The Intracellular Distribution of Free Nucleotides in the Tobacco Leaf

FORMATION OF ADENOSINE 5'-PHOSPHATE FROM ADENOSINE 5'-TRIPHOSPHATE IN THE CHLOROPLASTS

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1. A method of studying the free nucleotides of leaf tissue is outlined and the need for a metal ion-binding agent for the complete extraction of certain nucleotides by aqueous ethanol is established. 2. The method was used to study the effect of illumination or darkening of tobacco plants on the free nucleotides present in the chloroplast and non-chloroplast tissue components. 3. When plants that had been in the dark for a prolonged period were given 30 sec. of bright light there was a rapid phosphorylation of ADP to ATP in both chloroplast and non-chloroplast components of the tissue. 4. Where plants were moved into the dark from conditions suitable for rapid photosynthesis there was a rapid conversion of ATP into AMP and the AMP was formed only in the chloroplast fraction. In continuing darkness the AMP remained restricted mainly to the chloroplast fraction for at least 2 min., but eventually its concentration fell to the low value that is typical of tobacco leaves during conditions of constant illumination. If the plants were returned to the light for 30 sec. the AMP was rapidly rephosphorylated.

The purpose of this study was to determine the distribution of nucleotides in the tobacco leaf between chloroplast and non-chloroplast components and to obtain evidence as to their ease of movement through the chloroplast membranes. Nucleotides were determined by ion-exchange chromatography, which permits, not only a re-examination of the behaviour of AMP, ADP and ATP and the nicotinamide nucleotides that have been previously studied in higher plants by Santarius & Heber (1965) and Heber & Santarius (1965), but also an estimation of all the nucleotides occurring in concentrations greater than 10–20 $\mu\text{moles/g. dry wt. of tissue}$.

A method for the extraction of free nucleotides from freeze-dried plant tissue was described by Keys (1963) and Keys & Cornelius (1965). This method has now been extended and re-examined so that it may be successfully combined with a full ion-exchange column chromatography examination of the naturally occurring free nucleotides. Two factors are now more fully appreciated. These are first the high degree of stability of plant phosphatases and the extreme conditions under which they can hydrolyse nucleotides (Bielecki, 1964; Bielecki & Young, 1963), and secondly the failure of aqueous ethanol to extract certain nucleotides in

the absence of metal ion-binding agents. This latter point is discussed in relation to the particular method employed in the present study, though it is undoubtedly of much wider significance (Cole & Ross, 1966; Isherwood & Barrett, 1967).

MATERIALS AND METHODS

Special chemicals. The TTA* was the product of British Drug Houses Ltd. (Poole, Dorset), as was the enzyme glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP oxidoreductase, EC 1.1.1.49). Alcohol dehydrogenase (alcohol-NAD oxidoreductase, EC 1.1.1.1) was from Seravac (Pty) Ltd. (Maidenhead, Berks.). [$8\text{-}^{14}\text{C}$]ATP ($4\cdot8\ \mu\text{Ci}/\mu\text{mole}$) was obtained from The Radiochemical Centre (Amersham, Bucks.).

Plant material. Tobacco plants (*Nicotiana tabacum*), var. White Burley, were grown in a greenhouse under natural daylight during the summer and fluorescent artificial light during the winter. The freeze-dried leaf powders were prepared as described by Keys, Smith & Martin (1963) except that in certain cases conc. H_2SO_4 was used as a desiccant in the freeze-drier instead of anhydrous CaCl_2 . The dry powders were submitted to a gentle crushing action by placing them in an 8 in. glass mortar together with a stainless-steel ball 1.5 in. in diameter. The mortar was

* Abbreviation: TTA, 1,1,1-trifluoro-3-(2-thenoyl)-acetone.

carefully covered with polyethylene sheet to exclude moisture and was then placed on a rotary shaker for 24 hr. The speed of the shaker was set so that the ball moved gently round and round in the bottom of the mortar crushing the tissue. The powder was stored over P_2O_5 under high vacuum.

