BBA 56468

BIOSYNTHESIS OF LIPIDS IN GOLGI COMPLEX AND OTHER SUBCELLULAR FRACTIONS FROM RAT LIVER

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(Received February 25th, 1974)

Summary

1. Golgi complex, rough and smooth microsomes, plasma membranes, mitochondria and nuclei from rat liver were isolated and their purity assessed using specific marker enzymes.

2. The various subcellular fractions were assayed for the following processes: biosynthesis of sphingomyelin, CDPdiglycerides, phosphatidylinositol, phosphatidylserine, the conversion of phosphatidylserine into phosphatidylethanolamine, the formation of lecithin via N-methylation, and the activation of palmitic and octanoic acids.

3. None of these processes were found to be present in Golgi complex.

4. The endoplasmic reticulum appears to be the principal site in the cell for the synthesis of sphingomyelin, CDPdiglycerides, phosphatidylinositol, phosphatidylserine and the formation of lecithin. Interestingly, the biosynthesis of phosphatidylserine appears to be four times more active in rough than in smooth microsomes, which might suggest a ribosomal localization of this process.

5. Except for CDPdiglyceride synthesis, mitochondria do not contain any of the synthesizing activities described in 4. Mitochondria are, however, the only site in the cell where phosphatidylserine is decarboxylated. This activity appears to be localized in the inner membrane.

6. The activation of palmitate is localized predominantly in endoplasmic reticulum and mitochondria, though some activity was detected in plasma membranes as well. All other cell organelles, including Golgi and probably nuclei, did not contain significant palmitoyl-CoA synthetase activity. The sub-

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Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

cellular distribution of octanoyl-CoA synthetase resembled that of palmitoyl-CoA synthetase except that the former enzyme is more active in mitochondria than in microsomes.

Introduction

The Golgi apparatus in rat liver has been shown to be involved in the secretion of very low density lipoproteins which contain large amounts of lecithins and triacylglycerols [1]. In a previous paper on the metabolism of lipids in isolated Golgi complex of beef and rat liver, we found that this cell organelle does not possess CDPcholine: 1,2-diacylglycerol cholinephosphotransferase, EC 2.7.8.2. (cholinephosphotransferase) or acyl-CoA: 1,2-diacylglycerol O-acyltransferase, EC 2.3.1.20 (diacylglycerol acyltransferase). These enzymes appeared to be confined to rough and smooth microsomes [2]. The apparent absence of lecithin and triacylglycerol synthesis in Golgi complex indicates that the lipid portion of the lipoproteins secreted via the Golgi complex, is synthesized in the endoplasmic reticulum rather than in the Golgi apparatus. It seemed likely that both lipid and protein are transported to the Golgi apparatus and assembled into lipoproteins as suggested also by in vivo studies of Stein and Stein [3].
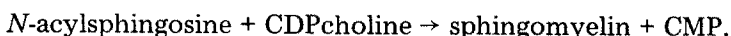
The possibility remained, however, that the Golgi complex might be involved in the biosynthesis of sphingomyelin or contribute to the biosynthesis of lecithin via the N-methylation of phosphatidylethanolamine. In the present study we have investigated further the subcellular localization of these enzymes in rat liver. We have also studied the localization of a number of other enzymes involved in the biosynthesis of phosphatidylserine, phosphatidylinositol and the activation of fatty acids.

Characterization of the subcellular fractions

Golgi apparatus, plasma membranes, rough and smooth microsomes, mitochondria and nuclei were isolated from rat liver. Marker enzymes were used to estimate the degree of contamination of each purified fraction with other subcellular fractions. This was carried out essentially as described previously [4,5]. Contamination with endoplasmic reticulum was estimated from rotenone-insensitive NADH—cytochrome c reductase and glucose-6-phosphatase activities. In some cases cholinephosphotransferase activity was also measured as an estimate of contamination [2]. Contamination with mitochondria was estimated from succinate—cytochrome c reductase measurements. These estimates are given in the tables where appropriate for the interpretation of the results. The purified cell fractions were also monitored by electron microscopy [4,5].

Biosynthesis of sphingomyelin

In 1958, Sribney and Kennedy [6] showed that in liver the biosynthesis of sphingomyelin can proceed according to the following equation:

N-acylsphingosine + CDPcholine $\rightarrow$ sphingomyelin + CMP.

