and *phenols* apparently is capable of conjugation to a limited extent.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOPHYSICS, FLORENCE R. SABIN LABORATORIES, AND THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF COLORADO MEDICAL CENTER]

# A Fractionation of D-Amino Acid Oxidase by Electrophoresis–Convection[1]

BY JOHN R. CANN AND WILHELM R. FRISELL

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Extracts of pig kidney cortex have been fractionated by electrophoresis–convection. Fractionations were carried out at *p*H 5.3, 7.0 and 8.1. Assay of the fractions for D-amino acid oxidase showed that considerable enrichment of the top fraction with respect to enzymatic activity was achieved in a single stage of fractionation at *p*H 7 after transport of 97% of the protein out of the top and into the bottom reservoir of the electrophoresis–convection cell. The specific activity of the top fraction, corrected for the effect of dilution upon enzymatic activity, was 3 to 5 times as great as that of the unfractionated extract. At *p*H 5.3 the specific activities of the top fractions were about 1.5 times that of the starting material, while at *p*H 8.1 very little if any fractionation was achieved.

Electrophoresis–convection is gaining extensive use in the fractionation of a variety of proteins.[2-5] This method of fractionation, first proposed and tested by Kirkwood and Nielsen,[6,7] is based upon the same principle as that of the Clusius column, except that electrophoretic transport, instead of transport by thermal diffusion, is superimposed on convective transport in a vertical convection channel. The separation of a heterogeneous protein into its constituents is accomplished by making use of small differences in their isoelectric points and mobilities. The details of construction and operation of the electrophoresis–convection apparatus have been described in detail by Cann and co-workers.[8,9a,b] Incidental to other studies in the purification of D-amino acid oxidase[10] we have found this method of fractionation to be useful in processing very crude extracts of the enzyme and believe that the study reported below should furnish a helpful direction for the application of electrophoresis–convection to enzyme purifications.

## Experimental

**Preparation of the Enzyme Extract.**—2250 g. of fresh pig kidney cortex was minced batchwise in a Waring blendor for one minute at room temperature and then stirred with 10.1 l. of *M*/60 sodium pyrophosphate at 12–13° for 40 minutes. The *p*H was maintained at 8.0 by the occasional addition of 6 *N* NaOH. This suspension was centrifuged cold and the insolubles discarded. To the supernatant, 8700 ml., was added 1827 g. of $(NH_4)_2SO_4$. After 24 hours at 5°, the suspension of the crude enzyme was centrifuged at 5° and the supernatant discarded. The precipitate was frozen immediately.

**Fractionation.**—For each fractionation an appropriate amount of the frozen precipitate was mixed with distilled water and dialyzed against cold tap water for 5 hours to remove the $(NH_4)_2SO_4$ and then against 2 or 3 changes of the desired buffer for 15 hours at 5°. This solution was centrifuged at 5° for about 3 hours and the supernatant used directly in the electrophoresis–convection cell.[11] Fractionations were carried out in phosphate buffer, ionic strength 0.1. Runs were made at *p*H 8.1 and 7.0 using a field strength of 2.0 volts/cm. and at *p*H 5.3 using 1.7 volts/cm. for times up to 48 hours. The progress of the fractionation was followed by withdrawing small samples from the top reservoir during the runs and analyzing them for protein content and enzymatic activity. These samples are designated according to the time of withdrawal from the apparatus, *e.g.*, fraction top 24 hr. is the sample withdrawn from the top reservoir 24 hr. after the start of the fractionation. The various fractions were stored frozen. For electrophoretic analysis, fractions were concentrated by precipitating the protein with $(NH_4)_2SO_4$ and redissolving the precipitate in buffer.

**Enzymatic Activity and Protein Assays.**—Enzymatic activities, expressed as $\mu$l. $O_2$/30 min./mg. protein, were determined manometrically with excess D,L-alanine as the substrate and in the presence of added excess flavin adenine dinucleotide. Protein concentrations were determined gravimetrically by precipitation of the protein from solution with an equal volume of 20% trichloroacetic acid.

