BLOOD AND PLASMA SPECIFIC GRAVITIES

AND

PLASMA FORMOL REACTIONS

IN

GENERAL MEDICAL CONDITIONS.

Thesis submitted to the University of Glasgow

by

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PREFACE.

The experimental work described herein was carried out in Wards 9 and 10, Western Infirmary, Glasgow, when I was a clinical assistant under Dr. D.S. Stevenson. I am indebted to Dr. Stevenson and his staff for their cooperation and encouragement.

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GENERAL INTRODUCTION.

The serum formol gel test (syn. Napier's aldehyde test, or the test of Gate and Papacostas) has been widely investigated; much less is known about the corresponding plasma formol gel test which has been applied mainly to rheumatic conditions. Accordingly the original intention here was to investigate the plasma formol gel test in general medical conditions.

Preliminary experiments made it clear that the plasma formol gel (PFG) test consisted of two reactions one of which was the formation of a gel while the other was the development of opacity. I have, therefore, divided the 'plasma formol (PF) reactions' into a 'plasma formol gel reaction' and, what I have called, a 'plasma formol flocculation (PFF) reaction'.

As the work progressed it became desirable to introduce other tests for correlation with the PF reactions. One such test assumed as much, if not more, importance than the original reactions. I refer to the copper sulphate method for estimating blood and plasma specific gravities which is described in Part One as a valuable addition to the methods employed in side-room haematology.

Apart from essential standardisation tests all the experimental work was done in general medical wards or in the side-room attached to the wards. Both parts of the work are, therefore, essentially clinical rather than laboratory investigations.

A system of reference letters is used to distinguish the blood samples of the series. The first letter of the reference indicates the main pathological group and the second letter indicates a pathological sub-group. Individual cases are distinguished by the third letter and individual blood samples by a number following the third letter (see Table A , Appendix B). It is realised that the classification is, in many cases, open to criticism (e.g. one case of Addisonian anaemia in Group X, the miscellaneous group) but the samples are grouped according to predominant clinical features rather than by strict aetiological criteria (in the case of Addisonian anaemia referred to above the main symptoms were those of an acute allergy to parenteral liver

Abbreviations and Symbols.

COHb -- carboxyhaemoglobin.
ESR -- erythrocyte sedimentation rate.
Gb -- specific gravity of whole blood.
Gc -- specific gravity of packed red cells.
Gm -- specific gravity of artificial mixture.
Gp -- specific gravity of plasma.
Gs -- specific gravity of serum.
Gw -- specific gravity of packed white cells.
H -- mm. packed red cells/100mm. blood.
Hb -- haemoglobin.
" (photo) haemoglobin in g./100 ml. by photoelectric method.
" (Sahli) " " " " Sahli method.
" (method A) " " " calculated from Gb*1
" (" B) " " " " " Gb & Gp*1
" (" C) " " " " " Gb, Gp, H*2
" (" D) " " " " " Gb, Gp, H & W*2
Hb-error -- estimated Hb less photoelectric Hb.
Hb-difference -- estimated Hb less Sahli Hb.
M.C.Hb.Conc. -- mean corpuscular haemoglobin concentration.
M.C.V. -- Mean cell volume.
M.R.C.V. -- mean red cell volume.
M.R.C.W. -- mean red cell weight.
M.W.C.V. -- mean white cell volume.
M.W.C.W. -- mean white cell weight.
PF -- plasma formol.
PFG -- plasma formol gel.
PFF -- plasma formol flocculation.
RBC -- red cells in millions/c.mm.
SF -- serum formol.
SFG -- serum formol gel.
SFF -- serum formol flocculation.
W -- mm. packed white cells/100 mm. blood.
WBC -- white cells/c.mm..

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*1 -- as described by Phillips et al (1945a).
*2 -- as developed in this work.

Main pathological groups and reference letters.

Group B -- BA -- addisonian anaemia.
BI -- iron deficiency anaemia.
BX -- miscellaneous anaemias.
BS -- splenic anaemias.
BH -- post-haemorrhagic anaemias.
BL -- lead absorption anaemia.
BP -- anaemia of pregnancy (physiological & pathological).

Group R -- RE -- lymphadenoma.
RL -- lymphatic leukaemia.
RM -- myeloid leukaemia.
RP -- myelomatosis.
RX -- thrombocytopenic purpura.

Group N -- all malignant tumours.
Group I -- infections.
Group X -- miscellaneous.

PART ONE.

BLOOD AND PLASMA SPECIFIC GRAVITIES

IN

GENERAL MEDICAL CONDITIONS.

BLOOD AND PLASMA SPECIFIC GRAVITIES IN GENERAL MEDICAL CASES.

The estimation of blood and plasma specific gravities by means of copper sulphate is fully described by Phillips, Van Slyke et al (1945 a & b). They give formulae and charts for converting the specific gravities into g. haemoglobin per 100 cc. blood, packed cell volume per cent blood volume, and g. protein per 100cc.plasma. The formulae are developed from normal bloods and their accuracy in abnormal bloods, is recorded separately in the original description. The method was designed to assist the Medical Officers of the Services to assess the transfusion requirements of battle casualties. For this purpose periodic variations in the specific gravities were more important than any absolute values calculated from them, and, in any event, it was reasonably safe to assume that the blood was normal before injury. As a war-time improvisation the method was widely adopted throughout British and American Forces in many theatres of war, and it would appear to have fulfilled its intended function satisfactorily.

Phillips et al record that the haemoglobin so estimated is accurate to within ±0.3g./100 cc. in normal bloods, but in abnormal bloods they found larger errors, up to ± 0.7g., an observation which attracted me particularly since the usual subjective methods for estimating haemoglobin do not attain to this degree of accuracy. This is amply proven by experiments described in Special Report No. 252, The Medical Research Council (1945). On expert advice the Committee on Haemoglobin Surveys adopted a Haldane-Gowers' method with a British Standards Institute (B.S.I.) colour standard subsequently found to be equivalent to 14.8g.Hb./100 ml. blood by iron analysis. To determine the/

the error due to personal variability 60 'practised observers' working under ideal conditions with strictly standardised apparatus and technique and with the knowledge that their personal variability was being compared with that of other observers, estimated the haemoglobin in ten standardised solutions of free haemoglobin. The results are expressed as percentages of the B.S.I. colour standard, 5% being equivalent to 0.74g.Hb/100 ml. blood. It was found that 258 out of the total 600 estimations differed by more than 5% (0.74g.Hb) from photoelectric estimations in the National Physical Laboratory; in 66 estimations the difference was more than 10% (1.48g.Hb). Individual observers were found to have personal variabilities up to 13.9% (2.0g.Hb) and the average personal variability of the 60 'practised observers' was ± 4.8% (0.71g.Hb). In clinical haemoglobinometry there are additional errors, e.g. instrumental deviation, poorly defined end-points, etc., and it seems certain that the error in clinical sideroom practice is in excess of ± 0.7g.Hb. , i.e. greater than the greatest error observed by Phillips et al in abnormal bloods.

In the copper sulphate method subjective error is minimal and the method should be particularly valuable in clinical work provided its accuracy can be confirmed in abnormal bloods. Accordingly the purpose of this part of the work was to explore the possibility of applying the copper sulphate method to clinical haemoglobinometry, and to clinical haematology in general.

Ashworth and Tigertt (1940)

Equation i .....

$$100Gb = HxGc + (100-H)Gp$$

which by transposition gives equation ii .....

$$H = \frac{100(Gb-Gp)}{Gc - Gp}$$

and equation iii .....

$$Gc = \frac{100(Gb-Gp) + HxGp}{H.}$$

Phillips et al (1945a)

Equation iv .....

$$H = \frac{100(Gc-Gp)}{1.097-Gp}$$

Equation v .....

$$Hb = \frac{33.9(Gb - Gp)}{1.097 - Gp}$$

Equation vi .....

$$Hb = 480.18xGb - 492.86$$

Phillips et al (1945b)

Equation vii .....

g.protein%plasma = 389(Gp.-1.0079)

and " " % serum = 389(Gs-1.0079).

SUMMARY OF THE LITERATURE.

The Original Description. (Phillips, Van Slyke et al 1945 a & b).

No alterations have been suggested in the original technique. The theoretical considerations underlying the original formulae and nomograms depend upon basic formulae credited to Ashworth and Tigertt (1940) - Equations i - iii, facing this page. Equation i expresses the weight of 100 ml. blood as the sum of the weights of the cells and the plasma.

Phillips et al examined blood from twenty normal American males, estimating Gb and Gp by the copper sulphate method. H was measured after spinning for 60 minutes at 3000 r.p.m. in a centrifuge, radius 18 cm., and the Hb was estimated by a method which measures the oxygen + carbon monoxide capacity of the blood (see page 33). The mean values so obtained were Gb=1.0595, Gp=1.0264, H=47% and Hb=15.9g./100 ml. Hence the mean M.C.Hb.Conc. = 33.9g./100 ml. packed red cells; the mean normal Gc calculated from these results by means of equation iii was 1.097. This mean normal Gc agreed with that found by Ashworth and Adams (1940) using the falling drop method of Barbour and Hamilton (1926).

Equation iv is developed by substituting 1.097 for Gc in equation ii. Accepting 33.9 as the M.C.Hb.Conc. and H as in equation iv, equation v is obtained.

Where Gp could not be estimated, as under war conditions, it is suggested that Gp should be assumed to be 1.0264. Substituting this figure for Gp in equation v, equation vi develops on simplification.

Equation vii opposite, is based on the mean Gp of the twenty normal bloods examined and on current standards of normal g. protein/100 ml. plasma by micro and macro-Kjeldahl methods. Equations iv, v, and vii are incorporated in their nomogram (Phillips et al 1945b).

The/

The originators state that the estimated specific gravities are accurate to within ±0.00005, and point out that an error ten times greater has very little effect on the accuracy of the calculated H and Hb. In normal bloods they found that H and Hb, calculated from Gb and Gp by means of equations iv and v, were accurate to within ±2.0% and ±0.3g.Hb% respectively; in abnormal bloods the corresponding error ranges were 9% for H and 0.7g. for Hb. Unfortunately they do not record the number of abnormal bloods examined or the nature of the abnormalities in the series. They do refer later to a series of abnormal bloods investigated by Atchley et al (1945) in which the Hb, calculated by equation vi from Gb alone, was accurate to within ±1.5g. in 48 out of the 50 bloods examined. In this series there was one case in which this modified method gave an error of 4.7g.Hb%; the plasma proteins amounted to 12.7g.% plasma in this case. The authors conclude that Hb calculated from Gb alone is accurate to within ±0.5g. "in bloods of the normal protein range." For the plasma proteins calculated from Gp by means of equation vi the error range in normal bloods was less than ±0.3g./100 ml. plasma; in abnormal bloods the greatest errors seen were 0.6g. in nephritic cases. "Larger errors" they state, "are unlikely even in abnormal bloods."

Subsequent Publications.

The copper sulphate method is outlined in the recent editions of several standard textbooks, e.g. "Recent Advances in Clinical Pathology" (1947), prepared by The European Association of Clinical Pathologists.

"Approved Laboratory Technic", Kolmer and Boerner (1945) 4th Edition.

"Chemical Methods in Clinical Medicine", Harrison (1947) 3rd Edition.

"Practical Physiological Chemistry", Hawk, Oser and Summerson 12th Edition. (1947)

"Clinical Hematology"/

"Clinical Hematology", Wintrobe (1947) 2nd Edition.

No more than a summary of the publications of Phillips et al is given in these books and in some instances the method is so simplified as to be almost misleading.

Hynes and Lehmann (1946) record an evaluation of Gb as a screening test for the detection of anaemia when large numbers of bloods have to be examined as quickly as possible. They chose 11.0g.Hb %. as the critical Hb level below which the bloods were to be classified as "anaemic". From equation vi 11.0g. corresponds to a Gb of 1.049. The proposed test therefore, consisted of allowing one drop of blood to fall into a copper sulphate solution of this specific gravity and observing its subsequent behaviour. Blood was collected from 270 Indian recruits; Gb, Gp and Gs were estimated in each blood by the copper sulphate method, and Hb by an alkaline haematin method. The Gb test classified 8 out of the 270 bloods wrongly according to the alkaline haematin method and it was concluded that 30% of all patients with Hb 10.0 - 10.9g.% are likely to have Gb equal to or greater than 1.049.

Hynes and Lehmann also used their results to assess the accuracy of the Hb calculated from Gb and Gp, and Gb and Gs. They made the following observations:-

1. There was no statistical difference between the mean Gp of their cases of microcytic and of macrocytic anaemia.
2. Gs was more reliable than Gp for the calculation of Hb. Gp was calculated by adding 0.001578 to Gs. This, they state, is equivalent to 0.28g. fibrinogen % plasma. It is important to note that they found ten cases in which Gp after correction for the anti-coagulant was less than Gs. Although Hynes and Lehmann do not attempt to explain this phenomenon it may well be related to the spontaneous precipitation, or/

or sedimentation of plasma protein discussed later in this work. The fact that their blood donors were Indian recruits raises the possibility of associated malaria and leishmaniasis. Spontaneous precipitation of plasma protein has been recorded in both conditions.

3. The accuracy of the Hb estimations relative to the alkaline haematin method is recorded thus:-

	Mean Difference.	Standard Dev.	Range of Difference.
Hb from Gb alone	-0.558g.%	0.9441	-2.2 to +2.7
Hb from Gb & Gp	-0.499	0.6650	-1.8 to +1.7
Hb from Gb & Gs	-0.410	0.6980	-1.7 to +1.7

The error of the alkaline haematin method is not stated.

Boynton (1946) investigated the value of Gb as a screening test for the detection of anaemia in would-be blood donors. For this purpose the critical Hb selected was 12.2g% and the critical Gb was accordingly 1.053. It was found that in 4% of would-be donors Gb was less than 1.053 although they had more than 12.2g. Hb%.

Miller (1947) examined blood from 131 cases of lobar pneumonia, estimating Gb and Gp by the copper sulphate method and Hb by means of a "Spekker" photoelectric colorimeter. The "Spekker" apparatus was calibrated against O_2 capacity estimations by "Van Slyke's method" but there is no indication whether the modified or the original method of Van Slyke (see page 38) was used. Gb and Gp were converted to Hb by means of the nomogram of Phillips et al. The results are shown in graphic form only; exact figures are not given. The coefficient of correlation is stated to be 0.9677 and from the graph it appears to me that the greatest error is just over 1.0g.Hb%., the photoelectric result being the higher in about

about 67% of the bloods.

Miller concludes with the recommendation that the copper sulphate method should be adopted generally for routine clinical estimations of Hb.

On the evidence presented alone this recommendation must be qualified. Lobar pneumonia is an acute illness of short duration and may well be associated with almost normal blood, particularly since the introduction of chemotherapy. Thus no evidence is advanced in Miller's paper to prove that the method is accurate in abnormal bloods.

O'Connor (1947) records his impressions after examining 250 normal and abnormal bloods by the copper sulphate method. Many important facts are not recorded, e.g. the number of normal bloods, the nature and frequency of the "discrepancies".

O'Connor converts Gb and Gp to Hb by the original nomogram of Phillips et al, and states that the results compare "reasonably well with the values found by a routine haematin method". On the other hand he rejects the corresponding H in the nomogram although the Hb figure is dependent on this value for H.

The importance of this paper lies in the fact that it gives the only estimated values for Gc in this country with which I can compare the values obtained herein. As in this work Gc was calculated from the estimated Gb, Gp, and H by means of equation iii (page 6). It should be noted that this equation is wrongly expressed in O'Connor's paper but his results indicate that the correct equation was/

was used in his calculations of Gc. Gc in normal bloods is stated to range from "1.090 to say 1.096"; in microcytic bloods Gc was low, down to 1.062, and in macrocytic bloods it was as high as 1.106.

O'Connor used Gc to calculate the Mean Cell Volume (M.C.V.) and hence the number of red cells in millions per c.mm.. His suggested method has an empirical basis and is here summarised in the formulae

$$M.C.V. = 100(Gc - 1.000)\text{c.u.}$$

and $$R.B.C. = \frac{10 \times H}{M.C.V.} = \frac{H}{100(Gc - 1.000)} \text{ mill./c.mm.}$$

The red cell "counts" so calculated "agree so tolerably well with the actual counts that the method is worthy of further study."

Although Phillips et al (1945a) record a maximum error of 0.7g.Hb% in "a series of abnormal bloods" they do not suggest that this is the greatest error likely to be found in abnormal bloods. They do not indicate the number of abnormal bloods examined nor do they state the nature of the abnormalities present in the bloods of their series. Hynes and Lehmann (1946) do not distinguish between their normal and their abnormal bloods and their maximum error is difficult to evaluate since the error of the alkaline haematin method is not stated. Miller (1947) and O'Connor (1947) have recommended that the copper sulphate method as described by Phillips et al, be introduced into routine clinical work as a means of estimating the Hb but they do not publish any evidence to confirm that the method is accurate in abnormal bloods. Thus it seems that errors as great as 0.7g.Hb% do occur, but there is no evidence to suggest that greater errors will not be found.

Several reports have been published since 1945 on the/

the accuracy of Gp as a means of estimating the plasma proteins and of Gs in the estimation of total serum proteins (g/100 ml. serum or plasma). Hock and Marrack (1945) discuss the accuracy of the Kjeldahl digest methods and state that for consistent results the digestion must continue for at least an hour, preferably for 3½ hours, and must be aided by a catalyst, selenium dioxide. They found that the best formula for converting Gs to g. protein % serum was ... Serum prot. = 364(Gs-1.006). This formula was accurate to within ±0.4 g. protein/100 ml. and gives results very similar to the formula of Phillips et al.

Atchley et al (1945), Abbott (1946) and Meyer et al (1947) found the plasma or serum proteins calculated from Gp or Gs and by chemical analysis to have a coefficient of correlation of at least 0.9. Meyer et al applied the method with similar accuracy to the plasma of dogs. Atchley et al found that Gp was independent of the relative concentrations of albumin and globulin in the blood. On the other hand Adams and Billou (1946) record a less satisfactory coefficient of correlation (0.74) and do not agree that albumin and globulin have the same effect on Gp. They found an inverse correlation between the ratios albumin: globulin and Gp: total plasma proteins.

Berry and Perkins (1947) reported unfavourably on the copper sulphate method as a means of estimating plasma and serum proteins. They compared it, however, with the turbidometric method of Looney and Walsh (1939) which has been shown to be inaccurate particularly in acute infections. The error of the turbidometric methods is discussed by Plimmer, and Lowry (1945) and by Stacey (1947).

Phillips, Yeomans et al (1946) describe a method for estimating total blood volume (±15%) from the decrease in blood specific gravity caused by the infusion of a known volume of isosmotic plasma or albumin solution. Gb is measured/

measured by the copper sulphate method before and after the infusion and the specific gravity of the fluid infused is also estimated by the copper sulphate method. They estimated Gb in venous blood and administered the plasma at the rate of 1200 ml. in about 45 minutes. The formula used for calculating blood volume is given on page 54 of this work.

Two observations by Phillips et al (1945a) are important to the present thesis although there is no mention of them in the subsequeht publications. In the first place they state that a blood sample packing to 51% after spinning for 60 minutes at 3,000 r.p.m. in a centrifuge of radius (axis to mid point haematocrit tube) 9 cm. will pack down to 47% in an 18 cm. centrifuge under the same conditions of time and speed.

Secondly, it is stated that the g.Hb per 100 c.c. packed cells is equal to 377(Gc - 1.007). This statement is in the form of a footnote. No explanation is given for the origin of the equation and no evidence is advanced to support it.

METHODS.

Only methods which apply particularly to this part of the work are described below. The general routine examination of the bloods is described in Appendix A.

SPECIFIC GRAVITY ESTIMATIONS.

The method described by Phillips et al (1945a) was used except that the stock copper sulphate solution of specific gravity 1.100 was prepared by a modification of their saturated solution method. Super-saturation was attained by heating the copper sulphate crystals and the water. The resulting solution was left at room temperature for 48 hours to crystallise out. Thereafter filtration and dilution were performed as described by Phillips et al. The specific gravity of the final solution was estimated on a light balance capable of weighing accurately to four decimal places. On each of the three occasions on which this method was used the final result was accurate to three decimal places and a little over 1.100 in the fourth decimal place. A calculated volume of water was added to correct this minor error (10-15 ml. water/litre).

STANDARDISATION TESTS.

Four normal bloods supplied the 24 samples included under this heading. Six samples were prepared from each blood as follows:-

I. The naturally occurring blood.
II. The 'concentrated' sample (plasma removed).
III-VI. 'Diluted' samples (plasma added).

The diluted samples were arranged to cover the range 3.0 to 12g.Hb%.

Hb was estimated in each of the 24 samples by the photoelectric method and by the Sahli method. Furthermore in/

in each of the four bloods Gb, Gp and H were estimated in samples 1 and 11 and Gb was estimated in the other four samples.

ARTIFICIAL SAMPLES.

Artificial samples were either (a) 'Concentrated' samples prepared by removing plasma from the natural blood or (b) 'Diluted' samples prepared by adding either plasma or normal saline to the natural blood. Gb, Gp and H were estimated in each sample.

WHITE CELL SPECIFIC GRAVITY (Gw).

A suspension of white cells in plasma was prepared from the centrifuged blood and the specific gravity of the suspension (Gm) was estimated by the copper sulphate method. The suspension was centrifuged to the 'no change' end point in the 18 cm. centrifuge at 3000 r.p.m. If W = packed volume of white cells per 100 ml. blood then .....

$$Gw = \frac{100(Gm - Gp)}{W.} + Gp.$$

MATERIAL.

All blood samples examined consisted of venous blood collected as described in Appendix A.

The total number of naturally occurring blood samples in the series is 103. Details of the cases from which the bloods were obtained are given in Appendix B.

126 samples were prepared artificially from the 103 bloods as follows:-

36 dilution samples (blood + saline or plasma)

66 concentrated samples (blood less plasma)

24 standardisation tests

The total number of blood samples investigated was therefore 229.

INTERPRETATION OF RESULTS AND DISCUSSION.

1. HAEMOGLOBIN ESTIMATION FROM BLOOD AND PLASMA SPECIFIC GRAVITIES.

The 24 samples examined by the photoelectric method will be referred to as the standardisation tests. In each of the 24 samples the Hb was also estimated by the Sahli method, by the methods of Phillips et al, and by the methods developed in the course of this work. Photoelectric estimations were not possible in the 103 bloods of the series.

