BIOORGANIC CHEMISTRY 11, 55–71 (1982)

Preparation, Structure, and Properties of Periodate-Oxidised ATP, a Potential Affinity-Labelling Reagent

PETER N. LOWE*,†,[1] AND R. BRIAN BEECHEY†

*Department of Biochemistry, Chelsea College (University of London), Manresa Road, London SW3 6LX; and †Shell Research Limited, Shell Biosciences Laboratory, Sittingbourne Research Centre, Sittingbourne, Kent ME9 8AG, United Kingdom

Received June 10, 1981

(1) Periodate oxidation of ATP yields a single product which has been purified and characterised. Periodate-oxidised ATP (o-ATP) behaves as a single compound during TLC analysis, but NMR spectral studies show that it exists in aqueous solution as an equilibrium mixture of three dialdehyde monohydrates and a dihydrate. Little free aldehyde is present. The dialdehyde monohydrates are in the form of diastereomeric cyclic hemiacetals. (2) The dialdehyde grouping of o-ATP can be reduced with sodium borohydride, producing a dialcohol. (3) o-ATP has been frequently used in attempts to affinity label nucleotide-binding sites on proteins. The proposed structure of o-ATP is discussed in relation to this use for o-ATP.

Oxidation of ribonucleotides by sodium periodate causes a cleavage of the vicinal diol group to form a dialdehyde derivative (*1*). In recent years the products have been increasingly used to affinity label nucleotide-binding sites on enzymes ((*2, 3*) and references therein).

Although these dialdehyde derivatives are prepared relatively easily, little is known about their structure, stability, and reaction chemistry, which constitute information relevant to their use in affinity labelling. Research into the structure of periodate-oxidised nucleotides has usually been limited to degradation reactions and to the production of derivatives, most of which have not been fully characterised (*4–9*). Some evidence pertaining to the structure of these compounds has been obtained from uv and ir spectra (*1, 10, 11*). NMR spectroscopy has been hitherto of little use due to the complexity of the spectra obtained.

In order to utilise periodate-oxidised nucleotides for the affinity labelling of enzymes it would seem essential to have more knowledge about their structure and chemistry. With this in mind we describe here the preparation of pure periodate-oxidised ATP (o-ATP).[2] This compound has been characterised and its structure determined by high-field-strength NMR spectroscopy of the compound and its borohydride-reduced derivative. This work extends the preliminary observations reported in Ref. (*12*).

[1] Present address: Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW, U.K.

[2] Abbreviations used: o-ATP, periodate-oxidised ATP, i.e., 9*H*-purine-9-acetaldehyde, 6-amino-α-(1-formyl-2-hydroxyethoxy)triphosphate; o-ATP/BH_4, borohydride-reduced o-ATP, i.e., 2,2′[1-(9-adenyl)-1′-(triphosphoryloxymethyl)]dihydroxydiethyl ether; o-ATP/B^2H_4, borodeuteride-reduced o-ATP.

0045-2068/82/010055-17$02.00/0

EXPERIMENTAL

Preparation and Purification of o-ATP

The reaction between ATP and sodium periodate is second order at 22°C with a rate constant $k_2 = 1.06 \times 10^3$ liters · min^{-1} · mol^{-1}. The time of reaction for preparation of o-ATP was adjusted for differing concentrations of reactants to give greater than 99% reaction. A typical large-scale preparation of o-ATP is as follows: adenosine 5'-triphosphate, disodium salt, trihydrate (113 mg, 0.187 mmol), dissolved in 0.9 ml of water and adjusted to pH 7 with 0.03 ml of 50% (w/v) NaOH, was oxidised with 1.1 ml of 0.2 *M* sodium periodate (0.22 mmol). After 1 hr in the dark at 4°C, excess periodate was destroyed by addition of 2 μl of ethane-1,2-diol. The whole reaction mixture was applied to a Sephadex G-10 column (1.6 × 78 cm) equilibrated with water at 4°C. The column was eluted with water at 4°C at a flow rate of 0.3–0.4 ml/min. The presence of solutes in the eluate was followed by measuring the absorbance at 227 and 260 nm and by testing for iodate. o-ATP was eluted first (46–70 ml). Up to an elution volume of 60 ml only adenine-containing compounds were eluted. After this, iodate appeared in the eluate. Iodate-free fractions were pooled and lyophilized. The resulting white powder was kept at −20°C over silica gel. The yield was 74 mg, 0.111 mmol quantitated from A_{260}. In general the overall yield was 60–75%.

Preparation of Borohydride- and Borodeuteride-Reduced o-ATP

The following procedures were carried out at 4°C. ATP, disodium salt (271 μmol), dissolved in 0.5 ml water, was adjusted to pH 7 with 35 μl of 50% (w/v) NaOH and then 1.59 ml of 0.2 *M* sodium periodate was added. After 75 min, 5 μl of ethane-1,2-diol was added and the solution was left for a further 40 min. Solid sodium borohydride (3 mmol) was added in small aliquots over a period of 1 hr with constant cooling in ice. Intermittently, 9 *M* H_2SO_4 was added to maintain the pH between 8 and 9. Twenty minutes after the final addition of borohydride, the pH was returned to 7 by further addition of H_2SO_4. During this procedure a thick white precipitate formed which was suspected to be inorganic material. This was removed by centrifugation. The supernatant contained 90% of the 260 nm-absorbing material. It was applied to a Sephadex G-10 column as for the large-scale preparation of o-ATP. The elution volume of the 260 nm-absorbing material was identical with that for o-ATP. The fractions containing high concentrations of o-ATP/BH_4 were pooled and lyophilized. The yield was 182 μmol (67%).

Borodeuteride-reduced o-ATP was prepared in the same way except $NaBH_4$ was replaced by NaB^2H_4.

Criteria of Purity for Oxidised Nucleotides

Four criteria have been used routinely to assess the purity of periodate-oxidised nucleotides.

Ultraviolet spectra. Spectra were measured at pH 7. At 227 nm the molar extinction coefficients for ATP, periodate, and iodate are 2500, 9200, and 1050

liters · mol^{-1} · cm^{-1}, respectively, whereas those at 260 nm are 15,000, 870, and 140 liters · mol^{-1} · cm^{-1}, respectively. Hence the ratio A_{227}/A_{260} can be used as a sensitive indication of the contamination of the nucleotide by iodate and periodate.

Dry weight per mole of adenine. Weighed samples of periodate-oxidised nucleotides were dissolved in water and adjusted to pH 7. The amount of adenine was quantified from the A_{260} of the solution.

Freedom from iodate and periodate. Iodate and periodate were detected in solutions using acidified starch–iodide paper. Samples of periodate-oxidised nucleotides which gave a positive reaction were not used.

