

PHYSIOLOGY OF PEA FRUITS

V. PHOSPHATE COMPOUNDS IN THE DEVELOPING SEED

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Summary

The changes in concentration of total phosphorus, total acid-soluble phosphorus, inorganic phosphorus, hexose phosphates, and adenosine pyrophosphates were measured in maturing seed of the pea (*Pisum sativum* L.) at different times from flowering.

The decrease in the value of the ratio inorganic phosphate/hexose monophosphate measured in the extracts of the seed preceded by some days the increase in the rate of starch synthesis. The rate of synthesis of starch in the seed was not closely related to the ratio inorganic phosphate/glucose 1-phosphate.

The accumulation of starch after 23 days from flowering was accompanied by decrease in concentration of hexose monophosphate. This decrease, presumably a direct consequence of the starch formation, would explain the accompanying loss of sucrose due to a reversal of the synthetic reactions in which it is formed.

The concentration of the phosphorus compounds did not appear to be closely related to the rate of protein synthesis or of respiration. The rate of respiration was not related to the ratio adenosine diphosphate/adenosine triphosphate.

Adenosine diphosphate, adenosine triphosphate, uridine triphosphate, and guanosine triphosphate were identified by paper chromatography in extracts from fresh pea seed.

I. INTRODUCTION

Previous workers have investigated changes in the concentration of carbohydrate fractions (Bisson and Jones 1932; McKee, Robertson, and Lee 1955; Danielson 1956; Turner and Turner 1957; Turner, Turner, and Lee 1957), protein and non-protein nitrogen (Bisson and Jones 1932; McKee, Robertson, and Lee 1955; McKee, Nestel, and Robertson 1955), inorganic and total phosphorus (McKee, Robertson, and Lee 1955), and in the rate of respiration (McKee, Robertson, and Lee 1955) in the maturing pea seed.

In the present paper, the concentrations of hexose phosphates, adenosine pyrophosphates, inorganic, total acid-soluble, and total phosphorus are reported in seeds sampled in two consecutive seasons, 1954 and 1955. The aim of the experiment was to look for correlations between changes in the concentration of compounds containing phosphorus and (1) changes in concentration of starch, sucrose, and protein nitrogen, and (2) changes in the rate of respiration.

In particular, it was intended to examine the hypothesis that the ratio adenosine diphosphate (ADP)/adenosine triphosphate (ATP), or the concentration of

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inorganic phosphate, limits the rate of respiration of tissue (Lardy 1952; Chance and Williams 1956). In experiments on the Pasteur effect in fresh pea seeds, Rowan, Seaman, and Turner (1956) have shown that the ratio ADP/ATP or the concentration of inorganic phosphate could limit the rate of aerobic respiration.

The mean seed mass and concentrations of starch reported here are those measured in replicate samples of seeds by Turner and Turner (1957), and those of sucrose by Turner, Turner, and Lee (1957).

II. MATERIALS AND METHODS

(a) *Sampling and Drying of Seed*

Seeds were first sampled at 12 days from full blossom; sampling continued at 2–5-day intervals until 30 days in 1954 and until 40 days in 1955. Turner and Turner (1957) have described the harvesting and drying of the seeds.

(b) *The Estimation of Phosphorus Fractions*

Samples containing 6–15 g of fresh seed were extracted by grinding with cold 1.5N HClO₄ and acid-washed sand, using a pestle and mortar (Rowan, Seaman, and Turner 1956; Rowan, unpublished data). This extract was used for the analysis of all acid-soluble fractions.

(i) *Inorganic Phosphorus*.—The method of Weil-Malherbe and Green (1951) was used.

(ii) *Total Acid-soluble Phosphorus*.—The method of Allen (1940) was used.

(iii) *Total Phosphorus*.—The dried seed was ground and samples of the powder analysed by the method of Berenblum and Chain (1938).

(iv) *Fructose 1,6-diphosphate (HDP), Hexose Monophosphate (HMP), ATP, and ADP*.—The enzymic method of Slater (1953) as modified by Rowan (1955 and unpublished data) was used. The concentration of hexose monophosphate measured was the sum of the concentrations of glucose 1-phosphate, glucose 6-phosphate, and fructose 6-phosphate.

(c) *Protein and Non-protein Nitrogen*

Total nitrogen was determined on dried material by a modified micro-Kjeldahl method (Turner 1949). Protein nitrogen was determined after extraction of the same material with 75 per cent. (v/v) ethanol, and soluble nitrogen calculated by difference.

