Since the isolation of myo-inositol from biological sources over a hundred years ago, little has been reported concerning its metabolism. Experiments in nutrition have implicated inositol variously as a vitamin (1) and as a lipotropic agent (2); and more recently inositol has been identified as a soluble factor required for the survival of human cells grown in tissue culture (3). The presence of inositol lipides in mammalian tissue in the form of diphosphoinositide (4) and monophosphoinositide (inositol phosphatide) (5) has been established. Recently, several investigators have confirmed the observation that the exchange of inorganic P32 with the phospholipides of tissue slices and homogenates occurs at a greater rate in the inositol lipide fraction than in the ethanolamine-, choline-, or serine-containing fractions (6–9).

In the present study, the metabolism of inositol was investigated by means of a spectrophotometric method for the determination of inositol and by the use of tritium-labeled inositol. A particulate preparation obtained from lyophilized guinea pig kidney mitochondria which catalyzed the incorporation of inositol into inositol phosphatide in the presence of cytidine nucleotide and Mg++ was examined in detail.

EXPERIMENTAL

Myo-inositol and scyllo-inosose were purchased from the Nutritional Biochemicals Corporation. Nucleotides were obtained from the Pabst Laboratories and from the Sigma Chemical Company, and Tween 20 (polyoxyethylene sorbitan monolaurate) from the Atlas Powder Company. Dimyristin, dimyristoyl-

The enzymatic synthesis of inositol phosphatidyl
dohe. Agranoff, Roy M. Bradley, and Roscoe O. Brady

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The final product contained a small amount of platinum and had a melting point of 224°C; the mixed melting point was 224°C. Microanalysis was as follows:

\[
\text{C}_{44}\text{H}_{116}\text{O}_1; \\
\text{Calculated:} \quad \text{C} 40.00, \text{H} 6.71 \\
\text{Found (corrected for residue). } \text{C} 40.09, \text{H} 6.82
\]

Inositol-H3 (randomly labeled) was prepared by the method of Wilzbach (13). 1 gm. of inositol was exposed to 2.0 curies of tritium gas for 2 weeks at room temperature. The product was lyophilized and reconstituted to constant specific activity. The purified inositol was completely stable and had a specific activity of 15 μc. per μmole. Tritium-labeled inositol and lipides were counted in steel planchets in a gas flow Geiger counter. The material to be counted was pipetted onto a planchet which was then flooded with ethanol and dried alternately three times in order to obtain uniform distribution of the sample. Satisfactory reproducibility and a counting efficiency of about 30 per cent was observed with samples weighing less than 10 μg, in an area of 8 cm². Self-absorption was measured by means of an internal standard (14). Reproducibility was poor when the self-absorption was over 60 per cent. Radioactivity in proteins was detected in a liquid scintillation counter. CMP was also labeled by the method of Wilzbach. A vial containing 40 mg. of CMP (sodium salt, Sigma Chemical Company) was exposed to 2 curies of tritium gas for 1 week. The product contained 245 mc. of tritium. Solution in water followed by evaporation reduced the total radioactivity to 14 mc. The product was then adsorbed on a column of Dowex 1-formate and was eluted with 0.1 M formic acid. The eluate was evaporated to dryness and subjected to distribution in the two phases of chloroform-methanol-water, 1:1:1, as described below.

**Determination of Inositol**—The reaction of inositol with sodium periodate was measured. The reduction of periodate was observed spectrophotometrically (15). A sample containing 0.01 to 0.3 μmole of free inositol in 1.0 ml. was mixed with 1.0 ml. of 0.3 N barium hydroxide and heated at 100°C for 15 minutes. This treatment was sufficient to oxidize sugars present to acidic derivatives. 1 ml. of 5 per cent zinc sulfate deproteinizing reagents were prepared as described by Nelson (11).

Preparation of Labeled Compounds—Inositol-2-H3 was prepared from scyllo-inosose by the method described by Posternak (12) with the exception that H2O was substituted for D2O.