**Electrophoretic Analysis.**—Electrophoretic analyses were carried out in a Perkin–Elmer Tiselius apparatus using the current regulating power supply described by Swingle.[12] The current was measured with a potentiometer circuit. Electrophoretic experiments were carried out in phosphate buffer *p*H 7.0 and ionic strength 0.1.

## Results and Discussion

The results obtained in some representative fractionations of crude enzyme extracts are presented in Table I, where $V$ is the volume of the fraction in ml., $c$ is the concentration of the fraction in mg. protein/ml., $A$ is the specific activity, $\mu$l. $O_2$/30 min./mg. protein, at concentration $c$, and $f$ is the ratio of specific activity of the fraction to the specific activity of the starting material adjusted to

(1) This work was supported in part by an Institutional Research Grant from the Damon Runyon fund and the American Cancer Society.

(2) (a) J. R. Cann and J. G. Kirkwood, *Cold. Spring Harbor Symposia Quan. Biol.*, **14**, 9 (1950); (b) J. R. Cann, D. H. Campbell, R. A. Brown and J. G. Kirkwood, THIS JOURNAL, **73**, 4611 (1951).

(3) R. A. Brown, J. B. Shumaker, Jr., S. N. Timasheff and J. G. Kirkwood, *ibid.*, **74**, 460 (1952).

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(5) R. J. Gibbs, M. Bier, J. A. Duke and F. F. Nord, Abstracts of Papers 122nd Meeting Am. Chem. Soc., Sept. 14–19, 1952, p. 49c.

(6) J. G. Kirkwood, *J. Chem. Phys.*, **9**, 878 (1941).

(7) L. E. Nielsen and J. G. Kirkwood, THIS JOURNAL, **68**, 181 (1946).

(8) J. R. Cann, J. G. Kirkwood, R. A. Brown and O. J. Plescia, *ibid.*, **71**, 1603 (1949).

(9) (a) J. R. Cann, R. A. Brown and J. G. Kirkwood, *J. Biol. Chem.*, **181**, 161 (1949); (b) J. R. Cann, R. A. Brown, J. G. Kirkwood and J. H. Hink, Jr., *ibid.*, **185**, 663 (1950).

(10) Unpublished work of W. R. Frisell.

(11) Fractionation of cloudy extracts resulted in the coating of membranes of the convection channel with colloidal material. These runs were accompanied by considerable osmotic transport of solvent into the electrophoresis–convection cell.

(12) S. M. Swingle, *Rev. Sci. Instrument.*, **18**, 128 (1947).

