affect the results. The results reported by no means cover all the lipids or even phospholipids in the red cell, and it may well be that other constituents are involved also. This, and the relationships these abnormalities have to the mechanism of the disease, form a problem under further investigation.

We thank Professor J. V. Dacie for kindly providing many of the samples of P.N.H. blood for this study.

REFERENCES

- Altman, K. I. (1953). *Arch. Biochem.*, **42**, 478.
 Bloor, W. R. (1916). *J. biol. Chem.*, **24**, 227.
 Braunsteiner, H., Gisinger, E., and Pakesch, F. (1956). *Blood*, **11**, 753.
 Crosby, W. H. (1953). *Ibid.*, **8**, 769.
 Dawson, R. M. C. (1954). *Biochim. biophys. Acta*, **14**, 374.
 Douglas, D., and Eaton, J. C. (1955). *Lancet*, **1**, 946.
 Haas, C. S., and Isherwood, F. A. (1949). *Nature (Lond.)*, **164**, 1107.
 Koch, F. C., and McMeakin, T. L. (1924). *J. Amer. chem. Soc.*, **46**, 2066.
 Mathes, M., Schuboth, H., and Lindemann, B. (1951). *Acta haemat. (Basel)*, **5**, 193.
 Muir, H. M., Neuberger, A., and Perrone, J. C. (1952). *Biochem. J.*, **52**, 87.
 Muller, G. L. (1930). *Medicine (Baltimore)*, **9**, 119.
 Prankerd, T. A. J. (1957). Unpublished.
 — and Altman, K. I. (1954). *Biochem. J.*, **58**, 622.
 Youngburg, G. E., and Youngburg, M. V. (1930). *J. Lab. clin. Med.*, **16**, 158.

RED-CELL SURVIVAL IN PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA

EFFECT OF PHENINDIONE AND CORTISONE

BY

F. P. MULDOWNY, M.B., M.R.C.P.Ed.

M. M. BLUHM, Ph.D., B.Sc., D.I.C., A.Inst.P.

AND

E. J. WAYNE, M.D., Ph.D., F.R.C.P., F.R.F.P.S.

From the University Department of Medicine, Gardiner
Institute, Western Infirmary, Glasgow, and the
Regional Physics Department, Glasgow

Paroxysmal nocturnal haemoglobinuria (P.N.H.) is a rare form of haemolytic anaemia in which the essential abnormality lies in the cells rather than in the plasma (Dacie and Firth, 1943; Dacie and Mollison, 1949; Crosby, 1953; Dacie, 1954). The only treatment of proved value in the condition is repeated blood transfusion, but it has been claimed that anticoagulant therapy may be beneficial. The basis for this form of treatment is the observation by Crosby and Dameshek (1950) that haemolysis is produced by a factor normally present in plasma which is activated by thrombin. A patient with P.N.H. was given dicoumarol and the degree of haemolysis diminished. Haemolytic crises, however, still occurred, and the authors conclude merely that "dicoumarol offers an intriguing possibility for treatment." They mention its additional value as a protection against the spontaneous thromboses to which these patients are so liable. In a later paper Crosby (1953) refers to three cases treated by dicoumarol, with apparent success in two.

Other cases subjected to anticoagulant therapy have been recorded. Kalant and Cyr (1952) noted that treatment with dicoumarol "coincided with a remission lasting five weeks." Perkins *et al.* (1955) report four cases, in two of which they thought the evidence was in favour of a diminished degree of haemolysis when the patient was receiving dicoumarol. Wang *et al.* (1956) describe a case in which both phenindione ("dindevan") and biscoumacetate ("tromexan") were thought to have extended the interval between transfusions given at a fixed haemoglobin level.

Both corticotrophin and cortisone have been used clinically in the treatment of P.N.H. Crosby (1953) states categorically that these substances are of no benefit. Fudenberg *et al.* (1954), however, describe a case in which the patient showed symptomatic improvement during six months of corticotrophin treatment. There was no evidence of intravascular thromboses over this period. Feichtmeir *et al.* (1955) discovered a patient suffering from P.N.H. who had present in his serum a haemolysin active against normal red cells and in whom cortisone produced a remission. Dicoumarol treatment had failed.