The results of Sribney and Kennedy [6] indicated that the enzyme catalyzing this reaction is highly specific, not only for CDPcholine but also for the type of N-acylsphingosine: only the N-acyl derivative of *threo*-sphingosine appeared to be reactive as a substrate despite the fact that naturally occurring sphingosine derivatives possess the *erythro* configuration. In a later study, Fujino et al [7] reported that rat liver mitochondrial and microsomal fractions can utilize both *erythro*- and *threo*-ceramides as substrates for sphingomyelin formation, if the ceramides were added to the reaction mixture as proper emulsions. In the present study, however, we confirmed the original observation of Sribney and Kennedy [6] that ceramides from brain were totally inactive as substrate, even when emulsified exactly according to the procedure of Fujino et al. [7]. Ceramides from brain tissue are characterized by the occurrence of very-long-chain fatty acids ($> C_{20:0}$), which make these compounds very difficult to emulsify even in the presence of detergents. However, $N\text{-}[1\text{-}^{14}C]$palmitoyl-*erythro*-sphingosine, synthesised by Dr H. Van den Bosch (Biochemistry Laboratory, State University of Utrecht, Utrecht) according to the procedure of Ong and Brady [8], was also inactive as substrate for the synthesis of sphingomyelin. (H. Van den Bosch, personal communication). In strong contrast, ceramides possessing the *threo* configuration are excellent substrates for conversion into sphingomyelin [6]. For the study on the subcellular localization of CDP-choline: N-acylsphingosine cholinephosphotransferase, EC 2.7.8.3 (ceramide cholinephosphotransferase) we used N-acetyl-*threo-trans*-sphingosine, generously donated to us by Dr E.P. Kennedy. (Harvard Medical School, Department of Biological Chemistry, Boston, Mass.). Table I shows that the biosynthesis of sphingomyelin is localized almost exclusively in rough and smooth endoplasmic reticulum. The relative specific activity of ceramide cholinephosphotransferase in plasma membranes (9.8%) corresponds to the microsomal contamination as

TABLE I

DISTRIBUTION OF THE BIOSYNTHESIS OF SPHINGOMYELIN FROM CERAMIDE AND CDP-CHOLINE AMONG VARIOUS SUBCELLULAR FRACTIONS OF RAT LIVER

The relative specific activity of an enzyme in a given fraction is defined as its specific activity in that fraction divided by the specific activity of that enzyme in rough microsomes. Relative specific activities × 100 are presented. Details of the assay are mentioned in Experimental.

Subcellular fraction	Spec. act. (pmoles/min per mg protein)	Relative spec. act.	Contamination with microsomes[*] (%)
Rough microsomes	724	100.0	—
Smooth microsomes	649	89.6	—
Plasma membranes	72	9.9	9
Nuclei	71	9.8	—
Golgi complex	27	3.7	8
Mitochondria	n.d.[**]		2
Supernatant	n.d.[**]		—

[*] In mitochondria estimated from glucose-6-phosphatase activity, in Golgi complex from glucose-6-phosphatase and rotenone-insensitive NADH—cytochrome c reductase, and in plasma membranes from glucose-6-phosphatase, rotenone-insensitive cytochrome c reductase and cholinephosphotransferase.

[**] Not detectable.

estimated by NADH—cytochrome c reductase and glucose-6-phosphatase (9%), whereas the relative specific activity in Golgi is even lower than that of microsomal marker enzymes. The sphingomyelin content of plasma membranes [9] and, to a lesser extent, of Golgi complex [10] is much higher than that of endoplasmic reticulum [9]. Nevertheless both plasma membranes and Golgi apparatus appear to depend on the endoplasmic reticulum for the supply of new sphingomyelins, possibly via a carrier protein and exchange process. Such exchange processes have been described by Wirtz and Zilversmit [11] and McMurray and Dawson [12] for lecithin and phosphatidylinositol. In this regard, it was shown recently by Lee et al. [13] that the sphingomyelin of plasma membranes has a lower turn-over than that of microsomal membranes. Rat liver mitochondria, which have a low sphingomyelin content [9], appear to be devoid of sphingomyelin synthesizing capacity. The sphingomyelin content of rough microsomes is also low [9], so that the content of this lipid in a membrane does not reflect the rate of synthesis by the membrane. The relative specific activity of ceramide cholinephosphotransferase in nuclei is rather low (9.8%). This activity may be referable to the outer nuclear membrane which seems to be continuous with and similar to the endoplasmic reticulum membrane [14,15].

