PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

5-Aminolaevulinic Acid Dehydratase: Structure, Function, and Mechanism [and Discussion]

D. Shemin and M. R. Moore

Phil. Trans. R. Soc. Lond. B 1976 **273**, 109-115
doi: 10.1098/rstb.1976.0004

Phil. Trans. R. Soc. Lond. B. **273**, 109–115 (1976) [109]
Printed in Great Britain

5-Aminolaevulinic acid dehydratase: structure, function, and mechanism

By D. Shemin
Department of Biochemistry and Molecular Biology,
Northwestern University, Evanston, Illinois 60201, *U.S.A.*

[Plate 1]

δ-Aminolaevulinic acid dehydratase catalyses the synthesis of porphobilinogen. The enzyme has a molecular mass of 285000 and is composed of eight similar subunits of molecular mass 35000. The N-terminal amino acid is acylated, and the number of peptides found on tryptic digestion equals the number of lysine and arginine residues per mass of 35000. The eight subunits are apparently arranged at the corners of a cube and therefore have dihedral (D_4) symmetry. The bovine liver enzyme which has been crystallized contains 4–6 atoms of zinc per mole of enzyme. The apo-enzyme obtained on prolonged hydrolysis can be reactivated by the addition of zinc or cadmium ions. The dialysed enzyme must be first treated with dithiothreitol. There are two very active SH groups in a total of 6–7-SH groups per subunit.

The substrate forms a Schiff base with the ε-amino group of a lysine residue. Reduction of the Schiff base with $NaBH_4$ should reveal the number of active sites per mole of enzyme. It appears that only four of the eight subunits form a Schiff base with the substrate indicating that the enzyme exhibits the phenomenon of either half-site reactivity or negative cooperativity.

The enzyme appears to have a strong subunit–subunit interaction for an immobilized preparation remained stable for at least a month. An immobilized enzyme preparation was treated in a manner so that it dissociated into tetramers. Both the eluate and the protein still attached to the Sepharose on a column were enzymically active. The bound enzyme could not reassociate under assay conditions but still contained about 50 % of the original enzyme activity. It would seem that the enzyme is active when composed with less than eight subunits.

In the biosynthesis of porphyrins, chlorophyll, and vitamin B_{12} the initial formation of an aromatic ring is the conversion of two molecules of δ-aminolaevulinic acid to the pyrrole, porphobilinogen (figure 1). This reaction is catalysed by the enzyme δ-aminolaevulinic acid dehydratase. Although this is not the first committed step in the synthesis of these tetracyclic compounds, it is quite probable that this enzyme which catalyses the aromatization reaction may play a role in the overall control concerned with the synthesis of these tetrapyrrolic and pyrrolic compounds. An analysis of the characteristics of the dehydratase reveals that the enzyme molecules isolated from bacteria, yeasts, plants, avian red cells, and mammalian tissues appear to have the same molecular mass yet different metal requirements for activation. It is also of interest to note that whereas the enzyme from *Rhodopseudomonas spheroides* requires K^+ or related ions for activation, the enzyme from eukaryotic cells do not have a metal requirement but are inhibited by EDTA (Shemin 1972). However, it appears that in all cases where studies have been carried out, the mechanism of pyrrole synthesis involves the intermediate formation of a Schiff base between the enzyme and the substrate (Nandi & Shemin 1968*b*). The mechanism postulated by Nandi & Shemin in 1968, shown in figure 2, indicates that only one of the two molecules of δ-aminolaevulinic acid in the reaction forms a covalent bond with the

enzyme. The reduction of this Schiff base with $NaBH_4$ permits one to determine the number of these active sites per mole of enzyme.

In view of some of the interesting differences among the enzyme preparations from different sources, the rather complex quaternary structure of the dehydratase (Nandi, Baker-Cohen & Shemin 1968; Nandi & Shemin 1968*a*; van Heyningen & Shemin 1971), its inhibition by hemin (Nandi *et al.* 1968), and the intrinsic interest of obtaining further understanding of the catalytic

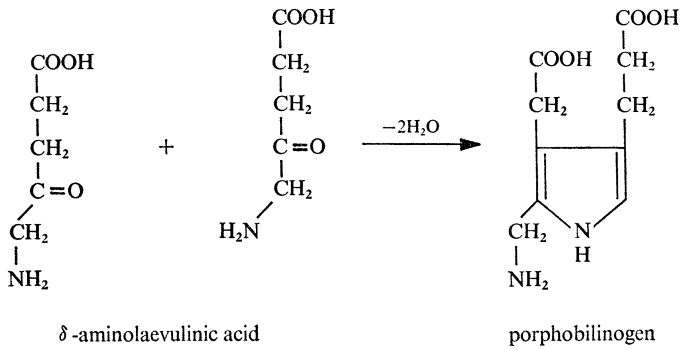


FIGURE 1. Porphobilinogen synthesis from δ-aminolaevulinic acid.

