Summary

The cases are presented of 24 patients with various diseases who had negative tuberculin reactions. Whilst treated with cortisone or prednisolone in moderate or low dosage the tuberculin reaction became positive at the same or higher dilutions in 20 of the cases.

The patients were mostly elderly, or showed frank signs of suprarenal cortical suppression.

The suggestion is made that steroid treatment, although able to suppress a positive tuberculin reaction when given in high dosage, may, given in lower dosage, actually restore a reaction which has been suppressed by age, infection, or suprarenal deficiency.

The implications of these findings and their possible application to the treatment of severe infections, especially in the elderly, are discussed.

I thank the physicians of the Aylesbury Hospitals for permission to investigate their patients, and in particular Dr. Wilfred Stokes and Dr. M. Saxty Good for much valuable advice and criticism. I also thank Dr. L. Nunn for the biochemical investigations and the nursing staff for assistance with the tuberculin reactions.

REFERENCES

- Citron, K. M., and Scadding, J. G. (1957). *Quart. J. Med.*, **26**, 277.
 Clarke, E. R., Zahn, D. W., and Holmes, T. H. (1954). *Amer. Rev. Tuberc.*, **69**, 351.
 Coste, F., Pierre-Bourgeois, Galmiche, P., Vic-Dupont, Mollon, R. H., Nahel, and Blatrix, C. (1951). *Rev. Tuberc. (Paris)*, **15**, 698.
 Even, R., Sors, C., Delaude, A., Roujeau, J., Trocmé, Y., and Commare, G., (1955). *Ibid.*, **19**, 1249.
 Favez, G., and Arnet, F. (1956). *Schweiz. med. Wschr.*, **86**, 843.
 Happ, W. M., and Casparis, H. R. (1922). *Amer. J. Dis. Childh.*, **23**, 527.
 Hart, P. D'A. (1932). *M.R.C. Spec. Rep. Ser. med. Res. Coun. (Lond.)*, No. 164.
 Houghton, L. E. (1954). *Lancet*, **1**, 595.
 — and Davies, D. (1954). *Tubercle (Lond.)*, **35**, 2.
 LeMaitre, C. A., Tompsett, R., Muschenheim, C., Moore, J. A., and McDermott, W. (1951). *J. clin. Invest.*, **30**, 445.
 Long, D. A., and Spensley, P. C. (1954). *Lancet*, **1**, 645.
 Long, J. B., and Favour, C. B. (1950). *Bull. Johns Hopk. Hosp.*, **87**, 186.
 McFarlane, C. N., and Truelove, L. H. (1957). *J. Obstet. Gynaec. Brit. Emp.* In press.
 O'Grady, F. (1957). *Brit. J. Tuberc.*, **51**, 74.
 Pilcher, J. D. (1930). *Amer. Rev. Tuberc.*, **21**, 669.
 Pyke, D. A., and Scadding, J. G. (1952). *Brit. med. J.*, **2**, 1126.
 Rose, Y., and Jauffret, Y. (1956). *Poumon*, **12**, 223.
 Schwartz, W. S., and Heise, F. H. (1931). *Amer. Rev. Tuberc.*, **24**, 479.
 Sulzberger, M. B., Sauer, G. C., Hermann, F., Baer, R. L., and Milberg, I. L. (1951). *J. invest. Derm.*, **16**, 323.
 Taylor, L. M., Smith, H. V., and Vollum, R. L. (1953). *Tubercle (Lond.)*, **34**, 296.
 Wilkins, E. G. (1956). *Brit. med. J.*, **1**, 883.

Speaking at a conference on mental deficiency at Manchester on November 7 and 8, Mr. RICHARD THOMPSON, Parliamentary Secretary to the Ministry of Health, said that the theme of the Royal Commission's proposals was based on the success of non-residential training and a growing tolerance of the public towards the mentally handicapped. To obtain results a closer co-ordination between health, welfare, and education services was essential, because the needs of defectives were chronic but not constant. If community care was to work public opinion must accept it, which meant abandoning some of the controls and restraints of present procedures. "We should develop a new outlook towards mental defectives not as hopeless outcasts, as has so often been the case hitherto, but more as we consider, for instance, blind people." Great progress had been made since the start of the Health Service in the organization for training mental defectives at occupation centres. In 1948 100 centres existed; to-day over 300. In 1951 just over 4,000 boys and girls under 16 were receiving training; to-day well over 9,000. Because most resources had been applied to mentally handicapped children, the more complicated problems of adult defectives had not yet been properly studied. With full employment and economic stringency it was difficult to provide staff and buildings, but this need not prevent a start being made. The conference was arranged by the National Association for Mental Health.

THE BINDING OF HAEMOGLOBIN BY PLASMA PROTEINS (HAPTOGLOBINS)

ITS BEARING ON THE "RENAL THRESHOLD" FOR HAEMOGLOBIN AND THE AETIOLOGY OF HAEMOGLOBINURIA

BY

A. C. ALLISON, D.Phil., B.M.

AND

W. ap REES, B.Sc.

Medical Research Council Unit for Research in Cell
Metabolism, Department of Biochemistry, University of
Oxford

Jayle and his colleagues (Jayle, 1939, *et seq.*, see Jayle and Boussier, 1955) have carried out an extensive series of investigations on serum proteins that have the striking property of combining with haemoglobin. These proteins have been termed haptoglobins, from the Greek root $\alpha\pi\tau\epsilon\iota\nu$, meaning "to hold fast." The haptoglobin migrates with the α_2 fraction on electrophoresis in the Tiselius apparatus and on filter paper, but when haemoglobin is added the haptohaemoglobin complex has a mobility corresponding to that of β_1 -globulin (Jayle, Boussier, and Badin, 1952). This observation has been confirmed by Wieme (1953), Tuttle (1955), and Reich (1956). Tuttle failed to find the haptoglobin in umbilical cord blood, although it was present in the blood of older children.