Biosynthesis of CDPdiglyceride and phosphatidylinositol

Table II shows the subcellular distribution of the enzymes involved in the biosynthesis of CDPdiglyceride and of phosphatidylinositol. Consistent with previous reports of Carter and Kennedy [16] and of Hostetler and Van den Bosch [17], the endoplasmic reticulum appears to be the main locus in the cell where CDPdiglyceride synthesis takes place. As found previously by Vorbeck and Martin [18] and by Hostetler and Van den Bosch [17], purified mitochondria can also synthesize CDPdiglyceride from phosphatidic acid and CTP: the relative specific activity of CTP: phosphatidate cytidylyltransferase, EC

TABLE II

BIOSYNTHESIS OF CDP-DIGLYCERIDE AND PHOSPHATIDYLINOSITOL IN THE VARIOUS SUB-CELLULAR FRACTIONS OF RAT LIVER

Relative specific activity is defined in Table I. Details of the assays are given in Experimental.

Subcellular fraction	CDPdiglyceride synthesis		Phosphatidylinositol synthesis		Contamination with microsomes (%)
	Spec. act. (pmoles/min per mg protein)	Relative spec. act.	Spec. act. (nmoles/min per mg protein)	Relative spec. act.	
Rough microsomes	520	100.0	13.9	100.0	—
Smooth microsomes	307	59.0	11.1	79.8	—
Mitochondria	64	12.3	n.d.*		2
Plasma membranes	26	5.0	1.3	9.3	9
Nuclei	28	5.4	2.2	15.8	—
Golgi complex	7	1.4	0.5	3.6	8
Supernatant	n.d.*		· n.d.*		—

* Not detectable.

2.7.7.41 (cytidylyltransferase) in mitochondria (12%) cannot be attributed to contaminating microsomes (only 2%).

The low specific activity in Golgi complex indicates that this cell organelle lacks the capacity to synthesize CDPdiglyceride. The low relative specific activity of cytidylyltransferase in plasma membranes (5%) can be accounted for entirely by the contamination with microsomes (9%).

The de novo synthesis of phosphatidylinositol from CDPdiglyceride and *myo*-inositol [19] also seems to be localized in the endoplasmic reticulum. In support of an earlier publication of McMurray and Dawson [12], no significant activity could be detected in purified mitochondria. The mitochondrial phosphatidylinositol is apparently renewed by an exchange of this phospholipid between mitochondria and endoplasmic reticulum. In vitro such an exchange of phosphatidylinositol between mitochondria and microsomes has been observed [12]. The Golgi complex and plasma membranes also do not possess significant amounts of the enzyme required for the conversion of CDPdiglyceride into phosphatidylinositol. In this context it might be relevant to mention the study of Michell et al. [20], on the subcellular localization of phosphatidylinositol kinase. These authors found that the synthesis of phosphatidylinositol phosphate, which requires phosphatidylinositol as substrate, appears to be localized primarily in the plasma membrane fraction.

Formation of lecithin by N-methylation

In addition to the CDPcholine pathway [21], lecithin can be synthesized from phosphatidylethanolamine by three sequential methylation reactions utilizing the methyl group of S-adenosyl-L-methionine [22,23]. Lecithin could be formed from exogenous phosphatidylmonomethyl- and dimethylethanolamine, but attempts to demonstrate the synthesis of lecithin from exogenous phosphatidylethanolamine have so far been unsuccessful [22—24]. Rehbinder and Greenberg [24] supplied evidence that the methylation of phosphatidylmonomethyl- and dimethylethanolamine is catalysed by a single enzyme in rat liver. Table III shows the subcellular distribution of the N-methylation pathway. In

TABLE III

FORMATION OF LECITHIN BY N-METHYLATION OF 1,2 DIPALMITOYL-PHOSPHATIDYL-N,N-DIMETHYL PHOSPHATIDYLETHANOLAMINE IN SUBCELLULAR FRACTIONS OF RAT LIVER

Relative specific activity is defined in Table I. Details of the assay procedure are presented in Experimental.

