Studies with Clearing Factor IV. Fatty Acid Exchange Reaction Catalyzed by Clearing Factor.* (31850)

A. N. PAYZA, H. EIBER, AND A. TCHERNOFF
Gilman Laboratory, Departments of Medicine and Biochemistry, New York Medical College, New York City

It was previously reported(1) that postheparin Clearing Factor catalyzes the exchange of fatty acids with triglycerides, diglycerides and monoglycerides. This exchange as measured by incorporation of C^{14} oleic acid, was postulated to take place only on the 1 and 3 position of the glyceride. It was thus suggested that the position specificity of Clearing Factor for the glyceride ester bonds was such that the exchange took place only in the 1, 3 positions. In these experiments no attempt was made to demonstrate that actual distribution of C^{14} fatty acids among the isomers of diglycerides and monoglycerides.

These conclusions were rejected later by Korn(7). He used adipose tissue Clearing Factor and demonstrated random hydrolysis of fatty acids from various natural triglycerides and chylomicrons. These results were possibly affected by the presence of β-monoglyceridase(8) and lipases other than the lipoprotein lipase in adipose tissue.

Similar exchange reactions with C^{14} fatty acids and dibutyrine, catalyzed by adipose tissue lipoprotein lipase, were demonstrated (5). In this report, it was claimed that 2 to 8 carbon fatty acids were more readily incorporated in the 3-position of 1,2-dibutyrine and ester bonds were formed. On the other hand, with long chain fatty acids exchange reaction occurred only at C-1. The exchange reaction and hydrolysis had an optimum at pH 7 with an apparent increase of activity with increasing pH.

The present report describes the exchange reaction of plasma Clearing Factor with C^{14} stearic and oleic acids and glyceride fractions of coconut oil and its hydrolysis products, *i.e.*, 1,2- and 1,3-diglycerides, α- and β-monoglycerides.

Methods and materials. The postheparin plasma Clearing Factor was prepared as previously reported(9). It had an activity of 16 μ E free fatty acids/ml postheparin plasma/hr. Stearic acid 1-C^{14}, with a specific activity 21.4 mC/mM, and oleic acid-1-C^{14}, 7.7 mC/mM were purchased from Volk isotopes. C^{14} fatty acids were purified by thin layer chromatography and by extraction with Dole's mixture(2) prior to use. These preparations showed one spot with iodine staining

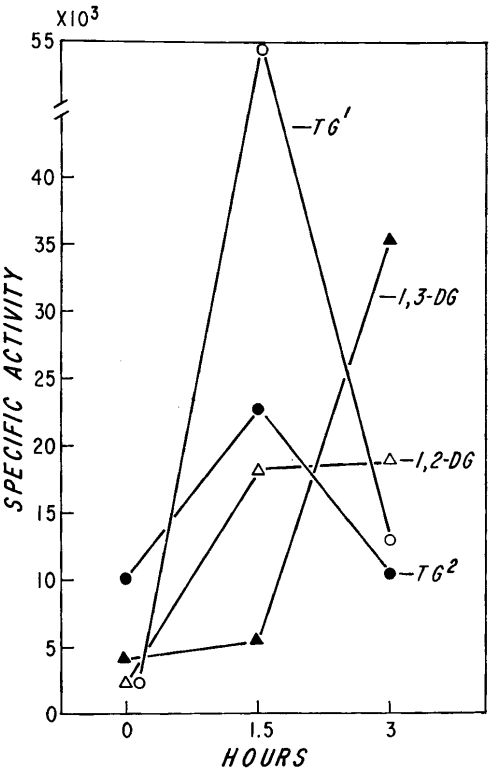

FIG. 1. Specific activity of triglycerides and diglycerides at 1.5 and 3 hr of incubation with C^{14} stearic acid and Clearing Factor. 1,2-Diglycerides; 1,3-Diglycerides. TG. 1. Triglycerides obtained from column fractions 3,4, and 5 (of separated lipid sample) had high specific activity. TG 2. Average specific activity of all triglycerides in lipid sample. See *Methods* for procedures for incubation mixture extraction of samples. See legend for Table I for fractionation and purification of lipid fractions. (Zilversmith, D. B., Entenman, C. and Fisherler, M. C., J. Gen. Physiol., 1943, v26, 323).

* Supported by USPHS grant RO1 HE 02823-10.

TABLE I. Incorporation of C^{14} Stearic Acid by Clearing Factor in the Fractions of Coconut Oil and Its Hydrolysis Products.

