of a divalent cation does not, however, appear to be of importance in studies of this kind with intact red cells.

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[1] M. E. GREIG AND W. C. HOLLAND, *Arch. Biochem.*, 23 (1949) 370.
W. C. HOLLAND AND M. E. GREIG, *Arch. Biochem.*, 26 (1950) 151.
W. C. HOLLAND AND M. E. GREIG, *Am. J. Physiol.*, 162 (1950) 610.
M. E. GREIG AND W. C. HOLLAND, *Am. J. Physiol.*, 164 (1951) 423.
[2] P. E. LINDVIG, M. E. GREIG AND S. W. PETERSON, *Arch. Biochem.*, 30 (1951) 241.
[3] W. N. ALDRIDGE, *Biochem. J.*, 46 (1950) 451.
[4] W. C. HOLLAND AND M. E. GREIG, *Arch. Biochem. Biophys.*, 32 (1951) 428.
[5] K.-B. AUGUSTINSSON, *Acta Physiologica Scand.*, 15, Suppl. 52 (1948).

Received February 23rd, 1955

The aconitase of *Aspergillus niger**

JACOBSOHN *et al.*[1] suggested that aconitase may consist of two enzymes, one catalyzing the reaction citrate $\rightleftharpoons$ *cis*-aconitate and the other *cis*-aconitate $\rightleftharpoons$ *iso*citrate. BUCHANAN AND ANFINSEN[2], however, obtained a 23-fold purification of aconitase with a relatively constant ratio of citric acid to *iso*citric acid being formed from *cis*-aconitic acid throughout the process. In contrast, RACKER[3] reported that an *iso*citrase-citrase ratio in crude heart extracts of 2.1, changed to 7.5 upon purification of the preparations. In his review of the literature on aconitase, OCHOA[4] concluded that the evidence to date suggested that only one enzyme was responsible for the equilibrium between citrate, *cis*-aconitate and *iso*citrate.

Preliminary studies with extracts of *Aspergillus niger* indicate that the aconitase system in this microorganism is made up of at least two enzymes. In the experiments undertaken citric acid was determined by a modification of the method of NATELSON *et al.*[5] and *d-iso*citric acid by the *iso*citric dehydrogenase method of OCHOA[6]. The protein content of the purified enzyme extracts was calculated from the optical density observed at 280 and 260 mμ in a model DU spectrophotometer. All enzyme fractionations were carried out in the cold. Crude enzyme extracts were prepared by sonic disintegration of the washed mycelium from 24-hour cultures of *Aspergillus niger* (72–4) grown on a rotary shaker in the medium of MARTIN[7]. 20 mg of cysteine were added, prior to disintegration, for each 100 ml of mycelial suspension. The crude sonicate was centrifuged at 14,000 r.p.m. and solid ammonium sulfate added to the supernatant to give 0.25 saturation. After centrifugation, the sediment was discarded and ammonium sulfate added to the supernatant to 0.75 saturation. The precipitate, after centrifugation, was suspended in *M*/100 phosphate buffer (pH 7.4), the pH adjusted to 7.4, and the solution dialyzed against the same buffer to which about 5 mg of cysteine per liter had been added. A protamine sulfate solution (20 mg/ml) was added to the dialysate until the ratio of absorption at 280 to that at 260 mμ reached 0.63. The mixture was then dialyzed as previously. After dialysis, the ratio at 280:260 mμ was 0.82. The dialysate was then treated with solid ammonium sulfate to give three successive fractions with 0.42, 0.55 and 0.75 saturation respectively. Each precipitate was suspended in buffer and dialyzed. The ratio of citric acid to *iso*citric acid as produced by the enzyme complex was determined on each of the fractions as follows: 0.05 *M* tris buffer (pH 7.4), enzyme solution, 1.5 μmoles *cis*-aconitate and water to 1.0 ml volume were incubated in a small pyrex test tube at 30° C in a water bath. The reaction was started by the addition of the *cis*-aconitate after the mixture had reached 30° C. 0.5 ml aliquots were removed at the desired times, usually 10 and 20 or 20 and 40 minutes and each was added to 0.15 volumes of 0.4 *M* acetate buffer (pH 3.8). The mixture was heated in boiling water to precipitate the protein, cooled and then centrifuged. The supernatant was adjusted to pH 7.4 with KOH. The citric acid and *iso*citric acid content of the supernatant was determined. The results for the 20 minute incubation period are given in Table I. A reaction mixture minus *cis*-aconitate served as an endogenous control and all results are corrected in this respect.

