PALMITATE ACTIVATION

Aspects of palmitate metabolism

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN
DOCTOR IN DE GENEESKUNDE
AAN DE MEDISCHE FACULTEIT TE ROTTERDAM,
OP GEZAG VAN DE DEKAAN DR. J. MOLL,
HOOGLERAAR IN DE FACULTEIT DER GENEESKUNDE,
TEGEN DE BEDENKINGEN VAN HET COLLEGE VAN DEKANEN
UIT DE FACULTEIT DER GENEESKUNDE TE VERDEDIGEN OP
WOENSDAG 8 DECEMBER 1971
TE 16.00 UUR DOOR

JAN WILLEM DE JONG

GEBOREN TE 's-GRAVENHAGE IN 1942

1971
Bronder-offset N.V. - Rotterdam

PROMOTOR: PROF. DR. W.C. HÜLSMANN

CO-REFERENTEN: PROF. DR. H.J. VAN DER MOLEN
PROF. DR. E.C. SLATER

Aan Lieneke,
Mart & Mansje

Contents

List of Appendix papers

Voorwoord

Bij het gereedkomen van dit proefschrift wil ik mijn ouders dankzeggen voor de stimulans die zij geweest zijn voor mijn studie.

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Dr. A.J. Meijer heeft de analyses uitgevoerd die vermeld zijn in Tabel I van het derde artikel van de Appendix.

Mejuffrouw C. Kalkman, beste Ina, ik ben je veel dank verschuldigd. Menig spannend experiment heb je meebeleefd. Jouw precisie en analytische vakkundigheid zijn van niet te onderschatten belang geweest bij dit onderzoek.

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T. van Gent, beste Teus, de onderzoekingen van het laatste halfjaar zijn mede door jou geassisteerd. Ik stel je ideeën en vaardigheid op hoge prijs.

Het voert te ver iedereen van de afdeling Biochemie hier te noemen. Toch heeft het werkklimaat bevruchtend gewerkt op het onderzoek. Alle medewerkers van genoemde afdeling die direkt of indirekt hebben bijgedragen tot dit proefschrift wil ik hiervoor hartelijk bedanken.

List of abbreviations and symbols*

ADP - Adenosine-5'-diphosphate
AMP - Adenosine-5'-monophosphate
ATP - Adenosine-5'-triphosphate
CoASH - Coenzyme A (reduced)
DNP - 2,4-Dinitrophenol
dpm - Disintegrations per minute
EDTA - Ethylenediamine tetraacetate
g - Relative centrifugal force
GDP - Guanosine-5'-diphosphate
GSH - Glutathione (reduced)
GTP - Guanosine-5'-triphosphate
K_i - Dissociation constant of inhibitor-enzyme complex
K_M - Michaelis constant
NAD^+ - Nicotinamide-adenine dinucleotide (oxidized)
NADH - Nicotinamide-adenine dinucleotide (reduced)
NADPH - Nicotinamide-adenine dinucleotide phosphate (reduced)
P_i - Inorganic orthophosphate
PP_i - Inorganic pyrophosphate
Q_{O_2} - Velocity of oxygen uptake (µl O_2/h per mg protein)
Q_{10} - Factor indicating the increase of reaction velocity when the temperature is elevated 10°.
RCOOH - Carboxylic acid
S.D. - Standard deviation
Tris - 2-Amino-2-hydroxymethyl-1,3-propanediol
U - Unit of enzyme activity (conversion of one µmole of substrate per minute)

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*For abbreviations and symbols used in this thesis, which are not mentioned in the above list, see: *Suggestions and Instructions to Authors in Biochimica et Biophysica Acta*, Elsevier, Amsterdam, 1965.

List of enzymes*

Number	*Systematic Name*	*Trivial Name*
1.1.1.27	L-Lactate:NAD oxidoreductase	Lactate dehydrogenase
1.1.1.35	L-3-Hydroxyacyl-CoA:NAD oxidoreductase	3-Hydroxyacyl-CoA dehydrogenase
1.1.1.37	L-Malate:NAD oxidoreductase	Malate dehydrogenase
1.2.4.1	Pyruvate:lipoate oxidoreductase (acceptor-acetylating)	Pyruvate dehydrogenase
1.3.99.3	Acyl-CoA:(acceptor) oxidoreductase	Acyl-CoA dehydrogenase
1.4.3.4	Monoamine:oxygen oxidoreductase (deaminating)	Monoamine oxidase
1.6.2.?	Reduced-NADP:ferri-cytochrome *c* oxidoreductase	NADPH cytochrome *c* reductase
1.9.3.1	Ferrocytochrome *c*:oxygen oxidoreductase	Cytochrome *c* oxidase
1.11.1.6	Hydrogen-peroxide: hydrogen-peroxide oxidoreductase	Catalase
2.3.1.16	Acyl-CoA:acetyl-CoA *C*-acyltransferase	3-Ketoacyl-CoA thiolase
2.3.1.?	Palmitoyl-CoA:carnitine *O*-palmitoyltransferase	Carnitine palmitoyltransferase

*The recommendations of the International Union of Biochemistry, *Enzyme Nomenclature*, Elsevier, Amsterdam, 1965, are followed where possible.

Number	*Systematic Name*	*Trivial Name*
2.3.1.?	Acyl-CoA:monoglyceride *O*-acyltransferase	Monoglyceride acyltransferase
2.7.1.1	ATP:D-hexose 6-phospho-transferase	Hexokinase
2.7.1.40	ATP:pyruvate phosphotransferase	Pyruvate kinase
2.7.3.2	ATP:creatine phosphotransferase	Creatine kinase
2.7.4.3	ATP:AMP phospho-transferase	Adenylate kinase
2.7.7.16	Ribonucleate pyrimidine-nucleotido-2'-transferase (cyclizing)	Ribonuclease
3.1.1.1	Carboxylic-ester hydrolase	Carboxylesterase
3.1.1.8	Acylcholine acyl-hydrolase	Cholinesterase
3.1.3.1	Orthophosphoric mono-ester phosphohydrolase	Alkaline phosphatase
3.1.3.2	Orthophosphoric mono-ester phosphohydrolase	Acid phosphatase
3.1.3.5	5'-Ribonucleotide phosphohydrolase	5'-Nucleotidase
3.1.3.9	D-Glucose-6-phosphate phosphohydrolase	Glucose-6-phosphatase
3.2.1.26	β-D-Fructofuranoside fructohydrolase	β-Fructofuranosidase (sucrase)
3.2.1.31	β-D-Glucuronide glucuronohydrolase	β-Glucuronidase
3.4.4.4		Trypsin
3.4.4.16		Subtilopeptidase A (Nagarse)
3.5.3.1	L-Arginine amidino-hydrolase	Arginase

Number	*Systematic Name*	*Trivial Name*
3.5.4.4	Adenosine aminohydrolase	Adenosine deaminase
3.6.1.3	ATP phosphohydrolase	ATPase
4.1.3.6	Citrate oxaloacetate-lyase	Citrate lyase
4.1.3.7	Citrate oxaloacetate-lyase (CoA-acetylating)	Citrate synthase
5.3.1.9	D-Glucose-6-phosphate ketol-isomerase	Glucosephosphate isomerase (phospho-glucose isomerase)
6.2.1.1	Acetate:CoA ligase (AMP)	Acetyl-CoA synthetase; short-chain acyl-CoA synthetase
6.2.1.2	Acid:CoA ligase (AMP)	Octanoyl-CoA synthetase; medium-chain acyl-CoA synthetase
6.2.1.3	Acid:CoA ligase (AMP)	Palmitoyl-CoA synthetase; long-chain acyl-CoA synthetase
6.2.1.?	Acid:CoA ligase (AMP)	Lauroyl-CoA synthetase
6.2.1.?	Acid:CoA ligase (GDP)	GTP-dependent acyl-CoA synthetase

CHAPTER 1

Introduction

Reaction mechanism of palmitate conversion

Palmitate, one of the important substrates to maintain life, is metabolically quite inert. However, it can be esterified with coenzyme A. In this activated form the acyl group can be used in various biochemical pathways, *viz.* oxidative degradation, the synthesis of esters (glycerides, phospholipids, cholesterol esters, waxes) and amides (glycolipids), or the formation of other fatty acids by elongation or desaturation of the carbon chain (Fig. 1).

The energy required for thioester formation is supplied by hydrolysis of ATP into AMP and PP_i[1,2] (Eqn. 1). The

$$C_{15}H_{31}COOH + ATP + CoASH \overset{Mg^{2+}}{\rightleftharpoons} C_{15}H_{31}COSCoA + AMP + PP_i \quad (1)$$

reaction is catalyzed by palmitoyl-CoA synthetase, also

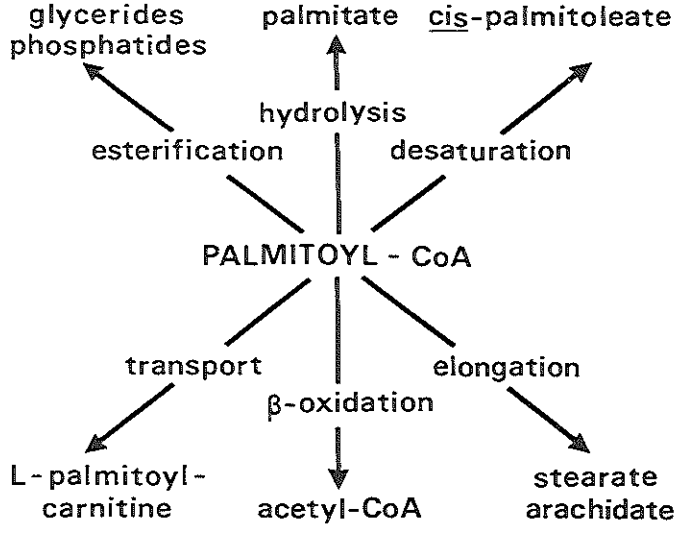


Fig. 1. Some routes of palmitoyl-CoA utilization.

referred to as palmitate thiokinase or long-chain fatty acid activating enzyme. The systematic name is acid:CoA ligase (AMP), EC 6.2.1.3. Unless explicitly mentioned otherwise the name palmitoyl-CoA synthetase in this thesis refers to the ATP-dependent enzyme.

For the reaction catalyzed by palmitoyl-CoA synthetase it is doubtful whether an enzyme-bound acyladenylate is formed, as found for short- and medium-chain acyl-CoA synthetases[3-7]. Two forms of palmitoyl-CoA synthetase in rat liver microsomes have been reported: an active and an inactive state. Incubation with CoASH and ATP results in activation of the enzyme; inactivation is caused by incubation with palmitoyl-AMP in the absence of CoASH. A CoA-enzyme complex is postulated as the active form[8]. Similar activation of palmitoyl-CoA synthetase activity by CoASH and ATP may be concluded from experiments with a triglyceride synthetase complex, isolated from hamster intestine[9,10]. An acyl-carrier protein is probably involved in fatty acid activation in intestine and some microorganisms[10,11].

Organ distribution and properties of palmitoyl-CoA synthetase

After the discovery of the palmitoyl-CoA synthetase in guinea-pig liver by KORNBERG AND PRICER[1] (*cf.* ref. 12), research has also been carried out on the enzyme in intestinal preparations from rat[13-18] (Appendix, Paper 4), cat[19] and guinea pig[19], adipose tissue from rat[20-24] and man[25], and rat kidney[26-28] and heart[16,26,28,29] (Appendix, Paper 4). High rates of long-chain acyl-CoA biosynthesis have been reported in rat liver[8,16,27,28,30-39] (Appendix, Papers 2 and 4), which was originally found to contain rather low long-chain acyl-CoA synthetase activity[1,40,41]. TRZECIAK[42] reported palmitoyl-CoA synthetase activity in swine aortic wall, whereas FARSTAD AND SANDER[43] recently found long-chain acyl-CoA synthetase activity in human blood platelets. The organ distribution has been studied in the rat by PANDE AND MEAD[26] and by AAS[28].

Palmitoyl-CoA synthetase activity has also been demonstrated in protists, in plants and in non-mammalian members of the animal kingdom. Long-chain acyl-CoA synthetase is found in *Bacillus megaterium*[44] and *Escherichia coli*[45-48], but not *Clostridium butyricum*[49]. *Candida tropicalis*, a yeastlike organism, which can be grown on *n*-tetradecane, exhibits palmitoyl-CoA synthetase activity[50-52]. Furthermore, the enzyme is found in mesocarp from avocado (*Persea americana*)[53] and in endosperm of castor bean (*Ricinus communis*)[54-56]. Long-chain acyl-CoA synthetase activity is present in eggs of the giant intestinal roundworm (*Ascaris lumbricoides*)[27], in flight muscle of the southern armyworm moth (*Prodenia eridania*)[57], and in skeletal muscle of the mackerel (*Pneumatophorus diego*)[58].

The organ distribution in mammals of palmitoyl-CoA synthetase[26,28], carnitine palmitoyltransferase[59], 3-hydroxyacyl-CoA dehydrogenase[60] and 3-ketoacyl-CoA thiolase[60] roughly correlate. Liver is most active, followed by moderate activity in heart and kidney, whereas lung, skeletal muscle and brain have little activity. The subcellular and submitochondrial localization of palmitoyl-CoA synthetase is discussed in Chapter 2.

Microbiological studies suggest that the expression of the linked structural genes of the acyl-CoA synthetase form a regulon, which is controlled by a regulator gene[46,47]. There is some evidence that the (mammalian) palmitoyl-CoA synthetase could be regulated by a soluble protein kinase[61-63] (contrast: ref. 2; see also ref. 64 for observations on the phosphorylation of medium-chain acyl-CoA synthetase).

Long-chain acyl-CoA synthetases have been (partially) purified from rat liver microsomes[2,8], *E. coli*[48] and *B. megaterium*[44]. The activity of the microsomal palmitoyl-CoA synthetase is found to be quite unstable after purification[2,8]. The increase in specific activity is very low. The molecular weight is estimated to be 250,000. BAR-TANA *et al.*[2] stated that this value is presumably exaggerated owing to the pres-

ence of unknown amounts of detergent bound to enzyme. The enzyme reacts with at least C_{12} - C_{18} saturated or unsaturated fatty acids[2]. A multienzyme complex, purified about 80 times from *E. coli*, has a molecular weight of 120,000 (ref. 48). The latter enzyme is active with C_4 - C_{18} fatty acids. Apparent K_M's for palmitate[2,34], ATP[2,34] and CoASH[2] of respectively 0.04 mM, 4 mM and 0.05 mM have been reported for the rat liver microsomal enzyme. The synthetase, demonstrated by KORNBERG AND PRICER[1] in the microsomal fraction of guinea-pig liver, shows no significant variation of activity in the pH range from 6.5 to 8.0. The inner membrane enzyme of rat liver mitochondria has comparable properties (unpublished observations). The purified palmitoyl-CoA synthetase from rat liver microsomes exhibits a sharp optimum at pH 9 (ref. 2), whereas the enzyme in rat gut microsomes has highest activity at pH 6.8 - 7.6 (ref. 13). A pH optimum of 8.5 has been reported for the *E. coli* enzyme[46].

The reaction rate of the microsomal (guinea-pig liver) enzyme at 40° is 2 to 3 times as rapid as at 25° (ref. 1). A higher value is found for palmitoyl-CoA synthetase in rat liver and heart mitochondria[65] (Appendix, Paper 6). Stimulation of the enzyme by high salt concentrations has been reported[66,67].

GTP-dependent acyl-CoA synthetase

An enzyme catalyzing acyl-CoA synthesis with GTP, according to Eqn. 2, has been demonstrated in several tissues and isolated from beef liver and rat kidney and liver mitochondria by ROSSI *et al.*[68-79]. The enzyme is able to acti-

$$RCOOH + GTP + CoASH \overset{Mg^{2+}}{\rightleftharpoons} RCOSCoA + GDP + P_i \qquad (2)$$

vate palmitate, although the K_M is quite high (2 - 3 mM). The substrate specificity of the synthetase is dependent on the isolation procedure. 4'-Phosphopantetheine is necessary for full activity. P_i and fluoride inhibit the isolated enzyme severely. The findings of the ROSSI group have not yet been confirmed by other laboratories, presumably due in part

to a difference in the rat strain used[79]. No or very low palmitoyl-CoA synthetase activity with GTP substituted for ATP was found in *B. megaterium*[44], *E. coli*[46], *C. tropicalis* mitochondria[51,52], castor bean endosperm[55,56], moth flight-muscle mitochondria[57], bovine brain mitochondria[80], rat liver[26,35,81,82] (Appendix, Papers 2 and 5), heart[26,83], kidney[26,83], intestine[26], adipose tissue[23,24,26,84] and other rat organs[26]. Whether the GTP-dependent acyl-CoA synthetase is of physiological importance has been doubted[35] (Appendix, Paper 2). It may, however, play a rôle in the synthesis of intramitochondrial GTP.

Scope of the present study

In the past decade some work has been done on the subcellular localization of palmitoyl-CoA synthetase in intestine. However, marker enzymes (to characterize subcellular fractions) were sometimes applied in a wrong way[13,85] or omitted from the investigation[26].

A number of schemes had been published concerning the (liver) mitochondrial compartmentation of enzymes involved in fatty acid breakdown[86-93]. The presumed loci of ATP and GTP-dependent acyl-CoA synthetases were often derived from oxidation experiments with liver mitochondria in which use was made of inhibitors. These sometimes lacked the assumed specificity. When it was found, for instance, that atractylate[94,95] and P_i[35,96] (Appendix, Papers 2 and 3) had more effects than accounted for in the schemes, new investigations were desirable.

A dearth of reports on myocardial palmitoyl-CoA synthetase was observed.

In the course of a study on the oxidation of ketone bodies an inhibitory effect of P_i, in the presence of DNP, was noticed (Chapter 3). Similar observations had been reported in the literature for fatty acid oxidation. The effect had been ascribed to an inhibition of the GTP-

dependent acyl-CoA synthetase (*vide ante*). We were not successful in demonstrating a GTP-dependent acetoacetyl-CoA synthetase in heart mitochondria. However, in our preparations GTP-dependent acyl-CoA synthetase activity was also very poor. When GTP was replaced by ATP high activities of the acyl-CoA synthetase were observed[35] (Appendix, Paper 2). Because the GTP-dependent activation of fatty acids was found to be relatively unimportant, we decided to reinvestigate the inhibition by P_i of fatty acid oxidation[35,96] (Appendix, Papers 2 and 3; Chapter 3).

Considerations, mentioned above, started our research on aspects of the activity, regulation, and subcellular and submitochondrial localization in rat heart, liver and intestine. In the course of this investigation it became clear from studies by us[16] (Appendix, Paper 4) and independently by others[97] that Nagarse, a peptidase used to facilitate the isolation of muscle mitochondria, rather selectively destroys palmitoyl-CoA synthetase activity (see Chapter 4). This finding was used in localization studies[37,65,82] (Appendix, Papers 5 and 6; Chapter 2). The discovery that adenosine is a strong inhibitor of palmitoyl-CoA synthetase[65,82] (Appendix, Papers 5 and 6) prompted a search for a possible rôle of this nucleoside in the regulation of the activity of the enzyme (Chapter 5).

CHAPTER 2

Localization of palmitoyl-CoA synthetase

Subcellular localization

Palmitoyl-CoA synthetase is partially soluble in procaryotic cells[44,46,48,49]. In eucaryotic cells the enzyme is membrane bound[28,33,34,36,38]. The "soluble" activity reported in the older literature[1,41,98] is probably due to incomplete sedimentation of microsomes. Palmitoyl-CoA synthetase activity is present in mitochondrial and microsomal fractions. The available data are given in Table I. The quite large range of specific activities observed for liver and intestine may be due to: (i) hormonal and dietary conditions[102,103]; (ii) differences in animal strain[79] and age[29,104]; (iii) the lability of palmitoyl-CoA synthetase (*e.g.* towards endogenous proteinases in the preparation[12], *cf.* Chapter 4); and/or (iv) different fractionation methods and activity determinations. To obtain high specific activities it is necessary to minimize the concentration of AMP (and adenosine formed through the 5'-nucleotidase action in the preparation), *e.g.* by using an ATP regenerating system[16,36] (Appendix, Paper 4). Adenosine and also AMP inhibit the palmitoyl-CoA synthetase[65,82] (Appendix, Papers 5 and 6; Chapter 5).

In rat liver about the same specific activity is found in the mitochondrial fraction (58 mU/mg protein) and microsomal fraction (73 mU/mg protein) (average values of 11 - 15 data reported by different authors, Table I). PANDE AND MEAD[34] communicated the highest specific activity in the liver cell membrane fraction. We were not able to reproduce this finding (ref. 16; Appendix, Paper 4) with either the modified carnitine assay[16,33] or the hydroxylamine method[34]. Recently, also LIPPEL *et al.*[38] were unable to detect appre-

TABLE I

LONG-CHAIN ACYL-CoA SYNTHETASE ACTIVITY IN MITOCHONDRIAL AND MICROSOMAL FRACTIONS

Species	*Organ*	*Mitochondria*		*Microsomes*		*Ref.*
		Spec.act. (mU/mg)	*Temp. (°C)*	*Spec.act. (mU/mg)*	*Temp. (°C)*	
C. tropicalis	-	27	37	-	-	52
Southern armyworm moth	Flight muscle	28	30	-	-	57
Rat	Liver	17-135	37*	17-145	37*	2,8,16,28, 30,32-38, 81,95,97, 99,100
Rat	Heart	27-37	37	38-60	37	16,26,28
Rat	Intestine**	11-20	37	3-119	37	13,15-17, 85
Rat	Skeletal muscle	18	37	3	37	26
Rat	Adipose tissue	50-184	37	95-640	37	21,24,26
Rat	Kidney	10	35	13	35	28
Rat	Brain	2	37	8	37	26
Guinea pig	Liver	-	-	1;10	37;40	12;1
Guinea pig	Intestine	-	-	20	37	19
Rabbit	Skeletal muscle	8-23	37	-	-	58,97
Cat	Intestine	-	-	54	37	19
Pig	Intestine	-	-	6	37	101
Beef	Heart	167	38	-	-	73

*A Q_{10} of 3.0 was used to recalculate palmitoyl-CoA synthetase activity if necessary[65] (Appendix, Paper 6).

The values of AILHAUD *et al.*[85] were included. It was assumed that **rat intestine was used in their experiments.

ciable palmitoyl-CoA synthetase in this fraction. At least in liver, heart and gut there seems to be a correlation between the palmitoyl-CoA synthetase activity present in the mitochondrial or microsomal fraction and the function of the organ[16] (Appendix, Paper 4). [Attention is drawn to the relatively small amount of microsomal protein in heart tissue[105,106] (*cf.* ref. 16; Appendix, Paper 4).] A comparable conclusion was drawn for (rabbit) skeletal muscle by PANDE AND BLANCHAER[58], who found that the activity of palmitoyl-CoA synthetase was twice as high in red as in white skeletal muscle. RODGERS AND BOCHENEK[17] measured the activities of palmitoyl-CoA synthetase and monoglyceride acyltransferase in rat duodenum, jejunum and ileum. The enzyme activities were significantly greater in the proximal small bowel, which is thought to be the major site of lipid absorption. The high activity of palmitoyl-CoA synthetase in adipose tissue is interesting as this tissue has a profound capacity for triglyceride synthesis. Likewise the activity in moth flight muscle is noteworthy as carbohydrate is not used for flight energy in this species, but rather converted into lipid, which is stored as a low-weight high-caloric fuel[107].

In mesocarp from ripening avocado palmitoyl-CoA synthetase is at least found in the microsomal fraction[53], whereas long-chain acyl-CoA synthetase in castor bean endosperm is located in the glyoxysomes[54-56].

It was reported earlier that no palmitoyl-CoA synthetase was present in the brush border fraction of rat intestine[16] (see Appendix, Paper 4). This correlates with recent findings of SCHILLER *et al.*[108], who do not find triglyceride synthetase activity in the brush border fraction from hamster and rat gut.

