Short Communications

Evidence for the Metabolism of Glycerol by Skeletal Muscle and the Presence of a Muscle Nicotinamide-Adenine Dinucleotide Phosphate-Dependent Glycerol Dehydrogenase

By C. J. Toews
Department of Biochemistry, Queen's University, Kingston, Ontario, Canada

(*Received* 13 *December* 1965)

The absence of a readily demonstrable glycerol kinase in muscle (Wieland & Suyter, 1957) has been generally regarded as indicating an inability of muscle to metabolize glycerol (Randle, Garland, Hales & Newsholme, 1963; Garland & Randle, 1964). There is, however, one report of the isolation of [^{14}C]glycogen from muscle that had been incubated in a medium containing [^{14}C]glycerol (Bloom & Foster, 1964).

In attempts to obtain further information on the ability of muscle to metabolize glycerol, [1,3-$^{14}C_2$]glycerol was incubated with rat diaphragm muscle and the radioactivities of the CO_2 produced during the incubation and of the lipids extracted from the tissue and medium after the incubation were measured.

In Table 1 the results of [^{14}C]glycerol metabolism in skeletal muscle are given. It is evident that some of the [^{14}C]glycerol has been converted into $^{14}CO_2$. If it is assumed that the respiratory quotient of muscle in the absence of added substrates (apart from glycerol) is 0·8 (Long, 1961), approx. 1·3% of the total CO_2 produced could have been derived from glycerol. The average incorporation of [^{14}C]-glycerol/hr./100mg. wet wt. of muscle into CO_2 is 0·113% and into lipids is 0·106%. In a few experiments liver slices were also incubated to determine the relative ability of the two tissues to metabolize glycerol to CO_2. In several experiments measurements were made of the concentration of glycerol in the incubation medium before and after the 60min. incubation. With the liver slices there was a slight fall in the glycerol concentration of the medium, whereas with diaphragm the glycerol concentration of the medium doubled after the 60min. of incubation. This indicates that glycerol was being released from the muscle during the incubation, presumably derived from triglyceride hydrolysis. Whereas much less $^{14}CO_2$ was formed from the glycerol in muscle than in liver (Table 1), the isotope dilution of glycerol in the medium containing muscle suggests that the rate of metabolism of glycerol to CO_2 in muscle is probably similar to the rate of its conversion into CO_2 in liver.

Though the results in Table 1 establish that glycerol is metabolized in muscle they provide no indication of any pathway for its metabolism. Several attempts to demonstrate the presence of a glycerol kinase in muscle were unsuccessful. However, during an investigation of all conceivable ways by which glycerol might enter the metabolic processes in muscle, the presence of an NADP-specific glycerol dehydrogenase was established. The enzyme was found predominantly in the non-particulate fraction of the cell. It is precipitated by between 50% and 70% saturation with $(NH_4)_2SO_4$. Partial purification was achieved by passing the resuspended 50–70% saturated $(NH_4)_2SO_4$ precipitate through Sephadex G-100. The R_F of muscle glycerol dehydrogenase on Sephadex G-100 was 2·04, suggesting a mol. wt. of approx. 15000 (Whitaker, 1963).

Because the equilibrium for muscle glycerol dehydrogenase was found to be in the direction of glycerol formation ($K_{eq.} = 1{\cdot}6 \times 10^{-3}$ at pH 7·0) most enzyme assays were made by measuring the oxidation of NADPH with glyceraldehyde as substrate. Kinetic measurements were made at 340mμ with a Unicam SP. 800 spectrophotometer equipped with a variable-speed slave recorder with a full-scale expansion of 0–0·1 extinction unit. The incubation was carried out at 25° in quartz cuvettes of 1cm. light-path. The incubation medium contained sodium phosphate buffer, pH 7·0 (50mM), in a total volume of 3·0ml. D-Glyceraldehyde, NADPH and glycerol dehydrogenase were present in various concentrations depending on the experiment. The control cuvette contained all reagents except D-glyceraldehyde. The reaction was started by the addition of enzyme to the cuvettes containing all the other components.