(d) *Respiration Rate*

The rate of output of carbon dioxide by samples of pea seed (10–20 g) was measured in air at 25°C by the Pettenkofer method (Turner 1949; McKee, Robertson, and Lee 1955). Rowan, Seaman, and Turner (1956) have shown that in mature seed at this temperature, both the seed coat and cotyledons provide a slight resistance to diffusion, and it was probable that a small part of the carbon dioxide was formed in anaerobic fermentation.

TABLE I
CHANGES IN FRACTIONS OF PHOSPHORUS AND IN PHOSPHORYLATED COMPOUNDS DURING THE DEVELOPMENT OF SEEDS, 1954

Days from Full Blossom	Mean Seed Mass (g)	No. of μ Moles/Gram Fresh Weight of:							Ratio ADP/ATP
		Inorganic Phosphorus	Total Acid- soluble Phosphorus	Unidentified Phosphorus	Hexose Mono- phosphate	Hexose Diphosphate	Adenosine Diphosphate (ADP)	Adenosine Triphosphate (ATP)	
12	0.033	10.6	19.5	6.1	0.846	0.149	0.198	0.374	0.53
15	0.082	12.1	18.6	3.6	0.944	0.179	0.243	0.291	0.84
17	0.119	11.4	18.4	2.1	3.154	0.226	0.131	0.356	0.44
19	0.179	10.4	16.9	3.0	6.328	0.278	0.328	0.392	0.83
21	0.323	8.3	15.8	0	6.242	0.290	0.471	0.406	1.19
24	0.380	8.2	20.9	5.8	2.244	0.536	0.542	0.849	0.63
28	0.369	3.8	27.0	18.0	1.027	0.426	0.725	0.605	1.09
30	0.400	3.3	28.1	20.0	1.168	0.335	0.358	0.743	0.48

III. RESULTS

Most of the results discussed in this paper are those from the longer experiment of 1955. The results from the shorter experiment of 1954 are given in Table 1 and are discussed only where they differ from those of 1955 or where the measurements were not made in 1955. The concentrations of metabolites are expressed both per seed and per gram fresh weight. Expressing concentrations per water content of seed (Danielson 1956) does not alter the conclusions to be drawn from the results.

(a) *Mean Seed Mass*

The mean seed mass of each sample is shown in Table 2.

TABLE 2
MEAN SEED MASS WITH TIME FROM FULL BLOSSOM IN 1955

Pick No.	Days from Full Blossom	Mean Seed Mass (g)	Pick No.	Days from Full Blossom	Mean Seed Mass (g)
1	12	0.034	7	25	0.504
2	15	0.114	8	27	0.605
3	17	0.174	9	30	0.601
4	19	0.216	10	33	0.599
5	21	0.335	11	36	0.558
6	23	0.450	12	40	0.412

(b) *Phosphate Esters*

(i) *Hexose Monophosphate*.—The concentration of hexose monophosphate per gram fresh weight increased sharply after 17 days (Fig. 1(a)). The concentration reached at 23 days was double that measured in the first picks. It then decreased until 30 days and increased again to a second maximum at 36 days. The first rise found in the experiment of 1954 was to six times the initial concentration (Table 1).

(ii) *Hexose Diphosphate*.—The concentration of hexose diphosphate per gram fresh weight remained approximately constant throughout the experiment (Fig. 1(a)).

(c) *Nucleotides*

(i) *Reactive Phosphate*.—The concentration of reactive phosphate ($\sim P$) is the sum of the β and γ phosphate groups of ADP and ATP, plus the γ phosphate group of uridine triphosphate (UTP) and guanosine triphosphate (GTP). It will be shown below that UTP and GTP are present in fresh pea seed in small concentration. Figure 1(a) shows that the concentration of reactive phosphate remained approximately constant between 12 and 17 days, rising to double the initial concentration at 27 days.

