2 ml of the resulting emulsion were then incubated with 1.8 ml of the ammonium buffer, 0.2 ml 60 % $CaCl_2$ and 100 mg pancreatin (Nutritional Biochemicals Corp., Cleveland) dispersed in 2 ml water at 37° in a water bath with continuous shaking. Incubation was continued until the lipolysis was about 40–60 % complete (followed by determination of the ester bonds, according to STERN AND SHAPIRO[9]), to obtain a fair proportion of monoglycerides. The glycerides were then extracted and isolated as described above, except that no additional separation of glycerides on silicic acid was undertaken. For the actual test, triglycerides were synthesized by mesenteria of rats previously starved for several days and refed on a high-carbohydrate diet, two days prior to the experiment. The tissues were incubated with [1-^{14}C]palmitic acid in a medium of 5 % albumin in Ca^{++}-free Krebs–Ringer phosphate solution. After 2-h incubation the triglycerides were isolated as described before and subjected to similar lipolytic degradation.

The results summarized in Table I show that while the glycerides obtained from triglycerides labeled by lipase-catalyzed exchange lost the major part of their activity upon subsequent lipolysis, the newly synthesized adipose tissue triglyceride underwent practically no reduction in specific activity by this procedure.

These results indicate that the triglyceride synthesis in adipose tissue follows mainly pathway (a), *i.e.* total esterification of α-glycerophosphate with three molecules of fatty acid.

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Department of Biochemistry, Hebrew University-Hadassah Medical School, Jerusalem (Israel)

B. SHAPIRO
M. STATTER
G. ROSE

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Enzymic synthesis of glutaryl-coenzyme A

The activation of succinate to succinyl-CoA[1,2] and of malonate to malonyl-CoA[3–5] by an ATP- and CoA-dependent reaction is well documented. We have found that glutaryl-CoA is synthesized in animal tissues from glutarate in analogous manner according to reaction (1).

$$\text{Glutarate} + \text{ATP} + \text{CoA} \rightleftharpoons \text{glutaryl-CoA} + \text{ADP} + P_i \qquad (1)$$

Abbreviations: CoA, coenzyme A; ATP, adenosine triphosphate; GTP, guanosine triphosphate; ITP, inosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; GSH, glutathione.

The system of KAUFMAN[1] (*cf.* Table I) was used to assay activation of dicarboxylic acids. It is seen that not only was glutarate converted to glutaryl-CoA in dog heart and muscle and rat and pigeon liver, but in all tissues the rate of glutarate activation was significantly greater than that of succinate and malonate.

TABLE I

ENZYMIC ACTIVATION OF DICARBOXYLIC ACIDS

The assay system[1] contained (in μmoles): Tris buffer, pH 7.5 (100), glutarate, malonate or succinate (100), crystalline ATP (10), CoA (0.2), GSH (20), $MgCl_2$ (8), neutralized $NH_2OH \cdot HCl$ (1000) and enzyme. Volume, 2.0 ml. Incubation, 30 min at 37°. Values are mμmole hydroxamic acid formed (measured as in ref. 1) per mg protein/30 min. Dog-heart and skeletal-muscle and pigeon-liver preparations were dialyzed 0.35–0.65 satd. $(NH_4)_2SO_4$ fractions of crude extract. Rat liver was the 54,000 × *g* supernatant of a 0.25-*M* sucrose homogenate.

Substrate	*Dog heart*	*Dog muscle*	*Pigeon liver*	*Rat liver*
Malonate	10.3	2.6	4.7 (0)*	11.5
Succinate	7.4	8.2	20.5 (3.4)	62.9
Glutarate	28.4	16.0	31.7 (45.6)	86.7

* After fractionation with ethanol, 40–70%, at —10°.

The glutarate-activating enzyme was shown to be a discrete enzyme, as expected from the reported inactivity of glutarate with purified succinate-activating enzymes[1,6] (P enzyme), by purification procedures. By ethanol fractionation, it was resolved completely from the malonate-activating enzyme and largely from the succinate-activating enzyme in pigeon liver (Table I). The glutarate enzyme was purified 5-fold from dog-skeletal muscle, with complete resolution of malonate and succinate activity, but became unstable, being inactivated after several days at —18°.

TABLE II

NUCLEOTIDE SPECIFICITY OF GLUTARATE-ACTIVATING ENZYME

Standard assay conditions. Pigeon-liver calcium phosphate gel eluate fraction (11.4 mg protein) specific activity 35 (glutarate), 8.8 (succinate). Values are rates relative to GTP. Values in parentheses refer to dog-muscle fraction.

Nucleotide	*Succinate*	*Glutarate*
GTP	100	100 (100)
ITP	74	85
ATP	15	92 (100)

The glutarate-activating enzyme, unlike the succinate-activating enzyme, is about equally reactive with ATP, GTP and ITP (Table II). The relative reaction rates for the succinate enzyme with GTP, ITP and ATP in pigeon liver and dog muscle are comparable to those (100:67:27) observed in guinea-pig liver[7] and to those measured in pig kidney and heart[2] (GDP:IDP = 100:44).

The glutarohydroxamic formed in the assay system was extracted under mild conditions using acetone[3] and placed on Whatman No. 1 paper. The chromatogram was developed in isoamyl alcohol–formic acid solvent[8] and after spraying with $FeCl_3$ one spot (R_F, 0.32) corresponding to authentic glutaromonohydroxamic acid

was found. With water-saturated butanol[3] as solvent, a single Fe-positive spot (R_F, 0.48) was again found (authentic glutaromonohydroxamic acid, 0.49).

Further evidence for reaction (1) was obtained by measuring acylation of substrate amounts of CoA in presence of ATP and glutarate (Table III). It appears that after several minutes a steady-state concentration of glutaryl-CoA was reached, probably reflecting opposing activation and deacylation reactions. The disappearance of CoA in presence of ATP and enzyme is not explained by oxidation.

TABLE III

ENZYMIC ACYLATION OF CoA BY GLUTARATE

Test system as in Table I (GSH and NH_2OH omitted) with 1.0 μmole CoA and dog-muscle fraction (5.4 mg protein). Sulfhydryl measured as in ref. 1.

System	$-\Delta$-SH		20 min
	5 min	10 min	
Complete	0.33	0.48	0.58
No glutarate	0.20	0.36	0.44
— Δ SH, corrected	0.13	0.12	0.14

The substrate specificity of the glutarate-activating enzyme was tested. Glutaconate reacted at 40 % of the rate of glutarate. The α-methyl, α,α-dimethyl, β-methyl, and β,β-dimethylglutarate derivatives were inert with the pigeon-liver enzyme and slightly reactive (30 %) with the dog-muscle enzyme. Adipate, muconate, β-hydroxyglutarate and β-hydroxy-β-methylglutarate were inert. (The latter gave positive hydroxylamine- and CoA-acylation tests in muscle and liver, probably due to some contaminant since the acylation product was not cleaved by purified β-hydroxy-β-methylglutarate-cleavage enzyme[9]. Neither was this product acetyl-CoA.)

These experiments show that the activation of malonate, succinate and glutarate is catalyzed by three different enzymes. The glutarate-activating enzyme presumably plays a role in initiating the oxidation of glutarate in tissues[10].

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Department of Pharmacology, School of Medicine,
Western Reserve University,
Cleveland 6, Ohio (U.S.A.)

GOVIND K. K. MENON
DANIEL L. FRIEDMAN
JOSEPH R. STERN

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