In an interesting series of experiments, Smithies (1955a, 1955b) observed that when mixtures of haemoglobin and serum are submitted to electrophoresis in starch gels the resulting patterns differ from subject to subject in a manner described fully below. Since the haptoglobins appear to be the only serum proteins that combine with haemoglobin, there is little doubt that Smithies was describing individual differences in haptoglobins. Smithies reported that the haptoglobins form a complex with about 125 mg. of haemoglobin per 100 ml. of plasma, any excess of haemoglobin above this figure remaining uncombined. Smithies, like Tuttle, was unable to find any haptoglobins in umbilical cord blood.

We have been able to confirm and extend the findings of Smithies and have obtained evidence that the binding of haemoglobin by plasma proteins has a bearing on the circumstances under which methaemalbumin is formed, on the problem of the so-called "renal threshold" for haemoglobin, and on the aetiology of haemoglobinuria.

Material and Methods of Investigation

Serum specimens from 12 normal subjects and 7 patients with haemoglobinuria have been studied. About 5 ml. of blood was allowed to run down the side of a centrifuge tube. The clot was allowed to form and retract for six hours at room temperature, and serum was removed after centrifugation. The serum proteins were submitted to electrophoresis in starch gels in borate buffer, pH 8.48, using the technique of Smithies (1955b). After electrophoresis the gels were sliced horizontally into two parts, one of which was stained with amidoschwartz B (Gurr) while the other was stained for haem proteins by benzidine (Franklin and Quastel, 1949) or

orthotolidine (O'Kelly and Kohn, 1955). Both the latter, especially the benzidine technique, are extremely useful, since they show quite clearly haem-protein bands that are scarcely visible or quite invisible when not specifically stained. Their application has enabled us to extend the observations of Smithies on the binding of haemoglobin by plasma proteins in normal subjects and to correct his description in some points of detail.

Oxyhaemoglobin solutions were prepared by collecting erythrocytes in 3% sodium citrate, and washing them three times in 0.9% sodium chloride. To each volume of packed cells two volumes of distilled water were added. The mixture was frozen twice to facilitate complete lysis of the cells, and the preparation was centrifuged at 10,000 *g* for 1 hour to sediment the erythrocyte stroma. The concentration of oxyhaemoglobin in the supernatant was measured in a Unicam SP.600 spectrophotometer at 539 *mμ* (Emm=59.6). Methaemoglobin was prepared by treating this oxyhaemoglobin solution with an equal volume of a 0.5% solution of $K_3Fe(CN)_6$ for two hours at room temperature. The excess ferricyanide was removed by dialysis and the concentration of methaemoglobin measured by the absolute extinction at 500 *mμ* (Emm=38.2). Methaemalbumin was prepared by the method of Fairley (1941). Purified haematin (B.D.H.) was dissolved in 0.01N NaOH (1 mg. per ml.), and one volume of this solution added to five volumes of serum to give a clear brown methaemalbumin solution. Absolute extinction values of all haem-proteins were obtained from iron analyses carried out by the method of Paul (1948).

Binding of Haemoglobin by Haptoglobins in Different Subjects

Varying quantities of oxyhaemoglobin (from 25 to 200 mg. per 100 ml.) were added to sera from different subjects and submitted to electrophoresis. The great majority of subjects fall into one of three groups with respect to the patterns obtained. For these groups the revised terminology of Smithies and Walker (1956) is used.

Subjects in group 1-1 have only one haptoglobin. In the absence of haemoglobin, this protein migrates in the fast α_2 position (Fig. 1 A). When small quantities of oxyhaemoglobin have been added, two haemoglobin-containing bands appear (Fig. 1, B). One of these bands is intermediate in mobility between the fast α_2 - and β -globulins while the other corresponds in mobility to β -globulin. When the quantity of added oxyhaemoglobin is raised, the first band disappears and the second band is intensified (Fig. 1, C). The interpretation of this observation will be discussed in detail elsewhere, but our tentative conclusion can be summarized here. Jayle, Boussier, and Tonnelat (1956) have produced evidence that each molecule of haptoglobin₁ (the type commonly observed in the blood) can combine with two molecules of haemoglobin. This suggests that the haptoglobin molecule might form a complex with either one or two molecules of haemoglobin, according to the equation:

where Hp stands for haptoglobin and HbO_2 for oxyhaemoglobin. We have concluded that the first band described above represents $Hp(HbO_2)$ and the second $Hp(HbO_2)_2$. As the quantity of added oxyhaemoglobin is raised, the second band increases at the expense of the first according to the phase rule.

When the quantity of added oxyhaemoglobin exceeds 135 mg. per 100 ml. another band appears (Fig. 1, D). This seems to represent uncombined oxyhaemoglobin, since it lies between the β - and the slow α_2 -globulins, which is the

position taken by oxyhaemoglobin when submitted to electrophoresis alone under comparable conditions. Smithies (1955b) states that the free oxyhaemoglobin band is intermediate between the β - and fast α_2 -globulins, but in all our experiments it has been in the position described, between the slow α_2 and β bands.

Subjects belonging to group 2-2 have three haptoglobin bands in the $\alpha\beta$ region (Fig. 2, A). When small quantities

FIG. 1.—Serum from a subject belonging to group 1-1. In A all the serum proteins are shown in the absence of haemoglobin. The protein bands are, in order from the origin (O): slow α_2 , post- β , β , fast α_1 , post-albumin, and albumin. The pre-albumin bands are not included in the photograph. When 50 mg. of oxyhaemoglobin is added per 100 ml. of serum, two haptoglobinaemia bands are shown by staining with benzidine (B). The faster-moving band appears to be $Hp(HbO_2)$ and the slower-moving $Hp(HbO_2)_2$. When the concentration of oxyhaemoglobin is raised to 135 mg. per 100 ml. the former disappears (C). When the concentration of oxyhaemoglobin exceeds 135 mg. free oxyhaemoglobin appears behind the haptoglobinaemia band (D).

