The Pathway of myo-Inositol 1,3,4-Trisphosphate Phosphorylation in Liver

IDENTIFICATION OF myo-INOSITOL 1,3,4-TRISPHOSPHATE 6-KINASE, myo-INOSITOL 1,3,4-TRISPHOSPHATE 5-KINASE, AND myo-INOSITOL 1,3,4,6-TETRAKISPHOSPHATE 5-KINASE*

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Inositol 1,3,4-trisphosphate (Ins(1,3,4)P₃) metabolism has been studied in liver homogenates and in 100,000 x g supernatant and particulate fractions. When liver homogenates were incubated in an "intracellular" medium containing 5 mM MgATP, equal proportions of Ins(1,3,4)P₃ were dephosphorylated and phosphorylated. Two inositol tetrakisphosphate (Ins₄P) products and an inositol pentakisphosphate (Ins₅P) were detected. The Ins₄P isomers were unequivocally identified as inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄) and inositol 1,3,4,6-tetrakisphosphate (Ins(1,3,4,6)P₄) by high performance liquid chromatography separation of inositol phosphates, periodate oxidation, alkaline hydrolysis, and stereospecific polyol dehydrogenase. Ins(1,3,4)P₃ 5-kinase is a novel enzyme activity and accounted for 16% of the total Ins(1,3,4)P₃ phosphorylation. Ins(1,3,4,6)P₃ was also shown to be further phosphorylated to inositol 1,3,4,5,6-pentakisphosphate (Ins(1,3,4,5,6)P₅) by a kinase not previously known to occur in liver. About 75% of Ins(1,3,4)P₃ kinase activities were soluble and were partially purified by anion-exchange fast protein liquid chromatography. The two Ins(1,3,4)P₃ kinase activities eluted as a single peak that was well resolved from Ins(1,3,4,5)P₄ phosphatase, Ins(1,3,4,6)P₄ 5-kinase, and Ins(1,3,4,5)P₄ 5-phosphatase activities. A further novel observation was that only 10% of Ins(1,3,4,5,6)P₅ inhibited Ins(1,3,4)P₃ kinase activities by 60%.

It is well known that receptor-mediated activation of phospholipase C promotes hydrolysis of phosphatidylinositol 4,5-bisphosphate to yield two intracellular messengers, Ins(1,4,5)P₃ and 1,2-diacylglycerol; the inositol phosphate mobilizes intracellular Ca²⁺ stores, and 1,2-diacylglycerol activates protein kinase C, and these effects are important in the regulation of cell function (see Ref. 1 for review). The metabolism of these intracellular messengers terminates the signaling response. Additionally, the complexities of the pathway by which Ins(1,4,5)P₃ is metabolized (see Ref. 2 for a review) has led to intensive research into the possibility that some of the ensuing products also have important cellular activities. For example, Ins(1,3,4,5)P₄, which is formed from Ins(1,4,5)P₃ by the action of a 3-kinase (3-5), may modulate cellular Ca²⁺ fluxes (6).

Among some of the recent developments in inositol phosphate metabolism has been the discovery by two independent laboratories that in liver (7) and adrenal glomerulosa cells (8) the dephosphorylated metabolite of Ins(1,3,4,5)P₄, i.e. Ins(1,3,4)P₃, is itself rephosphorylated. In order to understand the functions and control of these reactions, it is first necessary to characterize the products. The two groups of workers (7, 8) that initially reported Ins(1,3,4)P₃ could be phosphorylated noted that the resultant Ins₄P could be chromatographically resolved from Ins(1,3,4,5)P₄. Further experiments indicated that the Ins₄P did not have two adjacent hydroxyl groups (7, 8, 10) since it was resistant to oxidation by periodate (see also Refs. 11-13). The authors of these studies proposed that the Ins₄P was Ins(1,3,4,6)P₄ (7, 8, 10), but they made the untested assumption that there was no isomerization associated with the phosphorylation, so these identifications are not definitive. Subsequently, Stephens et al. (9) found that brain also had the capacity to phosphorylate Ins(1,3,4,4)P₄. These workers made no assumptions concerning isomerization, but because of their use of more elaborate techniques, they were able to prove that Ins(1,3,4,6)P₄ was formed from Ins(1,3,4,4)P₄. However, the proliferation of enzymes involved in inositol phosphate metabolism makes it unreliable to extrapolate results from brain and apply them to other tissues.