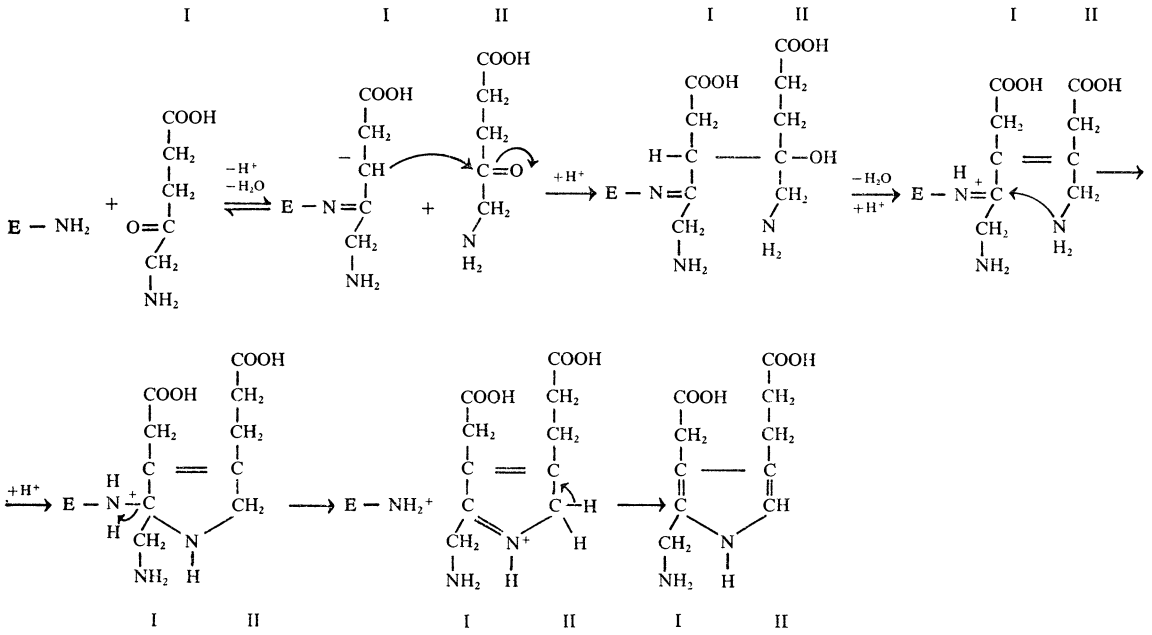
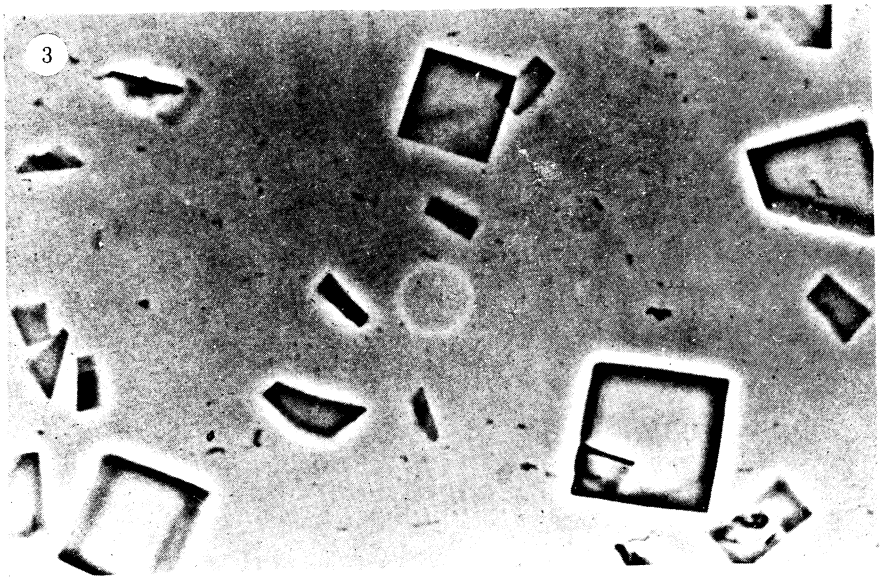
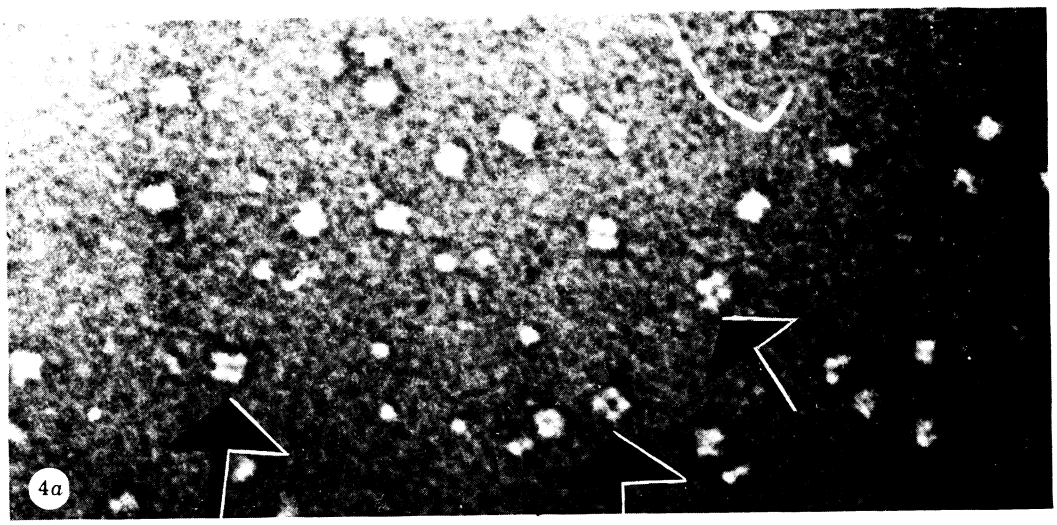


