EVIDENCE FOR TRANSAMINASE ACTIVITY IN THE SLIME MOLD, DICTYOSTELIUM DISCOIDEUM RAPER [1]

JEROME O. KRIVANEK AND ROBIN C. KRIVANEK

Department of Zoology, University of South Florida, Tampa, Florida 33620

As the developmental cycle of the slime mold, *Dictyostelium discoideum* Raper, proceeds from the myxamoeba stage to the mature sorocarp stage, there occurs a reduction in proteinaceous materials and an increase in polysaccharide carbohydrate, as shown by Gregg, Hackney and Krivanek (1954) and Gregg and Bronsweig (1956). On the basis of these quantitative studies, Gregg and his associates suggested that the protein components may serve as precursors not only for energy-source intermediates of development, but also for the synthesis of carbohydrate necessary for stalk formation. Attempting to define the metabolic mechanisms responsible for this inverse relationship, Krivanek and Krivanek (1959) chromatographically analyzed the amino acid components of the slime mold in both hydrolyzed and unhydrolyzed tissue. Their findings suggested that deamination may be one process relating protein degradation to carbohydrate synthesis. These authors did not exclude the possibility, however, that other metabolic mechanisms, *e.g.*, transamination, may be instrumental in this linkage.

Utilizing a spectrophotometric technique to observe the change in the characteristic absorption band of DPNH at 340 mμ, Wright and Anderson (1959) demonstrated the occurrence of "aspartic-pyruvic transaminase." However, these authors, as well as others (Meister, 1950; Aspen and Meister, 1958), have expressed the lack of definity of such an analytical technique because of its broad specificity.

In view of the important role which transaminase activity plays in relating protein and carbohydrate metabolism, more precise evidence than that thus far presented would seem desirable. It is therefore the intent of this paper to demonstrate that specific transaminase activities are indeed operative in the slime mold, *Dictyostelium discoideum*.

Materials and Methods

D. discoideum was cultured in the manner described by Bonner (1947), using *Escherichia coli* as the bacterial associate.

The following two transamination reactions were studied:

Reaction I: glutamate + pyruvate $\rightarrow$ α-ketoglutarate + l-alanine
Reaction II: glutamate + oxaloacetate $\rightarrow$ α-ketoglutarate + l-aspartate

The primary technique employed in studying these reactions was that of progressive chromatography as described by Hird and Roswell (1950).

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Individuals in the desired stage of development were harvested from the Petri plates and homogenized in ice-cold phosphate buffer, pH 7.4, using an homogenizer of the type described by Gregg *et al.* (1954). The final volume of the homogenate was 5 ml. The extent of homogenization was determined by microscopic inspection of the homogenate.

After centrifugation of the homogenate, the supernatant or soluble fraction (S) was withdrawn from the particulate centrifugate or insoluble fraction (I). Both fractions were then made up to the original volume of 5 ml.

One-ml. samples of each fraction were then transferred to five separate reaction tubes—five tubes for each fraction series. To the control tube in each series was added 1 ml. of phosphate buffer only. To each of the remaining four tubes in each series were added $\frac{1}{2}$ ml. glutamate and $\frac{1}{2}$ ml. oxaloacetate or pyruvate, depending upon the reaction under consideration. The concentration of the glutamate, oxaloacetate and pyruvate varied and will be discussed under Results. To this point all steps were carried out under ice-cold conditions.

It is conceivable that l-alanine may be formed from pyruvate, l-aspartate from oxaloacetate, and α-ketoglutarate from glutamate by reactions other than transamination. Therefore, supplementary controls were utilized to determine whether the appearance and disappearance of the appropriate substrates were mutually interdependent. In this particular control series, only one of the initial reactants, *i.e.*, glutamate, was added to the reaction tubes, with subsequent treatment of these controls being the same as for the phosphate buffer controls and the experimental series.

Immediately upon the addition of the last substrate, the reaction tubes were put into a closed anaerobic environment, consisting of gaseous nitrogen and pyrogallol, and the mixtures were allowed to react at a temperature of 37° C. Reaction tubes from both soluble and insoluble series were generally removed after 30, 120, 180 and 240 minutes and processed. Control tubes were processed in the same manner and for the maximum time interval.

