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RAPID PURIFICATION OF PLANT NUCLEOTIDE EXTRACTS WITH XAD-2, POLYVINYLPOLYPYRROLIDONE AND CHARCOAL*

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SUMMARY

A rapid chromatographic procedure is described for removing from plant extracts the phenols and other compounds that interfere with the chromatography and UV determination of nucleotides. XAD-2 sorbs some nucleotides, but at pH 2 it has a much higher affinity for phenols. Differences in the affinity of XAD-2 for nucleotides can be accounted for by differences in their electronic structures.

INTRODUCTION

The chromatographic separation and UV determination of nucleotides in plant extracts are seriously hindered, and in some instances prevented, by the presence of phenols. These compounds have chromatographic properties similar to those of nucleotides and they poison ion-exchange resins. Phenols strongly absorb UV below 400 nm; thus, in plant extracts, they can prevent detection, measurement, and characterization of nucleotides by UV absorbance. Phenols and nucleotides have not been separated satisfactorily by differential extraction or solvent-partitioning procedures, nor by conventional gel filtration, sorption chromatography or ion-exchange chromatography.

Loomis and Battaile[1] showed that some phenols are selectively sorbed by several water-insoluble polymers having hydrogen-bonding capability. Water-insoluble polyvinylpolypyrrolidone (PVP), a hydrogen acceptor[1,2], was especially effective. It sorbed phenols optimally at a pH of about 3.5 (ref. 2), but did not seem to bind nucleotides at pH 3 when it was used to remove phenols from plant nucleotide preparations[3]. PVP decreased the phenol concentration of plant extracts enough to improve the resolution of nucleotides on an anion-exchange column but, unfortunately, not enough to prevent phenol interference in the spectrophotometric determination and characterization of eluted nucleotides[3].

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A non-polar cross-linked polystyrene resin, Amberlite* XAD-2 also has been shown to be an efficient sorbent of phenols and other aromatic compounds in aqueous solution[4–8]. Loomis[5] reported that a combination of XAD-4 (chemically identical to XAD-2) and PVP removed more pigments from potato-tuber and walnut-hull extracts than either resin alone. Non-polar molecules or groups are bound to XAD-2 primarily by Van der Waals-type forces[4]; the polystyrene resin has no hydrogen bonding groups. Binding strength increases with decreasing polarity of the sorbed molecules, as in the case of a reversed-phase column packing. We are not aware of specific studies of nucleotide binding to XAD-2, but the resin has been shown to bind the purine caffeine[9], so it would be expected to have an affinity for nucleotides.

We have (1) investigated conditions that favor phenol binding to XAD-2 while suppressing nucleotide binding; (2) attempted to correlate the affinity of XAD-2 for nucleotides with their electronic structures; and (3) developed a procedure using XAD-2, PVP, and charcoal for rapid isolation of nucleotides in plant extracts.

EXPERIMENTAL

Materials

Amberlite XAD-2 (20–50 mesh) beads, was obtained from Rohm and Haas (Philadelphia, Pa., U.S.A.); PVP, from Calbiochem (Los Angeles, Calif., U.S.A.); activated charcoal, USP, from Mallinkrodt (St. Louis, Mo., U.S.A.); purines, pyrimidines, nucleotides (sodium salts), and sugar phosphates, from Sigma (St. Louis, Mo., U.S.A.) and Calbiochem. All common chemicals were analytical-reagent grade. Water was deionized-distilled-deionized. The UV absorbance (A) of solutions was measured with a Pye Unicam spectrophotometer, Model SP 1700.

Column preparation

XAD-2 and PVP were boiled in 3 *M* HCl for 1 h, stored overnight in 3 *M* HCl under vacuum, and washed on a sintered-glass funnel sequentially with water, 1 *M* NaOH, water, methanol, and water. The mean-size particles were selected by sedimentation in water. Each resin was poured as a thick aqueous slurry into glass chromatographic columns or plastic syringe barrels with a pad of pressed glass-cloth on the bottom. The columns were usually equilibrated with eluting solution before sample application.