FIGURE 2. Mechanism of synthesis of porphobilinogen from two molecules of δ-aminolaevulinic acid as catalysed by the enzyme (**E**). Notice the initial formation of a Schiff base (from paper of Nandi & Shemin 1968*b*).

mechanism concerned with aromatization and of the structure of the enzyme further studies on the dehydratase were carried out in my laboratory by my colleagues. These studies deal in part with the isolation and purification of the enzyme, the determination of the primary, tertiary, and quaternary structure, the nature and number of active sites, the mechanism of the reaction, the possible mechanism by which the catalytic activity of this enzyme may be controlled in the cell, and other pertinent studies which may aid in our understanding of this or





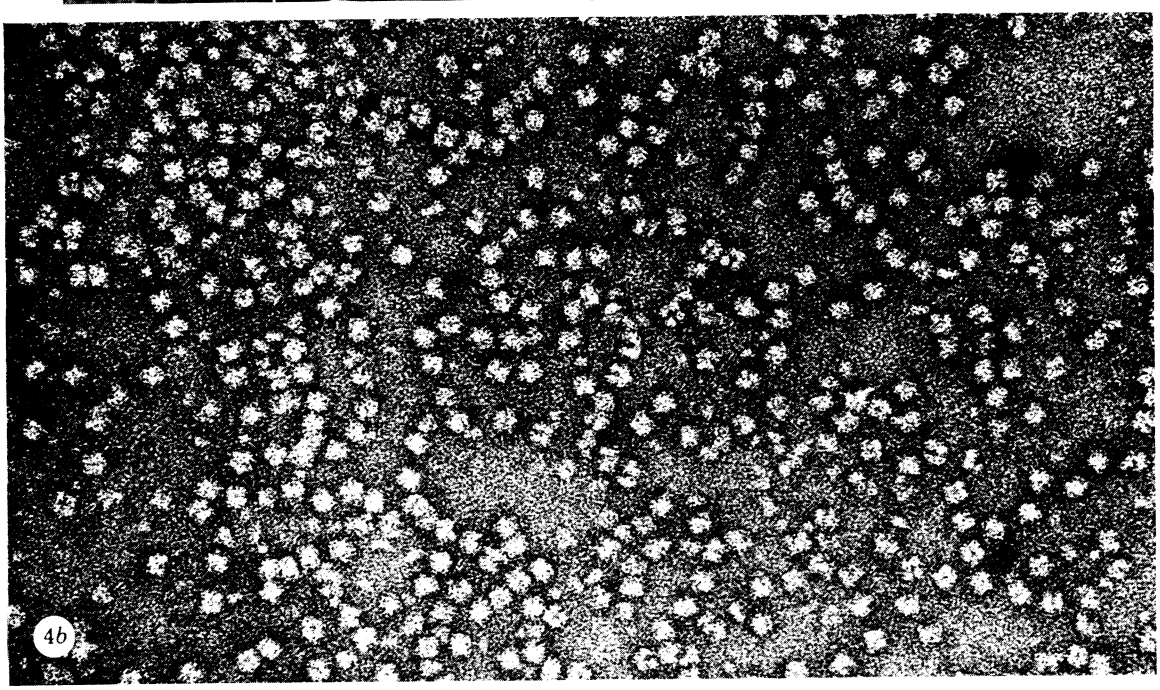


FIGURE 3. Crystals of δ-aminolaevulinic acid hydratase from bovine liver.

FIGURE 4. Electron photomicrograph of δ-aminolaevulinic acid dehydratase from bovine liver (from Wu *et al.* 1974).

(*Facing p.* 111)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

other enzymic reactions. In order to realize some of our aims we chose to study the enzyme from bovine liver, for one can obtain relatively large amounts.

The enzyme was isolated and purified by conventional methods to be published and appeared to be homogeneous by several criteria. The enzyme was crystallized by my collaborator, Dr Wen Wu (figure 3, plate 1). In collaboration with Dr Alan Edmundson of Argonne National Laboratory we are trying to grow larger crystals in order to determine the tertiary structure along with our studies on the amino acid sequence of the enzyme.

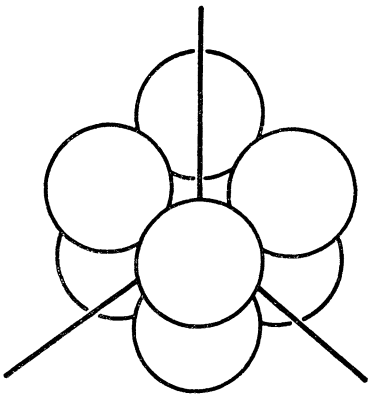


FIGURE 5. A model of the quaternary structure of δ-aminolaevulinic acid dehydratase from bovine liver (from Wu *et al.* 1974).