FIG. 2.—Serum from a subject belonging to group 2-2. In A all the serum proteins are shown in the absence of haemoglobin. They are, in order from the origin (O): slow α_2 , three $\alpha\beta$ bands, β , fast α_1 , post-albumin, and albumin. When 100 mg. of oxyhaemoglobin per 100 ml of serum is added, three haptoglobinaemia bands are observed (B). C shows serum from a subject belonging to group 1-2. The three $\alpha\beta$ -haptoglobinaemia bands have migrated further than in the serum from a group 2-2 subject. When 135 mg. of oxyhaemoglobin is added (D) four haptoglobinaemia bands are shown by staining with benzidine.

of oxyhaemoglobin are added, three rather faint benzidine-staining bands, all showing slightly slower mobility than the uncombined haptoglobin bands, are observed; these may represent complexes of $\text{Hp}(\text{HbO}_2)$. When the added oxyhaemoglobin is increased, three benzidine-staining bands, with a still lower mobility, are observed (Fig. 2, B). The slowest moving band is rather faint, so that it is difficult to see without specific staining, but the other two are more intense. It is believed that these represent the three $\text{Hp}(\text{HbO}_2)_2$ complexes. In addition, a fourth, rather faint, band with a slightly greater mobility is usually observed; this may represent $\text{Hp}(\text{HbO}_2)$. When the concentration of added oxyhaemoglobin exceeds 100 to 120 mg. (the exact level varying from subject to subject), an uncombined oxyhaemoglobin band appears in the usual position.

In the third group of subjects (group 1-2), all four haptoglobins are present (Fig. 2, C). Three of the haptoglobins lie in the $\alpha\beta$ position, although their mobility is slightly greater than that of the haptoglobins of subjects belonging to group 2-2. The fourth haptoglobin (which was overlooked by Smithies) migrates with the fast α_2 -globulin and corresponds to the haptoglobin in subjects belonging to group 1-1. On addition of oxyhaemoglobin patterns are obtained which resemble a combination of those previously described in group 1-1 and 2-2 sera (Fig. 2, D); however, the $\alpha\beta$ -haptoglobin complexes in group 1-2 subjects always show a somewhat greater mobility than those of group 2-2 subjects. Oxyhaemoglobin added in excess of 135 mg. per 100 ml. remains uncombined.

As pointed out by Tuttle and by Smithies, serum from umbilical-cord blood lacks haptoglobins altogether. A few older children and adults, apparently in good health, have been observed who have no demonstrable haptoglobins or who have only a single haptoglobin band in the $\alpha\beta$ region. Nevertheless, it can be concluded that the great majority of human subjects have proteins in their plasma (haptoglobins) that are capable of combining firmly with oxyhaemoglobin in concentrations up to 100 to 135 mg. per 100 ml. The electrophoretic mobility of the haptoglobin varies from subject to subject and allows the identification of three main groups of subjects. There is no doubt that these groups are genetically determined. The pattern of inheritance usually follows that postulated by Smithies and Walker (1955); the common haptoglobin types (1-1, 2-2, and 2-1) could be the three phenotypes expected in subjects homozygous and heterozygous for a single pair of allelomorphous genes. However, the existence of individuals showing other haptoglobin patterns, together with some of the findings in family studies, suggests that the mode of inheritance is more complex. This aspect of the problem will be discussed in detail elsewhere.

Methaemalbumin Formation

It has already been pointed out that when oxyhaemoglobin is added to serum in concentrations up to 100 mg. per 100 ml. in some subjects and 135 mg. per 100 ml. in others, all the haem-protein present, as shown by the benzidine and orthotolidine reactions, is in the form of the haptoglobin complex. As soon as oxyhaemoglobin is added in excess of the combining power of the haptoglobins, however, material staining with benzidine and orthotolidine appears in the albumin region as well as in the position of free oxyhaemoglobin (Fig. 3, A and B). The haem-protein in the albumin region is in all probability methaemalbumin, since it corresponds in electrophoretic mobility to methaemalbumin prepared artificially.

In addition to the large and dense methaemalbumin band, a rather faint haem-protein band in the position of the fast α_2 -globulins is usually observed. This band has a mobility quite different from methaemoglobin, and may represent a complex of haem and globulin. The concentration appears to be usually about 5% of that of methaemalbumin.

The identification of methaemalbumin by benzidine staining of serum proteins after filter-paper electrophoresis has been reported by Hensley and Blackburn (1952). This is a sensitive method for detecting the compound, but staining of starch gels, which contain more protein, further increases the sensitivity. Quantities of methaemalbumin of the order of one-hundredth of those detectable spectroscopically can be identified in this way. Methaemalbumin was observed in all our subjects when the added oxyhaemoglobin concentration was 150 mg. or higher. Fairley (1940) was able to

FIG. 3.—Serum from Case 1 with paroxysmal nocturnal haemoglobinuria (A) and from Case 6 with recurrent cold haemoglobinuria (B), stained with benzidine. Only uncombined oxyhaemoglobin (HbO_2) and methaemalbumin (Mhalb.) are visible.

recognize methaemalbumin spectroscopically after oxyhaemoglobin injections in human subjects when the concentration in the plasma exceeded 200 mg. per 100 ml.

In none of our experiments was methaemoglobin observed after addition of oxyhaemoglobin to serum. The electrophoretic mobility of methaemoglobin was determined by adding the compound to serum shortly before electrophoresis. Free methaemoglobin was found to have a mobility less than that of oxyhaemoglobin and slightly greater than that of the slow α_2 -globulins. Some of the methaemoglobin was bound by haptoglobins and some transformed into methaemalbumin.

These observations suggest that oxyhaemoglobin forms a relatively stable complex with haptoglobin. Free oxyhaemoglobin, on the other hand, is unstable and breaks down to liberate haem groups which are immediately oxidized and combine with albumin to form methaemalbumin. If methaemoglobin is an intermediary in the reaction, it must disappear rapidly, since it cannot normally be detected at all. Thus methaemalbumin is formed only when the concentration of oxyhaemoglobin in the plasma is greater than that which can be bound by haptoglobins.

