

The experiments reported in this paper, conducted over a period of nine weeks, indicate that different electrophoretic fractions of γ G-globulin possess different breakdown rates; the observed half-life of the entire γ G-globulin preparation being a mean of the individual values. The overall breakdown rate is non-linear and the half-life values increase with time. However, mean half-life values measured over the nine-week period were 25 days (J. W.) and 18 days (patient) respectively. These half-life values are in good agreement with those of Cohen and Freeman (1960), who reported half-life values of 21–26 days for γ G-globulins in the normal individual.

The half-life values obtained from the present experiment appeared to be characteristic of the catabolism of the individual and to be independent of the source of the labelled γ G-globulin.

The difference in the half-life values found for γ G-globulins in J. W. and the patient cannot be attributed to concentration dependence of γ G-globulin catabolism, since it was found that their serum γ G-globulin levels were 838 and 940 mg./100 ml. respectively. However, starch-gel electrophoresis experiments showed that in the patient the serum γ_2/γ_1 ratio was approximately 2:1, as compared with 1:1 in J. W. The 2:1 γ -globulin ratio would be expected to predispose the overall γ G-globulin catabolic rate in the cancer patient to be faster than that in the control subject with a 1:1 ratio, since it has been shown (Fig. 2) that the γ_2 fraction is broken down faster than the γ_1 fraction.

The possibility that the catabolic rates are affected by allotypic differences between the donor γ G-globulins and those of the recipient cannot be overlooked. The patient had had several transfusions during the course of operations for removal of his neoplasms. However, J. W. carried out a similar experiment to that reported in this paper on himself a year previously using homologous γ G-globulin from a normal individual. Two γ G-globulin preparations were used for this particular experiment. One was obtained from the normal donor directly and labelled with ^{125}I , the other was obtained from this donor after a "booster" injection of tetanus toxoid and labelled with ^{131}I . Both labelled γ G-globulin preparations showed the same heterogeneous breakdown pattern over a period of 15 weeks. The apparent biological half-life values were identical to those reported for J. W. in this paper. The results of this experiment

(Watkins and Tee, 1966), together with the present observations, indicate that the observed catabolic phenomena are not artifacts produced by immune response to the injected homologous γ G-globulin.

This work is now being extended to study the catabolism of γ G-globulins in other subjects, including both normal volunteers and patients with various pathological conditions.

Summary

The survival of radioiodine-labelled serum γ G-globulins was determined in a normal volunteer and in a cancer patient over a period of nine weeks.

It was found that different electrophoretic fractions of the γ G-globulin broke down at different rates. This catabolic heterogeneity of the γ G-globulins gave rise to an apparent time dependence of the observed half-life value. Mean half-life values, as measured over the entire nine-week period, were 25 days for γ G-globulins in the normal volunteer and 18 days for the same γ G-globulin preparation in the cancer patient. The breakdown rates in both individuals appeared to be independent of the source of the labelled γ G-globulins.

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Preliminary Communications

Demonstration of an Enzyme Variant in a Case of Congenital Methaemoglobinaemia

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In normal individuals methaemoglobin in the red cell is maintained at a very low level (<1%). Raised levels may be due to: (a) intake of certain drugs which overcome the normal mechanism for maintaining a high reduction potential in the cell—removal of the toxic influence results in disappearance of methaemoglobin; (b) presence of an abnormal haemoglobin M which is more susceptible to oxidation as well as being more resistant to reduction in the cell; and (c) deficiency of reduced nicotinamide adenine dinucleotide coupled methaemoglobin reductase.

Numerous cases of congenital methaemoglobinaemia due to deficiency of the reduced nicotinamide adenine dinucleotide linked "diaphorase" have been described, the affected individual showing very low enzyme activity in both haemolysates and whole cells, while the parents show intermediate levels of

activity (for review, see Jaffé and Heller, 1964). However, it has not been clear whether the defect was one of enzyme synthesis or of enzyme structure.

In the case of congenital methaemoglobinaemia here reported, it is shown by starch-gel electrophoresis of a partially purified red cell extract that the propositus possesses an electrophoretically distinct enzyme, indicating that the defect lies in the synthesis of a structurally altered enzyme protein. Normal individuals appear to possess at least two isoenzymes.