Quaternary structure

The molecular mass calculated from the sedimentation coefficient ($S^0_{20,w} = 11.75$) and the diffusion coefficient was found to be 289000. The molecular mass of the native enzyme determined by sedimentation-equilibrium was 282000. The subunit molecular mass of 34900 was obtained by sedimentation-equilibrium after dialysis of the enzyme against 6 M guanidine containing dithiothreitol. A value of 35000 was found for the subunit molecular mass on polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (Wu, Shemin, Richards & Williams 1974). The same molecular mass of the subunit was obtained in similar experiments after the protein was carboxymethylated. It would appear therefore that the native enzyme is composed of eight subunits. This conclusion was consistent with the appearance of the enzyme in the electron microscope carried out in collaboration with Dr Richards and Professor Williams. As can be seen in figure 4*a*, *b*, plate 1, the particles appear to consist of discrete lobes arrayed at the four corners of a square. The above conclusion that the dehydratase possesses eight subunits can be readily reconciled with the appearance of the enzyme in the electron microscope if it is postulated that the eight subunits are arranged at the corners of a cube as shown in figure 5. Therefore it would follow that the subunits are arranged with dihedral (D_4) symmetry. The diameter of the subunit is about 4.4 nm as determined from the electron micrograph. If the subunits are assumed to be spherical and possess a density of 1.36 g/cm^3, the molecular mass of the octamer can be calculated to be 292000 in reasonable agreement with results obtained by the methods mentioned above.

The number of subunits previously reported in the literature varied from six to a possible eight (van Heyningen & Shemin 1971; Doyle 1971; Gurba, Sennett & Kobes 1972; Wilson, Burger & Dowdle 1972; Cheh & Neilands 1973), This discrepancy is not due to the source of

the enzyme but probably to the lack of sufficient material and in the case of the bacterial enzyme to its particular properties of association and dissociation.

The eight subunits of the bovine liver dehydratase appear to be similar, for Dr Wu has found that the number of peptides liberated on hydrolysis with trypsin is in excellent agreement with the number of lysine and arginine residues we have found per mass of 35000. Consistent with this conclusion is the finding that the amino group of the N-terminal amino acid of all eight subunits is acylated.

Other characteristics of the enzyme

It has been observed for some time that the purified enzyme becomes inactive if the SH group is oxidized. On prolonged dialysis of the enzyme activity is restored by the addition of a sulphhydryl compound. It appears from the amino acid analysis that there are about 7 half cystines per subunit. On titration with Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) Bevan & Brown (1975) found that only two active sulphhydryl groups per subunit react rapidly. It is necessary to treat the protein with 4 M guanidine in order to titrate the remaining sulphhydryl groups. However, in the presence of laevulinic acid, a competitive inhibitor (Nandi & Shemin 1968*b*) only one sulphhydryl group per subunit reacts with Ellman's reagent (1959). It may mean that the second molecule of δ-aminolaevulinic acid may form a thiohemiketal with one of the active SH groups. This is consistent with the finding that inactivation of the enzyme from *R. capsulata* by *p*-chloromercuribenzoate, *N*-ethylmaleimide, and iodoacetamide is inhibited in the presence of the substrate (Nandi & Shemin 1973).

The state of oxidation of the SH groups may also play a role in the activation of the apo-enzyme by Zn^{2+} ions. In collaboration with Drs Bodlaender and Vallee from the Biophysics Research Laboratory of Harvard University it appears that the enzyme contains 4–6 atoms of Zn^{2+} per mole of enzyme. This is in agreement with the finding of Cheh & Neilands (1973). This number was arrived at by determining the number of atoms of Zn^{2+}/mole of enzyme necessary to restore full activity to the apo-enzyme. However, Bodlaender also found that the addition of Zn^{2+} was ineffective unless the enzyme was first reduced with dithiothreitol.

Number of active sites

Since the enzyme has eight subunits and forms a Schiff base with δ-aminolaevulinic acid the number of active sites per mole of enzyme can be determined directly. This can be done by reducing the covalent bond formed between the substrate and the enzyme with $NaBH_4$ to form a secondary amine. Drs B. Schearer and W. Wu reduced the enzyme in the presence of radioactive δ-aminolaevulinic acid and subsequently determined the radioactivity of the protein. It appears that out of the eight subunits only four react with the substrate to form a Schiff base. This is another example of half-site reactivity (Seydoux, Malhotra & Barnhard 1974).

The amino group which forms a Schiff base with the substrate has been identified. Dr Gurne of the laboratory synthesized the secondary amine formed between the carbonyl group of δ-aminolaevulinic acid and the ε-amino group of lysine by reduction. Dr Wu found that the radioactive material isolated by paper chromatography after hydrolysis of the enzyme, which had been reduced in the presence of radioactive δ-aminolaevulinic acid, had the same characteristics as the authentic sample. Besides the lysine residue at the active sites it appears from our previous work (Nandi *et al.* 1968) that there is another group carrying a positive charge. This was ascertained from our studies with the ester of the substrate.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Immobilized dehydratase and activity of subunits

It has been observed that the dehydratase is inhibited by the product of the reaction and that a concentration of approximately 2 mM porphobilinogen completely inhibits the enzymic activity (Jordan & Shemin 1973). Therefore it would seem that enzymic synthesis of quantities of porphobilinogen is not an attractive procedure unless one elects to work in very dilute solutions. In order to circumvent this property of the enzyme we considered the possibility of immobilizing the enzyme and thus minimizing product inhibition by continuous removal of the product as it formed in a column containing the immobilized enzyme. Dr Gurne successfully linked the enzyme of *Rh. spheroides* to Sepharose and found that an enzyme linked to the solid support was enzymically active (Gurne & Shemin 1973). A column containing 1 mg of enzyme was continuously operated for about a month with little loss of activity. Five grams of pure porphobilinogen were readily isolated over this period of time. The liver enzyme (20 mg) was subsequently attached and within 3 days 10 g of the pyrrole were isolated.