Charcoal was treated similarly but with 6 *M* HCl, washed on a büchner funnel, then mixed with water to form a thick paste. The paste was triturated, water was added, and the suspension of fairly uniform, small particles decanted. A column of charcoal-impregnated glass-cloth discs was packed in a 10-ml glass syringe barrel equipped with a PTFE stopcock as follows. Three discs of pressed glass-cloth, cut with a cork borer to fit the syringe barrel, were tamped, one at a time, under water, into the barrel. A 2-ml volume of the suspension of the fine charcoal was allowed to percolate through the discs followed by water to remove excess charcoal. This

* Trade names and company names are included for the benefit of the reader and do not imply any endorsement or preferential treatment of the product listed, by the U.S. Department of Agriculture.

process was repeated until the packed bed volume was 5 or 6 ml. The charcoal-impregnated glass discs were prevented from floating by a snug-fitting PTFE retainer ring pressed on top of the bed. The bed was washed with acetone, with ethanol–water–NH_4OH (65:35:0.3, v/v/v) eluting solution until the eluate absorbance at 220 and 260 nm was zero, with degassed water until the pH was below 7, and equilibrated with 0.01 *M* HCl. The ethanol–water–NH_4OH solution leaches UV absorbing material from plastic syringes and some plastic tubing; therefore, only glass and PTFE components were used for the charcoal column.

Analyses

Standard solutions, plant extracts, and eluates from columns or batch treatments were analyzed for phenols, nucleotides, and purine and pyrimidine bases by UV absorbance, for hexoses by the anthrone reaction[10], for total P by the Bartlett procedure[11], and for *ortho*-phosphate (Pi) by the Fiske–SubbaRow procedure[12].

Plant extract preparation

Samples of up to 15 g of fresh plant tissue were frozen in liquid nitrogen and pulverized under liquid nitrogen with a mortar and pestle. The frozen powder was triturated in the mortar with cold 0.5 *M* perchloric acid (2 ml per g fresh tissue). Insoluble material was pelleted by centrifugation at 0° and re-extracted with cold 0.2 *M* perchloric acid (2 ml per g fresh tissue). The two extracts were combined and neutralized with 1 *M* KOH containing 0.2 *M* $KHCO_3$. $KClO_4$ was removed by centrifugation and the neutral extract was stored at −20°. As needed, aliquots were thawed and centrifuged for removal of additional $KClO_4$ and pigments precipitated by freezing.

RESULTS

Sorption by XAD-2 and PVP

Batch XAD-2 treatment of a corn leaf extract and a 0.1 m*M* adenosine triphosphate (ATP) solution showed that the affinity of the resin for both phenols and ATP is strongly pH-dependent (Fig. 1). Aliquots (15 ml) of the solutions were adjusted to the desired pH with HCl or NaOH and gently shaken with 3 g of hydrated XAD-2 for 30 min at room temperature. Sorption was measured as a decrease in UV absorbance at 330 nm for phenols and 260 nm for ATP. Sorption of both phenols and ATP was maximal near pH 3. Fortunately, decreasing the pH to 2 markedly decreased sorption of ATP but had relatively little effect on sorption of phenols.

The recovery of nucleotides and several other compounds from a column of XAD-2 (22.5 × 1.5 cm I.D.; 40 ml bed vol.) is shown in Table I. A 5-ml sample of a standard solution was loaded onto the column, pre-equilibrated with eluting solution, and eluted with 0.02 *M* N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), pH 7.0, dilute HCl, or dilute HCOOH. At pH 7, Pi exists as a 2:1 mixture of divalent and monovalent anions and so should not be sorbed by XAD-2; it was completely recovered with 100 ml (2.5 bed vol.) of TES. Glucose, hexosephosphates, guanine, uracil, 5'AMP and 5'UMP also were completely recovered with 2 to 2.5 bed vol. of TES; so they were not sorbed or only weakly sorbed by XAD-2. Adenine and all of the adenine nucleotides except 5'AMP were eluted slowly, if at all, at pH 7.

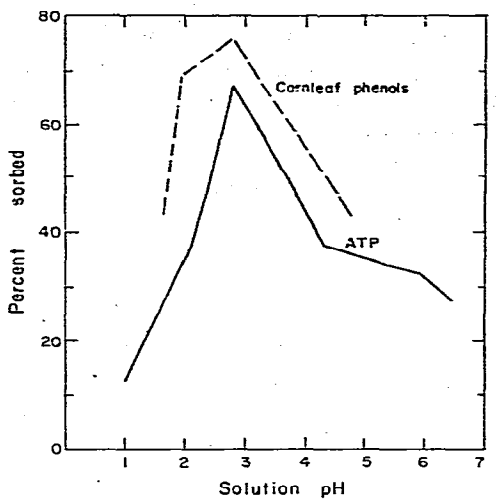

Fig. 1. The effect of pH on the sorption of corn-leaf phenols and ATP by XAD-2.