At the end of each incubation period, to each of the tubes were added two volumes of warm ethanol (60–70° C.) to precipitate the proteins. After centrifugation, the supernatant fluid was withdrawn and dried *in vacuo* at room temperature. The residue after evaporation was then resuspended in 1 ml. of distilled water, and identical fractions from each preparation were spotted on Whatman No. 1 filter paper for a uni-directional chromatographic separation of the amino acids. Among the various solvents used were: propanol-water (80:20), n-butanol-acetic acid-water (250:60:250), n-butanol-acetone-water (10:10:5), and n-butanol-acetone-water (5:4:1). Development of the spots was accomplished by means of spraying the chromatograms with a solution of 0.3% ninhydrin in 95% ethanol. Identification of the unknown spots was determined by means of positional comparisons between the unknown spots and spots of known amino acids applied to the same sheet.

The spot determination of the alpha-keto acids (oxaloacetate, alpha-ketoglutarate and pyruvate) was done by separating them as their 2,4-dinitrophenylhydrazones according to the method described by Block, Durrum and Zweig (1958). To samples of each of the reaction mixtures, after deproteinization with warm ethanol, was added 1 ml. of 0.5% 2,4-dinitrophenylhydrazine dissolved in 6 N HCl. After 30

minutes' standing at room temperature, the hydrazones were extracted in a separatory funnel with three 7½-ml. washes of a chloroform:ethanol (80:20) solution. The hydrazones, now in the latter solution, were then extracted with 7½ ml. of 1 N Na_2CO_3. After washing the hydrazone-containing Na_2CO_3 solution with 5 ml. of chloroform-ethanol solution, the Na_2CO_3 solution was then acidified with 2½ ml. of 6 N HCl in the cold. The resultant acidified Na_2CO_3 solution was further washed with three portions of the chloroform:ethanol solution totaling 10 ml. The 10 ml. of washings were then evaporated under a gentle air stream.

For a chromatographic separation of the 2,4-dinitrophenylhydrazones, the residue after evaporation was dissolved in 2 ml. absolute ethanol and spotted on Whatman No. 1 filter paper in 1-μl. amounts. Identification of the spots was determined by preparing samples of known alpha-keto acids, processing them in the same manner as the experimental series and spotting them on the same paper with the experimentals.

Detection of the hydrazones of the keto acids was accomplished by initially inspecting the chromatogram for yellow spots (distinctive of hydrazones), then spraying the paper with a 2% ethanolic KOH solution which imparts a red-brown color to the spots, and/or scanning the paper with a UV light which caused the spots to fluoresce.

Since the paper chromatographic method did not afford a clear separation between glutamate and aspartate, a paper electrophoresis procedure was utilized (Block, Durrum and Zweig, 1958). This method is specific in its separation of aspartate, glutamate, histidine, arginine, lysine, and the monoamino-monocarboxylic acids. A phthalate buffer, pH 5.9, was used in a Spinco paper electrophoresis apparatus usually run at 500 volts, 18 amperes for three hours.

Results and Discussion

A. Amino acids

Figure 1 shows the chromatographic results of Reaction I, *i.e.*, when tissue fractions, soluble and insoluble, were incubated in the presence of 1/40 *M* glutamate and 1/10 *M* pyruvate. It is to be noted that with lengthening periods of incubation (A is shortest, D is longest), color intensities of the glutamate spots decrease in both soluble and insoluble series. No corresponding spots were evident in the control series which were incubated for four hours—the maximum time for the experimental series. The appearance of alanine in the insoluble series lagged behind its appearance in the soluble series. Thus, after two hours' incubation alanine was first seen in the former series while only 30 minutes were necessary for it to appear in the soluble series.

