with 0.1 ml of borate-EDTA buffer containing an excess of hemoglobin; their electrophoretic patterns are shown in Fig. 2. The re-formation of the disulfide bonds in the presence of 0.004*M* ME was almost perfect, as judged by the hemoglobin-binding and electrophoretic properties of the re-formed polymers; yet in the absence of added ME the mixed disulfide of the "monomer" was completely stable.

The use of the reaction shown in Eq. 3 to couple side chains to protein thiol groups was tested with hemoglobin and diaminodiethyldisulfide (cystamine). Hemoglobins A and A_2 have six cysteine residues per molecule: two of the six are readily accessible to reagents combining with thiols; the other four are inaccessible under mild conditions (*12*). Treatment of hemoglobin with cystamine [0.1*M* solution of the dihydrochloride in borate-EDTA (*p*H 8.7) buffer] at room temperature for 30 minutes was consequently expected to attach aminoethylsulfide side chains to the two accessible protein thiol groups. Prior treatment with iodoacetamide (0.01*M* in the same buffer) should prevent the reaction by alkylating the accessible thiol groups. Figure 3 shows the results of an experiment to test these expectations.

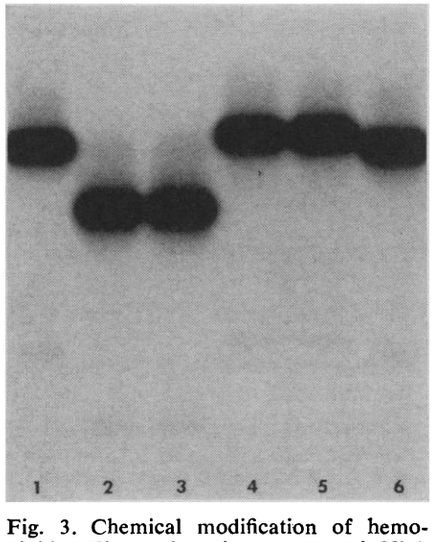

Fig. 3. Chemical modification of hemoglobin. Electrophoresis patterns of HbA and A_2 (the slower trace component) treated at room temperature as follows: (Sample 1) Borate-EDTA buffer alone; (sample 2) buffer plus diaminodiethyldisulfide (cystamine) for 30 minutes; (sample 3) cystamine for 30 minutes followed by iodoacetamide for 30 minutes; (sample 4) iodoacetamide for 30 minutes followed by cystamine for 30 minutes; (sample 5) iodoacetamide alone for 30 minutes; (sample 6) borate-EDTA buffer alone. Cystamine treatment attaches two positive side chains to both hemoglobins. Iodoacetamide prevents the coupling.

The iodoacetamide coupling slightly affects the electrophoretic mobilities of both hemoglobins A and A_2 (compare samples 5 and 6), presumably by affecting the dissociation of some of the ionizable amino acids in the proteins. Cystamine treatment has a considerable effect (samples 2 and 3 compared with 4 and 5), and the mobility changes are consistent with the addition of two positively charged groups per mole to both hemoglobins. (The chemically modified hemoglobin A migrates at a rate close to that of hemoglobin S, which has two fewer negatively charged residues than hemoglobin A.) Prior treatment with iodoacetamide prevents the cystamine coupling (no mobility differences between samples 4 and 5), and cystamine prevents iodoacetamide coupling (samples 2 and 3 have the same mobility). The results are completely consistent with expectations and show that 100-percent yields of an undenatured derivative of a thiol-containing protein can be obtained by mild treatment with a reagent disulfide under alkaline conditions.

These experiments also show that the determination of the electrophoretic mobilities of proteins after exposure to diaminodiethyldisulfide can be used to test for accessible thiol groups (preferably with diethanoldisulfide-treated material as the control). If the corresponding thiols are used in addition to the disulfides, the presence of accessible disulfide bridges in the proteins should also be detectable by still other mobility differences. Many variations in these procedures can be envisaged to investigate the relative accessibility of protein thiol groups or disulfide bridges under different conditions of *p*H, temperature, the presence of urea, and others.

