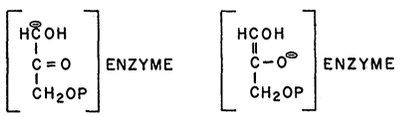
Spectrophotometric Evidence for Formation of a Dihydroxyacetone Phosphate-Aldolase Complex

Previous studies (*1, 2*) have shown that when dihydroxyacetone phosphate (DHAP) is incubated with muscle, aldolase labilization of a carbon-bound hydrogen occurs. On the basis of this observation the enzyme-DHAP complex was formulated as involving a carbanion or an enediol structure:



It was suspected that such structures would absorb in the ultraviolet region. For example, both dihydroxyacetone and D,L-glyceraldehyde in 1*N* sodium hydroxide show strong absorption in the ultraviolet with maxima at 220 mμ (*3*). The present investigation deals with the ultraviolet absorption characteristics of several combinations of protein and substrate.

Dihydroxyacetone phosphate, when it is dissolved in 0.002*M* phosphate buffer, *p*H 7.0, shows no absorption in the range 280 to 215 mμ. In the presence of muscle aldolase (Fig. 1, experiments 1 and 2), however, there is strong light absorption in the region between 250 and 233 mμ. The apparent decreases in optical density at lower wavelengths, as indicated by the broken lines, are artifacts caused by the fact that protein fluorescence contributes most of the light transmitted by the cuvettes (*4*). The absorption between 250 and 233 mμ increases with increasing concentration of both DHAP (experiment 1) and aldolase (experiment 2). With the highest concentration of DHAP and aldolase a tendency toward saturation is discernible. The shifting of the apparent peak in experiment 2 is an artifact caused by increased fluorescence consequent to increased protein concentration (*4*). The specificity of the enzyme-substrate combination was tested with substitutes for either the protein or DHAP. No absorption was observed in the system DHAP-albumin, or in the systems glucose-6-phosphate-aldolase and dihydroxyacetone-aldolase.

These results suggest a specific requirement for both DHAP and aldolase. D,L-Glyceraldehyde shows no absorption in the range 280 to 225 mμ in the presence of aldolase. This is consistent with the conclusion (*2*) that the aldehyde component of the condensation system is not activated by the enzyme in the absence of DHAP. Additional evidence that the absorption measured is produced by combination of the enzyme with its substrate is found in the obliteration of the absorption by the addition of D,L-glyceraldehyde (experiment 3, curve *J*). The presence of the aldehyde permits the catalysis of hexose-1-phosphate synthesis, and the equilibrium of this reaction leaves very little free DHAP to combine with the enzyme. The addition of excess DHAP (curve *K*) restores the absorption. These results indicate that the absorption observed in the low ultraviolet region is indeed a measure of an aldolase-DHAP complex, and they provide confirmation of earlier findings relating to the ability of muscle aldolase to activate DHAP in the absence of aldehyde and to the inability of the enzyme to activate the aldehyde acceptor.

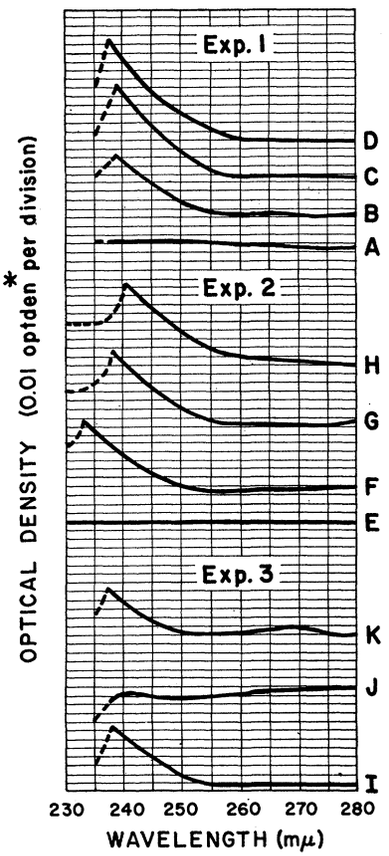


Fig. 1. Experiment 1: effect of DHAP concentration on the absorption spectrum of DHAP-aldolase. Curve *A* shows the absorption determined with 2 mg of crystalline aldolase in both test and reference cuvettes in 1.0 ml of 0.002*M* potassium phosphate buffer, *p*H 7.0. The test cell contained (curves *B, C,* and *D*), in addition, 0.02, 0.07 and 0.1 ml, respectively, of 0.01*M* DHAP, and the reference cell contained an equal volume of water. Experiment 2: effect of adolase concentration on the absorption by DHAP-aldolase. Curve *E* was obtained with 1.0 μmole of DHAP in 1.0 ml of 0.005*M* potassium phosphate buffer, *p*H 7.0. Curves *F, G* and *H* were obtained after the addition of 0.05, 0.15 and 0.25 ml of a 20-mg/ml aldolase solution to both the reference and test cells. Experiment 3: effect of D,L-glyceraldehyde on the absorption spectrum of DHAP-aldolase. Curve *I* was obtained when both cells contained about 2 mg of aldolase in 1.03 ml of water. The test cell contained, in addition, 0.5 μmole of DHAP. Curve *J* was obtained after the addition of 1.0 μmole of D,L-glyceraldehyde in 0.01 ml to both cells. Curve *K* was obtained after the addition of 2.0 μmole of DHAP in 0.12 ml to the test cell and the addition of an equal volume of water to the reference cell. In each experiment the successive curves have been displaced upward in order to simplify presentation. A Cary recording spectrophotometer, Model 14, was used for all measurements. Since use of the term *optical density unit* is technically incorrect, the term *optden,* defined as a unit of absorption measured in a 1-cm cuvette, is used. The optden has the dimension of log $(I_0/I) \times cm^{-1}$.