TABLE I

FRACTIONATIONS OF CRUDE ENZYME EXTRACTS

| Run | pH of fractionation | Fraction | V, ml. | c, mg. protein/ml. | A, μl. $O_2$/30 min./mg. protein | |
|---|---|---|---|---|---|---|
| 8 | 5.3 | Starting soln. | 100 | 3.6 | 46 | |
| | | Top, 24 hr. | 5 | 2.7 | 65 | 1.3 |
| | | Top, 48 hr. | 50 | 2.4 | 72 | 1.4 |
| | | Bottom, 48 hr. | 50 | 4.9 | 31 | 1.0 |
| 15 | 5.3 | Starting soln. | 100 | 8.3 | 20 | |
| | | Top, 24 hr. | 5 | 4.7 | 38 | 1.6 |
| | | Top, 48 hr. | 60 | 4.3 | 38 | 1.6 |
| | | Bottom, 48 hr. | 60 | 6.5 | 26 | 1.0 |
| 14 | 7.0 | Starting soln. | 100 | 9.4 | 20 | |
| | | Top, 24 hr. | 8 | 1.0 | 93 | 1.2 |
| | | Top, 48 hr. | 59 | 0.14 | 482 | 4.5 |
| | | Bottom, 48 hr. | 64 | 11.6 | 14 | 1.0 |
| 7 | 7.0 | Starting soln. | 100 | 7.2 | 28 | |
| | | Top, 24 hr. | 5 | 1.0 | 115 | 1.2 |
| | | Top, 48 hr. | 76 | 0.22 | 475 | 3.4 |
| | | Bottom, 48 hr. | 64 | 9.0 | 18 | 1.0 |
| 20[a] | 7.0 | Starting soln. | 200 | 8.8 | 23 | |
| | | Top, 24 hr. | 20 | 1.1 | 99 | 1.1 |
| | | Top, 48 hr. | 148 | 0.63 | 143 | 1.2 |
| | | Bottom, 48 hr. | 139 | 9.6 | 19 | 1.0 |
| 13 | 8.1 | Starting soln. | 100 | 16.4 | 14 | |
| | | Top, 8 hr. | 5 | 3.5 | 44 | 0.9 |
| | | Top, 22 hr. | 20 | 2.3 | 63 | 1.1 |
| | | Top, 33 hr. | 20 | 1.6 | 64 | 1.1 |
| | | Top, 48 hr. | 76 | 1.2 | 89 | 1.4 |
| | | Bottom, 48 hr. | 63 | 12.7 | 14 | 1.0 |
| 16 | 8.1 | Starting soln. | 100 | 10.0 | 21 | |
| | | Top, 6 hr. | 5.5 | 1.9 | 66 | 0.9 |
| | | Top, 24 hr. | 10 | 0.64 | 83 | 1.0 |
| | | Top, 48 hr. | 72 | 0.58 | 66 | 0.8 |
| | | Bottom, 48 hr. | 72 | 12.2 | 15 | 1.0 |

[a] This fractionation was carried out in duplicate and the results of activity and protein determination averaged.

the same total protein concentration as the fraction. These data show that at *p*H 7.0 and 8.1 between 86 and 94% of the total protein was transported from the top into the bottom reservoir of the electrophoresis-convection apparatus in 24 hours. 93–99% transport was obtained in 48 hours at *p*H 7.0. At *p*H 5.3, however, less than 50% of the protein was transported in 24 hours, a steady state having been established in the apparatus by that time. Previous studies[10] have indicated that the isoelectric point of D-amino acid oxidase is about *p*H 5.2 and that the enzyme is stable at this *p*H. One would expect electrophoresis–convection to provide the most efficient fractionation of the enzyme at its isoelectric point. However, the crude extracts apparently contained tissue proteins with isoelectric points about the same as that of the enzyme. As a result, the transport observed at *p*H 5.3 was low.

Assay of the various fractions for enzymatic activity revealed that at *p*H 7.0 considerable fractionation was obtained after transport of 97% of the protein out of the top reservoir. Thus, in the case of run 14, the top 48 hr. fraction possessed a specific activity 4.5 times as great as that of the unfractionated enzyme extract. At *p*H 5.3 the specific activities of the fractions were about 1.5 times greater than that of the starting material, while at *p*H 8.1 very little, if any, fractionation was achieved.

As first demonstrated by Krebs,[13] crude extracts of D-amino acid oxidase contain an inhibitor whose influence can be decreased by dilution of the enzyme. It was found that the mg. activities of the various fractions described in Table I could be greatly enhanced by dilution. For example, the activity of the starting solution used in run 14 increased from 22 μl. $O_2$/30 min./mg. at a concentration of 9.4 mg. protein/ml. to 108 μl. $O_2$/30 min./mg. when diluted to a concentration of 0.063 mg./ml., while the specific activity of the top 48 hr. fraction increased from 410 to 602 μl. $O_2$/30 min./mg. when diluted from 0.14 to 0.047 mg./ml. Because of the presence of this inhibitor it was necessary to compare the mg. activity of each fraction with the mg. activity of the starting solution when the latter had been adjusted to the same total protein concentration as that of each fraction. Only by taking into account this effect of dilution can any valid deductions be made concerning the efficiency of fractionation of enzymes in the presence of inhibitors. For example, direct comparison of the activities of starting material and top fractions would lead one to conclude that in runs 7 and 14, a 20–24 fold enrichment of the enzyme in the top reservoir was achieved in 48 hours of fractionation; but when the mg. activities of the top fractions are compared with those of the starting solution diluted to the protein concentration of each fraction, it is found that the enrichment of activity is 3–5 fold in these runs.[14]