Column fractions	1,2		3,4,5		6	7,8,9	
	CPM	SpA	CPM	SpA		CPM	SpA
Triglycerides							
0 hr	2,150	200	8,750	2,710	—	—	—
1.5 "	250	210	111,000	55,000	—	120	120
3 "	15,000	20,100	20,800	13,400	—	140	360
1,2-Diglycerides							
0 hr						2,400	—
1.5 "						10,400	18,400
3 "						7,280	19,850
1,3-Diglycerides							
0 hr						4,050	—
1.5 "						12,500	5,260
3 "						14,390	36,700
α-Monoglycerides							
0 hr						200	—
1.5 "						200	360
3 "						3,700	13,900
β-Monoglycerides							
0 hr						200	—
1.5 "						450	—
3 "						1,300	3,050

Incubation mixture(9) contained 4 parts of 10% bovine serum albumin adjusted to pH 8.5, 1 part of 0.5 M ammonium formate, 2 parts of 2% coconut oil emulsion (Ediol), 4 parts of postheparin plasma, and 0.1 mC stearic acid-1-C^{14}.

From the above incubation mixture 4 ml samples were extracted as described in text. Major lipid fractions were separated by silicic acid column (18 × 30 cm activated at 160°C) chromatography(4). Solvents used were 1) 350 ml of 1% ether in petroleum ether, 2) 60 ml of 4% ether:petroleum ether, 3) 120 ml of 4% ether:petroleum ether, 4) 140 ml of 4% ether:petroleum ether, 5) 200 ml of 8% ether:petroleum ether, 6) 450 ml of 8% ether:petroleum ether, 7) 250 ml of 25% ether:petroleum ether, 8) 300 ml ether, 9) 400 ml of methanol.

Lipids in each fraction were further purified by thin layer chromatography(3), designated by plates No. 1 to 9. Counting of the radioactivity on 1 ml samples was performed on a liquid scintillation counter, with premix M as phosphor. Standard counting error was 1%. The saponification of the glycerides and extraction of fatty acids were done as described in text. α- and β-monoglycerides were separated as described(3). CPM counts of C^{14} stearic acid in 1 ml sample (6 ml total sample). SpA counts per mg of fatty acids.

and autoradiography, on thin layer chromatography(3). In these experiments, the incubation mixture, in addition to the conditions previously reported(9), contained C^{14} fatty acid (0.1 mC/2 mg) dissolved in bovine serum albumin. From the above incubation mixture 4 ml samples were taken at zero time, 1.5 and 3 hours, and were extracted with 50 ml of ethanol:ether mixture (3:1). The extracts were filtered through sintered glass funnel, the precipitate was washed with hot methanol and solvent was evaporated. On these lipid samples column chromatography was performed according to Hirsch and Ahrens(4) and the 9 fractions obtained were further purified by thin layer chromatography(3). The fractions obtained by thin layer chromatography were saponified with 4 ml KOH solution (15%), overnight at 55°C and the resulting fatty acids were extracted with Dole's extraction mixture into 6 ml heptane. One ml of the extract was placed on planchets or absorbed on filter paper of known weights and radioactivity was counted by gas flow counter or a scintillation counter as indicated on the tables.

Results and discussion. The exchange and distribution of C^{14} fatty acids with coconut oil and its hydrolysis products are shown in Tables I and II. As will be seen from these data both C^{14} stearic and oleic acids are incorporated in the triglycerides. The highest exchange occurred at 1.5 hours and specific activity declined at the 3-hour incubation period because of the continued hydrolysis of triglycerides. The triglyceride fractions from column fractions 3,4,5 incorporated more fatty acids than did those from column fractions 7,8,9, indicating that certain triglycerides were better substrate for this reaction than others.

TABLE II. Incorporation of C^{14} Oleic Acid by Clearing Factor in Fractions of Coconut Oil and Its Hydrolysis Products.

Column fraction		3	4	5	6	7	8	9
Triglycerides								
0 hr	CPM	35	—	—	—	—	—	—
	SpA	—	—	—	—	—	—	—
1.5 "	CPM	4,290	1,160	—	—	1,300	—	—
	SpA	8,580	4,400	—	—	675	225	—
3 "	CPM	630	190	460	—	80	150	—
	SpA	1,370	300	875	—	1,445	270	—
1,3-Diglycerides								
0 hr	CPM					—	6,385	—
	SpA					—	—	—
1.5 "	CPM					445	78,170	95
	SpA					1,000	79,930	325
3 "	CPM					6,080	22,000	—
	SpA					12,160	33,800	—
1,2-Diglycerides								
0 hr	CPM					—	3,245	—
	SpA					—	—	—
1.5 "	CPM					425	22,050	495
	SpA					305	15,320	365
3 "	CPM					—	1,180	390
	SpA					—	3,580	3,810
Monoglycerides								
0 hr	CPM					—	—	465
	SpA					—	—	—
1.5 "	CPM					—	—	7,870
	SpA					—	—	4,670
3 "	CPM					—	—	4,850
	SpA					—	—	9,445

See legend for Table I for details of procedure. In this experiment a gas flow counter was used. Standard error of counting was 1%.

The triglycerides from fractions 3,4, and 5 had specific activity twice as high as the average specific activity of the triglycerides at 1.5 hours (Fig. 1). This difference which disappeared at 3 hours incubation indicated that triglycerides from fractions 1,2,7,8,9 were hydrolyzed faster and preferentially by Clearing Factor than the ones from 3,4, and 5.