Intramitochondrial localization

A number of studies have appeared in which the submitochondrial loci of acyl-CoA synthetases are indicated[81,91,109-111]. As mentioned before newer findings made ad-

ditional experiments desirable to gain insight in the localization of acyl-CoA synthetases[100,112]. The destructive action of Nagarse on palmitoyl-CoA synthetase[16,82,97] (Appendix, Papers 4 and 5; Chapter 4) was used as a tool to eliminate the very active hepatic outer membrane enzyme, which made earlier experiments difficult to interpret[31,113]. Our oxidation experiments[65,82] (Appendix, Papers 5 and 6) and the fractionation studies of VAN TOL AND HÜLSMANN[37], both performed with Nagarse-treated rat liver mitochondria, indicate that a palmitoyl-CoA synthetase is present in the inner membrane *plus* matrix fraction. This finding is in agreement with the conclusion of SKREDE AND BREMER[95], who eliminated the outer membrane activity by oxidizing CoASH with tetrathionate. The activity of the inner membrane *plus* matrix enzyme is 5 - 10 % of the total liver mitochondrial palmitoyl-CoA synthetase activity, which may be adequate to account for the oxygen uptake in the complete oxidation of palmitate. The contribution of the outer membrane palmitoyl-CoA synthetase is indispensable when the rate of fatty acid consumption is very high such as in ketogenesis[37]. Moreover, the outer membrane enzyme may be required for phospholipid synthesis[114-116].

Medium-chain acyl-CoA synthetases with broad specificity for fatty acids have been demonstrated in the inner membrane *plus* matrix fraction[28]. The pH and temperature dependence of palmitoyl-CoA and octanoyl-CoA synthesis in Nagarse-treated liver mitochondria seem to be different (unpublished observations), from which it could be concluded that the (inner membrane/matrix) medium-chain acyl-CoA synthetase is not involved in palmitate thioester formation (see, however, refs. 28 and 95). Possibly more information can be obtained by making use of differences in acyladenylate formation by substrate amounts of the two enzymes (see Chapter 1).

In rat heart mitochondria only evidence is found for an outer membrane localized palmitoyl-CoA synthetase[65,82] (Appendix, Papers 5 and 6).

CHAPTER 3

The inhibition of fatty acid oxidation by phosphate

In the course of an investigation of the oxidation of ketone bodies by heart mitochondria, it was noticed that P_i inhibits the respiration in the presence of DNP. In Fig. 2 the results of a manometric experiment are shown, in which rat heart mitochondria have been used to oxidize 3-hydroxybutyrate. After the addition of DNP, the presence of P_i inhibits the oxygen uptake. The same is observed when acetoacetate replaces 3-hydroxybutyrate (not shown). A possible ex-

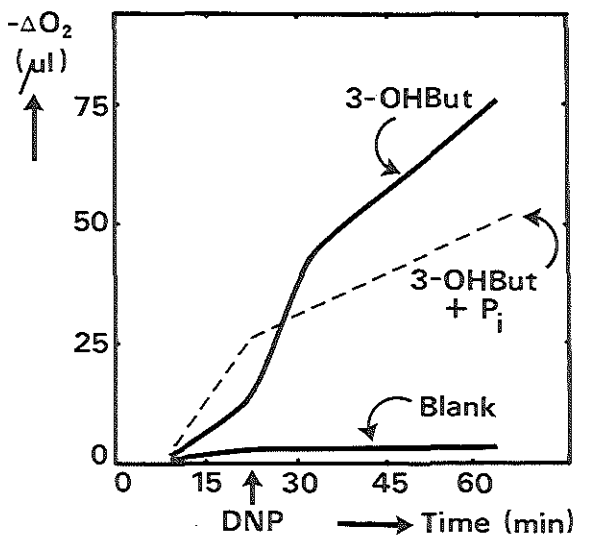


Fig. 2. Influence of P_i on 3-hydroxybutyrate oxidation by isolated rat heart mitochondria. Sarcosomes were isolated in the presence of Nagarse as described in ref. 16 (Appendix, Paper 4). Oxygen uptake was measured at 25° with differential manometers as described in ref. 117 (Appendix, Paper 1). The reaction medium contained 37.5 mM glucose, 25 mM KCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 0.4 mM DL-malate, 33 µM cytochrome *c*, 0.5 mM ATP, 25 mM sucrose, 75 mM Tris-HCl (pH 7.6) and, where indicated, 37.5 mM potassium phosphate (pH 7.6) and/or 20 mM sodium DL-hydroxybutyrate ("3-OHBut"). When P_i was included in the reaction medium the concentration of the Tris buffer was reduced to 25 mM. In the blank 3-hydroxybutyrate and P_i were absent. The reactions were started by the addition of sarcosomes (1.5 mg protein). After 7.5 min the contents of the flasks were considered to be equilibrated. The taps were closed and readings were taken at 2.5-min intervals. At the indicated time 50 µl DNP (in sucrose) was added from the side arm, giving a final volume of 1.0 ml. The final concentrations of DNP and sucrose were 50 µM and 37.5 mM, respectively.

planation could be that the inhibition by P_i is due to a lowering of the steady state concentration of succinyl-CoA[118,119] (see Eqn. 3), necessary for acetoacetate activation in heart[120] (Eqn. 4). In fact HATEFI AND FAKOUHI[118] showed that succinyl-CoA is a potent activator of acetoacetate oxidation in beef heart mitochondria, even when 90 % of the oxygen uptake is inhibited by P_i and AMP.

$$\text{succinyl-CoA} + \text{GDP} + P_i \rightleftharpoons \text{succinate} + \text{GTP} + \text{CoASH} \qquad (3)$$

$$\text{acetoacetate} + \text{succinyl-CoA} \rightleftharpoons \text{acetoacetyl-CoA} + \text{succinate} \qquad (4)$$

That P_i could inhibit the sarcosomal oxidation of ketone bodies in the presence of DNP, reminded us of a similar effect, discovered by VAN DEN BERGH[91,109,121], for fatty acid oxidation in rat liver mitochondria. Here also, fatty acid oxidation in the presence of DNP was inhibited by P_i.

The dissimilarity between the main mechanisms of acetoacetate activation in heart sarcosomes, and the activation of fatty acid in liver mitochondria, made us wonder whether the inhibitory effect of P_i on fatty acid oxidation were indeed to be ascribed to inhibition of a GTP-dependent activation process[74,75,91,109,121] (see Chapter 1). We showed in polarographic experiments that the oxidation of pyruvate or other acetyl-CoA donors such as acylcarnitines, was also inhibited by P_i addition[35,96] (Appendix, Papers 2 and 3). The inhibition of palmitoylcarnitine oxidation by P_i was confirmed by HOFFMANN AND VAN DEN BERGH[122]. Malate addition caused a relief of the P_i-induced inhibition of fatty acid, pyruvate or acylcarnitine oxidation[35,96] (Appendix, Papers 2 and 3). When P_i was added after malate no inhibition was observed[96] (Appendix, Paper 3).

The GTP-dependent acyl-CoA synthetase is said to be inhibited by P_i and fluoride, the latter inhibition being probably diagnostic for the activation in intact mitochondria[75]. We do not consider this phenomenon to be diagnostic at all. Under the conditions used for the study of the GTP-dependent

acyl-CoA synthetase, P_i and fluoride also inhibit the oxidation of pyruvate in intact rat liver mitochondria. The inhibitions are completely relieved by the addition of malate alone in the case of the P_i inhibition, and of malate *plus* P_i in the case of the fluoride inhibition (see Table II). This indicates that an interruption of the citric acid cycle, resulting in a deficiency of oxaloacetate, is the cause of the inhibition. P_i addition causes a loss of citrate and malate from the mitochondria[96] (Appendix, Paper 3). This extrusion of citric acid cycle intermediates occurs presumably by an exchange for P_i (see refs. 125-127, and KLINGENBERG[128] for a recent review on mitochondrial metabolite transport).

HÜLSMANN[129] discovered that fluoride inhibits the oxidation of 2-oxoglutarate in heart sarcosomes. The fluoride

TABLE II

INHIBITION OF PYRUVATE OXIDATION BY INORGANIC PHOSPHATE AND FLUORIDE IN RAT LIVER MITOCHONDRIA

Mitochondria were isolated as described in ref. 16 (Appendix, Paper 4). Oxygen uptake was determined with differential manometers[117] (Appendix, Paper 1). The medium consisted of 25 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 40 mM Tris-HCl (pH 7.4), 75 mM sucrose, 0.1 mM DNP, 10 mM sodium pyruvate, 7.5 mg mitochondrial protein and, where indicated, 10 mM potassium (L)-malate, 15 mM KF and/or 15 mM potassium phosphate (pH 7.4). For other conditions see legend to Fig. 2. After 53 min of incubation the reactions were stopped with $HClO_4$ (final concentration 4%). The contents of the flasks were transferred to tubes, centrifuged and neutralized with KOH. $KClO_4$ was removed after standing in the cold and acetoacetate and 2-oxoglutarate were determined[123, 124].

Additions	*Without malate*		Plus *malate*	
	*$-\Delta$Oxygen** (µatoms)	*Δ2-Oxo-glutarate* (µmoles)	*$-\Delta$Oxygen** (µatoms)	*Δ2-Oxo-glutarate* (µmoles)
None	13	0.1	17	0.2
+ P_i	5	0.1	18	0.2
+ F^-	4	0.1	12	1.1
+ P_i + F^-	5	0.0	19	0.2

*Oxygen uptake was corrected for acetoacetate formation (2 µatoms oxygen/µmole acetoacetate).

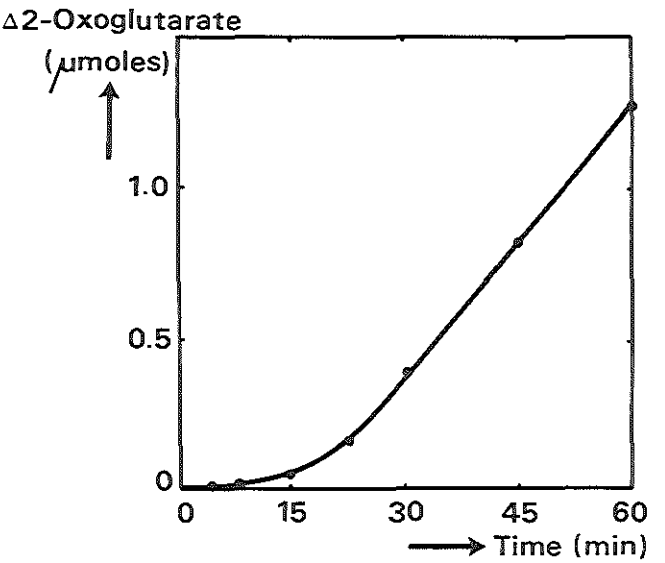


Fig. 3. Fluoride-induced 2-oxoglutarate accumulation in rat liver mitochondria, oxidizing pyruvate/malate. Incubations were carried out in test tubes under agitation. 60 mM Tris-HCl, 25 mM sucrose, 15 mM KF, 10 mM potassium (L)-malate and 6.0 mg mitochondrial protein were used. Other conditions were similar to those described in the legend to Table II (P_i was omitted). After incubation for the times indicated in the figure, reactions were stopped by $HClO_4$ addition. Analysis for 2-oxoglutarate was performed on deproteinized, neutralized samples (see legend to Table II).

inhibition of pyruvate oxidation is only partially relieved by malate alone, but completely by malate *plus* P_i (Table II). A possible explanation is the removal of P_i by fluoride (magnesium fluorophosphate formation), resulting in 2-oxoglutarate accumulation (Table II and Fig. 3) and oxaloacetate depletion.

CHAPTER 4

Effect of Nagarse on palmitoyl-CoA synthetase

Mitochondria isolated from different tissues differ in structure (shape, size, number of cristae) and functional properties[130,131]. By means of histochemistry and electron microscopy enzymatically and morphologically different mitochondria have also been demonstrated in one tissue simultaneously[130,132-135]. Separation of mitochondrial species from each other has been achieved from liver, kidney, heart and skeletal muscle[117,130,133-139] (Appendix, Paper 1).

From skeletal muscle of rat and man two types of mitochondria were isolated, one with loosely coupled oxidative phosphorylation localized probably subsarcolemmally, and another, more "deeply localized" with tightly coupled oxidative phosphorylation[117,136] (Appendix, Paper 1). Mild fragmentation of the muscle in a loose-fitting Potter-Elvehjem homogenizer preferentially released "subsarcolemmal" sarcosomes. By using a tight-fitting homogenizer and longer times to grind the tissue, more "deeply localized" mitochondria were found in the preparation. HÜLSMANN[137] extended these findings to rat heart. He showed that it was possible by treatment with Nagarse*, a subtilopeptidase A, to release "deeply localized" mitochondria from heart muscle homogenates depleted from "subsarcolemmal" mitochondria. The latter had typical properties of tightly coupled mitochondria. After CHANCE AND HAGIHARA[141,142] introduced the Nagarse digestion of tissue to facilitate the isolation of mitochon-

*The nomenclature of the subtilisins, serine proteinases from strains of *Bacillus subtilis*, has been changed several times. This has caused considerable confusion in the literature. Nagarse has been isolated from *B. subtilis* strain N'. The synonyms subtilisin BPN' and subtilopeptidase C are used. All subtilisins belong to the category EC 3.4.4.16 with the recommended trivial name subtilopeptidase A (see ref. 140).

dria from pigeon and rat heart, the use of Nagarse for the isolation of sarcosomal fractions has almost become a standard procedure. Mitochondrial fractions were obtained from Nagarse-treated hearts of a variety of avian and mammalian species[143-158], including man[157]. The method was also used for the isolation of mammalian skeletal muscle mitochondria[58,145,156,159-162]. The use of the proteinase gives mitochondrial preparations (i) in an improved yield; (ii) with less contamination; and (iii) with improved integrity according to the morphological and biochemical parameters tested. In oxidation studies mitochondria isolated with the Nagarse procedure showed oxidation rates (*e.g.* with pyruvate, succinate, 2-oxoglutarate and acylcarnitines), P/O and respiration-control ratios, which compared favorably with conventially prepared mitochondria. Similar observations were made when trypsin was used in stead of Nagarse[156,163].

Although mitochondria isolated from Nagarse-treated heart and skeletal muscle homogenates would appear to be better than those isolated without the use of a peptidase, this conclusion is premature. It was found by LINDENMAYER *et al.*[164] that heart mitochondria prepared by the Nagarse procedure rapidly utilized NADH. They concluded that the permeability of a mitochondrial membrane is increased. BODE AND KLINGENBERG[145,165-167] and MAKINEN AND LEE[159] reported that mitochondria from heart and skeletal muscle are able to oxidize palmitoylcarnitine, but not palmitate. It was concluded that palmitoyl-CoA synthetase in these organs is absent (see also ref. 168). These sarcosomal preparations were obtained by the Nagarse method. FRITZ and coworkers[87,110] as well as ourselves[65,82] (Appendix, Papers 5 and 6) showed, however, that rat heart mitochondria (isolated without the proteinase) were able to oxidize palmitate (*cf.* ref. 169). PETER AND LEE[170] isolated mitochondria from rat skeletal muscle by a new, improved technique. These particles were able to oxidize palmitate as well. Rat skeletal muscle mitochondria, isolated by orthodox methods, *e.g.* as

described in ref. 117 (Appendix, Paper 1), exhibited similar properties (data not shown). By direct assay PANDE AND MEAD[26], DE JONG AND HÜLSMANN[16] (Appendix, Paper 4) and AAS[28] demonstrated palmitoyl-CoA synthetase activity in mitochondria isolated from heart and skeletal muscle.

It was found independently by PANDE AND BLANCHAER[97] and ourselves[16] (Appendix, Paper 4) that Nagarse destroys palmitoyl-CoA synthetase activity in heart, liver and skeletal muscle. Furthermore, it was shown by BLANCHAER and collaborators (unpublished observations, see refs. 97 and 162) that Nagarse treatment of skeletal muscle mitochondria destroys the capacity to oxidize palmitate. We have made analogous observations for heart[65,82] (Appendix, Papers 5 and 6). Nagarse treatment does not impair hepatic fatty acid oxidation[65,82] (Appendix, Papers 5 and 6), because in rat liver mitochondria palmitate can be activated also in the inner membrane *plus* matrix compartment, contrary to muscle (see Chapter 2).

Palmitoyl-CoA synthetase in heart mitochondria is much less susceptible to Nagarse treatment when the proteinase is added to mitochondria oxidizing palmitate[65] (Appendix, Paper 6). Preincubation of mitochondrial sonicates of heart[65] (Appendix, Paper 6) or liver (Fig. 4) with palmitate, ATP and CoASH protects the palmitoyl-CoA synthetase to some extent against proteolytic action. The protection seems to be more pronounced in the case of heart mitochondria. The stabilizing action of ATP on palmitoyl-CoA synthetase during the fractionation of cells, reported by AAS[28], is possibly due to a similar effect. The presence of endogenous CoASH and palmitate together with added ATP may protect the enzyme against endogenous proteinases. KORNBERG AND PRICER[1] observed that their palmitoyl-CoA synthetase from guinea-pig liver microsomes was inactivated by 80 % by incubation with trypsin at 40° for 30 min.

Nagarse is reported to show a very wide substrate specificity[171]. The enzyme attacks about one third of the pep-

tide bonds in casein and a quarter of those in gelatin. In addition, the enzyme hydrolyzes the ester bond[172,173]. RICHARDS AND VITHAYATHIL[174] have used a subtilopeptidase A to split ribonuclease. It is quite surprising to read that in this case only one peptide bond is attacked, *viz.* between residues 20 and 21, without any loss of activity. Some specificity in inactivating action is also observed in our work with subcellular fractions. Monoamine oxidase, carnitine palmitoyltransferase, cytochrome *c* oxidase, carboxylesterase and glucose-6-phosphatase in isolated mitochondrial and microsomal fractions were relatively insensitive to Nagarse treatment[16,97] (Appendix, Paper 4). The activity of NADPH cytochrome *c* reductase disappeared from the microsomal fraction after proteolysis[16] (Appendix, Paper 4). It was shown by TAKESUE AND OMURA[175] that this was not due to inactivation, as the NADPH cytochrome *c* reductase was solubilized by

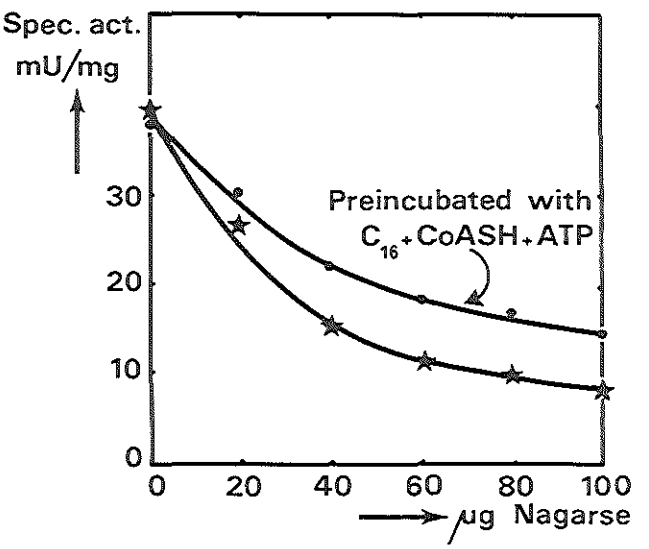


Fig. 4. Inactivation by Nagarse of palmitoyl-CoA synthetase in a rat liver mitochondrial sonicate. Preincubation of 1.0 mg mitochondrial protein was carried out in 0.21 M sucrose/0.01 M Tris-HCl (pH 7.4) at room temperature (22°) for 5 min in a volume of 0.24 ml. Where indicated 0.25 mM potassium palmitate/0.03 mM albumin, 1.2 mM ATP and 0.1 mM CoASH were added. The indicated amounts of Nagarse were added in a volume of 10 µl. Proteolysis took place at room temperature for 5 min. Samples of 0.10 ml were assayed for palmitoyl-CoA synthetase activity with the [^{3}H]-carnitine method[16] (Appendix, Paper 4). Incubations were carried out at 37° for 6 min in a volume of 1.0 ml.

the proteinase. Inside the mitochondria enzymes are presumably protected by the outer membrane against proteolysis[176]. However, also in ultrasonically or detergent disrupted mitochondria monoamine oxidase is perfectly able to resist Nagarse attack, as is carnitine palmitoyltransferase (experiments not shown). Very recently, BOND[177] compared the susceptibility of several soluble liver enzymes towards proteolytic attack. She found that arginase activity was increased by treatment with subtilisin (Nagarse?). The arginase activity was then stable for the 2-h incubation period at 37°. Lactate dehydrogenase activity, on the contrary, decayed quite rapidly[177].

We conclude that the vulnerability of enzymes to the action of Nagarse depends on subcellular localization and enzyme structure. From the foregoing it is evident that the Nagarse method for the isolation of sarcosomes is not without pitfalls.

CHAPTER 5

The inhibition of palmitate activation by adenosine

When the myocardial oxygen concentration diminishes (*e.g.* by hypoxia, a lower coronary blood flow or increased oxygen consumption) breakdown of nucleotides to adenosine takes place[178-180]. The mechanism of adenosine production from ATP by the myocardial cell is unknown[181]. During hypoxia cardiac blood flow is elevated presumably due to an adenosine-induced vasodilatation[182-184]. We considered the possibility that adenosine plays a rôle in the regulation of heart palmitoyl-CoA synthetase activity. When the oxygen level in the heart is low, it makes no sense that mitochondria synthesize palmitoyl-CoA. It may even be dangerous. Because β-oxidation in the heart is impaired under these conditions, carbohydrate is catabolized to meet the energy requirements. Palmitoyl-CoA is an effective detergent, which inhibits many enzymes *in vitro*[185-189]. Adenosine seems to be well-suited to control mitochondrial palmitoyl-CoA synthetase activity *in vivo*, because (i) quite low concentrations of adenosine inhibit the enzyme in mitochondrial sonicates; (ii) heart contains a large potential source of this nucleoside; (iii) it is formed in the heart under hypoxic conditions; and (iv) it is easy to get rid of the inhibitor, *e.g.* by deamination, phosphorylation or movement across the cellular membrane.

In sonicates of rat heart mitochondria a K_i of 0.1 mM is observed for the adenosine inhibition of palmitoyl-CoA synthetase[65,82] (Appendix, Papers 5 and 6). The inhibition is of the competitive type with respect to ATP. The specificity of the inhibition has been tested. The presence and the position of the amino group are important for the inhibition: guanosine and inosine are much less potent inhibitors[65,82] (Appendix, Papers 5 and 6). Under the conditions,

specified in these references, 1-methyl adenosine, 8-bromo-adenosine, 6-mercapto guanosine, 6-deoxyguanosine, purine riboside, 6-mercapto purine riboside and 6-methyl aminopurine-9-ribofuranoside inhibit less than 30 % (unpublished observations). Also the ribose moiety is necessary for the inhibition of palmitoyl-CoA synthetase. Omission or modification of the sugar by esterification usually results in loss of inhibitory power: adenine, 2'-deoxyadenosine and adenosine phosphates, including 3',5'-cyclic AMP (refs. 65 and 82; Appendix, Papers 5 and 6), as well as 6-methyl aminopurine, 2',3'-isopropylidene adenosine and 2',3'-diacetyl adenosine (unpublished results) are relatively poor inhibitors. Only the pseudo nucleotide adenosine sulfate is as effective an inhibitor as adenosine[65] (Appendix, Paper 6). Comparable results for the compounds mentioned above, were obtained on liver mitochondrial palmitoyl-CoA synthetase. It is concluded that the inhibition of heart mitochondrial palmitoyl-CoA synthetase by adenosine is quite specific.

The next step of our research was on [^{14}C]palmitate oxidation by isolated heart mitochondria. Adenosine (or adenosine sulfate) was able to inhibit oxygen uptake and $^{14}CO_2$ production in these preparations[65] (Appendix, Paper 6). Pyruvate oxidation, on the contrary, was not impaired on adenosine addition.

Proceeding towards the *in vivo* situation we tested the effect of adenosine on palmitate metabolism in the isolated, perfused rat heart (Langendorff preparation). In control experiments it became clear that adenosine or dipyridamole* [2,6-bis-(diethanolamino)-4,8-dipiperidinopyrimide (5,4-d) pyrimidine] caused an increase in flow rate of 2 - 4 times (Fig. 5). When dipyridamole is added together with adenosine, results are obtained comparable to Expt. 1 (Fig. 5). Dipyridamole (synonyms: Persantin, RA 8) is an inhibitor of adenosine deaminase[193]. It is thought to augment the endog-

*Dipyridamole was a generous gift from Propharma, Haarlem.