Sorption of adenine compounds at pH 7 increased in the approximate order 5′-AMP<adenine<5′ADP<NAD=NADP<5′ATP≤3′:5′ cyclic AMP. Sorption of these compounds was less at acid than at neutral pH and less in the presence of HCl than in the presence of HCOOH at the same pH. A strong proton donor evidently is required to prevent their sorption.

None of the compounds listed in Table I was sorbed by PVP in the pH range of 2 to 3. A sample of ATP loaded on a PVP column was completely recovered with one column volume of 0.01 *M* HCl. The PVP column had a smaller void volume than the XAD-2 column because the PVP particles were smaller and less porous.

The recovery of ATP from a corn leaf extract after treatment with XAD-2 was investigated. A 5-ml aliquot of the extract, containing a negligible amount of nucleotides, was spiked with 0.66 μmole ATP, adjusted to pH 1.5 with HCl, loaded onto an XAD-2 column, and eluted with 0.1 *M* HCl. The eluate showed two UV-absorbing peaks (Fig. 2). The first (max. at 40 ml, one bed vol.) was due to ATP as indicated by its UV absorption spectrum. The second was due to non-nucleotide material having a single UV absorption maximum in the region of 216 to 218 nm, a low non-specific, but significant, absorbance at 260 nm, and no absorbance at 300 nm. The lack of absorption at 300 nm indicates effective retention of phenols by XAD-2. The recovery of ATP in the column eluate was estimated from the absorbance of the eluate, pure ATP, and the non-nucleotide material at 260 and 230 nm. The simultaneous equations used for the estimate reduced to:

$$A_{260} \text{ due to ATP} = 1.02 \text{ (total } A_{260} - 0.08 \text{ total } A_{230}).$$

This "corrected" A_{260} indicated 97% recovery of the added ATP in the first 100 ml (2.5 bed vol.) of eluate. Analysis of the total P in the extract and column eluate indicated that at least 98% of the P in the sample also was recovered in the first 100 ml.

Tomato plants are especially rich in phenols that make nucleotide determinations difficult. XAD-2 and PVP each sorbed some of the UV-absorbing contaminants

TABLE I

RECOVERY OF COMPOUNDS FROM XAD-2 COLUMN

Compound	*Sample*		*Eluant**		*Recovery (%)*
	Amount (μmole)	*pH*	*pH*	*Volume (ml)*	
KH_2PO_4	100	7	7	100	100
Glucose	5	7	7	80	100
Glucose-6-P	40	7	7	100	100
Fructose-6-P	45	7	7	100	100
Adenine	0.6	7	7	170	100
Adenine	0.6	2.7	2.7	80	100
Guanine	0.8	7	7	90	100
Guanine	1	9.6	2.7	80	100
Uracil	0.9	7	7	80	100
Uracil	0.9	2.7	2.7	80	100
AMP (5′)	1	7	7	100	100
AMP (5′)	1	2.7	2.7	80	100
cAMP (3′:5′)	1	7	7	240	58
cAMP (3′:5′)	1	2.7	2.7	300	100
cAMP (3′:5′)	1	2.7	2.2	150	100
ADP (5′)	1	7	7	140	0
ADP (5′)	1.3	7	2.7	150	27
ADP (5′)	0.4	2.3	2.2	80	100
ATP (5′)	0.6	7	7	240	0
ATP (5′)	0.6	4	2.7	150	0
ATP (5′)	1.3	2.2	2.2	140	100
ATP (5′)	0.6	2	1.7	80	100
ATP (5′)	0.6	7	1.7	80	100
ATP (5′)	1.6	2	2**	300	100
UMP (5′)	0.4	7	7	80	100
NAD	1.7	2.2	2.2	100	100
NADP	1.1	7	7	300	86
NADP	1.1	2.7	2.7	200	53
NADP	1.1	2.2	2.2	100	100

* Neutral eluant = 0.02 *M* TES, acid eluant = HCl (except**); volume is that required for the indicated recovery.

** 0.16 *M* HCOOH.

in tomato plant extracts but not enough to unmask a nucleotide absorption peak. Treatment with both resins revealed a small peak at 263 nm (Fig. 3). The extract, equivalent to 10 g of fresh tomato leaves and roots, was adjusted to pH 2 with HCl and washed through tandem columns of XAD-2 and PVPs with 100 ml of 0.01 *M* HCl. The resins, 20 ml of XAD-2 and 10 ml of PVPs were packed in plastic syringe barrels. The PVP column received the out-flow of the XAD-2 column via a short section of Tygon tubing. Tests showed that during 24 h of exposure to HCl at concentrations up to 1 *M*, the syringe plastic and Tygon did not release any UV-absorbing contaminants. The UV absorption measurements (Fig. 3) were made with pre- and post-treatment samples that had been adjusted to pH 2 with HCl and diluted equally with 0.01 *M* HCl. The XAD-2 and PVP completely removed phenolic compounds absorbing above 310 nm and decreased absorbance at 260 nm by 80%.

