3 days after adjuvant injection, probably due to the inflammation produced by the Primol injection, and continued to increase up to the time of gross arthritic development. However, there was no marked change in any parameter either immediately before the appearance of gross lesions or after dexamethasone treatment. Furthermore, no convincing correlation between levels of any one parameter and gross arthritic development could be discerned from the data; in other words, no plateau appeared above which arthritis was invariably present.

A close correlation was noted among plasma inflammation units, sedimentation rates, and plasma glycoprotein concentrations, which apparently follow parallel courses during lesion development and regression, and any one parameter could conveniently serve as a reliable indicator of the disease. Since determination of inflammation units requires an amount of plasma which can easily be obtained from the living anesthetized animal, it is obviously the anti-inflammatory parameter of choice in following the progression of the arthritic state.

Summary. Inflammatory parameters were followed during the prearthritic stage of adjuvant-injected rats. Plasma inflammation units, erythrocyte sedimentation rates, and plasma sialic acid, glycoprotein and copper concentrations were raised 3 days post-dose and continued to increase up to 13 days, when gross lesions appeared. A prearthritic lymphoid hyperplasia occurred in the same manner. All these lesions regressed with dexamethasone treatment. It was concluded that none of the parameters investigated showed a unique sensitivity to the inflammatory state of the animal.

The authors thank Miss P. Palmer for her able technical assistance and Mr. N. C. Marston, Quality Control Department, Chas. Pfizer and Co., Inc., Groton, Connecticut, for performing the copper determinations.

1. Pearson, C. M., Proc. Soc. Exptl. Biol. Med. **91**, 95 (1956).
2. Pearson, C. M. and Wood, F. D., Arthritis Rheumat. **2**, 440 (1959).
3. Pearson, C. M. and Wood, F. D., Am. J. Pathol. **42**, 73 (1963).
4. Burnstein, N. A. and Waksman, B. H., Yale J. Biol. Med. **37**, 177 (1964).
5. Glenn, E. M. and Gray, J., Am. J. Vet. Res. **26**, 1180 (1965).
6. Glenn, E. M., Am. J. Vet. Res. **27**, 339 (1966).
7. Newbould, B. B., Brit. J. Pharmacol. **21**, 127 (1963).
8. Ward, J. R. and Cloud, R. S., J. Pharmacol. Exptl. Therap. **152**, 116 (1966).
9. Winter, C. A. and Nuss, G. W., Arthritis Rheumat. **9**, 394 (1966).
10. Graeme, M. L., Fabry, E., and Sigg, E. B., J. Pharmacol. Exptl. Therap. **153**, 373 (1966).
11. Glenn, E. M. and Kooyers, W. M., Life Sci. **5**, 619 (1966).
12. Warren, L., J. Biol. Chem. **234**, 1971 (1959).
13. Ayala, W., J. Clin. Invest. **30**, 781 (1951).
14. Jones, R. S. and Ward, J. R., Arthritis Rheumat. **6**, 23 (1963).

Received Feb. 12, 1968. P.S.E.B.M., 1968, Vol. 128.

Inhibition of Triglyceride Synthesis in Everted Intestinal Sacs* (33049)

GEORGE V. VAHOUNY, JON NELSON, AND C. R. TREADWELL
Department of Biochemistry, School of Medicine, The George Washington University, Washington, D. C. 20005

It has been reported that in the lymph-fistula rat, the concurrent administration of 2-ethyl-*n*-caproic acid inhibits the incorporation of oleic acid-1-^{14}C into lymph triglyceride (1). It was also shown that this branched-chain acid increased the portal absorption of oleic acid from 15 to 40% of the administered dose; the newly absorbed oleic acid in portal blood was predominantly in the form of the unesterified fatty acid. It was therefore suggested that 2-ethyl-*n*-caproate

* This study was supported in part by grants from the National Heart Institute (HE-02033) and the Washington Heart Association.

inhibits the esterification reaction and shifts the subsequent route of absorption of oleic acid from lymph to portal blood.

