

Phosphorus-31 Nuclear Magnetic Resonance of Insect Hemolymph Sera¹

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Sera from larval and pupal stages of the tobacco hornworm, *Manduca sexta*, have been investigated using phosphorus-31 pulsed Fourier transform nuclear magnetic resonance. Spectra of larval and pupal sera containing 5 mM EDTA were characterized by four major peaks and one or more minor resonances. A phosphorus-31 spectrum of dialyzed larval serum showed several weak signals which indicated the presence of some higher-molecular-weight phosphorylated compounds as well. None of those signals, however, corresponded to any of the ones seen with undialyzed sera. Three of the four prominent peaks and one minor peak in the whole larval serum had the same chemical shifts as those in the pupal samples. The pupal sera, in addition, displayed an extra peak well upfield from those of the larval stage. All of the low-molecular-weight resonances detectable in the hemolymphs have been identified and included four compounds not previously reported; trehalose-6-phosphate, phosphoarginine, phosphatidylcholine, and phosphatidylethanolamine. The phosphometabolites found at millimolar or higher concentrations in larval hemolymph were α -glycerolphosphate, phosphorylcholine, phosphorylethanolamine, inorganic phosphate, trehalose-6-phosphate, phosphatidylcholine, and phosphatidylethanolamine. All of the above compounds were found in pupal sera as well except for the addition of phosphoarginine and the deletion of phosphorylethanolamine. The levels of the phosphometabolites in common between the two stages of development, however, were quite different as were their stabilities after extraction. While the intensities of the larval phosphates remained virtually constant in the presence of EDTA at pH 7.8, those of the pupal sera changed rapidly. This was especially true for arginine phosphate which disappeared quickly.

High-resolution ³¹P nuclear magnetic resonance affords a unique, nondestructive way to observe phosphometabolites. Furthermore, the technique has been applied successfully to *in vivo* biological systems ranging from bacteria to beating hearts (1). We have begun a study of phosphate metabolism in insect tissues by determining the identities and titers of phosphometabolites in sera from the tobacco hornworm, *Manduca sexta* L. (Lepidop-

tera: Sphingidae). This animal was chosen because it is reared conveniently in the laboratory on a semisynthetic diet, grows to a large size (10 g) and undergoes complete metamorphosis (larva to pupa to adult) wherein developmental stages can be timed accurately for physiological studies.

MATERIALS AND METHODS

Manduca sexta eggs were a gift from Dr. J. Reinecke of SEA, USDA, Fargo, N.D., and the larvae were reared on a semiartificial diet (2) at 28°C and 69% relative humidity with a 15 h photophase. Hemolymph was collected by bleeding larvae and pupae through the abdominal horn and proboscis, respectively. After addition of phenylthiourea and centrif-

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ugation at 3200g and 4°C, the serum was transferred to an NMR² tube. The pH of the hemolymph was 6.6.

Phosphorus-31 NMR spectra were obtained using a Varian XL-100-15 instrument equipped for pulsed Fourier transform spectroscopy with a Nicolet 293A programmable pulse unit, quadrature-phase detection hardware, a 1180 time-sharing computer, and a Diablo 44A dual-drive disk unit. In general, a sample was prepared by adding 2.5 ml of pooled sera to a 12-mm NMR tube containing enough solid disodium EDTA to make a 5 mM solution. Upon dissolution of the EDTA the pH was adjusted to either 6.6 or 7.8 with 1 N NaOH and 5-mm concentric capillary containing 3 to 5 mM pyrophosphate in D₂O was inserted to provide the external ³¹P reference and deuterium lock signals. Sometimes the sera became cloudy during pH adjustments necessitating centrifugation prior to analysis. The precipitate obtained was partially soluble in chloroform:methanol (3:1) and a NMR spectrum of the solution showed two to three weak phosphorus signals with chemical shifts in the phospholipid region. In certain cases perchloric acid-precipitated sera (3) also were examined. Overall at least five analyses were performed on sera obtained from different stocks of animals. In addition, male and female sera were compared but no significant differences were found in their low-molecular-weight phosphometabolites.