The results relative to Reaction II are seen in Figures 2 and 3. In Figure 2, the spots of aspartate and glutamate in the soluble series are in close spatial relationship to each other, with aspartate trailing glutamate. The chromatographic technique did not clearly delineate the two compounds although development of the chromatogram with dicyclohexylamine did allow better interpretation than did ninhydrin. It is to be noted that aspartic acid increased in intensity in the soluble series. A corresponding decrease in the intensity of glutamate was also evident. In the insoluble series, no aspartate was apparent in either the chromatographic or

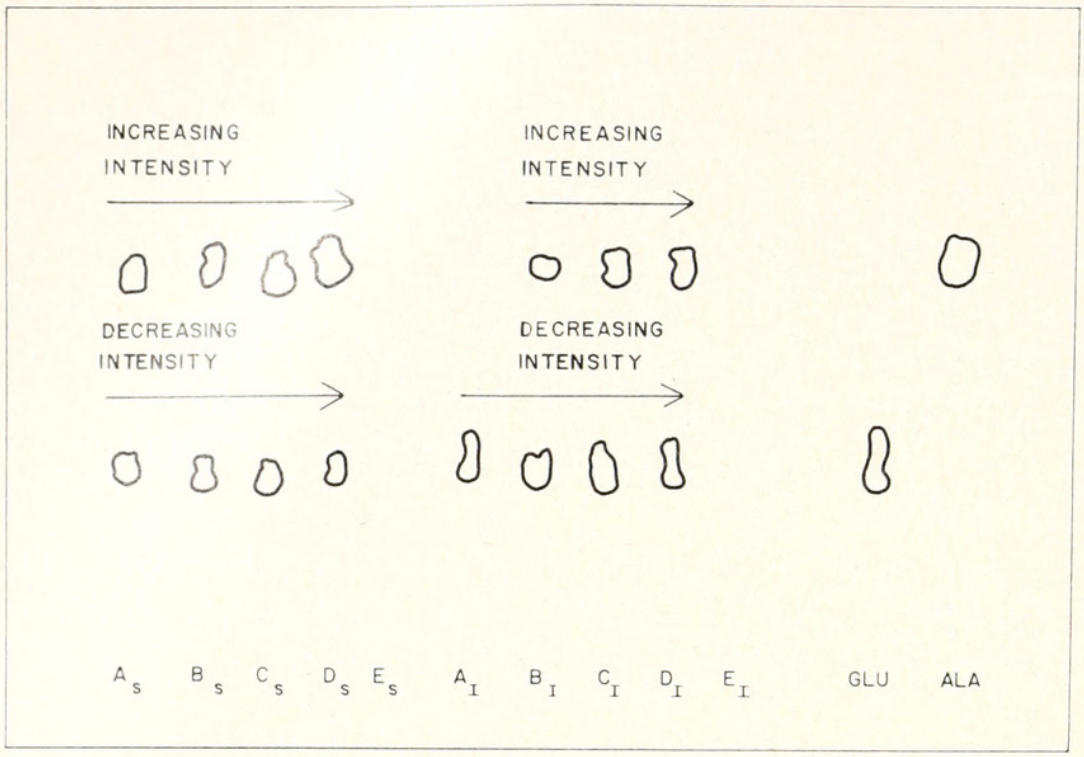

FIGURE 1. Exact reproduction of chromatogram showing amino acid results of Reaction I. Incubation times: A series, $\frac{1}{2}$ hour; B series, 2 hours; C series, 3 hours; D series, 4 hours; E series (phosphate control), 4 hours. "S" denotes soluble fraction, "I" denotes insoluble fraction, GLU = glutamate known; ALA = alanine known.