In the present study, the mechanism(s) of 2-ethyl-*n*-caproate inhibition of higher glyceride synthesis was investigated using the everted intestinal sac preparation and a mucosal microsomal system. With the sac preparation, significant inhibition of triglyceride synthesis from oleic acid-1-^{14}C occurred when sacs were preincubated for 5 min with 2-ethyl-*n*-caproate. The reduction of isotope incorporation into intestinal triglycerides was not accompanied by increased isotope levels in the diglyceride, monoglyceride, or phospholipid fractions. The microsomal preparation was used to study the effect of the branched-chain acid on activation of long-chain fatty acids to the corresponding acyl-CoA derivatives. A portion of this study appeared in a preliminary communication (2).

Materials and Methods. Oleic acid-1-^{14}C and oleic acid-9,10-^{3}H were obtained from Nuclear Chicago Corp., and carrier oleic acid (99% purity) was purchased from the Hormel Institute. These were checked for purity by thin-layer silicic acid chromatography and liquid scintillation counting as described below. 2-Ethyl (1-^{14}C)-*n*-caproic acid was synthesized using ethyl bromide-1-^{14}C and diethylbutyl-malonate as described previously (3). The purity of the final product was 98%, based on thin-layer silicic acid chromatography and gas-liquid radio-chromatography of the free acid on an ethylene glycol succinate polyester column (155°C, 20 psi).

Adult male rats from Carworth (Wistar strain, 150–250 gm) were fasted for 24 hours with free access to water. The animals were anesthetized with Nembutal; and the upper two thirds of the small intestine, beginning at the common duct, was carefully removed and placed in ice-cold, 0.154 *M* phosphate buffer, pH 7.0, containing 0.3% glucose. After washing with 50 ml of buffer, the intestine was everted and 4-cm sacs were prepared by the procedure of Wilson and Wiseman (4). The first two sacs which included the duodenum, were discarded.

The basic incubation medium was 0.154 *M* phosphate buffer and contained 0.3% glucose and 75 μmol of sodium taurocholate (25 μmol in early studies). Oleic acid, in amounts described in each experiment, was added to the medium in hexane and the solvent was evaporated at room temperature under nitrogen. The medium was sonicated (20 kc) for 5 min and was then oxygenated by bubbling with 95% O_2–5% CO_2 for 30 min at 37°C prior to use. Each sac was added to 5 ml of incubation medium in a stoppered 25-ml Erlenmeyer flask and incubation was carried out at 37°C in a Dubnoff metabolic shaker in an atmosphere of 95% O_2–5% CO_2. The use of stoppered flasks essentially eliminated any loss of 2-ethyl-*n*-caproate by volatilization.

After incubation, the sac was removed, rinsed with buffer and minced in 20 ml of ethanol–ether (3:1, v/v). This suspension was homogenized and extracted by bringing the mixture to a rolling boil. After cooling, the volume was adjusted to 25 ml and the extract was filtered. One ml of the extract was placed in a scintillation vial, 10 ml of scintillant mixture (4 gm of 2,5-diphenyl oxozole and 100 mg of 1,4-di-2-(5-phenyl) oxyzolyl)-benzene/liter of toluene) was added, and the radioactivity was determined in a Nuclear Chicago liquid scintillation counter. All counts were corrected to disintegrations per minute (dpm) and the values obtained were taken to represent total uptake from the medium.

Ten ml of the intestinal extract was evaporated to dryness under nitrogen, the residue was dissolved in 0.2 ml of hexane, and 0.1 ml was spotted on thin-layer silicic acid plates. Separation of major lipid classes was accomplished using a solvent system of n-hexane–acetone–acetic acid (89:11:3, v/v). The plates were air-dried, placed in an iodine atmosphere, and the lipid classes were identified by comparison against pure standards chromatographed simultaneously. The area corresponding to each lipid class was scraped directly into a scintillation vial, and 1 ml of methanol and 10 ml of scintillation mixture were added prior to counting. When samples contained both ^{14}C and ^{3}H, it was necessary to extract the lipids from

the silicic acid before scintillation counting. The silicic acid area corresponding to each lipid fraction was scraped into a centrifuge tube and 10 ml of methanol–ether (1:3, v/v) was added. The tube was then centrifuged, and the supernatant was removed. The extraction was repeated twice, and the supernatants were combined in a scintillation vial and evaporated to dryness under nitrogen. This procedure was followed for all fractions except phospholipids, which, because of their more polar nature, were extracted with methanol–ether (3:1, v/v). After the extracts were evaporated, the samples were counted as described above and all counts were corrected to dpm.