A preliminary report of this investigation was presented before the American Society of Biological Chemists at its Forty-ninth Annual Meeting at Philadelphia, April, 1958.

† Present address, Max-Planck Institut für Zellchemie, Munich, Germany.
consistent and was compensated for by an inositol standard taken through the same procedure.

1.0 ml of eluate was added to 1.0 ml of 1 m potassium acetate buffer, pH 4.7, in a 4.0 ml silica cuvette, followed by 0.3 ml of 0.01 m sodium m-periodate. A spectrophotometric reading was immediately taken at 260 mp. The reaction was allowed to continue at room temperature until there was no further decrease in optical density. This step generally took 20 to 30 minutes. The decrease in optical density represented oxidation of glycerol. Inositol was not oxidized under these conditions. The reaction mixture was then heated in the dark at 65° for 2 hours, cooled, and read again. The difference in the optical densities before and after heating was due to oxidation of inositol. 6 equivalents of periodate were consumed per equivalent of inositol. A linear relationship between optical density and concentration was observed (Fig. 2) when less than two-thirds of the periodate was consumed. Very little formaldehyde was produced. Since oxidation of inositol yields essentially only formic acid, the glycerol content could be estimated at this point by means of chromotropic acid determination of the formaldehyde present (16). A somewhat different technique was used for the determination of total free and combined inositol. The sample was first hydrolyzed with 6 N HCl for 48 hours (4), evaporated to dryness, and then passed through a mixed column of Amberlite IR-120 and IRA-400 (OH). Glycerol was decomposed by heating at 180° for 2 hours. The sample was then dissolved in water and passed through the same column, evaporated to a volume of 1.0 ml., and treated with periodate as above. Good agreement with the microbiological assay was obtained.

Preparation of Enzyme System—Four to five 200 gm. NIH-strain male guinea pigs were decapitated. The kidneys were homogenized in 4 volumes of a solution containing 0.13 M KCl, 0.003 M MgSO4, and 0.012 M potassium phosphate buffer, pH 7.4. The cell-free preparation was centrifuged at 600 X g for 1 minute and the overlying suspension was centrifuged at 7500 X g for 20 minutes. The sedimented particles were resuspended in a volume of the KCl-MgSO4-phosphate buffer equal to that of the discarded supernatant solution and recentrifuged. This washing procedure was repeated. The preceding steps were all performed at 2°. The sedimented fraction was resuspended in a small volume of water and lyophilized. The dried preparation was stored at -22° under vacuum. Enzymatic activity diminished slowly over a period of 2 weeks. The dried residue was prepared for incubation by trituration with 100 volumes of the KCl-MgSO4-phosphate solution, washing twice in the buffer by centrifugation, and finally resuspension in the buffer to give a concentration of 15 mg per ml of the lyophilized preparation. All incubations were performed at 37° under nitrogen.

Isolation of Lipides—Phospholipides were isolated by a modification of the method of Dawson (17). 1 ml of 1 per cent bovine serum albumin was added to 0.3 ml of incubation mixture followed by 2.0 ml of cold 20 per cent trichloroacetic acid. The mixture was filtered through Whatman No. 42 paper in a Buchner funnel and refiltered until the filtrate was clear. The precipitate was washed with 50 ml of cold 10 per cent trichloroacetic acid and finally with 100 ml of cold water. The filter paper containing the precipitate was transferred to a 10 X 50 mm. Soxhlet thimble and dried under vacuum for 1 hour. The thimble was then extracted with 15 ml of chloroform-methanol, 1:1, for 3 hours in a micro-Soxhlet apparatus. From 0.1 to 0.2 ml of the extraction mixture was plated and counted as described.

RESULTS

Uptake and Release of Inositol by Tissue Preparations—Many tissue slice preparations, particularly brain and testis, released inositol into the medium upon incubation. This release was depressed by the addition of exogenous inositol. Only in preparations of kidney tissue slices was it possible to demonstrate a net decrease in free inositol in the incubation medium (Table I). This tissue was therefore selected for further studies with isotopic inositol.