CASE REPORT

The patient, a boy aged 9, had a history of frequent upper respiratory infections and of becoming blue since birth. His milestones of development were slightly delayed, and he was not progressing well at school.

On clinical examination his lips were slightly greyish blue and his complexion was rather dusky; this finding suggested the possibility of methaemoglobinaemia as a diagnosis. There were no other abnormal physical signs. Investigations confirmed the presence of methaemoglobin in his blood, and this was again found on

numerous occasions. Chest x-ray films, routine urine analysis, and urinary chromatography for amino-acids were all normal. The tuberculin test and the Wassermann reaction were negative.

An educational psychologist, using the Wechsler Intelligence Scale, assessed the patient's full-scale I.Q. as 105 (verbal I.Q. 101, performance I.Q. 108), and noted that his "functioning fluctuates between near subnormal and very superior." Since that time, though slow and lacking in concentration, he has made reasonable progress with the aid of a remedial teacher.

Treatment with large doses of oral ascorbic acid had no obvious effect on the slight cyanosis, though his mother stated that he was better for it.

Family History.—Mother and two siblings are alive and well. There is a history of diabetes on the maternal side, and a cousin has suffered from acute haemolytic anaemia associated with glandular fever.

Investigations.—Haemoglobin 13.6 g./100 ml.; P.C.V. 40%; red cell count 4,700,000/cu. mm.; reticulocytes <1%; M.C.H.C. 34 g./100 ml.; M.C.H. 28 $\mu\text{g.}$; M.C.V. 85 cu. μ . The red cells appeared normal on the stained blood film. Serum iron 97 $\mu\text{g.}/100$ ml. Haptoglobins (type 2-2) present in plasma. The methaemoglobin present amounted to 4.4% of the total haemoglobin. Starch-gel electrophoresis of haemoglobin showed no abnormal haemoglobin; Hb-A₂ was within normal limits. The methaemoglobin A present migrated in the normal position for methaemoglobin A. Spectroscopic examination showed that the α -absorption band was between 630 and 635 $m\mu$. The starch-gel electrophoretic and spectroscopic properties of the methaemoglobin indicated that it was normal haemoglobin and not a type of haemoglobin M.

PREPARATION OF ENZYME EXTRACT FROM PATIENT'S RED CELLS

The separation of haemoglobin from other red cell proteins was carried out with diethylaminoethyl (D.E.A.E.) cellulose by means of a batch technique adapted from the methods of Hennessey *et al.* (1962) and Stansell and Deutsch (1965). Heparinized venous blood was centrifuged, plasma and buffy coat were removed, and the packed cells were washed three times with 0.9 g. of sodium chloride per 100 ml. Five millilitres of packed red cells were haemolysed in four volumes of ice-cold 0.005M tris (tris-(hydroxymethyl)-aminomethane) brought to pH 7 with phosphoric acid and centrifuged for 30 minutes at 35,000 g at 0° C. to remove stroma.

The decanted supernatant was passed through a Sephadex G-25 column (3 by 18 cm.), pre-equilibrated with the 0.005M tris-phosphate buffer, pH 7. The equilibrated haemolysate was collected direct into approximately 60 g. of D.E.A.E. cellulose (Whatman DE-52) equilibrated with the same buffer.

The haemolysate-cellulose suspension was stirred gently in the cold for three hours, then filtered under vacuum and washed with 0.005M trisphosphate, pH 7, until the washings were clear. This removed most of the haemoglobin and left the enzyme protein on the cellulose. The cellulose was then resuspended in the same buffer and packed under gravity into a column, 1.5 by 60 cm. The enzyme fraction was eluted from the column with 1M sodium chloride brought to pH 5.6 with phosphoric acid. This process concentrated the non-haemoglobin proteins to about 30 ml., and these were further concentrated to about 0.5 ml. by vacuum dialysis against the tris-borate-E.D.T.A. buffer, pH 8.6, used for electrophoresis. This concentrated extract had about 10 times the enzyme activity of packed red cells and was used for electrophoresis. The extract also contained a number of other red cell enzymes and proteins as well as a small amount of haemoglobin.