The spectra of samples containing EDTA routinely were acquired with 1K pulses over a 1K-Hz spectral width using a 60° flip angle, 2K data points, and no delay between pulses. The spectra of sera without EDTA, however, required longer accumulations to achieve an acceptable signal-to-noise ratio. Some spectra of each sample type also were collected over 5000-Hz spectral ranges to check for additional phosphorus signals, but none were detected. Upon acquisition the spectra were processed with no line broadening and were zero-filled to 4K time domain points before transformation. All spectra were obtained at 33 ± 1° and the chemical shifts are reported relative to 6 mM H₃PO₄ in 1 M HCl taken as 0 ppm. The chemical shift of 5 mM pyrophosphate in D₂O, pH 9.0 (meter reading), occurred 6.30 ppm upfield from that of H₃PO₄, and thereby provided a satisfactory secondary reference by avoiding any of the hemolymph signals.

The proton-decoupled fully relaxed ³¹P-NMR spectra used for serum concentration determinations

² Abbreviations used: NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; NOE, nuclear Overhauser enhancement; P_i, inorganic phosphate; PP_i, pyrophosphate; α-GP, α-L-glycerolphosphate; PE, phosphorylethanolamine; PC, phosphorylcholine; T6P, trehalose-6-phosphate; PA, phosphoarginine; Tris, tris (hydroxymethyl)amino-methane; tlc, thin-layer chromatography.

were acquired with 90° pulses and 41-s delays between pulses. This delay represented 4.5 spin-lattice relaxation times for inorganic phosphate which was almost certainly the slowest relaxing phosphate present (4). Concentration of a given compound was determined by comparing its digitally integrated peak intensity with that of a known amount of added pyrophosphate. Nuclear Overhauser enhancements for the serum phosphates also were determined. For these measurements 10 to 50 mM solutions of the authentic compounds in 0.01 M Tris-HCl, 0.15 M KCl, pH 7.8, containing 10 mM EDTA, were used and NOE were calculated from the ratio of each signal intensity with the decoupler on to that with it off (5). This resulted in a 3° change in sample temperature which was not anticipated to alter the intensity of a fully relaxed signal measurably. The NOE values were reproducible to within 6%.

Two-dimensional fingerprint maps were prepared from 80% methanol extracts of hemolymph on thin-layer plastic sheets, 20 × 20 cm, precoated with 100 μm cellulose. Two-dimensional separation was achieved by electrophoresis at pH 1.9 (8% acetic, 2% formic acid) at 200 V per plate for 2.5 h, followed by chromatography with butanol, pyridine, acetic acid, water (15:10:3:16). Phospholipids were extracted with chloroform:methanol (2:1) and separated on silica gel plates using chloroform:methanol:ammonium hydroxide:water (24:14:2:1). The amino acid derivatives were located by staining with ninhydrin-calcium acetate (6) and phosphorus-containing components with a molybdenum blue reagent as modified by Mann *et al.* (7) and lipids with saturated potassium dichromate in 55% sulfuric acid by heating at 180°C for 25 min.

Except as noted, all chemicals were reagent grade and obtained commercially. Trehalose-6-phosphate was a gift from Dr. Donald McDonald of Oregon State University, Corvallis. Most of the reagents were used as received except for some phosphates which had been obtained as relatively insoluble heavy metal salts that were exchanged immediately before use with Na⁺-Dowex 50.

