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DISTRIBUTION AND PROPERTIES OF CDP-DIGLYCERIDE:INOSITOL TRANSFERASE FROM BRAIN[1]

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Abstract—CDP-diglyceride is converted to phosphatidyl inositol by several particulate subcellular fractions of guinea pig brain, with highest specific activity in the microsomal fraction. Optimal conditions with respect to pH, metal ion concentration, and substrate concentrations have been determined. The reaction was stimulated by the addition of bovine serum albumin and by Tween 80.

Of several DL-CDP-diglycerides synthesized and used as substrates in a spectrophotometric assay for the enzyme, DL-CDP-didecanoin was the most active.

The enzyme showed a high selectivity for *myo*-inositol. Of a number of compounds tested, only *scyllo*-inosose and *epi*-inosose served as substrates. Three inositol isomers and three *myo*-inositol monophosphates inhibited the reaction slightly. The most potent inhibitor found was galactinol, a *myo*-inositol galactoside.

THE ENZYME CDP-diglyceride:inositol transferase (CDP-diglyceride:inositol 1,2-diglyceride phosphotransferase, EC 2.7.8) catalyses the reaction:

$$\text{CDP-diglyceride} + \textit{myo}\text{-inositol} \rightleftharpoons \text{phosphatidyl inositol} + \text{CMP}$$

The occurrence of this reaction in brain is well-documented (AGRANOFF, BRADLEY and BRADY, 1958; THOMPSON, STRICKLAND and ROSSITER, 1963). Phosphatidyl inositol is the precursor of the di- and tri-phosphoinositides (FOLCH, 1949; BROCKERHOFF and BALLOU, 1962; ROSSITER and PALMER, 1965), which are found predominantly in brain (DAWSON and EICHBERG, 1965). The enzymatic conversion of phosphatidyl inositol to diphosphoinositide by ATP was demonstrated in brain (COLODZIN and KENNEDY, 1964) and in guinea pig liver mitochondria (HAJRA, SEIFFERT and AGRANOFF, 1965). A recent study of developmental changes in rat brain enzymes concerned with inositol lipid metabolism showed that the increase in CDP-diglyceride:inositol transferase activity was not associated exclusively with any period of development, while phosphatidyl inositol kinase activity rose before myelination, and diphosphoinositide kinase and triphosphoinositide monoesterase activities rose during myelination (SALWAY, HARWOOD, KAI, WHITE and HAWTHORNE, 1968). The rapid turnover of

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the phosphoinositides during nervous activity points to a special role for their synthesis and breakdown in brain (HAWTHORNE, 1964; LARRABEE and LEICHT, 1965). The role of phosphatidyl inositol in the formation and maintenance of the various specialized membrane structures of brain is also of interest. Recently, WELLS and WELLS (1967) reported that the synthesis of both bound inositol and free inositol is inhibited in nervous tissue of rats on a high galactose diet, simulating the conditions of galactosemia. ELIASSON, SCARPELLINI and FOX (1967) found decreased levels of the phosphoinositides in the brainstem of rabbits with hereditary cerebellar ataxia; incorporation of inositol into these lipids was also decreased in brainstem slices from ataxic rabbits below that of controls. In view of the many unanswered questions concerning the physiological function of both inositol and the inositol lipids, the objectives of the present study were to characterize further the enzyme that incorporates inositol into lipid in brain and to determine the effects of various polyols and related compounds on the reaction.

MATERIALS AND METHODS

Chromatography systems. System A was used to separate and identify intermediates in the chemical synthesis of CDP-diglycerides. Thin-layer plates were prepared from a slurry of silica gel without binder (Kieselgel, Arthur Thomas Company) in 1×10^{-3} M-Na_2CO_3. The developing solvent was hexane–ether (1:1, v/v). Spots were detected with bromothymol blue, phosphate (DITTMER and LESTER, 1964), or hydroxamate (TATE and BISHOP, 1962) spray. The following R_F values were observed:

Fatty acid chlorides	0·90
Glycerol benzyl ether	0·75
Diglycerides	0·30
Phosphatidic acid diphenyl esters	0·45
Phosphatidic acid	0

System B was used to separate brain phospholipids and also various sugars and cyclitols. Thin-layer plates were prepared from Silica Gel G. The developing solvent was chloroform–methanol–acetic acid–water (50:25:7:3, by vol.). Lipids were detected with bromothymol blue or phosphate spray. The sugars and cyclitols were detected with silver nitrate spray (WALDI, 1965).

System C was used to separate and identify phosphatidic acid, CDP-diglyceride, and phosphatidyl inositol. Formaldehyde-treated paper was prepared as described by HÖRHAMMER, WAGNER and RICHTER (1959) with the modification of HENDRICKSON and BALLOU (1964). The papers were developed in a descending system for 10–14 hr with *n*-butanol–acetic acid–water (4:1:1, by vol.). Spots were visualized by dipping the papers in 0·005% Rhodamine 6G and viewing under ultra-violet light.

Chemical synthesis of CDP-diglycerides. DL-Benzyl glycerol ether was synthesized from DL-acetone glycerol as described by SOWDEN and FISCHER (1941). This product was condensed with palmitoyl chloride, docosanoyl chloride, or eicosanoyl chloride as described by SOWDEN and FISCHER (1941) to give the corresponding diglycerides. Didecanoin was synthesized from DL-acetone glycerol as described by BAER and MAHADEVAN (1959). DL-Phosphatidic acid was prepared from each diglyceride by condensation with diphenylphosphorochloridate and subsequent reduction with platinum oxide to remove the diphenyl ester group, following the method of BAER (1951). These phosphatidic acids were stored as the disodium salts at −20° *in vacuo*. Phosphatidic acid (Ca^{2+} salt) was prepared from egg lecithin by a procedure of KATES (personal communication) with cabbage phospholipase D (Calbiochem, Los Angeles). Methods of isolation or crystallization of all these various intermediates differed slightly depending on the chain length of the fatty acid. CDP-diglycerides were prepared by converting the phosphatidic acids to the free acid form and condensing them with CMP-morpholidate according to the method of AGRANOFF and SUOMI (1963). The CDP-diglycerides containing palmitic, docosanoic, or eicosanoic acids were isolated by the partition method (AGRANOFF and SUOMI, 1963). The CDP-diglycerides from didecanoin or egg lecithin were isolated on silicic acid columns as described below for the didecanoyl compound. Purity of all these compounds was checked by chromatography in System A or System C, by quantitative hydroxamate determinations (SNYDER and STEPHENS, 1959), and, where appropriate, by quantitative phosphate determinations (BARTLETT, 1959). Cytidine content in the CDP-diglycerides was determined spectrophotometrically.