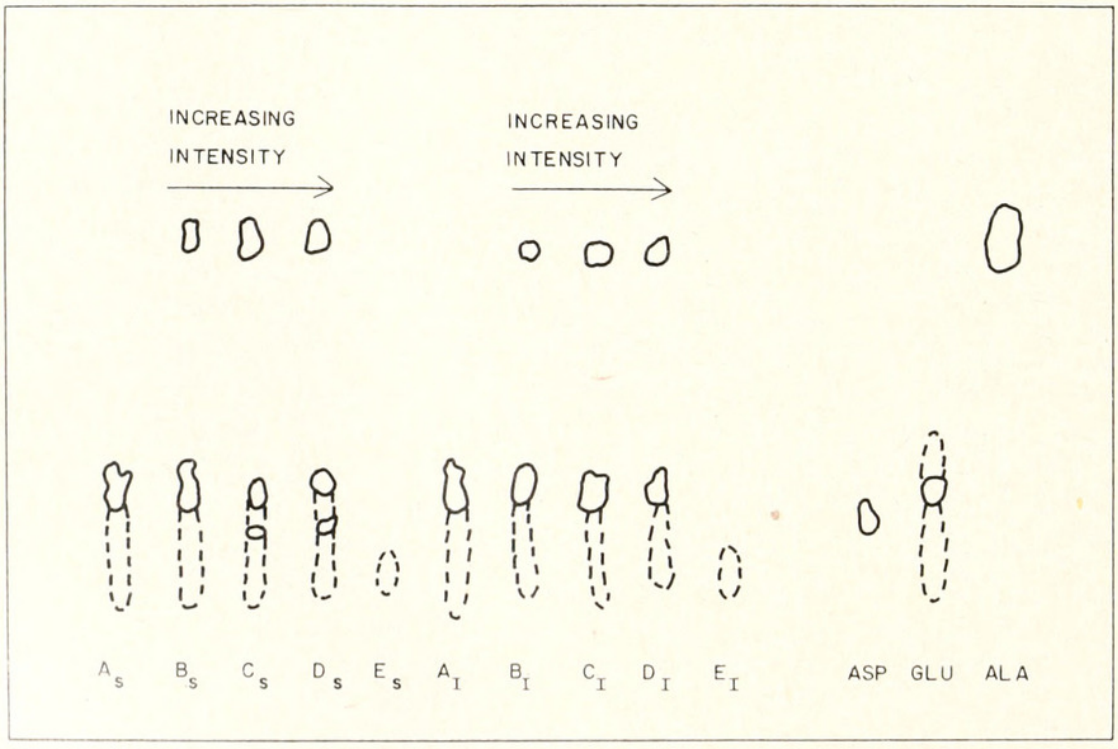

FIGURE 2. Exact reproduction of chromatogram showing amino acid results of Reaction II. Incubation times same as in Figure 1. ASP = aspartate known.