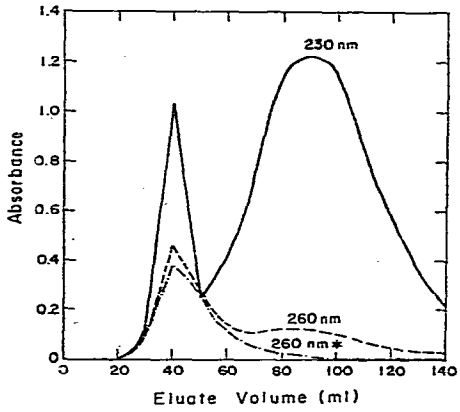

Fig. 2. The recovery of ATP added to a corn-leaf extract, loaded onto a column (1.5 × 22.5 cm) of XAD-2 and eluted with 0.1 *M* HCl. The top two curves show the A_{230} and A_{260} of the eluate. The bottom curve (260 nm*) shows the A_{260} of the eluate corrected for the absorbance of non-nucleotide compounds.

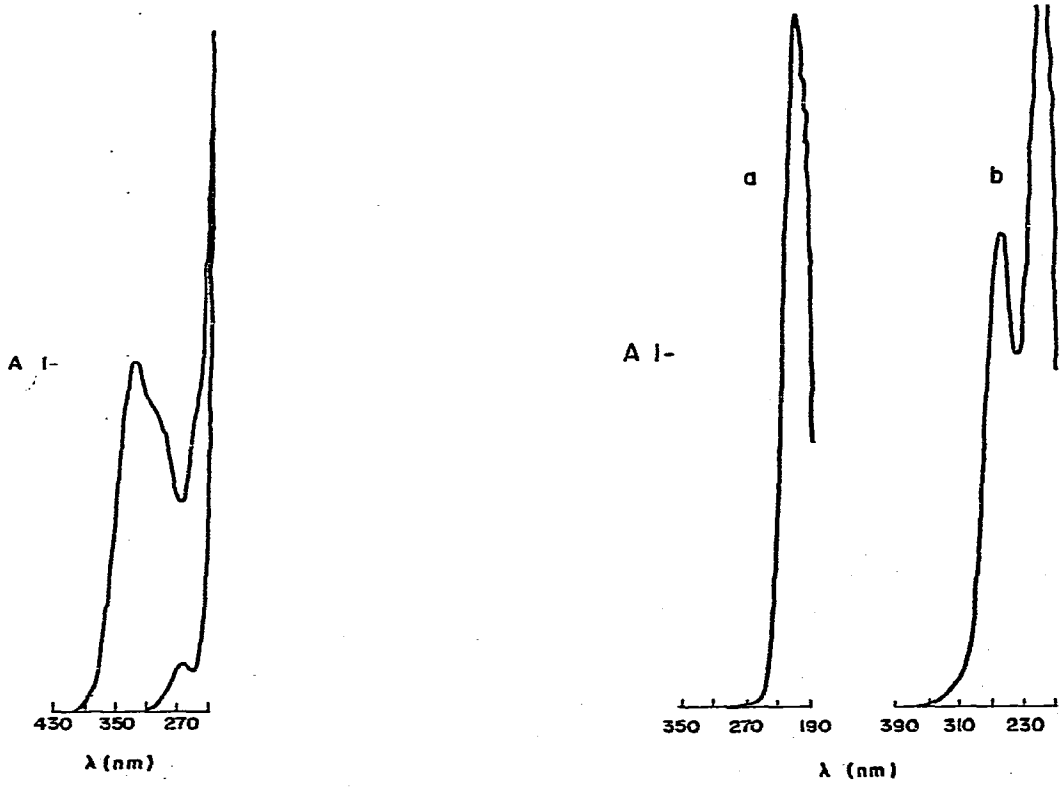

Fig. 3. UV absorption spectra of a tomato-plant extract before (upper curve) and after (lower curve) passage through XAD-2 and PVP.

Fig. 4. UV absorption spectra of (a) tomato-plant extract after passage through XAD-2, PVP, and charcoal; (b) tomato nucleotides retained by the charcoal and eluted with ethanol–water–NH_4OH.

