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COMPARISON OF THE PHOSPHORUS CONTENT, OPTICAL ROTATION, SEPARATION OF HEMES AND GLOBIN, AND TERMINAL AMINO ACID RESIDUES OF NORMAL ADULT HUMAN HEMOGLOBIN AND SICKLE CELL ANEMIA HEMOGLOBIN

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Introduction.—It has been shown that sickle cell anemia, a condition probably created[1,2] by homozygosity in a single gene, is associated with the presence in the red blood cells of a hemoglobin different from normal adult hemoglobin.[1] Striking differences exist between the solubilities of the reduced forms of the two hemoglobins, the tendency of the abnormal hemoglobin to aggregate being the cause of the severe troubles in this molecular disease. Electrophoretic measurements revealed another characteristic difference; sickle cell anemia hemoglobin carries a greater positive charge or smaller negative charge in the pH range 5.5 to 8.0. In cases of sickle cell trait, where the sickling of the blood cells is brought about much more difficultly and no clinical troubles arise, electrophoretic analysis demonstrated the presence of normal and sickle cell anemia hemoglobin in roughly equal amounts. Apparently in these cases one dose each of the sickle cell gene and its normal allele are present in the somatic cell. A careful quantitative amino acid analysis of both hemoglobins pointed to an almost identical overall composition.[3] The isolation of the same porphyrin dimethyl ester from both hemoglobins indicates that the protein parts are largely responsible for the difference. There were indications of a slight difference in the content of leucine and serine and perhaps also of valine and threonine. These might account for the difference in electrophoretic behavior in an indirect manner. It was therefore decided to make a more detailed comparison of normal adult and sickle cell anemia hemoglobin, in order to disclose the chemical background of the hereditary abnormality. A full account of the electrophoretic behavior of the globins from normal adult hemoglobin, sickle cell

anemia hemoglobin, and sickle cell trait hemoglobin will be given in the following paper.[4] In this publication we report on some complementary studies concerning the phosphorus content, the optical rotary power, the splitting into heme and globin, and the terminal amino acid residues in both hemoglobins and globins.

Phosphorus Content.—We first checked whether phosphate groups might be responsible for the difference in charge of the molecules. It is reported in the older literature that hemoglobins of fowl contain a characteristic amount of phosphorus, whereas the hemoglobins of some mammals do not.[5, 6] We confirmed the latter fact for normal human hemoglobin. Thirty milligram samples of hemoglobin prepared according to Drabkin[7] were digested with 0.5 ml. conc. nitric acid and 0.5 ml. conc. sulfuric acid in 50-ml. Kjeldahl flasks. After completion of the destruction the sulfuric acid was carefully neutralized with 10 *N* sodium hydroxide. Solutions were decanted from solid sodium sulfate, diluted with water, and acidified with 4 *N* sulfuric acid, so that 6 ml. samples 0.5 *N* in sulfuric acid were obtained. These were colored with 0.5 ml. molybdate and 0.4 ml. aminonaphtholsulfonic acid solution as described by King.[8] Blanks, standards, and determinations with known amounts of phosphorus added were run at the same time in exactly the same way. Comparison of the colors obtained after various time intervals led to the conclusion that about 8 γ of phosphorus was present in each of our 30 mg. samples; that is, about 0.6 atom of phosphorus per molecule. Since hemoglobin *a* is more acidic than hemoglobin *b*,[9] it is clear that the phosphate content cannot be the cause of the difference.

Optical Rotation.—It is well known that denaturation of a protein often causes a significant shift in its isoelectric point. Hence hemoglobin *b* might represent some kind of denatured normal hemoglobin. We were, therefore, curious to compare the specific optical rotary power of the two substances. In many cases this property is rather sensitive to denaturation, especially when this process brings about a change in the status of the sulfur-containing amino acids. A Schmidt and Haensch polarimeter was used that allows measurements with an accuracy of about 0.01°. Optical rotation was measured in 20-cm. tubes containing 1% solution of normal adult, sickle cell trait, and fetal carbonmonoxyhemoglobin, in 0.01 *M* disodium hydrogen phosphate solution. The specific rotations found, using the red light obtained from a cadmium lamp by use of a red filter, were −23.5° for the normal, −24° for the fetal, and −23° for the sickle cell trait. Investigations with more concentrated solutions at other wave-lengths and with the concentrated solutions of the globins rather than with the whole pigments eventually might reveal significant slight differences. However, for our problem at the moment, the results are definite to the extent that they provide no positive indication that hemoglobin *b* is a denaturation product of normal hemoglobin.