TLC analysis of periodate-oxidised nucleotides and their borohydride-reduced derivatives. TLC was performed on silica gel (F_{254}) analytical plates obtained from Merck AG and on polyethyleneimine-cellulose plates (Polygram CEL 300 PEI/UV 254, Machery-Nagel & Co.). Before use the polyethyleneimine-cellulose plates were washed by ascending elution with distilled water until the solvent reached the top edge of the plate. The plates were dried in air and stored at 4°C. This washing procedure removed yellow material from the running area of the plate.

The presence of adenine, adenosine, AMP, ADP, ATP, periodate-oxidised adenosine, AMP, ADP, ATP, and the adenine-containing elimination product (*12*), and tripolyphosphate could be detected by TLC on silica gel eluted by either 2 *M* LiCl, or propan-2-ol/water (1 : 1), acetonitrile/water (4 : 1), or acetonitrile/ethanol (1 : 1) or by TLC on polyethyleneimine-cellulose eluted with 1 or 2 *M* LiCl (*3*).

To facilitate analysis, some samples were reduced by the addition of a 3- to 20-fold molar excess of $NaBH_4$; after 15 to 30 min the samples were analysed.

Adenine-containing compounds were detected by fluorescence-quenching on irradiation with uv (254 nm) light. Phosphorus-containing compounds were detected by spraying with the reagent described in Dawson *et al.* (*13*). Aldehyde groups were detected by spraying with a solution of 2,4-dinitrophenylhydrazine (0.1 g dissolved in 15 ml of concentrated HCl and 15 ml of water and then diluted to 100 ml with water). Positive spots were red or yellow on a pale-yellow background.

Estimation of Phosphorus

Inorganic phosphate was estimated by the method of Lindberg and Ernster (*14*). Total phosphorus (combined organic and inorganic) was measured by the method of Lowry *et al.* (*15*).

NMR Spectroscopy

Aqueous solutions of either o-ATP, o-ATP/BH_4, or o-ATP/B^2H_4 were adjusted to pH 7 and then lyophilized. The dry powder was then dissolved in ^2H_2O.

^{1}H-NMR spectra were obtained at 360 MHz on a Bruker WH360 spectrometer at 25°C. The sample concentration was 4–8 mg/ml. The sample volume was approximately 0.5 ml. The probe diameter was 5 mm. ^{13}C-NMR spectra were

obtained using a Jeol PFT 100 spectrometer with a 10-mm-diameter probe at 25 MHz. The sample concentration was 30–200 mg/ml. The sample volume was 1–2 ml. Proton noise decoupled 40.5 MHz-^{31}P-NMR spectra were obtained on a Jeol PFT 100 spectrometer. Chemical shifts were referenced as stated in the figures and tables. The deuterium resonance of the solvents was used as a field lock. All the spectrometers were used in the Fourier-transform mode.

RESULTS

Characterisation of o-ATP

Ultraviolet spectrum. The spectrum of o-ATP, in aqueous solution at pH 7, is superimposable upon that of ATP. It is unlikely that significant amounts of free aldehyde are present in an aqueous solution of o-ATP, since free aldehyde groups absorb in the range 260–290 nm (*16*). The adenine moiety must be intact with N-9 still attached to C-1′ of the ribose moiety and the 6-amino group cannot have reacted with any aldehyde, since either of these modifications would result in a shift of the wavelength maximum (*17*). Elimination to the α,β-unsaturated aldehyde is also excluded, since this compound shows absorption maxima at 235 and 280 nm (*12, 16*).

Dry weight per mole of adenine. The dry weight of seven different preparations of o-ATP was determined to be (mean ± sd) 573 ± 47 g/mol of adenine. On the basis of the formula o-ATP, Na_3, the expected molecular weight is 572. Thus, o-ATP is effectively free from contaminating salts.

Phosphorus analyses. No significant amount of P_i was found in o-ATP which had been lyophilized, stored at −20°C for 5 weeks, and then dissolved in water. Freezing and thawing three times did not increase the P_i present. Assays for total phosphorus in o-ATP showed 3.0 ± 0.2 mol of P/mol of adenine.

TLC analysis. o-ATP chromatographs as a pure material on silica-gel TLC. However, on polyethyleneimine-cellulose plates o-ATP reacts with the amino groups of polyethyleneimine (*18, 19*). Subsequently, a slow elimination reaction can occur producing tripolyphosphate. Thus, after elution the adenine portion and most of the phosphorus of o-ATP remains at the origin while a small amount of tripolyphosphate can be detected. The tripolyphosphate cannot have been present before the o-ATP was applied to the polyethyleneimine-cellulose plate, since unpurified borohydride-reduced o-ATP chromatographs as a single phosphate- and adenine-containing compound, with no material remaining at the origin. No tripolyphosphate is detected.

Evidence that aldehyde group(s) are present in o-ATP. o-ATP reacts chemically as if it contains an aldehyde-functional group, since (a) it reacts with amino groups, e.g., with polyethyleneimine and N-α-acetylysine; (b) it reacts with 2,4-dinitrophenylhydrazine to produce a yellow–orange product; (c) it is easily reduced by $NaBH_4$ (see below); and (d) it reacts with dimedone to produce an addition compound. These aldehyde groups appear to be hydrated (see Fig. 1) since (a) the uv spectrum does not indicate the presence of free aldehyde groups,

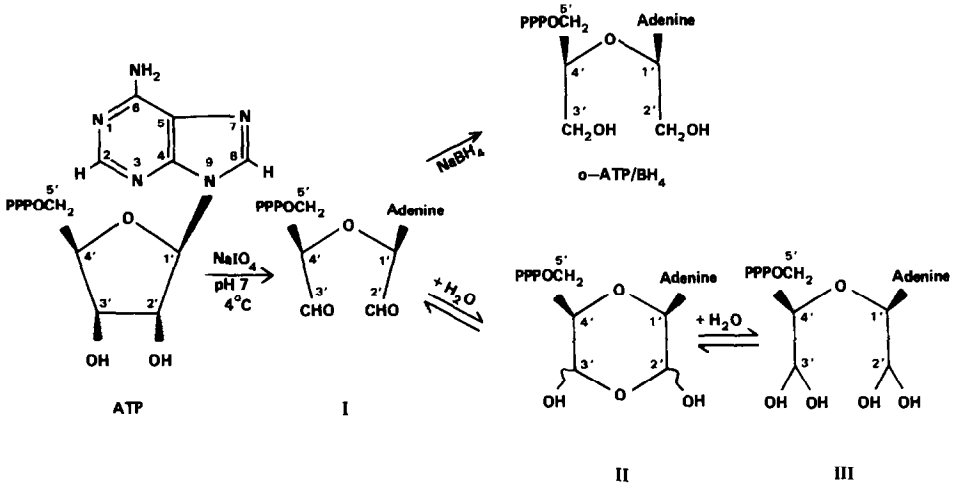


FIG. 1. Periodate oxidation of ATP, hydration of the dialdehyde product, and reduction with $NaBH_4$. Structures **I**, **II**, and **III** represent the different forms of o-ATP in aqueous solution. Structure **II** could exist as four diastereomers (**IIa–d**, Table 1) and as a polymer linked by C–O–C bridges.

and (b) the ^1H- and ^{13}C-NMR spectra of o-ATP show resonances typical of hydrated aldehydes (see below).

