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SEPARATION OF ENZYMES ON THE FILTER PAPER CHROMATOPILE*

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A recent report from this laboratory described a method for isolation of low molecular weight compounds by chromatography on a pile of filter paper disks (1). Preliminary experiments have now been completed on the application of this method to the separation of enzymes. It was expected that a mixture of enzymes could be placed near the top of a filter paper pile and fractionated by imposing a gradient (*i.e.* pH, organic solvent, or salt concentration) on the column, thus taking advantage of a continuous process. Following the observation of Tiselius (2) it also seemed probable that enzyme separations could be obtained on the filter paper pile by salting-out adsorption and adsorption displacement.

EXPERIMENTAL

Concentration Gradients—Although some separation of adenosine deaminase was obtained from amylase in taka-diastrase by means of a pH gradient in 0.1 M phosphate buffer, more promising results were derived from the use of an $(\text{NH}_4)_2\text{SO}_4$ concentration gradient. The following experiment is illustrative. Taka-diastrase (300 mg. of 525 per cent with respect to amylase activity; Parke, Davis and Company) was dissolved in 20 ml. of water and taken up on twenty sheets of 9 cm. Whatman No. 1 filter paper. After drying at room temperature, the twenty disks were incorporated at forty disks from the top of a 500 sheet pile (1). The entire apparatus and solvents were equilibrated at 10°. The solvent distributor and syphon were filled with 60 per cent saturated $(\text{NH}_4)_2\text{SO}_4$ solution (pH 6.5)¹ and the syphon tube placed in a beaker containing 100 ml. of the same $(\text{NH}_4)_2\text{SO}_4$ solution. The solvent surface was placed level with the top of the column. Distilled water was added from a drop-

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¹ All $(\text{NH}_4)_2\text{SO}_4$ concentrations are given for 10°. All pH values were determined without dilution with a Beckman pH meter.

ping funnel at such a rate as to maintain the solvent level in the beaker. A thorough mixing was obtained by a stirring motor. After 8 hours the column had taken up 275 ml. of solvent in 450 paper disks. The column was removed from the clamp and alternate sections of five disks each were removed and cut into 0.5×4 cm. strips for measurement of activity on five sheets at a time. Activities of amylase (3), adenylic acid phosphatase (3), and adenosine deaminase (4) were measured by methods

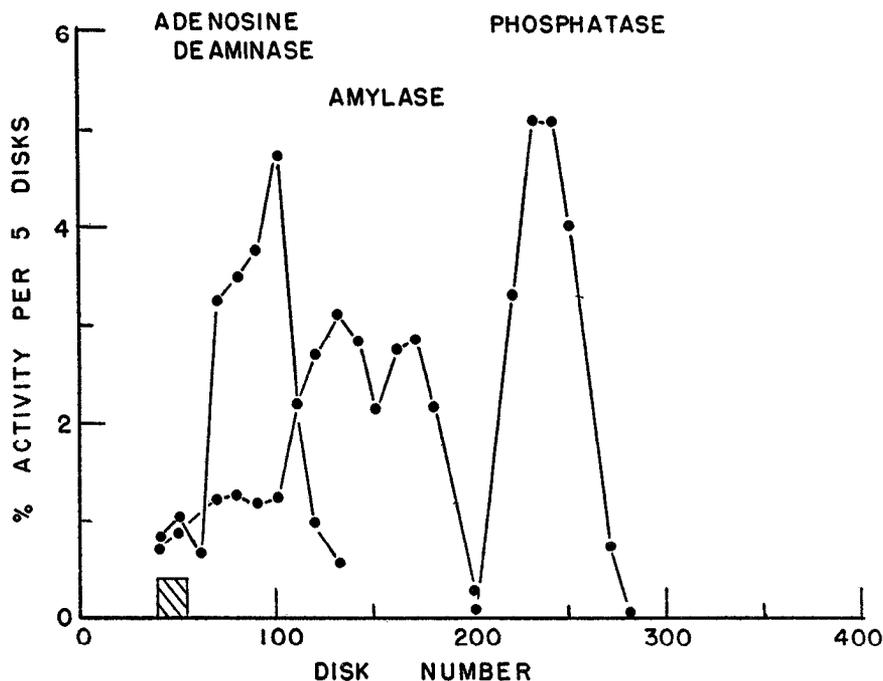


Fig. 1. Separation of three enzymes on the filter paper chromatopile with an $(\text{NH}_4)_2\text{SO}_4$ gradient at pH 6.5. Salt concentration in per cent saturation at 10° ; Disk 30, 14 per cent; Disk 120, 20 per cent; Disk 202, 40 per cent; Disk 280, 50 per cent.