In the studies on mucosal acyl-CoA synthetase, the small intestine was removed, washed as described above, and then split longitudinally. The mucosal layer was carefully scraped off and homogenized in 0.287 *M* mannitol. The microsomal fraction was prepared by sequential centrifugations at 900, 9500 and 100,000*g* in a Servall refrigerated centrifuge and Spinco ultracentrifuge. The assay system was essentially as described by Brindley and Hubscher (5) and the medium contained 0.66 *M* hydroxylamine, pH 7.4; 12 m*M* ATP; 0.17 m*M* CoA; 13.3 m*M* $MgCl_2$; 1.66 m*M* reduced glutathione; 13.3 m*M* potassium fluoride; 2 m*M* fatty acid; and 0.5 ml of microsomal enzyme (250 mg of original mucosa). The final volume was 3 ml. After 20-min incubation at 37°C, the reaction was terminated by addition of 0.2 ml of 75% perchloric acid. The tubes were cooled in an ice-bath, and 2.5 ml of chloroform (previously saturated with water) was added. The tubes were shaken for 10 min and centrifuged at 2500 rpm for 10 min. Two-ml aliquots were taken from the water and choloroform layers and the water-soluble and insoluble hydroxamates were assayed using the $FeCl_3$ reagent of Hill (6). Experiments were generally run in triplicate and the data represent means of triplicate determinations in typical experiments.

Results and Discussion. The optimum conditions for maximum incorporation of the labeled oleic acid into triglyceride during incubation of everted intestinal sacs was determined in preliminary experiments. These data are summarized in Table I and Fig. 1. In agreement with the results of Borgstrom *et al.* (7), it was found that the upper small intestine was more active in esterification of oleic acid than was the lower small intestine. In subsequent experiments, the upper one third of the small intestine was used.

TABLE I. Factors Affecting Uptake and Esterification of Oleic Acid by Everted Intestinal Sacs.

Expt.[a]	Oleic acid-1-^{14}C	
	Uptake (%)	Incorporation into triglyceride (%)
1. Small intestine (Medium pH 6.6)		
Upper	18.5	40.8
Lower	13.2	22.5
2. Medium pH (Upper small intestine)		
6.6	33.4	70.8
7.0	31.5	69.9
7.4	21.4	22.3

[a] In Expt. 1, everted intestinal sacs were prepared from the jejunum and lower (ileum) small intestine and incubated in 0.154 *M* phosphate buffer (pH 6.4), containing 0.3% glucose, 25 μmol of sodium taurocholate and 1 μmol of oleic acid-1-^{14}C. Incubations were for 30 min in an atmosphere of O_2:CO_2 (95:5 v/v). The conditions in Expt. 2 were similar except that sacs were prepared only from the upper small intestine, and the pH was varied as indicated. All values represent the means of triplicate incubations.

It was found that uptake and esterification of oleic acid by sacs prepared from the upper small intestine was equivalent at pH 6.6 and pH 7.0; at pH 7.4 both uptake and esterification of oleic acid was markedly reduced (see Table I). Due to the volatility of 2-ethyl-*n*-caproate in the un-ionized form (pH 6.4) subsequent studies were carried out at pH 7.0. It was also found that in the absence of O_2 or glucose in the incubation medium, there were marked decreases in the uptake and esterification of labeled fatty acid.

Figure 1 shows the relationship of triglyceride formation to duration of incubation,

TABLE II. Effect of 2-Ethyl-*n*-caproate on Oleic Acid Incorporation into Triglycerides in Everted Intestinal Sacs.