From the evidence in favour of these two forms of treatment it is clear that the chief difficulty in assessing their value lies in the tendency for spontaneous fluctuation in severity to occur in this disease. When conventional haematological criteria are used studies must necessarily be continued over weeks or months. A more precise index of improvement is the mean lifetime of red cells, and this can be measured by the use of red cells labelled with radioactive chromium (^{51}Cr). This method has the important advantage of providing a quick assessment of results. Accordingly, the effect of phenindione and of cortisone on the survival of ^{51}Cr -labelled cells was studied in a case of P.N.H. The survival time of the patient's cells transfused into a normal recipient and that of normal donor cells transfused into the patient were investigated at the same time.

Case Report

The patient, a 37-year-old man, was first seen by us in February, 1956. His presenting complaint was of dyspnoea on exertion. He had had no illness until March, 1951, when he had been investigated elsewhere for the same symptoms. He was then considered to be suffering from an iron-deficiency anaemia of undetermined aetiology, and thereafter received sporadic doses of oral iron, and two courses of intravenous iron therapy. He continued to feel moderately lethargic and short of breath, and these symptoms became worse after a bout of "influenza" in December, 1954. He then first noticed that his urine was at times dark in colour. He was admitted to the urological department, where routine urine tests, cystoscopy, and excretion pyelography revealed no abnormality. The haemoglobin was 9.5 g. per 100 ml. and the M.C.H.C. 29%. Occult blood was absent from the faeces, and barium meal and follow-through yielded negative results. Sternal marrow smears showed normoblastic hyperplasia. Free acid was present in the gastric juice. Nothing further was added on this occasion to the previous diagnosis of iron-deficiency anaemia of undetermined aetiology. The patient was discharged on oral iron therapy, and continued to feel unwell in a greater or less degree until his admission to the medical unit. His complaint was of breathlessness on exertion, tiredness, and weakness.

On examination there was obvious pallor of skin and conjunctivae. No enlargement of liver, spleen, or lymph nodes was noted. The results of laboratory investigations were: Hb, 5.9 g. per 100 ml.; P.C.V., 20.5%; M.C.H.C., 28.9%; W.B.C., 4,000 per c.mm.; reticulocytes, 8%; platelets, 80,000 per c.mm. No significant abnormality was detected in a stained film of peripheral blood. Occult blood was not present in the faeces. The urine contained urobilin, and tests for haemoglobin were strongly positive despite the absence of red cells on microscopical examination. The serum bilirubin was 0.6 mg. per 100 ml., and 24-hour faecal urobilinogen (mean of four-day period) was 280 mg. The Wassermann and Donath-Landsteiner reactions were negative. The direct Coombs test was negative on two occasions. Ham's acidified serum test (Ham, 1939) and Crosby's acid-serum-thrombin test (Crosby, 1950) were positive on each of three occasions. Spectroscopic examination of the

patient's serum revealed the typical absorption bands of oxyhaemoglobin and methaemoglobin. Methaemalbuminaemia was not detected.

Methods

25 ml. of the patient's blood was added to a sterile rubber-capped bottle which contained 30 μ C. of ^{51}Cr in the form of sodium chromate, and to which 5 ml. of acid-citrate-dextrose solution had been added immediately beforehand. The bottle was incubated for 45 minutes at room temperature, and the cells were washed three times with sterile isotonic saline. Then 20 ml. of this saline-cell suspension was administered to the patient by intravenous injection from a calibrated syringe, and two 1-ml. samples were taken from the remainder and diluted each to 50 ml. with distilled water. The packed cell volume of the injection suspension was determined, and the activity of the supernatant saline was measured by diluting 1 ml. to 50 ml. with distilled water and counting a 10-ml. aliquot. Blood samples were taken from a different vein 15 and 30 minutes after injection. Determinations of the packed cell volume were carried out on these samples, and the remainder was lysed with saponin and 10-ml. aliquots were taken for counting. The total red-cell mass was then determined from the following equation:

$$\text{Red-cell mass} = \frac{\text{Counts/min. injected} - \text{Counts/min. in supernatant}}{\text{Counts in blood/min./ml.} \times \frac{100}{\text{P.C.V.}}}$$