RESULTS

Serum ³¹P-NMR Spectra

Figure 1A shows a proton-decoupled ³¹P spectrum of larval serum as it appeared following extraction. It was evident that larval serum had large amounts of at least five phosphorus-containing components. However, the lines in this spectrum were quite broad which could result from partial immobilization, as might occur in a macromolecular environment, or from in-

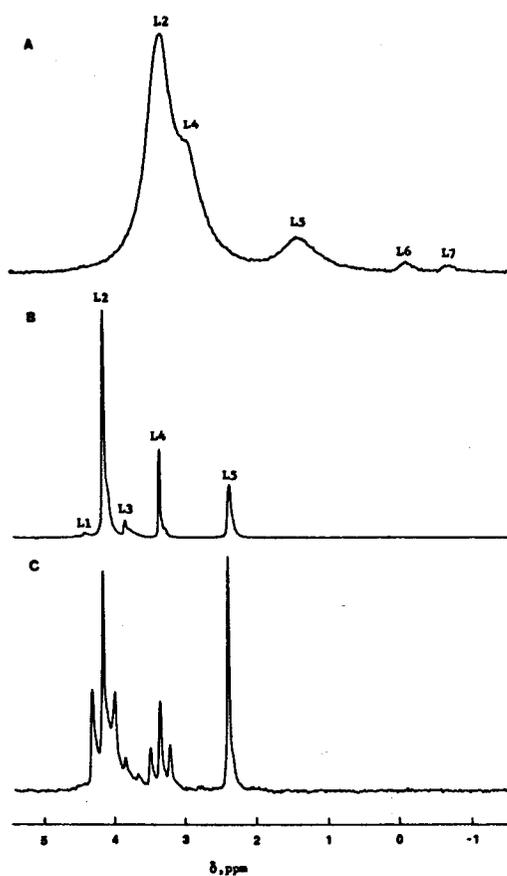


FIG. 1. (A) ^1H -Decoupled ^{31}P -NMR spectrum of *Manduca sexta* larval hemolymph sera, pH 6.6, 33°C. (B) ^1H -Decoupled ^{31}P -NMR spectrum of the neutralized supernatant from the perchloric acid precipitation of the sample in A made 5 mM in EDTA, pH 7.5, 33°C. All of the resonances were shifted downfield relative to those in A but remained in the same relative order (see text). (C) Proton-coupled ^{31}P -NMR spectrum of the solution in B, 33°C.

teractions of the phosphorus compounds with such paramagnetic metal ions as Cu (II) or Fe (III). Figure 1B shows the spectrum of perchloric acid-treated larval serum which was neutralized with K_2CO_3 and to which about 5 mM EDTA had been added. Comparison with Fig. 1A showed that all the resonances in the untreated sample were present in the perchloric acid-treated supernatant except L6 and L7, and that the lines were sharpened dramatically. The chemical shifts in Figs. 1A and B do not correspond because the pH

of the sample in 1B was raised to pH 7.5 to give sharper signals and improved resolution. Additional data gathered in connection with the pH dependence of the chemical shifts (Table 1) confirmed that the discernible resonances in Fig. 1A remained in the same relative order at pH 7.5. (L3, however, was not discernible in Fig. 1A.) The absence of L6 and L7 signals in the acid-treated serum suggested that they may be macromolecular, lipid, or highly labile phosphorus metabolites. Those resonances, however, were visible and remained relatively constant for several hours in serum to which EDTA had been added directly.

To see if macromolecular phosphorus-containing components were present in the serum, we exhaustively dialyzed a sample from larvae against 0.01 M Tris-HCl, 0.15 M KCl, pH 7.8, containing millimolar quantities of phenylthiourea and EDTA, using 2000 cutoff cellulose tubing. The spectrum of the dialyzed serum is shown in Fig. 2. The largest of the observable peaks was the D_3PO_4 external reference. The remaining two or three real peaks confirm the presence of several phosphorus-containing higher-molecular-weight components. None of the resonances corresponded to those of Fig. 1B. Furthermore, the higher-molecular-weight components were present at much lower concentration (probably $<500 \mu\text{M}$ in phosphorus) than the low-molecular-weight compounds shown in Fig. 1B. A chemical shift range of 6 to 12 ppm for the high-molecular-weight species was comparable with that of some phosphorylated proteins (8, 9) but not those of nucleic acids (10, 11), pyridine nucleotides (12, 13), or phospholipids (14). The phosphorylated proteins of *Manduca* hemolymph will be the subject of future studies.