For descriptive purposes the term 'error' refers to the Hb by any of these methods less the photoelectric estimation and 'difference' to the Hb less the Sahli estimation, Hb being measured in g. per 100 ml. blood in all cases. The standardisation tests, therefore, give both the Hb errors and Hb differences for each method in normal bloods.

The Sahli method was applied to the 103 bloods of the series most of which were abnormal. Preliminary experiments convinced the observer that he could define a more accurate end-point with this apparatus than even with the Haldane-Gowers haemoglobinometer. Special care was taken to define the end-point in terms of distance between the apparatus and the eye, and of the time and temperature of the blood-acid reaction. Readings were made at 30 minutes and again at 60 minutes. The apparatus used had bilateral identical glass standards; hence there was no necessity to change the standard from one side to the other as suggested in the Medical Research Council's Special Report No. 252 (1945). The detailed Sahli technique is described in Appendix A.

To compare the accuracy of the specific gravity methods in abnormal bloods with the accuracy in normal bloods, it is, therefore, necessary to compare the Hb differences in the 103 bloods with the differences in the 24 normal standardisation tests. The error in the 24 tests is known, but, before the error in the abnormal group can be/

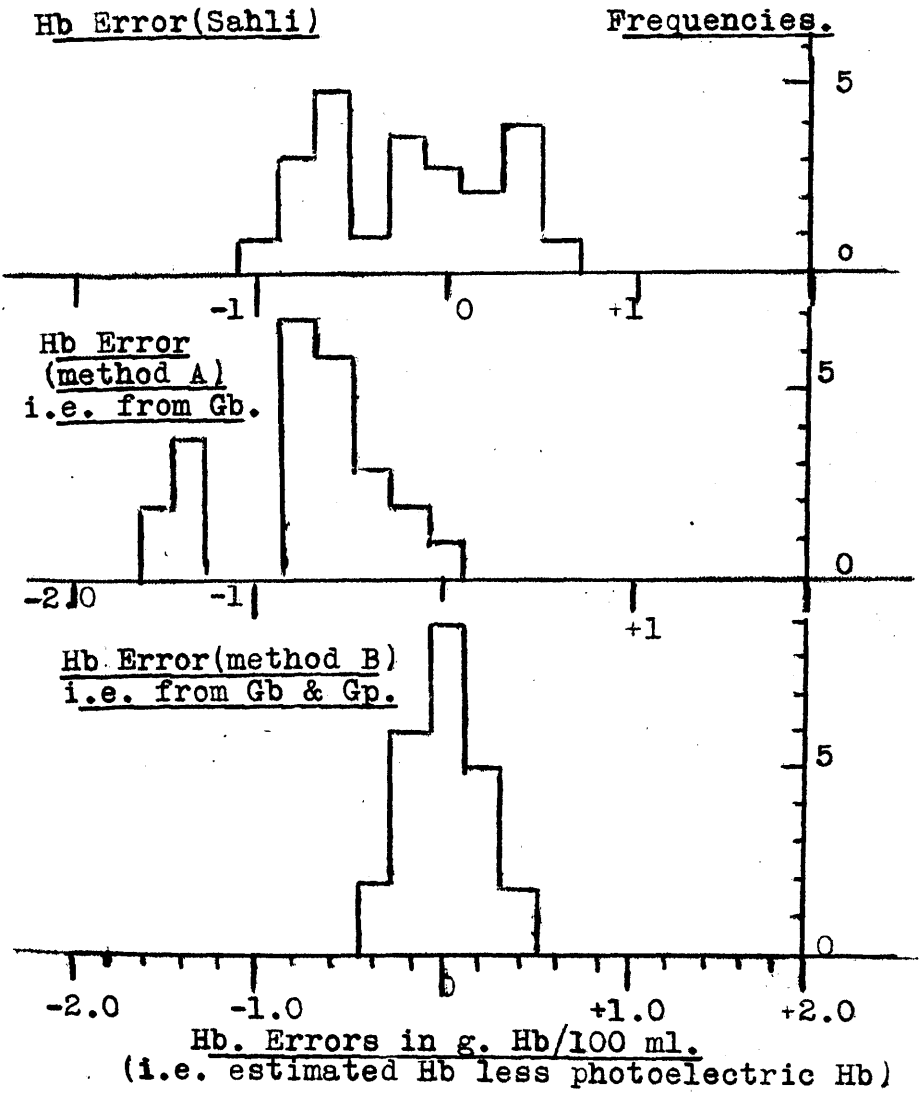


Fig. 1 :- The errors of the Sahli estimated Hb and Hb from the nomogram of Phillips et al(1945b) in the normal blood samples of the standardisation tests.

be assumed to be similar, the Hb differences must satisfy the criteria -

1. There must be no significant difference between the mean Hb differences in the two sets of results.
11. The distribution of the differences about the means in both series must be similar, i.e. the standard deviation of the differences about the mean difference must be similar.
111. The ranges of difference must also be similar.

If these criteria are fulfilled by the differences in the 103 bloods, it is then probable that the error in the abnormal bloods is no greater than the error in the 24 normal samples of the standardisation tests.

Error of the Sahli Method i.e. Hb-Sahli less photoelectric Hb, in the 24 normal bloods of the standardisation tests.

The error in each of the 24 bloods is shown in Fig. I opposite.

The mean error is +0.002g.Hb/100 ml. blood; the range of error is from -0.9 to +0.8g.Hb and the errors are distributed about the mean with a standard deviation of 0.486(±0.10).

Haemoglobin From The Nomogram of Phillips et al (1945b). (Methods A and B)

In this work 'Method A' refers to the modified method described by Phillips et al whereby Hb is calculated from Gb alone, Gp being assumed to equal 1.0264. 'Method B' refers to their original method using Gb and Gp. The nomogram

Hb.
g.%..
(Sahli)

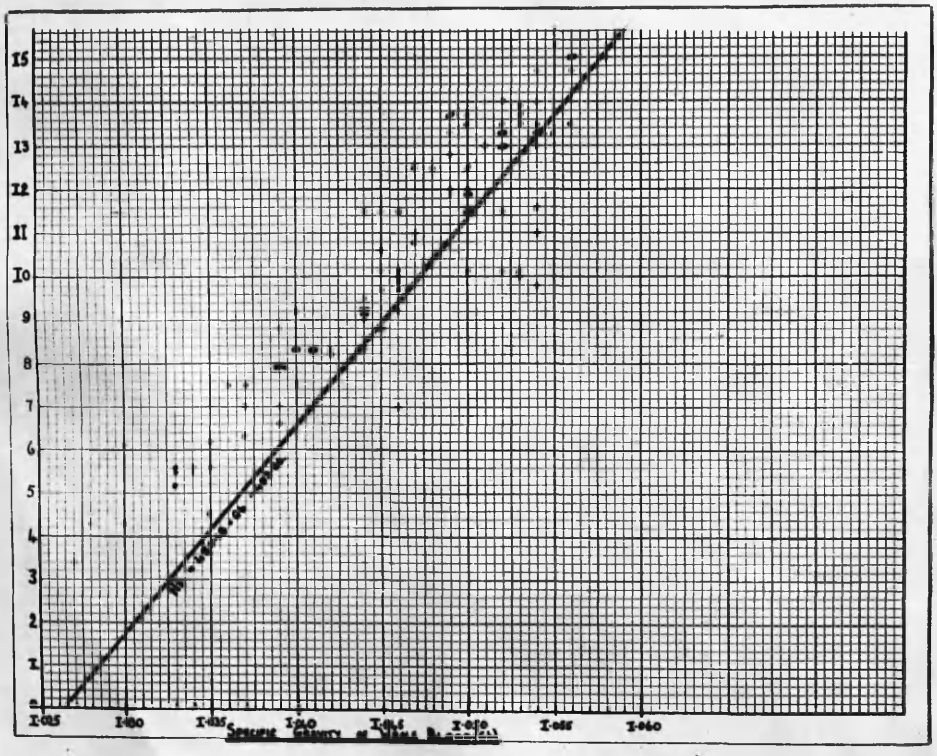


Fig.2 :-Whole blood specific gravity and Sahli estimated haemoglobin, in g./100 ml. blood, in 103 normal and abnormal bloods. The line...Hb= 480.18xGb-492.86..is shown.

(Phillips et al - 1945b) may be used for both methods. The graph Fig. 2, opposite, may be used for Method A.

Normal Bloods. The Hb-errors, Methods A and B, are shown in Fig. 1, opposite page 19.

The error range of Gb method A is greater than the ±0.5g. recorded by the originators in "bloods of the normal protein range." Method B is liable to an error which is very little greater than their error range for normal bloods, i.e. than ±0.3g. Hb%.

In The 103 Bloods of the Series:- This group is made up mainly of abnormal bloods but also contains ten normal control bloods. The various abnormalities present are recorded in Table A, Appendix B. The results are shown in Figs. 2 and 3; the Hb-differences are illustrated in Fig. 8, page 30, and summarised as follows:-

	Hb-Method A, i.e. from Gb alone.	Hb-Method B, i.e. from Gb and Gp.
(a) Mean Hb-difference	-0.92g.Hb%	-0.16g.Hb%
(b) Range of Hb-differences	-4.0g. to +3.5g.	-2.2 to +3.1g.
(c) Standard deviation of Hb-differences from mean Hb-difference.	1.10(±0.11)	0.845(±0.08)
(d) Percentage bloods with Hb-difference ±1.0g.Hb.	43.7%	80.6%

If the accuracy of any method in abnormal bloods is the same as in normal bloods, the maximum Hb difference permissible in the 103 bloods of the series should not exceed the sum of the maximum errors of the method and of the Sahli method in the standardisation tests. The sum of these/

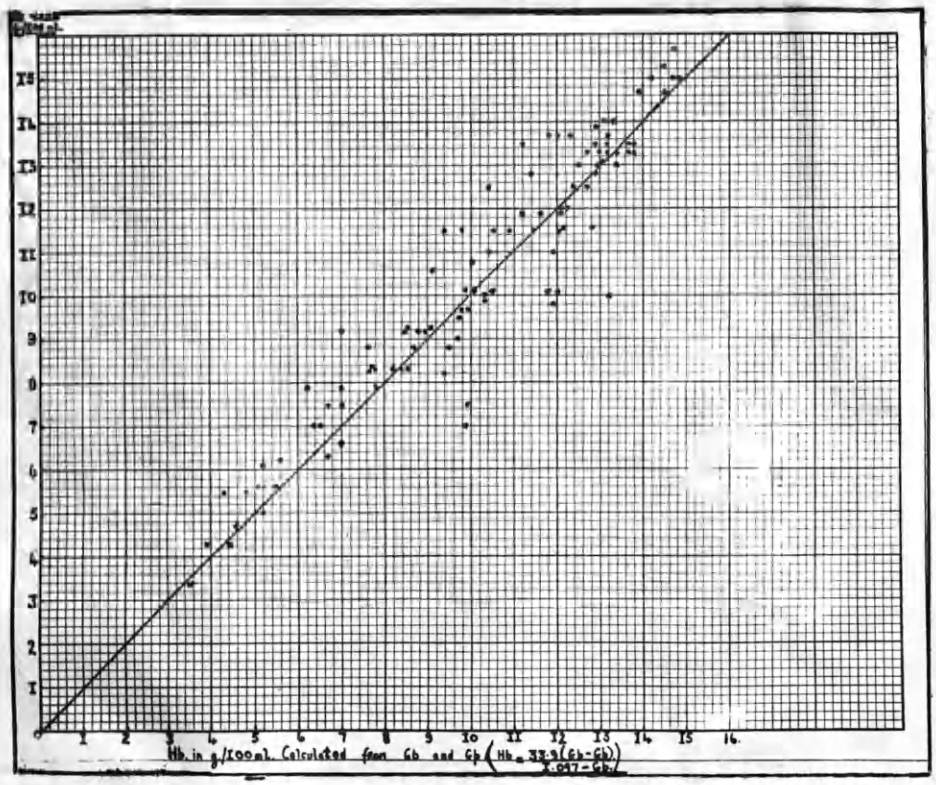


Fig. 3 :- Hb (Sahli) plotted against Hb from the nomogram of Phillips et al (1945b) using Gb & Gp(method B herein) in the bloods of the present series.

these maximal errors for Method A allows a maximum Hb-difference of -2.5 to +0.7g.Hb, and -1.4 to +1.2g.Hb for Method B. The actual difference range for both methods is much greater than the error in normal bloods.

Conversely if the maximum error of the Sahli method is subtracted from the range of Hb difference the resulting range represents the minimum actual error of the method in abnormal bloods. Thus the error of Method A in abnormal bloods must be at least -3.1 to +2.7g. and for Method B at least -1.3 to +2.3g.

The Artificial Samples have been used to confirm the accuracy of the estimated Gb, Gp and H in the original blood and to establish the consistent accuracy of the estimated specific gravities by the copper sulphate method. Exact figures are not quoted as they simply repeat the figures calculated from the Gb, Gp and H in the naturally occurring blood.

In each sample examined the estimated Gb, Gp and H were substituted in equation iii, page 6 , and Gc was calculated. Comparing Gc in the original and in the artificial samples, the results were within ±0.004 in the 66 concentrated samples, within ±0.001 in 38 and ±0.002 in 53 samples. The diluted samples were less satisfactory. In six bloods diluted with saline it was impossible to be certain of Gp owing to the rapid dispersal of the suspended drop in the copper sulphate solution. In the other 30 diluted samples the Gc was within ±0.006 of Gc in the original blood.

Specific Gravity of the White Cells (Gw):- Only one case with/

with a sufficiently high W to permit the direct estimation of Gw was encountered.

Results in Sample RMb .... Gp ... 1.025

	Spec. grav.	H.	W.
(a) Natural blood	1.046	20 ...	21
(b) Concentrated sample ...	1.049	23 ...	23
(c) White cell/plasma mixture	1.032 (Gm)	0 ...	27

Calculation of Gw

Applying the formula $Gw = \frac{100(Gm-Gp)}{W} + Gp$ (Page 15)

to (c) $Gw = \frac{100(1.032-1.025)}{27} + 1.025 = 1.051$

Checking Gw by calculating Gc.

Applying the results under (a) and (b) above to equation xii, page 31 , and substituting 1.051 for Gw, the values obtained for Gc are 1.1027 and 1.1032, mean Gc = 1.103.

DISCUSSION.

Haemoglobin Calculated by Method A i.e. from Gb alone. This method assumes that Gc = 1.097, M.C.Hb.Conc. = 33.9, and Gp = 1.0264.

In the normal bloods of the standardisation tests the Hb so calculated has an error of -1.6 to -0.1 g.%.. Phillips et al record an error by this method of ±0.5g. "in bloods of the normal protein range". From their nomogram it appears that "the normal protein range" is from 6.3g. (Gp = 1.0244) to 7.6g.% plasma (Gp = 1.028). The normal bloods of this series however, were found to have Gp as low as 1.023. The normal protein range in this country appears to be equivalent to a range of Gp from 1.023 to 1.028 with a mean of 1.0254 (see page 47). Substituting 1.0254 for 1.0264 in equation v the formula for converting Gb into Hb becomes:-

$$473.4 \times Gb - 485.3 = Hb$$

By this formula the range of Hb error in the standardisation tests becomes -0.8 to +0.3g.%.. Similarly if the mean Gp of the 24 normal bloods is substituted instead of 1.0254 the resulting formula gives a range of error from -0.7 to +0.4g.Hb%.. If the formula is further corrected by assuming Gc to have the mean value determined in these samples (1.0935) and the M.C.Hb.Conc. is similarly altered the final formula gives an error range of ±0.5 g.Hb as found by Phillips et al. Thus the excessive error in the normal bloods of this series can be accounted for by variations in Gp, Gc and the M.C.Hb.Conc. outwith the range accepted as normal by Phillips et al but probably within the range of normal for British bloods.

In the abnormal bloods Gp, Gc and the M.C.Hb.Conc. show still greater variations from the assumed normal values. Gp in the 103 bloods varies from 1.018 to 1.031, Gc from 1.067 to 1.110 and the M.C.Hb.Conc. from 23 - 39%. Such variations may cause errors in the Hb-method A of ±4.0g. %.

Haemoglobin Calculated by Method B i.e. from Gb and Gp by means of the nomogram of Phillips et al (1945b).

The error of this method in the normal bloods in this work is slightly greater than the error range recorded by Phillips et al (±0.3g.Hb). As in Method A, the difference is partly due to the difference between the assumed and actual Gc and M.C.Hb.Conc..

Considering the range of Gc and M.C.Hb.Conc. recorded above in the 103 bloods it is not surprising that the error in those bloods is much greater than in the 24 normal samples.

It has been observed that, allowing the maximum Hb error for the Sahli method, a minimum error of -1.3 to +2.3g.Hb remains in the abnormal bloods in this investigation. In 13 out of 103 samples the Hb-difference is greater than 1.5g. although the maximum Sahli error plus the maximum Method B error in normal bloods allows a maximum Hb difference of 1.4 g.%. It is therefore, concluded that the g.Hb% calculated by the nomogram of Phillips et al (1945b) from Gb and Gp is liable to an error which may exceed 2.0g.Hb in a proportion of abnormal bloods. This is a much greater error than that found by Phillips et al; their maximum error was 0.7g. but no comparison of the error ranges can be made unless the nature and extent of the various abnormalities in the bloods can also be compared.

It is difficult to assess the clinical value of the method in this form. Many methods are already available for approximate estimations of Hb but none of the existing methods are comparable as regards speed and lack of subjective error.

Errors in the Haemoglobin (Methods A and B) And Their Correction.

i. Variations in Cell Gravity and Haemoglobin Concentration Corrected by Method C.

The conversion of Gb and Gp to g.Hb% as described by Phillips et al (1945a) assumes constant values for the specific gravity of packed red cells (Gc) and for the M.C.Hb.Conc.. The first step towards any correction of the error due to variations in Gc and in the M.C.Hb.Conc. from the assumed values (1.097 and 33.9% respectively) was to calculate Gc and the M.C.Hb.Conc. in each of the 103 bloods of the series. Gc was obtained by means of equation iii, page , from Gb, Gp and H while the M.C.Hb.Conc. = $\frac{100 \times Hb(Sahli)}{H}$. It must be pointed out that H as used here differs from H as described by Phillips et al. They include W along with the packed red cell volume in their H, but in this work H stands for the packed red cell volume per 100 vols. blood. Hence H as used by Phillips et al = H+W herein.

Details of the method employed to estimate H are given in Appendix A but for comparison with the work of Phillips et al it was necessary to refer all values of H to the packed red cell volume after 60 minutes spinning at 3000 r.p.m. in a centrifuge of radius 18 cm., i.e. distance between the mid-point of the haematocrit tube and the axis = 18 cm.. A series of bloods in which H varied from 10 to 60% blood volume were centrifuged to the limit of packing in the side-room centrifuge, radius 9 cm., and thereafter in an 18 cm. laboratory centrifuge for 60 minutes at 3000 r.p.m.. In this way a correction chart was prepared giving H in the 18 cm. centrifuge from the packed red cell volume in the 9 cm. instrument. Later tests showed the corrected H to be accurate to within $\pm$1% blood volume.

When Gc was first plotted against the M.C.Hb.Conc. it was noticed that in three leukaemic bloods Gc was much greater/

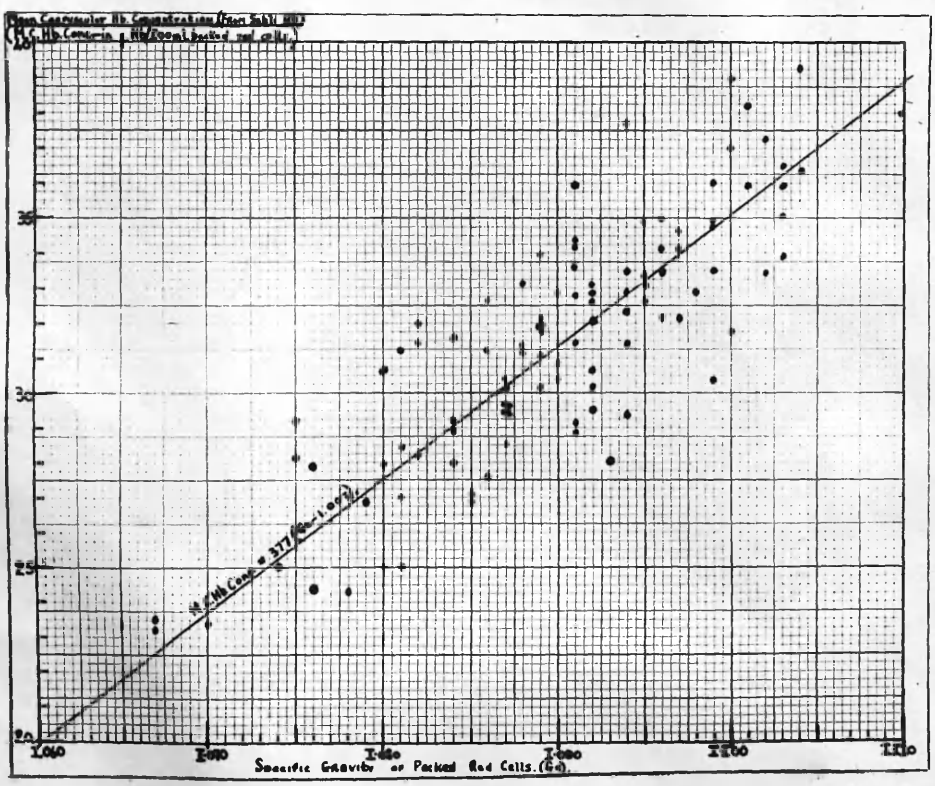


Fig.4:-Gc plotted against the M.C.Hb.Conc.(Sahli) in the bloods of the present series. Gc has been corrected for W and Gw but not for hyperproteinaemia. The line...y=577(x-1.007)...is shown.

greater relative to the M.C.Hb.Conc. than in the other bloods of the series. Hence a method had to be devised to allow for the weight and volume of the packed white cells (W). This correction was necessary before Gc and the M.C.Hb.Conc. could be correlated.

It was considered likely that some relationship existed between the Gc and M.C.Hb.Conc. as both are ratios of weight to red cell volume, the weights concerned being in the one case the packed red cell weight and in the other the weight of Hb in the packed red cells. The formula connecting the two ratios according to Phillips et al (1945a) is ... M.C.Hb.Conc. = 377(Gc - 1.007) ... Equation viii (see page 28).