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References

1. I. A. Rose and S. V. Rieder, *J. Am. Chem. Soc.* 77, 5764 (1955).
2. B. Bloom and Y. J. Topper, *Science* 124, 982 (1956).
3. Y. J. Topper and B. Bloom, unpublished observations.
4. A. H. Mehler *et al., Science,* this issue.

6 September 1957

Separation and Detection of Trifluoroacetyl Amino Acids

The need for separating and identifying trifluoroacetyl amino acids has arisen because of increased use of trifluoroacetic anhydride as an acetylating agent for the amino group during synthesis and study of peptides and proteins. Trifluoroacetyl amino acids do not form color complexes with ninhydrin, nor are the usual tests for fluorine adaptable for small quantities of these derivatives. However, the acetylated amino acids will undergo hydrolysis at a *p*H greater than 10 (*1*) with the liberation of the free amino acid. The free acids may then be detected with ninhydrin. Separation of mixtures of the trifluoro derivatives and the free amino acids is readily accomplished by means of paper electrophoresis.

Samples containing a mixture of either trifluoroacetyl glycine and glycine or trifluoroacetyl alanine and alanine were separated on a Reco electrophoresis apparatus (model E-800-2); potassium acid phthalate—sodium hydroxide buffer of *p*H 6.0 and of ionic strength 0.045 was used. The electrophoretic determinations were conducted at 600 v for 1 hour. At the end of this time the paper strips were dried, dipped in 1 percent alcoholic sodium hydroxide (1 ml of saturated sodium hydroxide solution in 99 ml of 95 percent ethanol), and dried. The color

was then developed with acidic-ninhydrin reagent (*2*).

Under the conditions described, a separation of 5.5 cm has been obtained between glycine and trifluoroacetyl glycine, the acetylated derivative migrating to the anode and the glycine remaining almost stationary. The use of the same technique afforded a separation of 4.5 cm between alanine and trifluoroacetyl alanine.

It is conceivable that the technique described may be of use in evaluating (i) the hydrolysis of trifluoroacetyl amino acids by enzymes (*3*), (ii) the solubilizing effect of trifluoroacetic acid on proteins (*4*), and (iii) the cleavage of peptides after trifluoroacetylation of the peptide bond (*5*). In addition, the purity of trifluoroacetyl amino acids may be determined by separating large quantities of these compounds electrophoretically and then checking for ninhydrin-positive compounds (the free amino acids are ninhydrin-positive without basic hydrolysis) (*6*).

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References and Notes

1. E. E. Schallenberg and M. Calvin, *J. Am. Chem. Soc.* 77, 2779 (1955).
2. J. F. Roland and A. M. Gross, *Anal. Chem.* 26, 502 (1954).
3. W. S. Fones and M. Lee, *J. Biol. Chem.* 210, 227 (1954).
4. C. Lenti, *Studi sassaresi* 34, 31 (1956); *Chem. Abstr.* 51, 4469g (1957).
5. F. Weygand, R. Geiger, U. Glöckler, *Chem. Ber.* 89, 1543 (1956).
6. This report has been authorized for publication as paper No. 2170 in the journal series of the Pennsylvania Agricultural Experiment Station.

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Occurrence of Plasmalogens in Lipides of Green Peas

The plasmalogens (*1*), a group of glycerophosphatides which contain a higher fatty aldehyde, have been reported to be constituents of various animal phosphatides (*2*). Recently Lovern (*3*) has shown that plasmalogens also occur in vegetable lipides. Commercial samples of the total phosphatide fractions of soybeans and peanuts possessed an aldehyde content equivalent to about 7 percent of palmitaldehyde. Plasmalogens are readily cleaved by acid and mercuric chloride, and the liberated aldehyde may be determined with fuchsin-sulfite solution (Feulgen reagent) (*4*).

The lipides of green peas (*Pisum sativum* L.) have been under investigation because of their probable role in the development of off-flavors during frozen storage of raw peas (*5*). Crude lipide material was extracted from lyophilized raw peas, Perfected Freezer variety, with chloroform-methanol, 2:1. The lipide components of the mixture were then separated by means of a solvent fractionation procedure (*6*). The various fractions were then analyzed for their plasmalogen content with Feulgen reagent. A reaction time of 4 hours at room temperature was employed. The color which developed was extracted from the aqueous reaction mixture with 4 ml of isoamyl alcohol and read at 572 mμ in a Beckman spectrophotometer, model DU, against reagent blanks. The amounts of plasmalogen were determined from a standard curve obtained with known amounts of palmitaldoxime treated as described above.