These uncertainties prompted a definitive analysis of the products of Ins(1,3,4)P₃ phosphorylation in liver. The metabolic pathway has been characterized by incubating liver homogenates, and subfractions derived therefrom, in media designed to imitate physiological ionic strength and pH. This led to the novel observation that there were two Ins₄P products, which were unequivocally determined to be Ins(1,3,4,5)P₄ and Ins(1,3,4,6)P₄.

When the Ins(1,3,4)P₃ phosphorylation pathway was discovered (7, 8), it was suggested (7) that it might produce precursors for Ins₄P. Such reactions may be physiologically important since Ins₄P has been detected in a number of cell types (e.g., Refs. 9, 14-19, and 31) and may be an extracellular signal (19). Both adrenal glomerulosa cells (20) and brain (9) can phosphorylate Ins(1,3,4)P₃ to Ins₄P. In brain this Ins₄P is Ins(1,3,4,5,6)P₅ (9); the isomer formed in the adrenal preparations has not yet been identified. The relationship between
InsP₃ synthesis and receptor-mediated inositol phosphate production has become more complicated following the observation that InsP₄ can also be formed by phosphorylation of Ins(3,4,5,6)P₄, the origin of which is currently unclear (16). In an effort to unravel some of these problems in liver, the importance of InsP₃ synthesis from Ins(1,3,4)P₄ has been studied in this report.

**EXPERIMENTAL PROCEDURES**

**Materials**—[¹⁴C]Xyitol and [³H]Ins, purchased from Amer sham International plc (United Kingdom). [¹⁴C]Ins(1,3,4,5)P₄ and [³H]Ins(1,3,4,5)P₄ were purchased from Du Pont-New England Nuclear. L-[¹⁴C]Altritol was produced from [³H]Ins(1,3,4,5)P₄ by periodate oxidation, reduction, and dephosphorylation (see below); up to 99% of the [³H]labeled products were altritol, indicating the original [³H]Ins(1,3,4)P₄ was not significantly contaminated. Samples of [³H]InsP₃ (isomer unknown) and periodate-sensitive [³H]InsP₄ were isolated from AR42J cells labeled with [³H]inositol. These were desalted as described below. This InsP₃ was the third of those eluting in the HPLC system used in Ref. 17 and may be [²H₅]Ins(1,3,4,5)P₄ (see Ref. 17 and “Results”).

Saponin and nonradioactive Ins(1,3,4,5)P₄ were purchased from Calbiochem. Dithiothreitol, t-tosyl-L-phenylalanine chloromethyl ketone, and bacitracin were purchased from Sigma. L-Altritol was produced from L-tallose with sodium borohydride (see below). Sodium periodate, periodic acid, and sodium hydrosulfite were purchased from Aldrich. Erythrocyte ghosts were prepared as described in Ref. 21.

**Preparation of Liver Homogenates and Subfractions—Liver homogenates** (30% w/v) were prepared from 200–250-g Sprague-Dawley rats as described previously (7), except that the homogenization medium was 0.25 M sucrose, 10 mM BisTris (pH 7.2 with NaOH), 5 mM sodium azide, 10 μM leupeptin, 4 mg/ml iodoacetamide, 0.1 mM trypsin inhibitor, and 20 μg/ml phenylmethylsulfonyl fluoride. 100,000 × g supernatants and pellets, each resuspended to a 30% concentration, were acid-quenched (7), and the [³H]InsP₃ was isolated by HPLC (9). Typical elution times were: Ins-l-P/Ins-3-P, 35 min; Ins-2-P, 40 min; Ins-5-P, 50 min; Ins-C-P/Ins-B-P, 53 min.

**Characteristics of Ins(1,3,4,5)P₄**—Samples were incubated with 10 nM [³H]Ins(1,3,4,5)P₄ in the absence of incubation buffer without dehydrogenase: either 6000 dpm of genuine d-[³H]altritol or 1000 dpm of [³H]altritol of unknown chirality (see “Results”). After a further 210 min reactions were quenched by their addition to 3 ml of Amberlite MB-3 resin. L-Altritol oxidation between 20 and 210 min was assessed by measuring NAD⁺ reduction at 340 nm, assuming a 1:1 stoichiometry. The Amberlite was washed with 4 × 1-ml aliquots of water, the eluates were combined and lyophilized, then added to [³H]altritol and chromatographed on the Polybore-Pb column (see Ref. 24).