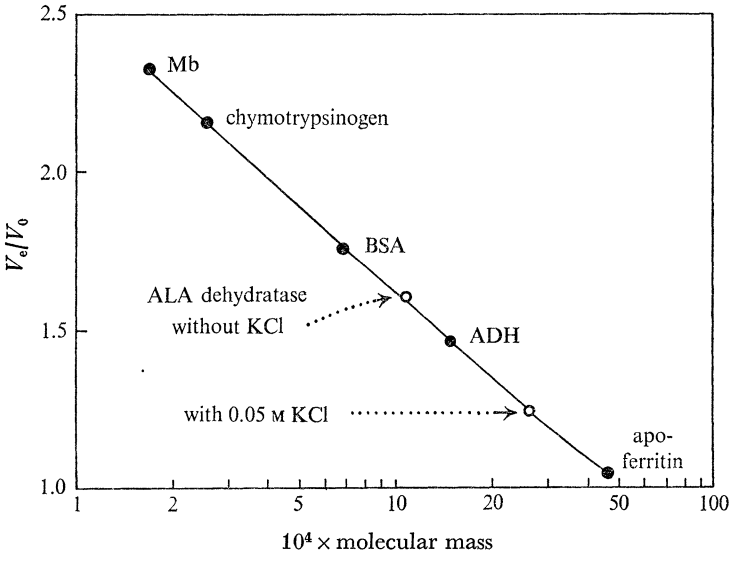


FIGURE 6. Determination of the molecular mass by gel filtration on Sephadex of δ-aminolaevulinic acid dehydratase from a strain of *Rhodopseudomonas spheroides*.

Presumably the enzyme was linked to the Sepharose via amino groups other than the reactive amino group which forms a Schiff base with the substrate. It would be reasonable to assume that even if the enzyme is active only when it retains its quaternary structure, 8 subunits per mole, that the enzyme is linked to the Sepharose only through 1, or at most 2, subunits. This value is arrived at on consideration of the probability of having both the required position of activated centres on the Sepharose and the amino groups on the enzyme spatially arranged in such a way for linkage to occur without distorting and thus inactivating the enzyme. If this is indeed so, it would appear that the binding forces among the subunits are exceedingly strong for, as mentioned above, the enzyme was kept at 36 °C for a month with very little loss of activity. However, another interpretation may explain the stability of the immobilized enzyme; namely that all eight subunits are not required for activity. It is indeed difficult to test this

hypothesis with an enzyme in solution, for it is difficult to ascertain the state of association or dissociation of the enzyme under assay conditions. It appeared feasible to test this hypothesis if one could immobilize the enzyme by covalently linking it to a solid support and subsequently dissociate subunits from the octamer and test for enzymic activity remaining on the solid support. This cannot readily be done however unless the enzyme can be dissociated under controlled conditions. Fortunately we had previously found that a strain of *R. spheroides* has a dehydratase which specifically requires Mg^{2+} ions for activation rather than K^+ or Mg^{2+} ions. Furthermore Dr Hayashi, while in my laboratory, found that whereas the enzyme requires Mg^{2+} ions it dissociates in the absence of K^+ ions. It can be seen from figure 6 that the molecular weight as determined by gel filtration on a Sephadex column is about one-half of that found in the presence of K^+ ions. It appears that the ocatamer dissociates into tetramers in the absence of K^+ ions. Therefore it became possible to test the hypothesis that less than eight subunits may have enzyme activity. Dr Gurne covalently linked the dehydratase of this strain of *Rh. spheroides* on to Sepharose in the presence of both Mg^{2+} and K^+ ions. The column was washed with tris buffer containing the metallic cations until no protein or enzymic activity could be detected in the eluate and then washed with another 2 l of the same buffer. After this washing procedure the column was treated with tris buffer, pH 8.0, containing no metallic cations. If the enzyme dissociates under these conditions, protein and enzymic activity should appear in the eluate. Indeed immediately after the void volume enzymic activity was found in the eluate. If tetramers were released by this treatment they could readily reassociate to form octamers in solution under assay conditions. However, since the protein still bound on the column cannot reassociate under assay conditions, any enzymic activity found on the column can probably be interpreted to mean that less than eight subunits, probably four subunits or perhaps less, can have enzymic activity. On determining the total activity on the column and in the eluate it was found that 55 % of the original activity was on the column, and about 40–45 % of the original activity was in the eluate.

Further experiments have to be carried out under other conditions to determine the minimum number of subunits that have enzymic activity and to determine if only one of two subunits form a Schiff base with the substrate.

The work described here is a summary of the contributions of my students and collaborators: David Bevan III, Charles E. Brown, Daniela Gurne, Norio Hayashi, Dhirendra Nandi, Bruce Schearer, and Wen Wu.