Disposition of Inositol-2-H3 in Vivo—After the injection of inositol-2-H3 into a rat, radioactivity was detected in trichloroacetic acid-soluble and trichloroacetic acid-insoluble tissue fractions. The acid-soluble fractions contained inositol and a radioactive anionic component which did not contain inositol. The acid-insoluble fractions contained a component extractable with methanol-chloroform as well as an unextractable component.

1 H. Eagle and B. W. Agranoff, unpublished observations.
The distribution of the latter three components in various organs is shown in Table II. In similar experiments, the acid-insoluble fractions were first extracted with ether, then ethanol, and finally with chloroform-methanol (1:1). Although this technique did not permit a quantitative separation of classes of lipides, striking differences were noted in solubility of lipides from different organs. About 70 per cent of the radioactivity in the kidney lipide was recovered in the ethanol-soluble fraction, whereas 90 per cent of the radioactivity recovered from liver was in the ether-extractable fraction. In each of the three categories listed in Table II, the greatest incorporation per gm. of tissue was in the kidney. Isolation of total inositol as described in "Experimental" revealed that the radioactivity present in the kidney lipide was present as inositol. The total radioactivity recovered from all of the fractions represented 2.28 per cent of the total radioactivity recovered from the incubation medium.

Identification of Anionic Material—The anionic component of the chloroform-acetic acid-soluble fraction was isolated as a single peak by gradient elution from a Dowex-1-formate column. The supernatant solution obtained after centrifugation of rat or guinea pig kidney at 100,000 X g for 30 minutes was dissolved in NaOH and treated with saturated lead acetate. The filtrate was cleared with barium acetate, applied to Dowex-1-formate, and eluted with 2 m formic acid.

The product was subsequently identified as glucuronic acid, in agreement with the observations of Charalambous (20). The residue insoluble in chloroform-methanol was then dissolved in Hyamine and counted by liquid scintillation techniques (18, 19). The acid-soluble fraction was neutralized with 10 N NaOH and treated with saturated lead acetate. The filtrate was cleared with barium acetate, applied to Dowex-1-formate, and eluted with 2 m formic acid.

The product did not indicate that this derivative was converted in vitro to inositol or to inositol lipide. The anionic product in liver has not been identified.

Incorporation of Inositol-3-3H into Inositol Lipide—Inositol incorporation into a lipide fraction was previously observed in homogenates of guinea pig kidney (21). Enzymes present in the particulate fraction which sedimented at 7500 X g catalyzed the rapid rate of incorporation during the 1st hour of incubation (Fig. 3). Nucleotide Requirements—Although the requirement for the cytidine moiety was specific, all of the cytidine nucleotides were effective to some extent. CDP-choline was the most effective nucleotide at the concentrations of nucleotide used (1.0 X 10⁻⁴ M). The relative efficacy observed in one typical preparation was: CDP-choline, 1.0; CMP, 0.39; CTP, 0.27; ATP, 0.17; cytidine, 0. However, these relative values were found to vary with the type of buffer used. In an experiment in which the residue was suspended in a buffer in which the phosphate was replaced with Tris, pH 7, CDP-choline and CMP stimulated inositol incorporation into lipide to the same extent as they did in the presence of phosphate buffer, whereas GDP and CTP were practically inactive. An increase in phosphate concentration (potassium phosphate buffer, pH 7) from 8 X 10⁻⁴ M to 8 X 10⁻³ M
The enzymatic activity of kidney preparations was variable. It was observed that the weight of lyophilized preparation obtained from a given number of guinea pig kidneys was a good index of the activity of the inositol incorporation system. A high yield corresponds with high specific activity of the enzyme preparation. The total weight as well as the enzymatic activity per unit weight of the lyophilized residue could be reduced several-fold by fasting the animals for 16 hours before they were killed. The preparations with low enzymatic activity were stimulated by the addition of the boiled residue from an active preparation, but the preparations having high activity were not further stimulated by the addition of the boiled material. In addition, stimulation by phosphatidic acids was more marked in contrast with analogous diglycerides, phosphatidic acids were found to stimulate incorporation of inositol. The experiment with Preparation 3, Table III, was performed after the optimal Mg++ and phosphate concentrations had been established. It is clear that the stimulatory effect of the phosphatidic acid is not derived from the release of inorganic phosphate.