Analysis of o-ATP by NMR Spectroscopy: ^1H-NMR Spectroscopy

The spectrum of o-ATP consists of a large number of resonances (Figs. 2 and 3). At 360 MHz the HO^2H signal does not appear to overlap with any resonances from o-ATP. The spectra of two different samples of o-ATP were recorded (Figs. 2a and 3). These spectra differ slightly in the chemical shifts of some of the resonances, presumably due to slight differences in the pH and concentration between the two samples. These differences proved useful in elucidating the fine structure of the spectra. The complexity of the spectrum is thought to be due to the presence of four different forms of o-ATP coexisting in the solution (cf. Fig. 1 and Table 1).

To facilitate discussion of the spectrum, it can be divided into three sections: that at lowest field (greater than 8δ) due to adenine protons and those at high (3.0–4.7δ) and low (4.7–6.2δ) field of the water signal, which are due to ribose-derived protons.

Lowest field section (greater than 8δ). The spectrum of o-ATP contains six peaks in the range 8.2–8.65 (resonances 1–6, Fig. 2a and Table 2). These resonances are ascribed to the protons (H-2 and H-8) of the adenine moiety. The chemical shifts are similar to those observed for the adenine ring protons in ATP (8.56δ, H-8; 8.36δ, H-2).

The combined area of resonances 1–4 is the same as that of resonance 5 plus resonance 6 (Table 2). The combined areas of resonances 7 to 18 and of 1 to 6 are in the ratio 3 : 2 (Table 2). Resonances 7 to 18 are interpreted as belonging to the three protons, H-1′, H-2′, and H-3′ (see below). The integration is thus consistent

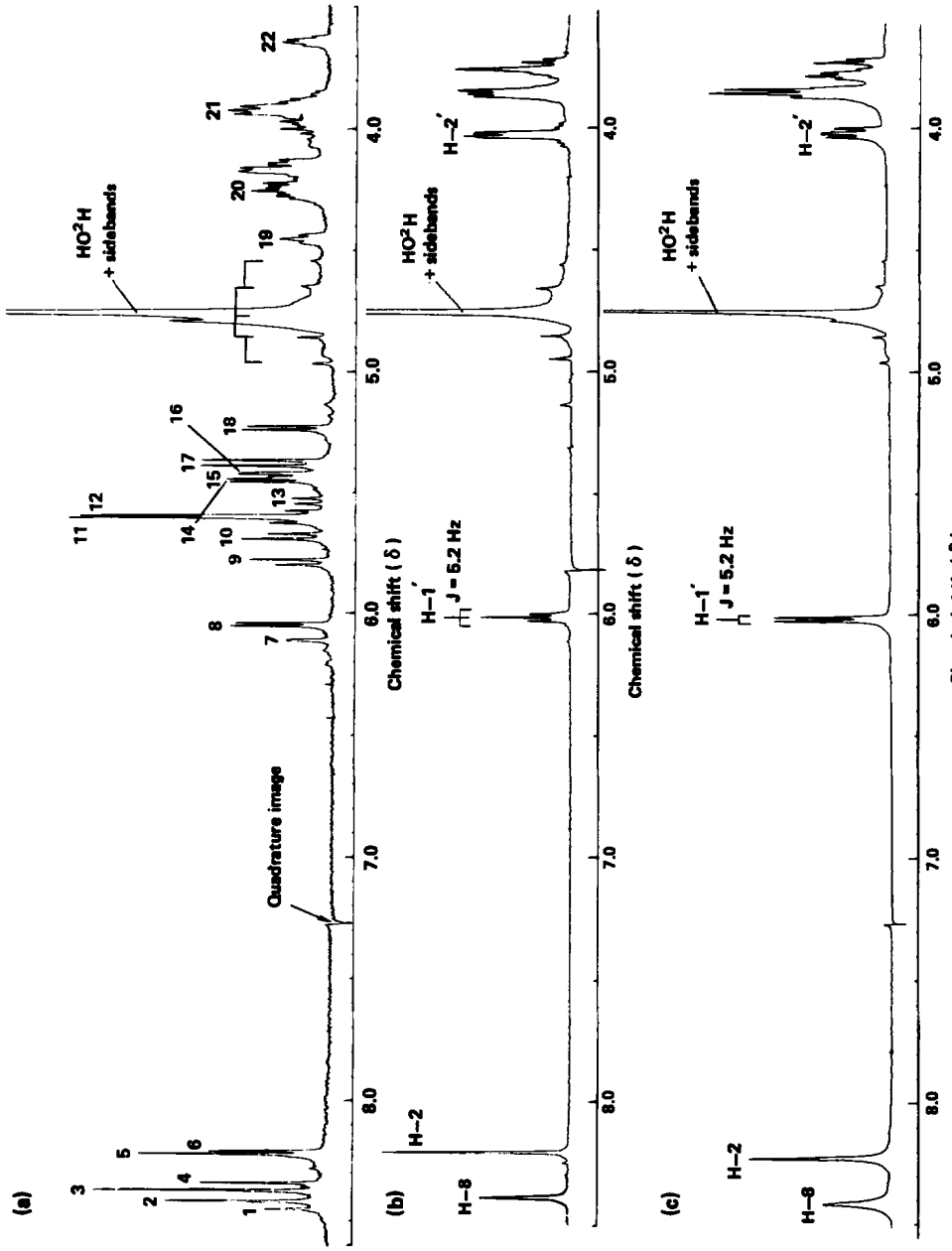


FIG. 2. ^{1}H-NMR spectra of o-ATP, o-ATP/BH_4, and o-ATP/B^2H_4. ^{1}H-NMR spectra of o-ATP(a), o-ATP/BH_4(b), and o-ATP/B^2H_4(c) were recorded in ^2H_2O at 360 MHz. The chemical shifts are referenced to sodium [2,2,3,3-$^2H_4$]2,2-trimethylsilyl propionate added after the spectra were recorded. Part of the spectrum of a different sample of o-ATP is shown in Fig. 3. The numbers above the resonances in a refer to those used in Tables 2 and 3.