Chloroplast fractionation. The method was based on that of Stocking (1959). With the freeze-dried powders described above it was not normally necessary to use a TenBroek homogenizer. In certain cases it was necessary to use a glass pestle in the polypropylene centrifuge tubes to further break up tissue powders in carbon tetrachloride-hexane mixtures. The powder (0.33 g.) was mixed at 0° with 15 ml. of the most dense mixture of carbon tetrachloride and hexane (b.p. $67-70^\circ$) (sp.gr. 1.34 or 1.36) in a polypropylene centrifuge tube. The lighter mixture (sp.gr. 1.32 or 1.34; 15 ml.) was carefully introduced above the suspension and an upper layer consisting of 1-3 ml. of a mixture of carbon tetrachloride and hexane with sp.gr. 1.05 was added. The tubes were centrifuged at 0° for 30 min. at 10000g (MSE High Speed 18 centrifuge) in the angle rotor (8×50 ml.). The chloroplast layer was taken from just below the upper layer of carbon tetrachloride-hexane (sp.gr. 1.05) and included material sticking to the top of the inner wall of the tube. The chloroplast fractions from two tubes, i.e. from a total sample of dry wt. 0.66 g., were mixed with an equal volume of hexane in a weighed glass centrifuge tube. The remaining chloroplast-depleted tissue in the two original centrifuge tubes was resuspended and the suspensions were combined; the volume was made to 100 ml. with hexane and, after mixing, a 20 ml. sample was pipetted into a weighed glass centrifuge tube. The glass tubes were centrifuged at 1200g for 5 min. at 0° in a bench centrifuge. After the supernatant liquid had been decanted the pellets were dried under vacuum, the air being readmitted several times followed by re-evacuation. Finally, the samples were dried in high vacuum over P_2O_5 for 24 hr. and weighed.

Extraction of nucleotides. Analyses were made of the chloroplast fraction, chloroplast-depleted fraction and a sample consisting of about 120 mg. of the whole tissue powder itself. The precipitates of the chloroplast fraction and chloroplast-depleted fraction were carefully broken up with a glass rod and all three samples were mixed with 0.5 g. of dry cellulose powder (Whatman Column Chromedia CF11). With some batches of cellulose powder it was easier to wash TTA from the cellulose powder columns (see Table 4) if the powder had been washed before use with water followed by 60% (v/v) ethanol and acetone. The tubes containing the tissue and tissue fractions were placed in a boiling-water bath for 1 min. and transferred to a bath at 80° , and to each was added 5 ml. of boiling ethanol. After 4 min. the tubes were removed and cooled in ice. When the tubes were cool 50 μ g. of phenazine methosulphate was added to each as a solution in 0.25 ml. of ethanol. The tubes were mixed and placed under a black cloth on a rotary shaker for 1 hr. The aim of this step (see Ogren & Krogmann, 1965) was to ensure that the nicotinamide nucleotides were all converted into the oxidized state. Separation of the nucleotides from pigments, lipids, sugars, glucosides and other compounds was achieved by using columns of cellulose powder as previously described (Keys, 1963; Keys & Cornelius, 1965).

Cellulose columns (1.1 cm. $\times$ 20 cm.) were prepared from 1 g. of cellulose powder suspended in acetone. The tissue

sample or fraction, suspended in ethanol, was introduced above the cellulose powder together with less than 2 ml. of ethanol used to complete the transfer. Excess of ethanol was drained through the column and the extraction was continued by the addition of portions of various solvents to the top of the column, each portion being allowed to drain to the upper surface of the tissue cellulose powder mixture before addition of the next. A total of 10 ml. of 95% (v/v) acetone was used to complete the extraction of chlorophylls; the concentration of these in the extract was determined by the method of Arnon (1949). To each cellulose column was added 2 ml. of chloroform-methanol (1:1, v/v) and the tissue-powder layer was carefully mixed. A further 3 ml. of the same solvent mixture was used followed by 5 ml. of methanol-TTA-aq. NH_3 (sp.gr. 0.88) (180:40:7, v/w/v), and a total of 3 ml. of methanol-1M-ammonium acetate, pH 7.5 (9:1, v/v), 10 ml. of 0.1M-ammonium acetate in methanol and 10 ml. of ethanol. Care was taken with the last three solvents to rinse the walls and tops of the column free from TTA.