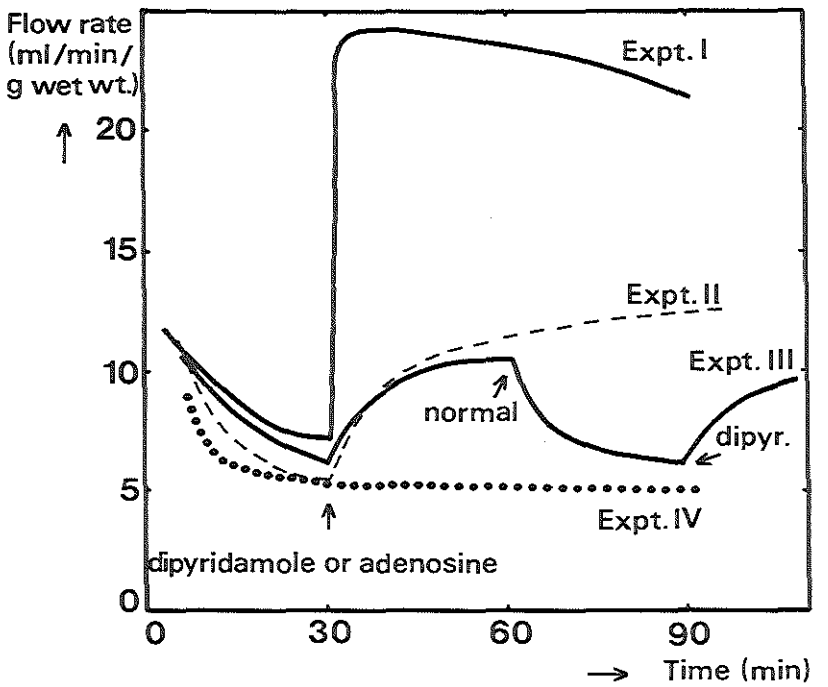


Fig. 5. Influence of adenosine and dipyridamole on the flow rate of the isolated, perfused rat heart. Fed male Wistar rats (220 - 280 g) were anesthetized with about 20 mg pentobarbital (Abbott) and heparinized with 500 I.U. Thromboliquine (Organon). Langendorff preparations, obtained as described by ZIMMERMAN *et al.*[190,191], were perfused at 37° with a Tyrode solution, modified according to MEIJLER[191,192], but containing 5 mM glucose. Use was made of a pace maker, set at 5 pulses/sec, 2 msec duration and 0.2 mA. At the indicated points the perfusion fluid was changed to Meijler's medium containing in addition: 0.5 mM adenosine (Expt. I) or 0.05 mM dipyridamole (Expt. II). In Expt. III the heart was perfused with dipyridamole (0.05 mM) enriched medium and Meijler's fluid ("normal") alternately. Expt. IV represents a control in which no drug was added.

enous adenosine concentration through action on this deaminase[193-196]. Other evidence indicates that dipyridamole inhibits the transport of adenosine across the myocardial membrane[197-199]. If this were true under the conditions of Fig. 5, it should have had a negative effect on the flow rate in the experiment shown, since adenosine formed in the myocardial cells must penetrate to the blood capillaries in order to change the flow rate. The observations on the flow rate are in agreement with those done on the open-chest dog myocardium[182,200-207] and the isolated, perfused heart of

the cat[208] and the guinea pig[182].

The application of the canula into the aorta takes 1.5 - 2 min. The heart is ischemic for this period. This causes accumulation of intracellular adenosine. The initial high flow rate is most probably due to endogenous adenosine that diffused across the cellular membrane. In further experiments a period of 30 min was taken for perfusion with glucose medium (see legend to Fig. 5). The height of contraction and the flow rate of the heart were usually constant after this period. Then perfusion was started with a [^{14}C]palmitate/albumin containing medium in a recirculating system. Palmitate was removed from the perfusion fluid at a rate of 0.5 μmole/min per g myocardial protein (Table III). A comparable value was reported by RODIS *et al.*[212]. Higher rates of extraction were published by CRASS *et al.*[213]. The disappearance of [^{14}C]palmitate from the perfusion fluid was stimulated twofold by the addition of 0.5 mM adenosine

TABLE III

EFFECT OF ADENOSINE ON PALMITATE EXTRACTION AND LACTATE ACCUMULATION BY THE ISOLATED, PERFUSED RAT HEART

Langendorff preparations were perfused as described in the legend to Fig. 5 for 30 min with Meijler's fluid, containing 5 mM glucose. Perfusion was continued in a recirculating system with 50 ml Meijler's medium (5 mM glucose) fortified with 0.5 mM [1-^{14}C]palmitate (0.13 μC/μmole)/0.17 mM dialyzed bovine serum albumin, and, where indicated, 0.5 mM adenosine. Foaming of this mixture, when equilibrated with 95% O_2/5% CO_2, was suppressed by application of a minimal amount of Antifoam A Spray (Dow Corning). The perfusion with palmitate/glucose medium took place for 30 min. Samples (0.5 ml) were taken at 5-min intervals and analyzed for disappearance of radioactive label (corrected for $^{14}CO_2$) and adenosine[209], and the formation of lactate[210] and inosine[211]. Mean values are given, with their S.D. where appropiate, whereas the number of experiments is given in brackets.

	(μmole/min per g myocardial protein)	
	Minus *adenosine*	Plus *adenosine*
$-\Delta$[^{14}C] Palmitate	0.47 ± 0.10 (6)	1.00 ± 0.28 (6)
ΔLactate	0.62 ± 0.43 (4)	2.29 ± 0.48 (6)
-ΔAdenosine	-	1.68 ± 0.32 (5)
ΔInosine	-	1.35 (3)

(Table III). Similarly, in the presence of dipyridamole or adenosine sulfate, which also caused an increase in flow rate, palmitate extraction was increased (not shown). Preliminary experiments indicate that also $^{14}CO_2$ production from palmitate is elevated, when adenosine is present in the perfusion fluid. Therefore, it seems doubtful whether the mitochondrial palmitoyl-CoA synthetase is inhibited by added nucleoside. Exogenous adenosine is unlikely to accumulate in the myocardial cell to a significant extent (*cf.* ref. 214). Adenosine deaminase is an active enzyme in heart[181,215-217] (*cf.* inosine formation, Table III). Furthermore, adenosine kinase rapidly phosphorylates adenosine in cat heart[208,218] and rabbit heart[219]. No net synthesis of adenine nucleotides under our conditions was observed (Table IV), but [8-^{14}C]adenosine was incorporated in the nucleotides (experiment not shown, compare ref. 219). From the drop in creatine phosphate content of the heart, following adenosine administration (Table IV), one could argue that transphosphorylation by the creatine kinase reaction is necessary to keep the ATP concentration at the control level. However, to support such a hypothesis, it would also be necessary to know the effect of adenosine on the myocardial creatine concentration.

TABLE IV

NUCLEOTIDES AND CREATINE PHOSPHATE IN RAT HEART AFTER ADENOSINE PERFUSION

Langendorff preparations were perfused as described in the legend to Table III. After 30 min of recirculation hearts were clamped at liquid nitrogen temperature and deproteinized in the cold with $HClO_4$. In neutralized extracts (*cf.* Table II) ATP[220], ADP[221], AMP[221] and creatine phosphate[222] were determined.

	μmoles/ g myocardial protein	
	Minus *adenosine*	Plus *adenosine*
ATP	18.1 ± 3.4 (5)	16.0 ± 4.2 (7)
ADP	4.2 ± 1.1 (5)	4.5 ± 1.2 (7)
AMP	0.5 ± 0.2 (5)	1.4 ± 0.8 (7)
ΣAdenine nucleotides	23.0 ± 3.8 (5)	22.0 ± 4.0 (7)
Creatine phosphate	30.4 (2)	18.1 ± 1.6 (4)

Recently, WIELAND *et al.*[223] observed with the isolated rat heart, perfused with a glucose-containing salt medium, that the addition of palmitate resulted in an augmented lactate level. It is likely that a stimulation of fatty acid extraction by added adenosine could result in an elevated lactate concentration, as is shown in Table III. WIELAND *et al.*[223,224] have presented evidence for their hypothesis that an increased supply of long-chain fatty acids is responsible for the conversion of active to inactive myocardial pyruvate dehydrogenase, resulting in lactate accumulation.

Is adenosine involved in the regulation of cardiac fatty acid metabolism *in vivo*? In the hypoxic rat heart triglyceride synthesis is increased[225]. Lipid accumulation in the heart also occurs in the clinical counterpart of myocardial hypoxia: myocardial infarction[226]. Obviously, adequate amounts of acyl-CoA can be generated for lipid synthesis under these conditions, where adenosine production is increased. The findings in isolated sarcosomes on the adenosine inhibition of palmitate activation and oxidation, are possibly restricted to the *in vitro* situation. From our findings with the isolated, perfused heart and the data in the literature the conclusion might be drawn that adenosine has probably nothing to do with myocardial palmitoyl-CoA synthetase *in vivo*, because the myocardial concentration of the nucleoside is likely to be always far below the K_i for the inhibition of palmitate activation. More studies are required on the activity and compartmentation of enzymes involved in adenosine and palmitate metabolism, before a definite statement, pertinent to the matter in question, can be made.

References*

1 A. KORNBERG AND W.E. PRICER, JR., *J. Biol. Chem.*, 204 (1953) 329.
2 J. BAR-TANA, G. ROSE AND B. SHAPIRO, *Biochem. J.*, 122 (1971) 353.
3 P. BERG, *J. Biol. Chem.*, 222 (1956) 991.
4 L.T. WEBSTER, JR., AND F. CAMPAGNARI, *J. Biol. Chem.*, 237 (1962) 1050.
5 L.T. WEBSTER, JR., *J. Biol. Chem.*, 238 (1963) 4010.
6 J. BAR-TANA, G. ROSE AND B. SHAPIRO, *Biochem. J.*, 109 (1968) 269.
7 J. BAR-TANA AND G. ROSE, *Biochem. J.*, 109 (1968) 275.
8 J. BAR-TANA AND B. SHAPIRO, *Biochem. J.*, 93 (1964) 533.
9 G.A. RAO AND J.M. JOHNSTON, *Biochem. Biophys. Res. Commun.*, 24 (1966) 696.
10 G.A. RAO AND J.M. JOHNSTON, *Biochim. Biophys. Acta*, 144 (1967) 25.
11 P.W. MAJERUS AND P.R. VAGELOS, *Advan. Lipid Res.*, 5 (1967) 1.
12 B. BORGSTRØM AND L.W. WHEELDON, *Biochim. Biophys. Acta*, 50 (1961) 171.
13 J.R. SENIOR AND K.J. ISSELBACHER, *Biochim. Biophys. Acta*, 44 (1960) 399.
14 J.R. SENIOR AND K.J. ISSELBACHER, *Fed. Proc.*, 20 (1961) 245.
15 J.B. RODGERS, JR., *J. Lipid Res.*, 10 (1969) 427.
16 J.W. DE JONG AND W.C. HÜLSMANN, *Biochim. Biophys. Acta*, 197 (1970) 127.
17 J.B. RODGERS, JR., AND W. BOCHENEK, *Biochim. Biophys. Acta*, 202 (1970) 426.
18 J.B. RODGERS, JR., *Amer. J. Clin. Nutr.*, 23 (1970) 1331.
19 D.N. BRINDLEY AND G. HÜBSCHER, *Biochim. Biophys. Acta*, 125 (1966) 92.
20 G. ROSE AND B. SHAPIRO, *Bull. Res. Counc. Israel*, Sect. A, 9 (1960) 15.
21 A.M. DANIEL AND D. RUBINSTEIN, *Can. J. Biochem.*, 46 (1968) 1039.
22 D.J. GALTON AND T.R. FRASER, *Anal. Biochem.*, 28 (1969) 59.
23 Z. DRAHOTA, A. ALEXANDRE, C.R. ROSSI AND N. SILIPRANDI,

*Title abbreviations of serials are according to rules of the "American Standard for Periodical Title Abbreviations" (see: *List of Serials with Title Abbreviations*, BIOSIS, Philadelphia, Penn., 1970).

Biochim. Biophys. Acta, 205 (1970) 491.
24 K. LIPPEL, A. LLEWELLYN AND L. JARETT, *Biochim. Biophys. Acta*, 231 (1971) 48.
25 D.J. GALTON, *J. Lipid Res.*, 9 (1968) 19.
26 S.V. PANDE AND J.F. MEAD, *Biochim. Biophys. Acta*, 152 (1968) 636.
27 C.W. WARD AND D. FAIRBAIRN, *Develop. Biol.*, 22 (1970) 366.
28 M. AAS, *Biochim. Biophys. Acta*, 231 (1971) 32.
29 B. WITTELS AND R. BRESSLER, *J. Clin. Invest.*, 44 (1965) 1639.
30 H.M. KORCHAK AND E.J. MASORO, *Biochim. Biophys. Acta*, 84 (1964) 750.
31 K.R. NORUM, M. FARSTAD AND J. BREMER, *Biochem. Biophys. Res. Commun.*, 24 (1966) 797.
32 D.W. YATES, D. SHEPHERD AND P.B. GARLAND, *Nature (London)*, 209 (1966) 1213.
33 M. FARSTAD, J. BREMER AND K.R. NORUM, *Biochim. Biophys. Acta*, 132 (1967) 492.
34 S.V. PANDE AND J.F. MEAD, *J. Biol. Chem.*, 243 (1968) 352.
35 A. VAN TOL, J.W. DE JONG AND W.C. HÜLSMANN, *Biochim. Biophys. Acta*, 176 (1969) 414.
36 A. VAN TOL AND W.C. HÜLSMANN, *Biochim. Biophys. Acta*, 189 (1969) 342.
37 A. VAN TOL AND W.C. HÜLSMANN, *Biochim. Biophys. Acta*, 223 (1970) 416.
38 K. LIPPEL, J. ROBINSON AND E.G. TRAMS, *Biochim. Biophys. Acta*, 206 (1970) 173.
39 P.B. GARLAND, D.W. YATES AND B.A. HADDOCK, *Biochem. J.*, 119 (1970) 553.
40 W.A. CREASY, *Biochim. Biophys. Acta*, 64 (1962) 559.
41 W.S. LYNN AND R.H. BROWN, *Arch. Biochem. Biophys.*, 81 (1959) 353.
42 W.H. TRZECIAK, *Acta Biol. Med. Ger.*, 24 (1970) 201.
43 M. FARSTAD AND J. SANDER, *Scand. J. Clin. Lab. Invest.*, Suppl. 118, 27 (1971) 51.
44 E.J. MASSARO AND W.J. LENNARZ, *Biochemistry*, 4 (1965) 85.
45 P. OVERATH, E.M. RANFRESS, W. STOFFEL AND W. ECKER, *Biochem. Biophys. Res. Commun.*, 29 (1967) 28.
46 P. OVERATH, G. PAULI AND H.U. SCHAIRER, *Eur. J. Biochem.*, 7 (1969) 559.
47 G. WEEKS, M. SHAPIRO, R.O. BURNS AND S.J. WAKIL, *J. Bacteriol.*, 97 (1969) 827.
48 D. SAMUEL, J. ESTROUMZA AND G. AILHAUD, *Eur. J. Biochem.*, 12 (1970) 576.
49 D. SAMUEL AND G. AILHAUD, *FEBS Lett.*, 2 (1969) 213.
50 J.M. LEBEAULT, B. ROCHE, Z. DUVNJAK AND E. AZOULAY, *Antonie van Leeuwenhoek J. Microbiol. Serol.*, 35 (1969) F33.
51 J.M. LEBEAULT, B. ROCHE, Z. DUVNJAK AND E. AZOULAY, *Arch. Mikrobiol.*, 72 (1970) 140.
52 Z. DUVNJAK, J.M. LEBEAULT, B. ROCHE AND E. AZOULAY, *Biochim. Biophys. Acta*, 202 (1970) 447.
53 T. GALLIARD AND P.K. STUMPF, *Biochem. Prep.*, 12 (1968)

66.
54 T.G. COOPER AND H. BEEVERS, *J. Biol. Chem.*, 244 (1969) 3514.
55 T.G. COOPER AND H. BEEVERS, *Fed. Proc.*, 29 (1970) 868 Abs.
56 T.G. COOPER, *J. Biol. Chem.*, 246 (1971) 3451.
57 E. STEVENSON, *Biochem. J.*, 110 (1968) 105.
58 S.V. PANDE AND M.C. BLANCHAER, *Amer. J. Physiol.*, 220 (1971) 549.
59 K.R. NORUM, *Acta Physiol. Scand.*, 66 (1966) 172.
60 O. WIELAND, D. REINWEIN AND F. LYNEN, in G. POPJAK AND E. LE BRETON, *Biochemical Problems of Lipids*, Butterworth, London, 1956, p. 155.
61 M. FARSTAD, *Biochim. Biophys. Acta*, 146 (1967) 272.
62 M. FARSTAD, *Acta Physiol. Scand.*, 74 (1968) 568.
63 J. BREMER, in F.C. GRAN, *Cellular Compartmentalization and Control of Fatty Acid Metabolism*, Universitetsforlaget, Oslo, 1968, p. 65.
64 L. HAREL, E. MENDOZA AND E. LE BRETON, in P. DESNUELLE, *The Enzymes of Lipid Metabolism*, Pergamon Press, London, 1961, p. 60.
65 J.W. DE JONG, *Biochim. Biophys. Acta*, 245 (1971) 288.
66 M. AAS, *Biochim. Biophys. Acta*, 202 (1970) 250.
67 M. AAS, *Abstr. Meet. Fed. Eur. Biochem. Soc.*, (1969) 204.
68 M. SACCHETTO AND C.R. ROSSI, *Experientia (Basel)*, 15 (1959) 28.
69 C.R. ROSSI AND M. SACCHETTO, *Experientia (Basel)*, 15 (1959) 64.
70 M. SACCHETTO AND C.R. ROSSI, *Experientia (Basel)*, 15 (1959) 65.
71 C.R. ROSSI AND D.M. GIBSON, *J. Biol. Chem.*, 239 (1964) 1694.
72 C.R. ROSSI, L. GALZIGNA AND D.M. GIBSON, *BBA Libr.*, 7 (1966) 143.
73 D.W. ALLMANN, L. GALZIGNA, R.E. MCCAMAN AND D.E. GREEN, *Arch. Biochem. Biophys.*, 117 (1966) 413.
74 C.R. ROSSI, L. GALZIGNA, A. ALEXANDRE AND D.M. GIBSON, *J. Biol. Chem.*, 242 (1967) 2102.
75 L. GALZIGNA, C.R. ROSSI, L. SARTORELLI AND D.M. GIBSON, *J. Biol. Chem.*, 242 (1967) 2111.
76 L. SARTORELLI, L. GALZIGNA, C.R. ROSSI AND D.M. GIBSON, *Biochem. Biophys. Res. Commun.*, 26 (1967) 90.
77 C.R. ROSSI, A. ALEXANDRE AND L. SARTORELLI, *Eur. J. Biochem.*, 4 (1968) 31.
78 L. GALZIGNA, L. SARTORELLI, C.R. ROSSI AND D.M. GIBSON, *Lipids*, 4 (1969) 459.
79 C.R. ROSSI, A. ALEXANDRE, L. GALZIGNA, L. SARTORELLI AND D.M. GIBSON, *J. Biol. Chem.*, 245 (1970) 3110.
80 D.S. BEATTIE AND R.E. BASFORD, *J. Biol. Chem.*, 241 (1966) 1412.
81 K. LIPPEL AND D.S. BEATTIE, *Biochim. Biophys. Acta*, 218 (1970) 227.
82 J.W. DE JONG AND W.C. HÜLSMANN, *Biochim. Biophys. Acta*,

210 (1970) 499.
83 S.G. VAN DEN BERGH, *Methods Enzymol.*, 10 (1967) 749.
84 K.J. HITTELMAN, O. LINDBERG AND B. CANNON, *Eur. J. Biochem.*, 11 (1969) 183.
85 G. AILHAUD, D. SAMUEL AND P. DESNUELLE, *Biochim. Biophys. Acta*, 67 (1963) 150.
86 I.B. FRITZ, *Physiol. Rev.*, 41 (1961) 52.
87 I.B. FRITZ AND K.T.N. YUE, *J. Lipid Res.*, 4 (1963) 279.
88 I.B. FRITZ, *Advan. Lipid Res.*, 1 (1963) 285.
89 J.B. CHAPPELL AND A.R. CROFTS, *Biochem. J.*, 95 (1965) 707.
90 D.W. YATES AND P.B. GARLAND, *Biochem. Biophys. Res. Commun.*, 23 (1966) 460.
91 S.G. VAN DEN BERGH, in E. QUAGLIARIELLO, S. PAPA, E.C. SLATER AND J.M. TAGER, *Mitochondrial Structure and Compartmentation*, Adriatica Editrice, Bari, 1967, p. 400.
92 G. DELISLE AND I.B. FRITZ, *Proc. Nat. Acad. Sci. U.S.A.*, 58 (1967) 790.
93 I.B. FRITZ, *Perspect. Biol. Med.*, 10 (1967) 643.
94 A. ALEXANDRE, C.R. ROSSI, L. SARTORELLI AND N. SILIPRANDI, *FEBS Lett.*, 3 (1969) 279.
95 S. SKREDE AND J. BREMER, *Eur. J. Biochem.*, 14 (1970) 465.
96 J.W. DE JONG, W.C. HÜLSMANN AND A.J. MEIJER, *Biochim. Biophys. Acta*, 184 (1969) 664.
97 S.V. PANDE AND M.C. BLANCHAER, *Biochim. Biophys. Acta*, 202 (1970) 43.
98 P.M. VIGNAIS, C.H. GALLAGHER AND I. ZABIN, *J. Neurochem.*, 2 (1958) 283.
99 K. LIPPEL, D.R. MANYAN AND A. LLEWELLYN, *Arch. Biochem. Biophys.*, 139 (1970) 421.
100 B.A. HADDOCK, D.W. YATES AND P.B. GARLAND, *Biochem. J.*, 119 (1970) 565.
101 G. AILHAUD, L. SARDA AND P. DESNUELLE, *Biochim. Biophys. Acta*, 59 (1962) 261.
102 M. SINGH, V.N. SINGH AND T.A. VENKITASUBRAMANIAN, *Biochim. Biophys. Acta*, 218 (1970) 183.
103 M. AAS AND L.N.W. DAAE, *Biochim. Biophys. Acta*, 239 (1971) 208.
104 B. WITTELS AND R. BRESSLER, *J. Clin. Invest.*, 44 (1965) 1113.
105 H.A.M. HULSMANS, *Verdeling van enzymactiviteiten bij fractionering van hartspierweefsel*, M.D. Thesis, Klein Offset Drukkerij Poortpers, Amsterdam, 1960.
106 H.A.M. HULSMANS, *Biochim. Biophys. Acta*, 54 (1961) 1.
107 E. ZEBE, *Z. Vergl. Physiol.*, 36 (1954) 290.
108 C.M. SCHILLER, J.S.K. DAVID AND J.M. JOHNSTON, *Biochim. Biophys. Acta*, 210 (1970) 489.
109 S.G. VAN DEN BERGH, *Biochim. Biophys. Acta*, 98 (1965) 442.
110 I.B. FRITZ AND N.R. MARQUIS, *Proc. Nat. Acad. Sci. U.S.A.*, 54 (1965) 1226
111 S.G. VAN DEN BERGH, C.P. MODDER, J.H.M. SOUVERIJN AND H. C.J.M. PIERROT, in L. ERNSTER AND Z. DRAHOTA, *Mitochon-*

dria, Structure and Function, Academic Press, London, 1969, p. 137.
112 P.K. TUBBS AND P.B. GARLAND, *Brit. Med. Bull.*, 24 (1968) 158.
113 D.S. BEATTIE, *Biochem. Biophys. Res. Commun.*, 30 (1968) 57.
114 W. STOFFEL AND H.-G. SCHIEFER, *Hoppe-Seyler's Z. Physiol. Chem.*, 349 (1968) 1017.
115 M. WAITE, P. SISSON AND E. BLACKWELL, *Biochemistry*, 9 (1970) 746.
116 L.N.W. DAAE AND J. BREMER, *Biochim. Biophys. Acta*, 210 (1970) 92.
117 W.C. HÜLSMANN, J.W. DE JONG AND A. VAN TOL, *Biochim. Biophys. Acta*, 162 (1968) 292.
118 Y. HATEFI AND F. FAKOUHI, *Arch. Biochem. Biophys.*, 125 (1968) 114.
119 J.W. DE JONG, A. VAN TOL AND W.C. HÜLSMANN, *Abstr. Meet. Fed. Eur. Biochem. Soc.*, (1969) 240.
120 D.E. GREEN, D.S. GOLDMAN, S. MII AND H. BEINERT, *J. Biol. Chem.*, 202 (1953) 137.
121 S.G. VAN DEN BERGH, *BBA Libr.*, 7 (1966) 125.
122 H. HOFFMANN AND S.G. VAN DEN BERGH, *Biochem. J.*, 116 (1970) 33P.
123 J. MELLANBY AND D.H. WILLIAMSON, in H.-U. BERGMEYER, *Methoden der enzymatischen Analyse*, Verlag Chemie, Weinheim, 1962, p. 454.
124 H.-U. BERGMEYER AND E. BERNT, in H.-U. BERGMEYER, *Methoden der enzymatischen Analyse*, Verlag Chemie, Weinheim, 1962, p. 324.
125 J.B. CHAPPELL AND A.R. CROFTS, *BBA Libr.*, 7 (1966) 293.
126 J.B. CHAPPELL AND K.N. HAARHOFF, in E.C. SLATER, Z. KANIUGA AND L. WOJTCZAK, *Biochemistry of Mitochondria*, Academic Press, London, 1966, p. 75.
127 A.J. MEIJER, *Anion translocation in mitochondria*, Ph.D. Thesis, Academic Service, Amsterdam, 1971.
128 M. KLINGENBERG, *FEBS Lett.*, 6 (1970) 145.
129 W.C. HÜLSMANN, *Over het mechanisme van de ademhalingsketen-phosphorylering*, M.D. Thesis, Klein Offset Drukkerij Poortpers, Amsterdam, 1958, p. 37.
130 J.K. POLLAK AND E.A. MUNN, *Biochem. J.*, 117 (1970) 913.
131 F.A. HOLTON, W.C. HÜLSMANN, D.K. MYERS AND E.C. SLATER, *Biochem. J.*, 67 (1957) 579.
132 A.B. NOVIKOFF AND W.Y. SHIN, *J. Microsc. (Paris)*, 3 (1964) 187.
133 V. KUBIŠTA, J. KUBIŠTOVÁ AND D. PETTE, *Eur. J. Biochem.*, 18 (1971) 553.
134 D. PETTE, *BBA Libr.*, 7 (1966) 28.
135 D. PETTE AND H. BRANDAU, *Enzymol. Biol. Clin.*, 6 (1966) 79.
136 W.C. HÜLSMANN, A.E.F.H. MEIJER, J. BETHLEM AND G.K. VAN WIJNGAARDEN, *Excerpta Med. Int. Congr. Ser.*, No. 199 (1969) 319.
137 W.C. HÜLSMANN, *Biochem. J.*, 116 (1970) 32P.