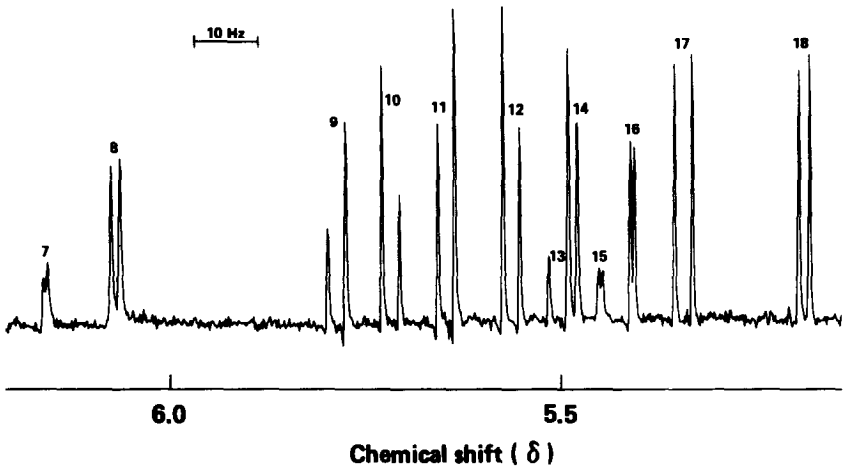
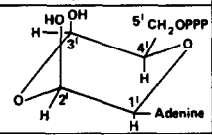
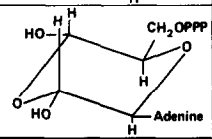
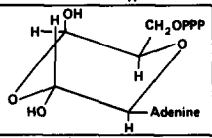
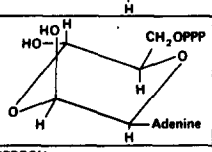
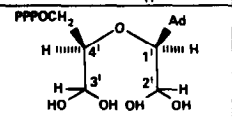


FIG. 3. Convolution-difference ^{1}H-NMR spectrum of o-ATP; low-field ribose-derived section. The ^{1}H-NMR spectrum of a sample of o-ATP in ^2H_2O was recorded at 360 MHz. The convolution-difference spectrum of the region between 5.2 and 6.2δ is shown above. It should be noted that the sample of o-ATP differs from that shown in Fig. 2 (see Results).

TABLE 1

CONFORMATIONAL ANALYSIS OF HYDRATED FORMS OF o-ATP; PREDICTED AND EXPERIMENTAL VALUES FOR COUPLING CONSTANTS

Structure	Theoretical: Conformation of hydroxyl groups 2'–OH	3'–OH	Predicted coupling constants* $J_{1'-2'}$ (Hz)	$J_{3'-4'}$ (Hz)	Experimental: Experimental coupling constants $J_{1'-2'}$ (Hz)	$J_{3'-4'}$ (Hz)
II a 	axial	axial	<4	<4	Not present	Not present
II b 	equatorial	equatorial	>6	>6	7.5	8.0
II c 	equatorial	axial	>6	<4	8.0	1.7
II d 	axial	equatorial	<4	>6	1.8	8.1
III 	average by rotation	average by rotation	3–6	3–6	4.1	5.0

* Williams and Fleming (*20*).

TABLE 2

CHEMICAL SHIFTS AND COUPLING CONSTANTS DERIVED FROM THE ^{1}H-NMR SPECTRUM OF o-ATP

Resonance number	Chemical shift, δ (ppm)	Integration	Multiplicity	Coupling constant, J (Hz)	Assignment: Proton	Assignment: Structure
1	8.51	~0.25 } 0.42	s	—	H-8	IIb or III
2	8.50	~0.17 }	s	—	H-8	IId
3	8.42	0.33 } 0.57	s	—	H-8	IIb or III
4	8.40	0.23 }	s	—	H-8	IIc
5	8.31	0.40 } 1.00	s	—	H-2	{ IIb, IIc, IId, and III }
6	8.28	0.60 }	s	—	H-2	
7	6.13	0.11	d	1.8	H-1′	IId
8	6.05	0.32	d	4.1	H-1′	III
9	5.77	0.25	d	8.0	H-1′	IIc
10	5.70	0.22	d	8.0	H-2′	IIc
11	5.63	0.32	d	7.5	H-1′	IIb
12	5.55	0.33 } 2.97	d	7.5	H-2′	IIb
13	5.50	0.10	d	8.1	H-3′	IId
14	5.47	0.33	d	4.1	H-2′	III
15	5.44	0.10	d	1.8	H-2′	IId
16	5.40	0.22	d	1.7	H-3′	IIc
17	5.34	0.35	d	8.0	H-3′	IIb
18	5.18	0.32	d	5.0	H-3′	III
19	~4.5		m	1.8, 5.0, 8.0	H-4′	IIc
20	~4.3	~3	m	Not resolved	{ H-5′, -H-5″ }	
21	~3.9		m		H-4′	
22	~3.7		m		H-4′	III

Note. The data in this table are derived from the spectrum of o-ATP, of which part is shown in Fig. 3. Similar data were obtained from the spectrum shown in Fig. 2a. The structures of o-ATP referred to are shown in Fig. 1 and Table 1.

with resonances 1 to 6 being due to 2 protons. Resonances 1 to 4 are assigned to H-8. The four resonances are due to different environments found in the various forms of o-ATP. Presumably H-2 also exists in four environments but two of the resonances are superimposed resulting in only two apparent signals (resonances 5 and 6).

There is no evidence for free aldehyde groups, since aldehyde protons resonate usually in the range 9–11δ. Furthermore, either a 2′- or 3′-aldehyde would couple with vicinal protons ($J \simeq 2$ Hz) (*20*); no such coupling is observed.

Low-field section (4.7–6.2δ). This section consists of 12 resonances (numbered 7 to 18, see Figs. 2a and 3 and Table 2). Each of these resonances is a doublet (J = 1.8–8.0 Hz) (Fig. 3). Evidence from decoupling experiments (see Table 3) shows that resonances 7, 8, 9, and 11 are coupled to resonances 15, 14, 10, and 12, respectively. The total integration of resonances 7, 8, 9, and 11 is equal to that of resonances 15, 14, 10, and 12 (see Table 2) and is also equal to the area under the resonances due to either H-8 or H-2 (see above).

TABLE 3

DATA FROM DECOUPLING EXPERIMENTS PERFORMED ON THE ^{1}H-NMR SPECTRUM OF o-ATP

Proton irradiated		
Resonance number	Chemical shift, δ (ppm)	Effects observed
7	6.13	Collapsed doublet 15 to a singlet
8	6.05	Collapsed doublet 14 to a singlet
9	5.77	Collapsed doublet 10 to a singlet
11	5.63	Collapsed doublet 12 to a singlet
13	5.50	Collapsed broad doublet at δ4.05 to a broad singlet
16	5.40	Changed AMNX system, 19, to a double doublet
18	5.18	Changed AMNX system, 22, to a double doublet
19	4.44	Collapsed doublet 16 to a singlet[a]
20	4.24	No effects to low field of HO^{2}H signal[a]
—	4.17	No effects to low field of HO^{2}H signal[a]
20/21	4.05	Collapsed doublet 13 to a singlet[a]
21	3.93	Collapsed doublet 17 to a singlet and multiplet 22 to a doublet
22	3.64	Collapsed doublet 18 to a singlet