Some samples of [³H]InsP₃ were incubated for 1 h at 37 °C with 50 μl of packed erythrocyte ghosts in 0.5 ml of medium containing 40 mM Hepes (pH 7.0 with NaOH), 0.2 mg/ml saponin. Incubations were stopped by adding 1 ml of medium containing 120 mM KCl, 20 mM Hepes (pH 7.2 with KOH), 10 mM MgSO₄, 5 mM ATP, 1 mM EGTA, and 0.2 mg/ml saponin. Supernatants and pellets, each resuspended to a 30% concentration (weight of original liver, volume), were prepared as described in Ref. 7. Routinely, elution times were 100% recovery of all [³H]polyols was determined by their absorbance at 200 nm. The [³H]polyols was determined by their absorbance at 200 nm.

**RESULTS**

**Inositol Phosphates—**Desalted samples of inositol phosphates were incubated at 25 °C for 40 h in the dark with either sodium periodate (for InsP₄) or periodate acid (for InsP₃, pH 3.0 with NaOH). Reduction and dephosphorylation were as described by Stephens et al. (9, 12), except that the elution through H⁺-Dowex was performed at 0–4 °C. The resultant polyl, in 10 μl of water added to 2000 dpm of [³H]inositol and 2000 dpm of [³H]xyitol, were applied to a Polybore-Pb column (4.5 × 220 mm, Pierce Chemical Co.) and eluted at 80 °C at 0.2 ml/min with water, which was degassed by sparging with helium. Fractions of 1 ml were collected. The identity of the [³H]polyol was determined by its Rᵥ values relative to [³H]inositol. The Rᵥ value of [³H]xyitol was typically 1.5; Rᵥ values for standards (100 μg) of altritol (1.25) and iditol (1.5) were also determined in separate runs from the absorbance of the eluant at 200 nm.

**Alkaline hydrolysis of InsP₃ and InsP₄** was as described by Stephens et al. (9, 12), except that the incubations with ammonia were performed for 120 h. The ammonia was removed by lyophilization, and then the inositol monophosphates were isolated using a Bio-Rad anion-exchange column (see above), desalted (see below), and separated by HPLC (9). Typical elution times were: Ins-1-P/Ins-3-P, 55 min; Ins-2-P, 40 min; Ins-5-P, 50 min; Ins-4-P/Ins-6-P, 55 min.

**Biochemicals**—Liver homogenates were transferred to ice and then the inositol monophosphates were isolated using a Bio-Rad anion-exchange column (see above) and separated by HPLC (9). Typical elution times were: Ins-1-P/Ins-3-P, 55 min; Ins-2-P, 40 min; Ins-5-P, 50 min; Ins-4-P/Ins-6-P, 55 min.

Polyl dehydrogenases were performed at 30 °C in 1 ml of 100 mM Tris (pH 8.3 with HCl), 20 mM NAD⁺, 50 μM L-altritol, and 1.2 Sigma units/ml polyol dehydrogenase. After a steady base line was attained (20 min), the following additions were made (in 0.09 ml of incubation buffer without dehydrogenase): either 6000 dpm of genuine l-[³H]altritol or 1000 dpm of [³H]altritol of unknown chirality (see “Results”). After a further 210 min reactions were quenched by their addition to 3 ml of Amberlite MB-3 resin. L-Altritol oxidation between 20 and 210 min was assessed by measuring NAD⁺ reduction at 340 nm, assuming a 1:1 stoichiometry. The Amberlite was washed with 4 × 1-ml aliquots of water, the eluates were combined and lyophilized, then added to [³H]altritol and chromatographed on the Polybore-Pb column (see Ref. 24).

Some samples of [³H]InsP₃ were incubated for 1 h at 37 °C with 50 μl of packed erythrocyte ghosts in 0.5 ml of medium containing 20 mM Hepes (pH 7.0 with NaOH), 0.2 mg/ml saponin. Incubations were acid-quenched (7), and the [³H]insP₃ was isolated by HPLC (see above) and then desalted as described above.

