Effect of Oleyl Alcohol on Esterification of Cholesterol in a Pancreatic Extract. (26497)

PAUL W. O'CONNELL (Introduced by M. H. Kuizenga)
Research Laboratories, The Upjohn Co., Kalamazoo, Mich.

In describing an enzyme in acetone powder of pancreas which esterifies vit. A, Pollard and Bieri(1) noted the similarities (nonspecificity of fatty acids and lack of requirements for coenzyme A activation) to cholesterol-esterifying enzymes of the pancreas(2,3). Employing a pancreatic extract with the same characteristics for study of cholesterol esterification, we have observed that addition of the lipid-soluble, unsaturated aliphatic alcohol, oleyl alcohol, resulted in its esterification and an increase in extent of sterol esterification.

Methods and materials. The standard reaction mixture was an emulsion of the fatty acid and cholesterol (one member of the substrate-pair being isotopically labeled) in 0.15 *M* potassium phosphate buffer, pH 6.2, containing sodium taurocholate (except where omission is noted in the tables), 10 mg per ml, and bovine serum albumin, 3 mg/ml. The fatty acid to cholesterol ratio was uniformly 2.5 to 1. The substrates were added to the buffered solution in a small volume of ether and the mixture was swirled in a warm water bath while a vacuum was applied to the flask.

Palmitic-1-C^{14} acid and cholesterol-4-C^{14} were purchased from Volk Radiochemicals and linoleic-1-C^{14} acid from Nuclear-Chicago. The unlabeled linoleic acid was obtained from Mann Fine Chemicals; palmitic acid was Eastman Kodak material. Commercial cholesterol was purified according to Hernandez and Chaikoff(3).

The enzymatic reaction was initiated by adding to 7 ml of the emulsion (equilibrated with shaking at 37.5°C) one ml of a pancreatic extract prepared by homogenizing fresh frozen pork pancreas in 5 volumes of cold 0.25 *M* sucrose (or, in some experiments, glycerol:water, 1:1) and centrifuging lightly. The fluid portion between a white surface layer and the centrifuged residue constituted the enzyme source. After a 15 min incubation, the reaction mixture was extracted twice with 15 ml volumes of hot chloroform. Where the production of cholesterol esters was anticipated, 10 mg of cholesteryl linoleate or palmitate were added as a carrier. The pooled chloroform extracts were concentrated to dryness and the residue taken up in Skellysolve B. In a single experiment, to be described, extraction was made with chloroform:methanol, 2:1 and the extract was washed with dilute alkali before subsequent Dowex-1 treatment. In early experiments, unreacted acid was removed by washing the Skellysolve solution with aqueous ammoniacal ethanol. Later it was found more effective to pass a wet ether solution of the total lipid extract over Dowex-1 (OH^- cycle), using the procedure of Savary and Desnuelle(4). An aliquot of the solution after resin treatment could be counted for estimation of fatty acid incorporated into total neutral lipid.

The chromatographic separation, "Scheme A" or "Scheme B", of Hirsch and Ahrens(5), scaled to 4 g silicic acid (Bio-Rad Laboratories) was used for isolation of the cholestreol esters. Fractions were collected in 10 ml volumes in vials used for the Packard liquid scintillation spectrometer. The cholesterol esters, or simple esters, are eluted by one per cent ether in petroleum ether (Skellysolve B) and will be referred to as the ester fraction. Glyceride esters are eluted in subsequent fractions. After evaporation of the eluting solvents, a sample was dissolved in 10 ml of toluene containing 3 g 2,5-diphenyloxazole and 0.1 g 1,4-di[2-(5-phenyloxazolyl)]benzene per liter and radioactivity measured in the spectrometer.

The oleyl alcohol employed in initial experiments was commercial material (Archer-Daniels-Midland, "Adol 85") stated to be 80% oleyl alcohol and the remainder as shorter chain saturated alcohols. In most of the experiments to be described, a purified sample, at least 95% pure, provided by Dr.

TABLE I. Effect of Oleyl Alcohol on Palmitate- and Linoleate-C^{14} Incorporation into Esters by Pancreatic Extracts.

Fatty acid substrate	Oleyl alcohol added (μmoles/flask)	Radioactivity ester fraction (counts/min.)
Palmitate	—	725 805
Linoleate	—	8,150
Palmitate	14	33,000 35,000
Palmitate	1.4	3,175
Linoleate	30	15,770
"	3.0	8,650

Each flask contained 7 ml of an emulsion of fatty acid (palmitate, 5 μmoles/ml, or linoleate, 10 μmoles/ml, containing 4×10^3 counts/min. of respective C^{14}-labelled acid/μmole), and cholesterol as described in *Methods*. The enzyme was added in 1 ml of extract of pork pancreas in glycerol-water, 1:1. Incubation, 15 min. at 37.5°C.

L. W. Beck, Procter and Gamble Co., was used.

Results. Varying amounts of oleyl alcohol were added to the palmitic acid-cholesterol and linoleic acid-cholesterol reaction mixtures, respectively, just before introduction of the pancreatic extract. The radioactivity appearing in the isolated ester fraction is shown in Table I.