previously described. Paper strips from each section were dropped directly into the substrate solutions without extraction of the enzymes from the paper or removal of the $(\text{NH}_4)_2\text{SO}_4$. Controls of unfractionated taka-diastase were carried out in the same fashion. The results of one experiment of this kind are summarized in Fig. 1. The figures for $(\text{NH}_4)_2\text{SO}_4$ concentration are calculated as per cent saturation at 10° based on the average volume of solvent per disk (0.61 ml.) in the pile. These values were determined by extraction of sheets in hot water, followed by a colorimetric determination of NH_3 by nesslerization. The calculated total

recoveries of enzyme activities were adenosine deaminase 85 per cent, amylase 120 per cent, and phosphatase 65 per cent. These values are based on controls with the original enzyme solutions absorbed on paper. Similar results were obtained by extraction of the enzymes from the paper before measurement of the activities, but this procedure was cumbersome and found to be unnecessary.

Although extensive investigations have not been carried out to determine the effects of various salts, the effect of a pH gradient in a constant

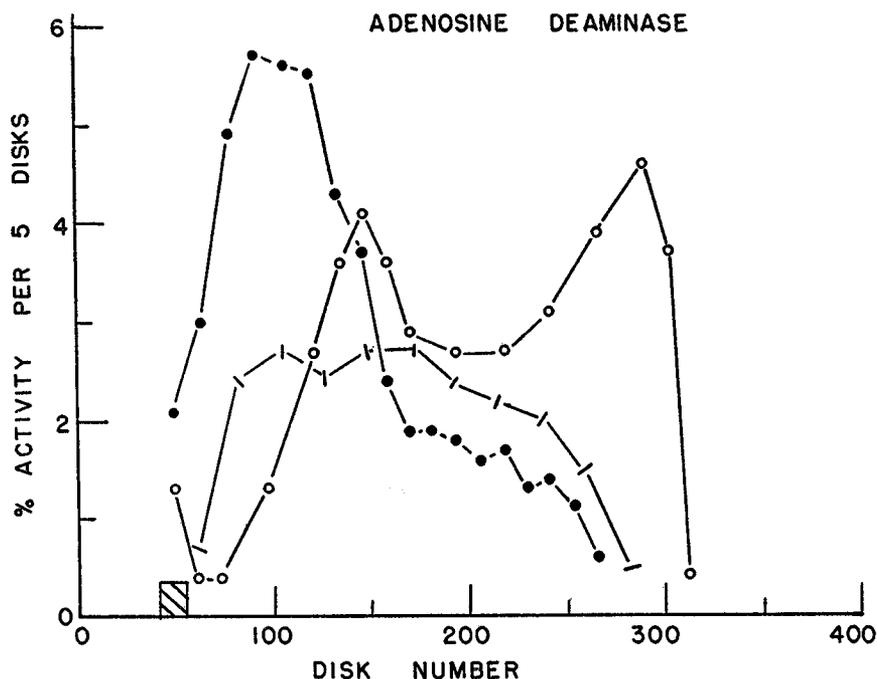


FIG. 2. Movement of adenosine deaminase in the chromatopile at different $(\text{NH}_4)_2\text{SO}_4$ concentrations and at pH 6.5. ●, 20 per cent saturation; ○, 31 per cent saturation; dash line, 45 per cent saturation.

salt concentration has been considered to a limited extent. These experiments were prompted by the finding that, in second runs with samples of twenty disks taken from activity peaks of amylase and adenosine deaminase from a preliminary pile fractionation, both enzymes failed to move significantly. The second runs were carried out at pH 6.5 in $(\text{NH}_4)_2\text{SO}_4$ concentrations as low as 10 per cent saturation. A pH gradient was therefore produced in 30 per cent saturated $(\text{NH}_4)_2\text{SO}_4$ solution by dropwise addition of the salt solution, 0.25 M in NH_4OH , to salt solution 0.1 M in phosphate buffer in the same fashion previously described for producing a salt gradi-

ent. Amylase activity was found to move satisfactorily in second runs above pH 7.3 but not in the range 4.9 to 7.3. Adenosine deaminase moved significantly at the highest pH attained (7.7) but not at lower pH values. Specific details on the simultaneous variation of salt concentration and pH will be considered subsequently. It is already clear that such variations will provide another means of obtaining protein separations.