**CHARACTERISTICS OF INOSITOL PHOSPHATES**—Ins(3,4,5)P₃ was isolated by HPLC (with a 4.6 × 250-mm AEndosorb AEX 3000-μm column, Alltech Associates, Deerfield, IL) and then desalted using a 1.5-ml column of Bio-Rad cation-exchange resin (200–400 mesh, H⁺ form), and the eluate was lyophilized. HPLC fractions containing either InsP₄, InsP₃, or InsP₅ were neutralized (23) and rechromatographed on a gravity-fed Bio-Rad anion-exchange column (see above). Samples were then desalted using a 0.1-mM anion-exchange column (see Ref. 24), except that the InsP₄ was obtained by washing the column with 3 × 1-ml aliquots of 0.1 M HCl and then eluting the InsP₄ with 3 × 1-ml aliquots of 2 M HCl. The acid was then removed by lyophilization.

**RESULTS**

**Characteristics of Ins(1,3,4,5)P₄**—Liver homogenates were incubated with 10 nM [³H]Ins(1,3,4,5)P₄ in the presence of 5 mM MgATP, and the reaction products were analyzed by HPLC. Fig. 1 shows data from a 5-min incubation, after which around half of the Ins(1,3,4,5)P₄ was metabolized by both dephosphorylation to InsP₃ and phosphorylation to InsP₄. About 1.3% of the original Ins(1,3,4,5)P₄ was phosphorylated to an InsP₅. This is the first demonstration that, 1 L. R. Stephens, personal communication.

2 K. Oliver and J. W. Putney, Jr., unpublished data.
in liver, Ins(1,3,4)P₃ can be phosphorylated to InsP₄, although the amount formed was small in comparison with the other routes by which Ins(1,3,4)P₃ was metabolized. Fig. 1 also reveals that there were two InsP₄ peaks. There are no previous reports that two InsP₄ isomers can be formed from Ins(1,3,4)P₃. In this experiment, the minor of the two InsP₄s accounted for 2% of total Ins(1,3,4)P₃ metabolites. This minor peak, which co-eluted precisely with Ins(1,3,4,5)P₄ standards (data not shown) is described hereafter as peak A. The earlier eluting and major InsP₄ is designated as peak B. A partial resolution of enzyme activities was achieved by anion-exchange chromatography (Fig. 3). Ins(1,3,4)P₃ kinase activities were well resolved from Ins(1,3,4,5)P₄, 5-phosphatase. The two kinase activities co-eluted, peaking at fraction 34, and these were resolved from Ins(1,3,4)P₃ phosphatase activity, showing that the substrate for phosphorylation was Ins(1,3,4)P₃ and not one of its metabolites. Fig. 4 shows that in fraction 34, 16% of total Ins(1,3,4)P₃ kinase activity produced the minor InsP₄ (peak B). Since no InsP₄ was formed in these incubations (Fig. 4), both InsP₄ isomers must be formed from Ins(1,3,4)P₃.

Fraction 34 in Fig. 3, which contained the peak Ins(1,3,4)P₃ kinase activity, was incubated as described in the accompanying legend with 0.5 μCi of [³H]Ins(1,3,4)P₃. The resultant InsP₄ isomers were isolated by HPLC and desalted as described under “Experimental Procedures.” The structural analyses were then undertaken, first by determining the sensitivity of the InsP₄ isomers to periodate. Under appropriate conditions, periodate will oxidize an InsP₄ only if it has two adjacent hydroxyls. Thus Ins(1,3,4,5)P₄ is resistant such that after incubation with periodate, followed by reduction and dephosphorylation, inositol is the resultant polyl (11-13). On the other hand Ins(3,4,5,6)P₄ is sensitive to periodate and should ultimately yield iditol (12). However, “periodate sensitivity” is a relative term; Ins(1,2,3,4)P₄, which is predictably periodate-sensitive, is not oxidized under some conditions (27). Thus Ins(1,3,4,5)P₄ and Ins(3,4,5,6)P₄ were used as controls that the periodate method was performing as expected.

In the experiments described by Table I, Ins(1,3,4,5)P₄ standards were virtually 100% periodate-resistant, and after reduction and dephosphorylation only inositol was formed. Standards of putative Ins(3,4,5,6)P₄ were oxidized by periodate, largely yielding iditol, although 24% of the resultant polyols were inositol. Thus either the original samples of Ins(3,4,5,6)P₄ were contaminated with another inositol phosphate or some of the putative Ins(3,4,5,6)P₄ had resisted attack by periodate. This problem was not investigated further. In any case the data indicate that the experimental conditions would detect at least 76% of a periodate-sensitive InsP₄. Both of the InsP₄ products of Ins(1,3,4)P₃ kinase yielded inositol after incubation with periodate followed by reduction and dephosphorylation (Table 1). However, a number of InsP₄ isomers are resistant to periodate (Table III) so the data in Table I by themselves do not identify the products of InsP₄ kinase.

