic plate covered with a thin layer of silicic acid and subjected to chromatography with the solvent system chloroform-methanol-aq. NH_3 (sp.gr. 0.88) (18:6:1, by vol.) (Davison & Graham-Wolfaard, 1964). After drying the chromatogram was divided into 1 cm. strips, which were separately removed from the chromatogram, eluted and counted, as described in the text. Only one well-defined peak was observed.

Fig. 8. Radiochromatography of the water-soluble fraction of the hydrolysate of polar lipids. Portions of the water-soluble constituents of the acid hydrolysate of polar lipids were chromatographed on 2 cm.-wide strips on Whatman no. 1 filter paper by using descending chromatography with four different solvent systems. After chromatography the chromatograms were scanned for radioactivity in a Baird and Tatlock Ltd. radioactive chromatogram counter. The following solvent systems were used: (a) 2-methylpropan-1-ol-85% (w/v) formic acid-water (8:1:1, by vol.); (b) butan-1-ol-ethylene glycol monoethyl ether-ethanol-water-aq. NH_3 (sp.gr. 0.88) (4:4:1.5:4:1.25, by vol.); (c) butan-1-ol-pyridine mixture prepared as described by Chargaff, Levine & Green (1948); (d) propan-1-ol-ethyl acetate-water (7:1:2, by vol.).

Fig. 9. Chromatographic identification of the radioactive component of the water-soluble fraction of the hydrolysate of polar lipids. 1, Hydrolysate+glycerol; 2, glycerol; 3, glucosamine; 4, galactose; 5, hydrolysate; 6, glucose; 7, inositol; 8, hydrolysate. The solvent was 2-methylpropan-1-ol-85% (w/v) formic acid-water (5:1:1, by vol.).

evidence that the total ^{14}C in the polar lipids was combined in only one water-soluble constituent of the lipid hydrolysate. The radioactive area of the chromatograms gave a positive reaction when treated with the silver nitrate reagent according to the procedure of Trevelyan, Procter & Harrison (1950), thus indicating that the substance was a carbohydrate or glycerol. The R_f indicated that it was glycerol. This was confirmed by the fact that on chromatography on paper in four different solvent systems (which are listed in Fig. 8) the substance showed a similar R_f to glycerol, but distinctly different R_f from sugars known to occur in lipids and used as reference standards (glucose, glucosamine, inositol and galactose). The radioactive water-soluble constituent also gave only one spot in four different solvent systems when subjected to chromatography together with glycerol. A tracing of one of these chromatograms is presented in Fig. 9. On the basis of these results I conclude that the radioactive water-soluble constituent obtained from the hydrolysate of polar lipids is glycerol.

DISCUSSION

Remarks on the chemical fractionation procedures. The experiments presented in this paper were performed on two groups of animals. The first group received only $[\text{U-}^{14}\text{C}]\text{glucose}$ (Figs. 1 and 2) whereas the second group was given an injection of insulin before the injection of $[\text{U-}^{14}\text{C}]\text{glucose}$ (Tables 1 and 2). The frozen carcass of the animals of the second group was subjected to a fractionation procedure (Scheme 2) that was a modification of the chemical fractionation procedure applied to the group 1 (Scheme 1). The modifications were introduced into the second procedure for the following reasons.

(a) No special effort was made to obtain a highly

purified nucleic acid fraction, as it was found (by using chemical fractionation procedure 1) that this fraction contained only a negligible proportion of the injected glucose carbon atoms (Fig. 2). In procedure 2 the ^{14}C of nucleic acids forms a part of the 'residual carbon'.