Subcellular fractions	Spec. act. (nmoles/min per mg protein)	Relative spec. act.	Contamination with microsomes (%)
Rough microsomes	2.50	100.0	—
Smooth microsomes	1.67	66.8	—
Plasma membranes	0.25	10.0	9
Nuclei	0.34	13.6	—
Golgi complex	0.09	3.6	8
Mitochondria	0.03	1.2	2
Supernatant	n.d.*		—

* Not detectable.

agreement with previous studies [22—24], the N-methylation is primarily localized in the microsomal fraction. Neiter mitochondria, nor Golgi complex, nor plasma membranes probably can synthesize lecithin through N-methylation of phosphatidylethanolamine, as indicated by the low relative specific activities in these fractions. It should be realized that 1,2-dipalmitoylphosphatidyl-N,N-dimethylethanolamine, which was used as substrate in the present study, is probably not the optimal substrate for this N-methylation. It has been shown that in vivo, hexaenoic phosphatidylethanolamines are used preferentially for N-methylation [25,26]. The question as to whether the use of a hexaenoic phosphatidyl-N,N-dimethylethanolamine as a substrate would have altered the subcellular distribution observed in the in vitro assay, remains unanswered.

The biosynthesis and decarboxylation of phosphatidylserine

The only pathway known for the biosynthesis of phosphatidylserine in mammalian tissues is the Ca^{2+}-dependent exchange between L-serine and phosphatidylethanolamine, detected in rat liver by Borkenhagen et al. [27]. Phosphatidylserine cannot be synthesized in mammalian tissues from CDPdiglyceride and L-serine as observed in bacteria [28]. The subcellular distribution of phosphatidylethanolamine-L-serine phosphatidyltransferase was studied recently by Dennis and Kennedy [29] and by Bjerve [30]. These authors, however, did not investigate rough and smooth microsomes separately, nor did they include plasma membranes or Golgi complex in their studies. Table IV shows the results obtained in the present study. In agreement with the reports of Dennis and Kennedy [29] and of Bjerve [30], microsomes appear to be the principal site of the cell where phosphatidylserine synthesis takes place. The activities found in mitochondria, Golgi and plasma membranes can be account-

TABLE IV

BIOSYNTHESIS AND DECARBOXYLATION OF PHOSPHATIDYLSERINE IN SUBCELLULAR FRACTIONS OF RAT LIVER

The relative specific activity is expressed with respect to rough microsomes for the biosynthesis of phosphatidylserine and with respect to mitochondria for the decarboxylation of phosphatidylserine.

Subcellular fractions	Biosynthesis of phosphatidylserine			Decarboxylation of phosphatidylserine		
	Spec. act. (pmoles/min per mg protein)	Relative spec. act.	Contamination with microsomes (%)	Spec. act. (pmoles/min per mg protein)	Relative spec. act.	Contamination with mitochondria[**] (%)
Rough microsomes	748	100	—	1.5	0.5	1
Smooth microsomes	180	24	—	6.5	2.1	3
Mitochondria	n.d.[*]		2	314	100.0	—
Plasma membranes	68	9.1	9	23	7.3	7
Nuclei	112	14.9	—	n.d.[*]		2
Golgi complex	70	9.3	8	8.5	2.7	3
Supernatant	n.d.[*]		—	n.d.[*]		1

[*] Not detectable.
[**] Estimated by succinate—cytochrome c reductase.

ed for by contaminating endoplasmic reticulum. On the basis of the relative specific activity in nuclei, it is difficult to rule out the possibility that some activity is present in this organelle. The finding that the specific activity of the exchange enzyme is a factor of 4 higher in rough than in smooth microsomes is particularly interesting. When the specific activities are normalized for the content of lipid-P per mg protein, this factor becomes 7.7. Recently Raetz and Kennedy [31] reported that in *Escherichia coli* the synthesis of phosphatidylserine from CDPdiglyceride and serine is associated with the ribosomes. In addition, Bjerve [32] reported that the formation of phosphatidylserine in liver proceeds in a small pool of the total phosphatidylserine in microsomes.

In view of these observations and the results concerning the pronounced enrichment of phosphatidylserine synthesis in rough microsomes, it is tempting to suggest that also in mammalian tissues, the formation of phosphatidylserine might be associated with the ribosomes. Studies to investigate this intriguing possibility are in progress. Phosphatidylserine is not an end product of phospholipid biosynthesis in mammalian tissues. It is rapidly decarboxylated to form phosphatidylethanolamine as shown by Borkenhagen et al. [27]. Kennedy [33] postulated that the formation of phosphatidylserine and subsequent decarboxylation to phosphatidylethanolamine comprises a decarboxylation cycle which represents the major pathway for conversion of serine into ethanolamine in vivo.