Fig. 4 , facing this page, shows Gc plotted against the M.C.Hb.Conc. (Sahli) in each of the 103 bloods of this series. The straight line of equation viii is also shown. The best correlating formula in these bloods is M.C.Hb.Conc. = 356.3(Gc - 1.0015) but the difference between the results by the two formulae is negligible. Equation viii is therefore applied without amendment throughout the remainder of this work. In this series the mean difference between the M.C.Hb.Conc. (from Gc) and the M.C.Hb.Conc. (Sahli) is -0.27 and the range of difference is from -4.9 to +4.4%. Such a range may well be due to the error of the Sahli method alone, e.g. if the maximum Sahli error coincides with the smallest H in the series the error in the M.C.Hb.Conc. becomes $\frac{0.9 \times 100}{15} = 6.0\%$.

The distribution of the differences about the mean difference is illustrated in Fig. 5 , overleaf.

By counting the number of points plotted within each square in Fig. 4 , the graph may be made into a correlation table. Let/

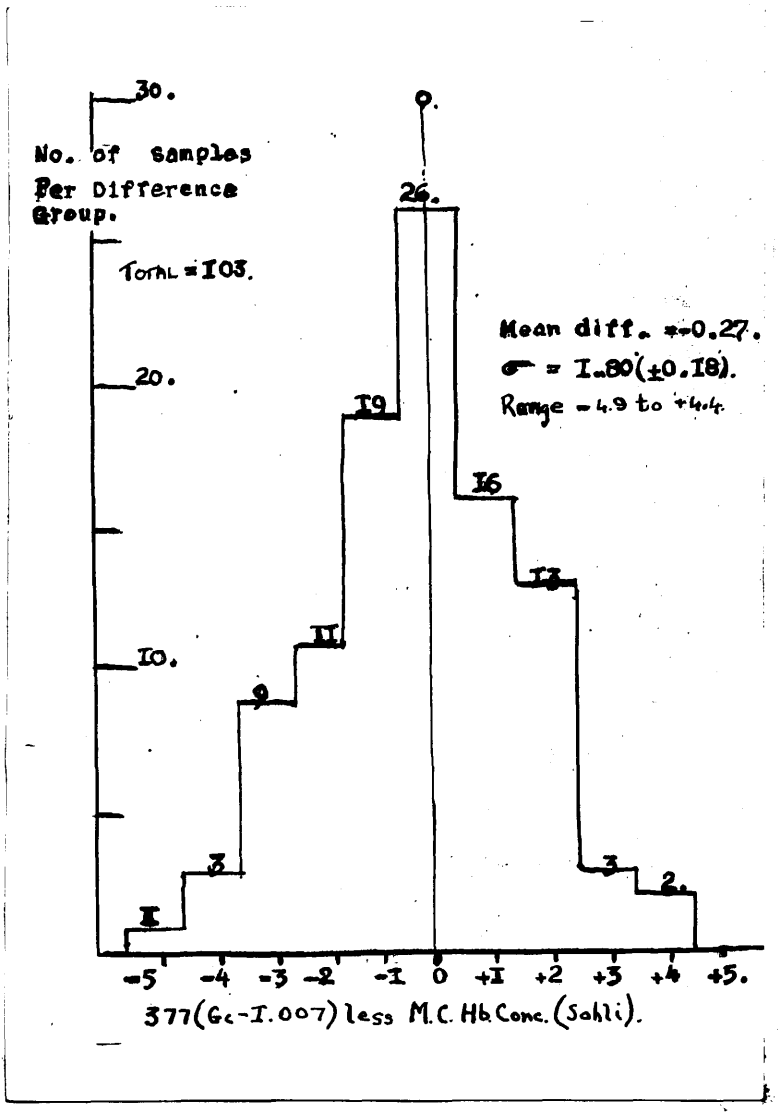


Fig. 5:- Frequency distribution chart for difference ....377(Gc -1.007) g. Hb less M.C.Hb.Conc.(Sahli)in g.Hb per 100 ml. packed red cells ...in the 103 bloods of the series. Difference groups = 1.0 g..

Let $\bar{Y}$ = the mean M.C.Hb.Conc.(Sahli), and $\bar{X}$ = the mean Gc, and let the symbol S stand for 'the sum of'.

It may be calculated that -

i) $\bar{Y} = 31.44$; $\bar{X} = 1.0897$

ii) $S(Y - \bar{Y})^2 = 1312$; $S(X - \bar{X})^2 = 0.007976$

iii) $S(Y - \bar{Y}) \times (X - \bar{X}) = 2.841$

Hence the coefficient of correlation between M.C.Hb.Conc. and Gc is 0.8785 (standard error $\pm$0.021). The best formula correlating the two variables is, by the method of least squares ....

M.C.Hb.Conc. = 356.3(Gc - 1.0015)

It was observed that the empirical formula - equation ix - M.C.Hb.Conc. = 350(Gc - 1.000) - gives M.C.Hb.Conc. results which are very similar to those given by the formulae above.

Theoretical Origin of Equation viii - M.C.Hb.Conc. = 377(Gc - 1.007).

The following explanation was found for equation viii during one of many attempts to find a theoretical formula relating Gc to the M.C.Hb.Conc.. It may be that Phillips et al have other reasons altogether for their foot-note.

It is assumed that 100 vols. packed red cells of Gc = 1.097 gain or lose V vols. of a fluid of specific gravity Gv.

Gc is then $\frac{100 \times 1.097 + V \times Gv}{100 + V}$, hence $V = \frac{100(1.097 - Gc)}{Gc - Gv}$;

the M.C.Hb.Conc. becomes $\frac{33.9 \times 100}{100 + V}$, and .....

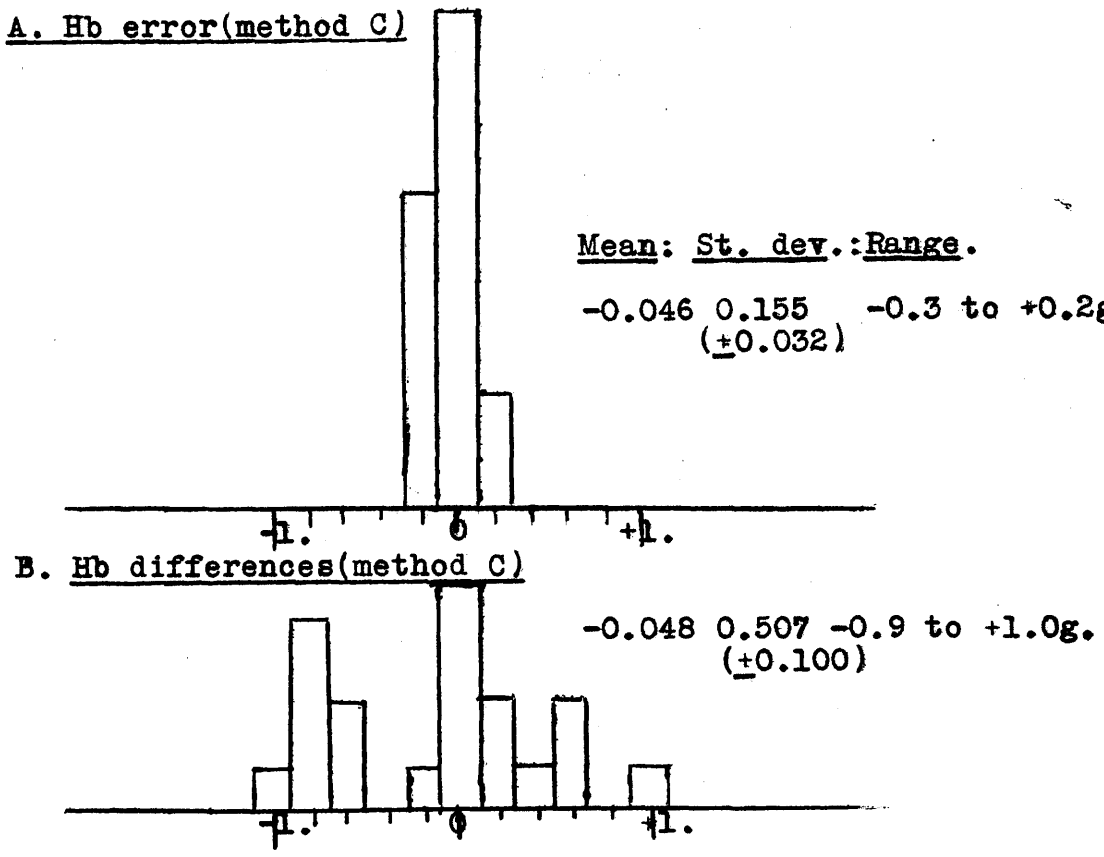
hence $V = \frac{100(33.9 - \text{M.C.Hb.Conc.})}{\text{M.C.Hb.Conc.}}$

$\therefore \frac{100(1.097 - Gc)}{Gc - Gv} = V = \frac{100(33.9 - \text{M.C.Hb.Conc.})}{\text{M.C.Hb.Conc.}}$

which on simplification gives

$$\text{M.C.Hb.Conc.} = \frac{33.9(Gc - Gv)}{1.097 - Gv}$$

If the fluid absorbed by the red cells is plasma ultra-filtrate then Gv, according to Phillips et al, equals 1.007/



Hb differences and errors in groups of 0.2g.Hb/100 ml..

Fig. 6:- Hb errors(calculated Hb less photoelectric Hb) and differences(calculated Hb less Sahli Hb) for method C(page 28) in the 24 normal blood samples of the standardisation tests. Since W is less than 2% and Gp is less than 1.028 in these samples the above results also apply to method D and the method underlying the nomogram developed in this work(Fig.B,App.C)

1.007, then

$$\text{M.C.Hb.Conc.} = \frac{33.9(Gc-1.007)}{0.090} = 377(Gc - 1.007)$$

i.e. equation viii.

Equation viii therefore expresses the variations in Gc and in the M.C.Hb.Conc. which result from an increase, or a decrease in some factor of the same specific gravity as plasma ultrafiltrate in the normal erythron of Phillips et al, i.e. Gc = 1.097 and M.C.Hb.Conc. = 33.9% (or g.Hb/100 ml. packed red cells). The fact that the formula applies to microcytic and macrocytic bloods in this work does not imply that the larger erythron of microcytic hypochromic anaemia differs only from the normal erythron in its content of fluid and diffusible salts. In the first place the formula cannot, on the evidence here available, claim to be more than a rough approximation. The true relationship probably varies in different pathological states. Secondly it is quite possible that variations in the cell content of lipoid and protein other than Hb may so alter the volume and weight of the erythron that the end result is similar to the effect of alterations in the water and salt content of the cells.

Calculating The Haemoglobin by Method C:- The method developed herein to allow for variations in Gc and the M.C.Hb.Conc. will be referred to as Method C. Besides Gb and Gp it is also necessary to estimate H.

Hb may be calculated in three stages, or in one stage using equation xi below.

The first stage makes use of equation iii to convert Gb, Gp and H into Gc ....

Equation iii - $Gc = \frac{100(Gb-Gp) + HxGp}{H.}$

Secondly Gc is converted to M. C.Hb.Conc. by means of equation viii- M.C.Hb.Conc. = 377(Gc - 1.007)
and finally the M.C.Hb.Conc. and H are applied to the formula/

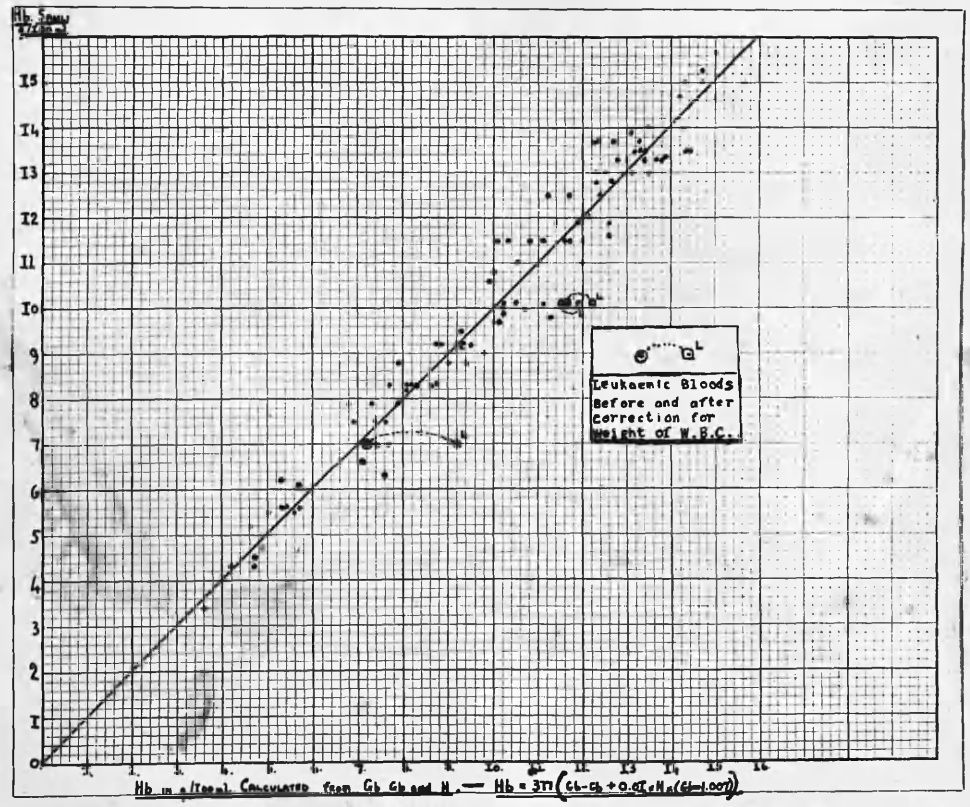


Fig. 7 :- The Sahli estimated Hb(OY) plotted against Hb calculated by methods C & D in the 103 bloods of the series.

formula below:-

$$Hb = 0.01 \times H \times M.C.Hb.Conc. \quad \ldots \text{ eqn. x.}$$

For the one stage calculation the formula was developed from eqn. x by substituting 377(Gc-1.007) for the M.C.Hb.Conc. (eqn. viii) and $\frac{100(Gb-Gp)}{H} + Gp$ for Gc (eqn. ii). This gives

$$\ldots\ldots Hb = 3.77((100(Gb-Gp) + H(Gp-1.007)) \ldots \text{ eqn. xi.}$$

The approximate eqn. ix, page 28, similarly gives ....

$$\ldots\ldots Hb = 350((100(Gb-Gp) + H(Gp-1.000)) \ldots \text{ eqn. xi (a).}$$

The nomographic charts developed herein, page 20, App. C, are based on eqn. xi (a) and may be used to convert Gb, Gp, H and W to g.Hb/100 ml. blood as well as to Gc and the M.C.Hb.Conc. as by the three stage method. The difference between the results by eqn. xi and eqn. xi (a) is negligible except that when Gc is very low, e.g. at Gc 1.060, the difference amounts to 0.1g.Hb/10mm. of H.

Effect of Errors in H on the Hb-method C:- According to eqn. xi an error in H of 1.0 mm./100 mm. blood will cause an error in the calculated Hb of 3.77(Gp-1.007)g.Hb%., i.e. between 0.04 and 0.09g.Hb. If a centrifuge calibrated against an 18 cm. pattern centrifuge is used the error in H is unlikely to exceed 4 mm/100 mm. blood, i.e. the difference between the 9 cm. and the 18 cm. centrifuges in a blood of 47 mm. H%, provided mixing of the cells and plasma is adequate. Thus errors due to H are unlikely to cause errors in Hb-method C of more than 0.16 to 0.36g. Hb% in the Gp range from 1.018 - 1.031. This error is much smaller than the subjective error of the Haldane-Gowers' method/

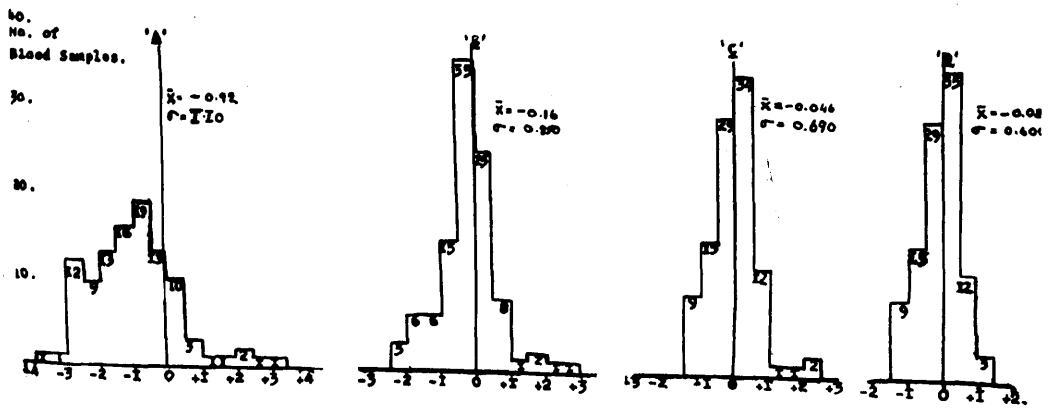


Fig. 8 :- The frequency distribution of Hb differences (calculated less Sahli estimated Hb) in the 103 bloods of the present series.

Methods A-D are shown in order from left to right.
'$\bar{X}$' = mean Hb difference in the 103 samples.
'σ' = standard deviation of the differences about the mean difference.

method in the hands of practised observers working under ideal conditions (see page 5).

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ii. Leucocytosis - Corrected By Method D.

If the original eqn. i (page 6) is modified to allow for the weight and volume of the packed white or buffy layer, it gives .....

$100Gb = H \times Gc + W \times Gw + (100-H-W)Gp$ .... eqn. xii, where W is the volume of packed white cells etc. expressed in mm./100 mm. blood and Gw is the specific gravity of this layer.

By transposition eqn. xii becomes .....

$$\text{..... } Gc = \frac{100((Gb-0.01xW(Gw-Gp)-Gp))}{H} + Gp$$

Substituting this value for Gc as for eqn. xi, page , the following equation results .....

..... $Hb = 377((100Gb-W(Gw-Gp) - 100Gp + H(Gp-1.007))$ .... eqn.xiii

This formula is identical to eqn. xi, page 30, except that W(Gw-Gp) must be subtracted at the outset from Gb x 100. Thus to correct method C for leucocytosis it is only necessary to substitute 'Gb x 100 - W(Gw-Gp)' for 100 x Gb in eqn. xi.

Gw was estimated in one case in this series and was found to be 1.051. No doubt the exact value of Gw varies with the nature of the cells, platelets, etc., contributing to W. In the case with Gw 1.051 it was observed that W was not fully packed until spinning had continued for 60 minutes. It was assumed, therefore, that W was lighter than usual in this case and the figure 1.055 was chosen as a suitable average value for Gw.

Substituting/

A. Standardisation tests (24 samples from normal bloods).

Hb errors (Calculated Hb less photoelectric Hb-g./100 ml.

Method	Mean	Range	Stand. dev.*1
a. Method A	-0.820	-1.6 to -0.1	0.456(±0.08)
b. " B*2....	-0.170	-0.5 to +0.4	0.212(±0.04)
c. " C*2....	-0.046	-0.3 to +0.2	0.155(±0.03)
d. Sahli method.	+0.002	-0.9 to +0.8	0.486(±0.10)
Hb differences (Calculated less Sahli estimation-g.%)			
e. Method C*2 actual.....	-0.048	-0.9 to +1.0	0.507(±0.11)
f. maximum (c+d above)....	-0.048	-1.2 to +1.0	0.510(±0.11)

*2 Method C in normal bloods is identical with method D.

B. 103 bloods of the series (mostly abnormal).

Hb differences			
g. Method A	-0.920	-4.0 to +3.5	1.100(±0.11)
h. Method B	-0.160	-2.2 to +3.1	0.845(±0.08)
i. Method C	-0.046	-1.4 to +2.2	0.690(±0.06)
j. Method D	-0.090	-1.4 to +1.5	0.606(±0.06)

*1..'Stand. dev.' = standard deviation of errors or differences about the mean error or difference; the standard error is given in bracquets.

~~Table~~ 1; Showing the Hb errors and differences for the various methods of estimating Hb in the 24 standardis-ation tests and in the 103 bloods of the series.

Substituting 1.055 for Gw and an average Gp of 1.025 for Gp in W(Gw-Gp) the result is 0.03 x W. No gross error will be produced if W(Gw-Gp) is assumed to be 0.03 x W, particularly when W is less than 10mm./100 mm. blood. The alternative involves estimating Gw by the method here described (page 15).

Equation xiii indicates that each mm.% of W will cause an error in the Hb, method C, of 3.77 x 0.03g. Hb%., i.e. 0.11g. Since Phillips et al included the normal W with their H in the calibration tests on which the original equations are based, there is little necessity to correct for W unless W equals 2mm. or more /100 mm. blood.

Thus summarising method D .....

a) when W is less than 2mm.%, method C is applied without modification.

b) W over 2 mm.%, subtract 0.03 x W from 100 x Gb and apply method C as before substituting the difference, 100Gb-0.03 W, for 100 Gb in eqn. xi or xi (a).

c) when W is very large, e.g. over 10% blood volume, it may be desirable to estimate Gw as on page 15 ; the resulting figure is used to calculate W(Gw-Gp) which is then treated like 0.03 x W in b) above.

Haemoglobin Calculated By Methods C and D in the Bloods of This Investigation.

The results are given in detail in Appendix C, illustrated in Figs. 6 - 8 and summarised in Table 1(opposite this page).

In the 24 normal standardisation samples the range of error is reduced to -0.3 to +0.2g.Hb.%. Adding the maximum

Sahli error, the maximum Hb-difference range compatible with this degree of accuracy in any series of bloods is from -1.2 to +1.0g.Hb.%.

Table 1 compares the Hb-difference in the 103 bloods of the series and in the standardisation tests. The range of Hb-differences is reduced by Method D to -1.4g. to +1.5g.Hb% which on subtracting the maximum Sahli error corresponds to a minimum Hb error in the abnormal bloods of from -0.5 to +0.7g. This is still larger than in the normal bloods but is much less than the minimum error found by the previous methods (-1.3 to +2.3g. for Method B). Furthermore, the mean Hb-difference in normal bloods is not 'significantly' different from the mean Hb-difference in the abnormal bloods (-0.046 and 0.09g.Hb. respectively, standard error of difference = 0.12, 't' = 0.35 Ξ P greater than 0.9). The distribution of the Hb-differences, judged by the standard deviations from the mean differences, are similar in the normal and in the abnormal bloods (standard deviation, normal bloods = 0.507, and in the 103 bloods = 0.606). It is probable therefore that the Hb error (Method D) in abnormal bloods is still slightly greater than the error in normal bloods.