To prepare CDP-DL-didecanoin, a mixture of 5·54 ml of DL-benzyl glycerol ether, 5·35 ml of dry pyridine, and 26·0 ml of benzene was added gradually with stirring to 12·72 ml of chilled decanoyl

chloride in a flask stoppered with a drying tube. After 18 hr at 37°, the product was isolated as described by BAER and MAHADEVAN (1959). The final ether solution was evaporated to a volume of 15 ml on a rotary evaporator. Upon addition of 5 ml of acetone and chilling in an acetone-dry ice bath, a heavy white precipitate appeared. This was dried to constant weight *in vacuo* at −20°. Yield: 14·1 g (90 per cent) of didecanoyl benzyl ether. Acyl ester content: 94 per cent of theoretical. Chromatography in System B showed the presence of a trace of benzyl glycerol ether in addition to the product, R_F 0·75.

The above product was shaken vigorously with 12 ml of glacial acetic acid and 2·5 g of palladium black in an atmosphere of hydrogen at 15 lb/in². After absorption of hydrogen ceased (1·5 hr), 40 ml of ether were added. The catalyst was sedimented by centrifugation and washed once with 20 ml of acetone. The supernatants were concentrated to a small constant volume on a rotary evaporator and dried *in vacuo*. The liquid was chilled to 0°, and large white crystals appeared. These were collected by centrifugation, recrystallized from a small volume of hexane–ether (1:1, v/v), and dried to constant weight *in vacuo*. Yield: 7·76 g (67 per cent) of DL-didecanoin, m.p.: 25–30°. Acyl ester content: 97 per cent of theoretical. Chromatography in System B showed a single spot, R_F 0·3.

A solution of 7·56 g of DL-didecanoin in 50 ml of dry pyridine was added slowly to 2·96 ml of diphenylphosphorochloridate in 50 ml of dry pyridine. After 20 hr at 30°, the product was isolated as described by BAER (1951). The final ether solution was evaporated to give 10·5 g of an oily, slightly yellow liquid. This was dissolved in 35 ml of absolute ethanol and kept at −20° overnight. The dense white crystals were collected on a sintered glass funnel at −20°. The mother liquor was evaporated to 20 ml, and a second batch of crystals was obtained. The batches were combined, recrystallized twice in ethanol, and dried to constant weight *in vacuo*. Yield: 8·39 g (70 per cent) of didecanoin-DL-glycerophosphoric acid diphenyl ester. Ratio of acyl ester:P was 0·96. Chromatography in System A gave a single spot, R_F 0·45.

To remove the diphenyl ester group, a mixture of 7·39 g of the above product with 1·5 g of platinum oxide and 100 ml of glacial acetic acid was stirred vigorously under H_2 gas. The catalyst was sedimented by centrifugation, and the supernatant solution was evaporated to a small volume. This was dried to constant weight *in vacuo* to give an oily liquid. Yield: 4·56 g (70 per cent) of didecanoyl-DL-phosphatidic acid. Ratio of acyl ester:P was 0·98. Chromatography in System A showed a major spot at the origin with a trace of didecanoin, R_F 0·30.

CDP-DL-didecanoin was synthesized by condensation of 0·71 g of didecanoyl-DL-phosphatidic acid with 1·11 g of CMP-morpholidate (Calbiochem). The isolation procedure described for CDP-dipalmitin (AGRANOFF and SUOMI, 1963) was not suitable for CDP-didecanoin owing to the greater solubility of the latter compound in methanol at low pH during the first partition step. After evaporation of pyridine from the reaction mixture, 45 ml of chilled chloroform were added to the residue, followed by enough 0·88% formic acid (about 1 ml) to bring the pH to 3·5 (pH paper). The mixture was transferred to a 50 ml ground glass-stoppered centrifuge tube and washed three times with 10 ml of 0·88% formic acid. Acidification and washing steps were done at 0–4°. The chloroform solution was concentrated to about 2 ml on a rotary evaporator with several additions of benzene to reduce foaming. The mixture was freeze-dried from benzene, taken up in 3 ml of chloroform, and placed on a Unisil column (17·5 g of Unisil in chloroform).

Stepwise elution was carried out with 200 ml of chloroform, then chloroform–methanol in the proportions of 95:5, 9:1, 8:2, and 7:3. Most of the CDP-didecanoin was in the last fraction, along with a trace of phosphatidic acid, as determined by chromatography in System C. This fraction was concentrated and freeze-dried from benzene and washed with ether containing methanolic ammonium hydroxide to remove phosphatidic acid. The residue was again freeze-dried from benzene. Yield: 0·58 g (50 per cent) of DL-CDP-didecanoin as the diammonium salt. Ratio of acyl ester:P:cytidine: 2·13:2·00:1·91. Chromatography in System C gave a single spot with R_F 0·45.

Preparation of subcellular fractions. For initial studies on subcellular localization of transferase activity, whole brains from adult male guinea pigs were homogenized in 4 vol. of 0·25 M-sucrose with an all-glass homogenizer. After centrifugation at 900 *g* for 10 min, the supernatant fraction was decanted and centrifuged at 12,000 *g* for 20 min to give a crude mitochondrial pellet. The resulting supernatant was centrifuged at 76,000 *g* for 80 min in the 40 rotor of a Spinco ultra-centrifuge to give a microsomal pellet. The pellets were resuspended in 0·25 M-sucrose by brief homogenization, and all fractions were dialysed overnight against 4 l. of 0·07 M-potassium phosphate buffer (pH 8·1) with one change of buffer. For subsequent experiments, the total homogenate was first dialysed, then centrifuged as above. The pellet resulting from the final centrifugation was resuspended in 0·25 M-sucrose (8 ml/g wet wt. of brain). In this form, the preparation could be stored at −20° for several months with little loss of activity. In all studies except those of subcellular localization the stored enzyme preparation was used.

Protein in the homogenates and subcellular fractions was determined by the method of LOWRY, ROSEBROUGH, FARR and RANDALL (1951).