Sorption by charcoal

Our plant extracts purified by treatment with XAD-2 and PVP still contained ions and organic compounds that interfered with the chromatography and measurement of nucleotides. We tested two different procedures for separating nucleotides from these contaminants: sorption of the nucleotides on (1) DEAE-cellulose (carbonate) at pH 7 and (2) charcoal at pH 2. In our experience, the concentration of ions, even in highly dilute plant extracts, was sufficient to prevent quantitative sorption of nucleotides on DEAE. Frequently the NAD in plant extracts was not retained at all on a 30-ml column of DEAE. On the other hand, a small (6 ml) charcoal column sorbed all of the nucleotides, including NAD, in extracts of up to 15 g of fresh plant tissue. To determine the recovery of nucleotides from the charcoal column, we loaded it with standard samples (0.5 μmole NAD, 2 μmole AMP, 1.5 μmole ADP, 1 μmole ATP) in 0.01 *M* HCl, pH 2, washed it with 100 ml of degassed water, and then eluted with ethanol–water–NH_4OH. Nothing absorbing at 260 nm came through the column in the sample effluent or water wash; absorbance at 200 nm was zero by the end of the water wash. Ninety percent of the nucleotide loaded onto the column was recovered in the first 50 ml of eluting solution and the remainder in the second 50 ml.

The sorption on charcoal and recovery of endogenous nucleotides in a plant extract was investigated. A tomato plant extract (100 ml, pH 2) that had been treated with XAD-2 and PVP (Fig. 3) was washed through a 6-ml charcoal column with 200 ml of degassed water. The column was eluted with ethanol–water–NH_4OH. The eluate was monitored by UV absorbance and collected in 10-ml fractions. The sample effluent and water wash showed a single UV absorption peak with a maximum at 222 nm and low tail-end absorption at 260 nm (Fig. 4a). They accounted for 70% of the A_{230} units loaded onto the column but only 8% of the A_{260} units. Their absorption spectra did not indicate the presence of nucleotides. The second fraction of the ethanol–water–NH_4OH eluate showed an absorption peak at 260 nm (Fig. 4b). This peak was reduced to a slight inflection by the tenth fraction. The first 10 fractions accounted for 19% of the A_{230} units put on the column and 76% of the A_{260} units. The second 10 fractions accounted for the remaining units but since these showed no specific absorbance at 260 nm, they probably contained no nucleotides.

The experiments described above led to the following procedure for isolating nucleotides from plant extracts. The neutralized and centrifuged perchloric acid extract was adjusted to pH 2 with HCl and passed, cold, at a rate of 1–3 ml/min through connected columns of XAD-2 (20 ml), PVP (10 ml), and charcoal (6 ml), all previously equilibrated with 0.01 *M* HCl. Next, the coupled columns were washed with at least 5 XAD-2 column volumes of cold 0.01 *M* HCl. The extract effluent and HCl wash were combined, neutralized, and saved for the analysis of Pi, sugars, and other aliphatic compounds. The charcoal column was disconnected and washed with cold degassed water until the A_{220} of the eluate was zero. The wash was saved for phosphate-ester analysis. (In some cases, it may be desirable to wash with 0.01 *M* bicarbonate or ammonia[13].) The column was then eluted at the rate of 1–3 ml/min with ethanol–water–NH_4OH at room temperature. The collecting vessel was kept on ice. Elution was continued until the A_{260} was negligible, usually 100–150 ml of eluant were required. The solvent was removed by vacuum distillation at 37°, and the residue was taken up in a minimum volume of water. At this stage the

nucleotide solution was usually suitable for anion-exchange chromatography. But if it still contained interfering pigment it was acidified and passed through a small mixed bed of XAD-2 and PVP.

Used XAD-2 and PVP were regenerated by Soxhlet extraction with methanol.

DISCUSSION

Passing plant extracts through successive columns of XAD-2, PVP and charcoal is a rapid and effective way to remove phenols and other compounds that interfere with the separation and measurement of nucleotides. Unlike PVP, XAD-2 strongly sorbs some nucleotides. However, loss of nucleotide on XAD-2 was avoided if the sample and wash were acidified with HCl; 0.01 *M* HCl, pH 2, seemed optimal (Fig. 1). Increasing the concentration of HCl decreased the wash volume required for recovery of the nucleotides (Table I) but it also increased the risk of hydrolyzing acid-labile phosphate groups. Lower concentrations required impracticably large volumes for nucleotide recovery, and phenols which are only weakly bound to XAD-2 above pH 3 were eluted with the wash. When the column was washed with 0.16 *M* formic acid, pH 2, phenols were eluted slowly from the resin while some nucleotides remained sorbed. Apparently a strong proton donor like HCl is required for retention of phenols and release of nucleotides.