The phosphate groups around an inositol ring can be eliminated by alkaline hydrolysis (9, 13, 16). If the reaction is stopped when inositol monophosphates are formed, the re-
FIG. 2. Time course of Ins(1,3,4)P₃ metabolism in the presence of ATP. Aliquots (60 μl) of either liver homogenates (●—●), 1000,000 × g supernatants (○—○), or particulate fractions (▲—▲) were incubated with 0.1 μCi of Ins(1,3,4)P₃, and the products were analyzed by HPLC as described under "Experimental Procedures." a, InsP + InsP₂; b, Ins(1,3,4)P₄; c, InsP₄ (peak A); d, InsP₆ (peak B); e, InsP₆. Data are means and standard errors from three to five independent experiments.

FIG. 3. Distribution of activities of Ins(1,3,4)P₃ kinase, Ins(1,3,4)P₃ phosphatase, Ins(3,4,5,6)P₄ kinase, and Ins(1,3,4,5)P₄ phosphatase during anion-exchange chromatography of a liver supernatant. A, anion-exchange chromatography and protein assays were performed as described under "Experimental Procedures." B, Ins((1,3,4)P₃ phosphatase activity (Δ—Δ) was determined by incubating 10-μl aliquots of fractions for 10 min with 10,000 dpm of [³H]Ins(1,3,4)P₃; samples were analyzed with gravity-fed columns. Ins(1,3,4)P₃ kinase activity was assayed with 0.1 μCi of [³H]Ins(1,3,4)P₃, and the reaction products were analyzed by HPLC: W, InsP₄ (peak A, i.e. earlier peak in Fig. 1); W, InsP₃ (peak B, i.e. later peak in Fig. 1). The reaction products of the remaining incubations were analyzed using gravity-fed columns. Ins(1,3,4,6)P₄ 5-phosphatase activity (Δ—Δ) was determined in incubations containing 80-μl aliquots of fractions incubated with 4000 dpm of [³H]Ins(1,3,4,6)P₄ for 1 h. The incubation media were supplemented with 10 mM phosphocreatine, 5 units/ml creatine phosphokinase, and 1 mM dithiothreitol. Ins(1,3,4,5)P₄ 5-phosphatase activity (Δ—Δ) was determined with 5-μl aliquots of fractions incubated for 10 min with 10,000 dpm of [³H]Ins(1,3,4,5)P₄, but the medium did not contain ATP. Recoveries of all enzymes exceeded 85%. Data are from a single experiment, typical of three.
The resultant mixture of isomers is indicative of the structure of the parent compound. Note that the nonchiral techniques used in this paper do not distinguish between enantiomeric pairs of monophosphates (i.e., Ins-1-P/Ins-3-P and Ins-4-P/Ins-6-P). Alkaline hydrolysis of the peak A InsP₄ yielded a mixture of Ins-1-P/Ins-3-P and Ins-4-P/Ins-6-P (Table II). Only one InsP₄ can product this pattern of monophosphates, i.e., Ins(1,3,4,6)P₄ (Table II). This compound would be predicted to be insensitive to periodate, which it is (see above). This is the first unequivocal structural characterization of this isomer in liver (see the Introduction). Note that the yield of Ins(1,3,4-P₄/Ins-1-P was rather less than Ins-4-P/Ins-6-P (Table II). The reason for this is unclear, but a disproportionate yield of monophosphates is a peculiarity of this technique (Table I). Polyols formed from various samples of [³H]InsP₄ incubated with periodate followed by reduction and dephosphorylation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>%H recovered in various polyols</th>
<th>% of total</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Inositol</td>
<td>Iditol</td>
</tr>
<tr>
<td></td>
<td>Putative Ins(3,4,5,6)P₄</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>InsP₄ (peak A)</td>
<td>99.9 ± 0.1</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>InsP₄ (peak B)</td>
<td>99.8 ± 0.1</td>
<td>0.1 ± 0.1</td>
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The conclusion of Ins(1-P/Ins-3-P, Ins-5-P, and Ins-4-P/Ins-6-P. Two possible periodate-resistant InsP₄ isomers could give this pattern of monophosphates: Ins(1,3,4,5)P₄ and Ins(1,3,5,6)P₄ (Table III). Both are equally plausible products, since they could be produced by either 5-kinase action on Ins(1,3,4)P₃ or by isomerization of Ins(1,3,4,6)P₄.