Comparing the results in g.Hb% by Method D (x) with the Sahli results (y) in the 103 bloods the coefficient of correlation is 0.9656($\pm$0.0067) and the regression lines have the equations .... $y = 0.995x + 0.15$

and ..... $x = 0.963y + 0.288$

a. Sample	b. Hb.g./100 ml.. Sahli	c. Meth.D	d. Corrected	e. Gp	f. (c -b)	g. (d-b)
RL2	9.8	11.3	10.05	1.031	+1.5	+0.25
RL3	10.1	11.6	10.85	1.029	+1.5	+0.75
REa2	11.0	12.0	10.75	1.031	+1.0	-0.25
BSa	10.1	11.1	10.35	1.029	+1.5	+0.25
RPa	10.1	11.6	11.1	1.028	+1.5	+1.0
RPb	11.5	11.1	10.6	1.028	-0.4	-0.9
RMb2	9.0	9.2	8.7	1.028	+0.2	-0.3
IM	11.6	12.6	12.1	1.028	+1.0	+0.5
Mean	10.4	11.31	10.56	1.029	+0.91	+0.16

Table 2 :- Results in the eight bloods with Gp over 1.027. The corrected Hb(column d) equals Hb by method D less 0.25 x 1000(Gp-1.026)g.Hb/100 ml..

iii. Error Associated with Hyperproteinaemia.

It was observed that Gp was 1.028 or more, in all samples with Hb-differences, by method D, of more than +1.0g. The individual results in all eight samples with Gp = 1.028 or more are shown in Table 2, facing this page. It will be observed that the range of Hb-differences is from -0.4 to +1.5g. and the mean difference is +0.91g./100 ml. blood. This mean difference and the distribution of the differences indicates that the error of method D in these samples is significantly greater than the error in the rest of the series and in the standardisation tests. No corresponding association between Gp and the Hb-differences was detected in any other range of Gp in this work.

On investigation it was found that the differences between the estimated M.C.Hb.Conc. and the M.C.Hb.Conc. calculated from Gc were similarly increased in these cases. Furthermore in the artificial samples prepared from these bloods the estimated Gc maintained the same relationship to the Gc figure, estimated in the original blood, as in the other bloods of the series. It was concluded, therefore, that in most of these cases, if not in all of them, the packed red cells contained an abnormal weight and volume of some substance other than haemoglobin. This substance could be incorporated in the cytoplasm of the red cells themselves, but for reasons indicated below it seems more likely that the unknown is packed around and between the red cells. Haemolysis could be excluded as a possible cause since the icteric index was normal in most of the samples concerned, and in any case haemolysis will give negative Hb differences.

In Part II of this work it is established that Gp = 1.028 or more is usually associated with hyperglobulinaemia which, in turn, is frequently associated with spontaneous cold precipitation of protein. It is, therefore, significant that the eight samples concerned came from patients suffering from chronic lymphatic leukaemia, multiple myelomatosis, lymphadenoma, malaria and chronic hepatic cirrhosis. Spontaneous protein precipitation has been reported in the literature in all of these conditions. I would, therefore, suggest that the unknown weight and volume packing with the red cells in the bloods with Gp greater than 1.027 may be cold precipitating protein which has either sedimented with the red cells or has been thrown down during the subsequent centrifuging; possibly some of this cold precipitating protein is clot-forming protein but the normal sedimentation rate in several of the bloods suggests that the bulk of the substance, or substances, is serum protein.

Hynes and Lehmann (see page 8) report that Gs was higher than Gp in ten samples out of 270 from Indian recruits. They concluded that Gs plus 0.001578 gave a more reliable figure for 'Gp' than the estimated Gp itself, but they do not explain the phenomenon. Many factors may be involved, e.g. the influence of refrigeration if it was used, the fact that plasma is generally separated by spinning while serum is not generally centrifuged etc., but one point seems clear and that is that among 270 Indian recruits one might reasonably find ten sub-clinical cases of chronic leishmaniasis, malaria, etc; in these/

	Hb difference-method D after adjustment for Gp over 1.027- in the present series(103 bloods)	Maximum*1 Hb difference-method C -in standardisation tests. (In normal bloods method C is the same as the modified method D).
i. Mean diff..	-0.03g.	-0.048g.
ii. Standard deviations of diff. about the mean	0.532(±0.05)	-0.510(±0.110)
iii. Range of diff..	-1.4 to +1.0g.	-1.2 to +1.0 g.

iv. Diff. between means in i above = 0.018; standard error of this diff. = 0.119; 't' = 0.150; P over 0.9

If the eight samples with Gp over 1.027 are omitted from the calculation of the best formula relating Gc and the M.C.Hb.Conc. the method of least squares gives .....

..... M.C.Hb.Conc. = 373(Gc - 1.005).

Table 3:- Effect of correction for Gp over 1.027 on the Hb results in the present series. Compare with Table 1, page

*1...See Table 1.

these conditions cold precipitating serum proteins are liable to be present.

On the basis of the eight samples here described the suggested method for correcting this error cannot be regarded as anything but a tentative approximation. No attempt is made to correct the calculated Gc or M.C.Hb.Conc; correction is applied directly to the calculated Hb result (by method D) by subtracting 250(Gp-1.026) or 1000(Gp-1.026)/4g.Hb/100 ml. from the Hb-method D. This correction is an empirical approximation to the best formula for correcting the results in the eight tests in this work. It may be, however, that the procedure described by Hynes and Lehmann whereby Gs is estimated in place of Gp may effectively correct this error in bloods with Gp greater than 1.027. On the other hand it may suffice in most cases to allow the plasma and cells to separate without the cooling effect of centrifuging. At present one can only suggest that, although the method D evolved in this work gives smaller Hb-differences than the original method of Phillips et al in these eight cases, any Hb figures calculated from specific gravities in bloods with Gp greater than 1.027 should be regarded as approximate.

Effect of 'Correction for Hyperproteinaemia' on the Hb Results in the 103 Bloods.

It will be seen from Table 3, facing this page, that the Hb-differences by method D corrected for abnormally high Gp, are similar in range and distribution to the Hb-differences obtained by method D in the standardisation tests where the Hb-error ranged from +0.2 to -0.3 g.Hb%. It follows, therefore, that/

that the error of method D so corrected, is very little greater than from +0.2 to -0.3g.Hb%.

iv. British and American Estimations of Haemoglobin.

The formulae for the conversion of Gb, Gp, etc., to g.Hb/100 ml. blood are based on estimations of oxygen capacity performed by Phillips, Van Slyke et al on normal American bloods. It is essential, therefore, to correlate their methods and results with those in this investigation and, if the copper sulphate method is to be used in this country, with the B.S.I. colour standard adopted by the Medical Research Council.

Methods Calibrated Against Iron Estimations (Delory - 1943)

The photoelectric procedure used to standardise the Sahli method in this work was calibrated by Bell et al (1945) against iron estimations.

King et al (1944), Macfarlane and O'Brien (1944) and King et al (1947) have shown the B.S.I. colour standard for the Haldane-Gowers method to be equivalent in colour to a blood of iron content 49.5 mgms.%, i.e., at 3.34 mgms. iron/1.0 g.Hb., 14.8g.Hb/100 ml. blood.

Hence -

100% Haldane-Gowers B.S.I. standard .... = 49.5 mgms. iron or = 14.8g.Hb/100 ml. = 14.8g.Hb% by the photoelectric and the Sahli methods in this work.

Methods Calibrated Against Oxygen Capacities.

The B.S.I. colour standard was also measured in terms of oxygen capacity by the workers referred to above. The 100% Haldane-Gowers blood was found to have an O_2 capacity of 19.3 ml.% which, at 1.34 ml.O_2 per 1.0g. blood, is equal to 14.4 g.Hb/100 ml. blood. The method used is stated to be that of Van Slyke and Neill (1924).

For the calibration of the copper sulphate method Phillips, Van Slyke et al modified the method so that the combined capacity for O2 and carbon monoxide was measured instead/

instead of the O_2 capacity alone. Furthermore they converted the O_2 + CO capacity to g.Hb on the basis of 1.36 ml. = 1.0g.Hb.. The following passages from their publication (1945.a)explain these modifications ... ".. the latter procedure" i.e. the original method, "fails to include the small amounts of CO in the neighbourhood of 0.3 volumes per cent, bound as HbCO that appears to be present in normal blood regularly, at least in New York. This normal CO is increased by smoking, which may raise it as high as 1 volume per cent." ... and "Bernhart and Skeggs" (1943) "have from apparently very accurate analyses, recently published the first consistent data on the amount of O_2 bound by 1 gram of haemoglobin. They find it to be 1.36 cc. of O_2." By this method Phillips et al found that their mean blood of Gc 1.097 and H 47 had an O_2 + CO capacity of 21.62 vols.% = 15.9g.Hb/100 ml.. The original method is stated to give a mean normal O_2 capacity of 20.7 vols. % and the difference is attributed by Phillips et al to the "negative error caused by small amounts of carbon monoxide in the blood, when only the O_2 is measured."

Correlation of the Methods and Results:-

If it is assumed that the correct Hb equivalent of the B.S.I. standard is 14.8 g.Hb%, it should follow that the equivalent in O_2 + CO capacity is 14.8 x 1.36 = 20.1 vols.%. The factor 1.36 is used to permit direct comparison with the results of Phillips et al. The actual O_2 capacity equivalent to the B.S.I. standard was found by the original method to be 19.3 ml.%.. There is, therefore, a difference of 0.8 vols.% which, like the 0.9 vols. difference of Phillips et al, is probably due to CO-Hb. If it is true that 14.8g.Hb by iron estimation combines with 19.3 vols. O_2 and 0.8 vols. CO% there should be 14.2g.Hb present to combine with O_2 and 0.6g. as CO-Hb/100 ml. blood. Applying the factor of Bernhart and Skeggs to the estimated O_2 capacity the Hb equivalent of the B.S.I. standard/

standard = 19.3/1.36 = 14.2 g.Hb%.

The findings of King et al (1947) give further evidence in support of this hypothesis. The CO capacity was estimated after reduction of the blood and the equivalent of the 100% B.S.I. standard was found to be 14.7g.Hb%. Thus it is highly probable that most of the "inactive" Hb postulated by King et al to account for the difference between their results by iron estimation and by oxygen capacity estimation is in the form of CO-Hb.

Furthermore, the CO-Hb content of blood is likely to vary according to the habits of the individuals examined. Phillips et al mention smoking as one factor which raises the blood CO-Hb, but it may be that motoring, working in factories or living in cities where the exhaust gases of furnaces pollute the atmosphere with low concentrations of CO, or the use of coal or coke stoves for heating, can cause a similar rise in CO-Hb in the blood. Thus blood from a donor who travels to the place of bleeding by car or bus may have more CO-Hb than that of a similar donor travelling by electrically propelled street car and, similarly, the smoker's last minute smoke before being bled may raise the CO-Hb content of the blood.

Such variation may well account for some of the contradictory reports published on an apparent sex difference in the relationship between the iron content and the O_2 capacity of the blood, the true difference being in the habits of the donors of each sex rather than between sexes. Thus it seems probable that in two similar groups, one male and one female, the male group will contain more smokers and more motorists than the female group. Hence more Hb may be present as CO-Hb in the males and the O_2 capacity may be reduced relative to the iron content. The extent of any such variation will itself vary according to the exposure of the individuals in the groups to CO.

This sex-difference in the iron to O_2 capacity relationship is recorded by Macfarlane and O'Brien (1944) and/

0.4 g. of CO-Hb. The normal blood of Phillips et al is now equivalent to 109% of the B.S.I. standard which itself is equal to 14.6g. Hb% by the copper sulphate method. If this factor is correct all the Sahli readings herein should exceed the copper sulphate estimations by 0.2 x 100/14.6 = 1.37% of the copper sulphate estimation. The mean Hb-difference should, therefore, be -0.13g..

From the above observations and results it is concluded that the copper sulphate method ought to give Hb results which agree to within 0.2g. with the current methods of iron estimation used in this country to estimate Hb. Hence the error of the method should be similar when compared with the Sahli and photoelectric methods in this work and with the B.S.I. standard (at 100% is = 14.8g.Hb%).

V. Miscellaneous Errors.

Theoretically any abnormality in the red cells which alters the relationship between cell weight/cell volume/cell haemoglobin is liable to cause an error in the haemoglobin (Method D). It is possible that such an error may occur if the blood contains a very large proportion of normoblasts, reticulocytes, punctate basophiles, siderocytes, spherocytes, etc.. In the present work no constant error was noted in bloods with 4 - 10% reticulocytes, or 4,000 - 6,000 punctate basophiles/c.mm.

The most important technical errors result from -

1) inadequate mixing of cells and plasma before the estimation of Gb and H. Gb should be confirmed by repeating the test with a second filling of the pipette;
2) The use of wet or improperly cleaned pipettes;
3) Free haemoglobin in the plasma is not estimated by the method and so Haemolysis will cause an error in the g.Hb% blood.

Blood Gravity as a Screening Test for Anaemia.

The/

and by Bell et al (1945) but denied by Gibson and Harrison (1946) and by King et al (1947). On examining the results in the last mentioned paper it is interesting to note in their Tables 2 and 3 that, after referring all bloods to the 100% Haldane-Gowers scale, although the O_2 capacities in the males and in the females averaged the equivalent of 14.4g.Hb, the average CO capacities were equivalent to 14.95g. in the males and 14.52g. in the females.

It is therefore, probable that the Haldane-Gowers B.S.I. standard is equivalent to an O_2 + CO capacity as estimated by Phillips et al of about 20.1 vols. %. Thus the normal blood of Phillips et al should be equal by colour comparison to $\frac{100 \times 21.62}{20.1}$% i.e. 107.5% of the B.S.I. standard. Conversely the 100% equivalent of the colour standard should equal 14.8g.Hb by the copper sulphate method as well as by the photoelectric and the Sahli methods used herein.

The mean American normal Hb would thus appear to be greater than the mean British normal Hb of 102% Haldane-Gowers for healthy males determined in the Hb Survey of 1943 (Special Report No. 252) but it was found in the survey that the mean Hb varied with the occupational group, laboratory technicians, civil servants and policemen having mean Hbs. from 105 to 110%. Phillips et al do not indicate the occupations of their 20 normal blood donors.

Wintrobe in the 1947 edition of his textbook "Clinical Hematology" states that Drabkin, D. (1945) has found that iron = 0.339% by weight of the Hb molecule. If this figure is used in place of 0.334 % the equivalent of the B.S.I. colour standard by iron estimation is equal to 14.6g.Hb%. This still equals 14.8g. by the photoelectric and the Sahli methods but should be equivalent to an O_2 + CO capacity as estimated by Phillips et al of 19.85 vols. %. 19.3 vols. of this result is the true O_2 capacity and accordingly 0.55 vols. should represent the amount of CO liberated by the blood Hence, the 14.6g.Hb must be made up of 14.2 g. free Hb and 0.4g./

Blood Gravity as a Screening Test for Anaemia.

The tests employed for this purpose are described on pages 8 and 9 . It has been shown in this work that the Hb calculated from Gb alone is liable to an error of ±3 - 4 g Hb% in abnormal bloods. The Gb test should therefore distinguish satisfactorily between bloods with 3.0g. Hb more or less than the critical haemoglobin level selected. Within the range ±3.0g.Hb from the critical level the numbers of wrongly grouped bloods are an indication of the number of abnormal bloods in the group rather than an indication of the accuracy of the method. Fundamentally the test is irrational in that, by means of Gb, it is hoped to spot those very conditions which are known to invalidate the formulae relating Gb to Hb. In the bloods of this series, omitting doubtful cases where the Hb-Sahli and Hb-Method D are themselves on opposite sides of the critical Hb level, 12.5% of the bloods are wrongly grouped by the standard Gb = 1.053 and by the standard Gb = 1.049, 2% are wrongly grouped. The latter standard has the disadvantage that several grossly abnormal bloods with Hb very close to the critical level pass as normal, e.g. blood from leukaemias, lymphadenoma and multiple myelomatosis.

The test, despite its shortcomings, is much better than no screening test at all. No method for estimating Hb more accurately can be employed in its place without adding considerably to the time, work, organisation and equipment required. Furthermore, any theoretical increase in accuracy may be concealed in practice by the introduction of additional possible causes of error, e.g. clerical and technical errors.

It seems probable that some revision of the formula relating Gb and Hb may be desirable in this country. The original equation vi was worked out from the 20 normal male bloods examined by Phillips et al in the United States. The normal Gp for blood donors, male and female in this country, has been shown to equal 1.0254 rather than 1.0264 as found by/

Calculation of Hb from Gb, Gp, H&W estimated as described on the opposite page.....

I. Correct Gb & Gp for the anticoagulant as described by Phillips et al (1945a)....For ammon. & pot. oxalate mixture subtract 0.0004 for each mg. mixture per ml. blood from Gb and Gp.

2. Apart from the few exceptions mentioned below eqn. xi or xia will convert Gb, Gp &H to g. Hb/100 ml. blood.

Eqn. xi...$Hb = 3.77((100(Gb-Gp)+H(Gp-1.007))$

" xia..$Hb = 3.5((100(Gb-Gp)+H(Gp-1.000))$

3. When W exceeds 2mm.%, the only difference is that Gb in eqn. xi & xia is replaced by Gb-0.0003xW.

If W is very high, e.g. over 10mm./100mm. blood, it may be advisable to estimate Gw as on page 15. Gb in eqn xi is then Gb-0.01xW(Gw-Gp).

4. When Gp is over 1.027, approximate correction can be made by subtracting 0.25g. Hb from the result as above for each 0.001 by which Gp exceeds 1.026.

The nomogram on page 20, App. C, is designed to facilitate the above calculation. It is based on eqn. xia.

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take about ten minutes excluding the time in the centrifuge. This estimate includes time for cleaning the pipettes and tubes.

Besides the Hb such a routine gives, without additional effort, the value for H, the M.C.Hb.Conc. and g. protein % plasma. Before the supernatant plasma is drawn off, the icteric index may be measured. The erythrocyte sedimentation rate is determined by leaving the filled haematocrit tube in a vertical position for one hour before spinning.

Advantages and Disadvantages of the Suggested Method:-

There are two main disadvantages, i.e. venupuncture is required and a centrifuge is also required. It is frequently possible to remove the necessary blood at the time of venupuncture for some other purpose, such as the W.R., sedimentation rate, Widal, blood culture, liver function tests, blood urea, blood grouping etc.. The side-room of general medical wards is generally provided with a power driven centrifuge: the type of centrifuge does not matter so long as it can be calibrated against an 18 cm. laboratory pattern centrifuge. Even if no centrifuge is available the work of the hospital laboratory will not be unduly increased if the filled haematocrit tube is sent for spinning.

The advantages are .....

1) subjective errors are avoided

2) the method is at least as accurate in most normal and abnormal/

abnormal bloods as the Haldane-Gowers' method at its best (see page 5).

3) the working time, i.e. excluding the time in the centrifuge is no more than the time taken by the usual subjective colorimetric methods.

4) without additional effort the following results are obtained in addition to the Hb .... packed cell volume, M.C.Hb.Conc., approximate plasma proteins, the icteric index, the crude and corrected sedimentation rate (Wintrobe's method).

5) no special apparatus is required and the cost is negligible.

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11. BLOOD AND PLASMA SPECIFIC GRAVITIES RELATED TO FACTORS OTHER THAN HAEMOGLOBIN.

The interpretation of Gb and Gp in normal and abnormal blood is fraught with mathematical possibilities. Some applications of the method are discussed briefly below.

A. THE PACKED CELL VOLUME.

The nomogram of Phillips et al converts Gb and Gp directly into H on the assumption that Gc = 1.097. The underlying formulae are .....

Eqn. (ii) ... $H = \frac{100(Gb - Gp)}{Gc - Gp}$ from which eqn. (iv) is developed thus .....

$$H = \frac{100(Gb - Gp)}{1.097 - Gp}$$

The error due to the assumption that Gc is constant may be illustrated by comparing three bloods with the same Gb and Gp but different Gc. If one of the three is the normal blood of Phillips et al then all three bloods by equation iv will have H = 47. This result is correct for the normal blood of Phillips et al. By equation ii if Gc in the other two bloods are 1.067 and 1.107 the true H values are 80.2 and 39.9% (errors +33 and -7% blood volume). It is not, therefore, surprising that in this series errors as large as 13% blood volume were found in abnormal bloods; in normal bloods the errors were as much as 7%.

An unsuccessful attempt was made to obtain a more accurate H by calculation from Gb, Gp, and the Hb in this work. The equation below is obtained by transposition from equation xi (page).

$$H = \frac{0.2653 \times Hb - 100(Gb - Gp)}{(Gp - 1.007)}$$

Difficulty arose from the fact that the Hb error

is/

B. Plasma Proteins from Gp.

Plasma Proteins in g. % Plasma.

A. From Gp	B. By Chemical Estimation	B - A
6.3	6.4	0.1
6.7	7.2	0.5
7.8	7.7	-0.1
8.2	8.1	-0.1

	A. From Gp	B. By Chemical Estimation of Serum +0.25g.	
	4.0	4.5	0.5
	4.4	4.6	0.2
	4.4	4.9	0.5
	6.3	6.7	0.4
	6.3	6.7	0.4
	9.0	9.2	0.2
	63.4	66.0	+2.6
Means	6.34	6.6	+0.26

Table 4:- Plasma proteins in g./100 ml. plasma by calculation from Gp and by chemical analysis in four blood samples. Chemical analyses were performed on serum in six samples; 0.25 has been added to the result in these cases to permit direct comparison.

is multiplied by from 12 to 25 in the calculated H. It follows that the Hb must be accurate to the second decimal place before the formula can be applied.

B. PLASMA PROTEINS.

Proteins were estimated chemically in ten of the bloods of the present series. Methods are described in Appendix A and the results are shown opposite this page in Table 4. The amended formula of Phillips et al (1945b) was used to convert Gp to g.protein % plasma or serum. The difference is less than ±0.5g. in the ten tests. This result is in agreement with the finding of Phillips et al (1945a) who consider that errors greater than 0.6g.protein % are very unlikely to occur even in grossly abnormal bloods (page 7).

Normal Gp in Great Britain.

No widespread survey of Gp has been carried out in this country. The normal values given below are based on the chemical estimation of total serum proteins in 353 unselected blood donors as recorded in Special Report No. 252 (Medical Research Council).....

