INCORPORATION OF LABELED ORTHOPHOSPHATE INTO NUCLEOSIDE DIPHOSPHATES*

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We wish to report on the presence of an enzyme in a particulate fraction of tobacco leaves which catalyzes the exchange of P^{32}-labeled inorganic phosphate into ADP, UDP, CDP and GDP but has little, or no ability, to incorporate C^{14}-labeled ADP or ATP into RNA. In addition attention is directed to the non-enzymatic labeling of nucleoside phosphates by impurities in P^{32}-orthophosphate, and a procedure for P^{32}-purification described.

Methods and Results:

Enzyme preparation Equal weights of tobacco leaves, quartz sand and homogenizing medium (0.04 M NH_4OH, 0.05 M Tris-free base and 0.005 M mercaptoethanol) were ground in a mortar and pestle. The slurry, pH approximately 8.6, was filtered through cheese cloth and centrifuged for 10 minutes at 7,000 X g. The supernatant fluid was filtered through paper, then centrifuged at 105,000 X g for 2 hours. The pellets were suspended in 75 ml of a solution of 0.005 M $MgCl_2$, 0.05 M Tris, pH 7.4, 0.005 M mercaptoethanol and centrifuged for 25 minutes at 50,000 X g. The resultant pellets were discarded and the supernatant fluid centrifuged for 2 hours at 105,000 X g. The pellets were collected and suspended in 4 ml of the above solution.

P^{32} purification Incubation of unpurified P^{32}-P_i with nucleoside diphosphates leads to incorporation of radioactivity into the diphos-

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phates. The radioactivity is adsorbable onto charcoal and co-chromatographs with ADP, GDP and UDP plus CDP using 0.1 M phosphate pH 6.8/ammonium sulfate/n-propanol (100:60:2) as the chromatographic solvent (Pabst, 1956). The labeling is temperature and time dependent and has a pH optimum at 5.0.

The impurity is eliminated as follows. To 3 millicuries of carrier-free P^{32}-P_i are added 3 μmoles of unlabeled P_i and HCl and water to make the final HCl concentration 0.1 N. This mixture is heated to 100° for 10 minutes. Following dilution to 0.02 N HCl the solution is passed through a 0.5 gm column of Dowex 50-X4-H^+. It is neutralized to pH 7 and adsorbed to a 0.5 gm column of Dowex 1-X8-Cl. After washing the column with H_2O, the radioactivity is eluted with 4 ml of 0.05 N HCl, discarding the first and last 10% of the radioactivity. After neutralization to pH 7 the P^{32}-P_i remains useable for at least 6 weeks at 4° and does not cause non-enzymatic labeling.

Assay Owing to the presence of phosphatases the following procedure was used to obtain the specific activity of the diphosphates and to demonstrate that all 4 were labeled. To a reaction mixture, such as that described for Fig. 1, was added 0.55 ml of N $HClO_4$ and the resultant precipitate removed by centrifugation. To 0.9 ml of the supernatant fluid was added 3.6 ml of 0.1 M phosphate, pH 5 and 100 mg of acid-EDTA washed charcoal. After stirring for 10 minutes, the charcoal was collected by filtration and washed with 25 ml of H_2O, 15 ml of 0.01 M phosphate, pH 5 and, finally, 10 ml of H_2O. The charcoal was eluted with a total of 5 ml of ethanol-NH_4OH-H_2O (50:1:49) using 3 successive elutions, and the combined supernatant solutions, evaporated and adsorbed onto a filter paper disk. The disk was stuck to Whatman 3 MM paper and subjected to electrophoresis using 0.01 M citrate buffer, pH 3.3. This procedure separates UDP and GDP, but CDP and ADP are coincident. For most assays, this was sufficient.

When it was desired to separate CDP and ADP and to remove traces of P_i, which moves only slightly ahead of UDP, chromatography (Pabst, 1956) with isobutyric acid/ammonia/water pH 4.3 (57:4:39) was carried out at 90° to the direction of electrophoresis. By this two-dimensional method and radio-autography, it was demonstrated that all 4 diphosphates were approximately equally labeled. For quantification, the UV absorbing areas were cut out, counted and then eluted with N HCl for spectral determination of purity and quantity. The mobilities and R_f values of the diphosphates, under our conditions, are shown below:

Compound	Mobility ($cm.^2 sec^{-1} volt^{-1}$)	R_f
ADP	8.7×10^{-5}	0.44
CDP	8.7×10^{-5}	0.38
GDP	12.4×10^{-5}	0.23
UDP	16.5×10^{-5}	0.20

Time Course The rate of incorporation of P^{32}-P_i into GDP, as a function of time, is shown in Fig. 1. This experiment was done by

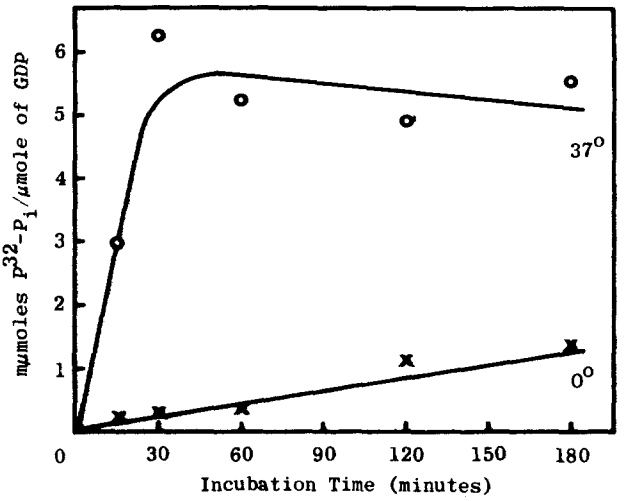

Fig. 1. Reaction rate as a function of time. Each tube contained in μmoles: Tris-acetate, pH 7.4, 10; Na Acetate, pH 7.4, 50; $MgCl_2$, 4; ADP, CDP, UDP and GDP, 0.4 of each; P^{32}-potassium phosphate, 5 (specific activity 5,370 c/mμmole/min^{-1}); enzyme, 0.2 ml and water to a total volume of 0.55 ml. Reaction terminated and diphosphates isolated as described in text.