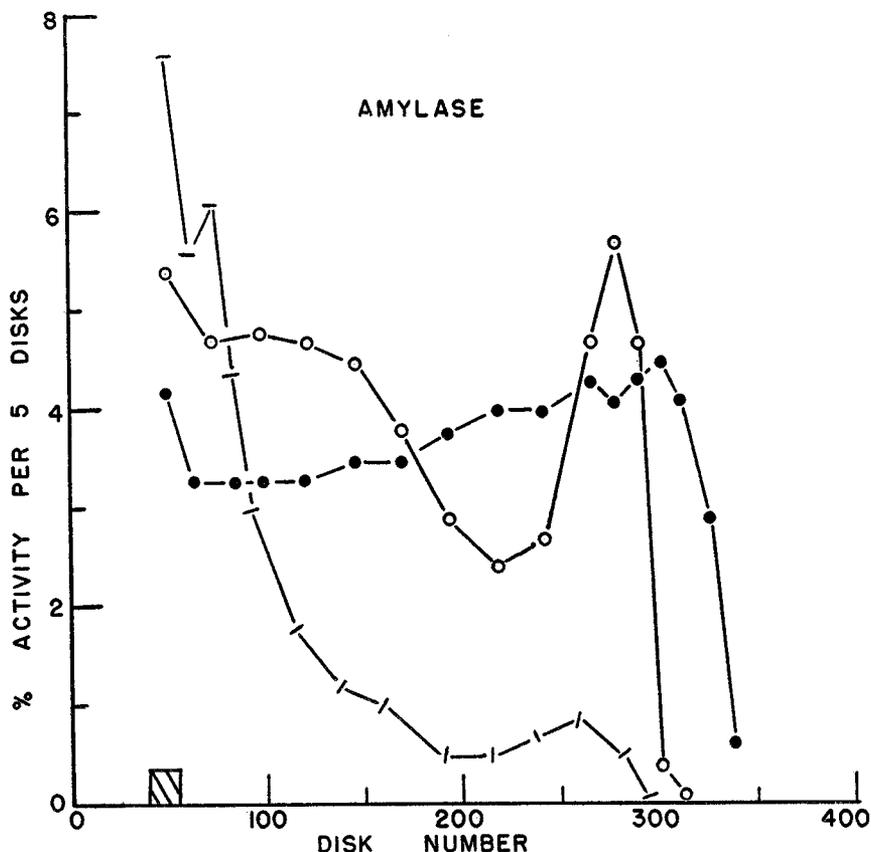


Fig. 3. Movement of amylase in the chromatopile at different $(\text{NH}_4)_2\text{SO}_4$ concentrations and at pH 6.5. ●, 20 per cent saturation; ○, 31 per cent saturation; dash line, 45 per cent saturation.

Constant Salt Concentrations—Amylase and adenosine deaminase activities were determined in four 350 sheet piles with 20, 30, 45, and 60 per cent saturated $(\text{NH}_4)_2\text{SO}_4$ respectively, as solvents. The experiments were carried out essentially as described above for the gradient pile. The results are summarized in Figs. 2 and 3. Data are not shown for the 60 per cent saturation level of $(\text{NH}_4)_2\text{SO}_4$, since both enzymes failed to move

significantly at this concentration level. It should be noted, however, that neither the adenosine deaminase nor the amylase of the crude mixture is precipitated from solution by 60 per cent saturation of $(\text{NH}_4)_2\text{SO}_4$.

DISCUSSION

It is evident from the experiments described that the chromatopile provides a convenient column for the fractionation of enzymes. Although the initial experiments were designed to separate enzymes simply on the basis of a continuous process involving solubility with a solvent gradient, it is clear that adsorption of the proteins on the paper plays a significant rôle and a gradient is not necessarily essential. In agreement with the report of Tiselius (2), the enzymes are strongly adsorbed by the paper at salt concentrations far below those required for precipitation. In addition, the movement of an enzyme in the pile is markedly influenced by the presence of other proteins. This may be interpreted in terms of displacement adsorption, as previously demonstrated (2). However, by appropriate adjustment of pH, displacement adsorption may become less significant, as indicated by repeated fractionation experiments. The data of Fig. 2 for adenosine deaminase show that the rate of movement of the enzyme goes through a maximum as the $(\text{NH}_4)_2\text{SO}_4$ concentration is increased. Although the picture is complicated by the presence of many proteins, it seems probable that this phenomenon is due in part to a simultaneous effect of the salt on the adsorbability of the protein and on the properties of the paper as an adsorbent.

Throughout the experiments that have been carried out there has been consistent evidence for a number of activity peaks with both amylase and the more specific enzyme adenosine deaminase (Figs. 2 and 3). Although these results suggest multiple molecular species for both enzymes, such a conclusion cannot be justified on the basis of the present experimental evidence. This question is being investigated further.

SUMMARY

1. It has been established that considerable enzyme resolution can be attained by use of the filter paper chromatopile.

2. Variations in salt concentrations and pH are shown to have a great influence on the movement of at least two enzymes in the pile. These variations with the many obvious ones that are yet to be investigated may provide some new criteria for protein homogeneity.

3. Sufficient quantities of proteins can be fractionated on the chromatopile to provide a means of isolation.

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