138 J.K. POLLAK AND M. WOOG, *Biochem. J.*, 123 (1971) 347.
139 J.J. CH'IH AND T.M. DEVLIN, *Biochem. Biophys. Res. Commun.*, 43 (1971) 962.
140 M. OTTESEN AND I. SVENDSEN, *Methods Enzymol.*, 19 (1970) 199.
141 B. CHANCE AND B. HAGIHARA, *Biochem. Biophys. Res. Commun.*, 3 (1960) 1.
142 B. CHANCE AND B. HAGIHARA, *Proc. Int. Congr. Biochem.*, 5 (1961) 3.
143 Y. HATEFI, P. JURTSHUK AND A.G. HAAVIK, *Arch. Biochem. Biophys.*, 94 (1961) 148.
144 L. SZARKOWSKA AND M. KLINGENBERG, *Biochem. Z.*, 338 (1963) 674.
145 C. BODE AND M. KLINGENBERG, *Biochem. Z.*, 341 (1965) 271.
146 E.J. DAVIS, *Biochim. Biophys. Acta*, 96 (1965) 217.
147 B. SOBEL, E. JEQUIER, A. SJOERDSMA AND W. LOVENBERG, *Circ. Res.*, 19 (1966) 1050.
148 D.D. TYLER AND J. GONZE, *Methods Enzymol.*, 10 (1967) 75.
149 G.P. BRIERLEY, *J. Biol. Chem.*, 242 (1967) 1115.
150 A.F. WHEREAT, F.E. HULL AND M.W. ORISHIMO, *J. Biol. Chem.*, 242 (1967) 4013.
151 J.B. WARSHAW, *J. Cell Biol.*, 41 (1969) 651.
152 K.J. KAKO, *Can. J. Biochem.*, 47 (1969) 611.
153 I.B. FRITZ AND R.E. BEYER, *J. Biol. Chem.*, 244 (1969) 3075.
154 J.B. WARSHAW AND M.L. TERRY, *J. Cell Biol.*, 44 (1970) 354.
155 J.B. WARSHAW AND R.E. KIMURA, *Biochem. Biophys. Res. Commun.*, 38 (1970) 58.
156 G. BULLOCK, E.C. CARTER AND A.M. WHITE, *FEBS Lett.*, 8 (1970) 109.
157 A.F. WILLEBRANDS, H.F. TER WELLE AND S.J.A. TASSERON, *Clin. Chim. Acta*, 32 (1971) 251.
158 S.V. PANDE AND M.C. BLANCHAER, *J. Biol. Chem.*, 246 (1971) 402.
159 M.W. MAKINEN AND C.-P. LEE, *Arch. Biochem. Biophys.*, 126 (1968) 75.
160 M.Y.A. RIGAULT AND M.C. BLANCHAER, *Can. J. Biochem.*, 48 (1970) 27.
161 C.H. LIN, A.J. HUDSON AND K.P. STRICKLAND, *Can. J. Biochem.*, 48 (1970) 566.
162 B.E. JACOBSON, M.C. BLANCHAER AND K. WROGEMANN, *Can. J. Biochem.*, 48 (1970) 1037.
163 B. KUYLENSTIERNA, D.G. NICHOLLS, S. HOVMÖLLER AND L. ERNSTER, *Eur. J. Biochem.*, 12 (1970) 419.
164 G.E. LINDENMAYER, L.A. SORDAHL AND A. SCHWARTZ, *Circ. Res.*, 23 (1968) 439.
165 C. BODE AND M. KLINGENBERG, *Biochim. Biophys. Acta*, 84 (1964) 93.
166 M. KLINGENBERG, *Ergeb. Physiol. Biol. Chem. Exp. Pharmakol.*, 55 (1964) 131.
167 M. KLINGENBERG AND C. BODE, in G. WOLF, *Recent Research on Carnitine*, MIT Press, Cambridge, Mass., 1965, p. 87.

168 R.J. BING, *Physiol. Rev.*, 45 (1965) 171.
169 G.V. VAHOUNY, S.L. RODIS, E. KOCH AND P. D'AMATO, in W. L. HOLMES, L.A. KARLSON AND R. PAOLETTI, *Drugs Affecting Lipid Metabolism*, Plenum Press, New York, N.Y., 1969, p. 279.
170 J.B. PETER AND L.D. LEE, *Biochem. Biophys. Res. Commun.*, 29 (1967) 430.
171 R.L. HILL, *Advan. Protein Chem.*, 20 (1965) 37.
172 T.E. BARMAN, *Enzyme Handbook*, Vol. 2, Springer-Verlag, Berlin, 1969, p. 631.
173 A.N. GLAZER, *J. Biol. Chem.*, 242 (1967) 433.
174 F.M. RICHARDS AND P.J. VITHAYATHIL, *J. Biol. Chem.*, 234 (1959) 1459.
175 S. TAKESUE AND T. OMURA, *J. Biochem. (Tokyo)*, 67 (1970) 259.
176 W.C. WERKHEISER AND W. BARTLEY, *Biochem. J.*, 66 (1957) 79.
177 J.S. BOND, *Biochem. Biophys. Res. Commun.*, 43 (1971) 333.
178 B. DEUTICKE AND E. GERLACH, *Pflügers Arch. Eur. J. Physiol.*, 292 (1966) 239.
179 E. GERLACH AND B. DEUTICKE, *Klin. Wochenschr.*, 22 (1966) 1307.
180 R.M. BERNE, R. RUBIO, J.G. DOBSON, JR., AND R.R. CURNISH, *Circ. Res.*, Suppl. 1, 28 and 29 (1971) I-115.
181 M.H. MAGUIRE AND S. STEGGALL, *Aus. J. Exp. Biol. Med. Sci.*, 47 (1969) P18.
182 R.M. BERNE, *Amer. J. Physiol.*, 204 (1963) 317.
183 H.G. RICHMAN AND L. WYBORNY, *Amer. J. Physiol.*, 207 (1964) 1139.
184 M. KATORI AND R.M. BERNE, *Circ. Res.*, 19 (1966) 420.
185 K. TAKETA AND P.M. POGELL, *J. Biol. Chem.*, 241 (1966) 720.
186 J. BREMER AND K.R. NORUM, *J. Biol. Chem.*, 242 (1967) 1749.
187 R.C. NORDLIE, T.L. HANSON AND P.T. JOHNS, *J. Biol. Chem.*, 242 (1967) 4144.
188 J.A. DORSEY AND J.W. PORTER, *J. Biol. Chem.*, 243 (1968) 3512.
189 H.R. SCHOLTE, *Het metabolisme van malonyl-CoA in mitochondriën*, Ph.D. Thesis, Mondeel-offsetdrukkerij, Amsterdam, 1970, p. 58.
190 A.N.E. ZIMMERMAN, *Substraat-geïnduceerde contractiliteitsremming en de "calciumparadox" bij het geïsoleerde, doorstroomde rattehart*, M.D. Thesis, Amsterdam, 1965.
191 A.N.E. ZIMMERMAN, W. DAEMS, W.C. HÜLSMANN, J. SNIJDER, E. WISSE AND D. DURRER, *Cardiovasc. Res.*, 1 (1967) 201.
192 F.L. MEIJLER, *Over de mechanische activiteit van het geïsoleerde volgens Langendorff doorstroomde zoogdierhart*, M.D. Thesis, Amsterdam, 1960, p. 16.
193 B. DEUTICKE AND E. GERLACH, *Arch. Pharmakol. Exp. Path. (Naunyn-Schmiedebergs)*, 255 (1966) 107.
194 E. GERLACH AND B. DEUTICKE, *Arzneimittel-Forschung*, 13

(1963) 48.
195 R.D. BUNAG, C.R. DOUGLAS, S. IMAI AND R.M. BERNE, *Fed. Proc.*, 22 (1963) 642.
196 R.D. BUNAG, C.R. DOUGLAS, S. IMAI AND R.M. BERNE, *Circ. Res.*, 15 (1964) 83.
197 W. KÜBLER AND H.J. BRETSCHNEIDER, *Pflügers Arch. Eur. J. Physiol.*, 280 (1964) 141.
198 K. PFLEGER, I. VOLKMER AND N. KOLASSA, *Arzneimittel-Forschung*, 19 (1969) 1972.
199 W. KÜBLER, P.G. SPIECKERMANN AND H.J. BRETSCHNEIDER, *J. Mol. Cell. Cardiol.*, 1 (1970) 23.
200 M.M. WOLF AND R.M. BERNE, *Circ. Res.*, 4 (1956) 343.
201 H.J. BRETSCHNEIDER, A. FRANK, U. BERNARD, K. KOCHSIEK AND F. SCHELER, *Arzneimittel-Forschung*, 9 (1959) 49.
202 T. HOCKERTS AND G. BÖGELMAN, *Arzneimittel-Forschung*, 9 (1959) 47.
203 J.W. WEST, S. BELLET, U.C. MANZOLI AND O.F. MÜLLER, *Circ. Res.*, 10 (1962) 35.
204 R. RUBIO, R.M. BERNE AND M. KATORI, *Amer. J. Physiol.*, 216 (1969) 56.
205 G. RABERGER, O. KRAUPP, W. STÜHLINGER, G. NELL AND J.J. CHIRIKDJIAN, *Pflügers Arch. Eur. J. Physiol.*, 317 (1970) 20.
206 S. AFONSO AND G.S. O'BRIEN, *Amer. J. Physiol.*, 219 (1970) 1672.
207 N. BITTAR AND T.J. PAULY, *Experientia (Basel)*, 27 (1971) 153.
208 M.I. JACOB AND R.M. BERNE, *Amer. J. Physiol.*, 198 (1960) 322.
209 H. MÖLLERING AND H.-U. BERGMEYER, in H.-U. BERGMEYER, *Methoden der enzymatischen Analyse*, Verlag Chemie, Weinheim, 1962, p. 491.
210 H.-J. HOHORST, in H.-U. BERGMEYER, *Methoden der enzymatischen Analyse*, Verlag Chemie, Weinheim, 1962, p. 266.
211 A. CODDINGTON, in H.-U. BERGMEYER, *Methoden der enzymatischen Analyse*, Verlag Chemie, Weinheim, 1962, p. 502.
212 S.L. RODIS, P.H. D'AMATO, E. KOCH AND G.V. VAHOUNY, *Proc. Soc. Exp. Biol. Med.*, 133 (1970) 973.
213 M.F. CRASS, III, E.S. MCCASKILL AND J.C. SHIPP, *Amer. J. Physiol.*, 216 (1969) 1569.
214 R. RUBIO AND R.M. BERNE, *Circ. Res.*, 25 (1969) 407.
215 H.P. BAER, G.I. DRUMMOND AND E.L. DUNCAN, *Mol. Pharmacol.*, 2 (1966) 67.
216 R. BURGER AND J.M. LOWENSTEIN, *J. Biol. Chem.*, 242 (1967) 5281.
217 J. COSTELLO AND T.G. BRADY, *Biochem. J.*, 119 (1970) 6P.
218 M.I. JACOB AND R.M. BERNE, *Proc. Soc. Exp. Biol. Med.*, 107 (1961) 738.
219 M.S. LIU AND H. FEINBERG, *Amer. J. Physiol.*, 220 (1971) 1242.
220 W. LAMPRECHT AND I. TRAUTSCHOLD, in H.-U. BERGMEYER, *Methoden der enzymatischen Analyse*, Verlag Chemie, Wein-

heim, 1962, p. 543.
221 H. ADAM, in H.-U. BERGMEYER, *Methoden der enzymatischen Analyse*, Verlag Chemie, Weinheim, 1962, p. 573.
222 C.F. BOEHRINGER UND SÖHNE, *Biochemica Catalogue*, 1968.
223 O. WIELAND, H. VON FUNCKE AND G. LÖFFLER, *FEBS Lett.*, 15 (1971) 295.
224 O. WIELAND, E. SIESS, F.H. SCHULZE-WETHMAR, H.G. VON FUNCKE AND B. WINTON, *Arch. Biochem. Biophys.*, 143 (1971) 593.
225 J.R. EVANS, *Circ. Res.*, Suppl. 2, 14 and 15 (1964) II-96.
226 L.H. OPIE, *Amer. Heart J.*, 76 (1968) 685.

Summary

1. The specific activity of palmitoyl-CoA synthetase (EC 6.1.2.3) in rat liver mitochondrial or microsomal fractions is approximately 0.1 µmole/min per mg protein (37°).

2. In rat heart palmitoyl-CoA synthetase activity is mainly localized in the sarcosomal fraction, whereas in intestine the enzyme is predominantly localized in the microsomal fraction.

3. There seems to be a correlation between the intracellular localization of the activity of palmitoyl-CoA synthetase and the function of an organ in lipid metabolism.

4. From oxidation studies it is concluded that in rat heart sarcosomes the palmitoyl-CoA synthetase is localized on the outside of the outer membrane. In liver mitochondria an additional locus of palmitoyl-CoA synthesis with a specific activity of some 5 % of the overall mitochondrial palmitoyl-CoA synthetase is present in the inner membrane *plus* matrix compartment. This activity can suffice for palmitate oxidation.

5. Only 3 - 6 % of the activity of acyl-CoA synthetase is found when GTP replaces ATP. It seems doubtful whether the GTP-dependent acyl-CoA synthetase plays an important rôle in fatty acid activation.

6. The inhibition by inorganic phosphate or fluoride can not be used to recognize the operation of a mitochondrial GTP-dependent acyl-CoA synthetase. Also pyruvate or acylcarnitine oxidation is inhibited by these agents. Phosphate addition causes extrusion of citric acid cycle intermediates from rat liver mitochondria. When pyruvate/malate is oxidized in the presence of fluoride, 2-oxoglutarate accumulation can be demonstrated.

7. Different species of mitochondria can be demonstrated in a single tissue. Separation from each other has been accomplished. The use of Nagarse (a subtilopeptidase A; EC 3.4.4.16) in an isolation procedure doubles the yield of mitochondria from muscle. On the basis of several biochemical parameters these mitochondria seem to be qualitatively better than those obtained with conventional homogenization techniques. However, Nagarse destroys palmitoyl-CoA synthetase activity. In sharp contrast, several marker enzymes for subcellular fractions do not loose their activity in the presence of the proteinase. When the substrates for fatty acid activation are added, palmitoyl-CoA synthetase is less susceptible to the Nagarse action.

8. The temperature dependence of rat liver and heart mitochondrial palmitoyl-CoA synthetase has been studied. A Q_{10} of 3 is found, which is quite high for an enzymatic reaction.

9. Adenosine inhibits palmitoyl-CoA synthetase activity in rat liver and heart mitochondrial sonicates. The nucleoside is a competitive inhibitor with respect to ATP with a K_i of 0.1 mM. The specificity of the inhibition is high. Of a variety of analogs only adenosine sulfate equals adenosine as an inhibitor. 2 mM Adenosine or 2 mM adenosine sulfate inhibit palmitate oxidation (in the presence of 2.5 mM ATP) in isolated rat heart mitochondria 60 - 90 %.

10. The flow rate of the isolated, perfused rat heart increases fourfold, when the perfusion fluid is enriched with 0.5 mM adenosine. Under these conditions the rate of palmitate extraction from the medium is doubled. It seems unlikely that the concentration of adenosine in the heart *in vivo* can be increased high enough to inhibit the palmitoyl-CoA synthetase.

Samenvatting

1. De specifieke activiteit van het palmitoyl-CoA synthetase (EC 6.1.2.3) in geïsoleerde rattelever mitochondriën of microsomen bedraagt ca. 0.1 µmole/min per mg eiwit (37°).

2. In rattehart is de activiteit van het palmitoyl-CoA synthetase overwegend gelocaliseerd in de sarcosomen, terwijl het enzym in de darm zich voornamelijk in de microsomen bevindt.

3. Er lijkt een verband te bestaan tussen de activiteit van het palmitoyl-CoA synthetase in de verschillende celfracties en de functie van een bepaald orgaan ten aanzien van de stofwisseling van lipiden.

4. Oxydatie-proeven wijzen uit dat in rattehart en -lever mitochondriën het palmitoyl-CoA synthetase zich aan de buitenkant van het buitenmembraan bevindt. In het binnenmembraan/matrix compartiment van lever mitochondriën bestaat een extra mogelijkheid palmitoyl-CoA te maken. De specifieke activiteit van dit tweede enzym bedraagt ca. 5 % van de waarde van de gehele mitochondriale palmitaat activering. Deze activiteit kan voldoende zijn voor palmitaat verbranding in lever.

5. Slechts 3 - 6 % van de activiteit van het acyl-CoA synthetase resteert als ATP vervangen wordt door GTP. Het is twijfelachtig of het GTP-afhankelijke acyl-CoA synthetase van belang is voor de activering van vetzuren.

6. De remming van de palmitaat oxydatie door anorganisch fosfaat of fluoride kan niet gebruikt worden voor het onderkennen van GTP-afhankelijke acyl-CoA synthetase activiteit in mitochondriën. De verbranding van pyruvaat of acylcarnitine wordt nl. ook geremd door deze stoffen. Door toevoeging van fosfaat aan rattelever mitochondriën vindt uittreding plaats van intermediairen van de citroenzuur cyclus. Als fluoride aanwezig is bij de verbranding van pyruvaat/malaat, hoopt

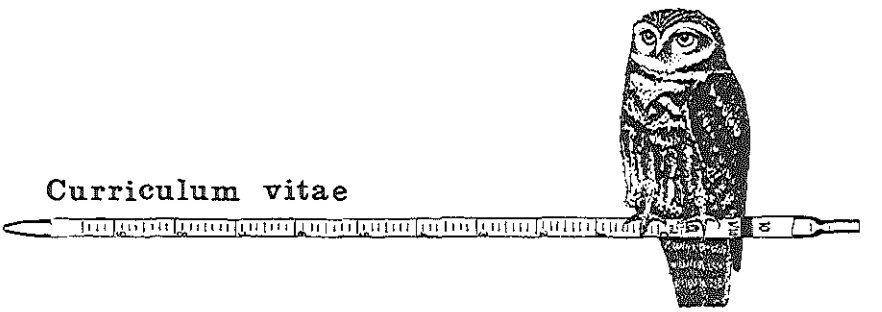
zich 2-ketoglutaraat op.

7. In een weefsel kunnen verschillende soorten mitochondriën aangetoond worden, die van elkaar gescheiden kunnen worden. Door Nagarse (een subtilopeptidase A; EC 3.4.4.16) tijdens de isolatie te gebruiken, verdubbelt de opbrengst aan mitochondriën uit spier. Te oordelen naar diverse biochemische parameters schijnen deze mitochondriën kwalitatief beter te zijn dan mitochondriën die geïsoleerd zijn m.b.v. conventionele technieken. Het is echter zo dat Nagarse de activiteit van het palmitoyl-CoA synthetase vernietigt. In schrille tegenstelling daarmee verliezen een aantal "gids" enzymen, gebruikt om subcellulaire fracties te karakteriseren, hun activiteit niet als ze in aanraking zijn met het proteinase. Als het palmitoyl-CoA synthetase voorzien is van zijn substraten, is het minder gevoelig voor de inwerking van het Nagarse.

8. Het palmitoyl-CoA synthetase in rattelever en -hart mitochondriën blijkt een (betrekkelijk hoge) Q_{10} van 3 te hebben.

9. Adenosine remt de palmitoyl-CoA synthetase activiteit in sonicaten van lever en hart mitochondriën. Het nucleoside remt competitief met betrekking tot ATP. De K_i is 0.1 mM. De specificiteit van de remming is hoog: van een groot aantal analoga van adenosine blijkt alleen adenosine sulfaat even goed te remmen als dit nucleoside. De palmitaat oxydatie door geïsoleerde rattehart mitochondriën, in aanwezigheid van 2,5 mM ATP, wordt door 2 mM adenosine of 2 mM adenosine sulfaat 60 - 90 % geremd.

10. De perfusiesnelheid van het geïsoleerde, doorstroomde rattehart neemt een factor vier toe als 0.5 mM adenosine aan het medium wordt toegevoegd. Palmitaat wordt onder deze omstandigheden tweemaal zo snel door het hart uit het medium geëxtraheerd. Het lijkt onwaarschijnlijk, dat de concentratie van adenosine in het hart *in vivo* dusdanig kan oplopen dat het palmitoyl-CoA synthetase geremd wordt.



Curriculum vitae

De schrijver van dit proefschrift behaalde in 1960 het diploma hogereburgerschool B aan het Christelijk Lyceum Populierstraat te 's-Gravenhage. Hij begon in dat jaar de studie (letter *f*) in de Faculteit der Wiskunde en Natuurwetenschappen van de Rijksuniversiteit te Leiden. In 1964 legde hij het kandidaatsexamen af. Het doctoraal examen met als hoofdvak biochemie en als bijvakken organische scheikunde en klinische chemie volgde twee jaar later. De auteur is sinds 1966 in dienst van de Medische Faculteit te Rotterdam, achtereenvolgens als student-assistent, wetenschappelijk medewerker en wetenschappelijk medewerker I. Op de afdeling Biochemie I heeft hij vanaf 1967 gewerkt aan zijn promotie-onderzoek, dat mede financieel gesteund is door de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.). In 1971 legde hij de ambtseed af voor Controleur-Vogelwet 1936.