Dennis and Kennedy [29] reported that phosphatidylserine decarboxylase is a mitochondrial enzyme. Their report is confirmed and extended by the results shown in Table IV. The activities found in plasma membranes, rough and smooth microsomes, Golgi and nuclei can be accounted for completely by contamination with mitochondria. The submitochondrial localization was also studied. Table V shows the ratio of the specific activity of phosphatidylserine decarboxylase in the inner membrane over that in the outer membrane. This ratio (2.12) is close to the corresponding ratio of succinate-cytochrome *c* reductase (1.69), which is known to be a marker enzyme for inner membranes [34], but is much higher than the ratio for monoamine oxidase (0.16), an outer-membrane enzyme [35]. No significant decarboxylase activity was found in the matrix. These results show that the decarboxylation of phosphatidylserine proceeds in the inner-membrane fraction of the mitochondrion. A similar submitochondrial distribution was found by Hostetler and Van den Bosch [17] for the synthesis of CDPdiglyceride and cardiolipin. The mitochondrial localiza-

TABLE V

SUBMITOCHONDRIAL LOCALIZATION OF PHOSPHATIDYLSERINE DECARBOXYLASE

Details of the assay procedures are mentioned in Experimental.

	Specific activity inner membrane / Specific activity outer membrane
Monoamine oxidase	0.16
Succinate-cytochrome *c* reductase	1.69
Phosphatidylserine decarboxylase	2.12

tion of phosphatidylserine decarboxylase might represent a mechanism for the production of mitochondrial phosphatidylethanolamine, which cannot be synthesized from diglycerides and CDPethanolamine. The substrate of this reaction, phosphatidylserine, is however predominantly formed in the rough endoplasmic reticulum. Since L-serine is very rapidly decarboxylated by intact liver slices [27], phosphatidylserine formed on the rough endoplasmic reticulum must be readily transported to the mitochondria for decarboxylation. Favouring the hypothesis [29] of phosphatidylserine being a potential source of mitochondrial phosphatidylethanolamine is the observation of Blok et al. [36] who found that phosphatidylethanolamine, unlike phosphatidylcholine and phosphatidylinositol, is involved to a limited extent only in the exchange processes between mitochondria and microsomes. Therefore, it might be possible that phosphatidylethanolamine synthesized in the endoplasmic reticulum only moderately contributes to the supply of mitochondrial phosphatidylethanolamine, whereas phosphatidylserine synthesized in the endoplasmic reticulum and subsequently transported to the mitochondria could be an major source of mitochondrial phosphatidylethanolamine.

Palmitoyl- and octanoyl-CoA synthetase

The subcellular distribution of these enzymes is shown in Table VI. In agreement with many reports [37—41], high acid: CoA ligase (AMP forming) EC 6.2.1.3. (palmitoyl-CoA synthetase) activities are found in the microsomal and mitochondrial fractions. The activity in the supernatant may be due to contamination with microsomes. The activity in Golgi complex, which had not been previously studied, can be accounted for entirely by contamination with mitochondria and microsomes. There is some disagreement in the literature about the presence of palmitoyl-CoA synthetase in plasma membranes. Pande and Mead [42] report that the specific activity of this enzyme is a factor of 1.7

TABLE VI

DISTRIBUTION OF ACYL-CoA SYNTHETASE ACTIVITY AMONG VARIOUS SUBCELLULAR FRACTIONS OF RAT LIVER

The relative specific activity is expressed with respect to rough microsomes. For details of the assay procedures, see Experimental.

Subcellular fractions	Palmitoyl-CoA synthetase		Octanoyl-CoA synthetase		Contamination with microsomes (%)	Contamination with mitochondria (%)
	Spec. act. (nmoles/min per mg protein)	Relative spec. act.	Spec. act. (nmoles/min per mg protein)	Relative spec. act.		
Rough microsomes	118	100	66	100	—	1
Smooth microsomes	109	93	66	100	—	3
Mitochondria	58.0	48	98	148	2	—
Plasma membranes	25.6	21.7	10	15	9	9
Nuclei	9.2	7.8	9.9	15	—	2
Golgi complex	3.4	2.9	2.9	4.4	8	3
Supernatant	4.5	3.8	6.3	9.5	—	1

higher in a crude nuclear fraction which was somewhat enriched in plasma membrane, compared to the microsomes. Lippel et al. [39], on the other hand, find a low specific activity in the plasma membrane, which cannot be accounted for completely by contamination with microsomes. In our plasma membrane the specific activity of palmitoyl-CoA synthetase is 22% of that in microsomes. About 14 of the 22% could be accounted for by microsomal and mitochondrial contamination of the plasma membranes. There seems therefore to be a small amount of the activity associated with plasma membranes. The conclusion that palmitoyl-CoA synthetase is an authentic plasma membrane enzyme is corroborated by in vivo and in vitro experiments of Wright and Green [43]. These authors showed that [^{14}C] palmitic acid is incorporated into plasma membrane phospholipids prior to entering into the interior of the liver cell. In contrast to the report of Creasey [44] that the specific activity of long-chain acyl-CoA synthetase is eight times higher in nuclei than in mitochondria, we found relatively low activities of this enzyme in nuclei which is in agreement with studies by other investigators [39—41].