The [³H]InsP₄ (peak B) now known to be either Ins(1,3,4,5)P₄ or Ins(1,3,5,6)P₄ (see above) was found to be dephosphorylated by incubation with erythrocyte ghosts (data not shown). Since the ghosts contain Ins(1,3,4,5)P₄ 5-phosphatase activity, it would be expected that if the unknown InsP₄ were Ins(1,3,4,5)P₄, then the InsP₄ product would be Ins(1,3,4)P₃. However, if the original InsP₄ were Ins(1,3,5,6)P₄, it is not inconceivable it might also be a substrate for 5-phosphatase. In such a case, Ins(1,3,6)P₄ would be the product. This is the enantiomer of Ins(1,3,4)P₄ and enantiomeric pairs of inositol phosphates are indistinguishable by nonchiral analytical techniques. Fortunately, Ins(1,3,6)P₄ yields l-altritol upon periodate oxidation, reduction, and dephosphorylation, whereas similar treatment of Ins(1,3,4)P₄ gives l-altritol. It was considered that polyol dehydrogenase might distinguish between enantiomers of altritol, since this enzyme oxidizes L-iditol 10 times more rap-
A.

Peaks are hereafter referred to as peaks 1 and 2, respectively.

Upon alkaline hydrolysis, yielded Ins-1-P/Ins-3-P, Ins-2-P, respectively, genuine \( ^{3}{}H \)altritol and genuine \( ^{3}{}H \)altritol dehydrogenase.

B.

By anion-exchange chromatography of liver supernatant (Fig. 0.04 pCi of Ins\((1,3,4,6)\)P\(_{4} \) for 1 h as described in the legend to Fig. 3. The resultant InsP\(_{4}\) was isolated, desalted, and hydrolyzed in ammonia (see "Experimental Procedures"). The proportion of \( ^{3}{}H \)altritol of unknown chirality was oxidized by polyol dehydrogenase (26%) was almost identical to that for \( ^{3}{}H \)altritol (Fig. 5); thus the unknown altritol must also have been the L-enantiomer. This identifies the parent InsP\(_{4}\) as Ins\((1,3,4)\)P\(_{4}\), and therefore the original InsP\(_{4}\) (peak B) was Ins\((1,3,4,5)\)P\(_{4}\).

Now that the two InsP\(_{4}\) isomers were characterized, 10,000 dpm of each were individually incubated in the "physiological" buffer (see "Experimental Procedures") plus 10 \( \mu l \) of fraction 34 (in Fig. 3) for 15 min. There was no isomerization of Ins\((1,3,4,6)\)P\(_{4}\) to Ins\((1,3,4,6)\)P\(_{4}\) or vice versa (data not shown), indicating that both InsP\(_{4}\) isomers were direct products of Ins\((1,3,4)\)P\(_{4}\) phosphorylation.