Range total serum proteins = from 5.8 to 7.9g./100 ml. (Mean = 6.56g.)

Allowing an average of 0.25g. fibrinogen /100 ml. this is equivalent to a normal plasma protein range extending from 6.0 to 8.2g.% with a mean of 6.8g.%.

According to equation vii the normal range of Gp is, therefore, from 1.023 to 1.028 inclusive and the average normal Gp = 1.0254. This is lower than the normal Gp found by Phillips et al (1.0264) but their figure is the mean Gp in 20 healthy male Americans, while the M.R.C. figures are from a mixed group of British males and females.

In the ten normal bloods in this series Gp ranged from 1.023 to 1.026 with a mean of 1.0249.

Gp/

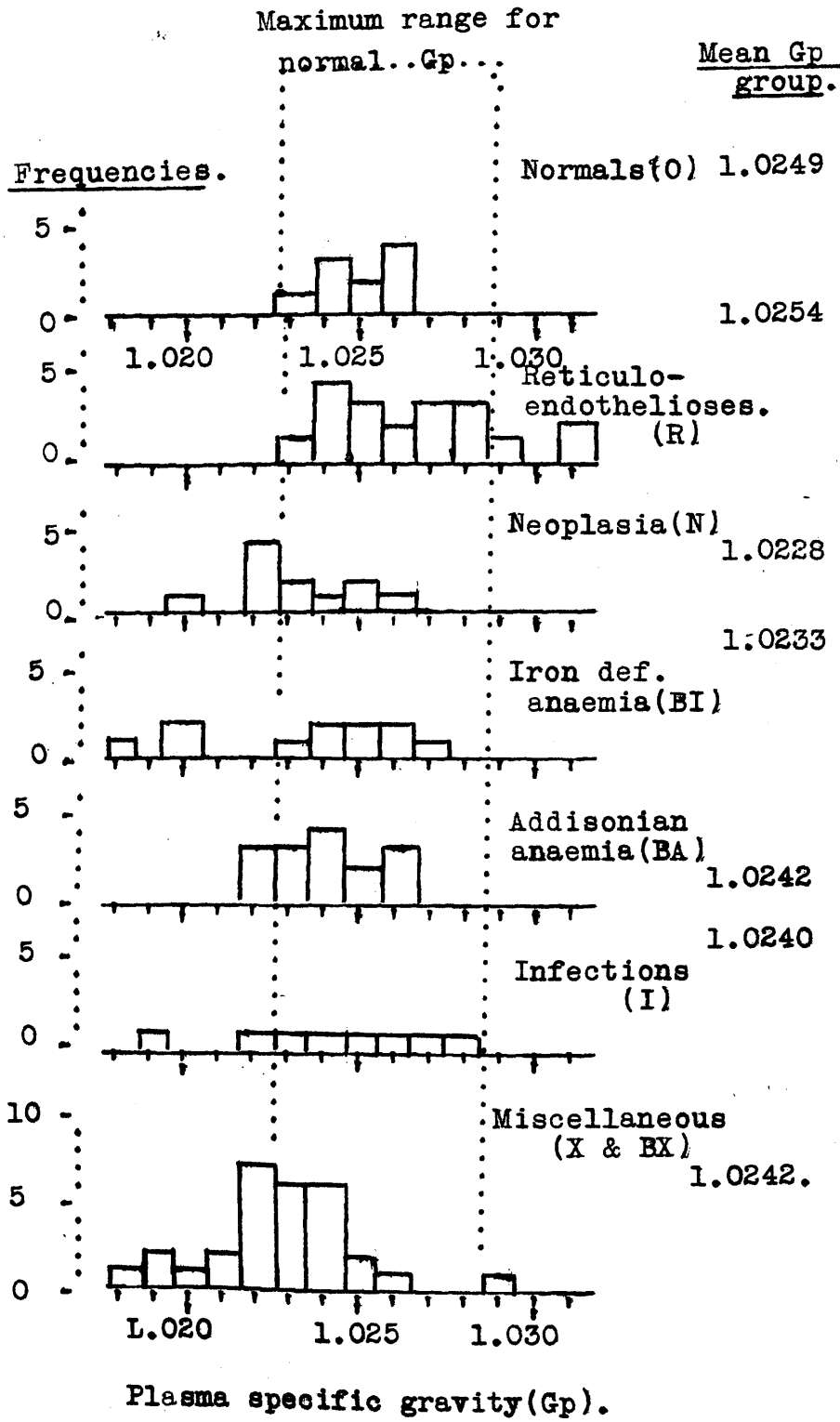

Fig. 9 :- Plasma specific gravity in each blood of the series arranged in pathological groups.

Gp in Abnormal Bloods in the Present Investigation.

The samples are arranged under pathological groups in Figure 9, opposite. The difference between the mean Gp of each pathological group and the mean of the normal group is tested for statistical 'significance' in Table overleaf. Only the group of 'neoplasia' has a mean Gp which is significantly lower than the mean Gp in the normal group. The difference is not 'significant' in either the microcytic or the macrocytic anaemia groups, nor is the difference between the means of the two groups 'significant'.. This is in agreement with the observation of Hynes and Lehmann (1946) who found no significant difference between the mean Gp in macrocytic anaemia and in microcytic anaemia.

It was considered likely that Gp and Hb might be related to one another, and in the 103 bloods of this series the coefficient of correlation between Gp and Hb is 0.346 (standard error ±0.088). Statistically this indicates a significant degree of correlation. Actually the correlation is most obvious in the post-haemorrhagic anaemias and in the group of neoplasia associated with chronic blood loss. It seems probable that Gp is related to Hb in those cases where red cells and plasma protein are lost simultaneously from the circulation. Possibly malnutrition can cause a parallel reduction in red cells and in plasma protein but the number of undernourished cases in this series is inadequate to permit any correlation in these cases.

C. SPECIFIC GRAVITY OF PACKED RED CELLS (Gc).

Gc, calculated from Gb, Gp, H and W by Method D, is shown for the 103 bloods of the series in Fig. 10, opposite page 50. It has not been possible to correct Gc in the bloods with Gp = 1.028 - 1.031 for the volume and weight of the unknown factor sedimented with the red cells (pages 33 - 35), but only the group of reticulo-endothelioses is seriously/

Group	a.	b.	c.	d.
Miscellaneous	0.00070	0.0007	1.01	2.609 NS*.
Neoplasm	0.0021	0.00054	3.84	2.093 Sig*.
Infection	0.0009	0.00095	0.91	2.120 NS..
Reticulo--endothelioses	0.0015	0.00085	1.76	2.052 "
Iron-def. anaemia	0.0016	0.0094	1.69	2.101 "
Addisonian anaemia	0.0007	0.00054	1.25	2.060 "

*NS.. not significant: Sig.'...significant.

Column a...Mean Gp of group less mean Gp of normal group.

" b.... Standard error of the difference between the means in 'a'.

" c. ..'t'(equal to 'a'/'b').

" d. ..value of 't' corresponding to P 0.05.

Table 5:- Statistical test of the significance of the difference between the mean Gp of the various groups and the mean Gp of the normal group(as described by Chambers(1940) on pages 35-38 of his book'Statistical calculations').

seriously affected.

In normal bloods ... Gc was between 1.089 and 1.097 and the mean was 1.0935. This mean differs significantly from that of Phillips et al (1.097) but their twenty bloods were from male Americans with an average of 15.9 g.Hb% while the bloods examined herein were from a mixed group of British males and females with an average Hb = 13.9g.%. O'Connor (1947) page 10 , found the normal Gc to range from 1.090 to 1.096.

In abnormal bloods ... Gc was between 1.067 and 1.110.

Correlation between Gc and the M.C.Hb.Conc. has already been discussed (page 27). It is not therefore surprising that in all the primary iron deficiency anaemias Gc is less than 1.086; in the Addisonian anaemias Gc was at least 1.090 with three exceptions. The three exceptions are all from the same patient (BAc). When the diagnosis was made the patient had received no treatment and Gc = 1.100 but after two weeks liver therapy, Gc had fallen to 1.080. Subsequent tests at two-weekly intervals gave Gc = 1.082 and 1.089 respectively. When Gc was 1.080 the blood was clearly hypochromic and a Price-Jones count of red cell diameters gave a mean red cell diameter of 7.0 u. Hence the three exceptions in the Addisonian anaemia group confirm the relationship previously noted between Gc and cell 'chromicity'.

The other significant finding is that in 8 samples from patients with Addisonian anaemia Gc was 1.099 or more. If the 'hyperchromic' appearance of the red cells in this condition is due to increase in cell depth as is commonly believed, Gc might reasonably be normal. Another factor to be considered is the amount of plasma enclosed within the packed red cells. Where the average red cell is large the space between the packed cells should be diminished and so 100 vols. of packed macrocytes will contain less intercellular plasma than 100 vols. of normocytes. The volume of plasma/

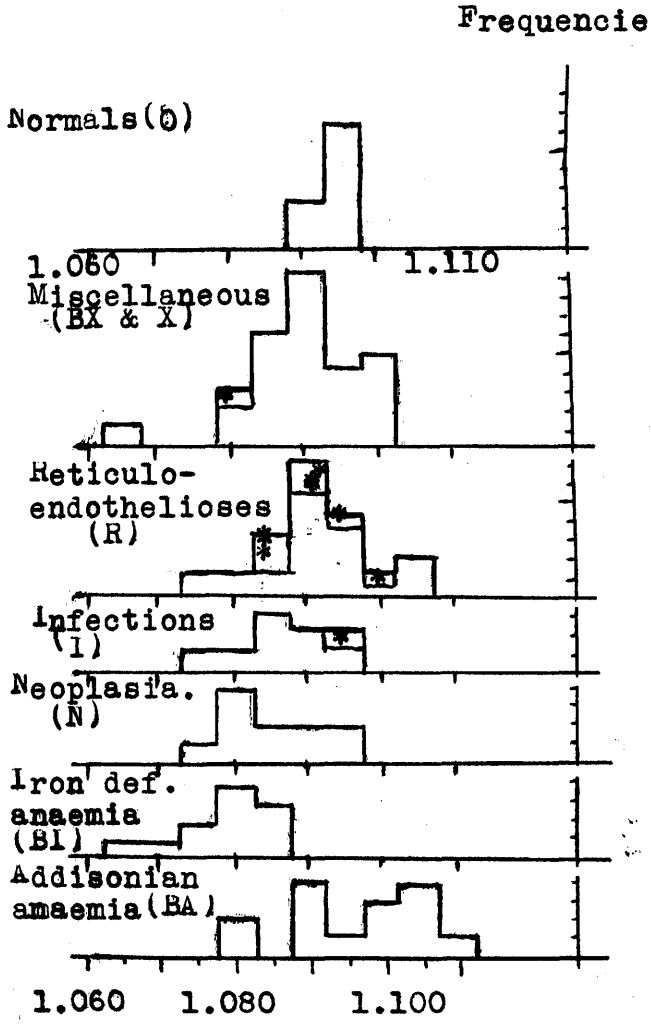

Fig. 10:- Gc calculated from Gb, Gp, H & W in the samples of the series arranged in pathological groups. No allowance has been made for hyperproteinaemia in samples with Gp over 1.027(indicated by an asterisk).

plasma enclosed with packed normal cells has been variously estimated at 2.25% (Maizels, 1945) and 7.0% (Chapin and Ross 1942). If 7% of plasma is taken from the normal packed erythron of Phillips et al Gc becomes 1.109. It is possible, therefore, that the higher Gc and M.C.Hb.Conc. in the Addisonian anaemias in this series is due to the fact that red cells of high normal 'chromicity' are packed with less intercellular plasma than in normal blood.

There are five samples in the series with Gc = 1.100 or more in which the erythron appears to be perfectly normal and there is no suggestion of any anaemia. One of the five requires to be corrected for Gp = 1.028 (Sample IM). The other four come from patients suffering from ... XN ... 'primary' nephrosis; XV ... peripheral neuritis apparently due to dietary deficiency of Vitamin B; ITa ... acute phthisis; and NGb ... gastric carcinoma with advanced hepatic involvement and a leuco-erythroblastic anaemia (5% normoblasts, no megaloblasts in the peripheral blood).

Hence it is concluded that if Gc is ....

i. less than 1.086 the blood is hypochromic;
ii. between 1.086 and 1.088 it is borderline normal / hypochromic;
iii. between 1.089 and 1.099 the blood is normochromic and may be either normocytic or macrocytic;
iv. greater than 1.099 the blood may be macrocytic and either normochromic or hyperchromic.

D. THE RED CELL COUNT.

O'Connor, 1947, (page 10) has suggested a relationship between the mean cell volume (M.C.V.) and Gc which may be expressed thus:-

$$M.C.V. = 1000(Gc - 1.000)$$

By substituting $\frac{10 \times H}{R.B.C.}$ for M.C.V. and by transposition this gives

$$R.B.C. = \frac{H}{100(Gc - 1.000)} \text{ millions /c.mm.}$$

In/

In this way O'Connor used Gc to calculate the red cell 'count'.

The statistical error of red cell counts made by one observer alone is too uncertain to permit correlation with Gc in this work and in any case, I do not consider that the formula above can give anything more than an index. Certainly the result can never be called a red cell count. The object of such an index is presumably to distinguish iron deficiency anaemia from macrocytic anaemia. It would seem more reasonable to relate Gc to the M.C.Hb.Conc. for this purpose. Both the M.C.Hb.Conc. and Gc are ratios of weights to volumes whereas the M.C.V. is a pure measure of volume.

O'Connor himself, admits discrepancies but gives no details of the frequency, extent or possible causes of the discrepancies. Two such discrepancies are apparent from the results herein .....

(a) M.C.Vs. as high as 130 c.U. were found in the macrocytic anaemias of this series but in no case was Gc over 1.110. O'Connor himself gives 1.106 as the highest Gc in the macrocytic anaemias examined by him.

(b) In the experiments described on pages 57 to 62 artificial venous constriction caused an increase in the M.C.V. but this was associated with a fall in Gc, not with a rise as the above relationship implies.

Inspite of these discrepancies it is probable that some correlation does exist between Gc and the M.C.V. since in iron deficiency anaemias the M.C.V. is low and the blood is hypochromic with a low Gc, while in Addisonian anaemia Gc may be normal or high and the M.C.V. is usually also high. The correlation is probably between Gc and the M.C.Hb.Conc. on the one hand, and between cell 'chromicity' and cell volume on the other, rather than a direct relationship between Gc and the M.C.V..

E. MEAN CELL WEIGHTS.

The mean red cell weight (MRCW) may be calculated from Gc by the formula ... $MRCW = \frac{10 \times H \times Gc}{R.B.C.}$ micro-micrograms or g.x 10^{-12} (millions/c.mm) Any error due to the plasma enclosed with the packed red cells should be very small.

In the normal bloods of this series MRCW is between 84 and 91, mean 89 g. x 10^{-12}. The iron deficiency anaemia samples have a range of 59 to 85 and a mean of 75 g. x 10^{-12}, and in the Addisonian anaemia samples the corresponding results are from 110 - 140, mean 124 g. x 10^{-12}.

Since the MRCW is the product of the M.C.V. and Gc and since these two factors vary in the same direction in most anaemias it is probable that any tendency towards anaemia will be readily detected by a rise or fall in the MRCW.

Similarly the mean white cell weight (MWCW) should be equal to $\frac{10^4 \times W \times Gw}{W.B.C.}$ g. x 10^{-12}. (thousands/c.mm.) On this basis the MWCW in case RMc = 580g. x 10^{-12}. The predominant cells in this blood are myelocytes and the promyelocytes. There seems little doubt that the value of the MWCW, as also the mean white cell volume (MWCV), will vary with the nature of the white cell preponderating. Measurements of mean white cell volume and weight may be of value in the differentiation of the primitive cells seen in the acute leukaemias. Alternatively, similarity in weight and volume may be additional evidence in favour of the common nature of such cells.

The mean white cell volume (MWCV) should be equal to $\frac{10^4 \times W}{W.B.C.}$ where W.B.C. is the white cell count in thousands per c.mm.. For this purpose W does not include the/

the superficial whitish platelet layer occasionally seen in myelogenous leukaemia and it is important to continue the spinning in an 18 cm. centrifuge to the 'no-change' end point for W. In sample RMc this required a full hour and it is quite likely that longer may be necessary in some cases. The following values for MWCV were found in leukaemic samples in this work ....

RLi ... 90% small, mature lymphocytes ... 75 c.u.

RL3 ... 30% lymphoblasts,
30% prolymphocytes,
20% mature lymphocytes .. MWCV = 420 c.u.

RMc ... 30% prolymphocytes
promyelocytes ..... MWCV = 553 c.u.

It is interesting to observe that Wintrobe - 'Clinical Hematology', 1947, 2nd edition, page 244, observes that 0.1 mm. of packed white cells in the centrifuged haematocrit tube represents an average of 2000 white cells per c.mm. when leucocytosis is marked. Hence by the above formula the average white cell volume = 500 c.u.. Similarly, Pines (1947) correlating W and W.B.C. in 11,485 cases finds the average W.B.C. for W = 2%, to be 40,000/c.mm..

Symbols Used in Discussion on Total Blood Volume.

Pages 54 to 56

Transfusion Cases .....

	Recipient Before Transfusion.		Recipient After Transfusion.		Blood Transfused.
Total volume of blood in mls.	V	:	V+T	:	T
Whole blood specific gravity.	Gb'	:	Gb"	:	Gt
Plasma specific gravity.	Gp'	:	Gp"	:	Gpt
Packed cells % blood volume.	H'	:	H"	:	Ht

Equation A:-

(i) Whole blood transfused:-

$$V = \frac{T(Gb"-Gt)}{Gb'-Gb"} = \frac{T(H"-Ht)}{H'-H"} = \frac{T((Gp"(100-H")-Gpt(100-Ht)))}{Gp'(100-H')-Gp"(100-H")}.$$

(ii) Plasma transfusion ... As in (i) but Ht=0 and Gt=Gpt.

Total Cell Volume and Total Plasma Volume:-

	Total Cell Volume	Total Plasma Volume
Pre-transfusion	0.01 x H'V.	0.01xV(100-H')
Post-transfusion	0.01xH"(V+T)	0.01(100-H")(V+T)

111. FURTHER THEORETICAL POSSIBILITIES AND PRACTICAL APPLICATIONS.

TOTAL BLOOD VOLUME.

The principles outlined below were originally developed in connection with the venous constriction experiments described later in this section, but they may also serve as a basis for several other practical applications of the copper sulphate method.

Total Blood Volume in Transfusion Cases.

If T ml. of fluid, specific gravity Gt, are mixed with V ml. of fluid, specific gravity Gb', and the specific gravity of the mixture is Gb", then, by equating the sum of the weights of each fluid to the weight of the mixture, it follows .....

$$Gb"(V + T) = VxGb' + TxGt$$

which by transposition gives .....

$$V = \frac{T(Gb" - Gt)}{(Gb' - Gb")}$$

(This formula was used by Phillips, Yeomans et al (1946). See page 12 .). Hence, provided the volume of fluid lost from the circulation during transfusion is small relative to the volume transfused, it should be possible to calculate the total blood volume (V) by means of the above formula from the following data .....

(1) The patient's blood gravity before (Gb'), and after (Gb") the transfusion.
(2) Gb of the blood transfused (Gt).
(3) The volume in ml. of blood given (T).

When plasma is given instead of blood the specific gravity of the plasma = Gt, and T = the volume administered. The error due to coincident fluid loss should be small except when the transfusion is given very slowly.

V may also be expressed in terms of H' and H", i.e. the pre-transfusion and post-transfusion packed cell volumes,/

volumes, and of Gp' and Gp", thus .....

$$V = \frac{T(Gb"-Gt)}{Gb'-Gb"} = \frac{T(H"-Ht)}{H'-H"} = \frac{T((Gp"(100-H")-Gpt(100-Ht))}{Gp'(100-H') - Gp"(100-H")} \quad \ldots.$$

(equation A.)

where Ht, Gpt are respectively the packed cell volume and the plasma specific gravity of the blood transfused. When plasma alone is given Ht = 0 and Gt = Gpt.

Since V is the pre-transfusion blood volume, the post-transfusion volume may be assumed to be V + T. Before transfusion the total cell volume = 0.01xVxH and the total plasma volume = 0.01xVx(100-H'); corresponding figures after the transfusion are respectively 0.01(V+T)H" and 0.01(V+T) (100-H").

It would appear therefore, that, given (a) the Gb', Gp' and H' of the original blood, (b) Gt, Ht, Gpt and the volume (T) of the blood transfused, (c) Gb" estimated at the end of the transfusion - it should be possible by means of equation A to calculate not only V but also H", Gp", and the total cell volumes and total plasma volumes before and after the transfusion.

Samson Wright in his text-book "Applied Physiology", 8th edition, page 349, states that estimations of total blood volume from venous blood are liable to error since the proportion of cells to plasma is higher in venous blood than in the general circulation. Finger blood seems to be more suitable. It may be possible to overcome this difficulty by examining both finger and venous blood before the transfusion, and finger blood alone after the transfusion. At the time of setting up the transfusion it is a simple matter to withdraw 2 ml. blood. This sample may be used to estimate the pre-transfusion Gp' and Gc' from which by means of equation ii, page 6 , and Gb', estimated in finger blood, the value of H' as in finger blood may be calculated.

The accuracy of equation A depends on the difference between/

Female; age 3 years; Aplastic Anaemia of 6 months' duration.

A. Calculation of blood volume etc. as described opposite:-
Essential estimated results ....
Gb ... Before transfusion (Gb')=1.0310; fluid transfused (Gt) = 1.0554; after transfusion (Gb")=1.0376, Gp'=1.025, Gpt=1.0204
H ... Before transfusion = 7.5% blood volume; blood transfused = 48% blood volume.
Volume of blood transfused = 400 ml.

Calculation.

i) $V=\frac{T(Gb"-Gbt)}{Gb'-Gb"}=\frac{400(1.0376-1.0554)}{1.0310-1.0376}$ = 1079 ml.

ii) $V=\frac{T(H"-Ht)}{H'-H"}$.·. $1079=\frac{400(H"-48)}{7.5-H"}$.·. H" = 18.28% (Estimated 18.5%) .

iii) $\frac{Gp"(100-H")-Gpt(100-Ht)}{Gp'(100-H')-Gp"(100-H")}$ $xT=1079=400\frac{(Gp"(100-H")-48x1.0204)}{92.5-1.025-Gp"(100-H")}$

.·. Gp" = 1.0224
(Estimated 1.0244)

iv) Final total cell volume 201 ml. and total plasma volume 878 ml.