XAD-2 is, in effect, a non-polar, reversed-phase-type sorbent; so its affinity for molecules decreases as their ionization and polarity increase. Ions (Pi) and polar compounds (glucose and hexose phosphates) were not sorbed. Of the three nitrogenous bases tested (adenine, guanine, and uracil), only adenine was sorbed at pH 7. These results agree with the electronic structures of the bases. Adenine has the lowest dipole moment[14] of the three. The adenine structure, having a pyridine type nitrogen at N_1, aromaticity of the ring, and lacking exocyclic (carbonyl) oxygen, permits greater delocalization of π-electrons than the structures of guanine and uracil; hence, adenine is the least polar of the three bases. All-valence electron calculations[14] indicate a shift of π-electrons toward the carbonyl oxygen (electron localization) in guanine and uracil; hence, the six-membered ring of either base is more positively charged than that of adenine.

Adenine sorption by XAD-2 was pH sensitive and decreased when the pH was lowered with HCl. At pH 2, adenine ($pK' = 4.1$) is fully protonated at the N_1 nitrogen[15,16] and carries a positive charge which prevents base stacking[16] and sorption by XAD-2. Adenine sorption was also affected by substituion of an electron-withdrawing ribose-phosphate group at the glycosyl nitrogen (N_9). Thus, as compared with adenine, 5'AMP was less readily sorbed, wheras 5'ADP, 5'ATP, and 3':5' cyclic AMP were more readily sorbed by XAD-2. X-ray-diffraction[17] and proton-magnetic-resonance[16] data indicate that 5'AMP is in the *anti* conformation with the phosphoryl group juxtaposed to the H_8 proton of the adenine ring. The phosphoryl group is close enough to cause specific deshielding of the H_8 proton and polarization of the C–H bond[16]. That it is the proximity of the negative phosphoryl group to the adenine ring rather than electron-withdrawal from the ring, that prevents sorption of 5'AMP, is indicated by the markedly greater sorption of 3':5' cyclic AMP; in this compound, the phosphoryl group is moved away from the ring

by cyclization. The decreased ease of protonating the cyclic AMP as compared to adenine, indicates decreased electron density on the ring; and the decrease would be expected to favor sorption.

Sorption of the adenosine 5'phosphates increased in the order 5'AMP, 5'ADP, 5'ATP. At pH 7, 5'ADP and 5'ATP were strongly sorbed, even though they are highly charged. At this pH, three of the four phosphoryl protons in ATP are fully ionized and the fourth is about 50% dissociated. Therefore, 5'ADP and 5'ATP could only be sorbed if the charged phosphoryl groups, unlike the phosphoryl group in 5'AMP, are restricted to a conformation that isolates them as much as possible from the adenine ring. The β and γ phosphoryl groups appear to stabilize such a conformation. Phosphoryl groups are known to exert a restraining effect on rotation of both the $C_{4'}$–$C_{5'}$ bond of ribose and the N_9–$C_{1'}$ glycosyl bond[17]. Furthermore, the close proximity of the negative charges borne by the phosphoryl groups would favor a linear conformation of these groups. The increased sorption, compared to adenine, caused by the addition of β and γ phosphoryl groups indicates decreased charge on the adenine ring, presumably caused by the electron-withdrawing effect of the phosphoryl groups. Decreased electron density on the ring is also indicated by the decreased ease of protonating ADP and ATP.

Charcoal has been used for many years to sorb nucleotides, but the usual recoveries have not been quantitative. Bartlett[13] compared several different types of charcoal for use in column chromatography of phosphates, including nucleotides, and concluded that the recoveries were best when the carbon was porous and friable. Two problems arise in the use of this type of carbon in column chromatography. If the particles are large enough to permit satisfactory flow-rates, the pores are so deep and tortuous that the solute they trap equilibrates slowly with the mobile solvent phase. On the other hand, small particles that promote more rapid equilibration pack so tightly that flow-rates, even under pressure, are unsatisfactory. To avoid these problems we have used small particles of highly porous carbon immobilized (sorbed?) on glass-cloth. In this way, equilibration time was short, and flow-rates were satisfactory (up to 5 ml/min). Nucleotides were completely retained by such a column and completely recovered.

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