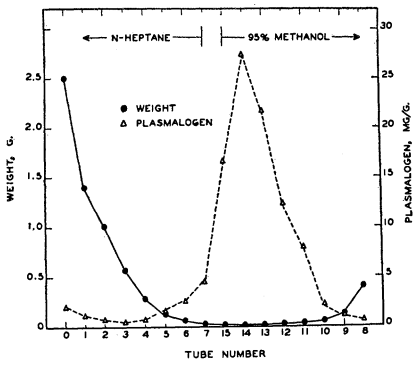

Fig. 1. Countercurrent distribution of acetone-soluble pea lipides between *n*-heptane and 95 percent methanol, showing distribution of weight (circles) and plasmalogens (triangles).

Crude pea lipides were separated into acetone-soluble and acetone-insoluble fractions, the latter primarily a mixture of phosphatides. Phosphatides may be classified on the basis of their solubility in glacial acetic acid and in 95 percent ethanol (*7*). Lecithin (phosphatidyl choline) and cephalin (phosphatidyl serine and phosphatidyl ethanolamine) are soluble in glacial acetic acid, whereas phosphatidyl inositol is not (*8*). Furthermore, lecithin is soluble in alcohol, and cephalin is not. The pea phosphatides (acetone insoluble lipides) were fractionated with glacial acetic acid and with 95 percent ethanol. The plasmalogen content of the phosphatide fractions (expressed as milligrams per gram of lipide) was as follows: alcohol soluble (lecithin), 3.9, alcohol insoluble (cephalin) 7.3, acetic acid insoluble (phosphatidyl inositol) 29.4. Thus the greatest concentration of plasmalogens would appear to be associated with the more complex inositol phosphatides which normally are insoluble in glacial acetic acid.

The acetone-soluble pea lipides were subjected to countercurrent distribution between *n*-heptane and 95 percent methanol in a seven-transfer system with single withdrawal (*6*) at a concentration of 13 percent with respect to each solvent (Fig. 1). It was anticipated that the bulk of the material would consist of triglycerides and that it would, therefore, be concentrated in the nonpolar solvent. In fact, over 75 percent of the total weight of lipides was found in the first three heptane fractions (tubes 0, 1, 2).

The plasmalogens were found to be distributed in the methanol fractions in the region of minimum weight distribution. The maximum amount was 27.5 mg/g in tube 14, which contained but 0.26 percent of the total weight. The tubes on either side contained lesser amounts of the plasmalogens, and only small amounts were present in the heptane fractions which comprised nearly all of the weight. Although phosphatides are normally considered to be insoluble in acetone, their presence in the acetone-soluble portion of a lipide mixture may be attributed to the well-known solubilizing powers of triglycerides.

Inasmuch as the separation of individual lipides from a naturally occurring mixture is always a difficult task, the apparent enrichment of plasmalogens by countercurrent distribution may provide a basis for the isolation and characterization of these unusual phosphatides in vegetable lipides. The lipides of green peas would appear to provide a convenient source of plant plasmalogens for such studies (*9*).

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References and Notes

1. R. Feulgen and K. Voit, *Arch. ges. Physiol. Pflüger's* 206, 390 (1924).
2. R. Feulgen and T. Bersin, *Z. physiol. Chem. Hoppe-Seyler's* 260, 217 (1939); S. J. Thannhauser *et al., J. Biol. Chem.* 188, 423 (1951); E. Klenk and P. Böhm, *Z. physiol. Chem. Hoppe-Seyler's* 288, 98 (1951); J. Olley and J. A. Lovern, *Biochem. J.* 54, 559 (1953); E. Klenk and H. Debuch, *Z. physiol. Chem. Hoppe-Seyler's* 299, 66 (1955).
3. J. A. Lovern, *Nature* 169, 969 (1952).
4. R. Feulgen and H. Grünberg, *Z. physiol. Chem. Hoppe-Seyler's* 257, 161 (1938).
5. A. C. Wagenknecht and F. A. Lee, *Food Research* 21, 605 (1956).
6. A. C. Wagenknecht, *J. Am. Oil Chemists' Soc.* 34, 509 (1957).
7. H. E. Carter *et al., J. Biol. Chem.* 206, 613 (1954).
8. A. C. Wagenknecht and H. E. Carter, *Federation Proc.* 16, 266 (1957).
9. Journal paper No. 1093, New York State Agricultural Experiment Station.

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Sex Chromatin and Sex Differentiation in Human Embryos

In the past, embryologists studying sex differentiation in man paid little attention to the fact that *chromosomally* there exists no indifferent stage because every fertilized egg is either XY-male or XX-female. Chromosome analysis was