Since the ratio of citric acid to *iso*citric acid formed by the enzyme solution, decreased markedly as the degree of saturation with ammonium sulfate was increased, further fractionation was undertaken. The solution of the 0.55–0.75 fraction was brought to 0.60 and then 0.75 saturation with solid ammonium sulfate, the respective precipitates were centrifuged, suspended in buffer and dialyzed. The ability to form citric and *iso*citric acids from *cis*-aconitate was then determined on each fraction. As may be seen in Table I, the ratio of citric to *iso*citric acids formed by the new fractions was lower

* Supported by a grant-in-aid from the National Research Council of Canada.

than that for the enzyme solution before the re-cutting procedure, but a difference in ratio was not observed between the new fractions.

TABLE I

FRACTIONATION WITH SOLID AMMONIUM SULFATE

Fraction	μM product/mg enzyme protein/20 min		Citrate/Isocitrate
	Citrate	Isocitrate	
0–0.42	0.621	0.0128	48.5
0.42–0.55	0.997	0.0478	20.3
0.55–0.75	0.617	0.237	2.6
0.55–0.60	0.184	0.166	1.25
0.60–0.75	0.404	0.283	1.43

In a further attempt to separate the enzymes, the 0–0.42 and the 0.42–0.55 fractions were combined and this solution was divided into three fractions by the addition of saturated alkaline ammonium sulfate (pH 7.5) to give 0.35, 0.42 and 0.55 saturation respectively. The precipitates were centrifuged, dissolved in buffer, and dialyzed and the ability to form citric and *iso*citric acids was then determined on each portion. As shown in Table II, the 0–0.35 fraction of the enzyme mixture gave a ratio of citric acid to *iso*citric acid of 300, whereas, the 0.42–0.55 fraction produced a ratio of 4.65.

TABLE II

FRACTIONATION WITH ALKALINE AMMONIUM SULFATE

Fraction	μM product/mg enzyme protein/20 min		Citrate/Isocitrate
	Citrate	Isocitrate	
0–0.55 (before fractionation)	0.845	0.0183	46.2
0–0.35	0.595	0.0019	313.0
0.35–0.42	0.975	0.0048	204.0
0.42–0.55	0.209	0.045	4.65

DICKMAN AND CLOUTIER observed that Fe^{++} ions and a reducing agent such as cysteine had an activating as well as stabilizing effect upon pig heart aconitase. When similar concentrations of Fe^{++} and cysteine were added to the aconitase of *Aspergillus niger*, only slight stimulation of activity was found. It must be noted, however, that DICKMAN AND CLOUTIER suspended their enzyme in water, whereas the enzyme of *A. niger* was dissolved in dilute phosphate buffer.

The data presented in Tables I and II show that the reaction citrate $\rightleftharpoons$ *cis*-aconitate $\rightleftharpoons$ *iso*citrate may be separated into two components by ammonium sulfate fractionation and indicate that the aconitase of *Aspergillus niger* is made up of at least two enzymes.

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[1] K. P. JACOBSOHN, M. SOARES AND J. TAPADINHAS, *Bull. soc. chim. biol.*, 22 (1940) 48.
[2] J. M. BUCHANAN AND C. B. ANFINSEN, *J. Biol. Chem.*, 180 (1949) 47.
[3] E. RACKER, *Biochim. Biophys. Acta*, 4 (1950) 211.
[4] S. OCHOA, in J. B. SUMNER AND K. MYRBÄCK, *The Enzymes*, Academic Press. Inc., New York, Vol. I, part 2, (1951).
[5] S. NATELSON, J. B. PINCUS AND J. K. LUGOVOY, *J. Biol. Chem.*, 175 (1948) 745.
[6] S. OCHOA, *J. Biol. Chem.*, 174 (1948) 133.
[7] S. MARTIN, *Can. J. Microbiol.*, 1 (1954) 6.
[8] S. R. DICKMAN AND A. A. CLOUTIER, *J. Biol. Chem.*, 188 (1951) 379.

Received March 21st, 1955