Relationship of the Binding of Haemoglobin by Haptoglobins to the "Renal Threshold" for Haemoglobin

A great deal of experimental work on dogs and on human subjects has established that when the concentration of haemoglobin in the plasma is below a threshold level no haemoglobin can be detected in the urine. In dogs the haemoglobin threshold varies from 80 to 150 mg. per 100 ml. plasma (Lichty, Havill, and Whipple, 1932; Monke and Yuile, 1932; Yuile, 1942) and in man it varies from 100 to 140 mg. per 100 ml., being in the majority of subjects about 135 mg. per 100 ml. (Ottenberg and Fox, 1938; Gilligan, Altschule, and Katersky, 1941; Blackburn, Hensley, Kerr Grant, and Wright, 1954). Once overt haemoglobinuria is established, however, in both dog and man it continues until the level of haemoglobin in the plasma falls to about 50 mg. per 100 ml., less than one-half of the original threshold level.

The explanation of Lichty *et al.* for the renal threshold is that haemoglobin passes through the glomeruli even at low plasma concentrations, only to be reabsorbed by the renal tubules. If the plasma concentration exceeds the threshold level the concentration of haemoglobin in the glomerular

filtrate exceeds the capacity of the renal tubules for reabsorption and haemoglobinuria occurs. This explanation has been generally accepted (Yuile, 1942; Smith, 1951; Dacie, 1954).

It has also been shown that shortly after haemoglobin injections above threshold levels the epithelial cells of the convoluted tubules of the kidney contain minute brown granules. These do not at first give the Prussian-blue reaction for iron, but can be stained by various methods which are more or less specific for haemoglobin (Fukuda and Oliver, 1923; Köster, 1937; Lison, 1938). After a few days the granules appear larger and stain readily for iron (Bogniard and Whipple, 1932). In chronic haemoglobinurias in man this process leads to a striking degree of siderosis of the kidneys (see Dacie, 1954). Even after very large injections of haemoglobin only part of the haemoglobin injected appears in the urine. There is thus no doubt that the convoluted tubules of the kidney can reabsorb and degrade haemoglobin.

However, a number of observations on record are difficult to reconcile with this explanation of the renal threshold for haemoglobin. In the first place, after repeated subthreshold injections of haemoglobin in dogs no deposits of iron-containing material can be observed in the kidney tubules (Bogniard and Whipple, 1932); at least some iron should be visible if haemoglobin had passed through into the glomerular filtrate and been reabsorbed. Second, the threshold level is reduced to less than one-half its original value by haemoglobinuria or repeated diminishing daily injections of haemoglobin into dogs (Lichty *et al.*, 1932). The lowering of the threshold has been explained by assuming that the tubular epithelial cells become filled up with haemoglobin and its degradation products to such an extent that their capacity to reabsorb haemoglobin is diminished. However, experimental results fail to support this view. Yuile *et al.* (1941) studied iron retention in the kidneys of dogs after injection of haemoglobin labelled with radioactive iron. Large injections, sufficient to raise the plasma concentration of haemoglobin to about 700 mg. per 100 ml., were given to dogs that had not previously received any haemoglobin and to others in which the threshold had been markedly lowered by repeated injections. When the perfused kidneys were analysed, only about 16% of the injected iron was present in the kidneys of the normal animals, in contrast with well over 20% in the kidneys of comparable animals with lowered thresholds.

Moreover, in both dogs and man the rate of haemoglobin excretion is proportional to the concentration of haemoglobin above the threshold level in the plasma up to very high concentrations (1,300 mg. per 100 ml.—see Yuile, 1942). It is difficult to reconcile this finding with an injury phenomenon increasing glomerular permeability or a process of tubular saturation leading to diminished reabsorption.

We have been led to an alternative explanation of the threshold for haemoglobin from consideration of the remarkable coincidence between the haemoglobin-combining power of the haptoglobins and the threshold level of haemoglobin in the plasma. We suggest that haemoglobin bound to haptoglobin does not appear in the glomerular filtrate at all. Jayle *et al.* (1956) have concluded that the common haptoglobin in plasma has a molecular weight of the order of 175,000 and that it does not appear in the urine of patients with renal disease. It is thus even less likely that the haptoglobin-haemoglobin complex (the molecular weight of which is about 310,000) would traverse the glomerular filter. Free oxyhaemoglobin, on the other hand, seems to pass into the glomerular filtrate relatively easily, which accounts for the proportionality between the rate of haemoglobin excretion and the concentration of haemoglobin above threshold level in the plasma.

The same observation implies that a nearly constant proportion of the haemoglobin present in the glomerular filtrate is reabsorbed in the renal tubules.

On this hypothesis, haemoglobinuria would be expected whenever the level of oxyhaemoglobin in the plasma exceeds the haemoglobin-combining power of the haptoglobins, which is in fact the case. Furthermore, haemoglobin injected to subthreshold concentrations would all be bound by haptoglobins, so that none would appear in the glomerular filtrate and be reabsorbed by the renal tubules. As stated above, no iron can be demonstrated in the renal tubules in these circumstances. The diminished threshold after repeated injections of haemoglobin would be due to the removal of haptoglobin from the plasma at a rate greater than the rate of replacement of haptoglobin. Hence, in animals receiving injections of haemoglobin after previous injections of the compound, less haemoglobin would be bound by haptoglobin and more would escape into the glomerular filtrate than in animals receiving a single haemoglobin injection. This would explain the comparatively high concentration of labelled iron found by Yuile *et al.* (1941) in the kidneys of animals receiving repeated haemoglobin injections.