Appendix

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Mitochondria with loosely and tightly coupled oxidative phosphorylation in skeletal muscle

From skeletal muscle of humans, suffering from muscle disease, mitochondria can be isolated which sometimes show loosely coupled oxidative phosphorylation, although the patients do not show increased basic metabolic rates[1,2]. This led us to conclude that our procedure of isolating mitochondria causes selection of loosely coupled mitochondria from a mixture of loosely and tightly coupled mitochondria, present in diseased muscle. The subsarcolemmal space of the affected muscle fibres shows the most pronounced morphological changes[1,2]. We concluded therefore that mild fragmentation of the muscle preferentially releases subsarcolemmal mitochondria.

The present paper shows that skeletal muscle from non-diseased white rats, on mild fragmentation, also preferentially releases mitochondria with a more loosely coupled state of oxidative phosphorylation when compared to the mitochondria released after more thorough homogenization of the muscle.

Mitochondria were isolated from 3 g of masseter muscle of rats. The muscles were chopped in two perpendicular directions with a McIlwain tissue slicer (0.1 mm distance between the cuts). The mince was then evenly suspended, by cutting with a pair of scissors, in 30 ml of isolation medium (pH 7.4), containing: 50 mM Tris–HCl, 100 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 1 mM EDTA and 0.5 mg of bovine serum albumin per ml. The mince was stirred for 5 min and part of the mince homogenized

TABLE I

OXIDATIVE PHOSPHORYLATION OF SKELETAL MUSCLE MITOCHONDRIA

The isolation of the mitochondria is described in the text. Oxygen uptake was measured with differential manometers. The reaction medium contained 25 mM glucose, 0.03 mM cytochrome *c*, 0.1 mM L-malate, 2.5 mM $MgCl_2$, 20 mM potassium phosphate buffer, 0.5 mM ATP, 0.5 mM EDTA, 50 mM KCl, 25 mM Tris–HCl buffer, 0.75 mg bovine serum albumin, about 1 mg of mitochondrial protein, and 15 mM glutamate (Expt. 1) or 15 mM pyruvate (Expt. 2). The centre well of the manometer vessels was provided with KOH and a filter paper. The side arm of the flasks contained 1.4 units (μmoles/min) of hexokinase (EC 2.7.1.1). The reaction volume was 1 ml, the temperature 25° and the pH 7.5. Readings were taken at regular intervals. After 20–24 min hexokinase was added from the side arm and after 20 min the reaction was stopped by the addition of perchloric acid (final concn. 4 %). The measurement of oxygen uptake and of phosphorylation, the calculation of the P/O ratio and the respiratory control index (RCI) were carried out as described before[1], except that phosphoglucose isomerase (EC 5.3.1.9) was included in the glucose-6-phosphate assay. Acid phosphatase activity was determined as described in ref. 3.

Expt. No.	*Type of homogenizer used*	Q_{O_2} (+ *hexokinase*)	*RCI*	*P/O*	*Yield of heavy mitochondria (mg protein/g muscle, wet wt.)*	*Relative acid phosphatase activity**
1	Loose	65	3.3	2.5	1.6	0.37
	Tight	87	5.4	2.6	2.5	0.24
2	Loose	98	2.6	2.6	3.4	0.20
	Tight	135	4.5	2.7	6.7	0.11

* For definition and unit see text.

in a loose-fitting homogenizer (5 strokes in a Potter–Elvehjem type homogenizer with a 'Teflon' pestle and a clearance of 0.25 mm (Expt. 1) or 3 strokes in an all glass homogenizer with a clearance of 0.15 mm (Expt. 2) and another part of the mince homogenized (5 strokes in Expt. 1; 20 strokes in Expt. 2) in a tight 'Teflon'-glass homogenizer (clearance, 0.10 mm). Particles sedimenting between 600 × *g* (3 min) and 4500 × *g* (10 min) were isolated by differential centrifugation and suspended in the complete isolation medium. All operations were carried out at 0–4°.

It can be seen from the experiments shown in Table I that the use of a tighter homogenizer not only improves the yield of the isolated muscle mitochondria, but also their quality, when a high respiratory control index may be used as a criterion for the intactness of the function of mitochondria. The release of the small amount of mitochondria by the use of the loose-fitting homogenizer was accompanied by the release of practically all of the β-glucuronidase (EC 3.2.1.31) and acid phosphatase (EC 3.1.3.2) activities from the minced muscle. The latter is reflected (Table I) by the relative acid phosphatase activity, defined as the total activity (in units) in the 600 × *g* supernatant divided by the amount of mitochondrial protein (in mg) isolated from the 600 × *g* supernatant. Since lysosomes are most concentrated in the perinuclear (subsarcolemmal) region, these findings indicate that mild fragmentation of muscle predominantly releases subsarcolemmal mitochondria.

Whether these mitochondria have a more loosely coupled oxidative phosphorylation due to aging[4], a process to which lysosomal activity could contribute[5], or whether the tightness of the coupling of oxidative phosphorylation is another difference between the various types of skeletal muscle mitochondria which can be distinguished (*cf.* ref. 6), still remains to be investigated.

Finally it may be of interest to note that SCHMALBRUCH[7] recently published that fibers of human larynx muscles show lateral dilatations of the sarcolemma, containing loosely disposed nuclei, lysosomes, lipofuscingranules and mitochondria, often with cristae arranged concentrically.

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1 G. K. VAN WIJNGAARDEN, J. BETHLEM, A. E. F. H. MEIJER, W. C. HULSMANN AND A. FELTKAMP, *Brain*, 90 (1967) 577.
2 W. C. HULSMANN, J. BETHLEM, A. E. F. H. MEIJER, P. FLEURY AND J. P. M. SCHELLENS, *J. Neurol. Neurosurg. Psychiat.*, 30 (1967) 519.
3 K. LINHARDT AND K. WALTER, in H. U. BERGMEIJER, *Methoden der Enzymatischen Analyse*, Verlag Chemie, Weinheim, 1962, p. 783.
4 C. R. ROSSI, L. SARTORELLI, L. TATO, L. BARETTA AND N. SILIPRANDI, *Arch. Biochem. Biophys.*, 118 (1967) 210.
5 A. MELLORS, A. L. TAPPEL, P. L. SAWANT AND I. D. DESAI, *Biochim. Biophys. Acta*, 143 (1967) 299.
6 D. PETTE, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, Elsevier, Amsterdam, 1966, p. 28.
7 H. SCHMALBRUCH, *Z. Zellforsch.*, 85 (1968) 343.

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SHORT COMMUNICATIONS

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On fatty acid activation in rat liver mitochondria

Two systems for the activation of fatty acids in liver mitochondria have been described, one catalyzed by ATP-dependent fatty-acid-activating enzymes[1] (acid: CoA ligase (AMP), EC 6.2.1.2 and EC 6.2.1.3) and a GTP-linked fatty-acid-activating system, discovered by ROSSI AND GIBSON[2].

The purpose of the present investigation was to evaluate the relative contributions of the two fatty-acid-activating systems. Rat liver mitochondria, isolated in 0.25 M sucrose, were suspended in 0.1 M Tris–HCl (pH 7.4), containing 2 mM reduced glutathione and 1 mM EDTA, prior to sonication for 60 sec at 3 A (Branson, Model S75) ar 0–4°. The sonicate was centrifuged for 10 min at 9500 × *g*, and the pellet was discarded. The supernatant was passed through a Sephadex G-25 column equilibrated with 0.1 M KCl before being tested for its ability to activate palmitate, oleate or octanoate.

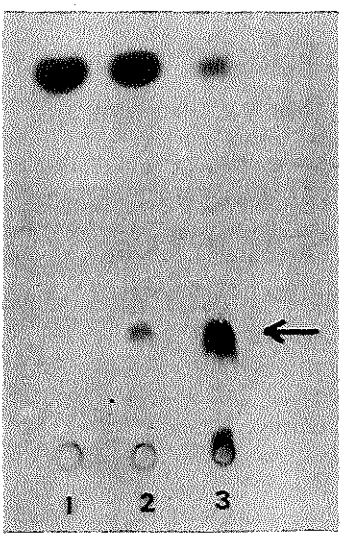

Fig. 1. Autoradiographic demonstration of palmitate activation. The reaction medium contained 100 mM Tris–HCL, 0.25 mM [1-^{14}C]palmitic acid (specific activity, 0.2 μC/μmole) complexed with 0.035 mM bovine serum albumin, 3 μg oligomycin, 10 mM DL-carnitine, 0.5 mM CoA, 2 mM $MgCl_2$, 30 mM KCl, 0.32 mg of purified palmitoyl-CoA:carnitine palmitoyltransferase and 0.2 mg of mitochondrial protein. The enzyme fractions were prepared as described in the text. Additions were in (2) 2 mM GTP and in (3) 2 mM ATP. The reaction volume was 1 ml, the temperature 37°, and the pH 7.4. After 10 min of incubation the reaction was stopped with HCl and the incubation medium extracted with *n*-butanol, as described by FARSTAD, BREMER AND NORUM[3]. 0.05 ml of each extract was applied to a thin-layer plate (silica gel). The chromatogram was developed with chloroform–methanol–water (70:30:5, v/v/v). Reference samples of [^{14}C]palmitate, [^{14}C]palmitoylcarnitine and [^{14}C]palmitoyl-CoA were chromatographed in the same way (not shown), indicating that the thick spots at the upper end of the autoradiogram are palmitate, those indicated by the arrow palmitoylcarnitine, and those just above the starting points palmitoyl-CoA. Agfa-Gevaert-Osray film was used (exposure time, 17 h).

The acyl-CoA formed was trapped as acylcarnitine by acyl-CoA:carnitine transferase, which is present in the sonicate. Addition of purified palmitoyl-CoA:carnitine palmitoyltransferase, prepared as described by FARSTAD *et al.*[3], enhanced the amount of acylcarnitine formed by about 50%, so that it was added routinely.

It can be seen from Fig. 1 that the addition of GTP or ATP is required to activate palmitic acid, as judged by the formation of [^{14}C]palmitoyl-CoA and [^{14}C]-palmitoylcarnitine, detected by autoradiography of thin-layer chromatograms. It can also be seen that ATP is a much more efficient source of energy than GTP. By scraping the radioactive materials from the thin-layer plates and measuring the radioactivity by liquid scintillation spectrophotometry, it was calculated that the specific activity (μmoles/mg protein per h) of the ATP-dependent reaction was 2.9 and that of the GTP-dependent reaction was 0.1.

When in other experiments palmitate was replaced by octanoate or oleate, the "GTP-system" had 3–6% of the activity of the "ATP-system".

Reports from the literature[4,5], on the other hand, suggested to us that in intact liver mitochondria, the "GTP-system" is about as active as the "ATP-system". In these studies fatty acids are oxidized by intact rat liver mitochondria in the presence of dinitrophenol and the absence of (inhibitory) P_i. The addition of dinitrophenol is required to stimulate the citric acid cycle by which, *via* oxidation of α-oxoglutarate, GTP can be generated. Under these conditions ATP is also formed[6]. Moreover the

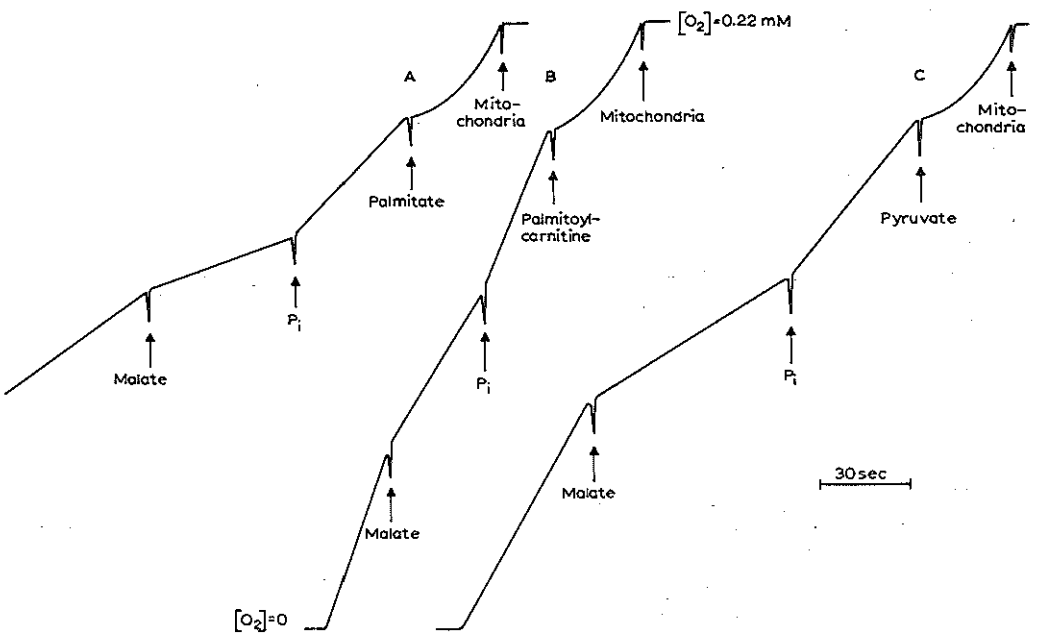

Fig. 2. Palmitate, palmitoylcarnitine and pyruvate oxidation, as measured with the Clark "oxygen electrode". The reaction medium contained 32 mM KCl, 4.5 mM $MgCl_2$, 73 mM Tris–HCl, 1 mM EDTA, 0.1 mM dinitrophenol, 12 mM sucrose and 4.4 mg of mitochondrial protein. 0.12 mM palmitic acid complexed with 0.015 mM bovine serum albumin, 0.24 mM DL-palmitoylcarnitine and 5 mM pyruvate were added as indicated in Expts. A, B and C, respectively. Other additions were, where indicated, 17 mM potassium phosphate and 4.5 mM DL-malate. The reaction volume was 2.2 ml, the temperature 37°, and the pH 7.5.

ADP present in the reaction medium[5] contributes to the formation of ATP through the myokinase reaction[6].

In the presence of dinitrophenol then, the "ATP-system" for fatty acid activation might still operate in addition to the "GTP-system". The inhibition of fatty acid oxidation by P_i in the presence of dinitrophenol, used as an argument in favor of the operation of the GTP-dependent fatty acid activation[4,5] in intact mitochondria, will therefore be analyzed further.

It can be seen from Fig. 2A that we can confirm the inhibitory action of P_i on palmitate oxidation in the presence of dinitrophenol. It may also be noted, however, that malate partially relieves the inhibition. This indicated to us that P_i at least partially inhibits the citric acid cycle. In agreement with this interpretation is the observation that substrates which do not need activation prior to oxidation, such as palmitoylcarnitine (Fig. 2B) or pyruvate (Fig. 2C), also show P_i-inhibited respiration which can be overcome by malate.

It may be concluded, then, that P_i inhibition of fatty acid oxidation does not necessarily mean the operation of the GTP-dependent fatty-acid-activation system in intact mitochondria.

We do not conclude from our data obtained with sonicated mitochondria (Fig. 1) that the "GTP-system" does not contribute significantly to fatty acid activation, since preferential inactivation of the "GTP-system" during isolation cannot be excluded. The "ATP-system", however, is so active (2.9 μmoles/mg protein per h) that in intact mitochondria, palmitate oxidation with a Q_{O_2} of 1478 could be expected if the activation reaction were rate limiting (in practice we never found Q_{O_2}'s for palmitoylcarnitine oxidation exceeding 150).

Future experiments will be required to elucidate the role of substrate-linked phosphorylation in fatty acid oxidation.

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1 H. R. MAHLER, S. J. WAKIL AND R. M. BOCK, *J. Biol. Chem.*, 204 (1953) 453.
2 C. R. ROSSI AND D. M. GIBSON, *J. Biol. Chem.*, 239 (1964) 1694.
3 M. FARSTAD, J. BREMER AND K. R. NORUM, *Biochim. Biophys. Acta*, 132 (1967) 492.
4 L. GALZIGNA, C. R. ROSSI, L. SARTORELLI AND D. M. GIBSON, *J. Biol. Chem.*, 242 (1967) 2111.
5 S. G. VAN DEN BERGH, *Biochim. Biophys. Acta*, 98 (1965) 442.
6 H. W. HELDT AND K. SCHWALBACH, *European J. Biochem.*, 1 (1967) 199.

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Phosphate-induced loss of citric acid cycle intermediates from rat-liver mitochondria

In previous reports[1,2] it was shown that the inhibition by P_i of palmitate oxidation by rat-liver mitochondria in the presence of 2,4-dinitrophenol is not specific for fatty acid oxidation. The oxidation of acylcarnitine and pyruvate is also impaired when P_i is present (Fig. 1A and ref. 1). If malate was added no inhibition could be observed. VAN DEN BERGH[3] earlier observed that malate prevents to some extent the inhibitory action of P_i on fatty acid oxidation by rat-liver mitochondria in the presence of 2,4-dinitrophenol.

The inhibition by P_i of fatty acid oxidation has been interpreted[4,5] as being diagnostic for the operation of the GTP pathway of fatty acid activation. However, from the results of our experiments we suggested that the P_i inhibition could be due to the removal, under the influence of P_i, of citric acid cycle intermediates from the mitochondria.

Such a P_i-induced removal would be expected to occur if P_i addition would allow the synthesis of phosphoenolpyruvate from oxaloacetate and GTP[6]. However, phosphoenolpyruvate formation in rat-liver mitochondria under the conditions of the assay is rather sluggish[6], whereas P_i inhibition of pyruvate, acylcarnitine or fatty acid oxidation sets in rapidly[1]. Moreover, when P_i is replaced by arsenate, during pyruvate oxidation, an inhibition is also observed, which can be overcome by malate addition (Fig. 1B). From these considerations the rapid loss of citric acid cycle intermediates by the formation of phosphoenolpyruvate becomes an unattractive hypothesis. Alternatively, the results obtained could be explained by an exchange of citric acid cycle intermediates with P_i (see refs. 7 and 8).

In the experiment of Table I, rat-liver mitochondria were incubated in the presence of 2,4-dinitrophenol, with or without pyruvate and P_i. The amounts of citrate and malate inside and outside the mitochondria were determined after 55 sec. The presence of P_i in the reaction mixture brought about an increase in the amount of citrate and malate outside the mitochondria. The total amount of citrate *plus* malate

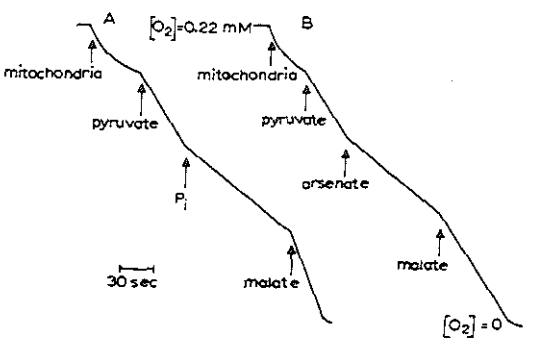

Fig. 1. The effect of P_i, arsenate and malate on pyruvate oxidation in the presence of 2,4-dinitrophenol in rat-liver mitochondria. Oxygen uptake was measured with the Clark "oxygen electrode" at 37°. The reaction medium contained 0.1 mM 2,4-dinitrophenol, 29 mM KCl, 4.8 mM $MgCl_2$, 77 mM Tris–HCl (pH 7.4) and 1 mM EDTA. Rat-liver mitochondria (1.6 mg protein), 2.4 mM sodium pyruvate, 14 mM potassium phosphate (Expt. A) or potassium arsenate (Expt. B), and 2.4 mM L-malate were added as indicated. The final volume was 2.1 ml.

was higher in the presence of P_i than in its absence, indicating withdrawal of these substances from intramitochondrial reactions. The fact that no malate could be detected in the mitochondria even in the absence of P_i indicates that it is rapidly converted to other tricarboxylic acid cycle intermediates when oxidation is allowed to occur. In freshly prepared, anaerobic mitochondria, considerable amounts of malate are present.

The inhibition by P_i of pyruvate oxidation can also occur in the absence of 2,4-dinitrophenol, if the P_i concentration is increased beyond that necessary for State 3 oxidation. Fig. 2 shows that increasing the P_i concentration from 1 to 18 mM resulted in a 25% inhibition of pyruvate oxidation. On the contrary when 1 mM L-malate was present from the beginning, the inhibitory action of excess P_i on

TABLE I

LOSS OF CITRATE AND MALATE FROM RAT-LIVER MITOCHONDRIA ON THE ADDITION OF P_i

Rat-liver mitochondria (5.4 mg protein) were incubated at 37° in the reaction medium mentioned in Fig. 1. Also present were 20 mM sucrose, and, where indicated, 2.4 mM sodium pyruvate and 16 mM P_i. The final volume was 1.22 ml. After 55 sec, 0.8 ml of the incubation medium was layered on silicon oil[9] (which floated on 15% $HClO_4$) and was centrifuged for 1 min at full speed in an Eppendorf "microfuge". 0.5 ml of the supernatant was immediately deproteinized. Citrate was determined with citrate lyase, NADH and malate dehydrogenase (*cf.* ref. 10), and malate with malate dehydrogenase, NAD^+, citrate synthase (see ref. 11) and acetyl-CoA, using the Chance-Aminco dual wavelength spectrophotometer.

Additions	*nmoles/mg protein*			
	Citrate in	*Citrate out*	*Malate in*	*Malate out*
None	0.7	1.3	0.0	1.7
P_i	0.2	4.2	0.0	3.7
Pyruvate	0.9	3.7	0.0	0.9
Pyruvate + P_i	0.2	8.8	0.0	1.7

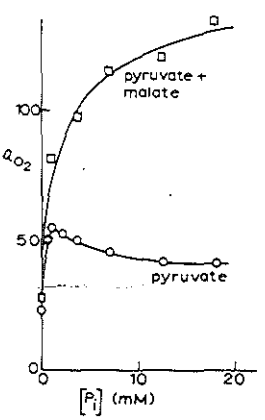

Fig. 2. Effect of P_i on pyruvate oxidation in coupled rat-liver mitochondria. Conditions as in Fig. 1, except that 0.85 mg mitochondrial protein was present. In addition, 1.2 mM ADP, and, where shown in the figure, L-malate (1 mM) and potassium phosphate were added. When less than 18 mM P_i was present, Tris buffer was added to compensate for the change in osmolarity. 60 sec after the addition of the mitochondria, 4.8 mM sodium pyruvate was added. Q_{O_2} values are expressed as μl O_2 per mg protein per h.

pyruvate oxidation was not seen. This suggests that also in coupled mitochondria excess P_i can result in inhibition of pyruvate oxidation, when the malate supply in the extramitochondrial space is limited.

The concentration of P_i in the mitochondrial and extramitochondrial compartment varies *in vivo*, depending on the conditions. The observations reported in this paper suggest that a P_i-induced exchange of citric acid cycle intermediates may play an important role in the regulation of metabolism *in vivo*.

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1 A. VAN TOL, J. W. DE JONG AND W. C. HÜLSMANN, *Biochim. Biophys. Acta*, 176 (1969) 414.
2 J. W. DE JONG, A. VAN TOL AND W. C. HÜLSMANN, *Abstr. 6th Meeting Federation European Biochem. Socs.*, Spanish Biochem. Soc., Madrid, 1969, p. 240.
3 S. G. VAN DEN BERGH, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, BBA Library, Vol. 7, Elsevier, Amsterdam, 1966, p. 125.
4 L. GALZIGNA, C. R. ROSSI, L. SARTORELLI AND D. M. GIBSON, *J. Biol. Chem.*, 242 (1967) 2111.
5 S. G. VAN DEN BERGH, *Biochim. Biophys. Acta*, 98 (1965) 442.
6 H. R. SCHOLTE AND J. M. TAGER, *Biochim. Biophys. Acta*, 110 (1965) 252.
7 J. B. CHAPPELL AND K. N. HAARHOFF, in E. C. SLATER, Z. KANIUGA AND L. WOJTCZAK, *Biochemistry of Mitochondria*, Academic Press and Polish Scientific Publishers, London and Warsaw, 1967, p. 75.
8 A. J. MEIJER, J. M. TAGER AND K. VAN DAM, in S. PAPA, J. M. TAGER, E. QUAGLIARIELLO AND E. C. SLATER, *The Energy Level and Metabolic Control in Mitochondria*, Adriatica Editrice, Bari, Italy, 1969, p. 147.
9 E. J. HARRIS AND K. VAN DAM, *Biochem. J.*, 106 (1968) 759.
10 W. GRUBER AND H. MOELLERING, *Biochem. Z.* 346 (1966) 85.
11 S. OCHOA, J. R. STERN AND M. C. SCHNEIDER, *J. Biol. Chem.*, 193 (1951) 691.

