We have reexamined the structure of inositol phosphates present in trophozoites of the parasitic amoeba \textit{Entamoeba histolytica} and show here that, rather than being \textit{myo}-inositol derivatives (Martin, J.-B., Bakker-Grunwald, T., and Klein, G. (1993) Eur. J. Biochem. 214, 711–718), these compounds belong to a new class of \textit{neo}-inositol phosphates present in which the cyclitol isomer is \textit{neo}-inositol. The structures of \textit{neo}-inositol hexakisphosphate, 2-diphospho-\textit{neo}-inositol pentakisphosphate, and 2,5-bisdiphospho-\textit{neo}-inositol tetrakisphosphate, which are present in \textit{E. histolytica} at concentrations of 0.08–0.36 mM, were solved by two-dimensional $^{31}$P-$^1$H NMR spectroscopy. No evidence for the co-existence of their \textit{myo}-inositol counterparts has been found. These \textit{neo}-inositol compounds were not substrates of 6-diphospho-\textit{myo}-inositol pentakisphosphate 5-kinase, an enzyme purified from \textit{Dictyostelium discoideum} that phosphorylates 6-diphospho-\textit{myo}-inositol pentakisphosphate and more slowly also \textit{myo}-inositol hexakisphosphate, specifically on position 5. Because preliminary data indicate that large amounts of the same \textit{neo}-inositol phosphate and diphosphate esters are also present in another primitive amoeba, \textit{Phreatamoeba balamuthi}, the occurrence of high concentrations of \textit{neo}-inositol polyphosphates may be much more general than previously thought.

Inositol phosphates are members of a large family of naturally occurring compounds that because of their complex role in cell signaling and homoeostasis have been intensively studied during the last two decades (1). Research has focused mainly on the commonly known \textit{myo}-derivatives, although many other naturally occurring inositol stereoisomers are known. The most abundant and ubiquitous member of the family, \textit{myo-InsP$_6$}, has been shown to reach an intracellular concentration close to 1 mM in \textit{Dictyostelium discoideum} (2, 3). Recently, new members of this class of compounds have been identified in cellular slime molds as 5-PP- and 6-PP-\textit{myo-InsP$_5$} and 3,5-bis-PP- and 5,6-bis-PP-\textit{myo-InsP$_4$} (4–6). The same or similar highly phosphorylated and diphosphorylated compounds have been detected in free living and parasitic amoebae and a number of mammalian cell types (7–11). The latter molecules may function in cell signaling (2, 12–14).

\textit{Entamoeba histolytica} is a human intestinal parasite that causes amoebiasis. Its trophozoites contain high amounts of inositol polyphosphates (8). \textit{Myo-InsP$_6$} is the major cyclitol building natural inositol polyphosphates, and other \textit{myo}-inositol polyphosphates are not only rarely found and in very low concentrations. Consequently, the two major inositol phosphates of \textit{E. histolytica} have been preliminarily identified as \textit{myo-InsP$_6$} and 5-PP-\textit{myo-InsP$_5$} (8). However, we subsequently noticed during comparative studies by high resolution anion exchange chromatography that the inositol polyphosphates from \textit{E. histolytica} were not eluted exactly with the same retention time as the \textit{myo}-inositols used as reference. This casted some doubt on the previous identification. Therefore, we have reinvestigated the structures of the inositol phosphates present in \textit{E. histolytica} by two-dimensional $^{31}$P-$^1$H NMR analysis after HPLC purification, and we show in the present study that \textit{E. histolytica} possesses high levels of several \textit{neo}-inositol polyphosphates.

**EXPERIMENTAL PROCEDURES**

Culture of Cells and Preparation of Acellular Extracts—\textit{E. histolytica} HM-1:IMSS (ATCC 30459) were cultured and perchloric acid extracts prepared as detailed in Ref. 8.

Isolation and Analysis of Highly Phosphorylated Inositol Phosphates—The HPLC-MDD analysis for inositol phosphates and their further purification were performed as described (5, 15).

Preparation of the 6-PP-\textit{InsP$_5$}, 5-Kinase—This enzyme was purified from \textit{D. discoideum} as described previously (4).

NMR Analyses—NMR spectra were recorded at room temperature on a Bruker AMX400 WB spectrometer at 400-MHz frequency for $^1$H, 162-MHz frequency for $^{31}$P, and 100.6-MHz frequency for $^{13}$C.