When an equimolar amount of oleyl alcohol replaced cholesterol in the emulsion, 11,000 cpm of palmitate were incorporated in the ester fraction during a 15 minute incubation, and 13,675 cpm in 30 minutes. A small amount of clear oil obtained from silicic acid column fractions corresponding to the peak of ester radioactivity had an infrared spectrum consistent with an ester containing a double bond, and formation of oleyl palmitate was thus inferred.

The same pancreatic extract was employed as the enzyme source for preparations in which the radioactive label was present in palmitic-1-C^{14} acid or cholesterol-4-C^{14}. The emulsions with radioactive cholesterol were prepared in presence and absence of sodium taurocholate. The results are summarized in Table II.

TABLE III. Effect of Sodium Taurocholate on Esterification of Palmitate-C^{14} with Oleyl Alcohol and Cholesterol, Alone or in Combination, by a Pancreatic Extract.

Alcoholic substrate	Sodium taurocholate (70 mg/flask)	Radioactivity (counts/min.) Total neutral lipid	Ester fraction
Cholesterol	—	1,675	430
	+	2,825	2,000
Oleyl alcohol	—	3,125	2,215
	+	7,725	2,800
Cholesterol + oleyl alcohol	—	13,100	10,000
	+	22,625	18,225

Conditions were same as in Table II.

Table III contains additional information on the effects of taurocholate. The emulsions were prepared without the bile salt and with the usual concentrations of palmitate-C^{14} and either or both oleyl alcohol and cholesterol. Taurocholate was added to the appropriate flasks as an aqueous solution during the equilibration period. Extraction of these reactions was carried out with chloroform:methanol, 2:1.

TABLE II. Esterification of Palmitic Acid with Oleyl Alcohol and Cholesterol, Effect of Sodium Taurocholate.

Alcoholic substrate (μmoles/flask) Cholesterol	Oleyl alcohol	Radioactive substrate	Sodium taurocholate (70 mg/flask)	Radioactivity of ester fraction (counts/min.)
14	—	Palmitic acid*	+	345
—	14	" "	+	2,955
14	14	" "	+	9,460
14	—	Cholesterol†	—	35
14	—	"	+	335
14	14	"	—	55
14	14	"	+	2,640

* 4×10^3 counts/min./μmole. † 5×10^3 counts/min./μmole.

Each flask contained 7 ml of an emulsion of palmitic acid (5 μmoles/ml) and appropriate alcoholic substrate as described in *Methods*. Enzyme was added in 1 ml of extract of pork pancreas in 0.25 M sucrose. Incubation, 15 min. at 37.5°C.

Discussion. The effects of additives on enzymatic reactions involving lipids must frequently be interpreted with consideration for the possibility that physical changes in the system have altered the availability of the substrate to the enzyme. In the present instance, several reasons support the idea that some interaction of the 2 alcoholic substrates increases the reactivity of each. In Table I, the effect of oleyl alcohol addition is more pronounced in stimulating palmitate esterification than esterification of the presumably more soluble linoleate. Palmitate esterification was greater when cholesterol was present together with oleyl alcohol but only a portion of this increase was due to stimulation of cholesterol esterification. Thus, the data of Table II show that oleyl alcohol increased the cholesterol esterified about 0.5 μmole, from 335 to 2640 counts per minute, whereas total palmitate esterified was increased about 2.2 μmoles, from 345 to 9460 counts per minute, and oleyl alcohol esterification alone amounted to about 0.75 μmoles. The mere physical presence of cholesterol was capable of increasing oleyl alcohol reactivity (Table III) since the sterol is essentially unreactive in absence of taurocholate.

Pancreatic extracts prepared on different days showed considerable variation in degree of activity. Quantitative comparisons could only be made between reactions run at the same time, and data of each table are from single runs. They are representative of many experiments in which qualitative relationships were always the same. Since all extracts were prepared from the same lot of tissue in frozen storage, day-to-day variations may well be related to physical conditions.

The reactions described here proceeded quite as well anaerobically as aerobically and, in agreement with Pollard and Bieri(1), were not affected by treatment of the pancreatic extract with Dowex-1. In addition to the saturated and polyunsaturated acids used as representative fatty acid substrates in the experiments described, oleic acid also could serve. Thus, there is a close similarity to the vit. A esterifying system. Pollard and Bieri could find no requirement for taurocholate, and the effect of the bile salt on oleyl alcohol esterification (Table III) is slight when compared with its action with the sterol.

The cholesterol-esterifying enzyme in pancreas was carried to a considerably higher degree of purity by Hernandez and Chaikoff (3). Although it was relatively non-specific for fatty acid substrates, they were able to elucidate several structural features of importance to the sterol specificity. If it is unlikely that the same pancreatic enzyme is catalyzing the esterification of several lipid-soluble compounds bearing a hydroxyl function, the experiments described here nevertheless establish an interrelationship.