Assays for CDP-diglyceride:inositol transferase. Transferase activity was measured by determining

the amount of CMP released from CDP-diglyceride (PAULUS and KENNEDY, 1960; AGRANOFF and BRADLEY, 1961). In initial experiments, the following large-scale assay, referred to hereafter as Assay A, was used. Incubation mixtures contained 350 nmoles of DL-CDP-didecanoin, 350 μmoles of tris–HCl buffer (pH 8·1), 1750 nmoles of *myo*-inositol, and 35 μmoles of $MgCl_2$ in a total volume of 2·0 ml. An incubation mixture without inositol served as a control to indicate release of CMP which was not dependent on the transferase. The CDP-diglyceride was added to the incubation tube in chloroform, dried with a stream of nitrogen, and suspended with buffer, inositol, and $MgCl_2$ by gentle sonication. In most experiments, bovine serum albumin was added to give a concentration of 5 mg/ml. The incubation mixtures were chilled, and enzyme was added. A portion was removed immediately, and the remainder was incubated at 37°. For each time point, 0·6 ml of the incubation mixture was pipetted into 1·0 ml of cold 7% TCA. After centrifugation, the pellet was washed once with 1·0 ml of 7% TCA. The TCA supernatants were combined and brought to a total volume of 3·0 ml with TCA. The amount of CMP released was determined spectrophotometrically by using the difference between the readings at 280 mμ and 310 mμ. A molar absorbancy of 13×10^6 $mole^{-1}$ cm^2 was used to calculate the amount of CMP. The difference in values between samples with and without inositol was used to calculate transferase activity.

A small scale assay (Assay B) was used in later experiments. Incubation mixtures contained 35 nmoles of DL-CDP-didecanoin, 35 μmoles of Tris–HCl buffer (pH 8·3), 280 nmoles of *myo*-inositol and 10·5 μmoles of $MgCl_2$ in a total volume of 0·2 ml. In most experiments, albumin was added as indicated. The analysis was the same as Assay A, except that a separate tube was prepared for each time point. A 0·4 ml portion of 7% perchloric acid was added to stop the reaction, and the supernatant fraction was assayed directly.

In experiments comparing the release of CMP with the incorporation of inositol into lipid, incubation mixtures were prepared as for Assay B, except twice the usual amounts were used, and [2-^{3}H]*myo*-inositol (1·9 μC/μmole) was substituted for unlabelled inositol. The final volume was 0·4 ml. After incubation, half of the sample was pipetted into perchloric acid as described above for the determination of CMP. Lipids were extracted from the remaining 0·2 ml by a modification of a procedure described by BLIGH and DYER (1959). An additional 0·2 ml of water was added, followed by 1·5 ml of chloroform–methanol (1:2, v/v), 100 μg of purified soybean phosphatidyl inositol, and 30 μl of concentrated HCl. After thorough mixing, another 0·5 ml of chloroform was added, followed by 0·5 ml of 2 M-KCl containing 0·2 M-inositol. After mixing and centrifugation, the lower layer was transferred to another tube with a capillary pipette. The upper layer was treated again with 1 ml of lower layer. The combined lower layers were washed three times with 1 ml portions of KCl-inositol. The lipid extracts were dried in counting vials and dissolved in 10 ml of scintillation fluid containing 0·5% PPO (2,5-diphenyloxazole) and 0·03% POPOP (1,4-bis-2(5-phenyloxazolyl)-benzene) in toluene–methanol (19:1, v/v).

Materials. Bovine serum albumin, Fraction V, was purchased from Armour, Chicago, Illinois. Detergents were obtained from several sources: sodium deoxycholate from Mann Research Laboratories, New York; Triton X-100 from Rohm and Haas, Philadelphia, Pennsylvania; Tween 80 and Tween 20 from Atlas Chemical Industries, Wilmington, Delaware; and Cutscum from Fischer Scientific Company, Chicago, Illinois. Sodium [^{3}H]borohydride was purchased from New England Nuclear Corporation, Boston, Massachusetts. PPO and POPOP were purchased from Packard Instrument Company, LaGrange, Illinois.

The following cyclitols were the generous gift of Dr. Laurens Anderson: the *muco-*, *epi-*, *neo-*, and *scyllo*-isomers of inositol; hydroxyisomytilitol (2-C-hydroxymethyl-*myo*-inositol); 2-O,C-methylene-*myo*-inositol; D-*myo*-inosose-1; the cyclohexanepentols (+)-quercitol and *scyllo*-quercitol; conduritol A; the O-methyl ethers (+)-pinitol, (−)-quebrachitol, bornesitol, sequoyitol, and dambonitol; the amino derivatives *scyllo*-inosamine, *neo*-inosamine-HCl, and streptamine; the 2-acetamido- and (±)-4-acetamido derivatives of *myo*-inositol.

Several cyclitols were contributed by Dr. S. J. Angyal: the hexa-acetate derivatives of *cis-*, *allo-*, and *muco*-inositols and the tri-*O*-cyclohexylidine derivatives of *epi-* and *neo*-inositols. The derivatives were converted to their free form by acid hydrolysis.

DL-*Epi*-inosose-2 was the gift of Dr. Theodore Posternak. [2-^{3}H]*Myo*-inositol was obtained by catalytic hydrogenation of the inosose (POSTERNAK, SCHOPFER and REYMOND, 1955; BENJAMINS, 1967). *Myo*-inositol-L-1-phosphate, *myo*-inositol-L-3-phosphate (dicyclohexylammonium salt) and *myo*-inositol-DL-4 phosphate were the gifts of Dr. Clinton Ballou.

Scyllo-inosose, glucitol, and DL-α-glycerol-phosphate were purchased from Sigma Chemical Company, St. Louis. *Levo*-inositol-3-phosphate, *myo*-inositol-2-phosphate, galactinol (1-O-α,D-galactopyranosyl-L-3-*myo*-inositol), and both D- and L-serine were purchased from Calbiochem, Los Angeles. Galactitol, D-galactonolactone, and *i*-erythritol were obtained from Mann Research Laboratories, New York. D-Galactose, 2-aminoethanol, and cyclohexylamine were purchased from Eastman, Rochester, New York, choline chloride was purchased from Merck, Rahway, New Jersey; and D-glucose from Allied Chemical, New York.

RESULTS

CDP-diglyceride:inositol transferase activity in dialysed homogenates of various guinea pig organs. The specific activity of the transferase was highest in heart and lung, while liver had the greatest total activity and lowest specific activity (Table 1). Liver was especially active in releasing CMP from CDP-diglyceride without added inositol. Since these preparations were well-dialysed, this may represent enzymatic hydrolysis of CDP-diglyceride to CMP or CDP.