One-dimensional $^1$H spectra of purified inositol phosphates from \textit{E. histolytica} dissolved in $\text{H}_2\text{O}$ were recorded in a 5-mm inverse probe head with an excitation pulse of 60°. Acquisition time was 2 s, and 32 scans were accumulated. The spectral width was set to 4032 Hz at a 8192 data size, which yields a digital resolution of 0.50 Hz/point. The $^1$H $\text{H}_2\text{O}$ signal was suppressed by a selective 2-s presaturation pulse. $^1$H chemical shifts are given relative to external tetramethylsilane at 0 ppm. For $^{31}$P-decoupled spectra, a WALTZ-16 sequence is used. Other NMR spectra were recorded in a 10-mm X-$^1$(H) probehead.

$^1$H-decoupled $^{13}$C spectra were recorded with an excitation pulse of 60°.
HCl gradient (10 mM) WALTZ-16 1H-decoupled, and 31P acquisition time was 0.4 s with 0.6 s correlation spectra were recorded with the following pulse sequence (16): RD-90°(1H)-1/2t1-180°(31P)-1/2t1-MLEV-17-2-acquisition (31P) with polarization transfer from 1H to 31P via JPOCH. The time interval D was set to 56 ms. The TOCSY mixing time consisted of a 104-ns MLEV-17 sequence. The $\Theta$ pulse of the DEPT transfer was 45°. Acquired spectra were WALTZ-16 proton-decoupled, and 31P acquisition time was 0.4 s with 0.6 recycling delay. Specific conditions are detailed in the legends of Figs. 3 and 6.

Quantification by NMR of E. histolytica Inositol Phosphate Content—Absolute amounts of inositol phosphates were calculated as described previously (8).

Standard Compounds—The standard compounds used for the comparative HPLC analysis of highly phosphorylated inositols were either isolated from D. discoideum (6-PP-myO-InsP5, myO-InsP4, myO-Ins(1,2,3,4,5)P5, myO-Ins(1,3,4,5,6)P5, and myO-Ins(1,2,3,4,5,6)P5) or obtained from Sigma (myO-Ins(1,3,4,5,6)P5).

RESULTS

HPLC-MDD Analysis of Highly Phosphorylated Inositol Phosphates in E. histolytica—The analysis by HPLC-MDD of a percolate extract from E. histolytica trophozoites showed three prominent peaks corresponding to two major compounds I and II and a minor compound III, with retention times corresponding to highly phosphorylated inositol phosphates (Fig. 1, trace...
A). Although the retention time of compound I was close to that of the various myo-inositol pentakisphosphates, it did not coincide with any of them. Compound II eluted with the same retention time as myo-InsP6, and compound III eluted slightly later than 6-PP-myO-InsP5 purified from D. discoideum (Fig. 1, trace B). This result suggested that the inositol phosphates present in E. histolytica might be different from the highly phosphorylated inositol phosphates presently known in D. discoideum. The intracellular amounts of the E. histolytica compounds I, II, and III were determined by 31P NMR to be about 0.11, 0.18, and 0.04 μmol/g wet cells, respectively. For further characterization, a perchloric acid extract was prepared from 18 g (wet weight) cells, and compounds I, II, and III were purified on an HPLC anion exchange column, with a 42–68% yield.

**Inositol Phosphates Extracted from E. histolytica Are Not Phosphorylated by 6-PP-InsP5 5-Kinase—**

The 6-PP-InsP5 5-kinase extracted from D. discoideum specifically phosphorylates position 5 of 6-PP-myO-InsP5 leading to 5,6-bis-PP-myO-InsP4 (4). This enzyme also phosphorylates more slowly position 5 of myo-InsP6 to form 5-PP-myO-InsP5.2 Compound II, although co-eluting from the HPLC column with myo-InsP6, was not phosphorylated by 6-PP-InsP5 5-kinase. This result suggested that the inositol phosphates present in E. histolytica might be different from the highly phosphorylated inositol phosphates presently known in D. discoideum.

**Fig. 3.** Two-dimensional 31P-H TOCSY-DEPT spectrum of neo-InsP6. HPLC-purified compound I was adjusted to pH 6.0. Acquisition conditions are detailed under “Experimental Procedures.” Spectral width was 320 Hz in the F2 domain (256 points) and 1290 Hz in the F1 domain (128 points), and the number of scans for an increment was 384. The two-dimensional contour plot was calculated by Fourier transformation after zero filling in F1 and F2 dimensions. The one-dimensional spectra correspond to the high resolution spectra and not to the projections of the two-dimensional map. The letters d and r refer to direct and relayed correlations, respectively.