This investigation was supported by the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, United States Public Health Service (grants AM 13091 and AM 16484) and by a grant from the American Cancer Society (BC-18D) and a grant from the National Science Foundation (GB-43916X).

References (Shemin)

Cheh, A. & Neilands, J. B. 1973 Zinc, an essential metal ion for beef liver δ-aminolevulinic acid dehydratase. *Biochem. biophys. Res. Commun.* **55**, 1060–1063.

Doyle, D. 1971 Subunit structure of δ-aminolevulinate dehydratase from mouse liver. *J. biol. Chem.* **246**, 4965–4972.

Ellman, G. L. 1959 Tissue sulfydryl groups. *Archs. Biochem. Biophys*, **82**, 70–77.

Gurba, P. E., Sennett, R. F. & Kobes, R. D. 1972 Studies on the mechanism of action of δ-aminolevulinate dehydratase from bovine and rat liver. *Archs. Biochem. Biophys*, **150**, 130–136.

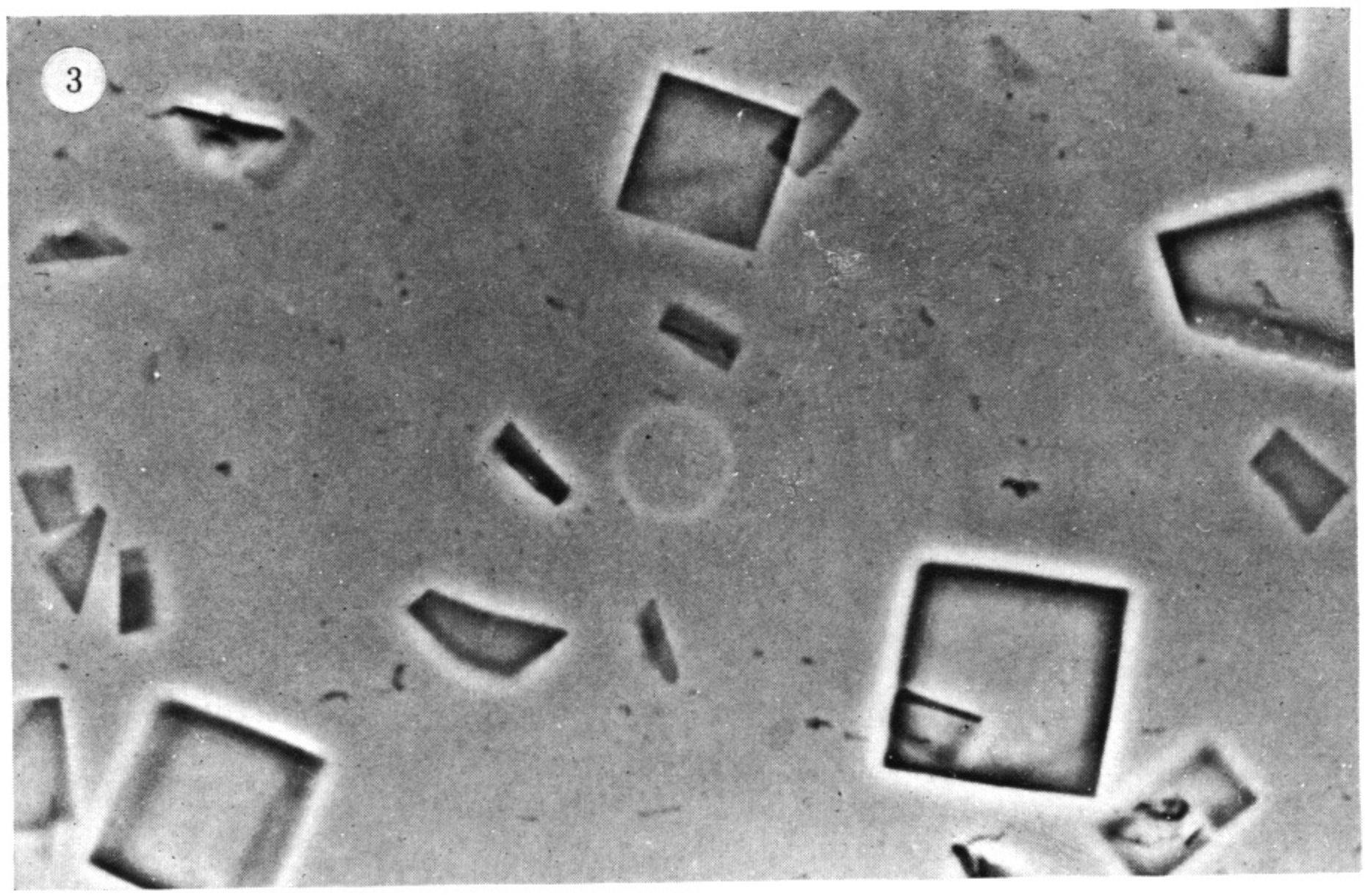
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

