The experiments reported in this paper, conducted over a period of nine weeks, indicate that different electrophoretic fractions of γG-globulin possess different breakdown rates; the observed half-life of the entire γG-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 γG-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 γG-globulin.

The difference in the half-life values found for γG-globulins in J. W. and the patient cannot be attributed to concentration dependence of γG-globulin catabolism, since it was found that their serum γG-globulin levels were 838 and 940 mg./100 ml. respectively. However, starch-gel electrophoresis experiments showed that in the patient the serum γ2/γ1 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 γG-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 γ2 fraction is broken down faster than the γ1 fraction.

The possibility that the catabolic rates are affected by allo-typic differences between the donor γG-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 γG-globulin preparations were used for this particular experiment. One was obtained from the normal donor directly and labelled with 131I, the other was obtained from this donor after a "booster" injection of tetanus toxoid and labelled with 131I. Both labelled γG-globulin preparations showed the same heterogeneous breakdown pattern over a period of 15 weeks. The apparent electrophoretic 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 γG-globulins in other subjects, including both normal volunteers and patients with various pathological conditions.

Summary

The survival of radioiodine-labelled serum γG-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 γG-globulin broke down at different rates. This catabolic heterogeneity of the γG-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 γG-globulins in the normal volunteer and 18 days for the same γG-globulin preparation in the cancer patient. The breakdown rates in both individuals appeared to be independent of the source of the labelled γG-globulins.

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References


Preliminary Communications

Demonstration of an Enzyme Variant in a Case of Congenital Methaemoglobinemia

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 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 methaemoglobinemia 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 methaemoglobinemia 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
lupus erythematosus.

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 μg.; M.C.V. 85 cu. μ. The red
cells appeared normal on the stained blood film. Serum iron
97 μg./100 ml. Hypoglobulins (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 methaemo-
globin A present migrated in the normal position for methaemo-
globin A. Spectroscopic examination showed that the α-absorption
band was between 630 and 635 μm. The starch-gel electrophoretic
and spectrophotometric 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 (1955) and Stansell and Deuch (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-(hydroxy-
methyl)-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
of the concentrated extract. 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 diaphoresis activity
could be seen on the gel as decolorized bands after approximately
15 minutes at room temperature. Photographs were taken with
transmitted light on microslide film (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
carbonmonoxo-derivatives of haemoglobin at 632 mμ. 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.

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

Methaemoglobin Reductase Activity in Haemolysates

Patient
Mother
Normal controls

| Change in optical density at 600 mμ/ 1 ml. packed red cells per minute |
|-----------------|-----------------|-----------------|
| Patient | 0.03 | 0.12 | 0.94 |
| Mother | 0.15 | 1.13 | 0.60 |

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.

![Starch-gel electrophoresis of enzyme extract prepared from red cells stained for reduced nicotinamide adenine dinucleotide-methaemoglobin reductase.](image)

**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 methaemoglobinemia.

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 methaemoglobinemic 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.

A 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 methaemoglobinemia 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 methaemoglobinemia 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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