TABLE 1.—TRANSFERASE ACTIVITY IN DIALYSED HOMOGENATES OF VARIOUS GUINEA PIG ORGANS

Organ	Wet wt. (g)	Protein (mg/g wet wt.)	Total activity/ organ (units)	Specific activity (units/mg protein)
Brain	4·2	97	19,200	49(19)
Liver	19·7	152	92,500	31(37)
Kidney	4·2	148	19,200	31(17)
Heart	1·9	108	17,300	76(15)
Lung	4·7	100	36,000	76(12)
Spleen	1·1	145	10,100	64(12)

Homogenates were prepared from similar amounts of each tissue and dialysed over night. Assay A was used, without bovine serum albumin. Values obtained when inositol was omitted are shown in brackets. CMP release in the presence of inositol minus CMP release without added inositol is considered transferase activity. One enzyme unit is that quantity of enzyme which releases 1 nmole of CMP in 1 hr. The values for protein and enzyme activity represent the means of duplicate determinations.

Localization of transferase activity in subcellular fractions of guinea pig brain. Initial studies on the localization of transferase activity gave variable results. In an effort to obtain more reproducible values, bovine serum albumin was added to each incubation mixture. This stimulated activity in all fractions to some extent, but especially in the pellet obtained after centrifugation at 76,000 *g*. Thereafter, all fractions were assayed in the presence of bovine serum albumin at a concentration of 5 mg/ml, unless otherwise noted. Of the total activity in the homogenate, 25–30 per cent could be recovered in each of the crude particulate fractions sedimented at 900 *g*, 12,000 *g*, and 76,000 *g*. Less than 5 per cent of the activity was recovered in the final supernatant fraction. The microsomal fraction (76,000 *g* pellet) had the highest specific activity, followed by the crude mitochondrial fraction (12,000 *g* pellet).

Dialysis of the total homogenate *before* preparation of the crude subcellular fractions resulted in an increase in both the total and specific activities of the transferase recovered in the 76,000 *g* fraction (Table 2). Dialysis before centrifugation reduced the amount of protein in this fraction to one-third of that obtained in the pellet dialysed after centrifugation. At the same time, the total activity in this pellet was increased 1·5 times. Consequently, this method was used to prepare the transferase for further characterization.

Fractionation of a crude mitochondrial preparation on a discontinuous sucrose density gradient (DE ROBERTIS, PELLEGRINO DE IRALDI, RODRIGUEZ DE LOREZ ARNAIZ and SALGANICOFF, 1962) indicated that none of the submitochondrial fractions is enriched in transferase activity. Fraction A, identified in the studies of DE ROBERTIS *et al.* (1962) as primarily myelin fragments, had a markedly lower specific activity than the other fractions. The crude mitochondrial fraction, the bands presumably enriched in nerve endings, and the final mitochondrial pellet all had similar specific activities.

TABLE 2.—DISTRIBUTION OF TRANSFERASE ACTIVITY IN BRAIN

Fraction	Protein (mg)	Total activity (units)	Specific activity (units/mg protein)
Centrifuged before dialysis			
Whole homogenate	369·0	46,000	125
12,000 *g* fraction	116·0	16,500	142
76,000 *g* fraction	45·7	14,100	309
Centrifuged after dialysis			
Whole homogenate	338·0	52,800	156
12,000 *g* fraction	130·0	24,000	276
76,000 *g* fraction	15·4	21,700	1400

A brain was homogenized in 9 vol. of 0·25 M-sucrose. One-half was centrifuged immediately (see Methods) and all fractions were dialysed. The rest of the homogenate was first dialysed, then centrifuged to prepare the subcellular fractions. Assay A was used, with bovine serum albumin (5 mg/ml) added. The values for protein and enzyme activity represent the means of duplicate determinations.

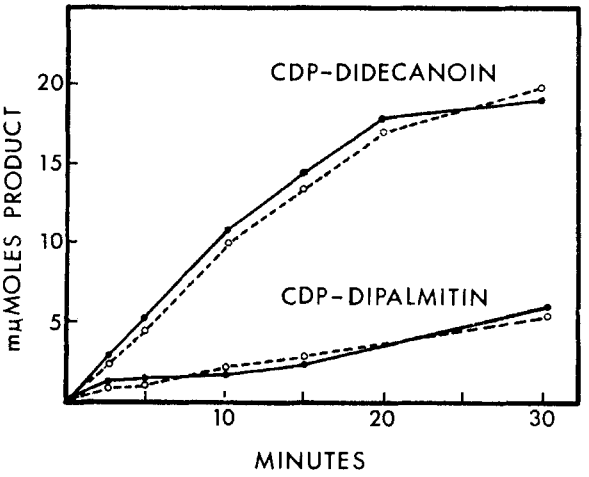

FIG. 1.—Stoichiometry of CMP release and incorporation of [2-^{3}H]*myo*-inositol. Microsomal fraction prepared from homogenates of brain after dialysis was incubated as described for Assay B, with bovine serum albumin (5 mg/ml). The solid lines indicate CMP release and the dotted lines inositol incorporation into lipid. The data are from a single typical experiment; each point represents the mean of duplicate determinations.

Stoichiometry of CMP release and incorporation of inositol into lipid. In the reaction catalysed by CDP-diglyceride:inositol transferase, one mole of *myo*-inositol was incorporated into phosphatidyl inositol for each mole of CMP released from CDP-diglyceride (Fig. 1). In these experiments, [2-^{3}H]*myo*-inositol was used, and release of CMP and synthesis of radioactive lipid were measured in duplicate samples of the same incubation mixtures. The exchange of inositol described by PAULUS and KENNEDY (1960) at high Mn^{2+} concentrations with chick liver microsomal fractions did not occur in these brain microsomal fractions with the assay conditions used.

In another experiment, with unlabelled *myo*-inositol, the conversion of CDP-diglyceride to phosphatidyl inositol was demonstrated by extraction of lipids and chromatography on formaldehyde-treated paper (System C). The amount of CMP released indicated that half of the DL-CDP-didecanoin, presumably the L-isomer,

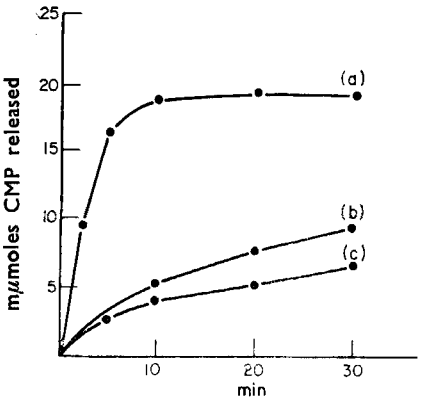

FIG. 2.—Comparison of three synthetic CDP-diglycerides as substrates. Conditions as in Fig. 1. Twice as much enzyme was used to obtain the two lower curves. (a) 37·5 nmoles of DL-CDP-didecanoin; (b) 37·5 nmoles of CDP-diglyceride prepared from egg lecithin; (c) 37·5 nmoles of DL-CDP-dipalmitin. Each point represents the mean of duplicate determinations. Assay B was used, with a total volume of 0·2 ml.

had reacted. The chromatogram showed about half of the CDP-diglyceride remaining, compared to the unincubated sample. A new spot appeared with an R_F corresponding to that of phosphatidyl inositol.