B. Blood volume by equations i, ii, iii above using the estimated H"(18.5) and Gp"(1.0244) ....
i) 1079 ml. ii) 1070 ml. iii) 1210 ml. Mean calculated total blood volume = 1130 ml.

Table 6 :- Calculation of blood volume from specific gravities is described on the opposite page. A. illustrates the procedure when H" Gp" are unknown, B. illustrates the different values for the blood by the different formulae.

between Gb' and Gt; as Gt approaches Gb' the accuracy of the calculated results diminishes. Since whole blood of specific gravity Gb' has no effect on the recipient's whole blood specific gravity, total blood volume cannot be calculated from equation A when Gb' equals Gt.

The example in Table 6, facing page , was investigated after the present series was completed. The specific gravities were estimated to the fourth place of decimals in this case. Copper sulphate solutions differing in specific gravity by 0.0005 were prepared by mixing equal parts of the solutions of specific gravity 0.0005 above and below the required figure.

In Cases Not Being Transfused. Equation A will apply whether the additional fluid is infused, or absorbed from the tissue fluid; it will also apply when fluid other than whole blood is lost from the circulation but in this event T will have a negative value. When no transfusion is given the value of T is unknown; V cannot therefore be calculated as in transfusion cases. Instead, equations B(i) and B(ii), facing this page, may be used to calculate the amount of fluid, other than whole blood, lost from, or taken up by the circulation in a given interval; this amount is expressed as a percentage of the blood volume within the circulation at the end of the given interval and is an index of haemodilution or concentration rather than an index related to the total blood volume, e.g. sustained haemorrhage does/

Equation B in Non-transfusion Cases.

Sample B' obtained at time B' and sample B" at time B".
Respective results Gb', Gp', H' and Gb", Gp", H".
T .... the change in total blood volume between B' and B", excluding whole blood lost or gained from the circulation.

V' ... the total blood volume at B'.
V" ... the total blood volume at B".
Gt ... specific gravity of the fluid, other than whole blood lost or gained.

Since whole blood loss has no effect on the specific gravities and T is a volume of fluid other than whole blood. Ht in eqn. A equals 0 and Gpt equals Gt. Thus by transposition eqn. A gives .....

$$\frac{100T}{V'} = \frac{100(H'-H")}{H"} = \frac{100(Gb - Gb")}{Gb" - Gt} = k.$$

$$\therefore \frac{100T}{k} = V'$$

If now t equals the volume change excluding whole blood lost or gained from the circulation between B' and B", and if t is expressed as a percentage of the final blood volume, then .....

$$t = \frac{100T}{V"} = \frac{100T}{V'+T} = \frac{100T}{\frac{100T}{k}+T.} = \frac{100k}{k+1}$$

$$\therefore t = \frac{100(H' - H")}{H'} \text{ ..... eqn. Bi.*}$$

and $t = \frac{100(Gb' - Gb")}{Gb' - Gt}$ which becomes ...

$$\ldots Gt = Gb' - \frac{100}{t}(Gb' - Gb") \text{ .... eqn. Bii}$$

* Note than $H" = \frac{(Gb" - Gp")100}{Gc - Gp"}$ where $Gc = \frac{100(Gb'-Gp')}{H'}+Gp.$

does not affect this figure but the subsequent haemodilution gives the figure a positive sign.

Equation B(ii) also gives the specific gravity of this fluid gained by, or lost from the circulation. Converting this specific gravity to g. protein % the result may be regarded as a measure of the permeability of the capillaries to macro-molecules.

Effects of Venous Constriction Studied By Specific Gravity Methods.

The experiments described below illustrate some of the practical applications of the principles outlined in the preceding section.

Intention. To assess the blood changes in limbs subjected to venous constriction as in the capillary resistance test of Hess. At the outset these five cases were intended to act as control tests; it was hoped that these results could be compared with the corresponding results in patients with diminished capillary resistance. No cases with abnormal capillary permeability were available.

Literature. The literature on the effects of venous constriction on the blood is summarised in the Medical Research Council's Special Report No. 252 (1945). Different investigators have used different pressures for various times, but it is generally agreed that constriction causes a rise/

Sample		Gb	Gp	H	Gc	Hb g.% by Gc	Hb g.% by Sahli	RBC x10^{-6}
RMb	A	1.044	1.026	27.0	1.092	8.7	8.3	3.2
	B	1.046	1.028	29.5	1.089	9.2	9.0	3.4
BAi	A	1.042	1.024	24.0	1.099	8.3	8.3	3.0
	B.	1.044	1.026	28.0	1.090	8.8	9.3	3.4
BAg	A.	1.049	1.022	33.0	1.104	12.0	12.0	4.0
	B.	1.050	1.024	36.0	1.096	12.0	12.1	3.9
XW	A.	1.054	1.025	41.5	1.095	13.8	13.3	4.9
	B.	1.056	1.027	47.0	1.089	14.4	13.5	5.5
HA	A.	1.047	1.025	35.0	1.083	10.5	11.0	4.4
	B.	1.049	1.027	40.0	1.082	11.3	12.8	4.8
Mean	A	1.047	1.024	33.0	1.096	10.7	10.6	3.9
"B-A		0.002	0.002	4.0	-0.006	0.48	0.76	0.26

'A'...no constriction . 'B'..after constriction.

Table 7 :- Results in five cases with normal capillary resistance as judged by the petechiae count. Venous constriction was applied as in the test of Hess.

rise in Hb and in plasma proteins. The extent of the rise is related directly to the constricting pressure and to the time for which this pressure acts.

Methods. i) The test of Hess was performed as described by Whitby and Britton in their text-book "Disorders of the Blood" 5th edition, page 306, 1947.

ii) While the venous constriction was acting on the left arm, blood sample A was taken from a vein in the right arm without the aid of any constriction. Sample B was taken from the left arm after exactly five minutes constriction and before the constriction was relieved. Both samples were oxalated and examined by the full routine described in Appendix A. The mixing of cells and plasma required to prevent sampling errors caused re-oxygenation of the bloods.

Material. Five patients were so examined. They suffered from the following conditions .....

Addisonian anaemia - 2 cases (BAi and BAg)
Chronic myeloid leukaemia - Case RMb
Chronic asthma during acute attack - Case HA
Tapeworm infestation - Case HW

Results. Capillary resistance, judged by the petechiae count, was normal in all five cases.

The haematological findings are recorded in Table 7 opposite this page. In each case the values for Gb, Gp and H were greater in sample B than in the non-constricted sample A, while the calculated Gc was lower in sample B than in sample A.

Discussion and Interpretation of the Results.

Several mechanisms appear to combine to produce the various changes noted between the A samples and the B samples. The fall in Gc is almost certainly due to the absorption of fluid by the red cells. This is discussed below/

below under the heading 'Intravascular Fluid Shift'; the fluid taken up by the red cells is taken from the plasma.

This fluid shift does not account for all the reduction in plasma volume; other processes are discussed under the heading 'The Extravascular Loss of Fluid'. This extravascular loss is made up of at least two component processes (i) whole blood loss as evidenced by the petechiae present in normal subjects; (ii) loss of plasma-fluid, responsible for the alterations in Gb and Gp and in H. Diffusion into the circulation of more than the usual complement of metabolites is considered under this heading for convenience.

The Intravascular Fluid Shift.

Stasis and anoxaemia cause an excess of CO_2, chloride ions and other diffusible crystalloids to accumulate within the red cells. Isotonicity is maintained by the passage of water from the plasma to the red cells. Thus, according to Wright's text-book "Applied Physiology" 8th edition, page 394, (1945), the cells of venous blood are more spherical in shape than the cells in arterial blood. When equilibrium is re-established the effect should be the same as though the red cells had absorbed plasma ultrafiltrate. The gravity of normal plasma ultrafiltrate is stated by Phillips et al (1945a) to be 1.007. Although this figure may be slightly raised in bloods subjected to venous constriction the calculations which follow are but little affected if figures as high as 1.015 are assumed for the specific gravity of plasma ultrafiltrate. Hence it is assumed that the specific gravity of the fluid and salts taken up by the red cells is 1.007.

If H' vols. of packed red cells suspended in (100-H') vols. of plasma absorb F vols. of a fluid of specific gravity 1.007 and the original packed red cell specific gravity (Gc') is altered to Gc", it follows that .....

$$Gc'' = \frac{H' \times Gc' + F \times 1.007}{H' + F.}$$

which/

	'F' in vols. per 100 vols. blood.	Gpf. (Eqn.F.)	H' + F (Eqn. E.)	H"
RMb ...	1.0	1.0263	28.0	29.5
BAi ...	2.6	1.0246	26.6	28.0
BAg ...	2.95	1.0235	35.95	36.0
HW ...	3.0	1.0260	44.5	47.0
HA ...	2.8	1.0258	37.8	40.0

Table 8 :- 'F' and 'Gpf' calculated as on the opposite page in each of the five non-constricted blood samples.

which by transposition gives .....

$$F = \frac{H'(Gc' - Gc'')}{Gc'' - 1.007} \qquad \text{equation E}$$

If furthermore the plasma specific gravity is altered from Gp' to Gpf, by the loss of F vols. of this fluid then ...

$$Gpf = \frac{(100-H')Gp' - F \times 1.007}{100 - H' - F} \quad \ldots \text{ equation F}$$

The calculated values for (H' + F) and for Gpf are shown in Table 8 facing this page.

It will be noted that in each case H" is greater than H' + F. It seems therefore that besides the loss of whole blood there must be also an extravascular loss of plasma fluid into the tissues.

Theoretically the rise in Hb is directly proportional to the additional volume of swollen red cells per 100 ml. blood, i.e. to H"-(H'+F). The error of the Sahli method is too great to estimate the difference between the A and the B samples but the Hb calculated by the final method developed herein follows the theoretical rise with accuracy (Table 7, page 58).

Extravascular Fluid Loss.

The petechiae which develop even in health testify to the escape of whole blood from the capillary bed during the period of constriction. As pointed out in the last section whole blood loss has no effect on the values of Gb, Gp and H. Since Gb, Gp and H are higher in the B samples than in the A samples it may be presumed that besides the loss of whole blood there is a loss of plasma fluid from the capillary bed. Co-incident with the fluid loss metabolites accumulating in the tissues/

	Volume of plasma fluid lost per 100 ml. final total blood volume. (t)	Specific gravity of the plasma fluid lost from the circulation.
RMb	-5.3 ml.	1.007
BAi	-5.3 ml.	1.004
BAg	0.	--
HW	-5.5 ml.	1.018
HA	-5.8 ml.	1.013

Table 9 :- Calculated volume and specific gravity of the plasma fluid, other than whole blood, lost from the capillary bed in the constricted limb in each of the five cases tested.

tissues diffuse back into the blood stream, but the effect of these additional metabolites on the specific gravities should be negligible. Thus the extravascular fluid loss consists of whole blood plus a non-cellular fluid derived from the plasma.

Equation B, page 57, may therefore be applied to determine the volume and the specific gravity of the fluid other than whole blood lost from the circulation into the tissues of the constricted part. The required volume is expressed as a percentage of the blood volume remaining in the circulation within the constricted area at the end of the period of the test. Provision must also be made for the intravascular fluid shift so that H' in the original equation B(i) is actually H'+F and Gpt is Gpf. The results so obtained in these five tests are shown in Table 9, facing this page.

In case BAg the constricting pressure was noted to have fallen to 40 mm. Hg. at the end of the period of constriction. The veins were still engorged and sample B was withdrawn without further constriction. In this case the difference between the A and B samples can be accounted for by the intravascular fluid shift alone. In the other four tests the volume offluid lost from the circulation was between 5.3 and 5.8 ml./100 ml. blood left in the circulation and the specific gravity of this fluid ranged from 1.004 - 1.018, i.e. from almost protein-free ultrafiltrate to about half of the plasma proteins. In these four tests the specific gravity of the fluid lost from the circulation appeared to be proportional to the packed cell volume.

Conclusions. Venous blood from a limb subjected to constriction as in the test of Hess gave higher values for H, Gb, Gp and Hb than did venous blood obtained simultaneously from the same patient without the use of constriction. Gc and the M.C.Hb.Conc. were lower in the constricted samples. The alteration in red cell haemoglobin content appeared to be due to the absorption of ultrafiltrate by the cells. The rest of the increase in H was due to the loss of fluid and other constituents of plasma from the blood stream. The calculated specific gravities of the plasma fluid escaping indicated a low protein content. Finally whole blood escaped from the circulation even when the capillary resistance was normal.

OTHER POSSIBILITIES.

The copper sulphate method offers a simple method for estimating the specific gravity of lyophilic protein solutions. The originators claim that it is accurate to within ±0.00005. A few of the uses to which such a method may apply are outlined below.

A method similar to that used herein to estimate white cell specific gravity may apply to small pieces of tissue such as scrapings, biopsy material, aspirated tissues, etc.. The variations in specific gravity of healthy and diseased tissues have yet to be worked out but it may be that some critical specific gravities may be found to distinguish between healthy tissue and fatty degenerate tissue on the one hand and between normal and hyperplastic/

hyperplastic or neoplastic tissues on the other.

Capillary resistance tests have in the past concerned themselves with the permeability of the capillaries to whole blood but the method outlined here appears to offer an opportunity to assess the permeability of the capillary bed to protein molecules. The investigation of cardiac and renal conditions by such a procedure may help to explain the functional pathology of these conditions and may help to assess the action of therapeutic agents employed in oedematous states. Smaller pressures would be used than in the tests here described, e.g. in some conditions it may even be that the difference between the horizontal and the dependent position may affect the amount of protein escaping into the tissues.

In burns, crushing injuries, pulmonary oedema, haematemesis, surgical shock, etc., the method outlined for estimating haemoconcentration and haemodilution may have experimental and clinical applications.

SUMMARY AND CONCLUSIONS - PART ONE.

The intention of this part of the work was to assess the value of the copper sulphate estimation of specific gravities of whole blood (Gb) and plasma (Gp) in side-room haematology, particularly as a means of estimating haemoglobin (Hb) in abnormal bloods.

The theoretical and experimental basis for the existing formulae and nomogram by means of which Gb and Gp may be converted to g. Hb./100 ml. blood, packed cell volume per cent blood volume, and g. protein/100 ml. plasma is described and the literature on the copper sulphate method of estimating specific gravities is summarised. It is pointed out that to date no evidence has been published to prove that these methods apply to the abnormal bloods commonly seen in general medical wards.

The total number of samples examined was 229 of which 103 were naturally occurring bloods and 126 were artificial cell-plasma mixtures prepared from the 103 bloods.

Gb and Gp were estimated by the copper sulphate method and a full routine haematological examination was carried out on each sample. Standard methods were used in this routine examination except that (a) after preliminary experiments with various haemoglobinometers a Sahli apparatus and a technique designed to avoid the usual errors of the acid haematin methods was adopted. The apparatus, the technique and the observer's judgement were simultaneously standardised by the examination of 24 normal blood samples in which the Hb was also estimated by/

by a photoelectric procedure calibrated against iron estimations (3.34mgms. iron = 1.0g.Hb.). (b) By spinning a series of bloods to the 'no change' end-point in the side-room 9 cm. centrifuge and then in an 18 cm. laboratory pattern centrifuge at 3000 r.p.m. for 60 minutes, a graph was prepared by means of which the apparent packed volume of red cells/100 vols. blood after spinning in the 9 cm. centrifuge was converted to within ±1.0% of the packed red cell volume (H) as estimated in the 18 cm. centrifuge.

Duplicate estimations of specific gravity on each sample confirmed the error of the estimated Gb, Gp and Gs to be less than ±0.0005.

Although the Sahli estimations were made with the greatest possible care the error range on comparison with the photoelectric estimations was from +0.8 to -0.9 g.Hb, (mean error +0.002 g.Hb%).

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1. Hb Estimation by the Copper Sulphate Method.

Hb Calculated From Specific Gravities By The Methods of Phillips et al (1945a and b). In normal bloods the Hb so estimated was within ±0.5 g. of the photoelectric estimation; the modified method using Gb alone was liable to an error of from -1.6 to -0.1g. (mean error -0.82g.Hb%).

In the 103 normal and abnormal bloods of the series these methods gave Hb values differing from the Sahli results by as much as 4.0g. when Gb alone was used, and by 3.0g. when Gb and Gp were used, respective mean Hb differences = -0.92 and -0.16g.

Error in Hb Calculated by the Methods of Phillips et al and Correction of the Errors.

i) When Gb is used alone, as in the modified method, error is introduced by variations in Gp from the assumed normal (1.0264). Such variations ranged from 1018 to 1031 in/

in the bloods examined. No correction, other than the estimation of Gp, was possible.

ii) Conversion of Gb and Gp to Hb by means of the nomogram of Phillips et al (1945b) assumes the red cell gravity (Gc) to be 1.097 and the mean corpuscular haemoglobin concentration to be 33.9%. In this work-Gc ranged from 1.067 to 1.110 and the M.C.Hb.Conc. from 23% to 39%.

The values for Gc and the M.C.Hb.Conc. (Sahli) were calculated for each blood of the series. By the method of least squares the best formula relating Gc and the M.C.Hb.Conc. was found to be 373(Gc-1.005) = M.C.Hb.Conc.. The calculated M.C.Hb.Conc. differs very little by this formula from the values given by the theoretical formula 377(Gc-1.007) = M.C.Hb.Conc.. A convenient approximation to both these formulae was found, i.e. M.C.Hb.Conc. = 350(Gc-1.000). Thus Gc, calculated from Gb, Gp and H, may be converted to the M.C.Hb.Conc. which with the aid of H gives the Hb corrected for variations in Gc and the M.C.Hb.Conc.,

iii) In the course of correlating Gc and the M.C.Hb.Conc it was observed that unusually large errors were present in three leukaemic bloods. A method was developed for the estimation of Gw(the specific gravity of the packed white cells). Correction was made for the volume of packed white cells (W) and for their weight (WxGw). Such correction was necessary only when W was greater than 2%. When W is less than 10% no significant error will result if Gw is assumed to be 1.055.

The Hb corrected as in ii and iii above differed from the Sahli estimated Hb by -1.4 to +1.5g.% (mean difference -0.08g.). The two sets of results have a coefficient of correlation/

correlation of 0.9656. In the 24 normal blood samples the error range was from -0.3 to +0.2g. (mean error -0.046g.) on comparison with the photoelectric results.

iv) Hyperproteinaemia. It was next observed that when Gp was 1.028 or more, the calculated haemoglobin was usually (seven cases out of eight) greater than the Sahli haemoglobin. It is suggested that in the bloods concerned the positive error may be due to spontaneous precipitation, or sedimentation, of part of the plasma protein along with the red cells. No attempt was made to correct Gc in these cases but the Hb was corrected empirically by subtracting 0.25 (Gp - 1.026)g. Hb from the result calculated as above.

With this final correction in the eight cases concerned the calculated Hb differs from the Sahli Hb in the 103 bloods by -1.4 to +1.0g.Hb% (mean difference = -0.03g.) and the Hb differences have a standard deviation about the mean difference of 0.532. Comparing the differences with those in the standardisation tests, it is concluded that the error of the Hb so corrected is very little greater in the abnormal bloods of the series than in the 24 normal bloods examined photoelectrically, i.e. very little greater than -0.3 to +0.2g.Hb.

v) The copper sulphate method was calibrated by Phillips et al (1945a) against estimations of oxygen plus carbon monoxide capacity in bloods from normal American males.

From recent publications an attempt was made to correlate/

correlate the method used by Phillips et al, and their results, with current British methods and standards and with the methods used in this investigation. It was decided that the Hb by the copper sulphate method should be within ±0.2g. of Hb by the photoelectric and Sahli methods herein, and within the same range of the Haldane-Gowers B.S.I. Standard allowing 14.8g.% as the Hb equivalent of the 100% Haldane-Gowers blood.

vi) The presence of large numbers of abnormal red cells, e.g. siderocytes, punctate basophils, reticulocytes, normoblasts, etc., may cause some error, but no constant error was associated with reticulocytosis or punctate basophilia in this work.

The Method Finally Recommended requires 3 - 4 drops of blood for the estimation of Gb and a haematocrit tube filled with blood (0.7 ml.) for the estimation of H and W. The supernatant plasma in the haematocrit tube is used for the estimation of Gp. Nomographic alignment charts differing in principle and in detail from those so far published were developed to facilitate the conversion of Gb, Gp and H to Gc, M.C.Hb.Conc., g. protein % plasma, and g. Hb% blood.

Technical errors result from (i) inadequate mixing of cells and plasma before the estimation of Gb; (ii) the use of pipettes which are not thoroughly dry; (iii) haemolysis.

The advantages and disadvantages of the method are compared with those of the colorimetric methods commonly used in the side-room.

Gb as a Test for Anaemia. Gb alone as a screening test for the detection of anaemia was shown to be unreliable and irrational, but its continued use seemed justifiable until a more accurate method of comparable simplicity and speed is available.

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11. Blood and Plasma Specific Gravities Related to Features Other Than Haemoglobin.

Packed Red Cell Volume (H). Estimations of H from Gb and Gp by means of the nomogram of Phillips et al (1945a or b) were liable to errors of 7.0 vols. % blood volume in normal bloods and 13% in abnormal bloods. An unsuccessful attempt was made to calculate H from Gb, Gp and the Hb. The necessary formula was developed, but it was found that the estimated Hb must be accurate to the second place of decimals.

Gp and the Plasma Proteins. Plasma or serum protein estimations were carried out by the biochemical laboratory as part of the routine investigation in ten cases. The g. protein % plasma calculated from Gp agreed with the chemical estimation to within ±0.5g. in the ten tests performed. From chemical estimations reported by the Medical Research Council, the normal range of serum proteins in Great Britain appears to be equivalent to a normal Gp range of 1.023 to 1.028 and a mean normal Gp of 1.0254 instead of 1.0264 as found by Phillips et al in their 20 male bloods. In the normal bloods of this series Gp ranged from 1.023 to 1.026, mean 1.0249. In the abnormal groups, Gp ranged from 1.018 to 1.031.

Comparing/

Comparing the mean Gp of each pathological group with the mean normal Gp the difference between the means is statistically 'significant' in the group of neoplastic diseases only.