Separation of Hemes from Globin.—The preparation of globin from hemoglobin deserves some comment; this process is of primary importance also in the dicussion of the electrophoretic measurements in a following paper.[4] The splitting of the carbonmonoxyhemoglobin into the porphyrin derivative and the globin was effected by acid acetone;[13] the process occurred more difficultly with hemoglobin *b* than with normal hemoglobin. This is of particular interest as it points to a difference between the two molecules in the region where the hemes are attacked. Such a difference was postulated by Pauling, Itano, Singer, and Wells[1] already in 1949 when they put forward a hypothesis on the mechanism of sickling in cells containing an appreciable amount of hemoglobin *b*.

The almost classical question of whether the splitting off of the heme from the globin part of the molecule is essentially connected with denaturation has been discussed over and over again. It is clear that certain changes take place in the protein when the heme is removed. However, it is our opinion, based on electrophoretic and other evidence, that if the splitting of carbonmonoxyhemoglobin is effected with great caution, a product can be obtained that still has the specific configurational features of the protein present originally in the hemoglobin molecule.[14]

Care should be taken to avoid the presence of oxygen during the reaction with dilute hydrochloric acid and acetone by working in a carbon monoxide atmosphere. Of special importance is the maintenance of a low temperature (0°C. or lower) throughout the preparation and the handling of the globin. Before electrophoretic or other measurements were performed with the globin, the solution of the hydrochloric acid salt of the globin in water was dialyzed for at least 24 hours at 4°C. against the buffer to be used. The buffer usually had a pH near the isoelectric point of the globin. During dialysis precipitation occurs of "irreversibly denatured" globin, the weight of which amounts to not more than 10% of the total in case the necessary precautions have been taken during the preparation. One has to be very careful with solutions of the globin even when the pH is near the isoelectric point and the molecules carry only a small charge. Keeping such solutions at 10 to 15°C. for a short period is sufficient to accelerate denaturation to such a degree that flocculation becomes visible. Addition of urea or of guanidinium chloride to a globin solution in concentrations of 5 to 10 *M* and subsequent removal of these reagents by dialysis in the cold leaves the protein strongly denatured, as becomes apparent from its insolubility at the isoelectric point. The globin from fetal hemoglobin proved to be especially prone to denaturation.

Terminal Amino Acid Residues.—A valuable method to check possible differences in chemical composition and structure between two proteins is the qualitative and quantitative determination of the terminal amino acid residues of the peptide chains of the protein molecules. For our

purpose we adopted the method of tagging the free amino end groups with 2,4-dinitrofluorobenzene, as has been worked out by F. Sanger.[10, 11] The separation and identification of the DNP amino acids obtained after hydrolysis was done by chromatography on columns of silicic acid and celite.[12]

Three pairs of determinations were made. In the first, the globins prepared from hemoglobins *a* and *b* were compared. One per cent solutions of the globins from hemoglobin *a* and from sickle cell trait hemoglobin ("*ab*") in 0.01 *M* disodium hydrogen phosphate were examined electrophoretically[4] before use, in order to establish the homogeneity of the sample in the one case and the presence of normal and abnormal globin in about equal amounts in the second case. The concentrations were estimated by measuring the absorption spectra in the ultraviolet region. Then 7.7 ml. of these solutions was treated with 0.1 ml. of dinitrofluorobenzene and kept in slow movement at 4°C. by mechanical rotation for 24 hours. Partial flocculation occurred in the course of time.[15] To the reaction mixture enough conc. hydrochloric acid was added to obtain a 6 *N* acid solution. Hydrolysis was effected by boiling under reflux for 20 hours. The terminal DNP-amino acids were extracted exhaustively with ether, and the combined ether layers were washed several times with 10^{-3} *N* hydrochloric acid and then evaporated to dryness *in vacuo*. Then a separation into groups was effected by chromatography.[12] Two main zones were observed in each case. The first ones in group III of the system[12] were identified as consisting mainly of dinitroaniline. The other, faster moving zones were purified and identified[12] as DNP-valine. For normal globin this result was to be expected on the basis of the analysis made by Porter and Sanger.[11] The zones were eluted with a mixture of alcohol and ether (1:4 by volume); the solutions were evaporated, and the DNP-valine was dissolved in glacial acetic acid. Measurement of the absorption spectra with the aid of a Beckman D.U. Spectrophotometer made it possible to calculate the amount of DNP-valine; $\epsilon_{max.}$ was assumed to be 1.6×10^4.