Ins\((1,3,4,6)\)P\(_{4}\), was itself incubated with fractions obtained by anion-exchange chromatography of liver supernatant (Fig. 3). This InsP\(_{4}\) was phosphorylated to InsP\(_{5}\), and two peaks of kinase activity were observed. The earlier and later eluting peaks are hereafter referred to as peaks 1 and 2, respectively. In Fig. 3, and two further experiments, peak 1 contained the majority of total activity (70 ± 4%). The products of Ins\((1,3,4,6)\)P\(_{4}\) kinase (Fig. 3) were also analyzed. Aliquots (100 \( \mu l \)) of fractions from both peaks were each incubated with 0.04 \( \mu Cl \) of Ins\((1,3,4,6)\)P\(_{4}\) for 1 h as described in the legend to Fig. 3. The resultant InsP\(_{5}\) was isolated, desalted, and hydrolyzed in ammonia (see "Experimental Procedures"). The InsP\(_{5}\) produced by peak 1 was hydrolyzed to the following monophosphates: Ins-1-P/Ins-3-P, Ins-5-P, and Ins-4-P/Ins-6-P (Table II), indicating that there was more than one InsP\(_{5}\) isomer, and possibly also an InsP\(_{6}\). The yield of Ins-2-P was relatively low (Table II), but because the monophosphates were not produced in equal proportions, it is not possible to quantify the amount of the isomer that gave rise to Ins-2-P. However, the formation of InsP\(_{6}\) by the enzymes in peak 2 was excluded since the products (data not shown) eluted as a single InsP\(_{5}\) peak during an HPLC procedure that is known to separate InsP\(_{5}\) from InsP\(_{6}\) (see under "Experimental Procedures"). Additionally, when 20,000 dpm of Ins\((1,3,4,5,6)\)P\(_{5}\) was incubated in the "physiological" medium with 100 \( \mu l \) of a 1:1 mixture of fractions 21 and 22 (which contained most peak 2 kinase activity), no further phosphorylation was observed (data not shown). The parsimonious interpretation of the data in Table II is that peak 2 contains both Ins\((1,3,4,6)\)P\(_{5}\), 5-kinase and Ins\((1,3,4,6)\)P\(_{4}\) 2-kinase. The latter enzyme has not previously been described, but it accounted for less than 30% of all Ins\((1,3,4,6)\)P\(_{5}\) kinase (Fig. 3). However, these analyses were performed under first-order conditions, so in vivo the putative Ins\((1,3,4,6)\)P\(_{4}\) 2-kinase may be more than the minor hepatic enzyme activity indicated by these data.

Effect of Ins\((1,3,4,5)\)P\(_{4}\) upon Ins\((1,3,4)\)P\(_{4}\) Kinase Activities—Fig. 6 shows that Ins\((1,3,4,5)\)P\(_{4}\) is a potent inhibitor of Ins\((1,3,4)\)P\(_{4}\) kinase. At concentrations between 3 and 10 \( \mu M \), Ins\((1,3,4,5)\)P\(_{4}\) reduced total Ins\((1,3,4)\)P\(_{4}\) kinase activities by between 40 and 60%. The products of the kinase activity were analyzed by HPLC, which revealed that both 5- and 6-kinase were inhibited to the same extent (data not shown).

CONCLUSIONS

In these studies, as in earlier work (7), liver homogenates and subfractions derived therefrom have been incubated in "physiological" media, and metabolism of inositol phosphates has been measured under first-order conditions. In should be noted that the fluxes of the various metabolites in vivo are likely to differ from those reported here, in particular because the concentrations of the intermediates are rather higher. At present it is difficult to assess the physiological metabolic fluxes, because there is little information on either the intracellular concentrations of inositol phosphates or kinetic parameters of the various enzymes. Nevertheless, the approach taken here is a valid means of identifying the pathways of inositol phosphate metabolism, and several important new observations have been made.
When Ins(1,3,4)P₃ phosphorylation was first observed (7, 8), it was only tentatively proposed that the resultant Ins₄P₄ was Ins(1,3,4,6)P₄, because it could not be excluded that isomerization of the Ins₃P₄ has occurred. Only in brain has Ins(1,3,4)P₃ phosphorylation to Ins(1,3,4,6)P₄ been definitively characterized (9). The methods used in the latter study have been utilized here, including HPLC, FPLC, periodate oxidation, alkaline hydrolysis, and stereospecific polyol oxidation. Thus for the first time it has been unequivocally demonstrated that, in liver, Ins(1,3,4)P₃ is phosphorylated to Ins(1,3,4,6)P₄. A further new observation in this work is the characterization of a second route of Ins(1,3,4)P₃ phosphorylation by a Ins(1,3,4)P₃ 5-kinase. The latter novel activity was not observed in earlier experiments with brain (9) or adrenal glomerulosa cells (8, 20), and it is not yet known if this enzyme is unique to liver.