The two groups of experiments which I have described here demonstrate the versatility and ease of control of the interchange reaction between a reagent disulfide and the disulfide bridges or thiol groups of proteins. Disulfide-bond cleavage, re-formation, and the chemical modification of proteins by the attachment of side chains can all be achieved by varying the reaction conditions (*13*).

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13. This work (paper 1040, Genetics Laboratory, University of Wisconsin) was supported in part by grants from NIH (GMO8217) and NSF (G 14240).

29 October 1965

Electron Microscopic and Biochemical Characterization of Fraction I Protein

Abstract. *Electron micrographs of high resolution have been obtained from fraction I protein of Chinese cabbage leaves. The protein, which has ribulose-1,5-diphosphate carboxylase activity, appears to be a cube with edge of about 120 angstroms. Substructure can be seen in individual particles, consistent with a model having 24 subunits, the number prescribed by the available physical and chemical data.*

When crude extracts of tobacco leaves were examined in the analytical ultracentrifuge over a decade ago, a major soluble-protein fraction, termed the fraction I, showed a sedimentation coefficient of about 18*S* (*1*). Subsequent work showed that the fraction I protein is located predominantly, if not exclusively, in the chloroplast (*2*). There, it comprises by weight at least 50 percent of the soluble proteins (*2*, *3*). Fraction I protein has been reported to have the following biological activities: (i) protochlorophyll holochrome: the protein to which protochlorophyll is at-

tached and which catalyzes the light-induced conversion of protochlorophyll to chlorophyll (*3, 4*); (ii) ribose-5-phosphate isomerase (*5*); (iii) ribulose-5-phosphate kinase (*5*); and (iv) ribulose-1,5-diphosphate carboxylase (carboxydismutase) (*6*). Trown has shown that the activities under (ii) and (iii) are readily separated from fraction I protein by gel filtration on Sephadex G200, whereas carboxydismutase activity (iv) is superimposable on the elution profile of fraction I protein (*7*). He concluded that fraction I protein and carboxydismutase were one and the same.

Trown found the molecular weight of carboxydismutase to be 515,000 by sedimentation equilibrium (*7*). He, as well as Markham *et al.* and Wildman *et al.*, found the 18*S* species prone to form linear aggregates, of which the dimer and trimer have sedimentation coefficients of 25*S* and 32*S*, respectively (*1, 7, 8*). The molecular weight, sedimentation coefficient, and aggregation characteristics of carboxydismutase are very similar to those of the *Escherichia coli* RNA polymerase (*9*).

A molecular weight of 515,000 is very high for a single polypeptide chain. Simple general considerations of the type that led Watson and Crick to the subunit model for the construction of protein shells of viruses suggest that carboxydismutase must be made up of subunits (*10*). If this is so, it becomes germane to inquire whether the rules governing the interaction of subunits are the same as those that apply in the case of small viruses (*10*). This is a realistic question in the case of carboxydismutase, since large amounts of purified material are readily available.

We therefore undertook a combined electron microscopic and biochemical investigation of fraction I protein to answer the following questions. (i) Is carboxydismutase composed of subunits? (ii) If so, what rules govern their interaction? (iii) Do morphological or functional relationships, or both, exist between carboxydismutase and RNA polymerase? (iv) Is fraction I protein carboxydismutase, and only carboxydismutase?

We now report an affirmative answer to (i), a tentative answer to (ii), a negative answer to (iii), and no answer to (iv).