(ii) *ATP and ADP*.—Estimations of ATP and ADP are available on the samples of seed harvested in 1954 only. No precautions were taken to reduce interference by UTP and GTP present in the extracts (Rowan, unpublished data). As shown

below, the concentrations of UTP and GTP in pea seed are much lower than that of ATP, and the correction required is not likely to be large. The concentrations of

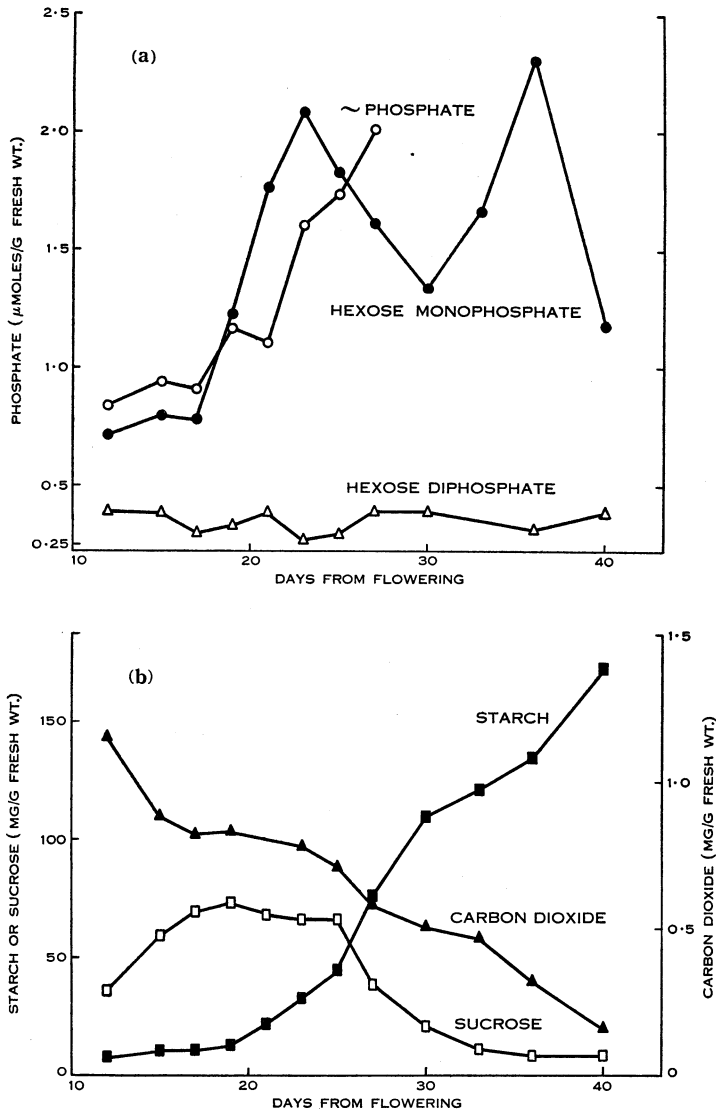


Fig. 1.—Changes in the concentration of (a) hexose monophosphate, hexose diphosphate, and reactive phosphate per gram fresh weight, and (b) starch and sucrose and in the rate of respiration per gram fresh weight with time from flowering.

both ADP and ATP tended to rise throughout the experiment, and no significant changes in the ratio ADP/ATP were observed (Table 1).

(iii) *UTP and GTP*.—UTP and GTP react in the enzyme system used to measure the concentrations of adenosine pyrophosphates (Rowan, unpublished data),

and, although it was not possible to extract nucleotides from the samples of seed used for the experiments described here, it was considered important to see if UTP and GTP were present in fresh seed.

Nucleotides were extracted from a sample of fresh seed by the method of Albaum, Ogur, and Hirshfeld (1950) and examined by the methods described by