The subcellular distribution of octanoate activation resembles that of palmitate activation except for plasma membrane where it is clear that this activity is insignificant. Little information is available from the literature on the subcellular distribution of medium-chain acyl-CoA synthetase activity in rat liver. The highest specific activity is found in the mitochondrial fraction (Table VI), which is in agreement with the results of Aas and Bremer [45], who used butyrate as a substrate. The activity of the microsomes when compared to mitochondria is higher with octanoate than with butyrate [45] as substrate. This is most probably due to the different substrate specificity of mitochondria and microsomes. In agreement with a report of Aas [40] it can be seen from Table VI that palmitate is a better substrate than octanoate in microsomes whereas the reverse is true in mitochondria.

Our studies clearly show that most of the lipid synthesizing enzymes, and thus lipid syntheses, are localized primarily in the endoplasmic reticulum. This is true for the synthesis of lecithin and phosphatidylethanolamine via the pathways involving transfer of CDPcholine or CDPethanolamine to diglyceride [2]. It is also true of triglyceride biosynthesis [2], N-methylation of phosphatidyl-N,N-dimethylethanolamine to form lecithin (Table III), sphingomyelin biosynthesis (Table I), phosphatidylinositol (Table II) and phosphatidylserine biosynthesis (Table IV). The phosphatidylserine synthetase is impressively higher in rough microsomes than in smooth microsomes. This difference is so large that it cannot be accounted for by smooth membranes referable to the Golgi complex or plasma membranes.

The biosynthesis of CDPdiglyceride is primarily localized in endoplasmic reticulum. However, there is a small but significant amount of this activity in mitochondria (Table II) which can be recognized due to the very high purity or our mitochondrial preparation [4]. The synthesis of phosphatidylethanolamine by decarboxylation of phosphatidylserine is localized in the inner membrance of mitochondria. Both long- and medium-chain acyl-CoA synthetases are quite active in both endoplasmic reticulum and mitochondria. The specific activity of these enzymes are 1—3 orders of magnitude greater than the other enzymes studied.

We find significant activity of the lipid synthesizing enzymes in the nuclear fraction. The activities are approximately an order of magnitude greater when normalized with respect to·phospholipid or membrane content of the nuclear fraction. These activities may be referable to endoplasmic reticulum in the sense that the outer nuclear membrane is continuous with and seems to be similar to endoplasmic reticulum [14,15].

Insofar as lipoprotein synthesis and assembly are concerned, it appears as though the main constituents of serum lipoproteins, i.e. polypeptides, sphingomyelin and lecithin, are all synthesized in the endoplasmic reticulum. Serum very low density lipoproteins, which are morphologically recognizable by electron microscopy are first observed in the Golgi saccules [46]. Thus it would appear that the assembly of protein and lipid constituents to form lipoproteins takes place in the Golgi complex.

Experimental

Isolation of Golgi and other subcellular fractions of rat liver

Plasma membranes, rough and smooth microsomes, nuclei, mitochondria and supernatant were prepared by a continuous procedure from the same rat liver homogenate [4]. A low speed sediment ($1000 \times g$) was used for the preparation of plasma membrane, nuclei and mitochondria. The mitochondria prepared in this manner have a very low level of contamination by lysosomes and peroxisomes and are only slightly contaminated with microsomes (2% or less by weight of protein). This mitochondrial preparation is particularly useful in order to evaluate the presence of enzymes which have high specific activities in the microsomal fraction (cf. Table II, cytidylyltransferase). The supernatant fraction is obtained after removal of the total microsome fraction by centrifugation at 40 000 rev./min for 1 h in a Spinco No. 40 rotor. The Golgi apparatus was prepared by a modification [5] of the step-gradient procedure described previously [47]. Inner and outer membranes of mitochondria were prepared by the method of Schnaitman et al. [35] as modified by Chan et al. [48].