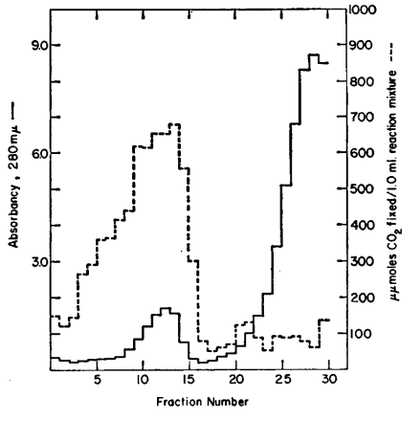

Fig. 1. Glycerol-gradient centrifugation of fraction I protein. One milliliter of a dialyzed fraction, precipitable with up to 50 percent (0 to 50 percent) saturated ammonium sulfate was layered on a glycerol gradient (5 to 30 percent) containing 0.01*M* tris sulfate and 0.001*M* glutathione, *p*H 7.7, and centrifuged for 24 hours at 4°C at 23,000 rev/min in the SW25 rotor of the model L Spinco. After centrifugation, 1-ml fractions were collected and assayed in a mixture containing 0.07 ml of the glycerol fraction, 1.5 μmole of $NaHC^{14}O_3$ (5.1 mc/mmole), 2 μmole of $MgCl_2$, 15 μmole of tris, *p*H 7.7, and 0.06 μmole of ribulose-1,5-diphosphate. The final volume was 0.20 ml, and the fractions were incubated at 25°C for 15 minutes. Glacial acetic acid (0.05 ml) was then added, and 0.05-ml portions were dried on planchets and counted (*17*).

Fresh, de-ribbed Chinese cabbage leaves were ground in the cold and filtered through several layers of cheesecloth. The extract was then centrifuged for 10 minutes at 12,000 rev/min in a Servall centrifuge, and the supernatant was made 50-percent saturated with ammonium sulfate. The precipitate was collected by centrifugation, dissolved in a small volume of 0.01*M* tris, *p*H 7.5, and dialyzed in the cold against the same buffer. One milliliter of the dialyzed preparation was then subjected to zone centrifugation on a glycerol gradient. Carboxydismutase activity is (Fig. 1) associated solely with the peak of ultraviolet-absorbing material at 18*S*. This result is consistent with two interpretations. The one favored by Trown is that the 18*S* protein is in fact carboxydismutase. Alternatively, it might be argued that carboxydismutase is a small molecule active only when associated with the 18*S* protein.

Each fraction in Fig. 1 was assayed for RNA polymerase activity with calf-thymus DNA as template under optimum conditions for the *E. coli* enzyme (*9*). No significant activity was found.

In some preparations, material having an absorption spectrum characteristic of reduced pyridine nucleotide was specifically associated with the 18*S* peak. These fractions were tested for triosephosphate dehydrogenase activity, but none was found. The significance of the bound pyridine nucleotide remains unknown.

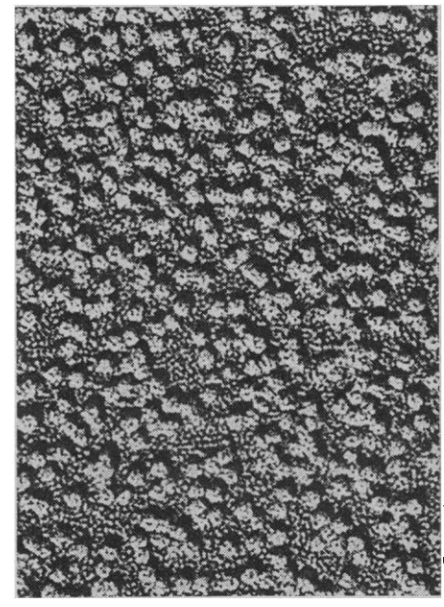

Fig. 2. Fraction I protein from Chinese cabbage leaves shadow-cast with platinum on thin carbon substrate (× 160,000).

Material from the peak of absorbancy in a gradient like that of Fig. 1 was then examined in the electron microscope.

The specimens were prepared for electron microscopy by shadow-casting and negative-staining techniques.

The specimens were examined in the native state shortly after preparation. The final concentration of the specimens was generally 0.1 mg of protein per ml in 0.01*M* tris or TMA ($10^{-2}M$ tris, $10^{-2}M$ magnesium acetate, and $2.2 \times 10^{-2}M$ ammonium chloride) at *p*H 7.15. Ammonium acetate buffer was used for the shadow-casting experiments. The specimens were mounted on extremely thin carbon films (10 Å to 50 Å thick) prepared by evaporation on freshly cleaved mica in a Varian Vac-ion pump 921 unit at a pressure of 10^{-8} to 10^{-9} mm-Hg. These ultrathin, practically structureless carbon films are supported on special fenestrated substrates (*11*).