Under conditions of limited substrate concentrations, 16% of total Ins(1,3,4)P₃ phosphorylation was attributable to a 5-kinase. This only became clear once the Ins(1,3,4)P₃ 5-kinase activity by anion-exchange chromatography. In supernatants, and more profoundly in particulate fractions and in homogenates, the Ins(1,3,4,5)P₄ that formed from Ins(1,3,4)P₃ was kept at low levels by the Ins(1,3,4,5)P₄ 5-kinase (7). In stimulated hepatocytes where substrate concentrations are much higher than were used in these experiments, significant levels of Ins(1,3,4,5)P₄ were detected (4, 26), so the 5-phosphatase activity must be exceeded by the combined activities of the Ins(1,3,4)P₃ 5-kinase and Ins(1,4,5)P₃ 5-kinase (3–5). Shortly after the termination of the cell's stimulation, as Ins(1,4,5)P₃ levels decay, the Ins(1,3,4)P₃ 5-kinase could contribute to prolonging intracellular levels of Ins(1,3,4,5)P₄ and any associated signaling activity (see Ref. 6). For example, Ins(1,3,4,5)P₄ may have a role in replenishing the mobilizable Ca²⁺ pool (6), which can only take place once Ins(1,4,5)P₃ has returned to near basal levels. The presence of Ins(1,3,4,5)P₄ 5-phosphatase and Ins(1,3,4)P₃ 5-kinase activities represents a "futile cycle" which in other circumstances has been shown to be a highly efficient control point in a metabolic pathway. However, there is no evidence yet that the 5-phosphatase activity is regulated by the hepatocyte (7, 25). Moreover, the activity of Ins(1,3,4)P₃ 5-kinase and (Ins(1,3,4)P₃ 6-kinase) in liver supernatants was not altered when the Ca²⁺ concentration of the medium was increased from 0 to 0.1 μM or 1 μM (data not shown and Ref. 26) nor when 100 μM cAMP was added (data not shown).

Further work will be necessary to unequivocally determine if Ins(1,3,4)P₃ 5- and 6-kinase activities are performed by a single enzyme, but several lines of evidence suggest that this is the case; both activities predominated in the soluble portion of the cell (Fig. 2), and they co-eluted during anion-exchange FPLC (Fig. 3). The most striking evidence is that both kinase activities were inhibited to the same extent by Ins(1,3,4,5)P₄. There are no direct measurements of Ins(1,3,4,5)P₄ levels in receptor-activated hepatocytes, but an indirect estimate of 0.7 μM (34) has been made, which is rather smaller than Ins(1,4,5)P₃ levels (2.5 μM (28)). At a concentration of 0.7 μM, Fig. 6 indicates that Ins(1,3,4,5)P₄ would inhibit Ins(1,3,4)P₃ kinase activity by 13%, which is a physiologically minor effect. However, in brain, the Ins(1,3,4,5)P₄ concentration appears to be up to 3-fold greater than that of Ins(1,4,5)P₃ (29). Inhibition of Ins(1,3,4)P₃ kinase by Ins(1,3,4,5)P₄ may be more important in this tissue and may partly explain why there was no detectable Ins(1,3,4,6)P₄ in receptor-activated brain slices (29), even though this tissue has an active Ins(1,3,4,5)P₄ 6-kinase (9).

This work has also shown for the first time that, in liver, Ins(1,3,4,6)P₄ can be phosphorylated to Ins(1,3,4,5,6)P₅. Brain contains a similar kinase activity (9). Adrenal glomerulosa cells also phosphorylate Ins(1,3,4,6)P₄, but the structures of the ensuing products have not been identified (20). No further phosphorylation of Ins(1,3,4,5,6)P₅ to Ins₆P₆ was detected in liver, but this tissue may contain more than 15 μM Ins₆P₆ (31). The possibility that liver may also synthesize Ins(1,2,3,4,6)P₅ (see above) needs further study, as does the possibility that this isomer may be a precursor for Ins₆P₆.

The work reported here has added new complexities and additional enzyme activities to a metabolic pathway that has already proved surprisingly convoluted; Ins(1,3,4)P₃ has now emerged as a particularly versatile intermediate, being a precursor for two Ins₄Ps as well as two Ins₅Ps (Fig. 7). This work has also revealed a relationship between the inositol phosphates produced by receptor activation and the synthesis of a compound, Ins₆Ps, that may be an extracellular signal (19). Liver has a second route of Ins(1,3,4,5,6)P₅ production, i.e. by phosphorylation of Ins(3,4,5,6)P₃ (16), although the origin of the latter is presently unknown. The interrelationship be-
between these two pathways of InsP₆ production is also unclear. An understanding of the factors that regulate fluxes of these and other inositol phosphates in vivo requires determinations of both the concentrations of the intermediates and the kinetics of the various enzymes.

REFERENCES