Proton-decoupled ^{31}P -NMR spectra of pupal sera are shown in Fig. 3. Line widths for the pupal serum without EDTA (Fig. 3A) were not nearly so broad as those seen for the larval serum (Fig. 1A), which suggested lower levels of paramagnetic ions at this stage of development. Similar to larval serum peaks the pupal resonances also sharpened upon the addition of EDTA

TABLE I
CHEMICAL SHIFT CHANGES BETWEEN pH 6.4 AND 7.9 OF *M. sexta* SERUM PHOSPHATES-COMPARED WITH THOSE OF AUTHENTIC COMPOUNDS

Peak	Compound	Serum (Δ ppm)	Authentic compound (Δ ppm)	Reference
L1	Trehalose-6-phosphate	1.45	1.43 ^a	(16)
L2	α -L-Glycerolphosphate	1.02	1.41	This study
L3	Phosphorylethanolamine	0.57	0.53	This study
L4	Phosphorylcholine	0.54	0.56	This study
L5	Inorganic phosphate	1.59	1.61	(16)
P7	Phosphoarginine	\sim 0	0.01	This study

^a Shift for glucose-6-phosphate.

to the sample (Fig. 3B). One signal, P7, disappeared during the process. It apparently represented a highly labile compound. All the other signals were present after EDTA treatment and several had identical chemical shifts with those of the larval samples: P1 and L1; P2 and L2; P3 and L4; P4 and L5; P5 and L6; as well as P6 and L7.

Identification of ³¹P Resonances

To begin the characterization of the low-molecular-weight phosphorylated metabolites, we obtained the proton-coupled ³¹P spectrum of larval hemolymph shown in

Fig. 1C. Clearly proton coupling splits peaks L2, L3, and L4 into triplets with *J* values of 7–10 Hz while leaving L5 unsplit and unbroadened. These observations combined with the chemical shift range of 3 to 5 ppm suggested that L2–L4 arose from mono-*O*-esterified phosphates containing equivalent protons on a β -carbon atom and that L5 was inorganic phosphate. Furthermore, the absence of any well-defined ³¹P doublets or higher multiplets in the proton-decoupled spectrum (Figs. 1B and 3B) precluded the presence of polyphosphate esters at sufficient concentration for detection. Finally, a chemical shift of 2.85 ppm at pH 7.85 for L5

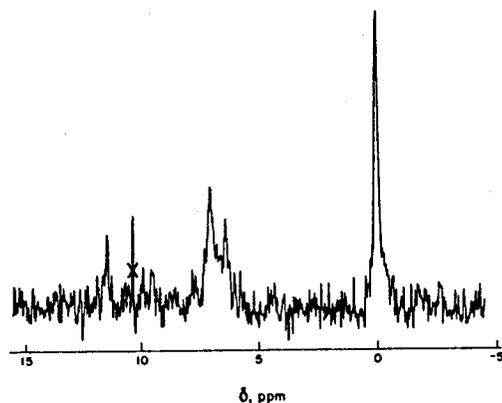


FIG. 2. ¹H-Decoupled ³¹P-NMR spectrum of the larval serum components of greater than 2000 dalton molecular weight, pH 7.8, resulting from 60,000 acquisitions using 60° pulses, 4K data points over a 2500-Hz spectral width, and no delay, 33°C. The spike marked with an X is an artifact.

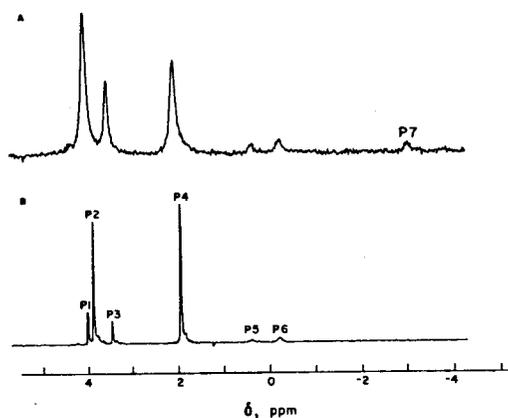


FIG. 3. (A) ¹H-Decoupled ³¹P-NMR spectrum of *Manduca sexta* pupal hemolymph without EDTA and 1–2 h after extraction, pH 6.6. (B) Spectrum of the same sample several hours later and after the addition of 5 mM EDTA, pH 6.5.

further linked this resonance with inorganic phosphate.