The microdroplet cross-spraying technique (*11, 12*) and a droplet technique (*13*) were used for negative staining (Fig. 3). By means of a special multiple-spraying device microdroplets of the specimen and of potassium phosphotungstate (1 or 2 percent) at *p*H 7.2 to 7.4 collide and interact very rapidly shortly before impinging on the

specimen grid. Positive staining with 1 or 2 percent uranyl acetate was also obtained with this technique.

A droplet of the specimen diluted with tris or TMA buffer was placed on the carbon-coated fenestrated films.

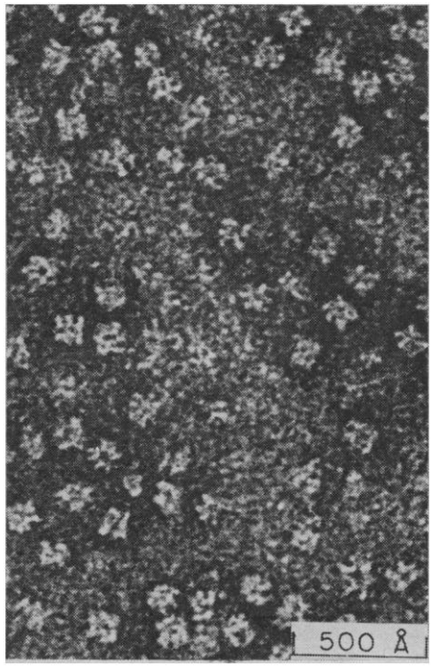

Fig. 3. Fraction I protein from Chinese cabbage leaves negatively stained with 2 percent phosphotungstate (*p*H 7.4) by microdroplet cross-spraying technique (× 360,000).

After 1 to 2 minutes, excess enzyme solution was removed by floating the grid upside down on TMA buffer for about 15 seconds. Then the specimen was stained by floating the grid on 0.5 to 1 percent neutralized potassium phosphotungstate or on 1 to 2 percent unbuffered uranyl acetate. After 1 to 2 minutes the specimen was dried by blotting the edge of the grid on filter paper.

Specimens fixed with 1.5 percent buffered glutaraldehyde prior to negative staining were also examined.

The electron-microscope studies were carried out in a facility designed to provide optimum conditions for consistent attainment of high resolution. Electron micrographs were recorded with Siemens Elmiskops I and Ia, at electron optical magnifications of 40,000 and 46,000 at 80 kv. These microscopes were operated with a highly regulated power supply and had pointed filaments of single-crystal tungsten (*14*, *15*) that provided microbeam illumination of high coherence, yielding enhanced contrast. For critical high-resolution analysis, through-focus series were taken in steps of 80 to 200 Å, and the measured astigmatism of the objective lens was 0.05 μ or less. A liquid-nitrogen anticontamination device was used while recording most of the micrographs. Calibrations were carried out with a diffraction grating replica of 21,600 lines per cm; these were checked by combined electron microscopy and electron diffraction of selected planes from copper phthalocyanine and chrysotile crystals.

M. W. Rees's data on the amino acid composition and number of tryptic peptides obtained from fraction I protein indicate that it consists of 24 *identical* subunits, each of molecular weight 22,500 (*16*).

Twenty-four subunits can be fitted into equivalent positions by placing them in groups of three on the vertices of a cube. Such a model for the enzyme can be ruled out on several grounds. First, the electron micrographs of concentrated droplets show no tendency toward aggregation in regular arrays. Secondly, attempts at crystallization from ammonium sulfate solutions have produced fibrous precipitates instead. Thirdly, when aggregates do occur, they are seen to be linear. Hence, the correct molecular model must be one which contains a unique twofold axis.