**Table I**

| Compound                  | H1   | H2   | H3   | H4   | H5   | H6   |
|---------------------------|------|------|------|------|------|------|
| neo-Insitol               | 3.65 | 3.94 | 3.65 | 3.65 | 3.94 | 3.65 |
| neo-InsP5                 | 4.42 | 4.90 | 4.42 | 4.42 | 4.90 | 4.42 |
| 2-PP-neo-InsP5            | 4.44 | 5.00 | 4.44 | 4.44 | 4.93 | 4.44 |
| 2,5-bis-PP-neo-InsP4      | 4.45 | 5.00 | 4.45 | 4.45 | 5.00 | 4.45 |

**NMR chemicals shifts (δ) and coupling constants (J) of neo-inositol phosphates**

All spectra were recorded at pH 6.0. NA, not applicable; ND, not determined.

| Compound                  | δP   | δP1 | δP2 | δP3 | δP4 | δP5 | δP6 | JPOP | JPOCH |
|---------------------------|------|-----|-----|-----|-----|-----|-----|------|-------|
| neo-InsP6                 | 0.8  | 0.6 | 0.8 | 0.8 | 0.6 | 0.8 | NA  | 6.8  | 10.4  |
| 2-PP-neo-InsP5            | 0.72 | -11.30 | 0.72 | 0.85 | 0.55 | 0.85 | -9.60 | NA   | 16.2  |
| 2,5-bis-PP-neo-InsP4      | 0.8  | -11.35 | 0.8 | 0.8 | -11.35 | 0.8 | -9.65 | -9.65 | 17.0  |

a δP1,3 and δP4,6 can be inversed.

b 13C NMR data was from Ref. 38, and 1H NMR data was from Ref. 39.

c δC1,3 and δC4,6 can be inversed; δC2 and δC5 can be inversed.
phosphorylated by this enzyme (Fig. 2, trace A), whereas added myo-InsP$_5$ was completely converted into 5-PP-mylo-InsP$_5$ (Fig. 2, trace B). Similarly, compound III, with an elution time from the HPLC column close to that of 6-PP-mylo-InsP$_5$, was not phosphorylated by the kinase (Fig. 2, trace C), whereas added 6-PP-mylo-InsP$_5$ was converted into 5,6-bis-PP-mylo-InsP$_5$ (Fig. 2, trace D). Compound I was no substrate for the kinase, either (data not shown). Taken together with the HPLC data, these results demonstrate that the highly phosphorylated inositol phosphates from E. histolytica were not identical to known myo-inositol phosphates. Compounds I, II, and III were further analyzed by NMR spectroscopy to solve their structures.

Identification of Compound I as neo-InsP$_6$—The one-dimensional $^1$H NMR spectrum of compound I showed two resonance lines at about 4.4 and 4.9 ppm in an intensity ratio of 2:1, respectively. No other proton peak was detectable in this $^1$H spectrum between 10 and 0 ppm, except for some residual water at 4.8 ppm (F$_1$ dimension, Fig. 3). Because the inositol ring has six $-$CH groups, this results indicates that compound I had just two groups of equivalent protons, one group consisting of four protons and the other group consisting of two protons. Similarly, in a $^{31}$P NMR spectrum, compound I gave two resonance lines at 0.8 and 0.6 ppm with the same intensity ratio of 2:1 or 4:2 (F$_2$ dimension, Fig. 3), suggesting an inositol molecule with either three or six phosphorus atoms. Only a 3-phosphate molecule is compatible with the symmetry of the myo-inositol ring. Assuming that myo-inositol phosphates were indeed present in E. histolytica, compound I was previously identified as myo-Ins (2,4,6)P$_3$ (8). The above data on its elution position from the HPLC column, the kinase assays, and the equivalence of the protons of the molecule suggested that this identification of compound I was wrong and that E. histolytica possessed an inositol isomer other than myo-inositol.