To facilitate identification of the metabolites, the pH dependence of several resonances was determined. The pH dependence of the L1-L5 resonances between 6.4 and 7.9 was compatible with the known behavior of phosphate and its mono-*O*-esterified derivatives since raising the pH from 6.6 to 7.4 shifted all the peaks downfield (Figs. 1A and B). In EDTA-treated larval sera at pH values near 6.6, L3 appeared as a downfield shoulder on the large L2 signal but became well separated at pH 7.5 (Fig. 1B) or higher due to its relatively smaller shift in this region. Most of the identifications of the larval resonances were performed at pH 7.8 to take advantage of the better resolution at higher pH.

Initial assignment of each resonance was accomplished where possible by adding small quantities of authentic phosphates directly to the serum in question. A probable identification was accepted when the intensity of a peak increased without detectable broadening of the EDTA-sharpened signal. At this point T6P had been found to match with L1 and P1, α -GP with L2 and P2, PC with L4 and P3, P_i with L5 and P4, as well as PA with P7. However, it was not possible to assign reasonably conclusively peak L3 by this procedure alone because serine-*O*-phosphate and PE both appeared to correspond to this resonance. Separate ^{31}P titrations showed virtually identical shifts for these compounds between pH 6.6 and 7.8 in 0.15 M KCl, 0.01 M Tris-HCl containing 10 mM EDTA. Other evidence (*vide infra*) suggested that L3 was probably PE. Furthermore, the chemical shifts of L6 and L7, and their pupal counterparts, P5 and P6, fall in the phospholipid region and their limited solubility prevented identification by direct additions. Instead, the major phospholipid components were identified by silica gel chromatography (Fig. 4) and the NMR resonances assigned tentatively from the chemical shifts of the corresponding pure compounds in chloroform:methanol (3:1) or from literature

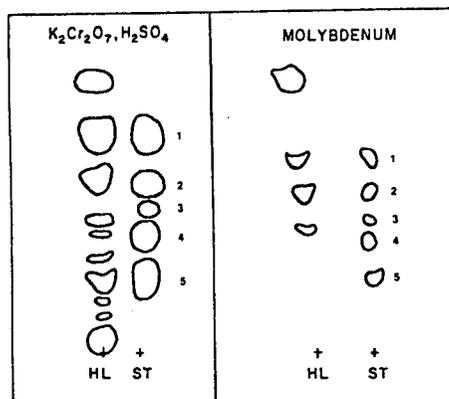


FIG. 4. Thin-layer chromatograph of methanol-chloroform extract of larval serum. Left: $\text{K}_2\text{Cr}_2\text{O}_7$ - H_2SO_4 sprayed. Right: Hanes-Isherwood molybdenum spray. HL, hemolymph serum; St, standards. 1, Phosphatidylethanolamine; 2, phosphatidylcholine; 3, lysophosphatidylethanolamine; 4, phosphatidylserine; 5, lysophosphatidylcholine.

chemical shift values of aqueous suspensions (14, 15).

To further confirm these probable identifications, the chemical shift changes with pH for most of the hemolymph resonances were compared with those of authentic compounds as assigned above (Table I). All of the shift changes (Δppm) agreed quite well except those for larval α -GP and thereby provided further verification of the probable assignments based on direct additions of compounds to the sera. Perhaps at the levels of α -GP found in larval serum (Table II) 5 mM EDTA does not completely free it from complexation. The chemical assignments of each of the numbered resonances in Figs. 1 and 3 are given in Table II. The chemical shifts for each metabolite also agree with those of the corresponding authentic compounds in 0.15 M KCl, 0.01 M Tris-HCl buffer within 0.2 ppm except for larval (pH 7.8) and pupal (pH 6.8) α -GP which are about 0.2 and 0.4 ppm too high, respectively, and the value for pupal P_i which exceeds that of the pure compound by a little over 0.5 ppm.