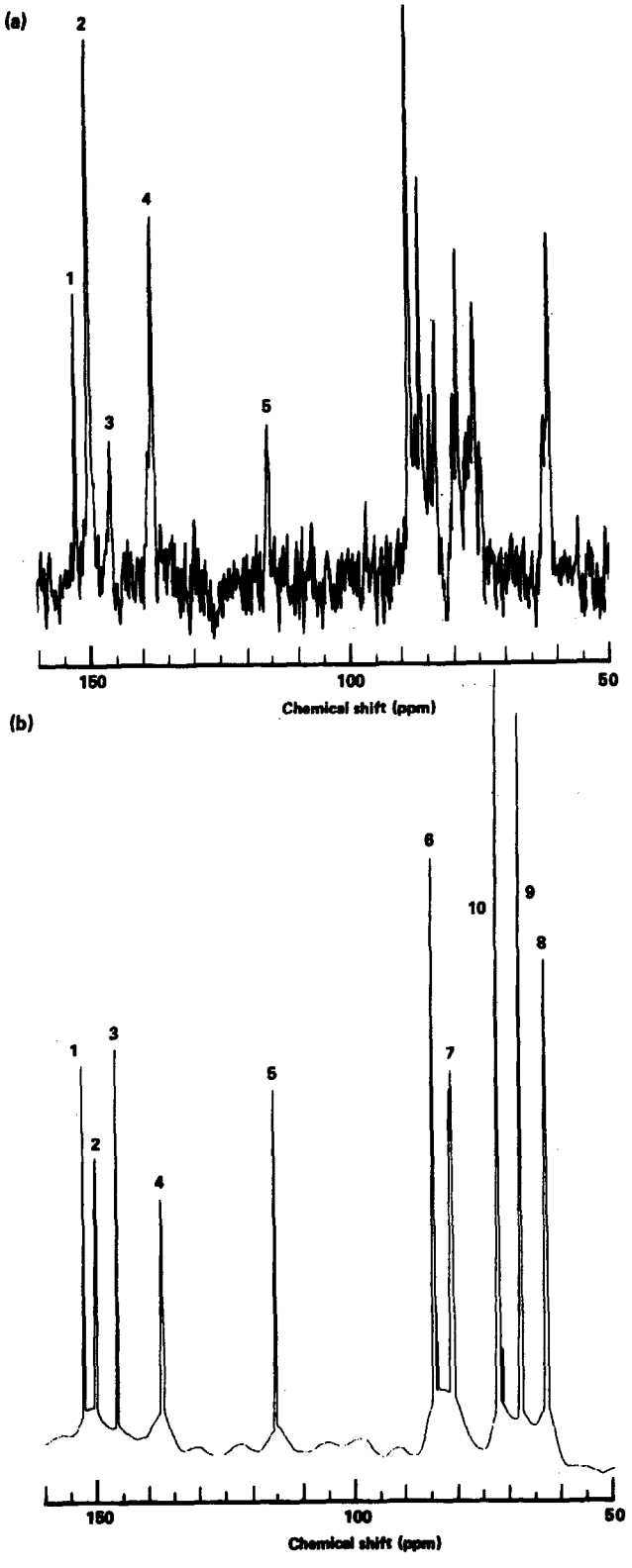
Note. No effects on protons resonating to low field (δ8–9) were observed. Irradiation of the signal due to HO^{2}H did not alter any of the other resonances.

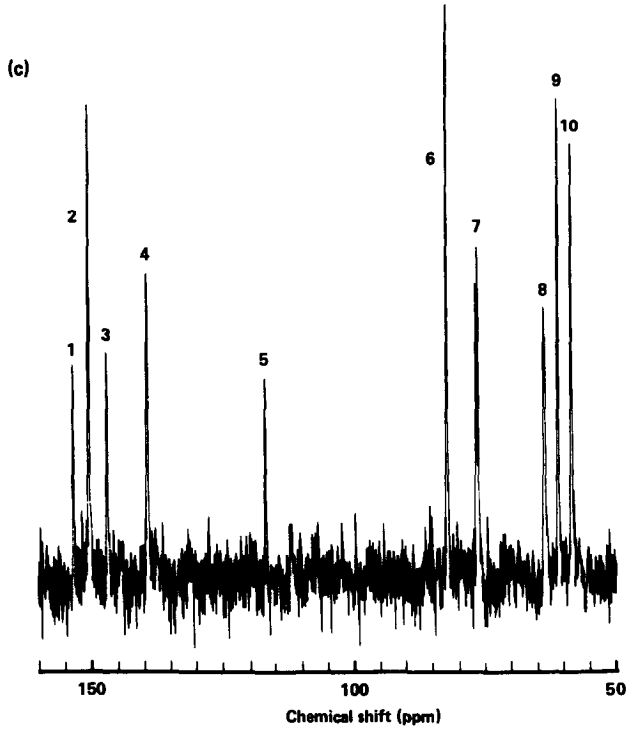
[a] Changes to the high-field region (δ3.6–4.5) occurred but are too complex to interpret.

The resonances at lower field (7, 8, 9, and 11) have been assigned to H-1′ and those at slightly higher field (15, 14, 10, and 12) to H-2′. These assignments are based on the assumption that H-1′ is more deshielded than H-2′ by the adenine moiety.

Resonances 13, 16, 17, and 18 form a group which are all coupled to protons resonating approximately 1 ppm upfield, i.e., to high field of the HO^{2}H signal (Table 3). The total area under these four lines is equivalent to one proton. The chemical shifts are similar to those of H-2′. These results are consistent with resonances 13, 16, 17, and 18 being due to H-3′, with o-ATP having either of the Structures **II** or **III** in Fig. 1. Thus H-3′ is coupled to H-4′ which would have resonances at higher field than H-3′ and the chemical shifts of H-2′ and H-3′ are both in the range typical for hydrated aldehyde groups (*19, 21*).

High-field section (3.0–4.7δ). One can see from Fig. 2a that this region is very much more complex than the regions discussed already. The two multiplets, resonances 19 and 22, are typical for AMNX systems. Irradiation of H-3′ resonances, 16 and 18, causes collapse of these two multiplets to double doublets (see Table 3), showing that they represent H-4′ resonances in different environments. The resonances are split into doublets by H-3′ and then again into doublets by magnetically nonequivalent H-5′ and H-5″. The remaining parts of the spectrum must be due to the two other H-4′ resonances and the corresponding H-5′ and H-5″ resonances. The situation is complicated, not only because four isomers of o-


(a)
1
2
3
4
5
150
100
50
Chemical shift (ppm)
(b)
1
2
3
4
5
6
7
8
9
10
150
100
50
Chemical shift (ppm)



ATP are present, but also because of the complexity of H-5′ (and H-5″) resonances of ribose 5′-triphosphates. The resonances of the H-5′ proton are coupled to the geminal proton H-5″ and the α- and β-phosphorus atoms, as well as to H-4′.