Effect of fatty acid composition of CDP-diglyceride on transferase activity. Initial studies indicated that DL-CDP-didecanion was a better substrate than DL-CDP-dipalmitin or CDP-diglyceride from egg lecithin (Fig. 2). No reaction was observed with DL-CDP-diglycerides containing C_{20} or C_{22} fatty acids under the conditions of Assay B. Omission of bovine serum albumin or replacement of the albumin with Tween 80 or Cutscum at several concentrations did not stimulate the reaction with the longer chain CDP-diglycerides nor did substitution of Mn^{2+} for Mg^{2+}. Addition of egg lecithin to give a concentration of 2·5 mg/ml inhibited the reaction with CDP-dipalmitin by about 25 per cent, and that with CDP-diglyceride from egg lecithin by about 90 per cent.

Optimal conditions for conversion of DL-*CDP-didecanoin to phosphatidyl inositol.* The rate of the transferase reaction was proportional to the amount of enzyme added over a range of 6 to 160 μg of protein/ml of incubation medium. The optimal pH was 8·6 (Fig. 3). Although Mn^{2+} stimulated best at low concentrations, it had a very sharp optimum (Fig. 4). Maximal stimulation with Mn^{2+} under these conditions was about two thirds of that obtained with higher concentrations of Mg^{2+}. Addition of EDTA abolished transferase activity. Ca^{2+} had no effect on the rate in the presence of Mg^{2+}. Other metals were not tested.

The apparent K_m of *myo*-inositol in this reaction was determined by following either the amount of CMP released or the amount of labelled inositol incorporated (Fig. 5). Similar values, around $1{\cdot}5 \times 10^{-3}$ M, were found in both cases. The Lineweaver–Burk plot was obtained by following the incorporation of labelled inositol into lipid, as described for the stoichiometry experiments.

When the effects of varying concentrations of CDP-didecanoin and CDP-dipalmitin were studied, CDP-didecanoin appeared to be inhibitory at concentrations above 187 nmoles/ml (Fig. 6). Apparent K_m values obtained were $1{\cdot}7 \times 10^{-4}$ M for DL-CDP-didecanoin and $5{\cdot}9 \times 10^{-4}$ M for DL-CDP-dipalmitin.

In the initial experiments on subcellular fractionation, bovine serum albumin consistently stimulated the microsomal fraction, but the degree of stimulation varied from three- to eight-fold. In later experiments with enzyme prepared after dialysis, the degree of stimulation was more constant (Fig. 7). With the optimal concentration of albumin, 5 mg/ml, a four- to five-fold stimulation was generally observed.

Various detergents were tested in the absence of bovine serum albumin to determine their effects on the transferase reaction. Cutscum, deoxycholate, and Triton X-100 at concentrations from 0·50 to 0·05 mg/ml had little effect on the reaction with CDP-didecanoin as substrate. Deoxycholate at the highest concentration inhibited slightly.

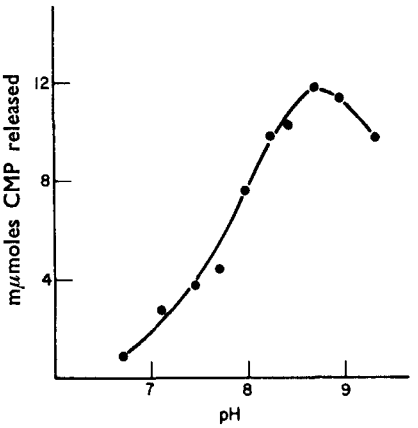

FIG. 3.—Effect of pH. Conditions as in Fig. 1, except unlabelled inositol and Assay A were used. Tris–HCl buffers were used; a similar optimum was observed with glycine–NaOH buffers. Each point represents the mean of duplicate determinations.

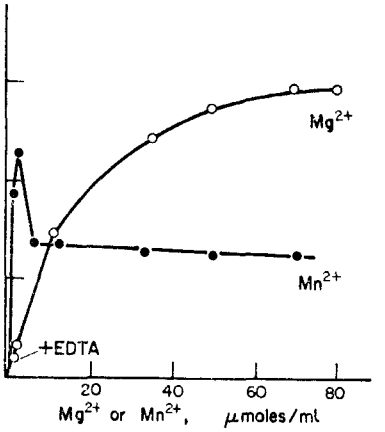

FIG. 4.—Effect of Mg^{2+} and Mn^{2+} concentrations. Conditions as in Fig. 3. Each point represents the mean of duplicate determinations.

Higher amounts of these detergents interfered with the spectrophotometric assay. Tween 20 inhibited the reaction by 40 per cent at a concentration of 5 mg/ml, but gave a two-fold stimulation at both 0·10 and 0·05 mg/ml. Tween 80 was the most effective detergent found, and its effect on transferase activity was studied over a wide range of concentrations, from 5 mg/ml to 5×10^{-5} mg/ml. At the higher concentrations, there was less transferase activity than that observed in the absence of detergent or albumin. At 5×10^{-3} mg/ml, it stimulated activity almost as much as optimal amounts of albumin (5 mg/ml). With Tween 80 at a concentration of 5×10^{-5} mg/ml, there was still a two-fold stimulation of activity. Preliminary experiments with Tween 80 at a concentration of 0·50 mg/ml indicated that it may act in part by solubilizing some of the microsomal membrane. When microsomal fraction was suspended in 0·25 M-sucrose and Tween 80 and held at 0–4° for 90 min, 30 per cent of the activity was recovered in the supernatant obtained following centrifugation at 76,000 *g* for 60 min. No release of enzyme activity into the supernatant was observed in similar experiments in which the microsomal fraction was frozen and thawed repeatedly, exposed to low ionic strength buffers, sonicated, or mixed with the other detergents mentioned above.