The packed cell volumes of the 15- and 30-minute samples were corrected for trapped plasma according to the figures of Chaplin and Mollison (1952). The number of counts injected was calculated from the count rates of the standard dilutions as follows:

$$\text{Counts/min. injected} = \text{Counts/min./ml.} \times \text{Dilution} \times \text{Volume injected}$$

The counts in the supernatant saline were determined in the following equation:

$$\text{Counts/min. in supernatant} = \frac{\text{Counts/min./ml.} \times \text{Dilution} \times \text{Volume injected} \times \frac{100}{100 - \text{P.C.V.}}}{100}$$

The red-cell survival curve was plotted on semi-logarithmic paper by expressing the count rate of the subsequent daily blood samples as a percentage of the count rate 24 hours after administration of the dose. Each daily count rate was corrected for changes in packed cell volume where this value differed from that of the 24-hour sample. No correction was made for elution of ^{51}Cr from the red cells, the results being expressed simply in terms of the time required for one-half of the ^{51}Cr activity to disappear from the circulation (Donohue *et al.*, 1955).

Total red-cell mass estimations were derived from measurements of plasma volume by means of the Evans

blue (cellulose extraction) method (Bedwell *et al.*, 1955). Haematocrit values were obtained with Wintrobe tubes spun at 3,000 r.p.m. for 55 minutes. Correction for trapped plasma was made according to the figures of Chaplin and Mollison (1952), and the true venous haematocrit was then corrected by the factor 0.91 to calculate total body haematocrit (Chaplin *et al.*, 1953). The red-cell mass was calculated as follows:

$$\text{Red-cell mass} = \frac{\text{Plasma volume} \times \text{Total body haematocrit}}{(100 - \text{Total body haematocrit})}$$

A preliminary study of the mean survival time of the cells was carried out before treatment with phenindione and cortisone. Dosage of the former was then adjusted so as to maintain the one-stage prothrombin activity between 15 and 20% in accordance with the recommendations of Crosby (1953). This regime was maintained for seven weeks, and during the period the effect of cortisone (200 mg. daily for 12 days) was studied. At the end of this time the administration of both phenindione and cortisone was continued and the patient was given the packed washed red cells of 4 pints (2.3 litres) of group A blood as recommended by Mollison (1951). There was no evidence of any reaction, and the haemoglobin rose from 7.0 to 10.1 g. per 100 ml. Five months later his haemoglobin had fallen to 7.4 g. per 100 ml. and he was given a further transfusion of six pints (3.4 litres) of washed red cells without a reaction. He returned to work, and 10 weeks later was still engaged as a welder. His haemoglobin was then 9.9 g. per 100 ml.

Results

The results of the cell-survival studies are shown graphically in Figs. 1-3. Each point represents one measurement, the limits of error being the standard deviations calculated from the count rates obtained. The statistical error in counting was by far the most important source of error, and the limits are chosen so that there is a 70% probability of the correct result lying within them. Each sample was counted in an Ekco scintillation counter type N.550 for long enough to give at least 1,000 counts (net) except for the last eight readings in Fig. 1(b) when 500 counts (net) were obtained.

Fig. 1(a) shows the red-cell-survival curve obtained in a normal subject, and gives a value of 25 days for the half-life survival time. This is in good agreement with other published estimates (Read *et al.*, 1954).

Fig. 1(b) shows the red-cell-survival curve in the patient, phenindione being given at the point indicated. Inspection of the curve suggests that the change in half-life, if any, is too small to be detected. To confirm this, the best straight line (based on the square of the difference between the observations and the straight line being a minimum) was fitted to the observations from the 1st to the 26th day, and

FIG. 1.—Comparison of survival of red cells in a normal subject (a) and in the case of P.N.H. before and during the administration of phenindione (b).

FIG. 2.—Survival of red cells in the case of P.N.H. during the continued administration of phenindione and later of phenindione and cortisone.