^{13}C-NMR Spectroscopy

The ^{13}C-NMR spectrum obtained at 25 MHz was not well resolved (Fig. 4). The adenine region of the spectrum from 110 to 160 ppm is generally similar to that for ATP, although the resonances for o-ATP are broader (see Fig. 4). The ribose region, 50–100 ppm, of the o-ATP spectrum is significantly different from that of ATP, and it is difficult to distinguish individual resonances. The broadening of the adenine resonances and the great complexity of the ribose-derived resonances in the spectrum of o-ATP is consistent with a model in which four similar compounds exist in solution.

The spectrum can be interpreted, in general terms, by comparison with spectra

FIG. 4. ^{13}C-NMR spectra of o-ATP, ATP, and o-ATP/BH_4. ^{13}C-NMR spectra of o-ATP(a), ATP(b), and o-ATP/BH_4(c) were recorded in ^2H_2O at 25 MHz. No internal reference was used but the chemical shifts on the spectra are directly comparable. All spectra are broad-band proton noise decoupled. The numbers above the resonances refer to those used in Table 4. It should be noted that no resonances were observed between 0 and 60 ppm and above 160 ppm.

TABLE 4

^{13}C-NMR SPECTRAL DATA FOR o-ATP/BH_4 AND ATP

Resonance number	Assignment	o-ATP/BH_4 Chemical shift, δ (ppm)	o-ATP/BH_4 Proton noise decoupled, multiplicity, J (Hz)	o-ATP/BH_4 Coupled, multiplicity, J (Hz)	ATP Chemical shift, δ (ppm)	ATP Coupled multiplicity
1	C-6	152.5	s	d, 10.7	152.5	d
2	C-2	149.5	s	d, 202.9	150.2	d
3	C-4	146.0	s	d, 10	146.2	d
4	C-8	138.3	s	d, 213.6	137.5	d
5	C-5	115.8	s	d, 10.7	115.6	d
6	C-1′	81.1	s	d, 160.2	85.1	d
7	C-4′	75.3	d, 9.2	d,138.9[a]	81.5	d
8	C-5′	62.4	d, 6.1	t, 151[a]	60.3	t
9	C-2′	59.7	s	t, 145	68.1	d
10	C-3′	57.2	s	t, 144	72.5	d

Note. ^{13}C-NMR spectra of o-ATP/BH_4 and ATP were recorded in solution in ^2H_2O at 25 MHz. No reference was used but the spectra were recorded under the same conditions so that the shifts are comparable. The ^{13}C-NMR spectra of ATP and o-ATP/BH_4 are shown in Fig. 4.

[a] The coupling to phosphorus was not resolved in the coupled spectrum.

for ATP and some model compounds. C-2′ and C-3′ of ATP resonate at 68.1 and 72.5 ppm, respectively (Table 4). In the spectrum of o-ATP, however, no lines are visible between 62 and 74 ppm, but resonances are present in the region 84–90 ppm. The carbon atoms of hydrated aldehydes resonate in this region, e.g., glyoxylic acid, 88.5; formaldehyde, 84–92; and glutaraldehyde, 93–97 ppm. The changes in the spectrum are thus consistent with periodate oxidation converting C-2′ and C-3′ into hydrated aldehyde carbons. Free aldehyde-carbon atoms which would resonate at 180–210 ppm are absent.

^{31}P-NMR Spectroscopy

Spectra of ATP and o-ATP were obtained at both pH 6.8 and 8.9. Chemical shifts are based on reported values for the α phosphorus atom of ATP (*22*). The following data were obtained:

ATP: δ (ppm) (^2H_2O, pH 6.8) 8.2 (d, J = 19 Hz, *γ-P*), 11.0 (d, J = 19 Hz, *α-P*), 22.0 (t, J = 19 Hz, *β-P*). (^2H_2O, pH 8.9) 5.7 (d, J = 19 Hz, *γ-P*), 10.9 (d, J = 19 Hz, *α-P*), 21.4 (t, J = 19 Hz, *β-P*).

o-ATP: δ (ppm) (^2H_2O, pH 6.8) 8.7 (broad, *γ-P*), 11.1 (d, J = 18 Hz, *α-P*), 22.3 (broad, *β-P*). (^2H_2O, pH 8.9) 5.8 (d, J = 20 Hz, *γ-P*), 10.9 (d, J = 19 Hz, *α-P*), 11.0 (d, J = 19 Hz, shoulder), 21.4 (t, J = 19 Hz, *β-P*).

It can be seen that the chemical shifts of o-ATP are very similar to those of ATP when measured at the same pH. Thus a linear triphosphate group is still present

attached to the ribose ring. Free tripolyphosphate, in contrast, has only two resonances and a larger coupling constant (*23*).

Evidence for the Structure of o-ATP Obtained by Reduction with $NaBH_4$

In order to show that o-ATP was the product of periodate oxidation of ATP, o-ATP was reduced with $NaBH_4$ to give o-ATP/BH_4, a dialcohol derivative. The uv spectrum of o-ATP/BH_4 is identical to that of o-ATP and ATP, demonstrating that the adenine moiety has not been reduced. o-ATP/BH_4 runs as a single spot on all the TLC systems used. o-ATP/BH_4 migrates away from the origin on polyethyleneimine-cellulose TLC plates eluted with LiCl, whereas unreduced o-ATP remains at the origin due to reaction of the polyethyleneimine with aldehyde groups. Further evidence that the aldehyde groups have been reduced to o-ATP/BH_4 is that the compound does not react immediately with 2,4-dinitrophenylhydrazine. Borodeuteride-reduced o-ATP was also prepared, and it had uv spectral and TLC properties identical to those of o-ATP/BH_4.

The structures of o-ATP/BH_4 (Fig. 1) and o-ATP/B^2H_4 have been confirmed by ^{1}H-NMR spectroscopy. The spectrum of o-ATP/BH_4 is much simpler than that of o-ATP (Fig. 2). As described by Von der Haar *et al.* (*8*) the low-field adenine region (Fig. 2b) shows only two singlets at 8.45δ and 8.26δ. As in the spectrum of ATP these are assigned to H-8 and H-2. H-1′ appears as a triplet (J = 5.2 Hz), at 6.0δ, which is similar in shift and coupling constant to H-1′ in ATP (d, J = 5.5 Hz). Since each of the protons H-8, H-2, and H-1′ gives only one resonance and the areas of each of these resonances are equal, one can conclude that only one major configuration is present.

The two protons H-2′ and H-2″ are not equivalent and appear at 4.1δ as an AB system further split by H-1′ to form an ABX system. Since H-1′ appears as a triplet, the coupling constant between H-2′ and H-1′ is identical with that between H-2″ and H-1′. Thus, it would seem likely that free rotation is possible around the C-1′ and C-2′ bonds. The protons at higher field were not well resolved. The H-5′, H-5″ system (3.8δ) is very different from that in ATP. In ATP an AB system is present, because the rigid ribose conformation make the two protons significantly nonequivalent. In o-ATP/BH_4, however, free rotation is possible around the C-3′, C-4′ bond, so that the two protons on C-5′ are nearer equivalence.