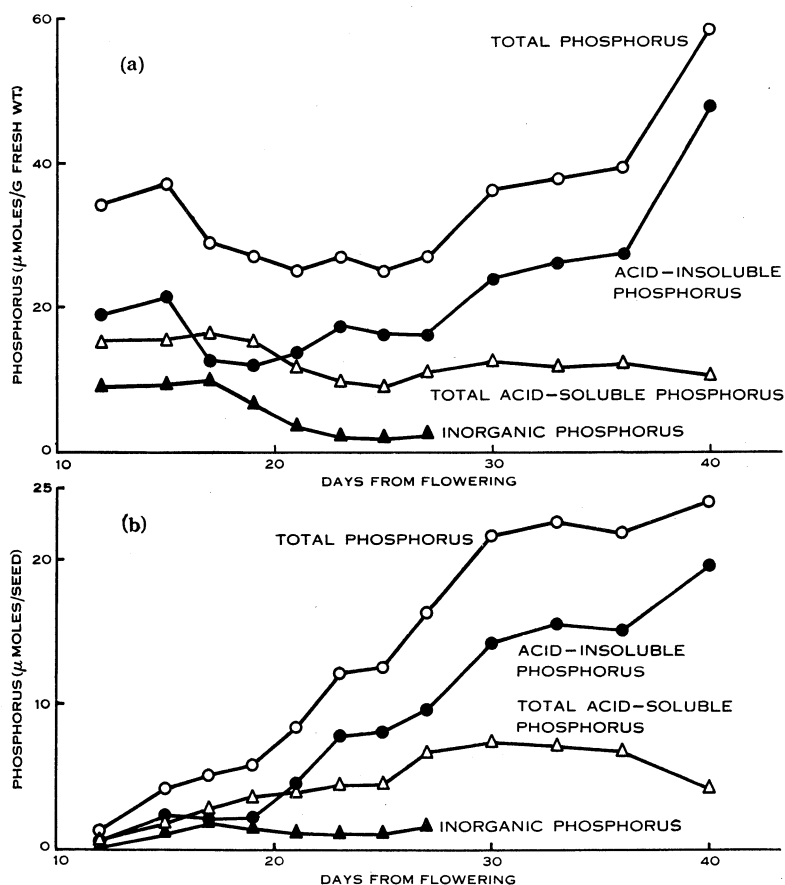


Fig. 2.—Changes in the concentration of total, acid-insoluble, total acid-soluble, and inorganic phosphorus (a) per gram fresh weight and (b) per seed with time from flowering.

Rowan (1957). The chromatogram on Plate 1 shows that GTP was present in addition to the nucleotides already found in young pea seedlings (Rowan 1957, Fig. 3). The lines containing ATP, ADP, UTP, and GTP were identified by running spots of authentic compounds on chromatograms. Samples of UTP and GTP were obtained by eluting the chromatograms and the γ phosphate groups shown to analyse as reactive phosphate in the method of Slater (1953).

The intensity of the lines of UTP and GTP on the chromatogram shown in Plate 1 is much less than that of ATP or ADP. Though extraction of nucleotides

by the method of Albaum *et al.* (1950) is not intended to be quantitative, it seems probable that the interference by UTP and GTP in the estimation of adenosine pyrophosphates will not be large.

(d) *Fractions of Phosphorus*

The changes in concentration of these fractions are shown in Figure 2. The main points in these results were:

(i) *Total Phosphorus*.—The amount per seed increased until the end of the experiment, though the rate of increase was small after 30 days. The concentration expressed as per gram fresh weight decreased until 25 days and then increased until the end of the experiment.

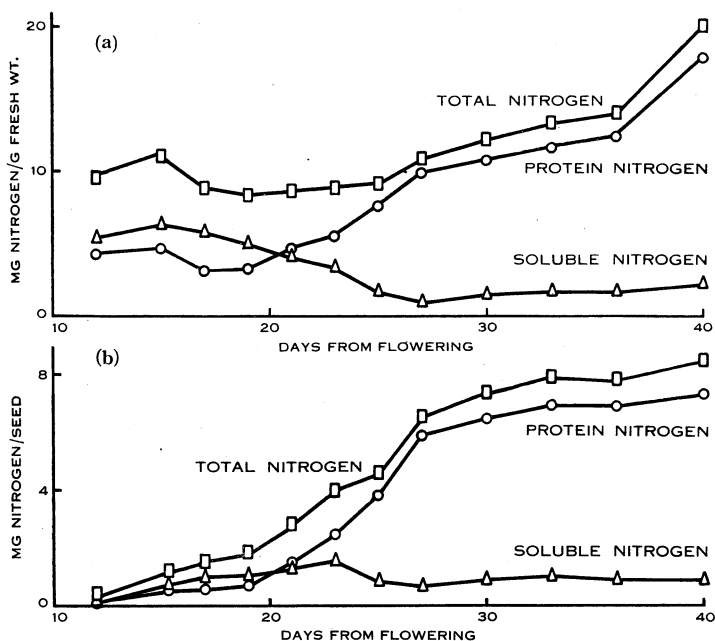


Fig. 3.—Changes in the concentration of total, protein, and soluble nitrogen (a) per gram fresh weight and (b) per seed with time from flowering.

(ii) *Acid-insoluble Phosphorus*.—The concentration per seed increased until the end of the experiment, when it was about 80 per cent. of the total phosphorus.