FIG. 3.—(a) Survival of normal red cells transfused into the case of P.N.H. (b) Survival of red cells from the case of P.N.H. transfused into a normal subject.

again for the observations from the 27th to the 53rd day. The half-lives calculated from these straight lines did not differ significantly (see Table).

On the 35th day it became necessary to give a further dose of labelled cells, and Fig. 2 shows the resulting survival curve and the effect on it of the administration of cortisone. Phenindione therapy was continued during this phase. Again no change was detected. A straight line was fitted to the observations during cortisone therapy from the 54th to the 66th day. Again the half-life did not differ significantly from the previous value (see Table).

Effect of Drugs on Survival Time of Red Cells

Day	Experiment	⁵¹ Cr Survival Time (Days) and Standard Errors
1-26 27-53	P.N.H.: patient: no therapy during phenindione	15.5 ± 0.5
54-66	P.N.H. patient: during phenindione and cortisone	14.4 ± 0.4
68-88	Transfused normal cells in P.N.H. patient	17.5 ± 2.3
68-76	Transfused P.N.H. cells in normal subject	24 days 15 "

The survival times of transfused washed normal red cells in the patient and of the patient's washed red cells in a normal subject were also measured. The resulting curves are plotted in Fig. 3, and the half-life values are given in the Table. It is seen that haemolysis of the patient's cells continued at the same rate in the normal subject, and the normal cells survived normally in the patient.

The total red-cell mass before treatment, determined by the ⁵¹Cr method, was 921 ml. compared with the estimate of 905 ml. derived from an Evans blue (cellulose extraction) determination. The theoretically normal red-cell mass, based on total body weight, was 1,870 ± 275 ml. (Huff and Feller, 1956). After treatment the red-cell mass determined by the Evans blue technique was 990 ml.

Discussion

Previous observers have suggested that phenindione, corticotrophin, or cortisone may reduce the degree of haemolysis in P.N.H. In these experiments the measure of the rate of haemolysis has usually been indirect and has not been related to the total red-cell mass. Moreover, observations have taken place over periods of time so long that spontaneous fluctuations in the rate of haemolysis may have occurred. The observations which we present here were designed so as to avoid these defects. The mean survival time of red cells has been found to be unchanged; the total red-cell mass altered by only 85 ml. from the beginning to the end of the studies; the reticulocyte count remained at the same level during administration of the drugs. It seems clear that in our case the administration of phenindione for 26 days and of phenindione and cortisone for 12 days had no demonstrable effect on the rate of haemolysis.

We should like to stress that whenever the survival of red cells is being studied estimates of the red-cell mass at the beginning and end of the set of observations should be made. This is discussed by Strumia *et al.* (1955). In our case this was done, and no correction for this effect was necessary, since there was no significant change in the red-cell mass during the course of the study. It is also necessary to relate the 24-hour excretion of urobilinogen in the faeces to the total red-cell mass, since this can be used as a quantitative measure of haemolysis only when viewed against the background of total circulating haemoglobin (Miller *et al.*, 1942). Our case provides an illustration of the importance of this qualification. The mean 24-hour faecal urobilinogen during a four-day period was 280 mg. This value is at the upper limit of the normal range of 40-280 mg. quoted by Whitby and Britton (1953). When, however, it is expressed as a function of total circulating haemoglobin the resulting figure for the haemolytic index is 106, compared with the normal range of 11-21.

The results of observations on the cross-transfusions between the patient and a normal donor show that the abnormality lies in the cells rather than in the plasma. This agrees with the results of Dacie and Mollison (1949), who used the differential agglutination technique. The survival curve obtained over a period of 26 days before the use of drugs in our patient (Fig. 1) shows no evidence of flattening. This is in contrast to the case studied by Dacie and Mollison in which some of the cells were remarkably resistant. Hinz *et al.* (1956) studied three cases, using ⁵¹Cr techniques, and in two found evidence of a mixed population of cells, some of which disappeared at a rapid rate and others at a rate approaching the normal. They suggest that the clinical severity of the disease is related to the proportion of cells with an extremely short life. They found that the haematological finding which correlated best with the mean half-life of the red cells was the reticulocyte count. On this view our case would not be regarded as severe, and it may be significant that Crosby (1953) thinks that the less severe cases are less likely to respond to anticoagulant therapy.