(b) Procedure 2 was adjusted to enable the incorporation of glucose carbon atoms into glycogen to be followed as closely as possible. It should be remembered that the state of glycogen in the tissues is still an open question. It has been suggested that glycogen occurs in the tissues only partly in a 'free' acid-soluble form, and that a considerable part is in a 'tissue-bound' acid-insoluble form. This acid-insoluble glycogen can be released only after treatment with hot (50°) trichloroacetic acid whereas the acid-soluble glycogen is released with cold trichloroacetic acid (Kits van Heijningen, 1957; Kits van Heijningen & Kemp, 1955). Recently Roe and his co-workers (Carroll, Longley & Roe, 1956; Roe, Bailey, Gray & Robinson, 1961) have questioned the existence of two forms of glycogen in animal tissues and conclude that the acid-insoluble glycogen is an artifact produced by insufficient extraction with cold acid and believe that the existence of two forms of glycogen in animal tissue has not been unequivocally established. On the other hand, Singh & Venkatasubramanian (1963) conclude from differences in the rate of incorporation of ^{14}C from $[\text{1-}^{14}\text{C}]\text{glycine}$ into the two forms of glycogen that the existence of two glycogen fractions has been clearly demonstrated.

These considerations and the necessity to examine more closely the conversion of glucose into glycogen after insulin injection led to the adoption of special precautions when dealing with glycogen. The extraction of the powdered mouse carcass with cold perchloric acid was performed five times (instead of three times as in chemical fractionation procedure 1), and each extraction was performed with the help of a high-speed homogenizer according to the procedure of Roe *et al.* (1961). The acid-soluble glycogen was isolated from the cold neutralized acid extract by precipitation with ethanol. A portion of the acid-insoluble tissue sediment (after extraction of lipids) was hydrolysed in potassium hydroxide and the hydrolysate precipitated with ethanol, as described in more detail in the Methods section. The results recorded in Tables 1 and 2 support the claim that at least two glycogen fractions exist, namely 'acid-soluble' and 'acid-insoluble' glycogen.

(c) It was observed in experiments performed according to fractionation procedure 1 that the purification procedure applied to the lipid fraction removed only a negligible amount of ^{14}C . In fractionation procedure 2 the measurements of radio-

activity were therefore performed only on the crude lipid extracts, but further measurements were performed on lipid fractions obtained after silicic acid chromatography. The conversion of 'crude lipids' into 'purified lipids' as described in procedure 1 was omitted in procedure 2.

Fate of glucose carbon atoms in vivo. [$U-^{14}C$]-Glucose, when injected into an intact animal, is eventually converted almost completely into $^{14}CO_2$; however, this process is not very rapid and it takes several hours before half the injected [$U-^{14}C$]glucose can be recovered in the expired carbon dioxide (Holt, Schmidt & Feldmann, 1961). An unestimated part of the glucose carbon is known to be incorporated into substances such as lipids, amino acids and proteins, which are not commonly included as intermediates in the generally accepted schemes of oxidation of glucose to carbon dioxide. It has also been shown that only a part of the expired carbon dioxide is formed by the direct oxidation of glucose; a considerable part of the respiratory carbon dioxide comes from what have been described as 'non-glucose sources' (Feller, Strisower & Chaikoff, 1950; Stetten, Welt, Jugle & Morley, 1951; Feller, Chaikoff, Strisower & Searle, 1951). A rapid and extensive incorporation of glucose carbon atoms into amino acids in the brain and other organs has been observed *in vivo* (Winzler, Moldave, Rafaelson & Pearson, 1952; Roberts, Flexner & Flexner, 1959; Busch, Fujiwara & Keer, 1960; Vrba, 1962; Vrba, Gaitonde & Richter, 1962; Gaitonde, Marchi & Richter, 1964), and it has been shown that glucose carbon is utilized for the synthesis of proteins and lipids in the brain and other organs. Although it is known that the utilization of glucose in various organs can be influenced by starvation, hypoglycaemia, drugs and other factors (Holt *et al.* 1961; Bachelard, Gaitonde, Richter & Vrba, 1962; Vrba, Bachelard & Krawczyński, 1963) it is not known how much of the injected glucose is actually assimilated *in vivo* into high-molecular cell constituents and what is the relative velocity of these processes.

In the experiments described above it was found that 1 hr. after the injection of [$U-^{14}C$]glucose 20–40% of the injected glucose carbon atoms had been converted into $^{14}CO_2$ (Fig. 1 and Table 1). This illustrates the importance of glucose as a substrate for oxidation. On the other hand, these results indicate that this is not the only or even the main role of glucose *in vivo* since the rate and extent of the anabolic reactions (conversion of glucose into molecules larger than glucose) were comparable with the rate and extent of the catabolic reactions: 1 hr. after injection of [$U-^{14}C$]glucose up to 35% of the glucose carbon atoms had been utilized for anabolic processes (Table 1). This means that a substantial proportion of the glucose carbon atoms

is rapidly assimilated *in vivo* into proteins, lipids and other high-molecular substances.