Additions to incubation media[a]	Oleic acid-1-^{14}C	
	Uptake (%)	Incorporation into triglyceride (mμmol)
Oleic acid-1-^{14}C (1 μmol)	5.1	27.0
Oleic acid-1-^{14}C (1 μmol) + 2-ethyl-*n*-caproate (1 μmol)	5.2	27.0
Oleic acid (0.1 μmol) + 2-ethyl-*n*-caproate (2 μmol)	4.8	2.4
Oleic acid (0.1 μmol) + 2-ethyl-*n*-caproate (5 μmol)	4.2	1.6

[a] The incubation medium consisted of 0.154 *M* phosphate buffer (pH 7.0) containing 0.3% glucose and 75 μmol sodium taurocholate. Incubations were carried out for 10 min in an atmosphere of O_2:CO_2 (95:5 v/v). Values represent the means of triplicate incubations.

and the linearity of fatty acid uptake during 20-min incubation using concentrations of 0.1, 0.5, and 1.0 μmol of oleic acid/5 ml of media. On the basis of these experiments, everted sacs were subsequently incubated in 0.154 *M* phosphate buffer (pH 7.0) containing 0.3% glucose in an atmosphere of O_2–CO_2 (95:5). Except where indicated, the concentration of oleic acid was 1 μmol/5 ml.

The effect of 2-ethyl-*n*-caproic acid on oleic acid-1-^{14}C uptake and esterification is shown in Table II. With equivalent amounts of the two acids, there was no effect on oleic acid uptake or incorporation into intestinal triglycerides; significant inhibition of labeled triglyceride formation was observed only when the ratio of 2-ethyl-caproate to oleate in the medium was 20:1 or greater.

Since the rate and extent of 2-ethyl-caproate absorption *in vivo* is inefficient compared to that of straight-chain acids (1), everted intestinal sacs were preincubated for 5 min in a buffer-glucose media containing 1 μmol 2-ethyl-*n*-caproic acid to allow accumulation of the branched-chain acid. One μmol of oleic acid-1-^{14}C in 1 ml of buffer was then injected through the stopper, and incubation was continued for an additional 10 min. As shown in Table III, under these experimental conditions, there was about 50% inhibition of isotope incorporation into the triglyceride fraction.

A further variation was to preincubate sacs for 5 min in buffer alone or buffer plus 2-ethyl-*n*-caproate, and subsequently transfer the sacs to fresh media containing oleic acid-1-^{14}C. Under these conditions (Table II) there was significant inhibition of oleic acid incorporation into triglyceride (25%), which was comparable to the extent of inhibition observed *in vivo* (26%).

When the intestinal lipids were extracted, separated into classes, and each lipid fraction counted, it was found that the inhibition of triglyceride synthesis by 2-ethyl-*n*-caproate

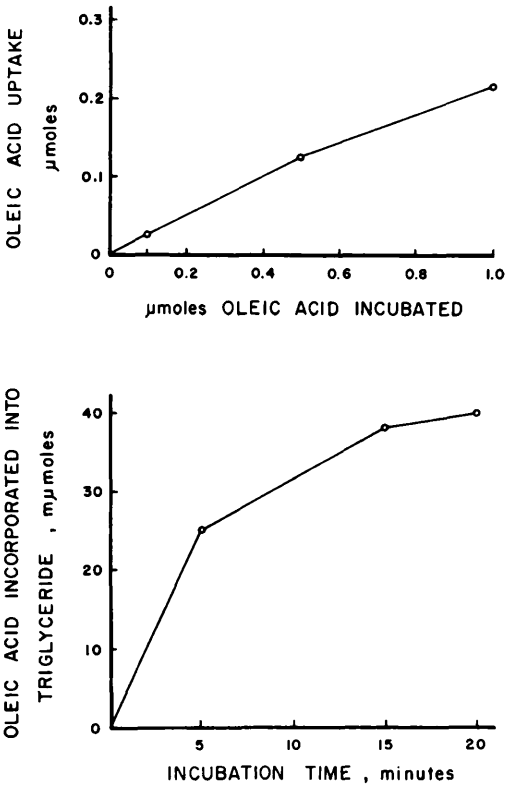

FIG. 1. Extent of oleic acid-1 ^{14}C uptake and incorporation into triglyceride during incubation with everted intestinal sacs. In the lower curve, sacs were incubated with 1 μmol of oleic acid-1-^{14}C in phosphate buffer (pH 6.4) containing 0.3% glucose and 25 μmol of sodium taurocholate.

TABLE III. Effect of Preincubation of Intestinal Sacs in 2-Ethyl-*n*-caproate on the Subsequent Incorporation of Oleic Acid-1-^{14}C into Triglycerides.