(iii) *Total Acid-soluble Phosphorus*.—The concentration changed in the same manner as total phosphorus until 30 days, when total acid-soluble phosphorus expressed both as per seed and per gram fresh weight decreased.

(iv) *Inorganic Phosphorus*.—The concentration per gram fresh weight fell between 17 and 25 days (Fig. 2(a)). The fall was sufficiently marked to be apparent when the concentration was expressed as per seed (Fig. 2(b)) and was thus similar to that found by McKee, Robertson, and Lee (1955). Reliable figures are not available after 27 days.

(e) Fractions of Nitrogen

The changes in protein and soluble nitrogen (Fig. 3) were similar to those found by McKee, Robertson, and Lee (1955). After 19 days, the concentration of protein nitrogen increased above that of soluble nitrogen and the rate of movement of nitrogen into the seed and the rate of synthesis of protein increased.

(f) Respiration

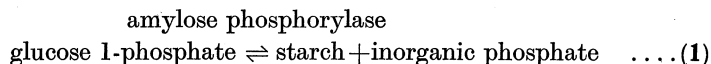
The changes in rate of respiration (Fig. 1(b)) were similar to those found by McKee, Robertson, and Lee (1955). The rate per fresh weight decreased throughout the experiment, except for a slight rise at 19 days.

IV. DISCUSSION

Although the fresh and dry weight of seeds from the harvest of 1954 were lower than those of 1955 (Turner and Turner 1957), the amount of the phosphate compounds per gram fresh weight did not differ significantly, except for the maximum concentration of hexose monophosphate, which in 1954 was three times higher than in 1955.

(a) The Relationship of Inorganic Phosphate and Hexose Monophosphate to Starch Synthesis

In vitro, the rate of synthesis of starch by the reaction



is independent of the concentration of starch formed but is a function of pH and the ratio inorganic phosphate/glucose 1-phosphate (Hanes and Maskell 1942). At equilibrium, this ratio falls from 11 at pH 5 to 2 at pH 7.

The rate of synthesis of starch in our experiment was calculated from tangents to a curve of best fit drawn through a graph of the concentration of starch per fresh weight plotted against time from full blossom (Fig. 1(b)). The ratio inorganic phosphate/hexose monophosphate decreased from approximately 13 to 2 some days before the rate of synthesis of starch increased to a maximum at 25 days (Fig. 4). Therefore, if we assume that glucose 1-phosphate was a constant fraction of the concentration of hexose monophosphate measured, it is unlikely that the rate of synthesis of starch at different stages of maturity of the seed was determined solely by the overall ratio inorganic phosphate/glucose 1-phosphate in the tissue.

Turner and Turner (1957) have shown a linear relationship between rate of synthesis of starch and the concentration of amylose phosphorylase, and it appears that the concentration of this enzyme, rather than the fall in the ratio inorganic phosphate/glucose 1-phosphate underlies the increase in the rate of synthesis of starch as the seed matures. The decrease in concentration of hexose monophosphate which occurred after 23 days is consistent with the increasing rate of utilization of hexose monophosphate in starch synthesis. Similarly, the rise in concentration of hexose monophosphate after 30 days is consistent with the decrease in rate of starch synthesis (Fig. 1(b)).

Figure 4 shows that some synthesis of starch occurred while the ratio inorganic phosphate/hexose monophosphate was above 11. Even if all the hexose monophosphate were glucose 1-phosphate and the pH at the site of synthesis were 5, no synthesis of starch would have occurred under these conditions *in vitro* (see eqn. (1)). Therefore, it is probable that the ratio inorganic phosphate/hexose monophosphate measured was not that at the site of synthesis of starch *in vivo*.