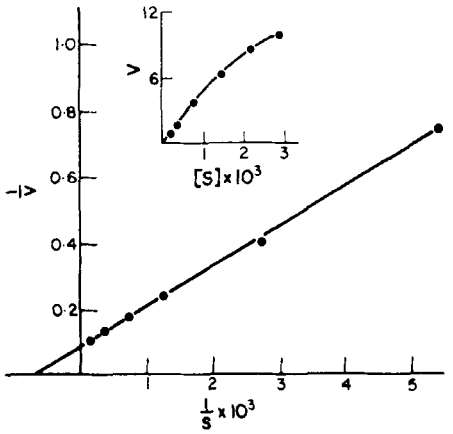

FIG. 5.—Apparent K_m of *myo*-inositol. Conditions as in Fig. 1. Incubations were terminated after 10 min, and the amount of [2-^{3}H]*myo*-inositol incorporated into lipid was determined. The data are from a single typical experiment; each point represents the mean of duplicate determinations.

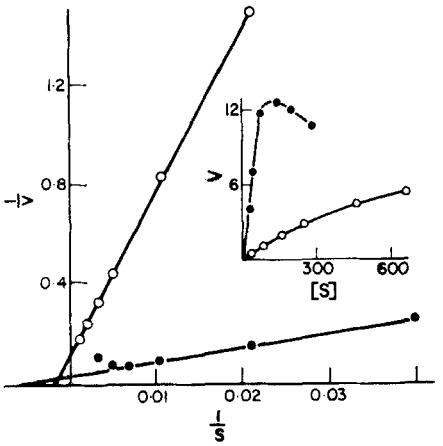

FIG. 6.—Apparent K_m of DL-CDP-didecanoin and DL-CDP-dipalmitin. Conditions as in Fig. 1, except unlabelled inositol was used. For DL-CDP-didecanoin (—●—●—), incubations were terminated after 2·5 min, and for DL-CDP-dipalmitin (—○—○—), after 10 min. The data are from a single typical experiment; each point represents the mean of duplicate determinations.

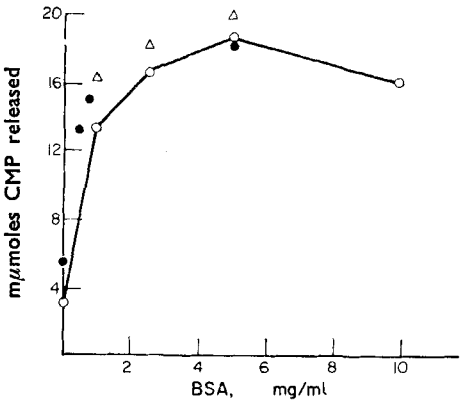

FIG. 7.—Effect of bovine serum albumin. Conditions as in Fig. 1, except unlabelled inositol was used. Triangles and circles represent results from three experiments with different batches of enzyme, and each point is the mean of duplicate determinations.

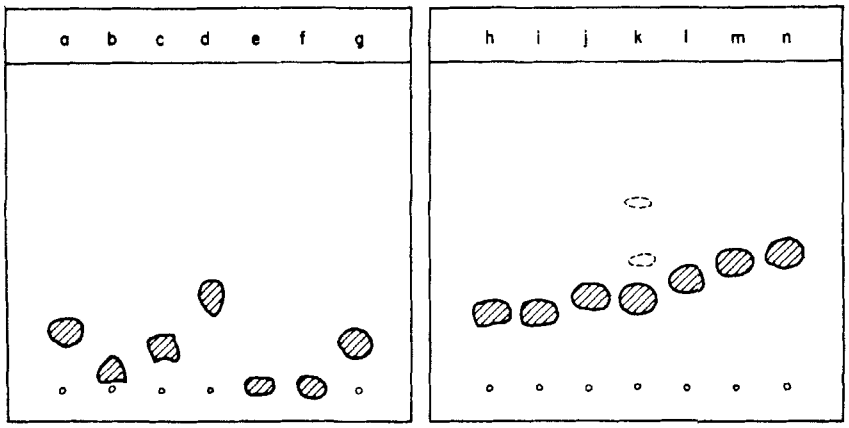

FIG. 8.—TLC of cyclitols. A sample of 40 μg of each compound was applied to a plate of silica gel G. The plates were developed in chloroform–methanol–acetic acid–water (50:25:7:3, by vol.), then visualized with silver nitrate. (a) *myo*-inositol, R_F 0·17; (b) *cis*-inositol, R_F 0·03; (c) *epi*-inositol, R_F 0·12; (d) *muco*-inositol, R_F 0·27; (e) *neo*-inositol, R_F 0; (f) *scyllo*-inositol, R_F 0; (g) *allo*-inositol, R_F 0·14; (h) *dextro*-inositol, R_F 0·22; (i) *levo*-inositol, R_F 0·22; (j) *scyllo*-inosose, R_F 0·24; (k) *epi*-inosose, R_F 0·24; (l) quercitol, R_F 0·32; (m) (+)-pinitol, R_F 0·37; (n) conduritol, R_F 0·40.

Specificity of the transferase reaction for cyclitol substrates. A number of cyclitols and related compounds were used to investigate the specificity of the transferase reaction. System B, used for TLC of lipids, was found, unexpectedly, to serve as a suitable solvent for separation of cyclitols. Subsequently, this TLC system was used routinely to check the purity of the various cyclitols (Fig. 8). Many of the compounds from Dr. L. Anderson were also checked for purity by GLC in his laboratory.

Most of the compounds were tested both as possible substrates for CDP-diglyceride:inositol transferase, and as possible inhibitors of the incorporation of *myo*-inositol into phosphatidyl inositol (Table 3).

Of all the compounds tested (see Materials), only *scyllo*-inosose and *epi*-inosose showed detectable activity as substrates under the conditions used. These two compounds were about 10 per cent as active as *myo*-inositol, based on CMP release.