one-dimensional electrophoresis and only GDP was measured. The reaction is linear with time up to 30 minutes and then levels off. At this time, P_i and diphosphates are not limiting nor has the enzyme been inactivated.

pH optimum The rates of incorporation of P^{32}-P_i into UDP, GDP, and ADP plus CDP are shown in Fig. 2. All 4 diphosphates are labeled at approximately the same rate for all H^+ concentrations tested. The pH optimum is about 7.4.

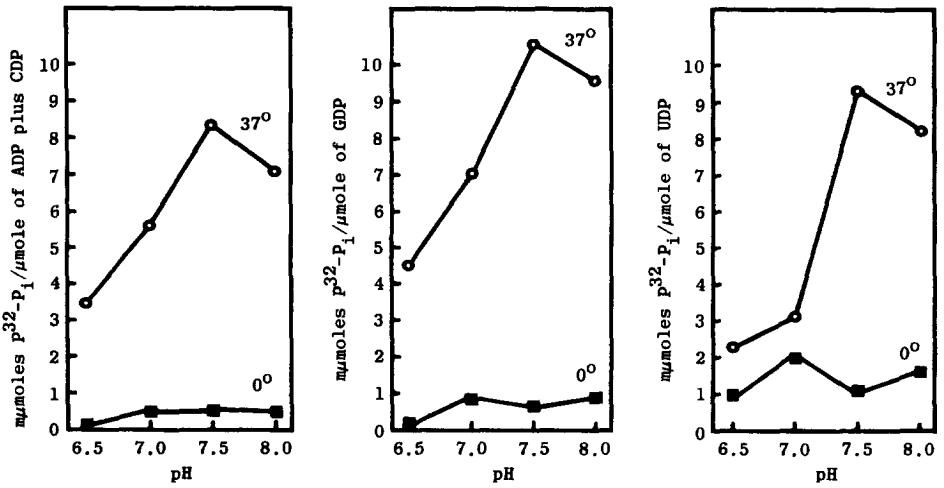

Fig. 2. Reaction rate as a function of pH. Conditions as for Fig. 1, except the pH was adjusted to the indicated value, and incubation was for 30 min.

Enzyme concentration Reaction rate as a function of enzyme concentration is shown in Fig. 3. Over the range tested, and the time intervals employed, the reaction is a linear function of enzyme concentration.

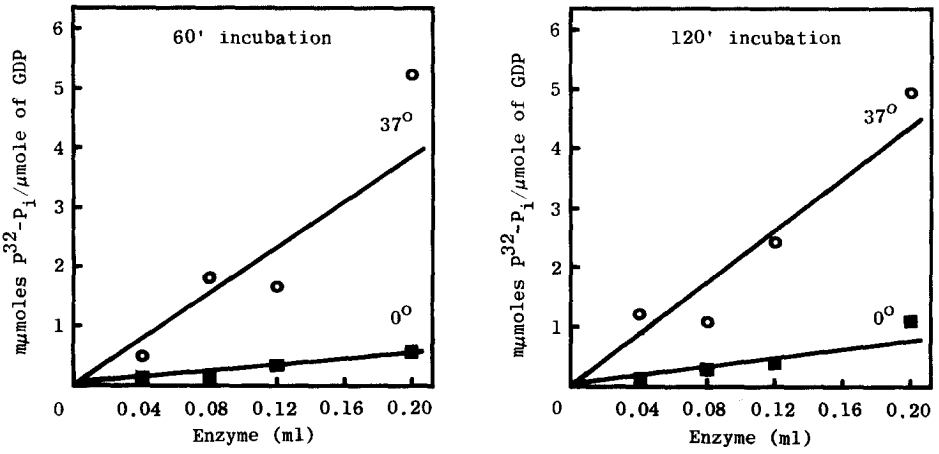

Fig. 3. Reaction rate as a function of enzyme concentration. Incubation conditions were as for Fig. 1, except enzyme concentration, as indicated. The optical density of the enzyme, prepared as described in the text, is 76 and 48 at 260 and 280 mu respectively.

Non-reactivity of 8-C^{14}-ADP and ATP A large number of experiments were performed in which C^{14}-ADP or ATP were incubated with the enzyme under varied conditions of time and presence or absence of the other three di- or triphosphates. The total radioactivity incorporated into the perchloric acid insoluble pellet ranged from zero to 0.02 mμmoles. This is negligible compared to the phosphorolysis reaction.

Discussion:

The enzyme preparation described, catalyzes incorporation of P^{32}-P_i into nucleoside diphosphates, but under our conditions, does not synthesize polynucleotide from C^{14}-ADP. An enzyme, which may be similar, has been obtained from yeast (Grunberg-Manago, 1963). Alternatively our preparation could be related to the polynucleotide phosphorylase-like enzymes previously described in plant and animal tissues (Brummond et al., 1957; Kessler and Chen, 1964; Harris, 1963 and Hilmoe and Heppel, 1957).

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