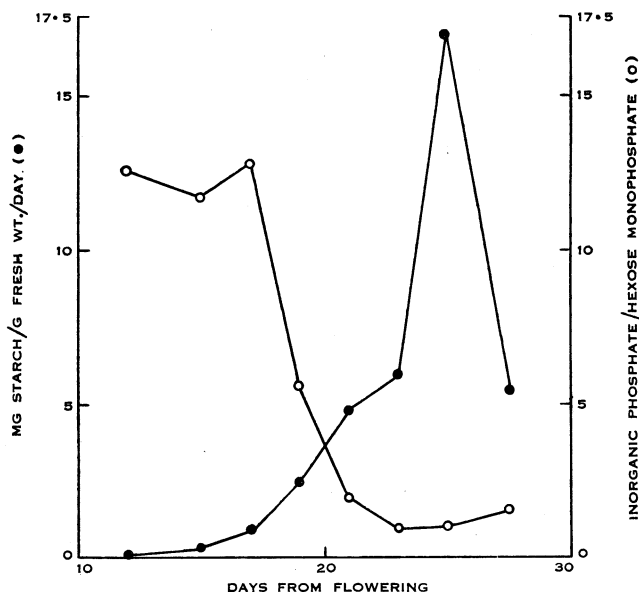
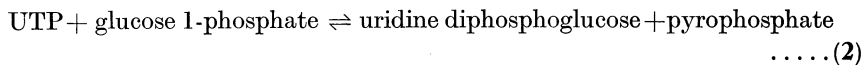


Fig. 4.—Changes in the rate of starch synthesis and in the ratio inorganic phosphate/hexose monophosphate with time from flowering.

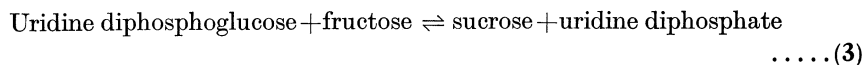
(b) *The Relationship of Hexose Monophosphate to Sucrose*

As mentioned already, the concentration of sucrose in the young seed was higher than that of starch, but subsequently the concentration of sucrose decreased, and carbohydrate, arriving at the seed by translocation as a sugar (Turner and Turner 1957), was stored as starch. In the present experiment, the decrease in concentration of sucrose occurred after 25 days (Fig. 1(b)), when the rate of synthesis of starch was at a maximum (Fig. 4).

Turner (1953, 1954), Cardini, Leloir, and Chiriboga (1955), and Turner and Turner (unpublished data) have shown that the reversible reactions shown in equations (2) and (3) occur in pea seed:



(Kalckar and Cutolo 1952; Munch-Petersen *et al.* 1953).



(Leloir and Cardini 1953).

The fall in concentration of hexose monophosphate between 23 and 30 days due to the rapid synthesis of starch could stimulate the reverse reactions of equations (2) and (3), and thus tend to bring about the conversion of sucrose to starch by this pathway in the later stages of development of the seed.

(c) *Phosphorus Fractions*

The important conclusions to be drawn from the changes in concentration of the fractions of phosphorus are as follows:

(i) Although the amount of total phosphorus per seed increased throughout the experiment (Fig. 2(b)) the movement of phosphorus into the seed did not keep pace with the increase in fresh weight, and the concentration per gram fresh weight decreased until 25 days (Fig. 2(a)).

(ii) The percentage of inorganic phosphorus fell from 25 to 8 per cent. of total phosphorus between 12 and 25 days.

(iii) After 25 days, the concentration of total acid-soluble phosphorus per gram fresh weight increased slightly to the end of the experiment. In 1954, this rise was more marked, and was caused by an increase in unidentified organic phosphorus (Table 1). It is probable that much of this unidentified phosphorus was phytin, which Fowler (1956) has isolated from pea flour.

(iv) After 25 days, the proportion of phosphorus found in the acid-insoluble fraction increased rapidly (Fig. 2(b)). A part of this increase was at the expense of the acid-soluble fraction.

(d) *Protein Synthesis*

The amount of protein nitrogen per seed increased rapidly after 19 days (Fig. 3); until this time, the net rate of synthesis of protein was low, and the concentration expressed per gram fresh weight decreased. The concentration of reactive phosphate increased at the time when the rate of protein synthesis increased (Figs. 1 and 3).

Webster (1954, 1955, 1956) has shown that ATP stimulated the incorporation of amino acids into protein in homogenates of plant tissue, and it is thus possible that the concentration of ATP is a factor governing the rate of synthesis of protein in pea seed. However, too few observations are available for a sensitive measurement of the correlation between the two variables. It is also possible that the concentration of metabolites reflected the concentration of the enzymic systems in which they were formed. Inspection of Figures 1(a) and 1(b) support this, for any correlation calculated between the rate of synthesis of protein and the concentration of hexose monophosphate or starch would be as close as that with reactive phosphate.