TABLE 3.—SUBSTRATES AND INHIBITORS OF THE TRANSFERASE REACTION

Substrates	Incorporation (%)	Inhibitors	Inhibition (%)
myo-inositol	100	galactinol	50
epi-inosose (*myo*-inososе-4)	10	*cis*-inositol	40
scyllo-inosose (*myo*-inososе-2)	10	*epi*-inositol	15
		scyllo-inositol	23
		myo-inositol-2-phosphate	17
		myo-inositol-L-3-phosphate	35
		myo-inositol-DL-4-phosphate	14

Other compounds tested as substrates or inhibitors and found to be inactive or of questionable activity

Isomers of *myo*-inositol	Derivatives of *myo*-inositol
allo-inositol*†	2-O,C-methylene-*myo*-inositol
neo-inositol*†	2-C-hydroxymethyl-*myo*-inositol*
muco-inositol*	*myo*-inosose-1
dextro-inositol	bornesitol (DL-1-O-methyl-*myo*-inositol)
levo-inositol	sequoyitol (5-O-methyl-*myo*-inositol)
	dambonitol (1,3-di-O-methyl-*myo*-inositol)
	2-acetamido-*myo*-inositol
	DL-4-acetamido-*myo*-inositol*
	myo-inositol-L-1-phosphate
	myo-inositol-5-phosphate
Miscellaneous	**Other cyclitols**
glucitol‡	quercitol (1,3,4/2,5 cyclohexanepentol)
galactitol‡	*scyllo*-quercitol
D-glucose‡	conduritol
D-galactose‡	quebrachitol (1-O-methyl-*levo*-inositol)
D-galactonolactone‡	pinitol (5-O-methyl-*dextro*-inositol)
lactose‡	*levo*-inositol-3-phosphate
i-erythritol	aminodeoxy-*scyllo*-inositol
D-serine	2-aminodeoxy-*neo*-inositol-hydrochloride
L-serine	streptamine (1,3-aminodeoxy-*scyllo*-inositol)
2-aminoethanol	
choline chloride	

Rates given are relative to *myo*-inositol in the absence of other substrates or inhibitors.

Compounds tested as possible substrates were substituted for *myo*-inositol in Assay B at a concentration of 2·8 μmoles/ml. To test for inhibition, the compound and *myo*-inositol were both added to the incubation mixture at a concentration of 1·4 μmoles/ml. In each case, samples with the same concentration of *myo*-inositol alone were incubated simultaneously. The reactions were stopped after 0, 5, 10, and 15 min, and the rates compared. Because of the limits of the sensitivity of the assay and the detection of minor impurities in the compounds, changes in rate of less than 3 per cent were not considered significant. If the change in rate was between 3 and 7 per cent, the effect was still considered questionable. The results for *epi*-inosose and *scyllo*-inosose represent the means of six experiments. The phosphates which inhibited were tested twice, and the other inhibitors three times.

* Questionable substrate.

† Questionable inhibitor.

‡ Not tested as substrate.

PAULUS and KENNEDY (1960) reported that these two inososes were about 25 per cent as active as *myo*-inositol with enzyme prepared from frozen chick livers; they used a similar spectrophotometric assay and also [^{14}C]-*epi*-inosose. Similar results were obtained in the present studies with enyzme from frozen chicken liver. With both chicken liver and guinea pig brain, the apparent incorporation of *epi*- and *scyllo*-inosose was verified in the present study by treating the lipid obtained following

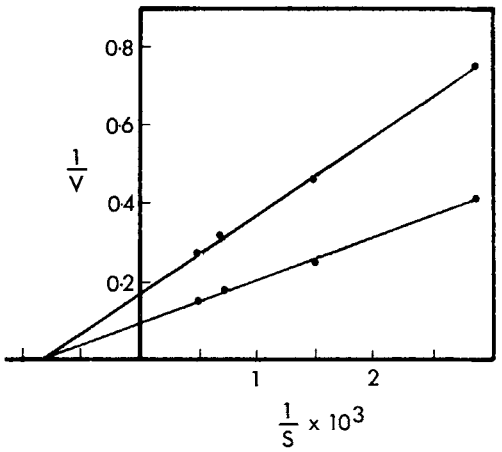

FIG. 9.—Inhibition kinetics of galactinol. Conditions as for Fig. 1, except unlabelled *myo*-inositol was used. The concentration was varied from 0·35 to 1·40 μmoles/ml (lower curve). The upper curve shows the results obtained when galactinol (1·4 μmoles/ml) was added. Each point represents the mean of duplicate determinations.

incubation with tritium-labelled sodium borohydride. A labelled lipid with chromatographic properties similar to phosphatidyl inositol was recovered from incubation mixtures containing either inosose, but not from unincubated inosose or incubation mixtures containing *myo*-inositol.

The compounds that inhibited the reaction were studied to determine the type of inhibition involved. Limited amounts (5–20 mg) of the cyclitols were available, so extensive studies were not possible. In these studies, the concentration of *myo*-inositol was varied, while that of the inhibitor was held constant. Galactinol showed non-competitive inhibition kinetics (Fig. 9). Smaller effects were seen with *epi*-, *scyllo*- and *cis*-inositol as well as the three *myo*-inositol monophosphates which inhibited. Both the K_m and V_{max} appeared to be affected by these compounds.

Since WELLS and WELLS (1967) reported that galactitol inhibited the conversion of phosphatidic acid to phosphatidyl inositol, the effects of this compound on the transferase reaction were examined in more detail. Galactose was used in these experiments for comparison. At concentrations 30 times that of *myo*-inositol, a very slight inhibition (about 5 per cent) was observed with both compounds. Preincubation of the enzyme with galactitol before addition of enzyme had no effect.

DISCUSSION

Studies on the distribution of CDP-diglyceride:inositol transferase activity in several guinea pig organs (Table 1) showed that the specific activities in the six tissues studied were similar. The greatest total activity was observed in liver, followed by lung, brain and kidney, heart and spleen. This can be compared with the data reported by AGRANOFF *et al.* (1958) for the incorporation of [2-^{3}H]*myo*-inositol in guinea pig *in vivo*. Total incorporation of radioactivity into a chloroform–methanol soluble fraction was greatest in liver, in agreement with the present spectrophotometric *in vitro* study. However, kidney had four times as much activity as lung *in vivo*. Heart and spleen had low activities, but the value for brain was very low, compared to

the present study. These differences may be due to differences in uptake and to dilution with endogenous pools *in vivo*.

CDP-diglyceride has been isolated from tissues only after preincubation with labelled precursors and addition of synthetic carrier, so that no information concerning its fatty acid substituents is presently available. The fatty acid content of phosphatidyl inositol from all tissues studied includes high amounts of stearic acid and arachidonic acid (HENDRICKSON and BALLOU, 1964; GLENDE and CORNATZER, 1966). This could very well resemble the fatty acid-content of naturally occurring CDP-diglyceride. Synthesis of CDP-diglyceride from egg lecithin provided a substrate with unsaturated fatty acids in the 2-position. The reported fatty acid content of egg lecithin is 36 per cent palmitic acid, 14 per cent stearic acid, 36 per cent linoleic acid and 14 per cent linolenic acid (TATTRIE, 1960). The observation in these studies that decanoate gives the higher activity suggests that the physical state of the substrate is of overriding importance in the *in vitro* system.