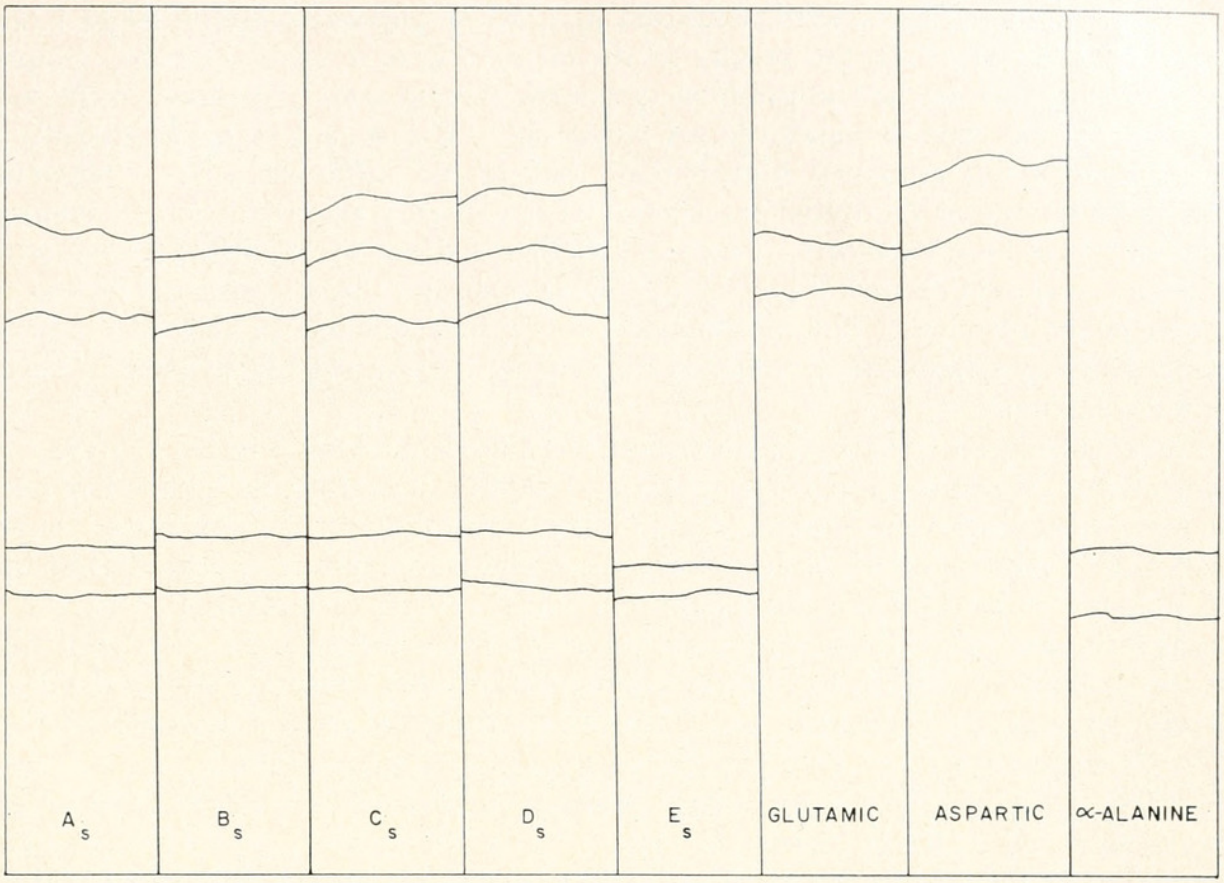

FIGURE 3. Exact reproduction of electrophoretic pattern showing amino acid results of Reaction II. Length of run: 3 hours; voltage: 500; amperage: 18 amps. Veronal buffer of pH 8.6. Letter notations same as in Figure 1.

electrophoretic determinations. It should be mentioned at this point that no decrease in color intensity of glutamate was observed when 1/20 *M* concentration was used. When 1/40 *M* glutamate was used, a perceptible decrease was evident. However, this decrease was less profound than in Reaction I.

In addition to the expected aspartate product of transamination Reaction II, an unexpected product—alanine—was also present in both soluble and insoluble series. No alanine was detected in the control series. The appearance of alanine could be accounted for by way of oxaloacetic acid being decarboxylated to pyruvic acid, with transamination subsequently occurring to form alanine. Such a transformation could be mediated only through the action of a decarboxylase.

The chromatographic separation of alanine, aspartic acid, and glutamate was supplemented by an electrophoretic separation. Using the electrophoretic technique previously described for the separation of monoamino-monocarboxylic amino acids, complete validation of the chromatographic results was accomplished as shown in Figure 3. A decrease in glutamate intensity and increase in alanine and aspartic acid intensities were noted.

B. Keto-acids

The keto-acid determinations essentially follow expectation if transamination is, in fact, operative in the slime mold.

When tissue extracts were incubated in 1/40 *M* glutamate and 1/10 *M* oxaloacetic acid, Reaction II, the results as shown in Figure 4 were achieved. Alpha-ketoglutarate, one of the end products of the reaction, is seen to increase in intensity with increasing time of incubation of the tissue. The degree of color intensity of the soluble series remained higher than that in the insoluble series. However, oxaloacetate, one of the initial reactants, did not display any reduction in intensity as might have been expected. Since our method did not distinguish between pyruvate and oxaloacetate (see Figure 4), pyruvate may have been generated during the course of the reaction. This would again not be unreasonable in view of the previously stated possibility that oxaloacetate may be decarboxylated to yield pyruvate in the slime mold.

Various concentrations of oxaloacetate were used in addition to the 1/10 *M* concentration. When a lower concentration (1/20 *M*) was used, no spot was evident at the oxaloacetate locus chromatographically. In addition, the enzymatic conversion of this substrate took place rapidly, for alpha-ketoglutarate appeared after only 15 minutes of incubation. Increasing the concentration of oxaloacetate to 1/5 *M* seemed to have an inhibitory effect on the reaction, as indicated by (1) large spots at the oxaloacetic acid locus, (2) extremely small ketoglutarate spots, and (3) no alanine or aspartic acid being formed. On the basis of these test concentrations, 1/10 *M* oxaloacetate was chosen as being the optimal concentration.