To determine the structure of compound I, we recorded a two-dimensional $^{31}$P-$^1$H relayed, correlative NMR spectrum on the HPLC-purified compound I. The phosphate atoms at 0.8 ppm were correlated with the four protons at 4.42 ppm, whereas the phosphate atoms at 0.6 ppm were correlated with the two protons at 4.90 ppm. Relayed cross-peaks were also detected between the four phosphate atoms and the protons at 4.90 ppm and between the two phosphate atoms at 0.6 ppm and the protons at 4.42 ppm. Because all protons of the inositol ring are correlated with phosphates (F$_1$ dimension, Fig. 3), the six hydroxyl groups must be phosphorylated. The peculiar $^{31}$P and $^1$H NMR spectra of compound I with only two resonance groups in the ratio 4:2 were also found with $^{13}$C NMR, with two lines (both of them broadened because of the multiple $^{13}$C-$^{31}$P coupling constants) at 72.8 ppm (intensity 2) and 75.3 ppm (intensity 1) (data not shown). This unique order of symmetry is only compatible, among the nine isomeric inositols, with compound I being neo-InsP$_6$. Chemical shifts and coupling constants are summarized in Table I. Both equatorial protons H$_2$ and H$_5$ had a δ of 4.90 ppm and a $J_{POCH}$ coupling constant of 10.4 Hz. The four axial protons H$_1$, H$_3$, H$_4$, and H$_6$ resonated at 4.42 ppm, and their $J_{POCH}$ coupling constant was 6.8 Hz.

Identification of Compound II as 2-PP-neo-InsP$_6$—Compound II co-eluted from the HPLC column with myo-InsP$_6$, after compound I identified just above as neo-InsP$_6$ (Fig. 3). Because the negative kinase assays eliminated the possibility of compound II being myo-InsP$_6$ (Fig. 2), we had a closer look at the structure of this component as well. Compound II had a $^{31}$P-decoupled $^1$H NMR spectrum with three singlets at 5.00, 4.95, and 4.44 ppm in the ratio 1:1:4 (Fig. 3B). All protons were coupled to a phosphorus atom with $J_{POCH}$ coupling constants around 10–11 Hz for the two downfield protons and 7–8 Hz for the four upfield protons (Fig. 4B and Table I). Again, these values of chemical shifts and $J_{POCH}$ coupling constants are an indication for the presence of the two equatorial and four axial protons characteristic of the neo-inositol isomer. The $^1$H-decoupled $^{31}$P NMR spectrum of compound II exhibited three singlets at 0.85, 0.72, and 0.55 ppm and two doublets at 9.60 and −11.30 ppm in the ratio 2:2:1:1:1 (Fig. 4C and Table I), suggesting a molecule with seven phosphate atoms among which is a diphosphate group responsible for the peak splitting in the two upfield doublets ($J_{POPH} = 16.2$ Hz). Its $^1$H-decoupled $^{31}$P NMR spectrum showed a splitting of each line into two, except for the doublet corresponding to the $b$P of the diphosphate group (Fig. 4D). This is an indication that each of the phosphate groups, except the $b$P, is coupled with one proton only. The HPLC elution order, the symmetry of the protons, the number of phosphate atoms, and the two doublets at −9.60 and −11.30 ppm were suggestive of PP-neo-InsP$_6$. The $c$-phosphate of the diphosphate group was correlated to an equatorial proton of the neo-inositol, which can be in position 2 or 5 (Fig. 5).

Identification of Compound III as 2,5-bis-PP-neo-InsP$_6$—The
1H-decoupled 31P NMR spectrum (F2 dimension, Fig. 6) of compound III exhibited a singlet at about 1 ppm and 2 doublets indicative of diphosphate bond(s) at −9 and −11 ppm in a 2:1:1 (or 4:2:2) ratio. This would be compatible with a total of either four or eight phosphate groups. The fact that compound III was eluted from the HPLC column after compound II identified above as a 2-PP-neo-InsP5 indicates that it must contain eight phosphate groups, a number that can only be reached if two diphosphate groups are present in the molecule. In a 31P-1H correlation map, the singlet and the doublet corresponding to the α-phosphate of the diphosphate groups are correlated to two groups of protons at 4.4 and 5.0 ppm in the ratio 2:1 (4:2). No other protons were detectable in a one-dimensional 1H NMR spectrum between 10 and 0 ppm, except for the signal of residual water at 4.8 ppm (F1 dimension, Fig. 6). These results are an indication that the two positions carrying equatorial protons (positions 2 and 5) were bearing the diphosphate groups. Compound III was thus identified as 2,5-bis-PP-neo-InsP4.