The ^{1}H-NMR spectrum of o-ATP/B^2H_4 is shown in Fig. 2c. This spectrum is further confirmation of the structure proposed for borohydride-reduced o-ATP. The resonance of H-1′ is now a doublet with J = 5.2 Hz, since one of the protons of the 2′-CH_2OH group has been replaced by a deuterium atom. Irradiation of the H-1′ signal leads to a simplification of the resonance at 4.1δ (H-2′) to a doublet with J = 7.5 Hz, which is the geminal coupling J_{H-C^2H}.

The ^{13}C-NMR spectrum of o-ATP/BH_4 is very similar to that of ATP, and is considerably simpler than that of o-ATP (see Fig. 4). The assignments of the adenine region are based on the coupled spectrum (not shown), and on the spectrum of ATP (see Fig. 4 and Table 4). Assignments of C-1′, C-4′, and C-5′ of the modified ribose are unequivocal because of the shifts, long-range phosphorus–carbon coupling, and the proton–carbon coupling. Thus C-1′ appears as a doublet

in the proton-coupled spectrum but shows no phosphorus coupling in the proton noise decoupled spectrum. C-4′ appears as a doublet in the coupled spectrum and as a doublet (J = 9.2 Hz) due to coupling with the α-phosphorus atom in the proton noise decoupled spectrum. C-5′ appears as a triplet in the coupled spectrum and as a doublet (J = 6.1 Hz) in the proton noise decoupled spectrum, again due to coupling with the α-phosphorus atom. As in ATP the four-bond coupling constant $J_{C-4'-P}$ is greater than the three-bond coupling constant $J_{C-5'-P}$ (*24*). C-2′ and C-3′ both appear as singlets in the proton noise decoupled spectrum but as triplets in the proton-coupled spectrum due to coupling with two protons in the $-CH_2OH$ groups. C-2′ and C-3′ have similar shifts; and the assignment chosen is based on the ^{1}H-NMR spectrum, in which H-2′ is more deshielded than H-3′. Relative to ATP all the ribose carbons are shifted upfield with C-2′, C-3′, and C-4′ showing the greatest effect. It is difficult to envisage any structure, other than that proposed, which is compatible with these observations.

CONCLUSIONS AND DISCUSSION

Although the preparation of o-ATP has been described previously, the product was not usually purified but was used crude in an aqueous solution (*8, 25, 26*). The method described here for the purification of o-ATP is based on that of Cory and George (*27*) and Easterbrook-Smith *et al.* (*2*). o-ATP prepared in this way was found to be chromatographically over 98% pure and to be essentially free of extraneous salts. No unreacted ATP could be detected by TLC or NMR spectroscopy. The availability of pure o-ATP enabled us to investigate its structure.

The Structure of o-ATP

We have shown that o-ATP contains an intact adenine ring (uv spectrum) and three atoms of phosphorus per mole of adenine (phosphorus estimation). The ^{31}P-NMR spectrum shows the presence of a linear triphosphate group. Alkaline degradation of o-ATP liberates tripolyphosphate (*12*), also indicating the presence of a linear triphosphate.

o-ATP would be expected to be a dialdehyde derivative. However, no free aldehyde groups are present in o-ATP, although chemically it reacts as an aldehyde. Neither the ^{13}C- nor the ^{1}H-NMR spectra of o-ATP show any resonances typical of free aldehydes (Figs. 2, 3, 4, and Table 2). Both spectra, however, show resonances typical for hydrated aldehydes, $H(R)C(OH)_2$, or hemiacetals, H(R)C(OH)OR.

The ^{1}H-NMR spectra of o-ATP are complex in that more than one resonance occurs for each proton or carbon atom. The ^{1}H-NMR spectrum of o-ATP (Figs. 2 and 3) is relatively well resolved and shows four resonances for each proton. The spectrum is interpreted on the basis that each set of four resonances represents the same proton in different environments, as would be expected if four major forms of o-ATP coexist in aqueous solution.

Other workers have also shown that various periodate-oxidised ribonucleosides

possess a hydrated dialdehyde group (*1, 10, 11*). o-ATP could exist as an equilibrium mixture of dialdehyde hydrates (Structures **I, II,** and **III,** Fig. 1), as has been proposed for periodate-oxidised adenosine (*1, 10*), periodate-oxidised α-D-glucopyranoside (*28*), and glutaraldehyde (*21, 29*). The cyclic hemiacetal (Structure **II**) could exist as four diastereomers (**IIa–d**, see Table 1), depending upon the orientation of ring closure between C-2′ and C-3′, and as a polymer. The polymeric form is thought not to be present, since o-ATP has a high mobility on silica-gel TLC plates eluted with 2 *M* LiCl and shows less multiplicity of resonances in the ^{1}H-NMR spectrum than would be expected in a polymer.

On the assumption that the adenine and CH_2OPPP moieties prefer to be equatorial, the chair forms of Structures **IIa–d,** shown in Table 1, would be the predominant conformers (*30*). Since 1,3-diaxial interactions of *O* substituents are unfavourable (*30*), Structure **IIa** would be less likely to be present. Those structures with axial electronegative substituents, **IIc** and **IId**, may be favoured since a tendency for hydroxyl groups to be in an axial position has been noted in 1,4-dioxans and sugars (*30, 31*).

The observed coupling constants $J_{H-1'-H-2'}$ and $J_{H-3'-H-4'}$ (see Table 1) are in agreement with this very simple conformational analysis. Thus Structure **IIa** would be expected to show two small couplings (about 2–3 Hz). No such set of couplings was observed (Table 1), indicating that form **IIa** does not exist under the conditions used to record the spectrum. Couplings consistent with Structures **IId, IIc,** and **IIb** being present in o-ATP are found (Table 1). Thus the two large couplings expected for the diaxial couplings in **IIb** are found (J = 7.5 and 8.0 Hz). Structures **IId** and **IIc** both would be expected to show one diaxial and one axial–equatorial coupling and would show large and small values for the two constants. We observed J = 1.8 and 8.1 Hz for **IId**, and 1.7 and 8.0 Hz for **IIc** (Table 1). This analysis confirms that the remaining signals in the spectrum are due to the dihydrate **III**, which would be expected to have coupling constants in the range 3–6 Hz (*20*); values of J = 4.1 and 5 Hz are observed.