Figure 5 shows the results of incubating homogenized tissue with 1/40 *M* glutamic acid and 1/10 *M* pyruvate. Here, as in the case of Reaction II, alpha-ketoglutarate increased in color intensity with increasing lengths of incubation times.

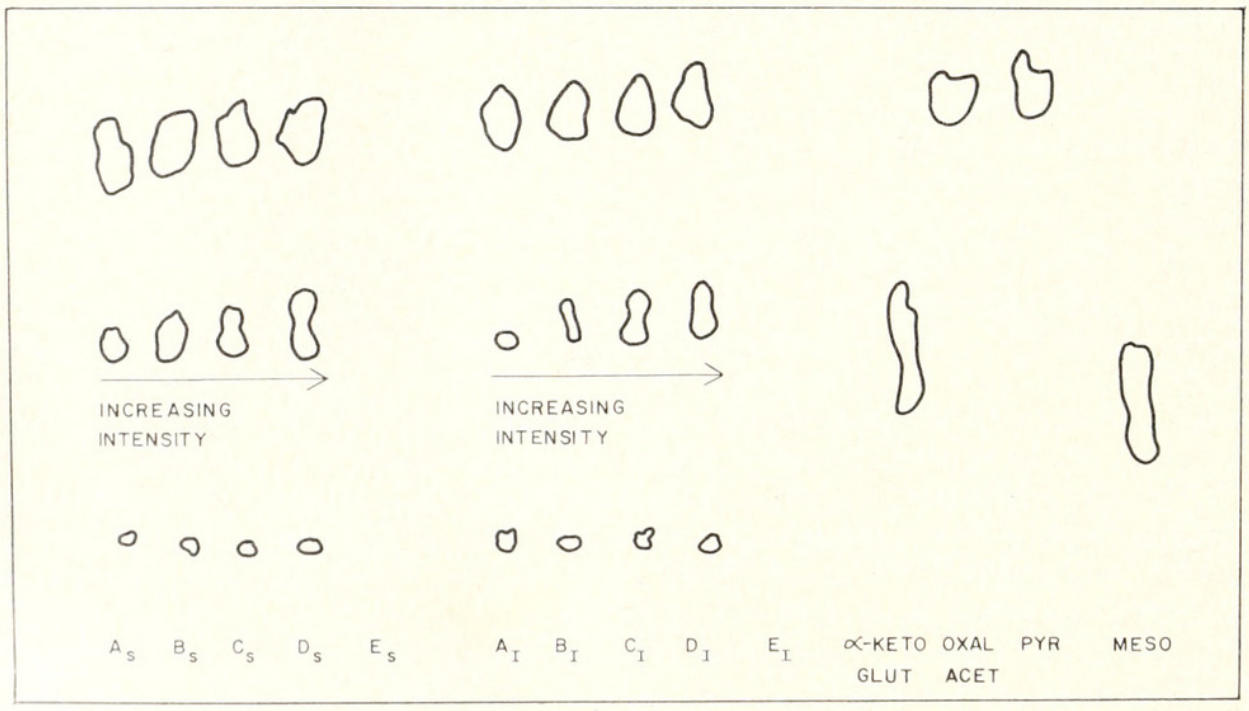

FIGURE 4. Exact reproduction of chromatogram showing keto-acid results of Reaction II. Incubation times same as in Figure 1. α-KETOGLUT = α-ketoglutarate known; OXALACET = oxaloacetic acid known; PYR = pyruvic acid known; MESO = mesoxalic acid known. Mesoxalic acid known was spotted to aid identification of unknown spots. Note loci of unknown "spot B" at points of spot application.