Preincubation (5 min) with 1 μmol of 2-ethyl-*n*-caproate[a]	Incubation (10 min) with 1 μmol of oleic acid-1-^{14}C	Oleic acid (%) incorporated into triglyceride
Expt. 1		
—	+	55.4 ± 2.3[b]
+	+	22.4 ± 3.7
Expt. 2		
—	+	61.2 ± 2.8
+	+	45.9 ± 3.5

[a] Preincubation medium contained 0.154 *M* phosphate buffer (pH 7.0) containing 0.3% glucose and 75 μmol of sodium taurocholate. Incubations were carried out in an atmosphere of O_2–CO_2 (95:5 v/v). In Expt. 1, 1 μmol of oleic acid-1-^{14}C in 1 ml of buffer was injected directly into the preincubation media. In Expt. 2, sacs were transferred from preincubation media to media containing only buffer, glucose, and oleic acid.

[b] Values represent means ± SE for 10 experiments.

was accompanied by a large increase in labeled unesterified fatty acids (Table IV). There was no accumulation of label in the phospholipid, monoglyceride, or diglyceride fractions, indicating that the effect of the branched-chain acid was probably not on enzymes involved in the monoglyceride pathway or in the acylation of α-glycerol phosphate.

The experiments shown in Table V indicate that a 5-min incubation of intestinal sacs with 2-ethyl (1-^{14}C)-*n*-caproate resulted in formation of 0.7 μmol of labeled triglyceride compared to 26.7 μmol formed from oleic acid-1-^{14}C. When either ethyl-2-^{14}C-*n*-caproate or oleic acid-1-^{14}C were present in the incubation medium, there was a reduction of the incorporation of oleic acid-9,10-^{3}H into triglyceride. In the case of the ethyl-*n*-caproate, this represented a net decrease in isotope incorporation. However, in the case of the oleic acids, the decrease in tritium incorporation was due entirely to dilution, since the total label incorporated into triglyceride (^{14}C + ^{3}H) was at least equal to that in control. Caproic acid-1-^{14}C was also poorly incorporated into mucosal triglyceride, and inhibited glyceride formation from oleic acid-9,10-^{3}H, indicating that both the straight- and branched-short chain acids had a similar mechanism of action.

The effect of 2-ethyl-*n*-caproate on hydrolysis of preformed triglyceride was studied. Nine everted sacs were each incubated in 1 μmol of oleic acid-1-^{14}C for 10 min and incorporation of isotope into triglyceride was determined on 3 sacs. The remaining sacs were transferred to control- or 2-ethyl-*n*-caproate-containing media and incubated for an additional 30 min. In both cases 4–6% hydrolysis occurred during the second 30-min incubation. Also there was no increase in the levels of labeled di- or monoglyceride.

Since the data suggested that the inhibition of triglyceride formation occurred early in the biosynthetic pathway, the activation of long-chain fatty acids by mucosal microsomes was investigated. The data in Table VI clearly show that both 2-ethyl-*n*-caproic and n-caprylic acids markedly inhibit formation of palmityl CoA by rat mucosal microsomes, as represented by the levels of water-insoluble hydroxamate formation. However, these acids are not themselves activated effec-

TABLE IV. Distribution of Isotope in Lipid Fractions of Intestinal Mucosa.[a]

Lipid fraction	Distribution of isotope from oleic acid-1-^{14}C	
	Without 2-ethyl-*n*-caproate	With 2-ethyl-*n*-caproate
Phospholipids	1	1
Monoglycerides	3	2
Diglycerides	12	10
Free fatty acids	22	40
Triglycerides	61	46
Cholesterol esters	1	1

[a] Intestinal sacs were preincubated in 0.154 *M* phosphate buffer (pH 7.0) containing 0.3% glucose and 75 μmol of sodium taurocholate with and without 1 μmol of 2-ethyl-*n*-caproic acid. The sacs were then transferred to buffered media containing 1 μmol of oleic acid-1-^{14}C and incubated for an additional 10 min. Following extraction of lipids, the lipid fractions were separated by thin-layer chromatography prior to liquid scintillation counting, as described in the text.