Anion-exchange resin (AG1; X4; -400 mesh) (Calbiochem Ltd., London, W. 1) in 0.35 cm. $\times$ 15 cm. columns was equilibrated with eluting buffer (see below) and washed thoroughly with water. The columns were sucked dry and then evacuated by means of a filter pump attached to their outlets. Ethanol was admitted to the top and was allowed to fill the column while the tube to the filter pump was clamped off. This process is referred to below as vacuum infiltration. The resin columns were connected to the outlets of the cellulose columns and nucleotides were transferred to the resin columns by passing a total of 22.5 ml. of 60% (v/v) ethanol through the pairs of columns under air pressure.

The ion-exchange columns were disconnected from the cellulose columns and vacuum-infiltrated with water.

Gradient elution of ion-exchange columns. Six ion-exchange columns were eluted simultaneously and the E_{254} value of the effluents was monitored continuously (A. J. Keys & T. A. Dyer, unpublished work). For elution, a concave gradient of formate and chloride ions at pH 2.5 was produced by means of a mixing vessel containing, initially, 1250 ml. of water with 0.5 ml. of 90% formic acid, in hydrostatic equilibrium with 250 ml. of eluting buffer (63 ml. of 90% formic acid, 13.5 g. of ammonium formate and 29.5 g. of NaCl in 1 l. of solution) in a suitable reservoir. The liquid in both vessels was kept at $35-40^\circ$. Eluent was distributed to the six columns through a six-channel peristaltic pump; the flow rates for individual columns (approx. 5.1 ml./hr.) were determined by measuring the weight of effluent obtained in a measured interval of time. The sensitivity of each of the six measuring units of the effluent monitor was checked before and after each elution with a standard solution of AMP. The measured flow rates and sensitivities, together with areas under peaks on the chart record, were used to obtain estimates of the amounts of the eluted nucleotides.

Characterization of eluted compounds. A highly reproducible and characteristic elution pattern was established for various tissues including tobacco leaf. The position for many nucleotides in this pattern was established by adding the various pure compounds, dried on to cellulose powder, to the tissue powder, extracting the nucleotides and comparing the elution diagram obtained with that for tissue without additions. This established the exact positions of

possible components. In addition, the actual components of the various peaks were recovered by adsorption on charcoal from combined effluent fractions, the charcoal was washed free from salts with water and the nucleotides were recovered by elution of the charcoal with ethanol-aq. NH_3 (sp.gr. 0.88)-water (50:1:150, by vol.). The identity was then established by a variety of means including paper chromatography, thin-layer cellulose powder chromatography, ultraviolet-absorption spectroscopy, phosphate analyses and identification of the products of acid hydrolysis. Identification of NAD and NADP was confirmed by their ability to act as substrates for the specific enzymes alcohol dehydrogenase and glucose 6-phosphate dehydrogenase respectively. There are undoubtedly many components that were not detected, and some that were detected but not yet identified. In general, where a measurement is offered it is known that the compound concerned is the major nucleotide in a particular peak on the elution diagram.

RESULTS

Method of analysis. With freeze-dried powders I have not observed any destruction of nucleotides during treatment with boiling ethanol. This is not the same situation as when fresh tissues are killed in boiling ethanol (Bielecki, 1964; Bielecki & Young, 1963). If instead of the treatment with boiling ethanol the tissue powders were shaken with

chloroform before extraction there was still no detectable hydrolysis of nucleotides. However, it is to be pointed out that the tissue was at no time exposed to a solvent richer in water than the 60% ethanol finally used to transfer the free nucleotides from the cellulose columns to the ion-exchange resin columns. The 60% ethanol was the highest concentration of ethanol that, when used as developing solvent for paper chromatography or cellulose column chromatography, gave acceptable rates of movement of nucleoside triphosphates. Though phosphatase action was still detectable in 60% ethanol it is likely that at this stage the substrate and enzyme are separated on the column. I have frequently used a stepwise increase in water content of aqueous ethanol mixtures to try to enhance any such tendency, but this does not seem to be necessary after the treatment with hot ethanol.