Packed Red Cell Specific Gravities (Gc). In the normal bloods of the series Gc was between 1.089 and 1.097, mean 1.0935. This differs significantly from the mean Gc determined by Phillips et al but they made their estimations on normal male bloods with a mean Hb of 15.9g.% whereas the normal group here was half male and half female and the mean Hb was 13.9g.%. In the abnormal bloods of the series Gc was between 1.067 and 1.110.

Mean Cell Weights. Formulae are given for the calculation of mean red and white cell weights. The mean red cell weights in normal bloods were between 84 and 91, mean 89 micro-micrograms (or g. x 10^{-12}). In the iron deficiency anaemias the range was from 59 to 85 and in macrocytic anaemias from 110 to 140 g. x 10^{-12}, the respective mean values being 75 and 124 g. x 10^{-12}.

In one case of leukaemia the mean white cell weight was 580 g. x 10^{-12}. Myelocytes accounted for about 80% of the white cells in this case.

111. Further Theoretical Possibilities and Practical Applications.

Theoretical considerations underlying the estimation of blood volume in transfusion cases and of changes in blood volume in non-transfusion cases by specific gravity methods are/

are discussed. The formulae so developed are applied to a short series of experiments designed to assess the changes produced in the blood by venous constriction. The results in these experiments serve to illustrate the value of the copper sulphate method in the investigation of fluid and protein shifts from the plasma to either the red cells or the extravascular tissues.

Finally a few, as yet, unexplored possibilities are suggested.

PART TWO.

PLASMA FORMOL REACTIONS

IN

GENERAL MEDICAL CASES

PART TWO.

INTRODUCTION AND INTENTION.

The clinical and pathological features associated with the plasma formol reactionswere investigated in this part of the work. The plasma formol gel (PFG) test has been reported positive in rheumatic subjects with accelerated erythrocyte sedimentation rates (ESR), but in acute gout the ESR was frequently accelerated although the PFG test was negative (Gibson and Richardson, 1938, and Gibson and Pitt, 1946). One object of this work was to determine the extent and the limitations of this association in non-rheumatic medical conditions. If gout alone gave negative PFG reactions with acceleration of the ESR, the PFG test might be a useful substitute for the 'corrected' ESR in anaemic patients. Alternatively, if the tests were related only in rheumatic conditions, the combined tests might have some diagnostic value. Between these extremes it was hoped that some feature common to the cases with positive PF reactions might be found to account for any partial correlation between the tests.

In addition to the main series a number of control tests were necessary. For example, the ESR was 'corrected' in anaemic bloods by means of charts based on standard methods of estimating the crude ESR and the packed cell volume (Whitby and Britton, 1946, and Wintrobe, 1946). The prescribed anticoagulant for these estimations is dry potassium and ammonium oxalate. Previous workers on the PFG/

PFG test have used a solution of potassium oxalate as the anti-coagulant. One series of control tests was required to determine the difference, if any, between the PFG reactions in ammon.-pot. oxalated plasma and in pot. oxalated plasma. Dry ammon.-pot. oxalate (see App. A) was used in the main series in this work.

In other investigations PFG test was positive if a solid gel formed within 24 hours; by this standard the test was inferior to the ESR as a test for rheumatic activity. Throughout this work the plasma-formol mixtures were inspected daily for seven days and the time taken to form a complete gel was noted; if no gel formed within a week the test was recorded as "PFG - 0". The final criteria for positive and negative PF reactions were not decided till the experimental work was finished.

Very little attention has been paid in the past to the opacities developed in formol gels. In this work the opacity in the plasma formol mixture was recorded after 24 hours whether a gel had formed or not. This opacity is referred to herein as the plasma-formol flocculation (PFF) reaction. The PFF and PFG reactions are correlated separately with the clinical and pathological findings.

As the work progressed additional tests were incorporated in the investigation to allow the PFG and PFF reactions to be related to certain physical properties of the plasma proteins. The tests selected are essentially side-room tests, e.g. the estimation of plasma specific gravity, the dilution/

dilution test and the Weltmann reaction. The estimated specific gravities gave an approximate figure for the g. total protein/100 ml. plasma; the plasma dilution test measured the amount of water insoluble protein present in the plasma; the Weltmann reaction measured the concentration of calcium chloride required to bring about heat coagulation of the proteins. Since the interest here was in the plasma proteins and their physical state before formol was added, the dilution test and the Weltmann reaction were applied to plasma oxalated, as in the formol reactions, with dry ammon.-pot. oxalate. The conventional dilution and Weltmann reactions are applied to serum. A second series of control tests in normal and abnormal bloods was required to determine the relationship between the serum and plasma tests, and to determine the significance of the results with plasma.

For the final correlation of the results in this work and in the literature an intensive study of the serum and plasma proteins in health and disease was necessary. From this part of the work a hypothesis was developed relating positive plasma and serum formol reactions with instability of the serum and plasma proteins. The hypothesis is extended to account for other manifestations of protein instability associated with positive formol reactions. It is suggested that the formol reactions, the erythrocyte sedimentation rate (ESR), the non-specific liver function tests and other tests should be grouped together as 'protein stability tests'.

SUMMARY OF THE LITERATURE ON THE FORMOL GEL TESTS.

The serum formol gel (SFG) test has been confused until recently with the plasma formol gel (PFG) test. Even yet some standard text-books describe "the formol gel test" without distinguishing between a serum and a plasma test. Harrison (1947) indicates in his text-book, "Chemical Methods in Clinical Medicine" that either serum or plasma may be used for the formol gel test.

Since many published observations on the SFG test apply also to the PFG test the literature on the serum test is included in this part of the work. Relevant articles on the other non-specific tests, performed on the plasma samples of this series, and on the serum and plasma proteins in general are described in the course of the discussion later in this thesis.

The serum formol gel test was first described by Gaté and Papacostas (1920). They added formol to pooled W.R. positive serum which they wished to preserve and found that by the following day the serum had set in a solid gel. After further investigation they concluded that this gel formation with formol was characteristic of syphilis and they described the serum gel test as a simple and reliable test for syphilis. Ecker (1921) found the test misleading and unreliable in this rôle. Almost at the same time Napier (1921) reported that opaque gels forming within 20 minutes were diagnostic for kala-azar. He attached similar significance to opaque gels forming within 24 hours but not to clear gels. In the 1946 edition of his text-book "Principles/

1. Rogers (1926)
2. Turkhud and Avari (1922)
3. Haseltine (1924)
4. Benhamon and Gille (1935)
5. Gutman et al (1935)
6. Napier (1921)
7. Pfeffer (1925)
8. Giraud et al (1935)
9. Trosier and Bariety (1934)
10. Taussig (1938)
11. Cantarow (1935)
12. Bauer (1935)
13. Faust and Meleney (1937)
14. Bing (1937)
15. De Vries (1939)
16. Vivoli (1931)
17. Schultze and Rose (1939)

"Principles and Practice of Tropical Medicine", Napier states that in 20,000 tests performed in Calcutta the number of opaque gels due to conditions other than kala-azar was negligible. Chopra (1936) reported positive results in 83.5% of untreated cases of kala-azar and stated that the test became negative under treatment.

Since 1921 the serum test has been reported positive in a number of conditions, but generally clear and opaque gels are classed together as positive formol gel tests. Some workers accepted only 24 hour gels; others allowed 48-72 hours. By such variable standards the test has been reported positive in the following conditions besides those already mentioned, (i.e. syphilis + kala-azar) — trypanosomiasis [1], leprosy [2,3], lymphogranuloma inguinale [4, 5], malaria [6], phthisis [6,7], subacute bacterial endocarditis [8,9], Still's disease [10], multiple myelomatosis [11,12], schistosomiasis (japonicum) [13], hepatic cirrhosis [14,15], lymphatic and monocytic leukaemia [14,15], and rheumatic endocarditis and carditis [16,17]. It appears therefore that the SFG test is positive in at least some subacute and chronic bacterial and protozoal infections, and in some conditions characterised by hyperplasia of the reticulo-endothelial tissues as well as in acute rheumatism which may be bacterial, virus or allergic in origin.

The SFG test has been widely investigated in relation to other laboratory and side-room tests. Napier, 1921, noted that it was positive in hyperglobulinaemia ('salting out methods') and commented on the great excess of protein precipitated from the/

the serum in cases of kala-azar by 33% sat. ammon. sulph. ("euglobulin" according to Napier and others mentioned below). Napier and Henderson (1931) observed some correlation between the ESR and positive SFG tests in kala-azar and pointed out that in kala-azar the ESR is frequently very high. Wise and Guttman (1937) reported positive gels when the total serum proteins exceeded 8.0g.% or when the globulin (precipitated by 50% sat. ammon. sulph.) exceeded 4.0g.% serum. Bing (1937) and Taussig (1938) related the test to an excess of "euglobulins" (defined as by Napier above). Bing noted that "euglobulin" in saline did not form a gel with formol but, if it was added to normal serum, the mixture readily gave formol gels. He also reported that formol gels formed most rapidly at 37°C and at pH 7.0. Gibson and Richardson (1938) found that the formol gel tests (serum and plasma) were unaffected by temperature differences between 37°C and 0°C. Bing also investigated the effect of a variety of substances on serum gel formation and concluded that urea, ammon. carbonate, ammon. oxalate, and amino-acids inhibited gel formation while ammon. sulphate, sodium sulphate, and sod. chloride accelerate gelation. Nattan-Larrier and Grimard-Richard (1934) working on serum gels in kala-azar reported that two factors were involved; one was responsible for gel formation and was removable on dialysis; the other was responsible for the opacity and was not removable on dialysis. Taussig (1938) describes the serum dilution test and concludes that the SFG test and the dilution test (serum) are both positive when there is an excess of "euglobulin". Bing states that plasma should not

be/

be used for the "formol gel test" as it is liable to give "false positive" results. I assume that "false positive" results in this respect mean positive formol gels in the absence of any excess of "euglobulin".

Modifications of the SFG test have been suggested as a means of estimating the serum globulin in g.% serum. Thus De Vries (1939) found the test positive when the serum globulins exceeded 3.7g.% serum. He outlines a method whereby the time, in hours, taken to form a solid gel indicates the approximate globulin content of the blood. Similarly Biguria and Foster (1941) report that serum gels, of sufficient solidity to prevent air bubbles rising to the surface, contain 3.0g. globulin % serum; they describe a method using serial dilutions of serum and formol. The mixtures are tested after 24 hours for the "stationary bubble" end point. From the dilutions of serum giving this end-point, the serum globulins are calculated.

Green et al (1939) described frequent positive SFG tests in rheumatic patients with acceleration of the ESR. Butterworth and Poindexter (1942) found no relationship between positive serum formol gels and the ESR, or cardiac lesions in rheumatic patients. Klein et al, 1941, applied the SFG and Weltmann tests to rheumatic patients and found them inferior to the corrected ESR as a test for rheumatic activity. In this respect the serum Weltmann reaction was intermediate between the SFG test and the ESR in sensitivity, but it had the advantage of distinguishing fibrotic from exudative lesions/

Packed Red Cell Volume % Blood Volume (P.C.V.)	Pos. 24 hrs. PFG. % P.C.V. Group.	% P.C.V. Group with ESR over30 mm.% * (Uncorrected)
50 and over	-	-
45 - 49	27%	100%
40 - 44	63%	94%
35 - 39	90%	95%
Less than 35	94%	96%

* ESR, converted to corresponding result by Wintrobe's method; equivalent to 30 mm. clear plasma after one hour.

Table 10:- Relationship between ESR, packed cell volume and PFG results in rheumatic cases. (Gibson and Pitt (1946)).

lesions (see Tab.II, page 84).

Most of the published work on the plasma formol gel test deals with rheumatic patients and normal controls. Miles and Salt (1941) report that the incidence of positive PFG tests in rheumatic patients increases as the packed cell volume decreases and as the crude ESR rises; they also found the PFG test to be more closely related to the crude than the corrected ESR. Gibson and Richardson (1938) and Gibson and Pitt (1946) also investigated the PFG and SFG tests in rheumatic patients, i.e., patients suffering from acute and subacute rheumatic fever, rheumatoid arthritis, osteoarthritis, gout, etc.. In more than 600 such cases the PFG reactions were always positive when the SFG reactions were positive, and frequently the PFG test was positive although the SFG test was negative. In each SFG positive blood the serum gels took longer to form than the plasma gels. They confirm the observation of Miles and Salt regarding the higher incidence of positive PFG tests in bloods with low packed cell volumes (see Table 10, facing this page) and in bloods with accelerated ESR. Correction of the ESR made little difference to their results however and they attribute the anaemia of chronic rheumatic states to hydraemia, and suggest that rheumatic cases may be divided into three groups corresponding to the chronicity of the condition thus:-

Group 1 - Early fibrinogen stage - PFG -ve ; SFG -ve
ESR high.

11 - Late fibrinogen stage - PFG +ve; SFG -ve.
ESR high

Group/

Group 111 - Globulin stage - PFG +ve; SFG +ve; ESR high

Gibson and Richardson (1938) report 14 cases in which the ESR and PFG results did not agree. In two of the 14 cases the ESR (method of Zecker and Goodwill, quoted from Gibson and Richardson (1938)) was normal although the PFG was positive. In one of these two cases the crude and corrected ESR was borderline normal/abnormal and gel formation was incomplete. Accelerated ESR were seen in 12 cases giving negative PFG tests--- in five the corrected ESR was normal by Wintrobe's chart. Three of the seven remaining cases had clinical gout. In eleven cases of gout in their series the test was negative although incomplete gels sometimes developed.

Certain differences between the methods used by Gibson and his colleagues and those used in this work must be discussed. They used a 10% solution of potassium oxalate as the anticoagulant instead of the dry ammon. pot. oxalate mixture used in this series. The tubes prepared for their blood samples contained 0.3 ml. oxalate solution which, they state, was "sufficient to prevent coagulation in 15 ml. blood." It is not clearly stated that 15 ml. blood was always added to this volume of solution. If 15 ml. normal blood is so diluted, the increase in plasma volume, excluding the additional fluid shift from red cells to plasma due to the absence of ammon. oxalate, is about 4% of the original plasma volume; if however, 5 ml. are added instead of 15 ml. the increase in plasma volume amounts to about 12%, i.e. sufficient to reduce 8.0g. protein %

to/

to 7.1g. protein %. According to Whitby and Britton (1946) the estimated packed cell volume where pot. oxalate alone is the anti-coagulant should be multiplied by 1.09 to allow for the fluid shift from the cells to the plasma. In normal bloods this represents a further increase in plasma volume of about 8% of the original volume. Hence the plasma tested by Gibson et al is a more dilute solution of the plasma proteins than the plasma examined in this work.

Gibson et al accept complete and incomplete gels forming within 18 hours as positive PFG tests. Here the criteria, defined after reviewing the results of the present investigation, allow up to 48 hours for positive PFG tests.

After the present experimental work was completed, I came across a paper published some months earlier in Norway* by Langé (1946). He applied the PFG, SFG, Takata-ara and erythrocyte sedimentation tests to a series of general medical cases and correlated the results with the protein fractions separated by salt precipitation from plasma. He confirms the occurrence of positive PFG reactions in all cases giving positive SFG reactions and relates PF gels, in the absence of SF gels, to increased fibrinogen in the plasma. SF gels, he states, form when there is an increase in globulin in the blood, particularly in "euglobulin" (the 33% sat. ammon. sulph. precipitable serum protein). Similarly the Takata-ara test is positive in relation to hypoalbuminaemia, hyperglobulinaemia or both. Langé gives little clinical information about the cases examined; his results are set out to show that these tests can, to a certain, extent, replace the more laborious methods of protein fractionisation.

* I am indebted to Dr. R. A. Shanks, Royal Hospital for Sick Children, Glasgow, for translating this paper from the original.

METHODS.

General haematological methods including the estimation of the ESR and packed cell volume are described in App. A.

Plasma specific gravities were estimated by the method of Phillips et al (1945a and b).

PF Reactions:- One ml. of haemoglobin free plasma was mixed with 0.1 ml. calcium-formol(see below) in a test-tube which was then stoppered and inspected after 20 minutes, 3 hours, 24 hours and thereafter daily for the next six days. The pH of the formol was standardised as suggested by Baker (1946) in his book "Cytological Technique", 2nd edition. He recommends the addition of solid calcium carbonate to the formol. This was done using liqu. formaldehyde (B.P.). Two drops of Universal Indicator (B.D.H.) were added to the 100 ml. bottle of formol. The calcium settled to the bottom of the bottle and the faint yellow colour of the indicator (pH=6.5) remained constant.

The PFG result expresses the time taken to form a solid gel .....

PFG 5+ ... solid gel within 20 minutes.

PFG 4+ ... solid gel within 3 hours.

PFG 3+ ... solid gel within 24 hours.

PFG 2+ ... solid gel within 48 hours.

PFG 1+ ... solid gel within 96 hours.

PFG 0 ... no solid gel within 7 days.

'Solid gel' here signifies that the tube may be inverted without any visible fluid movement in the gel.

The/

THE WELTMANN REACTION.

Technique:- 0.1 ml. serum is added to each of ten tubes containing 5 ml. of calcium chloride solution of the strength indicated below. The result is read after 15 min. in a bath of boiling water.

Tube no...	1	2	3	4	5	6	7	8	9	10
Conc. of $CaCl_2$...(g./100ml.)	0.10	0.09	0.08	0.07	0.06	0,05	0.04	0.03	0.02	0.01

Results:- In normal serum, tubes 1-6 show coagulation of the serum proteins; there is no coagulation in tubes 7-10. In such a case the coagulation zone is tubes 1-6. Abnormal sera may give prolonged coagulation zones, e.g. 1-8 when tubes 1-8 show coagulation, or they may give short coagulation zones, e.g. 1-3.

Significance of results:-

Shortened coag. zone.	Normal zone.	Prolonged coag. zone.
Exudative lesions.		Fibrotic lesions.
Acute abscesses.		Newborn.
Suppurative appendicitis,		Hepatic cirrhosis
" peritonitis.		" insufficiency.
Pneumonia		Endocarditis lenta.
(lobar & broncho..)		Malaria.
Tuberculosis...miliary,		Fibroid phthisis.
pleurisy with effusion.		
Acute leukaemia.		Chronic nephritis.
Nephrosis.		Tertiary syphilis.
	Fibrocaseous tuberculosis.	
	Obstructive jaundice.	

Table II:-The technique and interpretation of the Weltmann reaction(serum).....prepared from Levison & Macfate,1946.

The PFF result expresses the depth of the opacity of the plasma-formol mixture after 24 hours whether a gel develops or not. An arbitrary scale of opacities was adopted ranging from a white milk-like opacity (5+) to a faint but definite cloud (1+). Later it was possible to define these opacities in terms of the standard barium sulphate suspensions mentioned by Mackie and MacCartney (1942) on page 285 of their "Handbook of Practical Bacteriology", 6th edition .....

PFF 1+ ... opacity less than standard opacity no. 1.

PFF 2+ ... opacity equal to standard opacity no. 3.

PFF 3+ ... opacity equal to standard opacity no. 8.

PFF 4+ ... opacity greater than " " no. 10.

PFF 5+ ... milky white

These opacities were matched in the narrow tubes provided with the sets of standard opacities.

SF Reactions were performed exactly as for the PF reactions except that serum was used instead of plasma.

Dilution Tests. These were applied to both serum and plasma as described for serum by Taussig (1938). Doubtful results were compared with a 1/10 dilution of the serum or plasma in normal saline.

Weltmann Reaction. The technique outlined in tab. 11 facing this page, was used (Levison and Macfate (1946)). For the plasma test exactly the same technique was used. After the first twenty tests only tubes 4 - 8 inclusive were set up and tested; if all five tubes were coagulated, tubes 8, 9 and 10 were tested; if there was no coagulation tubes 1 - 4 were tested.

In the 130 bloods of the series the PF, dilution and Weltmann tests were applied to plasma containing ammon. and pot. oxalate as described in App. A. The corresponding serum tests were applied in a more limited series for control purposes.

In one group of control tests plasma was prepared with 10% solution of pot. oxalate as in previous work in rheumatic cases. The volume of solution added amounted to 2% of the volume of blood available, i.e. equivalent to 0.3 ml. per 15 ml. blood.

M A T E R I A L

The main series consists of 130 samples of plasma from general medical cases other than rheumatic cases. In addition to the PF reactions, clinical and pathological records were kept for each case; each sample of blood was subjected to a full routine haematological examination (see App. A) which included the estimation of packed cell volume and of the ESR. Corrected ESR were obtained from Wintrobe's chart and from Whitby and Britton's chart (see App. A). In 103 of the 130 samples the plasma specific gravity was estimated by the copper sulphate method (Phillips et al, 1945a and b), and the plasma dilution and plasma Weltmann tests were performed.

In 43 bloods of the series plasma was prepared using pot. oxal. alone as well as by the ammon.-pot. oxalate method. PF reactions were compared in these plasma samples.

In 53 blood samples serum formol reactions were compared with the PF reactions. SF, serum dilution and Weltmann reactions were performed on both serum and plasma in 49 of these bloods.

Other control experiments are described at appropriate places in the text.

R E S U L T S.

Results in individual samples are listed in App. D., except the plasma specific gravities in App. C.

In this section the results are given under .....

1. Control tests.
11. Criteria for positive PFF and PFG reactions.
111. Clinico-pathological features related to the PF reactions in this series.

I. CONTROL TESTS.

Influence of Anticoagulant on PF Reactions.

In this work dry pot. and ammon. oxalate was used as the anticoagulant instead of the 10% oxalate solution used by others investigation rheumatic cases. Theoretically the ammon.-pot. mixture does not cause dilution of the plasma; it raises the salt concentration in the plasma to a greater extent than does the pot. oxalate solution. Although much of the added ammonium is removed with the packed red cells, some increase in plasma ammonium probably occurs. Ammonium salts react with formol to form hexamethylene tetramine and in so doing may increase the acidity of the plasma-formol mixture. Thus at least three factors must be considered in relation to the effect of these anticoagulants on the PF reactions, i.e. i) effect of haemodilution, ii) effect of increased salt concentration, iii) effect of pH.

(a) Effect of dilution on PF reactions. In two PF positive samples of plasma it was observed that dilution of the plasma with saline and with water delayed and inhibited the PFG reaction and diminished the PFF reaction.

(b) Effect of dry salts on the PF reactions. The addition of further dry potassium or ammonium oxalate was found to accelerate the PFG reactions and accentuate the PFF reactions. The addition of relatively large amounts of these salts to normal plasma and serum induced 5+ PFF and PFG reactions, but the concentrations

required/

required were observed to precipitate some of the proteins from the serum and the plasma apart from the presence of formol. Dry ammonium sulphate had a similar effect.