Haptoglobins in Patients with Chronic Haemoglobinurias

It follows from this explanation of the "renal threshold" for haemoglobin that haemoglobinuria would be expected clinically in two circumstances. The first would be when an episode of severe acute intravascular haemolysis liberates enough haemoglobin to raise the concentration in the plasma above 135 mg. per 100 ml. Haemolysis of less than 1% of the circulating erythrocytes would be sufficient to achieve acute haemoglobinuria of this type, which might occur, for instance, in transfusion reactions or blackwater fever. Second, haemoglobinuria might occur in the presence of less severe intravascular haemolysis if for any reason the concentration of haptoglobin was much lower than usual.

For this reason it seemed worth testing the sera of patients with chronic haemoglobinuria for haptoglobins. Through the courtesy of Professor J. V. Dacie, Professor L. J. Witts, and Dr. Sheila Callender sera were obtained from four patients with paroxysmal nocturnal haemoglobinuria, one with paroxysmal cold haemoglobinuria, and two with acute cold haemoglobinuria.

Paroxysmal Nocturnal Haemoglobinuria

Paroxysmal nocturnal haemoglobinuria can be defined as a chronic haemolytic anaemia accompanied by attacks of haemoglobinuria occurring chiefly at night, associated with a characteristic defect in the erythrocytes. The abnormality of the erythrocytes is manifested by an increased susceptibility to haemolysis in acidified serum. This can be demonstrated conveniently by Ham's (1937) test. A description of the findings in four typical cases of paroxysmal nocturnal haemoglobinuria follows.

Case 1.—A female laundry worker, now aged 29, was admitted to the Radcliffe Infirmary, Oxford, in 1950, under the care of Professor L. J. Witts. She had a three-month history of lassitude, breathlessness on exertion, and increasing pallor. On examination she was strikingly pale, with a slight yellow tinge to the skin. There were no physical abnormalities; the liver and spleen were impalpable. The erythrocyte count was 860,000 per c.mm., the haemoglobin level 3.0 g. per 100 ml., and the reticulocyte count 10%. The peripheral blood film showed only polychromasia, with slight macrocytosis and anisocytosis. The bone-marrow film showed erythroid hyperplasia. The direct antiglobulin (Coombs), Wassermann, and Kahn tests were negative. The urine at this time showed only increased urobilinogen. Vitamin B₁₂ and folic acid were given for a fortnight without response, and she was then slowly transfused with the packed cells from 9 pints (5.1 litres) of blood.

Following discharge from hospital, the patient was well for two months, after which she had a shivering attack with

pain in the loins. The urine passed in the early morning was almost black, but it was paler during the rest of the day. This continued for five days, after which she was readmitted to the Radcliffe Infirmary. The erythrocyte count had fallen to 2,300,000 per c.mm. and the haemoglobin to 7.5 g. per 100 ml. The reticulocyte count was 6.4%. The plasma bilirubin concentration was 2.2 mg. per 100 ml., and spectroscopic examination of the plasma showed the presence of methaemalbumin. The urine contained urobilinogen in excess of normal, together with oxyhaemoglobin and methaemoglobin. No cells were seen on microscopical examination of the urine. Ham's test produced gross haemolysis, but Donath and Landsteiner's test for cold haemolysins (see below) was negative. Paroxysmal nocturnal haemoglobinuria was diagnosed and the patient was transferred to the Postgraduate Medical School under Professor J. V. Dacie, who confirmed the laboratory findings and diagnosis.

During the past seven years the patient has had 12 relapses with similar symptoms and has been admitted to hospital for transfusion with packed erythrocytes.

A serum specimen was obtained from the patient when she was visiting the out-patient department during a period of remission, when she had had no attack for three months and was feeling well. The haemoglobin was 11.9 g. per 100 ml. and the reticulocyte count 0.6%. The serum obtained was coloured faintly red and was submitted to electrophoresis without added oxyhaemoglobin. The resulting pattern is shown in Fig. 3, A. There was no evidence of haemoglobin binding by haptoglobin. On staining with benzidine, distinct free oxyhaemoglobin and methaemalbumin bands were evident.

Case 2.—A 55-year-old storeman had a nine years' history of pallor and weakness, aggravated at intervals when there was haemoglobinuria and slight jaundice. The condition was diagnosed in 1951, the patient being admitted to the Radcliffe Infirmary under Professor Witts. Two days before admission he had an acute febrile episode with pallor, slight jaundice, and dark urine. On admission he was found to have an erythrocyte count of 3,740,000 per c.mm. The plasma contained 2.5 mg. of bilirubin per 100 ml., and also oxyhaemoglobin and methaemalbumin. Haemoglobin and moderate amounts of albumin were found in the urine. The serum contained no demonstrable haemolysins or agglutinins, a finding confirmed by Professor J. V. Dacie. The diagnosis was paroxysmal nocturnal haemoglobinuria, and the patient was transfused with the packed erythrocytes of 3½ pints (2 litres) of blood. On four subsequent occasions the patient was readmitted to hospital with similar symptoms and treated by transfusions with packed cells. On the last two attendances at the out-patient clinic, when he had been symptom-free for 9 and 11 months, respectively, blood specimens were collected. The haemoglobin at these visits was 13.0 and 13.4 g. per 100 ml. respectively, and the reticulocyte count 1% on the latter occasion. The sera were faintly red and were submitted to electrophoresis in starch gels without added haemoglobin. On both occasions the results were very similar to those shown in Case 1. The oxyhaemoglobin migrated in the free position, with no sign of a haptoglobinaemia complex, and dense methaemalbumin bands were present.

Case 3.—This is a case of severe nocturnal haemoglobinuria in a man now aged 51. The history is described by Dacie (1954) in his report of Case 32 on page 442. The serum was distinctly red and was found on electrophoresis to contain moderate amounts of free oxyhaemoglobin and methaemalbumin but no bound haemoglobin.

Case 4.—The history is given by Dacie (1954) in his description of Case 33 on page 442. It is that of a typical, moderately severe case of paroxysmal nocturnal haemoglobinuria of 17 years' duration in a single woman now aged 52. The serum was faintly red, and was found on electrophoresis to contain free oxyhaemoglobin and methaemalbumin only.