The requirement for an agent forming complexes with metal ions was first observed in analyses of wheat leaves. At that time 0.85m-mole of EDTA included in the 30% (v/v) ethanol used to extract nucleotides from 1.5g. dry wt. of tissue on cellulose columns was found to be excessive; less efficient binders of metal ions such as iminodiacetic acid and nitrilotriacetic acid were not effective when used in equivalent quantity. It was concluded that

Fig. 1. Ion-exchange column chromatograms of nucleotides obtained from 150mg. of freeze-dried tobacco-leaf powder when various amounts of TTA were used during the extraction. The columns were 0.35 cm. $\times$ 15 cm. and contained anion-exchange resin (AG1; X4; -400 mesh) (Calbiochem Ltd.) and were eluted at approx. 5 ml./hr. at 25° with a concave gradient of 0.051M-NaCl in 1.7M-ammonium formate buffer, pH 2.5 (see the text). The amounts of TTA used were: 0 (a); 18.5 mg. (b); 27.8 mg. (c); 46.2 mg. (d); 55.5 mg. (e); 92.5 mg. (f).

Table 1. *Dependence of recovery of nucleotides on the amount of chelating agent used*

Values here were calculated for peaks on the elution diagram where the named nucleotide was the major one present. In the calculation account was taken of peak areas, sensitivity of the extinction measurement and the rate of flow of eluting buffer through the various ion-exchange columns.

Amount of chelating agent (mg./150mg. dry wt. of tissue) ... Nucleotide	Nucleotide extracted (mμmoles/g. dry wt. of tissue)					
	0	16.7	26.3	49.6	58.3	92.3
NAD ⁺ +NADH	162	165	159	137	145	158
AMP	152	156	158	147	132	146
NADP ⁺ +NADPH	21	44	67	72	65	74
ADP	30	69	245	308	280	323
UDP-glucose	515	634	683	717	650	728
ATP	10	19	89	551	508	578
UTP	—	—	17	110	96	107
GTP	—	—	7	77	73	58

Fig. 2. Effect of amount of metal ion-binding agent on the extraction of ATP. Samples of tobacco leaf freeze-dried powder were mixed with dry cellulose powder containing [¹⁴C]ATP and extracted as described in the text.

naturally occurring cations in the tissue were responsible for the effect. Because EDTA absorbs ultraviolet light (254mμ) and was eluted from the ion-exchange columns with ADP, a number of other chelating agents were considered. TTA was selected because both it and its complexes with metal ions are soluble in certain organic solvents and can be washed away before extraction of free nucleotides.

Unfortunately I found that a very high concentration of TTA was needed to ensure complete extraction of nucleotides from all tobacco-leaf fractions. The various nucleotides in plant tissues were not equally affected by a need for chelating agent. Fig. 1 and Table 1 show the effect of TTA concentration on nucleotides extracted. It was ADP,

ATP, UTP and GTP that were most markedly affected; in the absence of the complex-forming agent there was virtually no extraction of these compounds. NADP was slightly affected, and there was one unidentified compound (d in Fig. 1) that absorbed ultraviolet light and that was very strongly affected. Different plant tissues were not subject to the same requirements for complex-forming agent, and it would be wise to check the requirement for chelating agent for each new plant tissue studied. This check can conveniently be made by using [¹⁴C]ATP, as is illustrated for a series of experiments on a tobacco-leaf freeze-dried powder in Fig. 2. The [¹⁴C]ATP (0.08 μc in 0.025 ml.) was pipetted on to a 0.5g. sample of cellulose powder and after being dried in a vacuum desiccator this was mixed with a sample of the tissue to be extracted. The extractions were carried out as described except that the amount of TTA used was varied and that resin columns were not used, but the effluent from the cellulose columns during extraction with 60% ethanol was made to 25ml. This solution was assayed for radioactivity. Similar solutions from extractions where no [¹⁴C]ATP was added were used to prepare internal standards to obtain a correction for self-absorption by the samples. In this way the percentage of added ATP recovered in the 60% ethanol extract could be determined. Subsequent extraction of the cellulose columns with a solution of EDTA or with 0.1N-hydrochloric acid removed any ATP left behind. This could be detected by the measurement of radioactivity or by ion-exchange chromatography. It should be stressed that the ATP and other nucleotides left on the cellulose columns when the TTA concentration was inadequate were not broken down but could be recovered by using EDTA in 60% ethanol.