CDP-diglyceride:inositol transferase from guinea pig brain is similar to enzyme from other sources. The high pH optimum (8·6) may be compared to the optimum of 8·0 reported by PAULUS and KENNEDY (1960) for the synthesis of phosphatidyl inositol in a microsomal fraction from chick liver, with CDP-dipalmitin as substrate. PROTTEY and HAWTHORNE (1967) reported an optimal pH of 7·5 for the transferase in a microsomal fraction from guinea pig pancreas, with CDP-diglyceride prepared from bovine brain lecithin as substrate. In contrast to the results reported for chick liver or guinea pig pancreas, Mg^{2+} stimulates the transferase from brain to a greater extent than Mn^{2+}. PROTTEY and HAWTHORNE (1967) reported that bovine serum albumin stimulated the transferase from pancreas, as well as the acylation of α-glycerol-phosphate. The albumin may stimulate by removing inhibitory fatty acids as they suggest, or may exert a 'detergent-like' effect, allowing more effective combination of enzyme and substrates. The ratio of albumin to microsomal protein was about 20:1 in both the pancreas and present brain studies.

The cyclitol specificity of CDP-diglyceride:inositol transferase was first investigated by PAULUS and KENNEDY (1960) in a microsomal fraction from chicken liver, also using a spectrophotometric assay. Of the five cyclitols tested, *dextro*-inositol, *levo*-inositol, and pinitol had no activity as substrates while *scyllo*-inosose and DL-*epi*-inosose-2 each showed about 25 per cent as much activity as *myo*-inositol. L-Serine, choline, ethanolamine, glycerol, glucitol, L-iditol, D-mannitol, and ribitol had no activity in an assay in which the release of labelled CMP from cytidine-[^{32}P]-P-dipalmitin was measured.

In the present study, a large number of compounds was tested both as substrate and inhibitors of the transferase reaction in the microsomal fraction of guinea pig brain. The results show that the transferase is highly specific for *myo*-inositol, in agreement with the tissue culture studies of EAGLE, OYAMA, LEVY and FREEMAN (1957), who demonstrated specificity for *myo*-inositol in maintenance of culture growth. Inosose and DL-*epi*-inosose-2 could be demonstrated to serve as substrates in place of *myo*-inositol with the brain enzyme. Verification of the formation and possible occurrence of phosphatidyl inososes in brain would, of course, require isolation and identification of these compounds. *Scyllo*-inosose (*myo*-inosose-2) was found in rat sciatic nerve and calf brain (SHERMAN, STEWART, SIMPSON and GOODWIN, 1968).

Another possible substrate might be 2-C-hydroxy-methyl-*myo*-inositol, since WEINHOLD (1961) observed that this cyclitol derivative is incorporated into the lipid fraction of a number of organs in rat *in vivo*, although incorporation into brain lipids was not examined. This compound neither inhibited nor served as a substrate in the present study.

With guinea pig brain transferase, several compounds inhibited the incorporation of *myo*-inositol into lipid. The isomers of *myo*-inositol which were inhibitory were *cis*-, *epi*- and *scyllo*-inositol. These isomers differ from the five that did not inhibit in that they possess axial hydroxyls on only one side of the ring as does *myo*-inositol, a property which could allow a closer approach to the site of reaction on the enzyme than other inositols.

The most potent inhibitor of transferase activity found was galactinol, an inositol-α-D-galactopyranoside. *Myo*-inositol is glycosidically linked at its L-3 position to the galactose. The L-1 position is the one phosphorylated in phosphatidyl inositol, while the L-3 (D-1) position retains the phosphate group after cyclization of D-glucose-6-phosphate (EISENBERG, 1967). Galactinol was isolated from sugar beets (KABAT, MACDONALD, BALLOU and FISCHER, 1946) and enzymes which form it from *myo*-inositol and UDP-galactose are present in plants (FRYDMAN and NEUFELD, 1963). Its function is unknown. It is reported here for the first time to have an inhibitor property.

Another compound substituted in the L-3 position, *myo*-inositol-L-3-phosphate, inhibited the enzyme more than the other monophosphates. All of the *myo*-inositol monophosphates were tested as substrates, but none substituted for *myo*-inositol. For brain at least, inositol monophosphate does not therefore seem to provide an alternate pathway for phosphatidyl inositol phosphate. The finding that none of the inositol monophosphates served as substrates does indicate, however, that the microsomal enzyme preparation is relatively free of phosphatases which would liberate inositol to be used as a substrate. EISENBERG, BOLDEN and LOEWUS (1964) found a very active inositol phosphatase in the supernatant fraction of testis homogenates.

Studies on the brain transferase with galactose and galactitol gave no evidence of inhibition of incorporation of *myo*-inositol into lipids. Further investigation was prompted by the recently reported studies of WELLS and WELLS (1967) with rats on high galactose diets. They found that galactitol inhibited both the synthesis of inositol from glucose and the incorporation of inositol into phospholipid *in vivo*. In their studies, the incorporation of [2-^{14}C]inositol into lipids was investigated *in vitro* by incubating a microsomal fraction from rat brain with CTP and phosphatidic acid. WELLS and WELLS (1967) thus measured the combined activity of CTP:phosphatidate-cytidyl transferase and CDP-diglyceride:inositol transferase. In the presence of $1{\cdot}06 \times 10^{-4}$ M-galactitol, the incorporation of $4{\cdot}16 \times 10^{-4}$ M-inositol was inhibited by 50 per cent. Evidence for competitive inhibition was obtained. In our assay for CDP-diglyceride:inositol transferase in brain, only 5 per cent inhibition was observed with high concentrations of galactitol. Galactose caused a similar inhibition; this suggests a non-specific effect. It is possible that the inhibitory effect of galactitol is dependent on assay conditions, that a different enzyme is inhibited by galactitol (presumably CTP:phosphatidate transferase), or that a species or age dependency exists with respect to galactitol inhibition. Also, since the assay of WELLS and WELLS (1967) uses incorporation of labelled inositol into lipid, the incorporation observed may include inositol- phosphatidyl inositol exchange (PAULUS and KENNEDY, 1960)

and galactitol could inhibit this exchange rather than *de novo* synthesis of phosphatidyl inositol.

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