The shadowed preparations (Fig. 2) show uniform cubical particles often with rounded edges and a central depression which may be partly due to preparative (dehydration) effects. A model consistent with the views shown in electron micrographs (Figs. 3 and 4), as well as with the macroscopic properties of the protein, is that of a cube with three subunits along each edge, and one in the center of each of four faces. Particles resting on a side face will show three rows of three subunits (Fig. 4*A*). Particles resting on a top or bottom face will show a ring of eight subunits (Fig. 4*B*) corresponding to the side view with the central subunit missing. The subunits have a diameter of approximately 20 Å to 30 Å. Particles resting on an edge should show three rows, with the particle length in the direction of the rows up to 40 percent greater than its width perpendicular to the rows. Particles resting on a vertex should have a hexagonal appearance, with a central subunit and 12 subunits around the perimeter. Although rectangular or hexagonal particles have been observed, these are rare and could be due to distortion or association of particles on the substrate. Particles with hexagonal outline are seen occasionally, but these usually have one right-angle corner, and probably arise from partial embedding of the square face.

The diameter of a Stokes sphere of

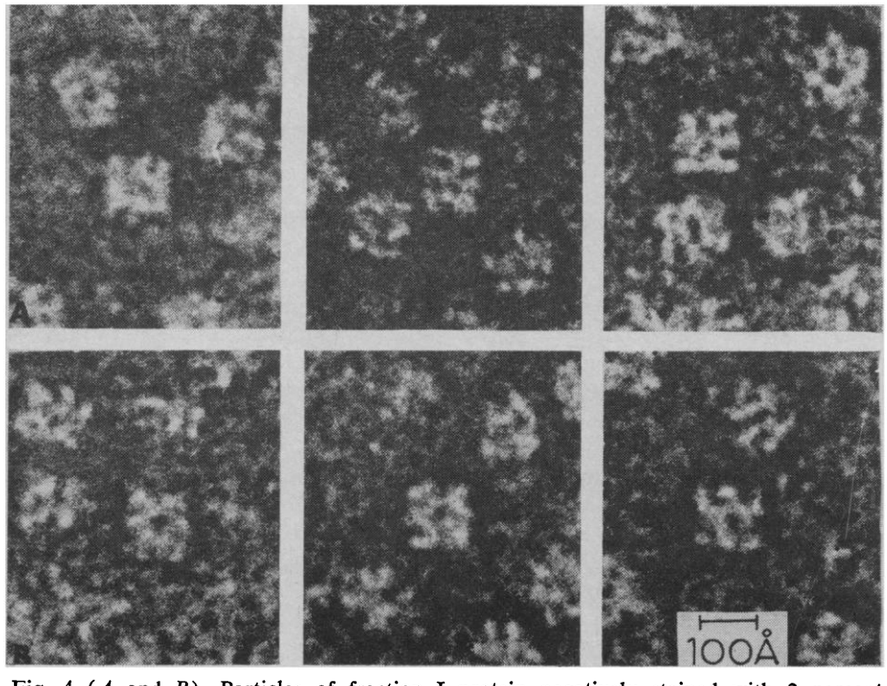

Fig. 4 (*A* and *B*). Particles of fraction I protein negatively stained with 2 percent phosphotungstate (*p*H 7.4) in different orientations (× 800,000).

molecular weight 500,000 and sedimentation coefficient 18*S* (*7*) is calculated as 120 Å. This value is in excellent agreement with the measured dimensions of the cubical particles (120 Å edge) as shown by electron microscopy using several preparation techniques.

Subunits in the model proposed are not related by the principle of quasi-equivalence that governs the structure of many small viruses (*10*). In the model proposed, subunits are found in three positions: on faces, edges, or vertices. We assume that the plane net, from which the structure may formally originate, consists of a square lattice. Then the distortion required to place a subunit on a vertex, maintaining the same bonding rule, becomes unacceptable (*10*). We are therefore obliged to propose either that subunits are capable of making several kinds of bond, or that subunits undergo considerable conformational changes as a result of their statistical location in the completed particle.