Phosphometabolite Concentration

Table II also lists concentrations of the phosphometabolites present in the two

TABLE II
PHOSPHORUS-CONTAINING METABOLITES IN LARVAL AND PUPAL SERA FROM *Manduca sexta*

Peak	Serum chemical shifts (ppm)	Compound	NOE	Concentration mM
Larval (pH 7.8)				
L1	4.95	Trehalose-6-phosphate	1.50 ^b	6
L2	4.67	L- α -Glycerolphosphate	1.47	177
L3	4.34	Phosphorylethanolamine	1.55	23
L4	3.82	Phosphorylcholine	1.41	59
L5	2.84	Inorganic phosphate	1.36	101
L6	0.10	Phosphatidylethanolamine ^a	—	~8
L7	-0.46	Phosphatidylcholine	—	~4
Pupal (pH 6.6)				
P1	4.03	Trehalose-6-phosphate	1.50 ^b	16
P2	3.90	L- α -Glycerolphosphate	1.47	24 ^c
P3	3.49	Phosphorylcholine	1.41	54 ^c
P4	2.05	Inorganic phosphate	1.36	22 ^d
P5	0.41	Phosphatidylethanolamine ^a	—	~2
P6	-0.17	Phosphatidylcholine ^a	—	~2
P7	-3.24	Arginine phosphate	1.43	22 ^c

^a Tentative assignments based on literature chemical shifts and the presence of these metabolites as shown by chromatography.

^b Value for glucose-6-phosphate.

^c Values obtained from extrapolation to time of extraction.

^d The initial concentration observed after extraction.

types of sera as determined from appropriate NMR spectra. Larval phosphate concentrations were determined on freshly prepared sera containing EDTA and adjusted to pH 7.8 to which known amounts of pyrophosphate had been added as described under Materials and Methods. No measurable change in phosphate concentration occurred over several hours under these conditions. Concentrations determined from even fully relaxed spectra can be systematically in error, however, due to differences in NOE arising from the simultaneous irradiation of proton resonances. Accordingly NOE of several of the hemolymph phosphates were determined and are listed in Table II. (Not reported in Table II is the NOE of PP_i , the internal reference, which is 1.39.) These enhancements in general agreed with those of Yeagle *et al.* (17) for the same or similar compounds. The concentrations of most of the larval and pupal serum phosphates listed

in Table II have been corrected for differences in NOE. They are estimated to be reproducible to within 10%. Because insufficient reference material was available, it was necessary to substitute the NOE of glucose-6-phosphate for that of T6P. It was not possible to measure the concentrations of the minor species as accurately as those for the major peaks and, accordingly, no efforts were made to apply NOE corrections to their intensities.

A similar approach for determining the concentration of phosphometabolites in pupal serum at pH 6.5 was not successful because of significant loss of most compounds over the first few hours after extraction. Just raising the pH to 7.8 in the presence of EDTA did not slow the degradation sufficiently either. Apparently pupal serum contained higher levels of phosphatase or other phosphate-utilizing enzymes than the larval samples. This activity even degraded the added pyrophos-