Case 5.—This patient, now aged 37, is described by Dacie (1954) as Case 35, on page 445. He had been treated for (aplastic ?) anaemia in childhood. In 1939 his blood was observed to undergo rapid spontaneous haemolysis, and this led to the discovery that his erythrocytes behaved *in vitro* in exactly the same way as paroxysmal nocturnal haemoglobinuria erythrocytes. However, he never had haemoglobinuria. Laboratory tests for the paroxysmal nocturnal haemoglobinuria abnormality were positive in 1942 but are now negative. The serum was clear, and 135 mg. of oxyhaemoglobin per 100 ml. was added before electrophoresis. All the haemoglobin was bound by haptoglobins, the pattern being typical of subjects belonging to group 1-2.

Paroxysmal Cold Haemoglobinuria

This is a haemolytic syndrome characterized by repeated episodes of haemoglobinuria following local or general exposure to cold. In 1904 Donath and Landsteiner showed that the haemoglobinuria is due to sudden intravascular haemolysis resulting from the action of a cold haemolysin present in the patient's blood. The haemolysin is absorbed by erythrocytes only at low temperatures, and haemolysis ensues when the cells are warmed and take up complement.

A description of the findings in a case of this type and in two cases in which there was a single episode of cold haemoglobinuria follows.

Case 6.—This is Case 3 of Ferriman, Dacie, Keele, and Fullerton (1951). The patient, a married woman aged 73, had chronic Raynaud's phenomena, haemolytic anaemia, and cold haemoglobinuria. High-titre cold haemolysins were present in her serum. Before electrophoresis 135 mg. of oxyhaemoglobin per 100 ml. was added to the serum. No binding of haemoglobin by haptoglobins was observed. The only haem-proteins present were free oxyhaemoglobin and methaemalbumin (Fig. 3, B).

Case 7.—The history is described by Dacie (1954) as Case 18, on page 288. The patient, a girl now aged 7, had two attacks of cold haemoglobinuria following measles. She was found to have in her serum a potent cold haemolysin of the Donath-Landsteiner type, which was active against normal erythrocytes as well as her own erythrocytes up to at least 25° C. The disappearance of the haemolysin over a period of three months coincided with her complete recovery, and there has been no recurrence of symptoms or abnormal laboratory findings. The serum specimen obtained was free from haemoglobin, and 100 mg. of oxyhaemoglobin per 100 ml. was added before starch gel electrophoresis. None of the haemoglobin was bound by haptoglobin, most of it migrating as free oxyhaemoglobin. A relatively small band of methaemalbumin was also present.

Case 8.—This is Dacie's (1954) Case 17, page 287. An 18-year-old soldier while undergoing military training in very cold conditions passed a specimen of urine which was dark red in colour. The haemoglobinuria lasted for about half the next day. He had never had this symptom before, nor has there been any recurrence. One month after this episode Dacie demonstrated the presence of a cold haemolysin of the Donath-Landsteiner type in the patient's serum; its activity *in vitro* slowly declined, but the antibody was still present three and a half years after the initial and only attack of haemoglobinuria. A recent specimen of serum contained no visible haemoglobin, and 100 mg. of oxyhaemoglobin per 100 ml. was added before electrophoresis. All the haemoglobin was present in the form of a complex with haptoglobin, the pattern being typical of that in subjects belonging to group 1-2.

Serum specimens from two other patients with haemolytic anaemias and high-titre cold haemolysins, but with no history of haemoglobinuria, were analysed. Both contained haptoglobins, the one belonging to group 1-2 and the other to group 2-2.

March Haemoglobinuria

A third recognized type of haemoglobinuria, march haemoglobinuria, is observed in a small proportion of subjects after severe exercise—for example, route marches or marathon races. The serum of one subject with this syndrome was available for analysis.

Case 9.—This subject, seen through the courtesy of Drs. R. G. Macfarlane and M. Shields, of Radcliffe Infirmary, Oxford, was a 22-year-old undergraduate. He was well until two years ago, when after running five miles in a marathon race he developed lower abdominal pain and nausea. A specimen of urine passed shortly afterwards was bright orange-red. These symptoms have recurred on two subsequent occasions. He can play ordinary games without difficulty.

No physical abnormalities were found on examination. The haemoglobin was 16 g. per 100 ml. and the white-cell count 5,700 per 100 ml. The blood film was normal, without increased polychromasia. The erythrocyte sedimentation rate (Westergren) was 6 mm. per hour. A specimen of serum was free from haemoglobin. The patient returned to the laboratory after running five miles. He had lower abdominal pain and nausea, and passed a specimen of urine distinctly red in colour. The characteristic absorption bands of oxyhaemoglobin at 540 and 570 $m\mu$ were found, and the benzidine test was strongly positive. The urine contained 300 mg. of protein per 100 ml. and only occasional red and white cells and hyaline casts. A second specimen of serum removed at this time was coloured distinctly red, and on electrophoresis showed an unusual pattern. Small bands of combined haemoglobin were present in the slow α_2 and β positions, and a third band of free oxyhaemoglobin was present between the two. A similar picture was obtained when 50 mg. of oxyhaemoglobin per 100 ml. was added to the specimen of serum obtained before exercise. When 100 mg. of oxyhaemoglobin was added to this serum a similar pattern was obtained but the band of uncombined haemoglobin was intensified and a distinct methaemalbumin band was observed. It can therefore be concluded that the haptoglobins in this subject are unusual in that only a single $\alpha\beta$ is present along with a small fast α_2 component, and that both can combine with less than 50 mg. of oxyhaemoglobin per 100 ml. as compared with the usual value of 120 mg. or more.