At 8 hr. after the injection of [$U-^{14}C$]glucose 82.5% of the labelled carbon had been oxidized to $^{14}CO_2$ and only 2.9% of the ^{14}C remained in the acid-soluble fraction (Fig. 1). Consequently, of the $^{14}CO_2$ produced by the total oxidation of the injected [$U-^{14}C$]glucose at least 14% must be derived from the oxidation of acid-insoluble substances including the lipids and proteins. These data show that glucose carbon atoms are utilized *in vivo* extensively and rapidly in the biosynthesis of proteins and lipids and that proteins and lipids are significant as intermediates in the process of oxidation of glucose to carbon dioxide. There is independent evidence that a part of the carbon dioxide eliminated from the brain is derived from oxidation of non-glucose substances, particularly from α -amino acids, lipids, proteins and nucleic acids (Geiger, 1958; Geiger, Horvath & Kawakita, 1960; Geiger, Kawakita & Barkulis, 1960; Allweiss & Magnes, 1958a,b).

Effects of insulin-induced hypoglycaemia on the fate of glucose carbon atoms. Injection of insulin did not increase the ^{14}C content of the expired air, of the acid-soluble glycogen, of the acid-insoluble 'tissue-bound' glycogen, of the protein or of the 'residual' carbon; but the rate of incorporation of ^{14}C into lipids was more than doubled (Table 1). It is concluded that the injection of insulin can influence glucose metabolism by changing the relative rates of catabolism to anabolism of glucose in favour of the latter. In the present experiments the injection of insulin increased the incorporation of glucose carbon atoms into certain lipid constituents in particular. These observations agree with the experiments of Stetten & Boxer (1944b), who showed that in alloxan-diabetic rats the synthesis of fatty acids from glucose was decreased to about 5% of the normal rate.

Another observation of Stetten & Boxer (1944a) is that only about 3% of the dietary glucose is converted into glycogen in the normal rat and that at least ten times as much of the dietary glucose is used to synthesize fatty acids as is used to synthesize glycogen. In the present experiments no evidence was obtained that insulin-induced hypoglycaemia in the mouse is connected with a net conversion of free glucose into glycogen: the rate of incorporation of glucose carbon into glycogen was not changed after insulin injection. This does not support the conclusion (Best, Hoet & Marks, 1926a; Best, Dale, Hoet & Marks, 1926b) that the glucose which disappears under the action of insulin is equal to the sum of the glycogen deposited in the muscles and the glucose equivalent of the oxygen absorbed. However, the experiments of Best *et al.* (1926a,b) were performed on spinal preparations of

the eviscerated cat. Possibly there are species differences in the mechanism of insulin action, or this mechanism may be disturbed by evisceration or use of drugs in connexion with the preparation of an eviscerated spinal cat. These problems call for further investigation.

The experiments described in this paper, which were performed on the whole animal, do not rule out the possibility that insulin may accelerate the local incorporation of some amino acids into protein in some particular organ, e.g. the diaphragm, on which a considerable number of experiments with insulin have been performed (Manchester & Young, 1958, 1960, 1961; Manchester & Krahrl, 1959). No evidence was obtained in my experiments to suggest that the main (or any measurable) metabolic effect of insulin-induced hypoglycaemia consists in an increased conversion of glucose into proteins, glycogen or carbon dioxide, considering the whole intact body of the mouse. However, it is possible that such effects may be of importance in individual organs.

In summary, the experiments reported here, which were performed *in vivo*, indicate the following: the main metabolic effect of injection of insulin into mouse (in amounts causing hypoglycaemia) is to channel increased amounts of glucose carbon atoms into two particular constituents of the lipids, one of which is the fatty acids and the other the glycerol combined in a glyceride. It has yet to be seen what is the anatomical site of these processes and if these results apply only to the mouse or have a more general validity.

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