ELECTROPHORESIS AND STAINING OF THE GEL

Electrophoresis was carried out on starch gels with tris-borate-E.D.T.A. buffer, pH 8.6 (see Huehns and Shooter, 1965). A current of 30 mA was passed for four hours.

The enzyme has about 9,000 times the activity with the dye 2:6-dichlorophenolindophenol as substrate than it has with methaemoglobin (Scott and McGraw, 1962). The property of reducing this dye in the presence of reduced nicotinamide adenine dinucleotide was therefore used in locating the different bands of enzyme activity on the gel. Chromatography paper (Whatman 3 MM) was dipped into a concentrated filtered solution of 2:6-dichlorophenolindophenol containing about 50 mg. of reduced

nicotinamide adenine dinucleotide per 100 ml. of 1M tris buffer, pH 7.5, with 0.3 mM E.D.T.A. The saturated paper was placed on the cut surface of the gel and the whole covered with a fine polyethylene sheet to exclude air. When sufficient dye had diffused into the gel to give a uniformly blue background the paper was removed and the polyethylene replaced. The diaphorase activity could be seen on the gel as decolorized bands after approximately 15 minutes at room temperature. Photographs were taken with transmitted light on microfilm (Kodak type 5453) with a deep yellow filter.

ASSAYS OF METHAEMOGLOBIN REDUCTASE

In whole cells the method of Jaffé (1959) was used, with adenosine as "nutrient" substrate. Methaemoglobin was estimated, utilizing the difference in spectral absorption of the met- and carbonmonoxy-derivatives of haemoglobin at 632 $m\mu$. This method is capable of measuring as little as 0.5% of the oxidized pigment. For haemolysates the enzyme was estimated as described by Scott (1960).

RESULTS

Whole Cells.—The results are shown in Fig. 1. The patient's cells show a very low rate of reduction of methaemoglobin compared with those of the mother and normal controls, which had almost completely re-formed oxyhaemoglobin after 17 hours. The mother falls within the range for normal individuals. In the absence of nutrient substrate there was a rise in the methaemoglobin level in the first three hours of incubation of about 10%, after which time the levels remained constant.

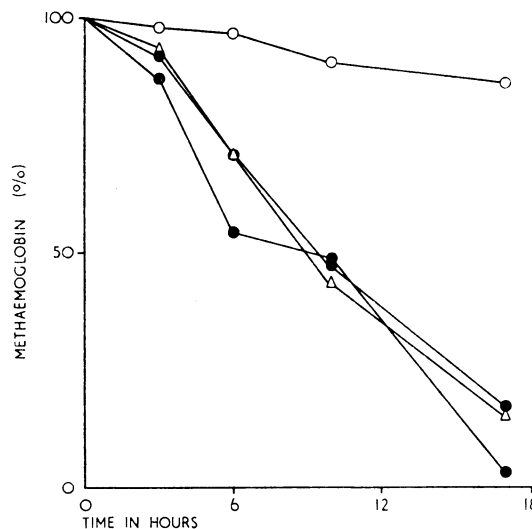


FIG. 1.—Reduction of methaemoglobin to haemoglobin during incubation of nitrite-treated red cells. The reduction of methaemoglobin is expressed as a percentage of the methaemoglobin content of the nitrated washed cells at the start of the incubation. ○—○ Patient with methaemoglobin reductase deficiency. Δ—Δ Mother of patient. ●—● Normal controls.

Haemolysates.—The results are shown in the Table. The proposition again shows very low activity while the mother falls within the normal range.

Methaemoglobin Reductase Activity in Haemolysates

	Change in optical density at 600 $m\mu$ /1 ml. packed red cells per minute
Patient	0.15
Mother	0.95
Normal controls (25)	1.37 \pm 0.40 (S.D.)