The total sample counts of the 1,3- and 1,2-diglycerides at 1.5 hours were approximately the same. The former had 3 times lower specific activity than the latter. At the 3-hour incubation, however, 1,3-diglycerides had twice the specific activity and total counts of the 1,2-diglycerides (Table I) indicating: 1) The first step of the hydrolysis was the formation of 1,2-diglycerides (Fig. 1). This might be a reversible step(5). 2) The equilibrium of the exchange reaction was towards the direction of the incorporation of C^{14} fatty acids into the 1,3-diglycerides, apparent by the high specific activity despite hydrolysis. The 1,3-diglycerides were better substrates for the exchange reaction with fatty acids than other glycerides. There was 3-to 4-fold higher concentration of 1,3-diglycerides than 1,2-diglycerides at 3 hours' incubation. The α-monoglycerides had 3 times the specific activity of β-monoglycerides with C^{14} stearic acid. Therefore, 1) C^{14} fatty acids were not in equilibrium with α and β positions. 2) The specific activities of β-monoglycerides and 1,2-diglycerides were lower than α-monoglycerides and 1,3-diglycerides. This demonstrated the incorporation of C^{14} fatty acids at a lower rate to the glycerides where the beta position of glycerol was already esterified.

It has been reported(6), similarly, that pancreatic lipase esterified 1,3 positions of 2-monoglycerides with free fatty acids at pH 5. At pH 8 and with the presence of bile salts, the equilibrium state was changed

towards hydrolysis.

Also, it is worth noting that the fraction with the highest specific activity observed with the incorporation of C^{14} stearic acid was 1/50th of the specific activity of the fatty acids in the same sample. On the other hand, when C^{14} oleic acid was incorporated to 1,3-diglycerides, this fraction had 4 times more specific activiy of fatty acids in the same sample at 1.5 hours and 1/2 at 3 hours incubation.

In a second exchange experiment with oleic acid, specific activity of 1,3-diglycerides was about 27% at 1.5 hours and 45% at 3 hours of the specific activity of free fatty acids in the same sample. For the 1,2-diglycerides these values were 3.1% and 6.7% respectively. This indicated that oleic acid was exchanged faster than stearic acid.

1. Borgstrom, B., Carlson, L. A., Biochem. Biophys. Acta, 1957, v24, 638.
2. Dole, V. P., J. Clin. Invest., 1956, v35, 150.
3. Payza, A. N., Eiber, H., Tchernoff, A., Proc. Soc. Exp. Biol. and Med., 1966, v122, 509.
4. Hirsch, J., Ahrens, E. H., Jr., J. Biol. Chem., 1958, v233, 311.
5. Lynn, W. S., Jr., Perryman, N. C., ibid., 1960, v235, 1912.
6. Borgstrom, B., J. Lipid Res., 1964, v5, 522.
7. Korn, E. D., J. Biol. Chem., 1961, v236, 1638.
8. Vaughan, M., Berger, J. E., Steinberg, D., ibid., 1964, v239, 401.
9. Payza, A. N., Eiber, H., Danishefsky, I., Biochem. Biophys. Acta, 1965, v111, 159.

Received October 25, 1966. P.S.E.B.M., 1967, v124.

Prevention of SV_{40} Virus Tumorigenesis by Irradiated, Disrupted and Iododeoxyuridine Treated Tumor Cell Antigens.* (31851)

J. H. COGGIN,† V. M. LARSON, AND M. R. HILLEMAN
Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pa.

Studies in these laboratories during the past several years have been directed toward the development of safe and practicable procedures for immunizing human beings against cancer utilizing animal model systems and based on the knowledge that new tumor antigens capable of inducing resistance to tumor appear in animal neoplasia. A principal aspect of the program has been the development of means for rendering tumor cells nonproliferative and for disruption of neoplastic cells without destroying the tumor antigen. The latter objective appears a requisite to tumor antigen purification with removal of normal cellular antigen components which might lead to autoimmune and other possible disorders upon application in immunization procedures.

Previous reports(1,2) by our group described the development of a model system for testing the efficacy of tumor immunization. In these studies, it was shown(2) that parenteral injection of x-irradiated SV_{40} tumor cells was highly effective in preventing SV_{40} virus tumor when the oncogenic virus was given to hamsters as newborns and the irradiated tumor vaccine was given in a single dose between day 34 and 76 following virus and prior to first appearance of virus-induced tumor. All protective activity was lost, however, when the untreated cells were disrupted by homogenization and the immunizing capacity was not preserved by prior treatment with formalin(3).

The present report describes the results of efforts to disrupt tumor cells without destroying immunizing ability and to devise additional methods for rendering tumor cells nonproliferative. In the studies, it was shown

* Research on cancer in our laboratories is supported in part by Contract PH 43-64-55 with the Viral Carcinogenesis Branch, Nat. Cancer Inst., Nat. Inst. Health, USPHS.

† Present address: Dept. of Microbiology, Univ. of Tennessee, Knoxville.