Chelating agent was still required where every precaution was taken to avoid contamination of

Table 2. *Distribution of some free nucleotides between chloroplast and non-chloroplast fractions of the tobacco leaf: effect on plants of exposure to 30 sec. of light (2000 ft.-candles) after 48 hr. in darkness*

Nucleotide	Distribution (mμmoles/g. of whole tissue)			
	After 48 hr. of darkness		30 sec. of light after 48 hr. of darkness	
	Chloroplast fraction	Non-chloroplast fraction	Chloroplast fraction	Non-chloroplast fraction
NAD ⁺ +NADH	69	210	68	221
NADP ⁺ +NADPH	235	218	146	115
ADP	298	283	189	147
UDP-glucose	237	1000	228	1070
ATP	197	808	411	918
ADP+ATP*	495	1091	600	1065
Calc. dry-wt. distribution (g./g. of whole tissue) ...	0.367	0.633	0.366	0.634
Chlorophyll in isolated chloroplast fraction (%) ...	4.98		5.03	

* Concentrations of AMP were negligible in these samples, but see Table 3.

Table 3. *Distribution of some free nucleotides between chloroplast and non-chloroplast fractions of the tobacco leaf*

The plants were kept in total darkness for 24 hr., placed in light (2000 ft.-candles) for 30 min. and treated as shown below.

Nucleotide	Distribution (mμmoles/g. of whole tissue)			
	30 sec. dark		30 sec. dark, then 30 sec. light	
	Chloroplast fraction	Non-chloroplast fraction	Chloroplast fraction	Non-chloroplast fraction
NAD ⁺ +NADH	94	141	71	142
AMP	496	-11*	47	20
NADP ⁺ +NADPH	189	69	167	59
ADP	300	177	312	77
UDP-glucose	355	761	260	720
ATP	337	543	581	748
ADP+ATP+AMP	1133	709	940	845
Calc. dry-wt. distribution (g./g. of whole tissue)	0.467	0.533	0.422	0.578
Chlorophyll in isolated chloroplast fraction (%)	3.74		4.11	

* The values given for nucleotides in the non-chloroplast fraction were obtained (see the text) by subtracting the value obtained for the chloroplast fraction from the value obtained for a whole tissue sample. When the concentration of a nucleotide in the non-chloroplast fraction is very low compared with its concentration in the chloroplasts, small errors very occasionally result in a negative value being obtained.

samples with metal ions. Freeze-drying over concentrated sulphuric acid instead of anhydrous calcium chloride did not affect the requirement for chelating agent. Tissue dropped fresh into boiling ethanol instead of being freeze-dried and subsequently ground in an all-glass homogenizer still required metal ion-chelating agents for the extraction of ATP and other nucleotides in detectable

amounts. In experiments with fractions of tissue produced by the non-aqueous technique it was found that the chloroplast-depleted tissue fraction required more agent forming complexes with metal ions than did an equal weight of whole tissue, and this was the basis on which approx. 1 g./150 mg. dry wt. of tissue was selected to give a safe margin for TTA.

Table 4. *Distribution of free nucleotides between chloroplast and non-chloroplast fractions of tobacco leaves*

The plants were destarched by 24 hr. of darkness and then exposed to bright light for 30 min. to established rapid photosynthesis. Analyses were made after transfer to the dark for the times indicated below. Each sample was a selected leaf from a separate plant.