Starch-Gel Electrophoresis.—In normal individuals at pH 8.6 two light bands appear migrating more slowly than Hb A towards the anode (Fig. 2). The patient has only one band of intermediate mobility and much lower activity as judged by the rate and intensity of decolorization of the dye. The patient's mother shows the two normal bands, as well as a weak band in the same position as

that of her son. This is not shown in the photograph, as the relative intensities of the bands made this technically difficult. No such band has been seen in 35 unrelated normal individuals studied in this way, and dilution of the normal concentrate did not alter the electrophoretic pattern.

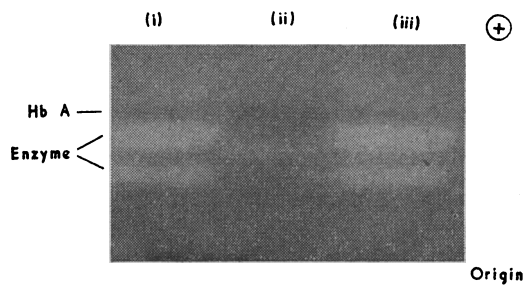


FIG. 2.—Starch-gel electrophoresis of enzyme extract prepared from red cells stained for reduced nicotinamide adenine dinucleotide-methaemoglobin reductase. (i) normal red cells, (ii) methaemoglobinaemic red cells, (iii) normal red cells.

DISCUSSION

The deficiency of "coenzyme factor I" (diaphorase, reduced nicotinamide adenine dinucleotide linked methaemoglobin oxidoreductase) described by Gibson (1948) has been confirmed in many patients with congenital methaemoglobinaemia.

The deficiency could be the result of two possible situations: (a) reduced rate of synthesis of a normal enzyme; and (b) normal rate of synthesis of an abnormal enzyme which is either stable, with a low specific activity, or unstable, resulting in non-uniform distribution of activity in old and young cells.

Scott (1962) and Scott *et al.* (1965) have partially purified reduced nicotinamide adenine dinucleotide methaemoglobin reductase from methaemoglobinaemic red cells. This enzyme shows altered properties with respect to pH and temperature stability compared with enzyme from normal individuals. Keitt *et al.* (1966) found that old red cells separated by ultracentrifugation from patients with this enzyme deficiency contained more methaemoglobin than young cells. These results suggest that in these patients the enzyme activity in the red cells is due to the presence of an abnormal unstable enzyme. The present finding of an electrophoretically abnormal enzyme is consistent with this idea. However, the defect present in this family may be different from that found by other investigators, because the amount of methaemoglobin in the patient was only 4.4% of total haemoglobin, whereas in other reported cases values range between 8 and 45% (Jaffé and Heller, 1964; Fialkow *et al.*, 1965; Jaffé *et al.*, 1966). Furthermore, the mother of our patient has an enzyme activity which falls within the normal range, whereas the heterozygotes reported by Scott (1960) have approximately half the normal enzyme levels. The finding of normal enzyme levels in the mother of our patient is consistent with the presence of both normal and abnormal enzymes. The presence of the abnormal enzyme protein in the mother allows the definite identification of the heterozygous state, which previously has only been presumptive, being based on the level of enzyme activity present. The father of our patient has so far not been studied.

The association of the synthesis of abnormal proteins or enzymes with disease has been well documented in, for example, the haemoglobinopathies (see Huehns and Shooter, 1965) and

in glucose-6-phosphate dehydrogenase deficiency (Marks, 1964). The present investigation suggests that congenital methaemoglobinaemia due to reduced nicotinamide adenine dinucleotide methaemoglobin reductase deficiency is also of this type, and study of other patients with this disease may show that it can also be caused by a number of electrophoretically distinct enzymes.

SUMMARY

A partially purified protein extract was prepared from the red cells of a patient with congenital methaemoglobinaemia and from normal individuals. Electrophoresis of these extracts at pH 8.6 and staining with a 2:6-dichlorophenolindophenol/reduced nicotinamide adenine dinucleotide system showed two decolorized bands of diaphorase activity in normal individuals and a single band of intermediate mobility in the patient. The mother of the patient showed both normal and abnormal enzymes. It is suggested that the enzyme deficiency in this patient is the result of the synthesis of an abnormal form of reduced nicotinamide adenine dinucleotide linked methaemoglobin reductase.

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