We found 2.04 μmoles of DNP-valine starting from 1.2 μmoles of normal globin and 1.87 μmoles of DNP-valine from 1.1 μmoles of sicklemic globin. Evidently there is no difference between the two proteins with respect to the amino end groups. Consideration of the losses due to destruction during hydrolysis (25%) and during isolation by chromatography and elution (10 to 15% for each chromatographic purification) leads to the value 4 as the number of terminal valine residues in each cases. There are indications in later experiments that the method of dinitrophenylation used did not effect a complete substitution of the amino groups, and the absolute value is hence unreliable; nevertheless the identity of the results obtained with normal and with sicklemic globin is important.

It was considered advisable to compare the blood pigments also by determination of terminal amino acids starting with the original hemoglobins.

The reaction with dinitrofluorobenzene was repeated with solutions of 250 mg. of normal and sickle cell anemia carbonmonoxyhemoglobin (the latter containing about 18% of fetal hemoglobin) in 7 ml. water to which 0.1 g. sodium bicarbonate had been added. After the reaction had gone on for 48 hours at 4°C. (precipitation occurred in the course of time) the substituted globins were set free and isolated by treatment of the reaction mixture with dilute hydrochloric acid and acetone. The preicpitates were washed with acetone until the washing liquid became colorless, and dried *in vacuo* over calcium chloride and potassium hydroxide (weight of the reaction products about 280 mg.). One hundred-fifty-milligram portions of the dinitrophenylated globins were hydrolyzed and analyzed in the usual way.

This experiment gave 25% more DNP-valine from the sickle cell anemia hemoglobin than from normal hemoglobin. The amounts could be estimated to correspond to 5 and 4 residues, respectively, per hemoglobin molecule. However, the dinitrophenylated globins were dissolved in dilute alkali and the ultraviolet absorption spectrum was measured; it was found that the extinction between 300 and 400 mμ, which must be ascribed to the whole of the dinitrophenyl groups in the proteins, and not only to those attached to the terminal valine residues, was also higher (by about 30%) with the sickle cell anemia preparation than with the normal material. Accordingly we conclude that the reaction with dinitrofluorobenzene proceeded somewhat more rapidly with hemoglobin *b* than with hemoglobin *a*.

An effort was made to carry out a complete dinitrophenylation. The reaction was carried out first by rotating 0.2 g. hemoglobin, 0.1 g. sodium bicarbonate, 0.1 ml. dinitrofluorobenzene, and 10 ml. water at 4°C. for 24 hours. Then 5 ml. of ethanol was added and the reaction was continued at 4°C. for 20 hours. Finally, the reaction mixture was brought to room temperature and mechanical rotation was continued for another 5 hours. The DNP-globins isolated were intensely yellow, suggesting the dinitrophenylation had proceeded well. From the hemoglobins *a* and *b* treated in this way the same amount of DNP-valine was isolated,[16] corresponding to about 5 terminal valine residues per hemoglobin molecule in each case.

Conclusion.—The evidence obtained in this investigation thus indicates a great similarity of the chemical composition of the protein of normal adult human hemoglobin and sickle cell anemia hemoglobin.

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[9] Following a proposal of H. A. Itano (these PROCEEDINGS, **37**, 775–784 (1951)) we shall, for brevity, designate normal adult hemoglobin as hemoglobin *a* and sickle cell anemia hemoglobin as hemoglobin *b*.

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[16] We got an indication of the presence of a small amount of DNP-methionine in the product from sickle cell anemia hemoglobin; more detailed investigations are needed to get definite information on this point.