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A COMPARATIVE STUDY OF PALMITOYL-CoA SYNTHETASE ACTIVITY IN RAT-LIVER, HEART AND GUT MITOCHONDRIAL AND MICROSOMAL PREPARATIONS

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SUMMARY

1. With the aid of marker enzymes, mitochondrial and microsomal fractions of rat-liver, heart and jejunum epithelium were characterized. In heart the palmitoyl-CoA synthetase activity was shown to be localized mainly in the sarcosomes; in intestine the microsomes were the site of long-chain acyl-CoA synthesis, while in liver mitochondria and microsomes were about equally active in catalyzing palmitoyl-CoA synthesis.

2. Nagarse treatment almost completely destroyed the cardiac and hepatic palmitoyl-CoA synthetase activity, in contrast to several other membrane-bound enzymes.

INTRODUCTION

In a previous study[1] the relative contribution of the ATP-dependent fatty acid activating enzymes[2,3] [acid:CoA ligase (AMP), EC 6.2.1.2 and EC 6.2.1.3] and the GTP-linked fatty acid activation system[4] was investigated. In sonicated rat-liver mitochondria the "GTP system" had 3–6% of the activity of the "ATP system", when octanoate, palmitate or oleate were used as substrates. We suggested that the "GTP system" was of minor importance in rat-liver mitochondria[1,5,6]. Further research was therefore focused on the ATP-dependent long-chain fatty acyl-CoA synthetase in mitochondrial and microsomal fractions of several tissues.

KORNBERG AND PRICER[2] found in 1953 biosynthesis of long-chain acyl-CoA as well in soluble as in insoluble fractions of guinea pig liver. BORGSTRØM AND WHEELDON[7] reported the occurrence of the enzyme in liver mitochondria and microsomes of the same species. VIGNAIS *et al.*[8] found the highest rate of palmitoyl-CoA production in rat brain in the soluble fraction. Later work revealed that also intestinal preparations from several species contained palmitoyl-CoA synthetase activity. Concerning the subcellular localization in intestinal epithelium contradicting results have appeared[9–12]. Several workers estimated the activation of palmitate in rat liver[1,5,13–19]. A wide range of specific activities in subcellular fractions (mostly mitochondria and microsomes) of liver was reported. Finally GALTON AND FRASER[20] communicated palmitoyl-CoA synthetase activity in adipose tissue homogenates.

There seemed to be a remarkable dearth of reports on long-chain acyl-CoA synthetase in muscle preparations, although fatty acid oxidation is the main source of energy in cardiac muscle, if the concentration of long-chain fatty acids in the coronary blood is sufficiently high. We therefore investigated the ability of rat-heart fractions to synthesize palmitoyl-CoA.

We measured the palmitoyl-CoA synthetase activity in liver, gut and heart by a method developed in our laboratory[14]. This assay, a modification of the method described by FARSTAD *et al.*[13], in which the palmitoyl-CoA formed was trapped with L-carnitine as palmitoylcarnitine, made it possible to avoid the hydroxylamine trap commonly used. While this work was in progress, we discovered the destructive effect of Nagarse (subtilopeptidase A, EC 3.4.4.16) treatment of heart homogenates on the palmitoyl-CoA synthetase activity.

EXPERIMENTAL

Reagents

From Serva Entwicklungslabor, Heidelberg, Nagarse was purchased. Other enzymes and nucleotides were obtained from C.F. Boehringer and Sons, Mannheim. Bovine serum albumin, Fraction V, was supplied by Sigma Chemical Co., St. Louis, Mo., or by Pentex Inc., Kankakee, Ill. Fatty acids were removed from albumin by charcoal treatment[21]. After dialysis and Millipore filtration the albumin was used as a clear, neutral solution. Albumin–fatty acid complexes in a 1:7 molar ratio were prepared by adding to the albumin an adequate amount of recrystallized potassium palmitate in water. Oligomycin and kynuramine were obtained from Sigma Chemical Co., rotenone from Penick and Co., New York, N.Y.

DL-[*Me*-^{3}H]Carnitine was kindly donated by Dr. J. Bremer (Oslo). The radioactive carnitine was diluted with L-carnitine chloride, supplied by Koch–Light Lab. Ltd., Colnbrook, to a specific activity of 0.032 mC/mmole L-carnitine.

Preparations

Male Wistar rats, weighing 200–250 g, used for liver and heart preparations, had free access to food and water. When intestine was needed, rats were treated *per os* one week before use with Metronidazolum [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole] (about 4 mg/24 h, for at least 5 days) to get rid of intestinal *Trichomonas vaginalis*[22]. The rats were fasted for about 48 h. All rats were killed by cervical fracture and subsequent bleeding. After removal of the organs care was taken to keep the preparations at 0–4°. Centrifugational g values refer to the bottom of the tube.

Liver was immediately chilled in 0.25 M sucrose–10 mM Tris–HCl (pH 7.4). The liver was weighed, cut into small pieces and washed with sucrose–Tris to remove blood constituents. A 10% (w/v) homogenate was prepared by grinding at low speed in a Potter–Elvehjem homogenizer with a Teflon pestle. The liver homogenate was subsequently centrifuged for 5 min at 900 × g in a Sorvall RC2-B centrifuge (Rotor SS-34) to remove nuclei, intact liver cells, erythrocytes and debris. The supernatant fluid was taken and centrifuged for 10 min at 5100 × g. The precipitate of (heavy) mitochondria was suspended in half the original volume of sucrose–Tris and centrifuged for 10 min at 12000 × g. The fluffy layer was removed from the mitochondrial pellet by gently shaking with small amounts of sucrose–Tris, and the mitochon-

drial fraction was resuspended in sucrose–Tris with the aid of an homogenizer. The 5100 × *g* supernatant fluid was also spun for 10 min at 12000 × *g*. The precipitate, consisting mainly of light mitochondria, was discarded. The supernatant fluid was centrifuged for 50 min at 135000 × *g* in a Beckman L2-65B ultracentrifuge (Rotor SW-27). The microsomal precipitate was washed with small amounts of sucrose–Tris and resuspended in this medium.

From two rat hearts the aortae and auricles were cut away. The hearts were opened, washed with 0.25 M sucrose–10 mM Tris–HCl (pH 7.4) to remove the blood, and weighed. The hearts were placed in 0.25 M sucrose–5 mM ATP–10 mM EDTA (pH 7.4). After chopping with a McIlwain tissue slicer[23], the mince (about 5 % w/v) was subjected to the (hand-operated) homogenizer mentioned above. The mince was magnetically stirred for 30 min and once more subjected to the homogenizer. The heart homogenate was centrifuged for 5 min at 500 × *g* in the Sorvall centrifuge. The supernatant fluid was centrifuged for 10 min at 12000 × *g*. The sarcosomal pellet was washed with a small amount of sucrose–Tris, carefully suspended in sucrose–Tris (about half the original volume) and recentrifuged for 10 min at 12000 × *g*. The sediment was suspended in sucrose–Tris after rinsing the pellet. A heart microsomal fraction was prepared from the first 12000 × *g* supernatant fluid by centrifugation for 50 min at 135000 × *g*.

From two rats the jejunal portions of each intestine were taken and rinsed with 0.15 M NaCl. According to the method of SJÖSTRAND[24] mucus was removed with 5 % (w/v) Ficoll–0.15 M NaCl, and intestinal epithelial sheets were prepared in 2 % (w/v) Ficoll–0.15 M NaCl–0.2 M sucrose (pH 7.4) and collected by centrifugation. The cell sheets were washed and finally homogenized. Further details of the preparation of the homogenate and the differential centrifugation will be described by IEMHOFF *et al.*[22]. In short the homogenate was centrifuged for 10 min at 1000 × *g*. The sediment was rehomogenized and again centrifuged. The combined 1000 × *g* supernatant fluids were centrifuged for 10 min at 15000 × *g*. The resulting mitochondrial pellet was washed with a small amount of sucrose–Tris, suspended and recentrifuged. The sediment was considered to be the intestinal mitochondrial fraction. The combined 15000 × *g* supernatant fluids were centrifuged for 60 min at 368 000 × *g* in the Beckman centrifuge (Rotor 65). The pellet was rinsed and suspended in sucrose–Tris and was designated the intestinal microsomal fraction.

Carnitine palmitoyltransferase was purified from calf-liver mitochondria as described by FARSTAD *et al.*[13].

Palmitoyl-CoA was synthesized according to SEUBERT[25].

Methods

Enzyme assays were carried out at 37°.

Palmitoyl-CoA synthetase [acid:CoA ligase (AMP), EC 6.2.1.3] activity was determined according to FARSTAD *et al.*[13], as modified by VAN TOL AND HÜLSMANN[14]. Palmitoyl-CoA formed from palmitate, coenzyme A and ATP by mitochondrial or microsomal fractions was converted to palmitoyl[^{3}H]carnitine by added [^{3}H]carnitine and carnitine palmitoyltransferase. The incubation mixture consisted of 1 mM potassium palmitate–0.14 mM albumin, 2 mM ATP, 0.2 mM coenzyme A, 5 mM [^{3}H]carnitine (specific activity, 0.032 mC/mmole L-carnitine), 0.3–0.5 mg purified carnitine palmitoyltransferase (0.04–0.1 U), 5 mM phosphoenolpyruvate, about 1.5 U

pyruvate kinase (EC 2.7.1.40), about 1.5 U adenylate kinase (EC 2.7.4.3), 20 mM KCl, 2 mM $MgCl_2$, 120 mM Tris–HCl buffer, 0.5 mM EDTA and 3 μg oligomycin. The reaction was started by the addition of 0.1–0.3 mg mitochondrial or microsomal protein, usually in 0.1 ml 0.25 M sucrose–10 mM Tris–HCl (pH 7.4). Incubation times up to 10 min were used. The final volume was 1.0 ml and the pH 7.4. The reaction was stopped by addition of HCl, and palmitoyl[^{3}H]carnitine was extracted into *n*-butanol[13]. Aliquots of 0.5 ml were transferred to counting vials. The scintillation mixture consisted of 10 ml toluene containing 50 mg 2,5-diphenyloxazole (Packard) and 3 mg 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene (Packard), while 0.4 ml NCS solubilizer (Amersham/Searle Corporation) was added to each counting vial. The counting efficiency for tritium was about 30 % as determined by the channels ratio method.

Glucose-6-phosphatase (EC 3.1.3.9) activity was assayed as described in ref. 26. Liberated inorganic phosphate was determined in deproteinized samples as described by SUMNER[27].

Monoamine oxidase (EC 1.4.3.4) activity was measured according to WEISSBACH *et al.*[28], using kynuramine as the substrate.

Cytochrome *c* oxidase (EC 1.9.3.1) activity was determined by the method of SOTTOCASA *et al.*[29] in which a Clark oxygen electrode was used. In the case of heart sarcosomes, freezing and thawing several times was necessary to obtain maximal activity.

Alkaline phosphatase (EC 3.1.3.1) activity was assayed at pH 10.5 with *p*-nitrophenylphosphate as the substrate[30].

NADPH cytochrome *c* reductase (EC 1.6.2.3) activity was measured in the presence of 0.15 μM rotenone by following the reduction of cytochrome *c* at 550 nm[29].

Carboxylesterase (EC 3.1.1.1) activity was measured manometrically as described by HÜLSMANN[31] (see also refs. 32 and 33). Glyceroltributyrate was used as the substrate. 10^{-6} M eserine was included in the incubation medium to inhibit the cholinesterase (EC 3.1.1.8)[33].

Carnitine palmitoyltransferase activity was assayed according to NORUM[34] with palmitoyl-CoA as the substrate. Protein was determined by the biuret method as described by JACOBS *et al.*[35].

RESULTS AND DISCUSSION

Liver

Table I shows the specific activity of the long-chain acyl-CoA synthetase in liver mitochondrial and microsomal preparations together with the activity of some enzymes from which the cross-contamination can be calculated. From the distribution of these marker enzymes it can be concluded that no gross amounts of mitochondrial inner membranes (marker: cytochrome *c* oxidase[36]) or outer membranes (marker: monoamine oxidase[36,37]) pollute the microsomal fraction. Comparatively more microsomes contaminate the mitochondrial fraction. It can be seen from Table I that the microsomal marker enzymes glucose-6-phosphatase[38], NADPH cytochrome *c* reductase[29,39] and carboxylesterase (*cf.* the review given by HAYASE AND TAPPEL[40]) in the mitochondrial fraction of liver have between 12 and 18 % of the specific activities of these enzymes in the microsomal fraction. DE DUVE *et al.*[38], FARSTAD *et al.*[13] and

VAN TOL AND HÜLSMANN[14] reported that the heavy *plus* light mitochondrial fraction had about the same protein content as the microsomal fraction. From the specific activities of palmitoyl-CoA synthetase in Table I it can be concluded that the long-chain acyl-CoA synthetase activity in rat-liver mitochondria and microsomes is approximately the same.

From refs. 1 and 13–17 it can be seen that quite different specific activities are obtained by various investigators for the palmitoyl-CoA synthetase in rat-liver mitochondria and microsomes. These discrepancies may be due to: (1) hormonal and dietary fluctuations, (2) the lability of acyl-CoA synthetase, as has been noticed by BORGSTRØM AND WHEELDON[7], and/or (3) different fractionation methods and activity determinations*.

The dual localization of palmitoyl-CoA synthetase agrees with the concept of the liver as an organ which consumes fatty acids by oxidative processes in the mitochondria, in addition to producing triglycerides and phosphatides mainly in the microsomes. After their synthesis in the microsomes the bulk of the lipids is passed into the systemic circulation.

Heart

Rat heart yields a relatively small amount of microsomes on homogenization (Table I)[32,41]. The rat-liver microsomal markers NADPH cytochrome *c* reductase[29,39] and carboxylesterase[32,40] exhibit also in heart the highest specific activity in the expected microsomal fraction (see Table I and ref. 32). From the specific activities of cytochrome *c* oxidase, a marker enzyme for heart sarcosomes[32,42], and the aforementioned microsomal markers in the sarcosomal and microsomal fractions, it can be concluded that a mutual contamination of 20–25 % exists. From Table I it can be seen that the specific activity of the palmitoyl-CoA synthetase is somewhat higher in the microsomal fraction. Hence it can be concluded that heart microsomes are able to activate palmitate. Their contribution to the total fatty acid activation of heart cells, however, is small, < 15 % of the mitochondrial activation.

Heart muscle, contrary to liver, mainly consumes fatty acids as substrates. In order to maintain an appropriate ATP level for contraction, acyl-CoA is broken down in the sarcosomes. The finding of a palmitoyl-CoA synthetase in the sarcosomes is therefore not unexpected. That heart microsomes do not contribute very much to fatty acid activation is in agreement with the function of the organ. The organ mainly generates mechanical work and does not, unlike liver, produce large amounts of substrates for the benefit of other organs.

Intestine

Table I shows the palmitoyl-CoA synthetase activity in mitochondrial and microsomal fractions of rat-intestinal epithelial cells. Cytochrome *c* oxidase has been used as a marker for intestinal mitochondria[9,43]. Carboxylesterase is a microsomal enzyme in rat liver[40] and heart[32]. NADPH cytochrome *c* reductase, which has been used by CLARK *et al.*[43] as a marker of the endoplasmic reticular fraction to differentiate it from membranes of the brush border portion of intestinal cells, shows essentially the same distribution pattern as carboxylesterase (Table I). The mitochondrial frac-

* Using either the carnitine- or the hydroxylamine-trapping method[15], in our hands the high acyl-CoA synthetase activity in the nuclear fraction, as reported by PANDE AND MEAD[15], is absent.

Biochim. Biophys. Acta, 197 (1970) 127–135

TABLE I

FATTY ACID ACTIVATION IN RAT-LIVER, HEART AND GUT FRACTIONS

Where indicated the following markers have been used: cytochrome *c* oxidase and monoamine oxidase for the mitochondria; glucose-6-phosphatase, NADPH cytochrome *c* reductase and carboxylesterase for the microsomes. Enzyme activities are given in nmoles/min per mg protein at 37°. Statistics refer to means ± S.D.; *n* = number of experiments.

	Liver				*Heart*				*Gut*			
	n	*Mitochondria*	*n*	*Microsomes*	*n*	*Mitochondria*	*n*	*Microsomes*	*n*	*Mitochondria*	*n*	*Microsomes*
Protein (mg/g tissue, wet wt.)	5	23.8 ± 5.5	4	25.3 ± 10.4	7	10.2 ± 1.8	3	1.48 ± 0.24	3	1.7 ± 0.2	3	2.3 ± 0.7
Palmitoyl-CoA synthetase (Δ palmitoylcarnitine)	6	82.3 ± 20.6	5	77.0 ± 13.7	7	26.8 ± 2.2	3	38.3 ± 4.8	3	10.8 ± 4.6	3	23.1 ± 6.6
Cytochrome *c* oxidase ($-\Delta O_2$)	5	808 ± 309	4	13 ± 16	7	1720 ± 280	3	600 ± 60	3	200 ± 47	3	12.7 ± 9.6
Monoamine oxidase ($-\Delta$ kynuramine)	4	9.7 ± 1.5	3	1.7 ± 0.8		—		—		—		—
Glucose-6-phosphatase (ΔP_i)	4	46 ± 12.8	3	250 ± 12.2		—		—		—		—
NADPH cytochrome *c* reductase (Δ cytochrome *c* reduced)	4	23 ± 8.8	3	182 ± 62	4	8.5 ± 1.0	2	34.2 ± 0.8	3	30 ± 8.3	3	34.2 ± 9.1
Carboxylesterase (ΔCO_2)	3	142 ± 57	3	1120 ± 281	1	65	1	269	3	7670 ± 509	3	11 400 ± 494
Alkaline phosphatase (Δ *p*-nitrophenol)		—		—		—		—	3	3.9 ± 0.8	3	43.5 ± 1.9

tion, when corrected for the apparent microsomal contamination, seems to lack palmitoyl-CoA synthetase. This conclusion is in agreement with the findings of SENIOR AND ISSELBACHER[10,11] and AILHAUD *et al.*[9]. These investigators made use of alkaline phosphatase as a microsomal marker[9,11]. EICHHOLZ AND CRANE[44], however, showed that at least in hamster this enzyme is localized in brush border membranes (see also refs. 45 and 46). The alkaline phosphatase activity in the microsomal fraction (see Table I) results, in part, from contamination with brush border fragments as can be concluded from the sucrase (EC 3.2.1.26) activity[43]. After separation of the brush border fragments from the microsomal fraction, it was shown that in the brush border almost no long-chain acyl-CoA synthetase activity was present (Dr. J. W. O. Van den Berg, personal communication). FORSTNER *et al.*[47] reported a specific activity of synthetase in the brush borders of 1/5 that of the microsomes. From our experiments it can be concluded that in rat-gut palmitoyl-CoA synthetase is mainly or even completely a microsomal enzyme. For preparative reasons intestinal fractions were obtained from fasted rats. SENIOR AND ISSELBACHER[11] stated that fasting did not influence the rat-intestinal palmitoyl-CoA synthetase.

The activity found by us, 23 nmoles/min per mg protein at 37°, is higher than that reported by SENIOR AND ISSELBACHER[11] (3–17 nmoles/min per mg protein). RODGERS[48], who used the method of SENIOR AND ISSELBACHER (fatty acyl-hydroxamate formation), measured a specific activity of 97 nmoles/min per mg protein.

In the digestive tract fat from nutrients is attacked by lipases of the succus entericus. Free fatty acids and monoglycerides are subsequently absorbed by the epithelium cells of the small intestine. Evidence has been presented that fatty acids are activated in the intestinal microsomes (Table I, refs. 9 and 10). The acyl-CoA is used for (microsomal) triglyceride and phosphatide synthesis[10–12], after which these

TABLE II

INFLUENCE OF NAGARSE TREATMENT OF RAT-HEART AND LIVER HOMOGENATES ON THE ACTIVITY OF SEVERAL MEMBRANE-BOUND ENZYMES

Mitochondrial and microsomal preparations were isolated from five rat hearts as described under *Preparations*. Before being stirred for 30 min at 0° 5 mg Nagarse were added to one half of the mince. In the case of liver, 15 mg Nagarse were added to one half (50 ml) of a 10% homogenate. Both homogenates were stirred for 30 min at 0° prior to differential centrifugation as described under *Preparations*.

Fraction	*Enzyme*	*Specific activity (nmoles/min per mg protein)*	
		− Nagarse	*+ Nagarse*
Heart			
Mitochondria	Palmitoyl-CoA synthetase	27.5	0.5
	Cytochrome *c* oxidase	1560	1410
Microsomes	Palmitoyl-CoA synthetase	40.0	0.8
	NADPH cytochrome *c* reductase	35.0	2.2
	Carboxylesterase	270	245
Liver			
Mitochondria	Palmitoyl-CoA synthetase	53.3	1.0
	Monoamine oxidase	9.2	10.3
Microsomes	Palmitoyl-CoA synthetase	66.7	0.0
	NADPH cytochrome *c* reductase	267	25
	Glucose-6-phosphatase	250	237

lipids enter the organism mainly *via* the intestinal lymphatics. In contrast to heart cells, the intestinal cells are mainly involved in the production of lipids for other organs. Therefore utilization of lipids for the proper function of intestinal epithelial cells is quantitatively of minor importance. Hence, it is understandable that in the gut the endoplasmic reticulum is the main site of fatty acid activation. This tubular system can function for large molecules or complexes like chylomicrons as a secretory pathway leading to extracellular spaces[49].

In earlier experiments with rat heart, employing the method of CHANCE AND HAGIHARA[50] for preparing sarcosomes (with Nagarse), it was not possible to find significant long-chain acyl-CoA synthesis. The purpose of the use of Nagarse was to obtain more sarcosomal protein. The sarcosomes showed excellent respiratory capacity, good oxidative phosphorylation and respiratory control ratio[51] with various substrates (*cf.* refs. 52–54). However, it can been seen from Table II that Nagarse treatment destroys the fatty acid activating enzyme. Mitochondrial inner membrane and matrix enzymes are probably not accessible to Nagarse. A certain specificity of Nagarse inactivation, which may be useful in localization studies, can be concluded from Table II. In the study of fatty acid oxidation Nagarse treatment could conceivably be used to eliminate the mitochondrial outer membrane and microsomal long-chain fatty acid activating enzyme.