Comment

Thus in all four patients with paroxysmal nocturnal haemoglobinuria studied, and two of the three patients with cold haemoglobinuria, the haptoglobins were absent or present only in quantities too small to be detected. In the single case of march haemoglobinuria studied the haptoglobin concentration was less than half the normal value. It is inferred that the susceptibility of these subjects to haemoglobinuria may be related to the low level of haptoglobins in the plasma, which would be expected to reduce the "renal threshold" for haemoglobin.

There are two obvious explanations for the low level of the haptoglobins in these subjects: first, that the rate of formation or liberation into plasma is markedly reduced, for genetic or other reasons; or second, that because of continuous intravascular haemolysis the haptoglobins combine with oxyhaemoglobin as soon as they are released into the plasma and are then rapidly removed, so that their concentration at any time is low. It is not possible to decide between these alternatives on the available evidence. In paroxysmal nocturnal haemoglobinuria the well-known defect in the erythrocytes would seem to be the primary abnormality, and the low plasma haptoglobin level may be secondary. To suppose that a coincidence of defective erythrocytes and congenital absence of haptoglobins is required for full development of the haemoglobinuric syndrome does not seem to be an economical hypothesis. However, this is not impossible, since cases are known—for example, Case 5—in which the typical erythrocyte defect

was present without haemoglobinuria and in which the haptoglobin level was high when tested later.

In cold haemoglobinuria low plasma haptoglobin may be one of the primary aetiological factors. Many subjects show haemolysis both *in vivo* and *in vitro* in the presence of cold antibodies in high titre without any signs of haemoglobinuria (Dacie, 1954). In the two such subjects tested by us haptoglobins were present, as they were in two subjects with severe acquired haemolytic anaemias with warm antibodies. Haemoglobinuria might be expected when the intravascular haemolysis was so severe that enough oxyhaemoglobin would be released to exceed the combining power of the haptoglobins, as in the single episode of haemoglobinuria in Case 9, whose serum was later shown to have normal haptoglobins. Less severe intravascular haemolysis would produce haemoglobinuria if the haptoglobin level was already low. This may be the explanation of the absence of haptoglobins in Case 7 long after the patient ceased to show signs of intravascular haemolysis. A similar mechanism presumably operates in march haemoglobinuria. After severe exercise some subjects show well-marked haemoglobinuria (Gilligan, Altschule, and Katersky, 1943). Haemoglobinuria might occur in a small proportion of these if the concentration of liberated oxyhaemoglobin in the blood rose above the normal combining power of the haptoglobins or if the concentration of haptoglobin in the plasma was originally low, as in Case 9.

These observations provide a clue to the physiological and genetical significance of the haptoglobins. For the binding of haemoglobin in the plasma prevents the loss of this substance from the body through the kidneys and must play a part in iron retention, upon which the critical iron balance depends.

Summary

Proteins (haptoglobins) exist in human plasma that combine specifically with oxyhaemoglobin when the concentration of the latter is up to about 135 mg. per 100 ml. Four distinct haptoglobins, occurring in three common and a number of rare combinations, have been distinguished by electrophoretic analysis by previous workers and the authors.

The complex of oxyhaemoglobin with haptoglobin is relatively stable. When the concentration of oxyhaemoglobin in serum or plasma exceeds the combining power of the haptoglobins, however, the uncombined oxyhaemoglobin is rapidly broken down, with the formation of methaemalbumin.

The haptohaemoglobin complex probably does not pass into the glomerular filtrate, which free oxyhaemoglobin does readily. Hence haemoglobinuria is observed only when the level of oxyhaemoglobin in the plasma exceeds the combining power of the haptoglobins. In the sera of four cases of paroxysmal nocturnal haemoglobinuria and two out of three cases of cold haemoglobinuria studied, no haptoglobins could be demonstrated. In one case of march haemoglobinuria the concentration of haptoglobin was considerably below half the normal value. It is concluded that the susceptibility of these subjects to haemoglobinuria is related to the low level or absence of haptoglobins in the plasma.

Specimens of serum from patients with haemoglobinuria and haemolytic anaemia were obtained through the courtesy of Professor J. V. Dacie, of the Postgraduate Medical School, London, and Professor L. J. Witts, Dr. S. Callender, Dr. R. G. Macfarlane, and Dr. M. Shields, of the Radcliffe Infirmary, Oxford. We are indebted to Professor H. A. Krebs, Professor Dacie, and Dr. Macfarlane for reading and commenting on the typescript of this paper.

ADDENDUM.—After this paper was submitted for publication a paper by C. B. Laurell and M. Nyman has appeared

(*Blood*, 1957, 12, 493) suggesting that haptoglobins determine the renal threshold for haemoglobin. However, no observations were made on patients with haemoglobinurias.