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diet, as well as those enzymes which catalyze the incorporation of inositol into lipide.

Formation of Cytidine-Lipide—CMP-H₃ was incubated with the kidney residue preparation in the absence of inositol. Radioactivity was recovered in the chloroform-methanol extract of the trichloroacetic acid-insoluble fraction of the incubation mixture (Table IV). Solvent distribution studies revealed that this radioactive material, soluble in chloroform methanol, represented a previously undescribed form of cytidine nucleotide (Table V). After alkaline hydrolysis (6) radioactive material was found to be distributed preferentially into the aqueous phase of the two-phase system. The radioactivity in the aqueous phase was readily adsorbed by charcoal from a dilute acetic acid solution (pH, 3 to 4) and was eluted with ethanol-water-1 N NH₄OH (3:3:1). Electrophoresis on paper of the eluate in ammonium acetate buffer indicated that radioactivity migrated with CMP and occasionally, but not consistently, there was also a radioactive spot which migrated with CDP-glycerol.

DISCUSSION

Experiments performed in vitro indicated that there was a rapid uptake of inositol from the incubation medium by guinea pig kidney tissue slices. Isotopic studies revealed that the cause of this uptake was the conversion of inositol to glucuronic acid as well as the incorporation of inositol into a phosphatide and rapid uptake of inositol from the incubation medium by guinea pig kidney tissue slices. Isotopic studies revealed that the cause of this uptake was the conversion of inositol to glucuronic acid as well as the incorporation of inositol into a phosphatide and

**Table III**

**Stimulation of Incorporation of Inositol into Lipide by Phosphatidic Acids**

Incubation mixtures contained 0.2 ml. of the enzyme preparation in KCl-MgSO₄-phosphate buffer, 0.2 µmole of CDP-choline, and 0.2 µmole of inositol-H₃ (2.0 X 10⁶ c.p.m. The lipide esters were added to Tween 20 and water to yield a suspension containing 6 mg. of lipide and 5 mg. of Tween 20 in 0.2 ml., of which 0.03 ml. was added to the incubation mixtures as indicated. For the Tween 20 control incubations, 0.03 ml. of a solution containing 25 µg. of Tween 20 in 1.0 ml. of water was added. Preparations 3 contained in addition 10 µmoles of MgSO₄ and 20 µmoles of potassium phosphate buffer, pH 7.0. Total incubation volume in each case was 0.3 ml.

<table>
<thead>
<tr>
<th>Preparations</th>
<th>Radioactivity in Lipide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation 1</td>
<td>c.p.m.</td>
</tr>
<tr>
<td>None</td>
<td>14,440</td>
</tr>
<tr>
<td>Choline, 1.0 µmole</td>
<td>15,020</td>
</tr>
<tr>
<td>Glycerol, 1.0 µmole</td>
<td>17,060</td>
</tr>
<tr>
<td>L-α-glycerophosphate, 1.0 µmole</td>
<td>12,940</td>
</tr>
</tbody>
</table>

Preparation 2

<table>
<thead>
<tr>
<th>Preparation 2</th>
<th>Radioactivity in Lipide</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10,720</td>
</tr>
<tr>
<td>Tween 20</td>
<td>9,990</td>
</tr>
<tr>
<td>Tween 20 + dimyristoyl-α-glycerophosphate</td>
<td>16,100</td>
</tr>
<tr>
<td>Tween 20 + d-α,β-dimyristin</td>
<td>8,390</td>
</tr>
<tr>
<td>Tween 20 + inositol phosphate</td>
<td>2,250</td>
</tr>
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</table>