Our results show that at least in one case of P.N.H. neither phenindione nor cortisone had any effect on the mean survival of the patient's red cells. The previously recorded evidence of positive results is not convincing when one takes into account the notorious variability in the course of the condition. We present these observations because the disease is rare and so far as we are aware no similar studies have been reported. We should like to point out the desirability of investigating the effects of anticoagulants on further and more severe cases of P.N.H., using the ⁵¹Cr technique. The administration of anticoagulants should be considered in all cases, since 50% of deaths have been attributed to thrombotic incidents (Crosby, 1953). We can confirm the value of transfusion with washed normal erythrocytes.

Summary

The results of studies of the blood volume and of the mean survival time of red cells with ⁵¹Cr-labelled cells in a case of paroxysmal nocturnal haemoglobinuria are presented.

Phenindione and cortisone produced no effect on the rate of haemolysis, on the total red-cell mass, or on the reticulocytosis. Transfusion with washed normal red cells preceded a long remission. The merits of the labelled-red-cell technique in the study of the treatment of haemolytic states are pointed out.

We are grateful to Professor G. M. Wilson and Dr. G. A. Tudhope, of the Department of Pharmacology and Therapeutics, University of Sheffield, for helpful advice.

REFERENCES

- Bedwell, G. A., Patterson, J., and Swale, J. (1955). *J. clin. Path.*, **8**, 61.
 Chaplin, H., and Mollison, P. L. (1952). *Blood*, **7**, 1227.
 — and Vetter, H. (1953). *J. clin. Invest.*, **32**, 1309.
 Crosby, W. H. (1950). *Blood*, **5**, 843.
 — (1953). *Ibid.*, **8**, 769.
 — and Dameshek, W. (1950). *Ibid.*, **5**, 822.
 Dacie, J. V. (1954). *The Haemolytic Anaemias*. Churchill, London.
 — and Firth, D. (1943). *Brit. med. J.*, **1**, 626.
 — and Mollison, P. L. (1949). *Lancet*, **1**, 390.
 Donohue, D. M., Motulsky, A. G., Giblett, E. R., Pirzio-Biroli, G., Viranuvatti, V., and Finch, C. A. (1955). *Brit. J. Haemat.*, **1**, 249.
 Feichtmeir, T. V., Boulware, J. M., Willett, F. M., and Perkins, H. (1955). *J. Lab. clin. Med.*, **46**, 443.
 Fudenberg, H., Palmer, W. L., and Kirsner, J. B. (1954). *Amer. J. med. Sci.*, **227**, 32.
 Ham, T. H. (1939). *Arch. intern. Med.*, **64**, 1271.
 Hinz, C. F., Hurley, T. H., and Weisman, R. (1956). *J. Lab. clin. Med.*, **48**, 495.
 Huff, R. L., and Feller, D. D. (1956). *J. clin. Invest.*, **35**, 1.
 Kalant, N., and Cyr, D. P. (1952). *Blood*, **7**, 607.
 Miller, E. B., Singer, K., and Dameshek, W. (1942). *Arch. intern. Med.*, **70**, 722.
 Mollison, P. L. (1951). *Blood Transfusion in Clinical Medicine*. Blackwell, Oxford.
 Perkins, H. A., Boulware, J. M., Feichtmeir, T. V., Thayer, W. W., and Spaet, T. H. (1955). *Ann. intern. Med.*, **43**, 1218.
 Read, R. C., Wilson, G. W., and Gardner, F. H. (1954). *Amer. J. med. Sci.*, **228**, 40.
 Strumia, M. M., Taylor, L., Sample, A. B., Colwell, L. S., and Dugan, A. (1955). *Blood*, **10**, 429.
 Wang, I., Craig, J., and Wallace, J. (1956). *Scot. med. J.*, **1**, 177.
 Whitby, L. E. H., and Britton, C. J. C. (1953). *Disorders of the Blood*, 7th ed., p. 307. Churchill, London.