REFERENCES

- Blackburn, C. R. B., Hensley, W. J., Grant, D. Kerr, and Wright, F. B. (1954). *J. clin. Invest.*, 33, 825.
 Bogniard, R. P., and Whipple, G. H. (1932). *J. exp. Med.*, 55, 653.
 Dacie, J. V. (1954). *The Haemolytic Anaemias*. Churchill, London.
 Donath, J., and Landsteiner, K. (1904). *Münch. med. Wschr.*, 51, 1590.
 Fairley, N. H. (1943). *Brit. med. J.*, 2, 213.
 — (1941). *Quart. J. Med.*, 10, 115.
 Ferriman, D. G., Dacie, J. V., Keele, K. D., and Fullerton, Jane M. (1951). *Ibid.*, 20, 275.
 Franklin, A. E., and Quastel, J. H. (1949). *Science*, 110, 447.
 Fukuda, Y., and Oliver, J. (1923). *J. exp. Med.*, 37, 83.
 Gilligan, D. R., Altschule, M. D., and Katersky, E. M. (1941). *J. clin. Invest.*, 20, 177.
 — (1943). *Ibid.*, 22, 859.
 Ham, T. H. (1937). *New Engl. J. Med.*, 217, 915.
 Hensley, W. J., and Blackburn, C. R. B. (1952). *Aust. J. Sci.*, 15, 66.
 Jayle, M. F. (1939). *Bull. Soc. Chim. biol. (Paris)*, 21, 14.
 — and Boussier, G. (1955). *Expos. ann. Biochim. méd.*, 17, 157.
 — and Badier, J. (1952). *Bull. Soc. Chim. biol. (Paris)*, 34, 1063.
 — and Tonneat, J. (1956). *Ibid.*, 38, 343.
 Köster, H. (1937). *Beitr. path. Anat.*, 100, 100.
 Lichty, J. A., Havill, W. H., and Whipple, G. H. (1932). *J. exp. Med.*, 55, 603.
 Lison, L. (1938). *Beitr. path. Anat.*, 101, 94.
 Monke, J. V., and Yuile, C. L. (1940). *J. exp. Med.*, 72, 149.
 O'Kelly, T., and Kohn, J. (1955). *J. clin. Path.*, 8, 249.
 Ottenberg, R., and Fox, C. L., jun. (1938). *Amer. J. Physiol.*, 123, 516.
 Paul, K. G. (1948). *Acta chem. scand.*, 2, 430.
 Reich, M. (1956). *Aust. J. exp. Biol. med. Sci.*, 34, 343.
 Smith, H. W. (1951). *The Kidney*. Oxford Univ. Press, New York.
 Smithies, O. (1955a). *Nature (Lond.)*, 175, 307.
 — (1955b). *Biochem. J.*, 61, 629.
 — and Walker, N. F. (1955). *Nature (Lond.)*, 176, 1265.
 — (1956). *Ibid.*, 178, 694.
 Tuttle, A. H. (1955). *Science*, 121, 701.
 Wieme, R. J. (1953). *Experientia (Basel)*, 9, 380.
 Yuile, C. L. (1942). *Physiol. Rev.*, 22, 19.
 Steinman, J. F., Hahn, P. F., and Clarke, W. F. (1941). *J. exp. Med.*, 74, 197.

SOME PAEDIATRIC PROBLEMS OF UGANDA

BY

JOSEPH LUDER, M.D., M.R.C.P., D.C.H.

Senior Registrar, the Hospital for Sick Children,
Great Ormond Street, London, seconded to Uganda
Medical Service, 1954-6

This paper deals specifically with problems of paediatrics that were met with during my experience in Uganda from 1954 to 1956 as the first liaison registrar to go there from Great Ormond Street. Many of these problems, however, are common to other parts of the tropics and subtropics. It is often overlooked that a majority of the children in the world are living in these underdeveloped regions, and that the bulk of the childhood mortality and morbidity is occurring there.

Whilst the diseases of temperate climates are largely present, indeed in florid form, many of the problems are conditioned by different genetic backgrounds, environments, food habits, customs, superstitions, and disease vectors, and the result is not merely a number of unfamiliar "tropical" diseases, but a pattern of child life, health, and disease which differs greatly from that of temperate areas. The whole subject of tropical paediatrics has been largely neglected in medical practice and teaching in these regions and even more so in the advanced countries. It is with the object of casting some light upon these problems that this paper is presented.

Background

Uganda is a somewhat under-populated country taken as a whole, supporting some five million people. With

a good average rainfall and mainly fertile soil, it could undoubtedly support far more. Sir Harry Johnston as early as 1900 considered twenty millions as a reasonable figure. The effects of disease and deprivation, however, have kept the population static, and until recently it was declining, owing to the combined effects of relative infertility and premature adult death.

There are some ten major tribes and many smaller ones, but from their way of life they can be divided into two main groups—those living mainly by settled cultivation of plantains (non-sweet bananas), cassava (tapioca), sweet potato, millet, and maize, and those living off their herds of cattle, of which the blood and milk are taken but rarely the meat. The agricultural peoples have a largely carbohydrate diet, with little protein and fat, and protein malnutrition is rife. The herdsmen have the protein at hand, but the children may still suffer through ignorance. In many areas cash incomes are very low, and there is a large population of immigrants from Ruanda-Urundi working for very low wages, so that very few Africans can afford to buy foods such as meat or milk.

Numerous customs, superstitions, and taboos exist in these peoples, many of them connected with ill-health. The subject must be approached with caution, since, as Jelliffe (1955) emphasizes, not all are harmful, and some may be beneficial, as, for instance, the widespread practice of prolonged breast-feeding in many parts of the tropics where cow's milk is not available. Others, however, such as female circumcision and many native remedies, are harmful. Medical practice is hampered by the great suspicion of blood transfusion and of taking blood samples.

Vital statistics are most unreliable owing to lack of trained personnel, inaccessibility of dwelling-places, lack of proper machinery, concealment of information, etc. The proportion of children is undoubtedly much higher than in more advanced countries because of the greatly reduced expectation of life. The infant mortality figure can only be guessed at, but most observers think it is well over 100 per 1,000 live births, and may reach 300 in places. Furthermore, the infant mortality does not give a good indication of the total child wastage, as many deaths occur in the early years of childhood. The main causes of child death are malnutrition of all forms, malaria, respiratory disease, gastro-enteritis, and sickle-cell anaemia. There is often a fatalistic acceptance of death in childhood which militates against cases being brought early for advice. It is probable that half the children die before reaching adolescence.

Medical Services

When the Uganda Medical Service was instituted the two major diseases to be faced appeared to be syphilis and sleeping sickness. The first proved to be less formidable than expected, but trypanosomiasis caused vast epidemics around Lake Victoria some fifty years ago, and a major achievement has been its reduction to the occasional clinical curiosity or diagnostic puzzle. Pathological services have been largely conditioned by the prevalent diseases, malaria and helminthiasis, except in the main centres.

The majority of sick persons, adult and children, are seen first or entirely by African personnel called medical assistants who have done a three-year course in elementary medicine and nursing. Some of these invaluable persons are in sole charge of rural dispensaries with up to 30 or 40 in-patient beds, with only monthly visits by a doctor.