Because the double-reciprocal plots of initial velocity versus concentration of D- or DL-glyceraldehyde could be superimposed when the calculation for the DL-racemate was made for the D-isomer only, it was concluded that the enzyme was specific for D-glyceraldehyde. Dihydroxyacetone was reduced at 5% of the rate of D-glyceraldehyde. The rate with NADH was only 10% of that with an equivalent amount of NADPH. The pH optimum

Table 1. *Formation of* $^{14}CO_2$ *and* ^{14}C*-labelled lipids from* [^{14}C]*glycerol*

The diaphragm pieces from male hooded rats (Quebec Breeding Farms, St Eustache, Quebec, Canada) were incubated in Warburg flasks containing: 2·5ml. of a low-calcium (55mM) Krebs–Ringer phosphate buffer, pH7·4 (Krebs & Henseleit, 1932), glycerol (3·3mM), 1μc of [1,3-$^{14}C_2$]glycerol (0·16mc/mg.; New England Nuclear Corp., Boston, Mass., U.S.A.) and rat tissue slices (100–200mg.) in a total volume of 3·0ml. The glycerol substrate was in one side arm and 0·2ml. of 50% (w/v) citric acid in the other. The filter paper in the centre well was moistened with 0·1ml. of 33% (w/v) KOH. After 15min. of preincubation at 37° the glycerol was added from the side arm. The contents of the control flasks were identical with those of the experimental flasks, but the tissue was boiled before its addition to the flasks. Oxygen uptake was measured at 5min. intervals for 60min. Citric acid was then added from the side arm to liberate all the CO_2 from the buffer. The contents of the centre well were quantitatively transferred to the main compartment of another Warburg flask containing 0·2ml. of Hyamine hydroxide (1·0M-di-isobutylcresoxyethoxyethyldimethylbenzylammonium hydroxide in methanol; Rohm and Haas Co., Philadelphia, Pa., U.S.A.) in the centre well and 0·2ml. of 7·1N-H_2SO_4 in the side arm. After the system was closed the H_2SO_4 was added to the main compartment and the flasks were shaken for 40min. The Hyamine hydroxide was then transferred to a scintillation vial containing Liquifluor–toluene [Pilot Chemicals Inc., Watertown, Mass., U.S.A.; the Liquifluor was diluted in toluene to give a concentration of 4g. of 2,5-diphenyloxazole and 50mg. of 1,4-*p*-bis-(5-phenyloxazol-2-yl)benzene/l.] scintillation solution. Lipids were extracted with chloroform–methanol (Folch, Lees & Sloane-Stanley, 1957). After evaporation to dryness the lipids were dissolved directly in 10ml. of Liquifluor–toluene scintillation fluid. The samples were counted in a Nuclear–Chicago scintillation counter (model 724). The efficiency was determined by the channels-ratio method (Bush, 1963).

Tissue slices in experimental flasks	Radioactivity in CO_2* ($\mu\mu$c)		Percentage of [^{14}C]-glycerol converted into $^{14}CO_2$	Percentage of total CO_2 derived from glycerol	Radioactivity in muscle lipids* ($\mu\mu$c)	
	Experimental flask (per 100mg. of tissue)	Control flask			Experimental flask (per 100mg. of tissue)	Control flask
Diaphragm (200mg.)	945	45	0·09	0·98	—	—
Diaphragm (280mg.)	367	45	0·032	0·36	—	—
Diaphragm (110mg.)	1990	183	0·18	1·3	—	—
Diaphragm (120mg.)	1460	183	0·13	1·0	—	—
Diaphragm (170mg.)	1340	226	0·11	1·3	—	—
Diaphragm (100mg.)	520	264	0·03	0·4	—	—
Diaphragm (80mg.)	704	3	0·07	2·6	—	—
Diaphragm (160mg.)	1560	3	0·16	4·6	—	—
Diaphragm (190mg.)	340	212	0·0012	0·16	186 (0·017)‡	14
Diaphragm (230mg.)†	1480	212	0·012	0·67	1470 (0·14)‡	14
Diaphragm (100mg.)†	—	—	—	—	1670 (0·16)‡	58
Diaphragm (170mg.)	4320	37	0·43	—	—	—
Liver (220mg.)	2190	169	0·21	3·2	—	—
Liver (190mg.)	3440	167	0·32	3·0	—	—
Liver (190mg.)	8850	303	0·85	—	—	—

* Counts/min. were calculated to 100% efficiency by dividing by the efficiency obtained by channels-ratio counting.

† Glycerol 0·8mM.

‡ Percentage of [^{14}C]glycerol incorporated into lipids.

is 7·0 for D-glyceraldehyde reduction and 9·0 for glycerol oxidation. The K_m values for the reactants are: D-glyceraldehyde, $4·5 \times 10^{-5}$M; NADPH, $1·39 \times 10^{-5}$M; glycerol, 0·1M; NADP, $1·25 \times 10^{-5}$M. The K_m values, however, are somewhat dependent on the concentration of the fixed substrate. No bivalent metal requirement for the enzyme could be demonstrated, and EDTA is not inhibitory at a concentration of 10mM. Glycerol dehydrogenases that are NADP-dependent have been described in micro-organisms (Chiang & Knight, 1959; Rao, Ramakrishnan & Sirsi, 1960) and also in rat liver (Moore, 1959).