Before the effects are regarded as solely an *in vitro* phenomenon, it is worthy of note that Channon and Collinson(6) observed that oleyl alcohol fed to rats increased the non-saponifiable liver lipids and a substantial portion of the increase was cholesterol. In terms of certain present-day concepts of cholesterol absorption(7), the presence of oleyl alcohol in the intestinal mucosa together with the pancreatic enzymes required for cholesterol esterification could facilitate esterification and, hence, absorption. In view of the similar reactivities of oleyl alcohol and vit. A, it is tempting to speculate that vit. A might similarly play a role in cholesterol esterification and absorption. Available information, however, makes it obvious that the vitamin is involved in sterol metabolism in a more complex relationship. Morton and associates (8) established that, under certain conditions, cholesterol feeding in rats may lower liver vit. A stores, but dietary vit. A deficiencies did not have direct effect on cholesterol levels. During these same studies, the first observations were made which adumbrated the finding that vit. A is involved in mevalonic acid utilization and other aspects of sterol metabolism(9,10).

Summary. Addition of oleyl alcohol to a pancreatic extract capable of cholesterol esterification resulted in esterification of the aliphatic alcohol and stimulation of cholesterol esterification. Correspondingly, the extent of the oleyl alcohol reaction was greater in presence of cholesterol. Some possible relationships of the findings to *in vivo* processes are discussed.

1. Pollard, C. J., Bieri, J. G., *Arch. Biochem. Biophys.*, 1960, v87, 9.

2. Swell, L., Treadwell, C. R., *J. Biol. Chem.*, 1955, v212, 141.

3. Hernandez, H. H., Chaikoff, I. L., *ibid.*, 1957, v228, 447.

4. Savary, P., Desnuelle, P., *Bull. Soc. Chim. Fr.*, 1954, 936.

5. Hirsch, J., Ahrens, E. H., *J. Biol. Chem.*, 1958, v233, 311.

6. Channon, H. J., Collinson, G. A., *Biochem. J.*, 1928, v22, 391.

7. Swell, L., Trout, E., Hopper, J., Field, H., Treadwell, C. R., *J. Biol. Chem.*, 1958, v233, 49; Treadwell, C. R., Swell, L., Vahouny, G., Field, H., *J. Am. Oil Chemists' Soc.*, 1959, v36, 107.

8. Kantienga, N. L., Morton, R. A., *Biochem. J.*, 1955, v60, 28; Green, B., Lowe, J. S., Morton, R. A., *ibid.*, 1957, v67, 223, Green, B., Horner, A. A., Lowe, J. S., Morton, R. A., *ibid.*, 1957, v67, 235.

9. Gloor, U., Wiss, O., *Arch. Biochem. Biophys.*, 1959, v83, 216.

10. Van Dyke, R. A., Wolf, G., Johnson, B. C., *Biochem. Biophys. Research Commun.*, 1960, v3, 123.

Received January 6, 1961. P.S.E.B.M., 1961, v106.

Modification of Glucagon-Induced Hyperglycemia in Rats by 17-ethyl-19-nortestosterone. (26498)

ROBERT L. HAZELWOOD AND KEVIN D. O'BRIEN*
Dept. of Physiology, Boston University School of Medicine, Boston, Mass.

Considerable data are available indicating that 17-ethyl-19-nortestosterone (Nilevar) possesses potent myotrophic and anabolic properties as well as demonstrating a high anabolic to androgenic ratio(1,2,3). Recently, Nilevar was demonstrated to reduce the hyperglycemia associated with injection of glucagon (HGF) in human subjects(4). The specific mechanism by which Nilevar exerts its suppression of a glucagon-induced hyperglycemia is unknown. Blockade of glycogenolytic pathways, decreased gluconeogenesis, mediation through the pituitary or direct action on target endocrine tissues which affect any of the former, or a combination of the foregoing can be cited as the most likely possibilities.

Reported herein are studies carried out to determine more precisely the nature of Nilevar's reduction of glucagon-induced hyperglycemia. The ability of Nilevar to modify the glucagon tolerance test was compared with that of testosterone; additionally, castrated rats were compared with intact animals in their response to the hyperglycemic factor. Finally, hypophysectomized animals pretreated with Nilevar were subsequently injected with glucagon to weigh the possible influence of hypophyseal secretions on the mode of action of Nilevar as an anti-hyperglycemic agent.

Methods. All rats employed in these studies were male descendants of the Sprague-Dawley strain, were fed Purina laboratory chow *ad libitum*, and were kept at a room temperature of 74-78°F. Rats, hypophysectomized at a body weight of 200-210 g, were purchased from the Charles River Breeding Laboratories and were used 20 days postoperatively. All injections were given daily at 9:00 a.m. for 5 consecutive days; the terminal injection was given 2 hours before taking the first blood sample. Both Nilevar and testosterone† were suspended in peanut oil such that 0.15 mg of either steroid was contained in 0.25 ml of vehicle. Control rats received peanut oil only in a volume equal to experimental groups. Cortisone acetate was suspended in saline such that 2.5 mg were contained in 0.25 ml.

The glucagon tolerance test (GTT) was performed on rats fasted 4 hours (except in the study with hypophysectomized rats which were fed *ad libitum* to insure adequate liver

* Holder of a N.S.F. Medical Student Research Fellowship.

† Generously provided by Dr. V. A. Drill, G. D. Searle Co., Chicago, Ill.