Time in the dark (sec.) Nucleotide Fraction		Distribution (mμmoles/g. of whole tissue)			
		0	15	30	120
AMP	Chloroplast	(87)*	308	324	171
	Non-chloroplast	(38)*	66	103	56
ADP	Chloroplast	514	428	431	351
	Non-chloroplast	92	182	140	220
ATP	Chloroplast	474	296	252	260
	Non-chloroplast	578	536	474	490

* No values were obtained in this experiment for AMP at zero time because of interference from the TTA. The values in parentheses are from a comparable experiment.

Intracellular distribution of free nucleotides. The various nucleotides were assigned to the chloroplast or non-chloroplast tissue components on the basis of the distribution of chlorophyll between the tissue fractions analysed (Stocking, Williams & Ongun, 1963) and assuming that the chloroplast fraction isolated was composed of pure chloroplasts.

Results are presented comparing the distribution of free nucleotides between chloroplast and non-chloroplast components of samples of tissues obtained from corresponding half-leaves of tobacco plants that were given different successive light treatments. A half-leaf taken after a long dark period was compared (Table 2) with the other half of the same leaf given the long dark period followed by 30 sec. of light. The results shown are typical for samples of this type. A decrease in ADP as a result of the short illumination was accompanied by an increase in ATP. The changes in the concentrations of the two nucleotides were apparent in both the chloroplast and non-chloroplast components of the leaf.

A more interesting comparison concerns leaves that, after being destarched by 24 hr. of darkness, were given 30 min. of strong light followed by 30 sec. of darkness. At this stage one half-leaf was taken and then after subsequent exposure to 30 sec. of bright light the other half-leaf was taken. The comparison of two such samples is shown in Table 3. The significant feature is the accumulation of AMP only in the chloroplast fraction after 30 sec. of darkness. After a further period of 30 sec. in light the AMP had been rephosphorylated mainly to give ATP, which appeared both inside and outside the chloroplasts. It is to be noted that AMP was not a major constituent of the tobacco leaf at the end of a long dark period or during a long period of light. Only under the circumstances with which Table 3 is

concerned were there ever readily measurable amounts of AMP.

Table 4 gives a few measurements showing what happened to the AMP when the plants were left in the dark. It disappeared relatively slowly and was still a major component after 2 min.

DISCUSSION

Some earlier results of the work were described in an abstract (Keys, 1967). The results at that time indicated no rapid synthesis of ATP from ADP in the non-chloroplast fraction of tobacco leaves transferred from the dark into bright light. With improvements in technique a significant formation of ATP from ADP was found in both chloroplast and non-chloroplast leaf components. This type of observation, which was originally made by Santarius & Heber (1965), was interpreted by them on the hypothesis that the chloroplast membrane is readily permeable to ADP and ATP. In the absence of reproducible reciprocal changes in the sum of the adenosine nucleotides in each component there is no acceptable evidence either for or against the hypothesis from these purely analytical studies.

Santarius & Heber (1965) found that AMP was formed within 3 min. in the chloroplasts of *Beta vulgaris*, *Coleus shirensis* and *Spinacea oleracea* when the plants were transferred from light to darkness. They suggested that an unknown reaction generated AMP in photosynthesis. Changes of nucleotide concentrations in non-chloroplast components could not be assessed because of analytical difficulties. By the method now described a complete analysis of nucleotides is possible, and it was found that AMP was formed only in the chloroplasts of tobacco plants. The AMP was formed from ATP (Table 4) without a significant change in ADP

(other than a slow equilibration between the chloroplast and non-chloroplast tissue fractions). Though the AMP had begun to disappear after 2 min. there was no indication of movement into the non-chloroplast fraction. It is concluded that the chloroplast membranes cannot be readily permeable to AMP.

Phosphorylation of 3-phosphoglyceric acid and of ribulose 5-phosphate in the photosynthetic carbon cycle and of UDP in sucrose synthesis result in ADP formation. When plants are darkened these reactions would be expected to result in an overall conversion of ATP into ADP, but not into AMP, when intermediates of the photosynthetic cycle become depleted. However, on turning off the light the concentration of phosphoglyceric acid rises rapidly and ribulose 1,5-diphosphate (Bassham, Shibata, Steenberg, Bourdon & Calvin, 1956) is depleted; ATP is then not used for further processing of 3-phosphoglyceric acid or ribulose 5-phosphate and may become available for a reaction leading to AMP formation. This might be a reaction activating carbon dioxide, prior to the carboxylation reaction, that might involve the formation of an adenylyl-carbon dioxide derivative and the elimination of pyrophosphate. There is insufficient information available for a single species to show whether the amount of AMP formed is closely related to the amount of phosphoglyceric acid formed when plants are transferred to darkness.