Preparation 3

<table>
<thead>
<tr>
<th>Preparation 3</th>
<th>Radioactivity in Lipide</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>61,150</td>
</tr>
<tr>
<td>Tween 20</td>
<td>19,880</td>
</tr>
<tr>
<td>Tween 20 + dioleoyl-α-glycerophosphate</td>
<td>42,750</td>
</tr>
<tr>
<td>Tween 20 + d-α,β-diolein</td>
<td>23,820</td>
</tr>
</tbody>
</table>

**Table IV**

**Incorporation of CMP-H₃ into lipide extract**

Incubation mixtures contained 0.2 ml of enzyme in the homogenizing buffer to which were added 10 µmoles of MgSO₄, 20 µmoles of potassium phosphate buffer, pH 7, and 0.3 µmole of CMP-H₃ (4.2 X 10⁶ c.p.m.) in a total volume of 0.3 ml. Incubation conditions and lipide isolation techniques were those described under "Experimental."

<table>
<thead>
<tr>
<th>Length of incubation</th>
<th>Radioactivity in chloroform- methanol extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td>c.p.m.</td>
</tr>
<tr>
<td>0</td>
<td>999</td>
</tr>
<tr>
<td>30</td>
<td>1047</td>
</tr>
<tr>
<td>60</td>
<td>1508</td>
</tr>
<tr>
<td>120</td>
<td>1733</td>
</tr>
</tbody>
</table>

**Table V**

**Solvent distribution ratios of radioactivity after incubation of CMP-H₃ with kidney enzyme preparation**

An incubation mixture 5-fold the quantity of that described in Table IV was incubated for 2 hours, treated with trichloroacetic acid, and extracted with chloroform-methanol, 1:1, as described previously. The extract was shaken for 15 minutes with ⅔ volume of 0.03 M potassium phosphate buffer, pH 7. The two phases were separated by centrifugation and aliquots of the upper (aqueous) and lower (chloroform) layers were counted. The upper phase was replaced with a new aqueous layer which was obtained from a previously equilibrated solvent system. The mixture was again shaken and centrifuged to obtain a new distribution ratio. This step was repeated and the purified chloroform layer was taken to dryness and subjected to mild alkaline hydrolysis with methanolic NaOH (6). The hydrolysate was passed through a column of Dowex 50, evaporated to dryness, extracted with ether to remove unhydrolyzed material, and again subjected to distribution in the chloroform methanol-aqueous phosphate buffer mixture.

<table>
<thead>
<tr>
<th>Step</th>
<th>Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. CMP-H₃*</td>
<td>400:1</td>
</tr>
<tr>
<td>2. Chlороform-methanol extract after incubation with CMP-H₃</td>
<td>8812</td>
</tr>
<tr>
<td>3. Chlороform-methanol extract after replacement of aqueous phase</td>
<td>8688</td>
</tr>
<tr>
<td>4. Chlороform-methanol extract after second replacement of aqueous phase</td>
<td>7452</td>
</tr>
<tr>
<td>5. Ether extract after hydrolysis</td>
<td>1338</td>
</tr>
<tr>
<td>6. Hydrolysate</td>
<td>3024</td>
</tr>
<tr>
<td>7. Hydrolysate after replacement of chlороform phase</td>
<td>2439</td>
</tr>
</tbody>
</table>

* Solvent distribution ratios were also obtained with nonisotopic compounds by comparison of ultraviolet absorption of the two phases. Ratios of 101:1 to 465:1 were observed for all derivatives examined: cytosine, cytidine, CMP, CDP, CDP-choline, and CDP-glycerol.
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FIG. 5. Proposed relationship of the pathways for inositol phosphatide and lecithin synthesis. CDP-choline may react with either phosphatidic acid to produce a liponucleotide or with diglyceride to form lecithin. The liponucleotide and inositol may then react to form inositol phosphatide.