As shown in Table II, the total recovery of enzymatic activity ranged from 66 to 100% of the total activity of the starting material and nearly always paralleled the recovery of protein. Electrophoresis–convection is a mild method of fractionation of proteins, and the losses of material in these experiments were almost entirely incurred during manipulation of the fractions.

TABLE II

MATERIAL AND ACTIVITY BALANCE

| Run | pH of fractionation | Total mg. protein | | Total activity corrected for dilution effects, ml. $O_2$/30 min. | |
|---|---|---|---|---|---|
| | | Starting material | Recovered | Starting material | Recovered |
| 8 | 5.3 | 362 | 380 | 74.5 | 72.3 |
| 15 | 5.3 | 834 | 672 | 83.0 | 98.8 |
| 14 | 7.0 | 937 | 784 | 80.6 | 63.4 |
| 7 | 7.0 | 722 | 598 | 101 | 66.1 |
| 20 | 7.0 | 1760 | 1452 | 202 | 188 |
| 13 | 8.1 | 1640 | 986 | 115 | 112 |
| 16 | 8.1 | 998 | 938 | 105 | 80.5 |

The electrophoretic patterns of the crude enzyme extract and of a composite of top fractions obtained by fractionation at *p*H 7 for 48 hours are shown in Fig. 1. The pattern of the crude extract is resolved into three major electrophoretic components designated as $C_1$, $C_2$ and $C_3$. The apparent distribution

(13) H. A. Krebs, *Biochem. J.*, **29**, 1620 (1935).

(14) The total oxygen consumed when a D-amino acid is acted upon by D-amino acid oxidase may be twice as great in the absence of catalase as in its presence.[15] All the fractions contained catalase; and therefore the large enrichment of mg. activity in runs 7 and 14 was not due to the removal or denaturation of catalase.

(15) E. Negelein and H. Brömel, *Biochem.*, **300**, 225 (1939).

of the components is 13% $C_1$ (mobility of $-0.5 \times 10^{-5}$ cm.$^2$ sec.$^{-1}$ volt$^{-1}$), 12% $C_2(-1.6 \times 10^{-5})$ and 75% $C_3(-3.8 \times 10^{-5})$. The spreading of the peak corresponding to $C_3$ indicates the presence of additional components. The top fraction analyzed 71% $C_1(-0.68 \times 10^{-5})$, 19% $C_2(-1.92 \times 10^{-5})$ and 10% of a new component $C_4(-2.8 \times 10^{-5})$. The small amount of component $C_3$ in the top fraction has been included with $C_4$. Fractionation by electrophoresis-convection resulted in an increase in the relative concentration of $C_1$ and $C_2$ by factors of 5.5 and 1.6, respectively. Unpublished results of one of us[10] show that the enzyme has a mobility of about $-2 \times 10^{-5}$ at *p*H 7, which would correspond to component $C_2$. Although the relative concentration of $C_2$ was increased by a factor of 1.6 on fractionation, the specific activity of the enzyme in the top fractions was as much as 3 to 5 times that of the crude extract. However, this large enrichment factor was obtained only after transport of 97% of the protein out of the top reservoir, transport of 86–93% of the protein yielding an enrichment factor of only 1.2. Since a large increase in the enrichment factor would not be expected to occur during the transport of the last few per cent. of material out of the top reservoir, it would appear that considerable separation of enzyme from its inhibitor was accomplished during the latter stages of fractionation. Of course, there is no reason to believe that component $C_2$ is composed entirely of the enzyme, and it is possible that fractionation resulted in an enrichment of $C_2$ in the enzyme.