Presence of neo-Inositol Polyphosphates in Phreatamoeba balamuthi—P. balamuthi is a free living amoeba that lacks mitochondria (20, 21). It also contains unusual diphospho-inositol polyphosphates in large quantities (9). An HPLC-MDD analysis of the three inositol-containing compounds present in P. balamuthi indicated that they exactly co-eluted with neo-InsP6, 2-PP-neo-InsP5, and 2,5-bis-PP-neo-InsP4 purified from E. histolytica (data not shown). We conclude from this that P. balamuthi probably contains the same set of neo-inositol metabolites.

DISCUSSION

Distribution of myo- versus neo-Inositols—Our data show that E. histolytica trophozoites contain phosphate and diphosphate esters of neo-inositol: neo-InsP6, 2-PP-neo-InsP5, and 2,5-bis-PP-neo-InsP4 at cytosolic concentrations of 0.22, 0.36, and 0.08 mM, respectively. So far, the most abundant inositol isomer encountered in nature has been myo-inositol, whereas neo-inositol is only a relatively rare isomer. Neo-InsP6 has first been recognized as a soil constituent (22). Mixtures of myo-, scylo-, chiro-, and neo-inositol are indeed present in soil as their pentakisphosphate and hexakisphosphate esters (23). L-neo-Inositol 1-phosphate has been found in the brain, heart, testis, and spleen of rat in micromolar concentrations (24). Phosphorylated neo-inositol has been identified besides polyphosphates on the cell surface of the freshwater carnivorous Amoeba discoides (25).

In vitro experiments show that E. histolytica trophozoites are sensitive to micromolar concentrations of exogenously added myo-inositol phosphates. E. histolytica possesses specific and separate binding sites for myo-Ins (1,4,5)P3 and myo-Ins (1,3,4,5)P4, and independent calcium stores are releasable by each of these myo-inositol phosphates (26, 27). The presence of high concentrations (~100–400 μM) of neo-inositol polyphosphate derivatives suggests that neo-inositol metabolism may be regulated independently from myo-inositol in this amoeba. Comparable cases are found in nature where the myo-isomer does not represent the major inositol in terms of concentration. High concentrations of scylo-inositol have been found in human brain, breast tumors, and the skate Raja erinacea (28–31), and chiro-inositol has been identified in bovine liver and mouse brain (32).

Bio synthesis of neo-Inositols—Two biosynthesis pathways by which neo-inositol might be formed have been proposed: 1) the cyclization of mannose 6-phosphate into neo-inositol 1-phosphate by l-myo-inositol 1-phosphate (d-myo-inositol 3-phosphate) synthase, the same enzyme that cyclizes glucose 6-phosphate into myo-inositol 1-phosphate (24) and 2) the epimerization of myo-inositol into neo-inositol at C-5 by a de-
hydrogenation to the 5-keto compound (myo-inosose-5) followed by reduction of the carbonyl group (33). Both processes have been proven to proceed in bovine brain (24, 34). In support of the first pathway, a gene encoding L-myoinositol 1-phosphate synthase from *E. histolytica* has been cloned, and the native enzyme from trophozoites has been purified and characterized (35).

**Function of Diphospho-neo-inositols**—Among the neo-inositol phosphates found in *E. histolytica*, two bear diphosphate groups. Diphospho-myoinositol phosphates have been found recently in Dictyostelids and a number of mammalian cell types (4–7, 10, 11). The wide phylogenetic spectrum of distribution of these diphosphate cyclitols is taken as an indication for a fundamental physiological function. Diphosphoinositols have been suggested to be a new form of phosphate donor (2) or serve as intracellular second messengers (13, 14). Alternatively, *in vitro* studies suggest a regulatory function of vesicle trafficking (36, 37). A high turnover rate of diphospho-myoinositol phosphates has been revealed, because treatment with fluoride, which inhibits the bisphosphatase involved in their breakdown, elevated their levels up to 10-fold at the expense of myo-inositol pentakisphosphate and myo-InsP₆ (7, 10). By contrast, treatment of *E. histolytica* with 10 mM NaF, although drastically reducing the levels of nucleotide diphosphates and triphosphates, had no significant effect on the levels of neo-InsP₄, 2-PP-neo-InsP₄, and 2,5-bis-PP-neo-InsP₄ (data not shown). This suggests that the diphospho-neo-inositols are stable end products that are not involved in serving as energy stores or in regulating vesicle traffic. Whatever their function, it is clear that the neo-inositol phosphates found in *E. histolytica* and strongly suspected to be present in *P. bala-
muthi* represent yet another class of inositol metabolites and that nature exploits the full versatility of the inositol moiety.

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