The present findings indicate that glycerol can be metabolized in skeletal muscle. The presence of a glycerol dehydrogenase suggests that the initial step of glycerol metabolism may be the formation of D-glyceraldehyde. Fructose 1-phosphate might then be formed by the action of the muscle aldolase to condense D-glyceraldehyde with dihydroxyacetone phosphate. It could then be possible for fructose 1,6-diphosphate, a normal

glycolytic intermediate, to be formed by the action of a specific fructose 1-phosphate kinase (Slein, Cori & Cori, 1950). It is, however, difficult to speculate on the activity of the enzyme under conditions obtaining in intact muscle owing to the lack of knowledge of steady-state concentrations of glyceraldehyde and of glycerol in muscle cytoplasm. In phosphate buffer, pH 7·4 (0·05M), with saturating concentrations of D-glyceraldehyde and NADPH the glycerol dehydrogenase in homogenates of skeletal muscle metabolized 2800mμmoles of D-glyceraldehyde/hr./g. of muscle. Under optimum conditions, without provision for the removal of the glyceraldehyde, the rate of the reaction in the reverse direction (glycerol to D-glyceraldehyde) is one-tenth of this. The rate of [^{14}C]glycerol conversion into $^{14}CO_2$ (113mμmoles/hr./g. of muscle) and incorporation into lipids (29mμmoles/hr./g. of muscle) in diaphragm slices (Table 1) is therefore apparently of a similar order of magnitude to the activity of glycerol dehydrogenase in skeletal-muscle homogenates.

I thank Dr P. B. Hagen for his advice and criticism during these investigations, which were supported by a grant from the Medical Research Council of Canada. The work was carried out during the tenure of a Post-Doctoral Fellowship of the Muscular Dystrophy Association of Canada.

Bloom, B. & Foster, D. W. (1964). *J. biol. Chem.* **239**, 967.
Bush, E. T. (1963). *Analyt. Chem.* **35**, 1024.
Chiang, C. & Knight, S. G. (1959). *Biochim. biophys. Acta*, **35**, 454.
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
Garland, P. B. & Randle, P. J. (1964). *Biochem. J.* **93**, 678.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Long, C. (1961). *Biochemists' Handbook*, p. 804. London: E. and F. N. Spon Ltd.
Moore, B. W. (1959). *J. Amer. chem. Soc.* **81**, 5837.
Randle, P. J., Garland, P. B., Hales, C. N. & Newsholme, E. A. (1963). *Lancet*, i, 785.
Rao, G. R., Ramakrishnan, T. & Sirsi, M. (1960). *J. Bact.* **80**, 654.
Slein, M. W., Cori, G. T. & Cori, C. F. (1950). *J. biol. Chem.* **186**, 763.
Wieland, O. S. & Suyter, M. (1957). *Biochem. Z.* **329**, 320.
Whitaker, J. R. (1963). *Analyt. Chem.* **35**, 1950.

Biochem. J. (1966) **98**, 29c

The Conversion of 7-Dehydrocholesterol into Cholesterol

By D. C. Wilton, M. Akhtar and K. A. Munday
Department of Physiology and Biochemistry, University of Southampton

(*Received* 25 *November* 1965)

Previous investigations from several Laboratories have established the sequence of reactions leading to synthesis of cholesterol (II) from acetate (Clayton, 1965). The mechanisms of a number of the reactions, especially those between acetate and lanosterol, have also received attention (Clayton, 1965). However, details of the reactions that result in the formation of cholesterol from lanosterol are still obscure. In the biosynthesis of cholesterol from lanosterol three methyl groups at positions 14, 4α and 4β are replaced by three hydrogen atoms, the $\Delta^{24,25}$-double bond is reduced and the $\Delta^{8,9}$-double bond is replaced by one between $C_{(5)}$ and $C_{(6)}$.

Some recent reports suggest that the last intermediate in the biosynthesis of cholesterol (II) is 7-dehydrocholesterol (I) (Dempsey, Seaton, Schroepfer & Trockman, 1964; Dvornik, Kraml & Bagli, 1964); however, it has not yet been unambiguously established that 7-dehydrocholesterol is an obligatory intermediate in the biosynthesis of cholesterol.

The present communication describes the origin and stereochemistry of the two hydrogen atoms at positions 7 and 8 during the conversion of 7-dehydrocholesterol (I) into cholesterol (II) with rat-liver homogenates by the enzyme 7-dehydrocholesterol reductase. Optimum conditions for the conversion of 7-dehydrocholesterol (I) into cholesterol (II) were studied by using 7-dehydro[19-^{3}H]cholesterol (Akhtar & Gibbons, 1965). In all experiments