The studies with enzyme preparations represent another example of the implication of cytidine nucleotides in the formation of phospholipides. A striking characteristic of the cytidine nucleotide requirement for the enzyme system described here is the fact that many cytidine nucleotides are effective. The participation of CDP or CTP requires the presence of inorganic phosphate. Since inositol phosphatide is actively formed in the absence of phosphate, e.g. in Tris buffer, and since CDP-choline was more effective than CMP in both Tris and phosphate buffer, it would seem that CDP-choline is the reactant nucleotide in the inositol-incorporating system. The effectiveness of CMP can be explained by its conversion to CDP-choline by diglyceride transferase (31) in the presence of lecithin in the kidney residue.
Chromatographic evidence revealed that the inositol lipide formed was the monophosphatide similar to that isolated from plant sources and from horse liver (5).

In contrast to the enzymes involved in the biosynthesis of choline and phosphatidyl ethanolamine (27), inositol appears to be incorporated into lipide without previous phosphorylation. No evidence for the presence of CDP-inositol was found in experiments with allicic or cell-free preparations. Furthermore, the addition of d-α,β-diglycerides did not stimulate the incorporation of inositol as would be expected by analogy with the reaction of CDP-choline (27), whereas phosphatidic acids did stimulate the system.

The participation of phosphatidic acids as lipide acceptors in this system suggested that the primary reaction was a transphosphorylation of CDP-choline and a phosphatide acid to form CDP-d-α,β-diglyceride as follows: CDP-choline + phosphatide acid → CDP-d-α,β-diglyceride + P-choline. The CDP-d-α,β-diglyceride then would react with a hydroxyl group of inositol in a pyrophosphorolytic reaction analogous to the reaction of CDP-choline with the free hydroxyl group of the diglyceride in the “glyceride transferase” reaction (27): CDP-d-α,β-diglyceride + inositol → inositol phosphatide + CMP.

Evidence for a cytidine-lipide was demonstrated by the solvent distribution properties of a product obtained after incubation of CMP-IP with the enzyme preparation. Hydrolysis yielded CMP and probably CDP-glycerol.

The metabolic role of phosphatidic acid has remained questionable since its identification as the product of an enzymatic digestion of di-glyceride (27). The rapid incorporation of Pa2 into the inositol lipides and phosphatidic acid, which has been observed in vitro (6–9), might be explained by the presence of Mg++ at higher concentrations than is found in vivo. In addition, the phosphorus of phosphatidic acid and inositol phosphatide are derived from glycerophosphate, whereas the phosphorus of lecithin is derived from the phosphorylated base, phosphoryl choline, or phosphoryl ethanolamine. The metabolic interrelationship is summarized in Fig. 5.

SUMMARY

A spectrophotometric method for the determination of inositol is described. Studies in vitro indicated active utilization of inositol by kidney tissue. Experiments with tritium-labeled inositol demonstrated the incorporation of inositol into the lipides of all tissues studied.

An enzyme system present in the insoluble residue of guinea pig kidney mitochondria is described which catalyzes the incorporation of inositol into phosphatidic acid in the presence of Mg++ and cytidine di-phosphate-choline or cytidine-5′-phosphate. The incorporation of inositol was stimulated by the addition of phosphatidic acids, but not by diglycerides. A mechanism is proposed for the synthesis of inositol phosphatide.

REFERENCES


4. Deacylation of the CDP-diglyceride would yield CDP-glycerol. Attempts at deacylation in methanolic NaOH as well as under milder conditions converted over 80 per cent of authentic CDP-glycerol to CMP. The relative rates of pyrophosphate cleavage and deacylation may account for the varying yields of CDP-glycerol from hydrolysis.
The Enzymatic Synthesis of Inositol Phosphatide
Bernard W. Agranoff, Roy M. Bradley and Roscoe O. Brady


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