Marker enzyme assays

Mg^{2+}-stimulated ATPase, rotenone-insensitive NADH-cytochrome c reductase and succinate-cytochrome c reductase activities were carried out as described previously [49]. Glucose-6-phosphatase was determined by a modification [2] of the method of Swanson [50]. Galactosyltransferase was determined as described previously [5]. Protein was determined by the method of Lowry et al. [51] using crystalline bovine serum albumin as a standard. Monoamine oxidase (substrate kynuramine) was assayed as described by Weissbach et al. [52] and succinate—cytochrome c reductase of the mitochondrial subfractions was determined by the method reported by Tisdale [53].

Substrates

Egg lecithin was isolated and purified as described by Singleton et al. [54] and converted into phosphatidic acid by hydrolysis with phospholipase D (Sigma, St Louis, U.S.A.) following the method of Davidson and Long [55]. CDP-diglyceride was prepared from phosphatidic acid and cytidine-5-monophospho-

morpholidate (Sigma) as described by Agranoff and Suomi [56] and modified by Prottey and Hawthorne [57]. Phosphatidyl-[1′-^{14}C]serine was synthesized from CDPdiglyceride and [1-^{14}C]serine using a particulate preparation of *E. coli* as enzyme source [28]. CTP-5′-[5-^{3}H]triphosphate (spec. act. 500 Ci/mole), *myo*-[2-^{3}H]inositol (spec. act. 4.48 Ci/mmole and [1-^{14}C]octanoic acid (spec. act. 22.5 Ci/mole) were purchased from the Radiochemical Centre (Amersham, Great Britain). *S*-Adenosyl-L-[*Me*-^{14}C]methionine (spec. act. 53.1 Ci/mole, DL-[3-^{14}C]serine (spec. act. 12.5 Ci/mole), DL-[1-^{14}C]serine (spec. act. 8.2 Ci/mole), [1-^{14}C]palmitic acid (spec. act. 12.5 Ci/mole) and CDP-[*Me*-^{14}C]choline (spec. act. 40 Ci/mole) were products from N.E.N.-chemicals (Dreieichenhain, Germany). Non-labelled CDPcholine, *myo*-inositol, L-serine, palmitic and octanoic acids and ATP were obtained from Sigma, CTP and coenzyme A from Boehringer (Mannheim, Germany), and phosphatidylserine and phosphatidyl-*N,N*-dimethylethanolamine from Koch—Light (Colnbrook, Great Britain). *N*-Acetyl-*threo-trans*-sphingosine was a generous gift of Dr E.P. Kennedy (Harvard Medical School, Boston, U.S.A.).

Assay for the biosynthesis of sphingomyelin

This assay was carried out in a medium of the following composition: 0.3 mM *N*-acetyl-*threo-trans*-sphingosine, 0.8 mM CDP-[*Me*-^{14}C]choline (spec. act. $0.5 \cdot 10^{6}$ dpm/μmole), 4 mM MnCl$_2$, 5 mM glutathion, 50 mM Tris, pH 7.4, and the appropriate amounts of the various subcellular fractions in a total volume of 0.1 ml. After 0, 15, 30 and 60 min incubation at 37°C in a shaking water bath, the reactions were terminated by the addition of 0.4 ml water and 2 ml of methanol—chloroform (2:1, v/v). The reaction conditions were chosen such as to give a linear response to both protein concentration and to time. After extraction of the lipids according to the procedure of Bligh and Dyer [58], sphingomyelin was isolated via thin-layer chromatography on 0.5-mm silica plates which were run in chloroform—methanol—water (65:35:4, by vol.). The sphingomyelin-containing area was then scraped from the plate and directly transferred to scintillation vials containing 1 ml of water and 10 ml of Packard Instagel (Packard Instruments, U.S.A.). Scintillation counting was performed in a Packard Tricarb model 2425B liquid scintillation counter. Alternatively, since sphingomyelin appeared to be the only phospholipid which was significantly labelled when incubated in the presence of ceramide, the formation of sphingomyelin was also assayed by the filter disc method introduced by Goldfine [59]. Both assay methods yielded essentially identical results.

Biosynthesis of CDPdiglyceride

The formation of CDPdiglyceride was assayed in a medium basically similar to that described by Hostetler and Van den Bosch [17]. It contained the following components: 250 μg of phosphatidic acid, 100 mM Tris, pH 7.4, 3 mM [5′-^{3}H]CTP (spec. act. $5.5 \cdot 10^{6}$ dpm/μmole), 40 mM MgCl$_2$ and the required amount of the various subcellular fractions chosen such as to give a linear response to protein and time. The total volume of the incubation mixture was 0.33 ml. The incubation was carried out at 37°C for 0, 5, 10 and 20 min. Formation of radioactive CDPdiglyceride was measured by the filter disc method [59], since it was demonstrated that CDPdiglyceride was the only radioactive product formed during the incubation.