(e) *Respiration Rate*

The rate of respiration per gram fresh weight decreased throughout the experiment in a manner similar to that found by McKee, Robertson, and Lee (1955). The concentration per gram fresh weight of the phosphate compounds and fractions analysed did not follow closely the plot of the rate of respiration (Figs. 1(a) and 1(b)),

though the decrease in the concentration of total phosphorus, total acid-soluble phosphorus, and inorganic phosphorus between 15 and 25 days corresponded approximately to the fall in the rate of respiration.

The fall in respiration rate was not reflected in any change in the concentration of hexose diphosphate, which remained constant throughout the period. In spite of the increase in synthetic rate after 19 days and the fall in respiration rate, the concentration of reactive phosphate increased, showing that energy production by respiration did not limit phosphorylation at any stage.

The ratio ADP/ATP, measured in 1954, fluctuated between 0.44 and 1.19 (Table 1), but did not appear to follow the drift in the rate of respiration. Also, throughout the preliminary experiment on the variety Shaster (Rowan 1955), the ratio remained constant. Therefore, it appears that the rate of respiration of the tissue of the seed is limited by a factor or factors other than those investigated in this experiment.

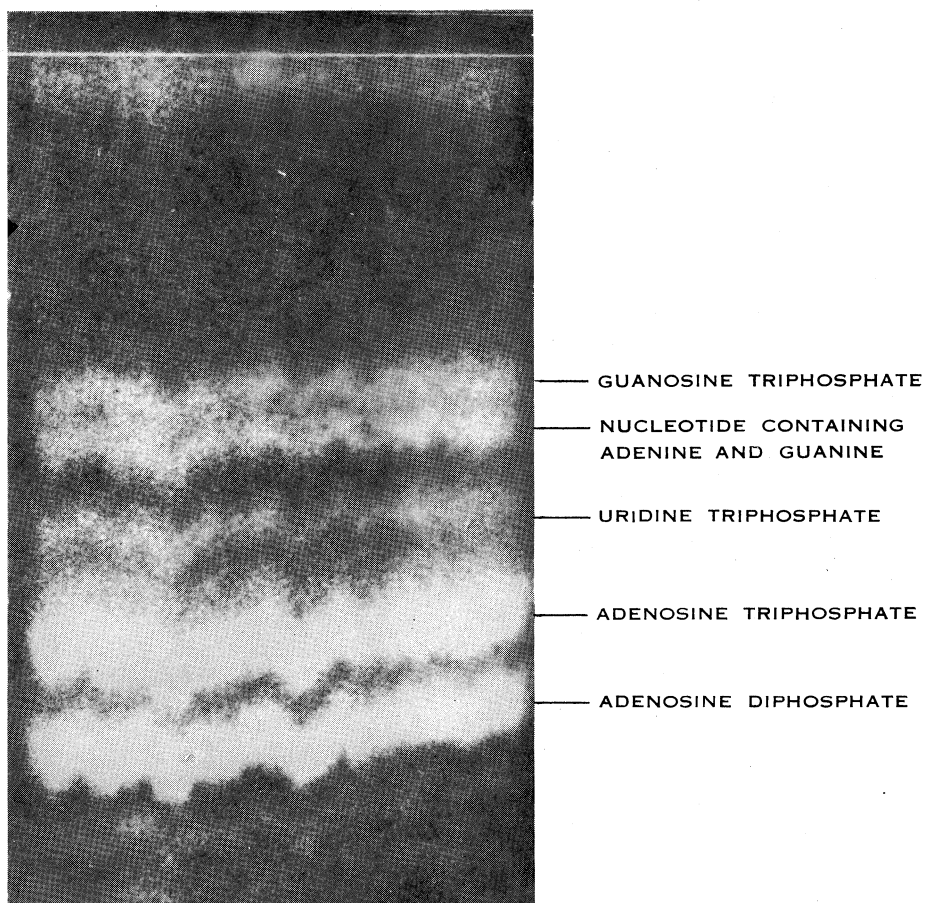
V. ACKNOWLEDGMENTS

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PHYSIOLOGY OF PEA FRUITS. V



Chromatogram of an extract of nucleotides from fresh pea seed run as a line in *n*-propanol-ammonia (sp. gr. 0.880)-water (60 : 30 : 10 v/v) (Hanes and Isherwood 1949). The chromatogram was photographed with ultraviolet light by a modification (Rowan 1957) of the method of Markham (1955). Running time 40 hr on acid-washed Whatman No. 3 filter paper.

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