From the foregoing, fraction I protein consists of a cube, about 120 Å along each edge, containing 24 subunits as seen in high-resolution electron micrographs. The protein contains all the carboxydismutase activity recoverable from glycerol gradients. It has the same sedimentation coefficient and molecular weight as bacterial RNA-polymerase, but lacks the latter's activity. The particle structure does not appear to be governed by the same rules that apply in the case of protein shells of small viruses. We believe this to be the first occasion upon which the substructure of a protein enzyme of about 120 Å diameter has been resolved in electron micrographs.

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18. We thank Misses C. M. Runner and D. A. Meddoff for assistance in specimen preparation, C. L. Hough for assistance with photographic reproductions, and Mrs. L. Deloria for technical assistance.
19. Supported by USPHS grant AI-04448 and AI-06279, NIH grants NB-04267 and GM-13243, NASA grant NsG 441-63, AEC contract AT (11-1)-1344, the L. Block Fund, and the Otho Sprague Memorial Fund of the University of Chicago.
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11 October 1965

X-Linked 6-Phosphogluconate Dehydrogenase in Drosophila: Subunit Associations

Abstract. *6-Phosphogluconate dehydrogenase (PGD) subunits have been dissociated and reassociated in vitro. The intermediate PGD of the heterozygous phenotype is reproduced by reassociation in vitro of subunits derived from the two types of PGD from homozygous flies. This result has a bearing on gene action in the diploid organism and on the mechanism of dosage compensation in* Drosophila melanogaster.

Dosage compensation in mammals is probably accomplished by inactivation of one X chromosome in each female somatic cell (*1*). In the female, when one allele of an X-linked gene determines the structure of the wild-type protein and the other allele produces a mutant protein, each cell contains either the wild type or the mutant type of protein, but not both. Although dosage compensation has been repeatedly demonstrated in *Drosophila*, a specific mechanism is unknown (*2*). Evidence from position-effect variegation at the white locus suggests that both X chromosomes are active in female cells (*2*). We now report that both alleles of the X-linked gene, which determines the structure of 6-phosphogluconate dehydrogenase (PGD), function in those cells in which this enzyme is synthesized in the *D. melanogaster* female.

Young has located the PGD structural gene (*Pgd*) at approximately 0.9 on the X chromosome (*3*). Certain strains have a single PGD band whose electrophoretic mobility is greater than that of the single PGD band of other strains. These phenotypes were called PGD A and PGD B, respectively. The mating of a PGD A individual with a PGD B fly yielded a third phenotype in all female offspring, which was characterized by the PGD A and PGD B bands along with a more-densely staining PGD band that was precisely intermediate in mobility; the intermediate band was designated PGD I^{AB}.

The molecular configuration that best accounts for the single intermediate band in the heterozygous female is that of a dimer. In the cells of this fly two different subunits, designated s^a and s^b (*4*), could assemble to produce three dimers. PGD A, PGD B, and PGD I^{AB} would then be composed of s^as^a, s^bs^b, and s^as^b, respectively. Random combination of two different subunits would yield two molecules of s^as^b for every s^as^a or s^bs^b. It also follows that each allele of the *Pgd* gene would specify either an s^a or s^b subunit. Demonstration that the intermediate band is indeed composed of subunits contained in the PGD A and PGD B enzymes would require dissociation of the subunits of those enzymes and their reassociation to produce the PGD I^{AB}. Dissociation was accomplished by treatment of a partially purified PGD enzyme with propanedithiol, a compound that breaks disulfide bonds; reassociation occurred after dialysis and concentration of the dialyzate. All purification procedures were carried out at 4°C.

Twenty grams of adult flies (about 25,000) mixed with 3.6 g of Norit A were homogenized in 400 ml of 0.02*M* phosphate buffer, *p*H 6.2 and $10^{-4}M$ with respect to ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at 18,000*g* for 75 minutes and the precipitate was discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant to give a 46-percent $(NH_4)_2SO_4$ solution which was stirred for 30 minutes and centrifuged; the precipitate was discarded, and the supernatant was adjusted to 58 percent $(NH_4)_2SO_4$ by addition of the salt. After another 30 minutes of mixing, this precipitate, which contained