Thus o-ATP exists in aqueous solution as a mixture of four major compounds. These are thought to be three diastereomers of the cyclic hemiacetal (Structures **IIb, IIc, IId,** Table 1) and the dihydrate (Structure **III**, Fig. 1). These forms are proposed to be in equilibrium with each other and with a very small amount of free aldehyde, since (a) sodium borohydride reduces the mixture to a single compound, (b) the chemical reactivity indicates the presence of groups reacting like aldehydes, and (c) the compounds all run as a single spot in a number of different TLC systems.

The results are in agreement with those of Jones *et al.* (*10*), who found that o-adenosine consisted of aldehyde hydrates (unidentified) but not the polymeric or free aldehyde forms. In contrast, Hansske *et al.* (*1*) also studied o-adenosine and suggested that a proportion of the compound existed as a polymer, both as a solid or in solution. Borchardt *et al.* (*19*) examined the ^{1}H-NMR spectra of periodate-oxidised adenosine and *S*-adenosyl-homocysteine and found that three or four forms of these compounds existed at equilibrium. They proposed that these were all hydrated aldehydes. No work on the structure of o-ATP in solution has been previously reported.

The Use of o-ATP as an Affinity Label

The use of o-ATP as an affinity label for ATP-binding sites on proteins stems from the reactivity of the dialdehyde group within the molecule and from its supposed resemblance to ATP. The results presented above show that whereas it reacts chemically as if it contains a dialdehyde grouping, in aqueous solution the aldehyde groups are in fact almost completely hydrated. Hydrated o-ATP exists in four major forms (see Table 1). The dihydrate (Structure **III**, Fig. 1) constitutes about 32% of o-ATP (Table 2). This compound does not contain a sugar ring and its conformation is likely to be very different from that of o-ATP. The remaining forms (Structures **IIb, IIc,** and **IId,** Table 1) are monohydrates existing in the form of cyclic hemiacetals, i.e., substituted 1,4-dioxanes; these structures more closely resemble ATP with the five-membered ribose ring converted by periodate oxidation to a six-membered ring. These three forms of o-ATP differ one from another in the configuration of the substituents on the 1,4-dioxane ring. Presumably different diastereomers would interact differently with an ATP-binding site, and thus the concentration of the species interacting at a site need not necessarily be the same as the concentration of added o-ATP.

The chemical shifts and coupling constants observed in the ^{31}P-NMR spectrum and their variation with pH are similar to those found for ATP. This correspondence suggests that the triphosphate groups of o-ATP and of ATP are both in similar environments.

ACKNOWLEDGMENTS

P.N.L. acknowledges support from the Science Research Council (CASE). We thank Dr. D. P. Leworthy, Dr. P. D. Regan, and Mr. D. M. Barnett of Shell Research Limited for recording ^{1}H- and ^{13}C-NMR spectra and C. Brown of the University of Kent for recording ^{31}P-NMR spectra. We are grateful to Drs. P. E. Linnett and L. Mulheirn for useful discussions.

REFERENCES

1. F. Hansske, M. Sprinzl, and F. Cramer, *Bioorg. Chem.* **3,** 367 (1974).
2. S. B. Easterbrook-Smith, J. C. Wallace, and D. B. Keech, *Eur. J. Biochem.* **62,** 125 (1976).
3. P. N. Lowe, Ph.D. Thesis, University of London, 1980.
4. D. M. Brown, M. Fried, and A. R. Todd, *J. Chem. Soc.,* 2206 (1955).
5. J. X. Khym, *Biochemistry* **2,** 344 (1963).
6. D. M. Brown and A. P. Read, *J. Chem. Soc.,* 5072 (1965).
7. A. Steinschneider, *Biochemistry* **10,** 173 (1971).
8. F. Von der Haar, E. Schlimme, M. Gomez-Guillen, and F. Cramer, *Eur. J. Biochem.* **24,** 296 (1971).
9. F. Hansske and F. Cramer, *Carbohydr. Res.* **41,** 366 (1975).
10. A. S. Jones, A. F. Markham, and R. T. Walker, *J. Chem. Soc. Perkin Trans. 1,* 1567 (1976).
11. F. Hansske and F. Cramer, *Carbohydr. Res.* **54,** 75 (1977).
12. P. N. Lowe, H. Baum, and R. B. Beechey, *Biochem. Soc. Trans.* **7,** 1131 (1979).
13. R. M. C. Dawson, D. C. Elliott, W. H. Elliott, and K. M. Jones, "Data for Biochemical Research," 2nd ed., p. 575. Oxford Univ. Press, London, 1969.

14. O. LINDBERG AND L. ERNSTER, "Methods of Biochemical Analysis" (D. Glick, Ed.), Vol. III, pp. 6–7. Interscience, New York, 1956.
15. O. H. LOWRY, N. R. ROBERTS, K. Y. LEINER, M. WU, AND A. L. FARR, *J. Biol. Chem.* **207,** 1 (1954).
16. J. G. GRASELLI, "Atlas of Spectral Data and Physical Constants for Organic Compounds," p. A-64. Chemical Rubber Co., Cleveland, 1973.
17. P. GACESA AND W. J. WHISH, *Biochem. Soc. Trans.* **5,** 1407 (1977).
18. J. W. BODLEY AND J. GORDON, *Biochemistry* **13,** 3401 (1974).
19. R. T. BORCHARDT, Y. S. WU, AND B. S. WU, *Biochemistry* **17,** 4145 (1978).
20. D. H. WILLIAMS AND I. FLEMING, "Spectroscopic Methods in Organic Chemistry," 3rd ed. McGraw–Hill, New York/London, 1980.
21. E. B. WHIPPLE AND M. RUTA, *J. Org. Chem.* **39,** 1666 (1974).
22. M. COHN AND T. R. HUGHES, *J. Biol. Chem.* **235,** 3250 (1960).
23. M. M. CRUTCHFIELD AND R. R. IRANI, *J. Amer. Chem. Soc.* **87,** 2815 (1965).
24. D. E. DORMAN AND J. D. ROBERTS, *Proc. Nat. Acad. Sci. USA* **65,** 19 (1970).
25. I. R. LEHMAN, M. J. BESSMAN, E. S. SIMMS, AND A. KORNBERG, *J. Biol. Chem.* **233,** 163 (1958).
26. R. LAMED, Y. LEVIN, AND A. OPLATKA, *Biochim. Biophys. Acta* **305,** 163 (1973).
27. J. G. CORY AND C. B. GEORGE, *Biochem. Biophys. Res. Commun.* **52,** 496 (1973).
28. C. D. HURD, P. J. BAKER, R. P. HOLYSZ, AND W. H. SAUNDERS, *J. Org. Chem.* **18,** 186 (1953).
29. A. H. KORN, S. H. FEAIRHELLER, AND E. M. FILACHIONE, *J. Mol. Biol.* **65,** 525 (1972).
30. H. R. GREENBERG AND A. S. PERLIN, *Carbohydr. Res.* **35,** 195 (1974).
31. C. ALTONA, C. ROMERS, AND E. HAVINGA, *Tetrahedron Lett.* p. 16 (1959).