The bottom fractions obtained at *p*H 7.0 still possessed considerable activity. With the hope of recovering some of this activity and thereby increasing the over-all yield of purified enzyme, an attempt was made to refractionate at *p*H 7.0 the bottom fraction from run 14. Although 90% of the protein of the starting material was recovered in this experiment, only 5% of the activity was recovered. Similar results were obtained when a composite of bottom fractions was refractionated at *p*H 8.1. As shown in Table II, no serious loss of activity was encountered on fractionation of the crude enzyme extracts. At present no explanation can be given for the loss of activity on refractionation, although large changes in ratio of enzyme to inhibitor in the various fractions might lead to an apparent loss of enzyme.

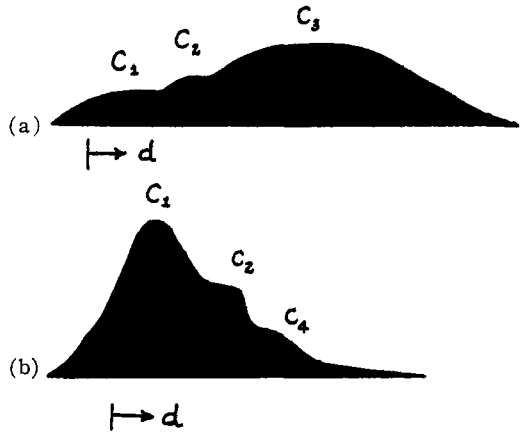


Fig. 1.—Electrophoretic patterns of (a) the crude enzyme extract and (b) a composite of top fractions obtained by fractionation at *p*H 7.0 for 48 hours.

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# The Surface Areas of Proteins. V. The Mechanism of Water Sorption[1]

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The sorption of water by solid proteins is characterized by the evolution of heat, a peculiar hysteresis, swelling of the solid and an increase in the BET surface area of the solid. Heat evolution is of the order of magnitude of the heat of vaporization of water and in conjunction with the poor thermal conductivity of the protein acts to slow the sorption considerably. The hysteresis is unusual in that it is almost constant over the entire range of partial pressures from about $p/p_0 = 0.05$ to 0.90 and independent of temperature. Although the hysteresis loop is quite reproducible (*i.e.*, in both branches) the sorption–desorption curves do not correspond to states of thermodynamic equilibrium and thermodynamic data calculated from them are apt to be quite anomalous. It is observed that the surface areas of proteins as measured by the BET method show an increase after water sorption which is reproducible and can be interpreted in terms of particle dimensions to give an apparent molar density for water which is anomalously high and indicates rather unusual packing efficiency in the solid. It is proposed that the hysteresis is associated with binding on the free basic groups of the protein. The existing data on sorption do indeed show an excellent correlation between number of free basic groups and the maximum amount of hysteresis and a hypothesis is suggested for such a correlation.

**I. Hysteresis.**—Recent work in these laboratories[2] has shown that whereas dry, solid proteins are capable of adsorbing non-polar gases[3] in a manner characteristic of general physical adsorption processes, polar gases[4,5] fall into a distinctly different category displaying characteristics ranging from chemical reaction to solvation.[5,6] The sorption of water, while similar in many ways to the other polar gases, shows some special features, in particular that of complete reversibility and much

(1) The contents of this paper were presented in part at the Symposium on Colloid Chemistry held by the Division of Colloid Chemistry of the American Chemical Society at Los Angeles, June, 1952.

(2) The authors wish to express their appreciation to the Research Corporation and to the Office of Naval Research for grants which made the present work possible.

(3) S. W. Benson and D. A. Ellis, THIS JOURNAL, **70**, 3563 (1948); **72**, 2095 (1950).

(4) S. W. Benson, D. A. Ellis and R. W. Zwanzig, *ibid.*, **72**, 2102 (1950).

(5) S. W. Benson and J. M. Seehof, *ibid.*, **73**, 5053 (1951).

(6) S. W. Benson and J. M. Seehof, unpublished work, presented in part at National Colloid Symposium, June, 1952.