phate. Consequently phosphate concentrations in pupal sera were found from a set of spectra obtained on a sample that had been made 105, 14, and 12.9 mM in NaF, EDTA, and PP_i , respectively, and adjusted to pH 7.8 immediately after extraction and while still chilled. These conditions were selected to inhibit acid and alkaline phosphatase activity (18, 19). The solution was quickly placed in the spectrometer, warmed to probe temperature (33°C) and three fully relaxed proton-decoupled spectra were collected over the next 6 h. A fourth spectrum was taken at 20 h. This procedure slowed the activities adequately and allowed all except the minor components to be observed in each spectrum. PC and PA decreased steadily over the observed interval with PC showing the steeper decline in the inhibited hemolymph samples. α -GP and PP_i , on the other hand, remained essentially unchanged throughout. The loss of phosphorus intensity by the choline and arginine derivatives, however, did not immediately appear as increased P_i concentration. Indeed, P_i decreased initially only to rise above the original intensity sometime between 7 and 20 h. The *in vivo* pupal concentrations of α -GP, PC, and PA were predicted by extrapolating a first-order plot of intensity vs time of extraction. The zero-time intensities were converted to concentrations through the known concentration of PP_i and corrected for NOE differences as described above. The P_i concentration given in Table II for the pupal hemolymph is that obtained from the first spectrum and therefore represents an average value between 1 and 3 h after extraction. Subsequently the P_i dropped to 13.4 mM at 7 h and finally rose to 43.0 mM by 20 h.

DISCUSSION

The phosphate compositions of the hemolymph sera summarized in Table II generally agree with, and extend the results of earlier purely chemical approaches (20-24). It is evident from the present study that *M. sexta* pupal serum

has a total phosphate concentration of about 150 mM and larval serum of nearly 400 mM. This is in marked contrast to human plasma or red blood cells whose total low-molecular-weight phosphate content is only a few millimoles (25, 26). A value of 150 mM, however, agrees reasonably well with the 122 ± 22 mM total phosphate concentration reported by Jungreis (24) for pharate *M. sexta* pupae reared on the same semisynthetic diet. Clearly phosphates must make a very substantial contribution to the osmolarity of insect hemolymph, especially at the larval stage.

In regard to *M. sexta* larval hemolymph, the ^{31}P -NMR analyses showed α -GP to be the major phosphorus-containing compound in complete agreement with the chemical studies of Wyatt *et al.* (21). α -GP was also a substantial, if not the major, phosphate of *Galleria mellonella* hemolymph (22). α -GP is an end product of glycolysis as well as lipolysis and, therefore, probably serves as a substrate for mitochondrial oxidation in muscle (27-29) and as a precursor for the glycerol that accumulates during diapause in many insects (30).

Besides α -GP, Wyatt and co-workers reported finding only inorganic phosphate in hemolymph from full-grown *M. sexta* larvae but apparently that organism was not investigated as thoroughly as *Hyalophora cecropia* in which PC and PE were present along with other unidentified phosphates. Levels of PC and PE in silkworm hemolymph, however, were many times higher than those found in the hornworm or in several other species (21). It was primarily on this basis that L3 was identified as PE instead of serine-*O*-phosphate despite their nearly identical chemical shifts at the pH values investigated. Furthermore, serine-*O*-phosphate apparently has never been found in any insect hemolymph. Consequently, it appears that α -GP, PC, PE, and P_i were the major phosphorus-containing metabolites of larval hemolymph in most species thus far investigated. The notable exception to this was *Bombyx mori*, which almost completely substituted sorbital-6-phosphate

for α -GP. Efforts to locate sorbital-6-phosphate or glucose-6-phosphate in *M. sexta* larval sera by ^{31}P -NMR analyses were unsuccessful as they apparently had been by chemical means (21). It is interesting that the four major phosphates of larval hemolymph were precursors of phospholipids. Apparently absent at detectable levels, however, were glycerolphosphorylcholine and glycerolphosphorylethanolamine.