TABLE V. Incorporation of Oleic Acid-9,10-^{3}H into Intestinal Triglyceride *in Vitro*.[a]

Additions to 5 min preincubation	Oleic acid-9, 10-^{3}H addition to 10 min incubation	Uptake (%) by everted sac		Triglyceride formed (mμmol)		Decrease of ^{3}H incorporation into triglyceride (%)
		^{14}C	^{3}H	^{14}C	^{3}H	
None	+	—	7.8	—	31.2	
Oleic acid-1-^{14}C	+	4.2	8.1	26.7	19.5	34
Ethyl-2-^{14}C-*n*-caproic acid	+	1.1	8.1	0.7	22.9	26
Caproic acid-1-^{14}C	+	0.9	8.2	1.1	19.5	34

[a] Intestinal sacs were preincubated for 5 min in 0.154 *M* phosphate buffer (pH 7.0) containing 0.3% glucose, 75 μmol of sodium taurocholate, and 1 μmol of each acid as indicated. The sacs were then transferred to fresh media, as above, containing 1 μmol of oleic acid-9,10-^{3}H, and incubation continued for 10 min.

TABLE VI. Fatty Acid Activation by the Microsomal Fraction of Rat Small Intestine.

Additions to basic media[a] (6 μmol)	Fatty acyl CoA formed (μmol/20 min)	
	Chloroform-soluble[b]	Water-soluble
Palmitic acid	1.45	0.0
Palmitic acid + caprylic acid	0.67	0.11
Palmitic acid + 2-ethylcaproic acid	0.79	0.08

[a] The medium contained 0.66 *M* hydroxylamine, pH 7.4; 12 m*M* ATP; 0.17 m*M* CoA; 13.3 m*M* KF; 0.5 ml of microsomal suspension, and the fatty acids in the amount indicated. The final volume was 3 ml and incubations were carried out for 20 min at 37°C. Reaction was terminated by addition of 0.2 ml of 72% perchloric acid (5).

[b] After addition of 2 ml of chloroform to the reaction mixture and shaking for 10 min, 2-ml aliquots of the chloroform and aqueous layers were assayed for acyl hydroxamates (6). By this procedure long-chain acyl hydroxamates are extracted completely into chloroform.

tively as shown by the levels of water-soluble hydroxamates formed.

In vivo, caprylic acid is absorbed efficiently and rapidly and probably does not accumulate to any significant extent in intestinal mucosa. It also has no effect on mucosal triglyceride synthesis. However, 2-ethyl-*n*-caproate transport and clearance is less efficient (1). Since the effect of 2-ethyl-*n*-caproate on triglyceride synthesis both *in vivo* (1) and *in vitro* is not immediate, it appears likely that accumulation of the branched-chain acid in the mucosal cell must occur before the effect on glyceride synthesis is apparent.

Summary. Optimal conditions for synthesis of triglyceride from oleic acid-1-^{14}C in everted intestinal sacs were determined. Using several experimental conditions, it was found that 2-ethyl-*n*-caproate inhibited oleic acid incorporation into triglyceride by 25–50%. When inhibition of triglyceride synthesis occurred, there was a concomitant increase in mucosal unesterified fatty acids but no increase in the phospholipid, mono- or diglyceride fractions. The inhibition of glyceride synthesis in the intestinal mucosa by 2-ethyl-*n*-caproate appears to be at the level of long-chain fatty acid activation.

1. Hyun, S. A., Vahouny, G. V., and Treadwell, C. R. Biochim. Biophys. Acta 137, 306 (1957).

2. Nelson, J., Vahouny, G. V., and Treadwell, C. R., Federation Proc. 26, 472 (1967).

3. Hyun, S. A., Vahouny, G. V., and Treadwell, C. R. Biochim. Biophys. Acta 137, 296 (1967).

4. Wilson, T. H. and Wiseman, G., J. Physiol., (London) 123, 116 (1954).

5. Brindley, D. N. and Hubscher, G., Biochim. Biophys. Acta 125, 92 (1966).

6. Hill, U. T., Anal. Chem. 19, 932 (1947).

7. Borgstrom, B., Dahlquist, A., Lundh, G., and Sjovall, J., Clin. Invest. 36, 1521 (1957).

Received Feb. 16, 1968. P.S.E.B.M., 1968, Vol. 128.