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REFERENCES

1 A. VAN TOL, J. W. DE JONG AND W. C. HÜLSMANN, *Biochim. Biophys. Acta*, 176 (1969) 414.
2 H. R. MAHLER, S. J. WAKIL AND R. M. BOCK, *J. Biol. Chem.*, 204 (1953) 453.
3 A. KORNBERG AND W. E. PRICER, Jr., *J. Biol. Chem.*, 204 (1953) 329.
4 C. R. ROSSI AND D. M. GIBSON, *J. Biol. Chem.*, 239 (1964) 1694.
5 J. W. DE JONG, A. VAN TOL AND W. C. HÜLSMANN, *Abstr. 6th Meeting Federation European Biochem. Soc.*, Spanish Biochem. Soc., Madrid, 1969, p. 240.
6 J. W. DE JONG, W. C. HÜLSMANN AND A. J. MEIJER, *Biochim. Biophys. Acta*, 184 (1969) 664.
7 B. BORGSTROM AND L. W. WHEELDON, *Biochim. Biophys. Acta*, 50 (1961) 171.
8 P. M. VIGNAIS, C. H. GALLAGHER AND I. ZABIN, *J. Neurochem.*, 2 (1958) 283.
9 G. AILHAUD, D. SAMUEL AND P. DESNUELLE, *Biochim. Biophys. Acta*, 67 (1963) 150.
10 J. R. SENIOR AND K. J. ISSELBACHER, *Federation Proc.*, 20 (1961) 245.
11 J. R. SENIOR AND K. J. ISSELBACHER, *Biochim. Biophys. Acta*, 44 (1960) 399.
12 D. N. BRINDLEY AND G. HÜBSCHER, *Biochim. Biophys. Acta*, 125 (1966) 92.
13 M. FARSTAD, J. BREMER AND K. R. NORUM, *Biochim. Biophys. Acta*, 132 (1967) 492.
14 A. VAN TOL AND W. C. HÜLSMANN, *Biochim. Biophys. Acta*, 189 (1969) 342.
15 S. V. PANDE AND J. F. MEAD, *J. Biol. Chem.*, 243 (1968) 352.
16 D. W. YATES, D. SHEPHERD AND P. B. GARLAND, *Nature*, 209 (1966) 1213.
17 H. M. KORCHAK AND E. J. MASORO, *Biochim. Biophys. Acta*, 84 (1964) 750.
18 J. BAR-TANA AND B. SHAPIRO, *Biochem. J.*, 93 (1964) 533.
19 K. R. NORUM, M. FARSTAD AND J. BREMER, *Biochem. Biophys. Res. Commun.*, 24 (1966) 797.
20 D. J. GALTON AND T. R. FRASER, *Anal. Biochem.*, 28 (1969) 59.
21 R. F. CHEN, *J. Biol. Chem.*, 242 (1967) 173.
22 W. G. J. IEMHOFF, J. W. O. VAN DEN BERG, A. M. DE PIJPER AND W. C. HÜLSMANN, *Biochim. Biophys. Acta*, submitted for publication.
23 W. C. HÜLSMANN, J. W. DE JONG AND A. VAN TOL, *Biochim. Biophys. Acta*, 162 (1968) 292.
24 F. S. SJÖSTRAND, *J. Ultrastruct. Res.*, 22 (1968) 424.

25 W. SEUBERT, *Biochem. Prep.*, 7 (1960) 80.
26 A. E. HARPER, in H.-U. BERGMEYER, *Methoden der enzymatischen Analyse*, Verlag Chemie, Weinheim, 1962, p. 788.
27 J. B. SUMNER, *Science*, 100 (1944) 413.
28 H. WEISSBACH, T. E. SMITH, J. W. DALY, B. WITKOP AND S. UDENFRIEND, *J. Biol. Chem.*, 235 (1960) 1160.
29 G. L. SOTTOCASA, B. KUYLENSTIERNA, L. ERNSTER AND A. BERGSTRAND, *J. Cell Biol.*, 32 (1967) 415.
30 K. LINHARDT AND K. WALTER, in H.-U. BERGMEYER, *Methoden der enzymatischen Analyse*, Verlag Chemie, Weinheim, 1962, p. 779.
31 W. C. HÜLSMANN, *Over het mechanisme van de ademhalingsketen-phosphorylering*, M. D. thesis, Poortpers, Amsterdam, 1958, p. 31.
32 H. A. M. HULSMANS, *Biochim. Biophys. Acta*, 54 (1961) 1.
33 D. K. MYERS, *Studies on Selective Esterase Inhibitors*, Ph. D. thesis, Excelsior, The Hague, 1954, pp. 9, 91.
34 K. R. NORUM, *Biochim. Biophys. Acta*, 89 (1964) 95.
35 E. E. JACOBS, M. JACOB, D. R. SANADI AND L. B. BRADLEY, *J. Biol. Chem.*, 223 (1956) 147.
36 C. SCHNAITMAN, V. G. ERWIN AND J. W. GREENAWALT, *J. Cell Biol.*, 32 (1967) 719.
37 P. M. VIGNAIS AND J. NACHBAUR, *Biochem. Biophys. Res. Commun.*, 33 (1968) 307.
38 C. DE DUVE, B. C. PRESSMAN, R. GIANETTO, R. WATTIAUX AND F. APPELMANS, *Biochem. J.*, 60 (1955) 604.
39 A. H. PHILLIPS AND R. G. LANGDON, *J. Biol. Chem.*, 237 (1962) 2652.
40 K. HAYASE AND A. L. TAPPEL, *J. Biol. Chem.*, 244 (1969) 2269.
41 F. A. HOLTON, W. C. HÜLSMANN, D. K. MYERS AND E. C. SLATER, *Biochem. J.*, 67 (1957) 579.
42 K. W. CLELAND AND E. C. SLATER, *Biochem. J.*, 53 (1953) 547.
43 M. L. CLARK, H. C. LANZ AND J. R. SENIOR, *Biochim. Biophys. Acta*, 183 (1969) 233.
44 A. EICHHOLZ AND R. K. CRANE, *J. Cell Biol.*, 26 (1965) 687.
45 A. EICHHOLZ, *Biochim. Biophys. Acta*, 163 (1968) 101.
46 J. H. HOLT AND D. MILLER, *Biochim. Biophys. Acta*, 58 (1962) 239.
47 G. F. FORSTNER, E. M. RILEY, S. J. DANIELS AND K. J. ISSELBACHER, *Biochem. Biophys. Res. Commun.*, 21 (1965) 83.
48 J. B. RODGERS, JR., *J. Lipid Res.*, 10 (1969) 427.
49 E. W. STRAUSS, *J. Lipid Res.*, 7 (1966) 307.
50 B. CHANCE AND B. HAGIHARA, *Biochem. Biophys. Res. Commun.*, 3 (1960) 1.
51 B. CHANCE AND M. BALTSCHEFFSKY, *Biochem. J.*, 68 (1958) 283.
52 Y. HATEFI, P. JURTSHUK AND A. G. HAAVIK, *Arch. Biochem. Biophys.*, 94 (1961) 148.
53 G. P. BRIERLEY, *J. Biol. Chem.*, 242 (1967) 1115.
54 B. HESS, in H.-U. BERGMEYER, *Methoden der enzymatischen Analyse*, Verlag Chemie, Weinheim, 1962, p. 43.

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PRELIMINARY NOTE

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Effects of Nagarse, adenosine and hexokinase on palmitate activation and oxidation

In a previous investigation[1] it was demonstrated that Nagarse (subtilopeptidase A, EC 3.4.4.16) almost completely destroyed rat heart and liver mitochondrial palmitoyl-CoA synthetase [acid:CoA ligase (AMP), EC 6.2.1.3] activity (see also ref. 2). Nevertheless Nagarse-treated liver mitochondria showed intact palmitate oxidation, in contrast to similarly treated heart sarcosomes (Table I).

TABLE I

INFLUENCE OF NAGARSE TREATMENT ON PALMITATE OXIDATION IN LIVER AND HEART MITOCHONDRIA

Mitochondrial fractions were prepared in the presence or absence of Nagarse as described before[1]. O_2 uptake was measured with differential manometers at 25° as given in ref. 3. Reaction vessels contained 0.25 mM [1-^{14}C]palmitate (0.2 μC/μmole), 0.04 mM dialysed bovine serum albumin, 1.0 mM L-malate, 2.5 mM ADP, 25 mM potassium phosphate (pH 7.4), 25 mM D-glucose, 5.0 mM $MgCl_2$, 20 mM KCl, 1.0 mM EDTA, 75 mM Tris–HCl buffer (pH 7.4), 2.4–3.8 mg mitochondrial protein and 25 mM sucrose. Further additions were dialysed hexokinase (3.2.I.U.) and 2.5 mM L-carnitine as indicated below. The reaction was terminated by the addition of 0.05 ml 70% $HClO_4$ after 42.5 min (liver) or 41 min (heart). Shaking was continued for 30–60 min. The KOH soaked paper in the centre well was transferred with 10 ml scintillation mixture[1] to a counting vial. After addition of 1 ml 1 M hydroxide of hyamine in methanol (Packard) and vigorously shaking, $^{14}CO_2$ was measured with a counting efficiency of approx. 70%. Total O_2 consumption was calculated by extrapolation.

Hexokinase:	*O_2 uptake (Σ μl/mg protein)*				*$^{14}CO_2$ production (counts/min per mg protein)*			
	−Nagarse		*+Nagarse*		*−Nagarse*		*+Nagarse*	
	−	+	−	+	−	+	−	+
Liver								
No palmitate added	7	11	6	12	—	—	—	—
Palmitate	8	25	8	25	98	2887	78	2628
Palmitate+carnitine	9	29	10	23	65	3314	82	2917
Heart								
No palmitate added	7	7	12	5	—	—	—	—
Palmitate	7	4	15	6	86	38	146	36
Palmitate+carnitine	43	3	16	5	6328	43	687	30

The oxidation of palmitate by liver mitochondria was stimulated by the addition of hexokinase (EC 2.7.1.1), in contrast to heart mitochondria where this addition was strongly inhibitory. Furthermore, when ADP or AMP were added as phosphate acceptor instead of hexokinase, a stimulation of hepatic palmitate oxidation was observed, whereas these nucleotides, and also adenosine, were inhibitory when heart mitochondria were used. Other substrates like palmitoylcarnitine or

pyruvate exhibited in both tissues much higher oxidation rates on ADP or AMP addition (not shown).

Estimation of palmitoyl-CoA synthetase activity in sonicated heart sarcosomes showed that, in the presence of hydroxylamine, ADP practically did not inhibit, but that AMP and adenosine, in contrast to, *e.g.* inosine, clearly inhibited (Table II). The strong inhibition by adenosine was also observed when liver mitochondria were used instead of heart sarcosomes (not shown). In heart and in liver mitochondrial sonicates a K_i for adenosine inhibition of palmitate activation of 0.1 mM could be

TABLE II

ADENOSINE INHIBITION OF HEART SARCOSOMAL PALMITOYL-CoA SYNTHETASE

Rat heart sarcosomes, prepared as described before[1], were sonicated. Palmitoyl-CoA synthetase activity was determined at 37° according to PANDE AND MEAD[4,5] with 0.94 mg sarcosomal protein, 15 mM ATP and 1.2 mM CoASH. The incubation time was 30 min.

Addition (2 mM)	*Specific activity (nmoles/min per mg protein)*	*Inhibition (%)*
None	33.8	—
5′-ADP	32.5	4
5′-AMP	28.5	16
3′,5′-cyclic AMP	31.0	8
Adenosine	4.4	87
Deoxyadenosine	21.9	35
Inosine	34.5	0
Adenine	28.1	17

calculated. When liver mitochondria were prepared in the presence of Nagarse (which destroyed about 95% of the palmitoyl-CoA synthetase activity[1,2]), the residual palmitoyl-CoA synthetase was hardly sensitive to adenosine. This residual activity could be sufficient for fatty acid oxidation, since palmitate oxidation in liver mitochondria was not impaired by the addition of 2 mM adenosine.

It is concluded that under the conditions described in Table I, palmitate oxidized by liver mitochondria is presumably activated by a palmitoyl-CoA synthetase localized in the inner membrane–matrix compartment, because (i) Nagarse treatment does not effect liver palmitate oxidation (Table I); (ii) hexokinase addition causes an increase of liver palmitate oxidation (Table I), although at the locus of the outer membrane palmitoyl-CoA synthetase, the ATP level can be expected to be extremely low; (iii) no inhibition of liver palmitate oxidation is observed on addition of adenosine, although the outer membrane, Nagarse-sensitive, palmitoyl-CoA synthetase is severely inhibited by adenosine; (iv) carnitine hardly stimulates liver palmitate oxidation (Table I). The palmitoyl-CoA which is used for oxidation, is probably generated by an ATP-dependent system rather than a GTP-dependent one[6], since substitution of GTP for ATP (see also ref. 7) in the palmitoyl-CoA synthetase assay[4], employing Nagarse-treated liver mitochondria, results in an 80% drop of activity.

In heart sarcosomes palmitate is activated on the outside, which makes palmitate oxidation Nagarse-, adenosine- and hexokinase-sensitive and strongly carnitine-dependent (Tables I and II). Whether the effect of adenosine, reported in this paper, is of physiological significance, remains to be investigated. It may be of interest to note that BERNE[8] reported adenosine release in the hypoxic heart.

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1 J. W. DE JONG AND W. C. HÜLSMANN, *Biochim. Biophys. Acta*, 197 (1970) 127.
2 S. V. PANDE AND M. C. BLANCHAER, *Biochim. Biophys. Acta*, 202 (1970) 43.
3 W. C. HÜLSMANN, J. W. DE JONG AND A. VAN TOL, *Biochim. Biophys. Acta*, 162 (1968) 292.
4 S. V. PANDE AND J. F. MEAD, *J. Biol. Chem.*, 243 (1968) 352.
5 S. V. PANDE AND J. F. MEAD, *Biochim. Biophys. Acta*, 152 (1968) 636.
6 C. R. ROSSI AND D. M. GIBSON, *J. Biol. Chem.*, 239 (1964) 1694.
7 A. VAN TOL, J. W. DE JONG AND W. C. HÜLSMANN, *Biochim. Biophys. Acta*, 176 (1969) 414.
8 R. M. BERNE, *Am. J. Physiol.*, 204 (1963) 317.

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INFLUENCE OF ADENOSINE AND NAGARSE ON PALMITOYL-CoA SYNTHETASE IN RAT HEART AND LIVER MITOCHONDRIA

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SUMMARY

1. A Q_{10} of about 3 for palmitoyl-CoA synthetase (EC 6.2.1.3) in rat heart and liver mitochondria is found.

2. In heart mitochondria Nagarse (EC 3.4.4.16) destroys the ability to activate palmitate. When, however, heart mitochondria are oxidizing palmitate, they are protected from the inactivating action of Nagarse.

3. Although treatment of liver mitochondria with Nagarse causes the loss of about 95 % of the palmitoyl-CoA synthetase activity, no influence is observed on palmitate oxidation.

4. Adenosine inhibits palmitoyl-CoA synthetase in liver and heart mitochondria. Adenosine is a competitive inhibitor with respect to ATP with an apparent K_i of 0.1 mM. The residual palmitoyl-CoA synthetase in Nagarse-treated liver mitochondria is much less sensitive to adenosine.

5. 2 mM adenosine or 2 mM adenosinesulfate inhibit palmitate oxidation (in the presence of 2.5 mM ATP) in heart mitochondria 60–90 %.

6. The data obtained are consistent with the concept of a palmitoyl-CoA synthetase localized on the outside of the outer membrane of rat heart and liver mitochondria, with an additional locus of (ATP-dependent) palmitoyl-CoA synthesis in the inner membrane matrix compartment of liver mitochondria.

INTRODUCTION

It was shown in a preliminary note[1] that adenosine strongly inhibited ATP-dependent palmitoyl-CoA synthetase (acid:CoA ligase (AMP), EC 6.2.1.3) activity in rat heart and liver mitochondrial sonicates. In the present communication further details are given about the specificity of this inhibition. Furthermore, the influence of adenosine on palmitate oxidation by mitochondrial preparations is shown.

DE JONG AND HÜLSMANN[2] and PANDE AND BLANCHAER[3] independently observed that Nagarse (subtilopeptidase A, EC 3.4.4.16), often used to isolated mitochondria from cardiac muscle, acted destructively on palmitoyl-CoA synthetase in heart mitochondria. The same was found in liver mitochondrial and heart and liver microsomal preparations[2]. The available evidence indicates that the highly active palmitoyl-CoA synthetase on the outside of the outer membrane of mitochondria is

destroyed[1,4]. Whether there is a separate ATP-dependent palmitoyl-CoA synthetase in the inner membrane matrix compartment, is still a matter of discussion. VAN TOL AND HÜLSMANN[4] and SKREDE AND BREMER[5] noticed a small, but distinct, ATP-dependent long-chain fatty acid activation in the inner membrane matrix fraction of rat liver mitochondria (see also VAN DEN BERGH *et al.*[6] and LIPPEL AND BEATTIE[7]). However, in a very recent paper AAS[8] did not find conclusive evidence for activation of palmitate in the inner membrane or matrix fraction of liver mitochondria. ALLMANN *et al.*[9] performed localisation studies with beef heart mitochondria (but see ref. 10).

In this paper some experiments are presented which are in agreement with the concept of an ATP-dependent palmitoyl-CoA synthetase situated on the outside of the outer membrane of rat heart and liver mitochondria, with an additional locus of (ATP-dependent) palmitoyl-CoA synthetase in the inner membrane matrix compartment of liver mitochondria. The kinetic properties of the two differently localized enzymes in liver mitochondria provide further indirect support of two separate palmitoyl-CoA synthetases.

EXPERIMENTAL

Reagents

Nagarse was furnished by Serva Entwicklungslabor, Heidelberg. Adenosine (puriss.) and other nucleosides were purchased from Koch–Light Lab., Colnbrook, Bucks. Adenosinesulfate, 2′-AMP and 3′-AMP were obtained from Schuchardt, Munich. Cyclic 2′,3′-AMP was purchased from Sigma Chemical Co., St. Louis, Mo., whereas other nucleotides and enzymes were from C. F. Boehringer und Söhne, Mannheim. Coenzyme A and sodium pyruvate (Boehringer) were solved just before use. Bovine serum albumin (from Pentex Inc., Kankakee, Ill.) was defatted by charcoal treatment[11] and dialyzed. [1-^{14}C]Palmitic acid was supplied by The Radiochemical Centre, Amersham, diluted with potassium palmitate and complexed to albumin in a 7:1 molar ratio as described before[2]. In the same reference the source of DL-[*Me*-^{3}H]carnitine is acknowledged.

Preparations

Rat heart and liver mitochondria were isolated as described before[2]. For the experiments shown in Table IV and Figs. 4 and 5 the heart mitochondria were not subjected to the 12000 × *g* centrifugation step. Sonicates of the mitochondrial fractions were prepared at 0–5° with a MSE 100-W Ultrasonic Disintegrator (Measuring and Scientific Equipment Ltd., London), operated with a microtip at 21 kcycles/sec (amplitude 6.5 μm, peak to peak) for 60 sec/ml of suspension, at least when the mitochondrial protein concentration was below 20 mg/ml. Otherwise the sonication time was doubled.

Carnitine palmitoyltransferase (palmitoyl-CoA:carnitine *O*-palmitoyltransferase, EC 2.3.1.—) was purified from calf liver mitochondria as described by FARSTAD *et al.*[12].

Methods

Palmitoyl-CoA synthetase activity was assayed either as the formation of palmitoylhydroxamate according to PANDE AND MEAD[13,14] or as the synthesis of [^{3}H]-

palmitoylcarnitine in the presence of [^{3}H]carnitine and carnitine palmitoyltransferase. The latter method was described by FARSTAD *et al.*[12], and modified in our laboratory[2,15].

Oxygen uptake was determined in a vessel equipped with a Clark oxygen electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio) and a Micrograph BD5 recorder (Kipp en Zonen, Delft) or manometrically with a differential respirometer (Gilson Medical Electronics, Villiers-le-Bel, France). Calibration of the electrode was performed with NADH and catalase (*cf.* ROBINSON AND COOPER[16]).

Protein was measured by the biuret method as described by JACOBS *et al.*[17].

Radioactivity was estimated in a Nuclear-Chicago 720 liquid scintillation counter. $^{14}CO_2$ was counted with an efficiency of about 65 % in 10 ml of the mixture of toluene, Triton X-100 and ethanol (containing PPO and POPOP) described by PATTERSON AND GREENE[18].

RESULTS AND DISCUSSION

Kinetics of the inhibition of palmitoyl-CoA synthetase by adenosine

Fig. 1 shows the inhibition of palmitoyl-CoA synthetase by various concentrations of adenosine with isolated heart mitochondria. With these mitochondria a normal Dixon plot (concentration of inhibitor *versus* the reciproke value of the activity at two ATP concentrations, *cf.* ref. 19) is obtained. The K_i for adenosine is 0.1 mM (two separate experiments). The inhibition is competitive with respect to ATP.

In preparations of liver mitochondria the Dixon plot obtained for the inhibition of palmitoyl-CoA synthetase by adenosine does not give one straight line (Fig. 2). In this case a K_i of 0.1 mM (two experiments) is found when the concentration of inhibitor is less than about 1 mM (see Fig. 2, insert). This value was reported in the preliminary note[1] and confirmed by VAN TOL AND HÜLSMANN[4], who used a different assay. The inhibition is of the competitive type with respect to ATP. A second, much higher K_i can be found by extrapolation of the values in the Dixon plot obtained for

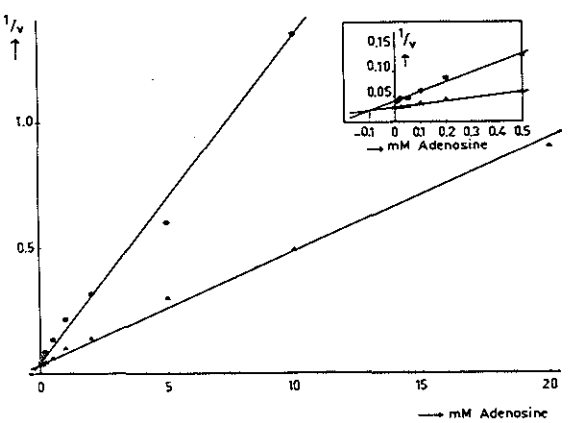


Fig. 1. The Dixon plot of inhibition by adenosine of palmitoyl-CoA synthetase in sonicated rat heart mitochondria. Palmitoyl-CoA synthetase was assayed with hydroxylamine according to PANDE AND MEAD[13,14]. The concentration of ATP was 4 mM (●—●) or 15 mM (▲—▲). 2 mM palmitate and 1.2 mM CoASH were present initially. The incubation was carried out with 0.54 mg mitochondrial protein for 30 min at 37°. V = munits/mg protein.

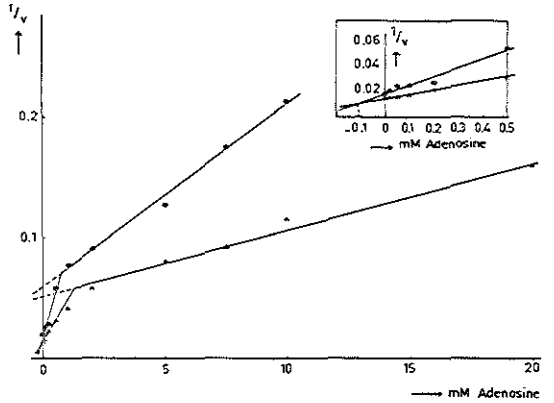


Fig. 2. The Dixon plot of inhibition of palmitoyl-CoA synthetase in sonicated rat liver mitochondria. In the assay (hydroxylamine method[13]) 0.38–0.70 mg mitochondrial protein was used. The concentration of ATP was 4 mM (●—●) or 15 mM (▲—▲). Further conditions as indicated in the legend to Fig. 1.

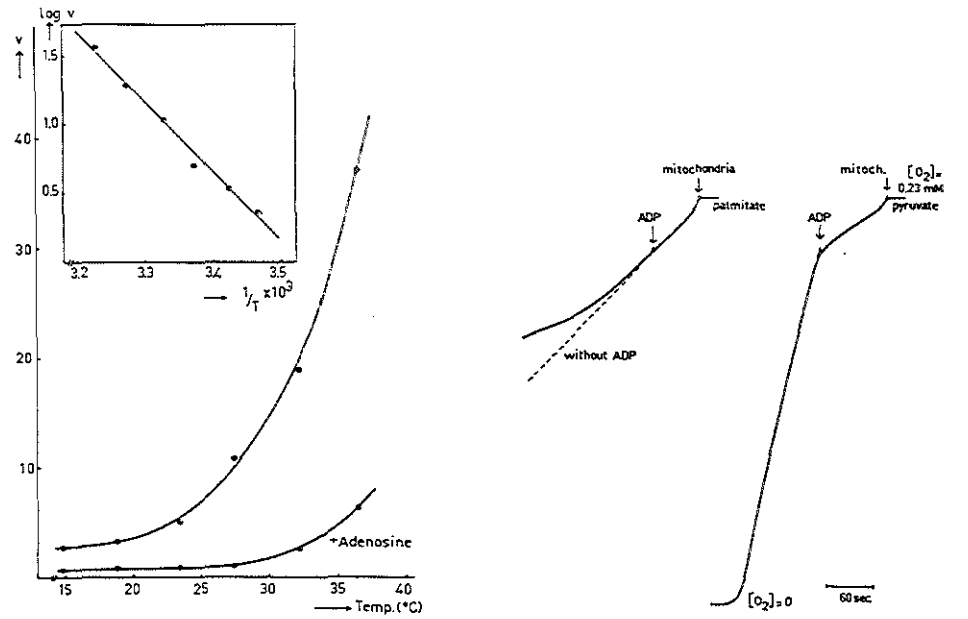


Fig. 3. Temperature dependence of palmitoyl-CoA synthetase in a heart mitochondrial sonicate. Palmitate activation was determined with the hydroxylamine method[13,14] in the presence of 2 mM potassium palmitate, 15 mM ATP, 1.2 mM CoASH and 0.26–1.0 mg mitochondrial protein. The incubation time was 30–60 min. Where indicated, the assay was carried out in the presence of 2.0 mM adenosine. The insert shows the Arrhenius plot (reciproke value of absolute temperature *versus* log (munits/mg protein)) for palmitoyl-CoA synthetase without inhibitor.