In addition to the four compounds described above, the present study has identified T6P, phosphatidylcholine, and phosphatidylethanolamine in *M. sexta* larval hemolymph. Identification of T6P was by direct additions of the compound to the serum while the phospholipids were assigned from their chemical shifts and their known presence as confirmed by silica gel tlc analysis. The occurrence of T6P at millimolar levels seemed reasonable since trehalose (and T6P because the earlier studies did not distinguish between them) ranged from <10 to >100 mM depending on the species, development stage, and sex (24, 31, 32). Although the presence of an enzyme capable of hydrolyzing T6P had been demonstrated previously in insects (33-35), to our knowledge this metabolite previously had not been identified directly. In *Manduca* larvae, the trehalose titer was also rather large (30 to 50 mM) but based on the concentration of T6P (Table II), only 12-20% was phosphorylated. The T6P level in larval sera treated with EDTA, however, remained quite constant with time after extraction indicating the absence of phosphatase or other enzymatic activities utilizing T6P as a substrate. Neither was there an apparent active synthesis of T6P after extraction. The phospholipids, on the other hand, were labile in the sense that as the sample aged, they tended to precipitate. This may indicate that they were stabilized in solution by interactions with proteins or other macromolecules which were themselves susceptible to enzymatic or denaturing factors. Alternatively, the removal of divalent metal ions by EDTA may be responsible for their precipitation since it

was during this step that the solutions first became cloudy.

Fewer data were available on phosphorus-containing metabolites of pupal hemolymph than on larval. In the one previous detailed study of phosphates in pupal hemolymph Wyatt *et al.* (21) found *H. cecropia* pupae to have a higher overall acid-soluble phosphorus content than the corresponding fifth stadium larvae. In *M. sexta* just the opposite situation was obtained. From the concentrations listed in Table II it can be seen that pupal serum has only one-half the total phosphate content of larval. The distribution of compounds also differed in the two stages. In fresh pupal sera α -GP had been replaced as the major component by PC. In terms of concentration, pupal serum PC was about the same as that of larval while α -GP was only one-seventh as much. P_i concentrations also differed considerably with larvae having five times the amount in pupae. Another distinguishing feature of *M. sexta* pupal hemolymph is the absence of PE. Again, this differs from *H. cecropia* in that PE not only was present but it occurred at concentrations about one-third that of PC. It is possible, however, that PE was even more labile in extracted pupal sera than PC and it may have escaped detection by our present methods, especially if it occurred at very low concentrations.

In addition to the above compounds, ^{31}P -NMR analyses allowed two previously unreported *M. sexta* pupal serum phosphates, T6P and PA, to be identified. The concentration of serum T6P in pupae was nearly three times that found in fully developed larvae and represented the fifth most abundant phosphometabolite. The ratio of T6P to total trehalose in pupal sera, however, differed widely from that in larval. From 60 to 100% of the disaccharide must be phosphorylated in pupae since the concentration of T6P is 16 mM and the total trehalose concentration was only half that found at the larval stage (32). This difference in titer no doubt reflected the changing metabolic needs of the individual developmental stages.

The presence of surprisingly large

amounts of phosphoarginine was confirmed by ^{31}P -NMR after direct addition of the authentic compound to pupal sera and by fingerprint analysis of 80% methanol serum extracts on thin-layer cellulose sheets, which revealed the presence of two major phenanthrenequinone- and ninhydrin-positive zones with identical mobilities to those of arginine and phosphoarginine (36). Furthermore, the lack of a chemical shift between pH 6.6 and 7.8 (Table I) further confirms the metabolite. PA was the third or fourth most abundant phosphate of pupal serum from *M. sexta* but it was highly labile. Indeed, it was not detected in the initial ^{31}P -NMR studies and was found only when special efforts were made to obtain spectra quickly in the presence of phosphatase inhibitors. Phosphoarginine no doubt serves as a reservoir of phosphate bond energy in *Manduca* as has been suggested for other insect species (37-39). Phosphoarginine also has been found by ^{31}P -NMR to be a major low-molecular-weight phosphate of barnacle muscle (40) and in the muscles of the marine invertebrate, *Tapes watlingi* (41).

In summary, ^{31}P -NMR has proved to be a rapid, convenient, nondestructive method to study phosphorus physiology in a problem concerning developmental biology. Four kinds of phosphometabolites have been detected in insect hemolymph at reasonable levels of sensitivity (>0.5 mM) including inorganic phosphate, low-molecular-weight *O*-phosphoesters, higher-molecular-weight phosphoesters, and a guanidinophosphate.

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