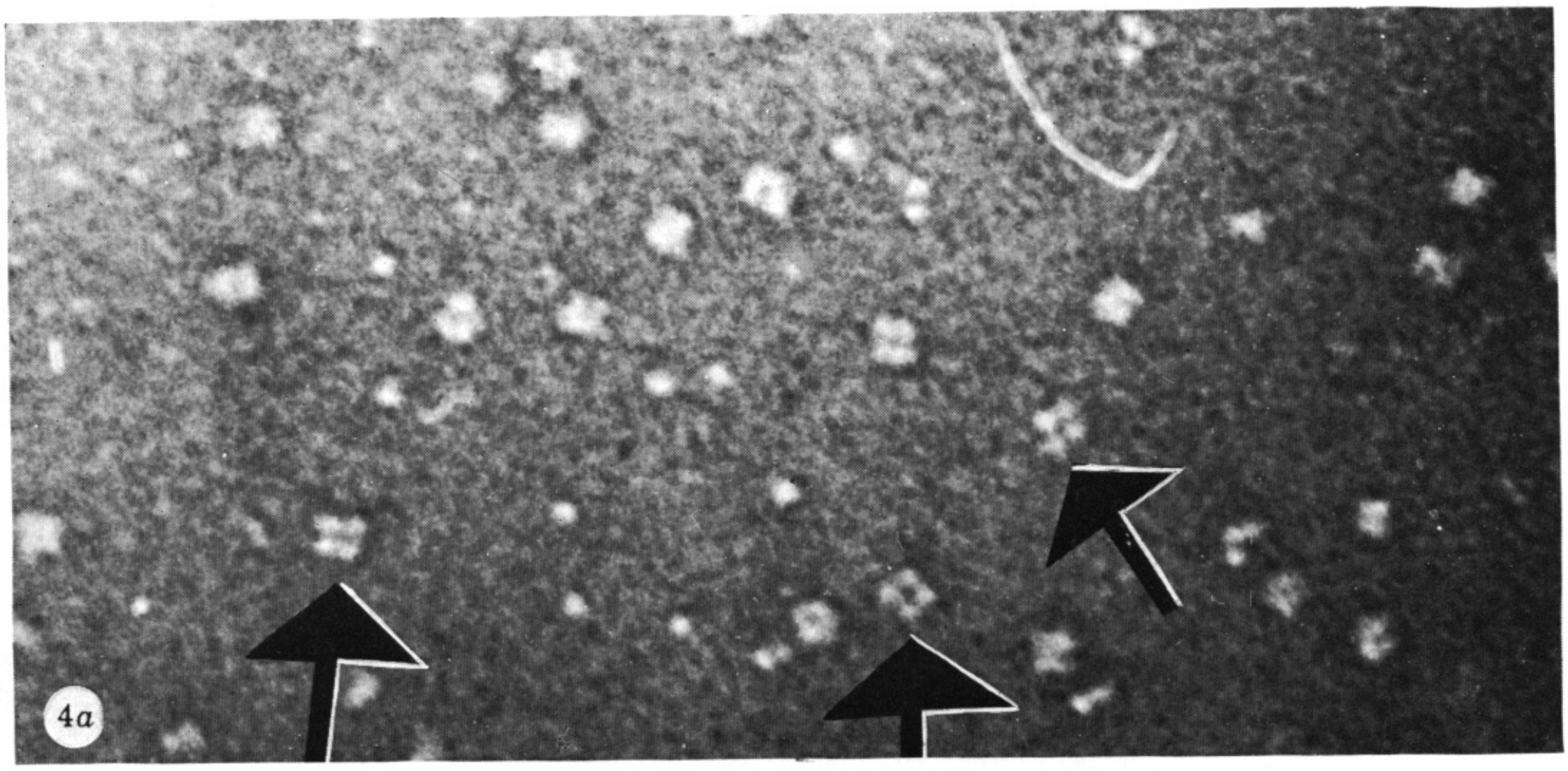
Gurne, D. & Shemin, D. 1973 Synthesis of the pyrrole porphobilinogen by sepharose-linked δ-aminolevulinic acid dehydratase. *Science, N.Y.* **180**, 1188–1190.
Jordan, P. & Shemin, D. 1973 Purification and properties of uroporphyinogen I synthetase from *Rh. spheroides*. *J. biol. Chem.* **248**, 1019–1024.
Nandi, D., Baker-Cohen, K. F. & Shemin, D. 1968 δ-Aminolevulinic acid dehydratase of *Rh. spheroides*. I. Isolation and mechanism. *J. biol. Chem.* **243**, 1224–1230.
Nandi, D. & Shemin, D. 1968*a* δ-Aminolevulinic acid dehydratase of *Rh. spheroides*. II. Association to polymers and dissociation into subunits. *J. biol. Chem.* **243**, 1231–1235.
Nandi, D. & Shemin, D. 1968*b* δ-Aminolevulinic acid dehydratase of *Rh. spheroides*. III. Mechanism of porphobilinogin synthesis. *J. biol. Chem.* **243**, 1236–1242.
Nandi, D. & Shemin, D. 1973 δ-Aminolevulinic acid dehydratase of *Rh.* capsulata. *Archs. Biochem. Biophys.* **158**, 305–311.
Seydoux, F., Malhotra, P. O. & Bernhard, S. A. 1974 Half-site reactivity. *CRC Crit. Rev. in Biochem.* **2**, 227–257.
Shemin, D. 1972 δ-Aminolevulinic acid dehydratase. In *The enzymes*, 3rd ed. vol. VII (ed. P. Boyer), pp. 323–337. New York: Academic Press.
van Heyningen, S. & Shemin, D. 1971 Quaternary structure of δ-aminolevulinate from *Rh. spheroides*. *Biochemistry* **10**, 4676–4682.
Wilson, E. L., Burger, P. E. & Dowdle, E. B. 1972 Beef liver δ-aminolevulinic acid dehydratase. *Eur. J. Biochem.* **29**, 563–571.
Wu, W., Shemin, D., Richards, K. E. & Williams, R. C. 1974 The quaternary structure of δ-aminolevulinic acid dehydratase from bovine liver. *Proc. Natn Acad. Sci. U.S.A.* **69**, 2585–2588.