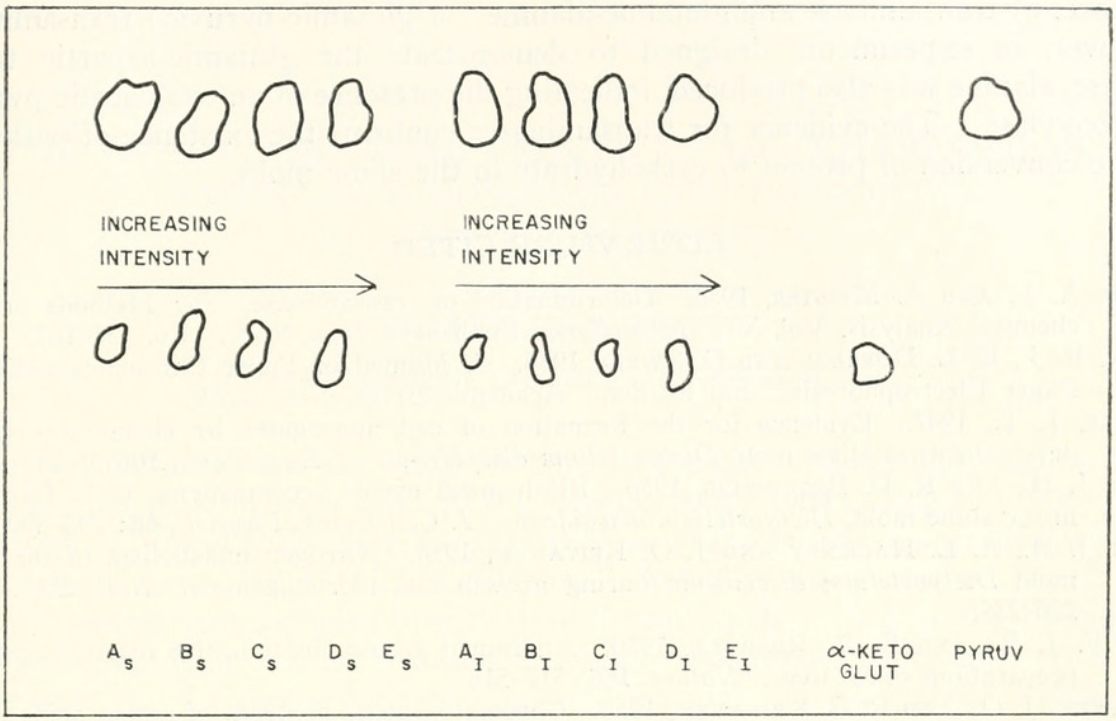

FIGURE 5. Exact reproduction of chromatogram showing keto-acid results of Reaction I. Incubation times same as in Figure 1. Abbreviations same as in Figure 4.

With pyruvate, as with oxaloacetate at this concentration, no progressive diminution in color intensity occurred with increasing lengths of incubating times. Similar reasoning and possible concentration effects can be applied to this event as were applied to the oxaloacetate results.

Brief mention should be made of two unidentified spots evident only in Reaction II experiments. Their possible importance lies in the fact that they occurred only in the experimental series and were not evident in the controls. The locus of "spot A," when present, was invariably midway between the point of origin (point of application of the test solution on chromatogram) and the alpha-ketoglutarate locus. Although every attempt was made to reproduce exact conditions between each run, "spot A" was not always detected, occurring more times than not. It appeared when using 1/20 *M* and 1/10 *M* concentrations of oxaloacetic acid.

The second unidentified spot, "spot B," was consistently present, its locus being at or slightly above the points of application of the experimental samples on the chromatogram (see Figure 4). The various concentrations of oxaloacetic acid did not affect its appearance. Not only was it present in all test series, but there was also a tendency for it to increase in color intensity with increasing incubation times. Its absence from the controls and from the point of application of a known oxaloacetic acid solution is to be especially noted. The significance, if any, of unidentified "spots A and B" is at present not apparent.

SUMMARY

Progressive chromatography and paper electrophoresis techniques have demonstrated qualitatively the occurrence of two transaminating systems in the slime mold, *Dictyostelium discoideum*. These systems are glutamic-aspartic (or glutamic-

oxaloacetic) transaminase and glutamic-alanine (or glutamic-pyruvic) transaminase. However, in experiments designed to demonstrate the glutamic-aspartic transaminase, alanine was also produced, indicating the presence of an oxaloacetic-pyruvic decarboxylase. The evidence for transaminases confirms the existence of pathways for the conversion of protein to carbohydrate in the slime mold.

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