Pedersen, Kirk & Bassham (1966a) have, however, shown that the concentration of PP_1 in *Chlorella* increases when the cells are illuminated, whereas the opposite has now been observed with respect to AMP. The two effects might be completely unrelated. PP_1 is formed by reactions leading to sucrose and polysaccharide synthesis in which no AMP is formed. Changes in PP_1 concentration during inhibition of photosynthesis in *Chlorella* by fatty acids and esters (Pedersen, Kirk & Bassham, 1966b) do indeed seem to follow changes in the rate of incorporation of $^{14}CO_2$ into sucrose. However, my own experience on the need for chelating agents in the extraction of nucleotides by aqueous organic solvents suggests that the results of ^{32}P distribution in ATP, ADP, PP_1 and UTP reported by Pedersen *et al.* (1966a,b) should be viewed with extreme caution because their extractions may have been incomplete. Since those phosphate esters (e.g. ATP, ADP) that themselves form stable metal ion complexes are difficult to extract it would be expected that PP_1 might present as great an extraction problem as the nucleotides.

In the light (Table 3) a system exists in chloroplasts that rapidly rephosphorylates AMP as well as ADP. There may also be a mechanism of photophosphorylation for the direct combination of PP_1 with AMP to form ATP, which may be

important if photosynthesis does indeed involve production of large amounts of PP_1 .

The purity of the chloroplast fraction isolated in the non-aqueous system determines the degree of certainty with which various compounds may be assigned to chloroplast or non-chloroplast leaf components. No attempt was made to determine directly the amount of contamination, but Tables 2 and 3 include values showing the proportion of dry weight assigned to the chloroplasts in the tissue as a result of the fractionation and chlorophyll determination. The value for the percentage of chlorophyll in the dry chloroplast fraction is also given. These values show that the chloroplast fractions obtained from leaves that had been exposed to 30 min. of light (Table 3) were less pure than the fractions obtained after the prolonged dark period. In Table 3 the differences in chloroplast purity could account for an apparent movement of adenosine phosphates between chloroplast and non-chloroplast components suggested by the values given for the sum of AMP, ADP and ATP. Though the chloroplast fractions were not as pure as might be wished, the formation of AMP from ATP shown in Table 3 is reasonably assigned to the chloroplasts.

The results obtained serve to illustrate the value of the analytical methods that have been devised. No precise measures of the accuracy of the method are given; various experiments that have been carried out while the method was being elaborated showed the recoveries of added nucleotides to be better than 90%. The reproducibility is satisfactory with respect to the bigger changes, but does require some further improvement. The chief merit of the method is that it permits a number of extractions to be made simultaneously at normal room temperature. With the facility for measuring effluents from six ion-exchange columns simultaneously a routine means of comparing the free nucleotide content of plant tissue samples is available. Both acidic and alkaline conditions are avoided in the extraction stage.

The recently published methods of Isherwood & Barrett (1967) employ the principle of cellulose column chromatography for the purification of plant phosphate esters. The solvents used are completely different from those proposed here. Use of propan-1-ol-ammonia-water mixtures after the acid extraction would lead to complete breakdown of both oxidized and reduced nicotinamide nucleotides. Though it is possible to use the acid breakdown products as measures of the reduced coenzymes (Heldt, Klingenberg & Papenberg, 1965) this is not necessarily the case with alkali breakdown products of the oxidized coenzymes. Because of the importance of the nicotinamide coenzymes and the particular problems they present in analyses by chromatographic methods it has been of special

interest in my own studies of free nucleotides in plants to try at least to obtain measures of the total amounts present.

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