Discussion

Dr M. R. Moore (*Department of Materia Medica, University of Glasgow, Stobhill General Hospital, Glasgow G*21 3*UW*). In studies in our laboratories we have found that Zn^{2+} activates the human erythrocyte ALA dehydratase. We have also found that Al^{3+} activates this enzyme and this activation is additive to the Zn^{2+} activation.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES





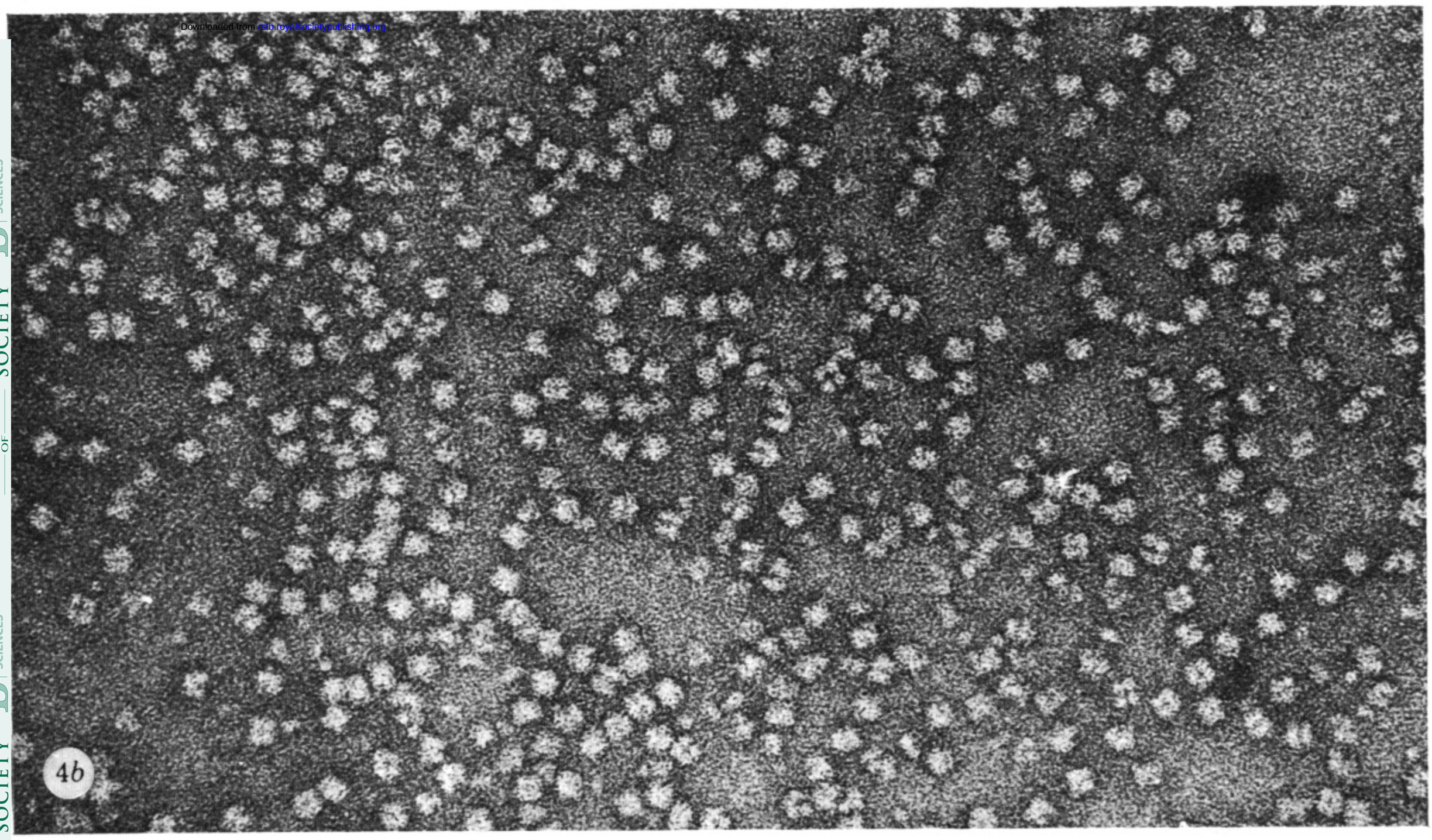


FIGURE 3. Crystals of δ-aminolaevulinic acid hydratase from bovine liver.

FIGURE 4. Electron photomicrograph of δ-aminolaevulinic acid dehydratase from bovine liver (from Wu *et al.* 1974).