Biosynthesis of phosphatidylinositol

The formation of phosphatidylinositol was followed in an incubation mixture similar to that described by Paulus and Kennedy [19] : 0.2 mM CDPdiglyceride (200 μl pipetted from a sonicated emulsion of 1 mM CDPdiglyceride in water), 2 mM MnCl$_2$, 100 mM Tris, pH 8.4, 2 mM *myo*-[2-^{3}H]inositol (spec. act. 4.4 · 10^6 dpm/μmole) and the appropriate amounts of the various subcellular fractions in a total volume of 1.0 ml. The incubations were carried out at 37°C in a shaking water bath. At 0, 2.5, 5, 10 and 20 min 50-μl aliquots were taken from the reaction mixture for assay of radioactive phosphatidylinositol using the filter disc method [59]. To the remaining incubation mixture (0.75 ml 3.0 ml of methanol—chloroform (2:1, v/v) was added and the lipids extracted [58]. The chloroform phase was washed twice with 1 ml of 50 mM inositol followed by two washes with distilled water. Thin-layer chromatography on silica plates (developing system chloroform—methanol—water (65:35:4, by vol.)) showed that phosphatidylinositol was the only radioactive lipid formed during the incubation.

Conversion of phosphatidyl-N,N-dimethylethanolamine into lecithin

The methylation of phosphatidyl-N,N-dimethylethanolamine into lecithin was studied as follows: 10 μmoles of phosphatidyl-N,N-dimethylethanolamine were suspended in 5 ml of a 0.2 M Tris solution (pH 8..2) containing 20 mg of sodium deoxycholate, by sonication using a MSE-sonifier (four times for 30 s at 20 kHz). 50 μl of this suspension, containing 0.1 μmole of phosphatidyl-N, N-dimethylethanolamine were added to an incubation mixture containing: 1 mM S-adenosyl-L-[*Me*-^{14}C]methionine (spec. act. 2.2 · 10^5 dpm/μmole), 50 mM Tris, pH 8.4, and the required amounts of the various subcellular fractions. The formation of radioactive lecithin was determined at 0, 10 and 20 min using the filter disc method [59]. Incubations without addition of phosphatidyl-N,N-dimethylethanolamine were carried out to correct for methylation of endogenous phosphatidylethanolamine.

The Ca^{2+}-dependent biosynthesis of phosphatidylserine

The formation of phosphatidylserine was assayed essentially as described by Dennis and Kennedy [29]. The incubation system consisted of: 2 mM [3-^{14}C]serine (spec. act. 5 · 10^5 dpm/μmole), 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.8, 10 mM CaCl$_2$ and the appropriate amounts of the various subcellular fractions in a total volume of 0.4 ml. The formation of radioactive phosphatidylserine, which was shown by thin-layer chromatography to be the only labelled lipid, was monitored after 0, 2.5, 5 and 10 min of incubation by means of the filter disc method.

Conversion of phosphatidylserine into phosphatidylethanolamine

The decarboxylation of the phosphatidylserine was measured using phosphatidyl-[1'-^{14}C]serine as substrate [28]. The assay was carried out in 25 ml Warburg flasks. The center-well of the flask contained 0.1 ml 10 M KOH and the side-arm 0.2 ml 5 M H$_2$SO$_4$. The assay system consisted of 1 mM EDTA, 0.2% (w/v) Triton X-100, 50 mM HEPES, pH 7.1, and 0.2 mM phosphatidyl-[1'-^{14}C]serine (spec. act. 2 · 10^5 dpm/μmole), which was added last to start

the reaction. The incubations were conducted for 15 and 30 min. The reaction was terminated by the addition of the 0.2-ml H_2SO_4 from the side-arm. Shaking was then continued for an additional 30 min, after which the KOH solution in the centre-well was transferred with several portions of distilled water (total amount 1.8 ml) into a scintillation vial containing 15 ml of scintillation fluid (Unisolv, Koch—Light) for measurement for $^{14}CO_2$.

Palmitoyl- and octanoyl synthetase

The activity of these enzymes in the various subcellular fractions was assayed exactly as described by Batenburg and Van den Bergh [60], except that 0.1% Triton X-100 (w/v) was included in the reaction mixture.

Acknowledgements

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