Fig. 4. Inhibition of palmitate oxidation in rat heart mitochondria by ADP. For the isolation of mitochondria see *Preparations*. Oxygen uptake was measured with a Clark oxygen electrode. The incubation medium contained 12.5 mM potassium phosphate, 50 mM KCl, 1.0 mM ATP, 2.5 mM $MgCl_2$, 0.02 mM CoASH, 0.2 mM GSH, 80 mM Tris–HCl, 4.0 mM EDTA, 0.05 mM cytochrome *c*, 0.25 mM L-malate, 0.062 mM palmitate/0.01 mM albumin and 1.0 mM L-carnitine. In the control 7.5 mM sodium pyruvate was substituted for palmitate + carnitine. Where indicated 0.98 mg mitochondrial protein (+ 25 μmoles sucrose) and 10 μmoles ADP were added. The final volume was 2.0 ml, the pH 7.4 and the temperature 30.0°.

adenosine concentrations higher than 1 mM. In liver mitochondria a palmitoyl-CoA synthetase with high activity is located on the outside of the mitochondrion[1,4]. This activity is lost on treatment of the liver mitochondria with Nagarse[1,2,4]. For Nagarse-treated liver mitochondria a K_i value of 0.2 mM has been reported[4]. When the hydroxamate assay is used instead of the carnitine assay (see *Methods*), K_i values of 2–10 mM are found. Also in this case the inhibition is competitive with respect to ATP[1,4].

When the inhibition of palmitoyl-CoA synthetase by adenosine was investigated at several temperatures a very high Q_{10} for the activation of fatty acid was found. In the literature no experiments were found concerning the temperature dependence of palmitoyl-CoA synthetase activity. Fig. 3 shows the activity of palmitoyl-CoA synthetase at different temperatures in the presence or absence of 2.0 mM adenosine

TABLE I

TEMPERATURE DEPENDENCE OF PALMITATE ACTIVATION IN HEART MITOCHONDRIAL SONICATES

Mitochondrial fractions were prepared as described before[2]. Palmitoyl-CoA synthetase was determined either with hydroxylamine[13] or with [^{3}H]carnitine[12], as modified in ref. 15. The amounts of mitochondrial protein and the incubation time for the former are mentioned in the legend to Fig. 3. In the carnitine assay 0.2–0.4 mg enzymatic protein, 1.0 mM palmitate/albumin, 2.0 mM ATP and 0.2 mM CoASH were used. The incubation time was 6 min in this case. The results were arranged in an Arrhenius plot (*cf.* Fig. 3), from which Q_{10} and energy of activation were calculated.

	Energy of activation (*kcal·mole*$^{-1}$)	Q_{10} 20–30°	30–40°
Hydroxylamine assay			
Expt. I	23.6	3.98	3.64
Expt. II	19.5	3.13	2.90
[^{3}H]Carnitine assay			
Expt. I	18.0	2.87	2.68
Expt. II	19.3	3.03	2.88

TABLE II

INHIBITION OF PALMITOYL-CoA SYNTHETASE IN SONICATED LIVER AND HEART MITOCHONDRIA BY NUCLEOSIDES

Mitochondrial fractions were prepared as described before[2]. The hydroxylamine assay was carried out at 30° as given in the legends to Figs. 1 and 2 with 15 mM ATP and 2.0 mM nucleoside. Average values were calculated for 3–6 experiments. The specific activity of the enzyme without inhibitor was 31.3 munits/mg protein (liver) and 14.6 munits/mg protein (heart).

Nucleoside + NH_2 group	*Inhibition* (%)		*Nucleoside − NH_2 group*	*Inhibition* (%)	
	Liver	*Heart*		*Liver*	*Heart*
Adenosine	79.2	82.1	Inosine	0.2	3.3
Deoxyadenosine	25.1	27.0			
Guanosine	18.2	1.6	Xanthosine	7.1	8.9
Cytidine	24.4	4.9	Uridine	9.4	0.0
			Thymidine	10.9	8.4

in a heart mitochondrial sonicate. From the Arrhenius plot [reciproke value of absolute temperature *versus* log(activity), *cf.* ref. 19] a Q_{10} of about 3 could be calculated (Fig. 3, insert). Table I shows the data obtained with different preparations using two different assay systems to estimate long-chain fatty acid activation. This Table also gives the energy of activation for palmitoyl-CoA synthetase as calculated from the Arrhenius plots. Similar results were obtained with liver mitochondria (not shown).

The specificity of adenosine as an inhibitor of palmitoyl-CoA synthetase

In a preliminary note[1] the inhibition of palmitoyl-CoA synthetase in heart mitochondria by adenosine and some analogs was shown. Table II shows the activity of the enzyme in sonicates of liver and heart mitochondria in the presence of several nucleosides. From this Table it can be concluded that the inhibition by adenosine is somewhat more specific in heart than in liver. In liver mitochondria the inhibition of the enzyme by the analogs tested seems to be dependent on the presence of an aminogroup.

PANDE AND MEAD[12] reported the inhibition of palmitoyl-CoA synthetase in rat liver microsomes by AMP (and to a smaller extent by ADP and cyclic AMP). They mentioned an AMP inhibition of palmitate activation by the mitochondrial fraction without giving details. In Table III the inhibition of palmitoyl-CoA synthetase in heart and liver mitochondrial sonicates by a variety of phosphate esters of adenosine is shown. As can be seen from Table III the inhibition by adenosine is several times greater than that obtained by the nucleotides. Adenosinesulfate is as effective an inhibitor as adenosine. This compound could be useful in the study of the regulation of palmitate catabolism.

TABLE III

INHIBITION OF PALMITOYL-CoA SYNTHETASE IN SONICATED LIVER AND HEART MITOCHONDRIA BY ADENOSINE, ADENOSINEPHOSPHATE ESTERS AND ADENOSINESULFATE

Conditions were similar to those described in the legend to Table II.

Inhibitor (2.0 *mM*)	*Inhibition* (%)	
	Liver	*Heart*
Adenosine	79.2	82.1
5′-ADP	10.5	8.8
Cyclic 3′,5′-AMP	12.4	13.5
Cyclic 2′,3′-AMP	8.6	7.7
5′-AMP	22.8	24.2
3′-AMP	19.3	7.0
2′-AMP	9.4	8.6
Adenosine sulfate	78.4	83.0

Only adenosine and adenosinesulfate are able to inhibit oxygen consumption and CO_2 production clearly when palmitate is oxidized by heart or liver mitochondria. Details are given in the next paragraph.

Effects of adenosine and Nagarse on mitochondrial palmitate oxidation

In previous experiments conducted with the Clark oxygen electrode with rat

heart mitochondria it was often noted that ADP inhibited palmitate oxidation. A typical tracing is shown in Fig. 4. We expected to find a stimulation by ADP, like we observed with substrates like palmitoylcarnitine and pyruvate (see Fig. 4), because of the transition of the mitochondria from State 4 to State 3. WARSHAW AND TERRY[20] showed (in their Fig. 5) that palmitate oxidation in calf heart mitochondria was impaired by ADP addition, without discussing the effect.

Addition of AMP or adenosine, breakdown products of ADP, also inhibited palmitate oxidation. This led us to think that adenosine as it could be generated from ADP or AMP, might be responsible for the inhibition. The effects, however, were quite variable in the short-lasting experiments with the oxygen electrode. Therefore, we

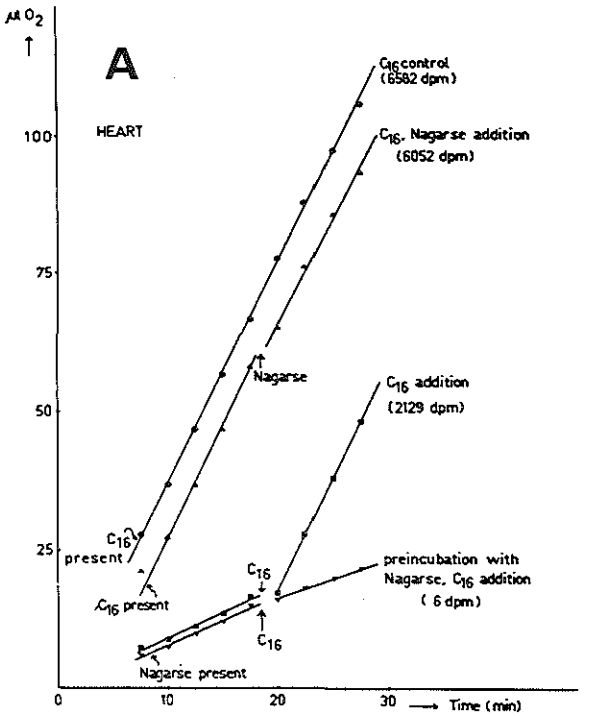
TABLE IV

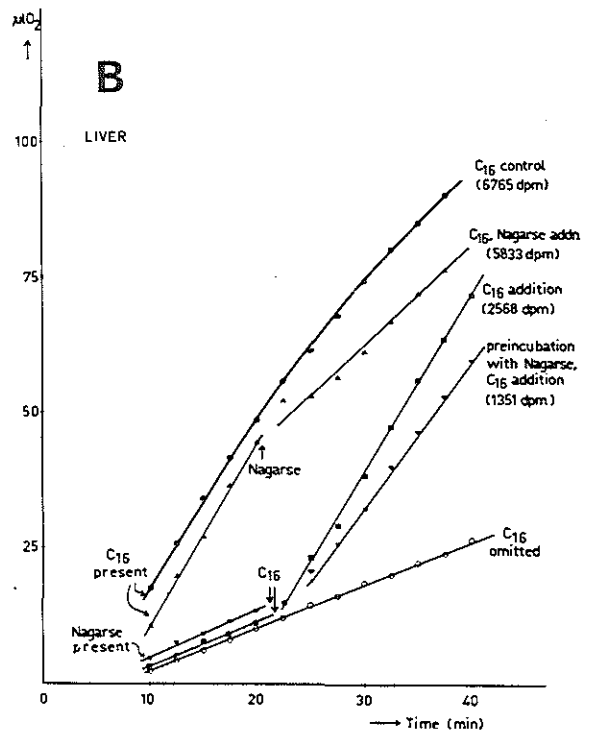
INFLUENCE OF ADENOSINE AND SOME ANALOGS ON PALMITATE OXIDATION IN HEART MITOCHONDRIA

Heart mitochondria were isolated as described under *Preparations*. Oxygen uptake was measured with a differential respirometer at 25.0°. Reaction vessels contained 0.25 mM [1-^{14}C]palmitate (0.040 μC/μmole)/0.04 mM albumin, 1.0 mM L-malate, 2.5 mM ATP, 2.5 mM L-carnitine, 0.1 mM CoASH, 10 mM potassium phosphate (pH 7.4), 5.0 mM $MgCl_2$, 30 mM KCl, 1.1 mM EDTA, 75 mM Tris–HCl buffer (pH 7.4), about 2.8 mg mitochondrial protein and 25 mM sucrose. Adenosine and analogs were tested in a final concentration of 2.0 mM. The reaction volume was 1.0 ml. All constituents were added from stock solutions, if necessary neutralized with KOH. The centre well of the vessels was provided with 0.05 ml 2.5 N KOH and a filter paper. The side arm of the flasks contained 0.05 ml 70 % $HClO_4$. The reaction was started with the mitochondria. Readings were taken at 2.5 min intervals. After 30 min the reaction was terminated by addition of $HClO_4$. Shaking was continued for 30–60 min. The KOH-soaked paper in the centre well was transferred with 10 ml scintillation mixture to a counting vial. For details of the measurement of $^{14}CO_2$ see *Methods*. Total O_2 consumption was calculated by extrapolation.

Condition	*Total O_2 uptake (Σ μl/mg protein)*		*$^{14}CO_2$ production (disint./min per mg protein)*	
	Expt. I	*Expt. II*	*Expt. I*	*Expt. II*
Palmitate/albumin omitted	10	10	—	—
Palmitate albumin	46	49	2438	2548
+ adenosine	20	23	523	721
+ deoxyadenosine	—	46	—	2535
+ inosine	42	49	2362	2466
+ guanosine	41	—	2150	—
+ xanthosine	44	—	2328	—
+ 5′-AMP	47	—	2871	—
+ 5′-IMP	42	—	2195	—
+ adenosinesulfate	18	21	441	601
+ adenine	42	—	2160	—
+ hypoxanthine	—	46	—	2475

Fig. 5. The effect of Nagarse on [1-^{14}C]palmitate oxidation in heart mitochondria (A) and liver mitochondria (B). (A) 2.7 mg heart mitochondrial protein was present; further conditions were as indicated in the legend to Table IV. In experiment (B) 3.3 mg liver mitochondrial protein was used. The palmitate oxidation was stimulated in this case by the addition of 25 mM D-glucose and 3 I.U. dialyzed hexokinase (EC 2.7.1.1) (*cf.* ref. 1). 25 mM potassium phosphate (pH 7.4), 20 mM KCl, 60 mM Tris–HCl buffer (pH 7.4) were included in the medium. The reaction was started by the addition of mitochondria. Other conditions for liver were identical to those given in the legend to Table IV. Where indicated in (A) and (B) Nagarse (0.5 mg) or [1-^{14}C]palmitate were present from the start or added after preincubation. The reactions were stopped after 30 min (heart) or 40 min (liver) and $^{14}CO_2$ was counted (see Table IV). The amounts of $^{14}CO_2$ present at the end of the experiment are indicated in (A) and (B). dpm = disint./min.


μl O2
A
HEART
100
75
50
25
C16 control (6582 dpm)
C16, Nagarse addition (6052 dpm)
Nagarse
C16 addition (2129 dpm)
C16 present
C16
preincubation with Nagarse, C16 addition (6 dpm)
C16 present
C16
Nagarse present
0
10
20
30
Time (min)


μl O2
B
LIVER
100
75
50
25
C16 control (6765 dpm)
C16, Nagarse addn (5833 dpm)
C16 addition (2568 dpm)
preincubation with Nagarse, C16 addition (1351 dpm)
Nagarse
C16 omitted
C16 present
C16
Nagarse present
0
10
20
30
40
Time (min)

turned to manometry, which enabled us to use radioactive labelled palmitate to differentiate between endogenous and added substrate. Table IV shows the oxidation of [1-^{14}C]palmitate by heart mitochondria. In the medium 0.1 mM coenzyme A is included, because it is found that coenzyme A is easily washed out from the mitochondria. Leakage of coenzyme A from rat liver mitochondria is demonstrated by SKREDE AND BREMER[5]. Several compounds are tried for their ability to inhibit palmitate oxidation. When tested in a concentration of 2.0 mM only adenosine or adenosinesulfate are inhibitory to a significant extent. The same compounds are powerful inhibitors of palmitoyl-CoA synthetase (Tables II and III). The discrepancy of oxygen uptake and $^{14}CO_2$ production as far as the degree of inhibition is concerned, is probably due to the oxidation of endogenous substrates (mainly fatty acids). For instance lauroyl-CoA synthetase (personal communication of H. R. SCHOLTE) and octanoyl-CoA synthetase (unpublished observation) are relatively insensitive to the inhibitors. When instead of heart mitochondria liver mitochondria were tested the inhibition of palmitate oxidation was found to be less. In those experiments (not shown) adenosine and adenosinesulfate inhibited oxygen uptake and $^{14}CO_2$ production only half as strong. In those experiments the conditions used were not the same. In the experiments with heart mitochondria phosphate acceptor was generated, from the 2.5 mM ATP present, by Mg^{2+}-stimulated ATPase. In the experiments with liver mitochondria phosphate acceptor was generated by the addition of hexokinase and glucose. The conditions used for palmitate oxidation by liver mitochondria are given in the legend to Fig. 5. It should be emphasized that in this case the ATP concentration in the vessels is very low, contrary to the experiments conducted with heart mitochondria. When it is kept in mind that the inhibition by adenosine of palmitoyl-CoA synthetase is competitive with respect to ATP (Figs. 1 and 2), one could argue that cardiac palmitate oxidation is even more sensitive to adenosine than hepatic palmitate oxidation. It is quite difficult to oxidize palmitate under identical conditions as was shown before[1].

In an earlier publication it was demonstrated that in mitochondria isolated from homogenates of liver and heart treated with Nagarse palmitoyl-CoA synthetase activity was almost completely lost[2]. Palmitate oxidation was severely impaired in the case of Nagarse-treated heart mitochondria as was shown by DE JONG AND HÜLSMANN[1]. The effect of Nagarse on isolated mitochondria was studied in greater detail. Addition of quite large amounts of Nagarse to heart mitochondria oxidizing palmitate, failed to produce the expected inhibition. If, however, the fatty acid is added after Nagarse no palmitate can be oxidized (Fig. 5). Apparently Nagarse is not able to destroy the palmitoyl-CoA synthetase in heart mitochondria when conditions are favorable for fatty acid activation, suggesting a change in conformation. This was confirmed by experiments (not shown) in which sonicates were preincubated with Nagarse in the presence of palmitate, ATP and coenzyme A. Palmitoyl-CoA synthetase was found to be quite insensitive to the Nagarse action under this condition. Preincubation with either palmitate, ATP or coenzyme A was much less effective to protect the enzyme. Also palmitoyl-CoA (0.2 μM) was not able to protect the palmitoyl-CoA synthetase from Nagarse digestion. Under the conditions specified in Fig. 5, palmitate oxidation by liver mitochondria is much less sensitive to preincubation with Nagarse. The palmitoyl-CoA synthetase present in the outer membrane, is destroyed, but the inner membrane enzyme (insensitive to Nagarse treatment[4]), is able to provide adequate amounts of palmitoyl-CoA[1].

CONCLUSIONS

The effects of Nagarse, hexokinase, adenosine and carnitine on palmitate activation and oxidation in liver and heart mitochondria are summarized in Table V. We conclude that in the oxidation experiments with liver mitochondria, as shown in this communication and in the preliminary note[1], palmitate is activated mainly in the inner membrane matrix compartment. BEATTIE[21] found that 95 % of the total palmitate oxidation activity (measured as formazan formed from tetrazolium salt) of rat liver mitochondria was recovered in the inner membrane matrix fraction, as obtained by digitonin treatment. LIPPEL AND BEATTIE[7] concluded from this experiment that palmitoyl-CoA synthetase was localized in the inner membrane fraction. This conclusion does not seem justified since the preparation contained approx. 11 % outer membrane[21], which has a very high palmitoyl-CoA synthetase activity[1,4–6,8,22]. VAN TOL AND HÜLSMANN[4] showed that during hepatic fatty acid oxidation at low concentrations of palmitate (*i.e.* below 40 μM), with carnitine present, the outer membrane acyl-CoA synthetase is operative.

TABLE V

SUMMARY OF THE EFFECTS OF NAGARSE, HEXOKINASE, ADENOSINE AND CARNITINE ON LIVER AND HEART MITOCHONDRIAL PALMITATE ACTIVATION AND OXIDATION

The results were compiled from experiments shown in Figs. 1, 2 and 5, Table IV and the preliminary note[1] (Table I).

	Palmitate oxidation		*Palmitate activation*		
	Liver	*Heart*	*Liver*		*Heart*
			Inner membrane matrix	*Outer membrane*	
Nagarse	No effect	Inhibition	No effect	Inhibition	Inhibition
Hexokinase	Stimulation	Inhibition	No effect	Inhibition	Inhibition
Adenosine	Small inhibition	Inhibition	Small inhibition	Inhibition	Inhibition
Carnitine	No effect	Obligate	—	—	—

In heart mitochondria we find only evidence for a palmitoyl-CoA synthetase located on the outside of the outer membrane (see also the preliminary note[1]).

Adenosine is a potent vasodilator as has been shown a long time ago by DRURY AND SZENT-GYÖRGYI[23] (*cf.* also ref. 24). Many workers reported the formation of adenosine in the heart by breakdown of adenine nucleotides under hypoxic conditions (see, for instance, refs. 25 and 26). KATORI AND BERNE[27] emphasized the importance of adenosine as the possible metabolite responsible for autoregulation of coronary blood flow. It is possible that adenosine reduces mitochondrial palmitoyl-CoA formation, when the cardiac oxygen level is low, so that adenosine not only functions as a regulator of blood flow contributing to oxygenation and lactate removal, but also as a metabolic inhibitor.

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REFERENCES

1 J. W. De Jong and W. C. Hülsmann, *Biochim. Biophys. Acta*, 210 (1970) 499.
2 J. W. De Jong and W. C. Hülsmann, *Biochim. Biophys. Acta*, 197 (1970) 127.
3 S. V. Pande and M. C. Blanchaer, *Biochim. Biophys. Acta*, 202 (1970) 43.
4 A. Van Tol and W. C. Hülsmann, *Biochim. Biophys. Acta*, 223 (1970) 416.
5 S. Skrede and J. Bremer, *Eur. J. Biochem.*, 14 (1970) 465.
6 S. G. Van den Bergh, C. P. Modder, J. H. M. Souverijn and H. C. J. M. Pierrot, in L. Ernster and Z. Drahota, *Mitochondria, Structure and Function*, Academic Press, London, 1969, p. 137.
7 K. Lippel and D. S. Beattie, *Biochim. Biophys. Acta*, 218 (1970) 227.
8 M. Aas, *Biochim. Biophys. Acta*, 231 (1971) 32.
9 D. W. Allmann, L. Galzigna, R. E. McCaman and D. E. Green, *Arch. Biochem. Biophys.*, 117 (1966) 413.
10 J. M. Smoly, B. Kuylenstierna and L. Ernster, *Proc. Natl. Acad. Sci. U.S.*, 66 (1970) 125.
11 R. F. Chen, *J. Biol. Chem.*, 242 (1967) 173.
12 M. Farstad, J. Bremer and K. R. Norum, *Biochim. Biophys. Acta*, 132 (1967) 492.
13 S. V. Pande and J. F. Mead, *J. Biol. Chem.*, 243 (1968) 352.
14 S. V. Pande and J. F. Mead, *Biochim. Biophys. Acta*, 152 (1968) 636.
15 A. Van Tol and W. C. Hülsmann, *Biochim. Biophys. Acta*, 189 (1969) 342.
16 J. Robinson and J. M. Cooper, *Anal. Biochem.*, 33 (1970) 390.
17 E. E. Jacobs, M. Jacob, D. R. Sanadi and L. B. Bradley, *J. Biol. Chem.*, 223 (1956) 147.
18 M. S. Patterson and R. C. Greene, *Anal. Chem.*, 37 (1965) 854.
19 M. Dixon and E. C. Webb, *Enzymes*, Longmans, Green and Co., London, 2nd ed., 1964, Chapters IV and VIII.
20 J. B. Warshaw and M. L. Terry, *J. Cell Biol.*, 44 (1970) 354.
21 D. S. Beattie, *Biochem. Biophys. Res. Commun.*, 30 (1968) 57.
22 K. R. Norum, M. Farstad and J. Bremer, *Biochem. Biophys. Res. Commun.*, 24 (1966) 797.
23 A. N. Drury and A. Szent-Györgyi, *J. Physiol.*, 68 (1929) 213.
24 S. Afonso and G. S. O'Brien, *Amer. J. Physiol.*, 219 (1970) 1672.
25 B. Deuticke and E. Gerlach, *Pflügers Arch.*, 292 (1966) 239.
26 W. Kübler, P. G. Spieckermann and H. J. Bretschneider, *J. Mol. Cell. Cardiol.*, 1 (1970) 23.
27 M. Katori and R. M. Berne, *Circ. Res.*, 19 (1966) 420.