(c) Effect of pH on the PF Reactions. By adding graduated quantities of dry acid sodium phosphate and of basic sodium phosphate to portions of the plasma-formol mixtures, it was seen that gel formation was accelerated and PFF reactions were intensified in proportion to the amount of acid salt added. The basic salt was less effective in this respect but if relatively large amounts of the basic salts were added to the plasma, immediate gelation could be induced. Such alkaline gels were much less opaque than the acid gels.

In 45 normal and abnormal bloods no striking differences were observed between the formol reactions in ammon.-pot. oxalated plasma and in plasma prepared with the pot. oxalate solution. In PFF 1+ or 2+ samples it was noticed that the ammon.-pot. oxal. plasma gave a slightly greater opacity than the pot. oxal. plasma but the difference was not sufficient to alter the recorded PFF results. Even in normal plasma a cloud developed with ammon. pot. oxalate but it was much less than the 1+ PFF reaction. Similarly the PFG reactions were frequently faster in the ammon. pot. oxal. plasma but the difference was never sufficiently great to influence the recorded PFG result.

Thus although ammon. pot. oxalate mixture by diminishing/

	SFG 3+	SFG 2+	SFG negative	Totals
SFF 3+	2 (RL2,NGc2).	1 (REa2)		3
SFF 2+	2 (REa2,RL3)	2 (RPa,IE)	1 (NKa)	5
SFF 1+		2 (RPb,BSa)		2
SFF 0			43	43
Totals	4	5	44	53

Table 12:- Frequency of positive SFF and SFG reactions in 53 serum samples.

diminishing plasma dilution, by increasing the salt concentration and by increasing the acidity of the plasma-formol mixtures, should accelerate and accentuate the PF reactions by comparison with pot. oxal. solution, the difference was negligible in the 45 bloods in which both forms of plasma were tested. It may be that ammonium oxalate, in the concentrations concerned, has an inhibitory action on gel formation as reported by Bing (1937); such an inhibitory effect may counteract the other accelerating features.

Serum Reactions and the Plasma Reactions.

SF and PF Reactions. In 53 bloods of the series serum was examined as well as plasma. In 31 samples the PF reactions were positive; in 10 the SF reactions were positive; 4 of the 10 gave 24 hour serum gels, 5 gave 48 hour gels and 1 gave no gel but a definite opacity after 24 hours.

The PF reactions were invariably faster and stronger than the SF reactions except in NKa, where the plasma and serum reactions were similar. In the other 9 SF positive bloods the PF reactions were either 4+ or 5+.

In RL 2 & 3, REa2, and BSa the plasma specific gravity was at least 1.029 indicating total plasma proteins over 8.0g.%; in RPa and RPb the plasma specific gravity (1.028) indicated total plasma proteins just under 8.0g.%; in NGc 1 and 2 Gp (1.025) was within the range of normal. Gp was not estimated in IE. Only one sample (IM) with Gp 1.028 or more gave negative SF reactions; PF reactions were positive in/

in this instance.

It was observed that even when the SF reactions were negative some bloods gave PF reactions of maximal intensity.

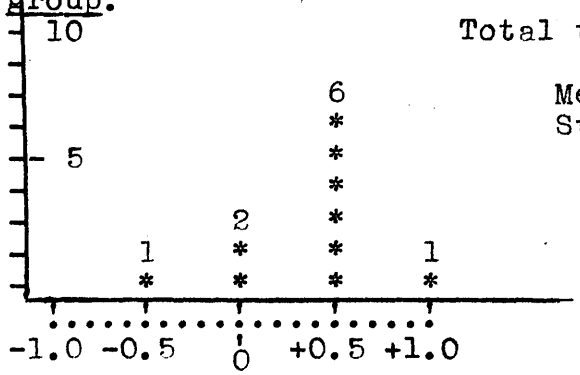
Serum and plasma dilution tests. As observed by Taussig (1938) the serum dilution test followed the SF reactions very closely. It was positive in eight of the nine SF positive samples tested. It was also positive in four SF negative samples .... REc, NGb, IP, and XVb ... all of which had some impairment of hepatic function. (See pages 140 - 141).
In all serum dilution positive samples the plasma dilution test was positive; in other eight of the 53 bloods the plasma dilution was positive and the serum dilution was negative. In five of the eight the PF reactions were 4+ or 5+ and in the other three they were 3+ or 2+.

It appears therefore that like the PF and SF reactions, positive serum dilution tests usually associate with positive plasma dilution tests although the converse does not apply. Furthermore, both dilution tests are apparently related to the corresponding formol test.

Serum and plasma Weltmann reactions. The end-point for the coagulation zone was more distinct with serum than with plasma. Partial coagulation was never seen in more than one tube with the serum test, but it was occasionally present in two and even three tubes with plasma. The end-point for the plasma reaction in this work was, as in the serum test, the last tube showing approximately half as much coagulation as in tubes 1 - 4. In four normal bloods and in six bloods

from/

A. In Normal Bloods.
Frequency per diff. group.

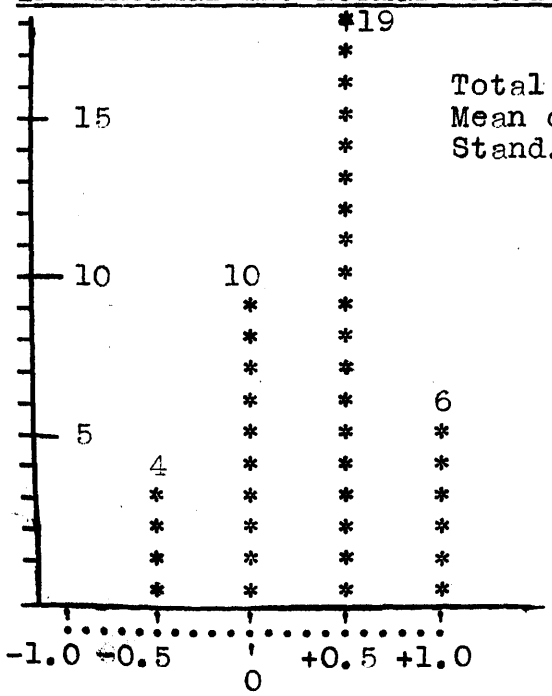


Total tests 10.

Mean diff. = +0.35.
Stand. dev.= 0.39.

Plasma coagulation zone less serum coagulation zone.

B. In Abnormal and Normal Bloods.



Total samples 39.
Mean diff. = +0.34.
Stand. dev.= 0.42.

Plasma coagulation zone less serum coagulation zone.

Fig. 11 :- Showing the frequency of differences between the plasma and the serum Weltmann reactions in A. Ten normal bloods and B. in other 39 bloods from the series.

from healed duodenal ulcer patients, the serum coagulation zones ranged from 5.5.to 6.5 while the corresponding plasma zones ranged from 5.5 to 7.0. The maximum difference amounted to 1.0 tube; the differences are shown in fig.11 facing this page. The differences in abnormal bloods are shown in fig. also. Generally the plasma zone is longer by about 0.5 tubes than the serum zone but the differences ranges from -0.5 to +1.0 tubes. Thus plasma coagulation zones over 7.0 or under 5.5 probably differ significantly from normal. There was no definite correlation between the difference between the serum and plasma results and the ESR or hepatic insufficiency.

To ascertain the effect of the anticoagulant on the coagulation zone additional oxalate was added to oxalated plasma samples giving normal, long, and short coagulation zones and to serum giving similar zones. In each case sufficient oxalate was added to double the concentration of oxalate in plasma, and to produce approximately the normal plasma concentration of oxalate in serum; in the presence of this additional oxalate the coagulation zones were shortened by approximately 0.5 tube.

From this it is argued that, since the total average difference between plasma and serum reactions is an increase of about 0.5 tubes in plasma, the average effect of the coagulable substances in plasma on the coagulation zone must amount to an extension of about 1.0 tubes.

Normal Plasma Controls.

The series contains fourteen samples from normal healthy blood donors. The fastest gel in these samples was formed/

11. CRITERIA OF POSITIVE PF REACTIONS.

After the main series was completed the recorded results were correlated with such features as abnormal packed cell volumes and abnormal ESR. Assuming that the optimal criteria for positive PF reactions are those which give most positive reactions in abnormal bloods and the fewest positive reactions in normal bloods, the criteria adopted are as follows .....

'PFF positive' ... includes PFF 2+ to 5+ but not PFF1+.

'PFG positive' ... includes PFG 2+ to 5+ but not PFG1+.

PFF and PFG 1+ reactions are therefore included as 'PFG and PFF negative' hereafter, unless stated otherwise.

By such standards the reactions were negative in all the normal bloods examined in this work (page xxvii) and the associations between positive PF reactions and abnormal packed cell volumes and accelerated ESR are optimal (pages 106 and 107).

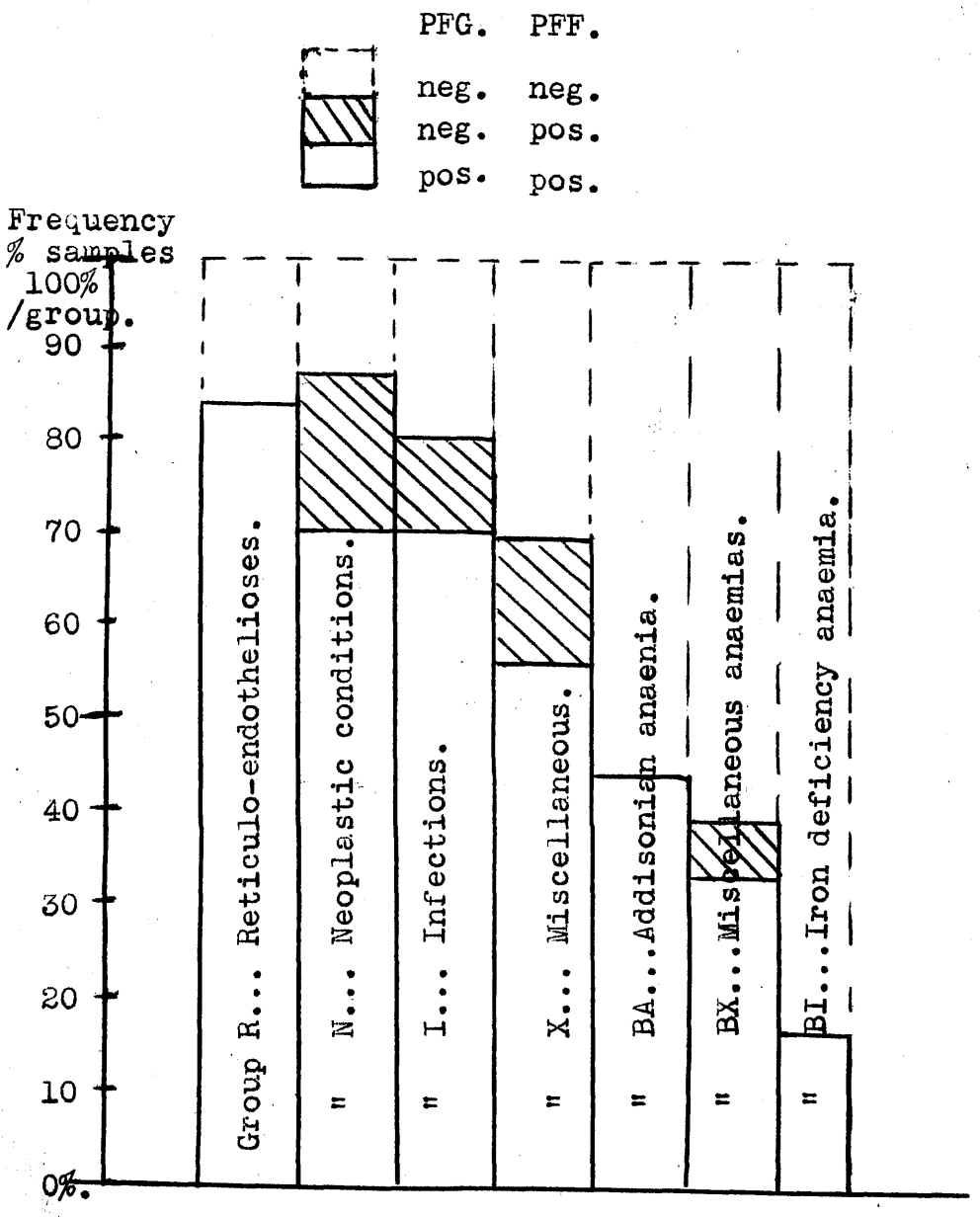


Fig.12:- The frequency of positive plasma formol reactions, i.e. PFG &PFF 2+ to 5+, in the various pathological groups of the series.

111. CLINICO-PATHOLOGICAL FEATURES RELATED TO PLASMA FORMOL REACTIONS.

Pathological Groups.

See fig. 12 facing this page.

Group R--Reticulo-endothelioses. In this group the incidence of positive PF reactions is greater than in any of the other groups of this series. Both the PFG and the PFF reactions were positive in 20 out of 24 samples examined. Positive reactions occurred in cases of chronic lymphatic and myeloid leukaemia, myelomatosis, lymphadenoma, and in one sample from a case of thrombocytopenic purpura with splenomegaly (sample RXi). This case recovered spontaneously within ten days of the onset and test RX2, at a time when the purpura was cleared up but the spleen was still palpably enlarged, was PFF and PFG negative. The other three negative reactions were in cases RMc and REd. RMc was suffering from myeloid leukaemia; symptoms had been present for only three weeks; the peripheral blood contained more primitive white cells than the other leukaemia bloods of this series. Case REd was similarly more acute than the other cases of lymphadenoma in the series. The symptoms, pruritis and enlarged glands, had been present for three weeks at the time of REdi and for five weeks at the time of REd2. There was generalised erythrodermia at the time of the tests. The diagnosis was established by lymph-node biopsy. There was no response to X-ray therapy and he died six months after the onset of the symptoms.

Group I--Infections. PFG and PFF reactions were positive in seven/

seven out of the ten samples of the series; one case (IP) gave a positive PFF reaction but a negative PFG reaction; negative reactions occurred in one case of infective hepatitis and one apparently quiescent case of pulmonary tuberculosis as judged by the X-ray appearances, negative sputum and normal ESR. Positive PF reactions were seen in lobar pneumonia, active pulmonary tuberculosis, malaria, infective endocarditis, sarcoidosis and diffuse lupus erythematosus. IP suffered from chronic hepatic cirrhosis and had developed an acute pyogenic septicaemia at the time of the test.

Group N-Neoplastic Conditions. The PFF and PFG reactions were positive in eight reasonably early cases os gastric, bronchial and renal carcinoma and in one neurofibromatosis with histological evidence of sarcomatous change. One case, NP, gave an unusual reaction; the plasma formol mixture was perfectly clear after 24 hours but after 48 hours a perfectly translucent pale green gel had formed. The patient had obstructive jaundice due to a small localised carcinoma of the head of the pancreas. Icteric plasma usually became green with formol but the unusual feature here was the formation of a gel in the absence of a 24 hour positive PFF and the striking translucency of the gel. Six months after the test this gel was unchanged in appearance. Two of the remaining four tests gave PFF positive, PFG negative results (NGa, NKa); in both cases there was gross hepatic involvement, icterus, extensive metastatic tumours of bone, gross emaciation and moderate anaemia. In cases NH and NX the PF reactions were both negative. NX was admitted in a moribund state with a palpable tumour/

Sample.		PFG	PFF	Plasma Spec. Grav.
BAci	Before treatment	1	0+	1.022
" 2	Partially treated	5+	4+	1.023
" 3		0	1	1.024
" 4	Hb 100%	3	3	1.026

Table 13 The PF reactions in one case of pernicious anaemia before and during treatment.

tumour in the left iliac fossa, gross hepatic enlargement, severe anaemia and emaciation; she died within a few days but no post-mortem examination was permitted. NH (male, aet 20) was perfectly well until two weeks before test, when he became jaundiced. He gave no history of any previous jaundice but stated that he "missed it when his sister had it ten years ago." He died ten days after the test and at post-mortem his liver showed the nodular hyperplasia which follows subacute hepatic necrosis; in addition there was a large primary carcinoma in the right lobe of his liver. No metastatic tumours were found.

Group BI-- Primary Iron Deficiency Anaemias. The PF reactions were negative in eleven out of thirteen samples in this group. The positive cases were BId and BIb. BIb was grossly emaciated but had no specific vitamin deficiencies; she recovered rapidly on iron and general nutritional measures. BId presented the opposite picture in that she had recently become excessively stout and presented with the appearance of a pituitary dysfunction in addition to the anaemia as described by Snapper et al (1937) and Watkinson (1947).

Group BA- Addisonian Anaemia. One case is grouped in the miscellaneous group (case XB) because the chief clinical manifestations were allergic in nature; in this case the PFG reaction was 4+ and PFF 5+. Of the other 17 samples, 8 gave positive and 9 gave negative PF reactions. Three of the nine negative samples came from untreated, uncomplicated cases; three came from one case which was refractory to parenteral liver but later responded to proteolysed liver by mouth (BAg); one was from a patient/

patient who had had a few injections of liver from her doctor but had received none for four weeks. The two remaining negative samples are BAh and BAc3. BAc is discussed below. BAh also suffered from diabetes mellitus and was comatose as well as severely anaemic on admission. She died before there was sufficient time for the usual effects of liver therapy to be evident. Thus in eight out of nine PF negative cases there was either no clinical response to liver therapy or no treatment had been given. The eight positive cases had all been treated with liver for at least two weeks, but other factors complicate the picture. Six of the eight positive samples came from patients who had recently developed subacute combined degeneration of the cord with either muscle wasting or cystitis. Serial tests in one case (BAc) were made at two weekly intervals from BAc1, before any liver treatment had been given, to BAc3 and 4 where the haemoglobin was 100% again. The results are shown, Table 13, page 97, with the plasma specific gravity results.

Group BX - Miscellaneous Anaemic States. In four post-haematomesis samples from patients with no previous dyspeptic symptoms and in three cases of lead absorption anaemia the PF reactions were invariably negative. Four tests in three healthy pregnant women (7-8 months) gave positive PFF and PFG reactions; a fourth patient (in the seventh month of pregnancy) was icteric and had normoblasts in the peripheral blood and macro-normoblasts in the marrow. She later responded well to liver therapy but gave negative PF reactions before treatment was started. There are/

are three samples from cases diagnosed as splenic anaemia in this group. One, (BSa), was subjected to splenectomy after massive transfusions three weeks before the test. At operation she was noted to have marked cirrhosis of the liver. In this case PFG and PFF were both 5+. The second case, BSb, had typical Banti's syndrome. BSbi was performed, before treatment, and gave PFF2+, PFG1+; 24 hours later he was given one pint of whole blood intravenously and five pints of fluid were removed from his abdomen; five days later at the time of test BSb2, the ascitic fluid had reaccumulated, and the PFG reaction was 4+ and the PFF reaction 2+.

Group X - Miscellaneous Conditions. Eight tests on duodenal and gastric ulcer patients gave negative PF reactions. Five tests on three patients whose chief complaints were allergic in nature (XB, XA and XW) gave PF reactions of 3+ - 5+. Four tests on coronary thrombosis patients were positive with one exception. The exception was tested two days after the onset; the other three patients were tested one week after the original attack. Positive PF reactions were also seen in cases of constrictive pericarditis with ascites and pleural effusion, amyotrophic lateral sclerosis, clinical nephrosis, and malnutrition.

	Absolute frequency		Totals	n*	X^2*	P*
	PFF pos.	PFG pos.				
A. Males	26	23	49			
Females	28	23	51	1	0.1	0.9+
				not significant		
B. Age groups						
60 and over	4	3	7	4	0.77	
50 - 59	16	14	30			0.9+
40 - 49	16	11	27			
30 - 39	9	8	17			
Under 30	9	10	19	not significant		
C. Febrile	37	3	40			
Afebrile	17	43	60	1	35.6	0.01+
				significant.		
D. Loss of weight	22	3	25			
No loss of weight	32	43	75	1	13.0	0.01-
				significant		
Totals (A, B, C or D)	54	46	100	Subjects (86 patients, 14 healthy)		
E. Chronicity						
Over 12 months.	20	6	26	2	6.5	0.05
1 - 12 months	25	17	42	significant		
0 - 1 month	8	10	18			
Total (E)	53	33	86	Patients		

* Square contingency test... Fisher, 1946.
(n = degrees of freedom: X^2 = square contingency;

P = probability that association is coincidental.

P = less than 0.05 indicates a significant association, i.e. the chances are less than 1 in 20 that such an association has occurred by coincidence.

Table 14:- Absolute frequency of positive PFF reactions in the cases of the present series arranged in groups according to age, sex, etc.. Where more than one test was performed on any patient the strongest PF reaction is recorded.

Clinical Features and the PF Reactions.

The absolute frequency of positive PFF reactions in the samples of the series arranged in various groups are shown in Table 14, facing this page, along with statistical confirmation of the observations recorded below.

In this series there was no evidence of any variation in the incidence of positive reactions in different age and sex groups. Positive reactions however, were more common in febrile cases, in patients who were losing weight, and in chronic conditions, than in the corresponding groups of afebrile cases, patients not definitely known to be losing weight, and acute and subacute conditions. Thus 92% of febrile and 27% of afebrile patients gave positive reactions; 88% of patients with loss of weight and 43% without definite loss of weight were positive. In chronicity groups the frequency diminishes as the duration of relevant symptoms diminishes, ...

.... with symptoms for over one year 78% PFF +ve;
" " " 1 mth. to one year 59% PFF +ve;
" " " less than 1 mth. 44% PFF +ve.

Effect of Venous Constriction. In five subjects venous constriction as for the test of Hess (see pages 57 to 58) accelerated and accentuated the PF reactions. The results are shown in Table overleaf. In one PF negative blood the reactions were also negative after constriction. Attempts to assess the effect of such constriction on normal bloods were thwarted by haemolysis which invariably developed in the constricted samples, even when no ammon. pot. oxalate was added. It seems probable, however, that in a proportion of borderline bloods, otherwise negative reactions may be altered/

Sample.	PFG	PFF	Dilution (plasma)	Weltmann (plasma)	Crude ESR Wintrobe	Corrected ESR Wintrobe & Whitby
XAi	3+	3+	+	6.0	normal	normal
XA2*	5+	4+	+	6.0	normal	normal
XWi	3+	3+	neg.	5.5	12	9 (normal)
XW2*	5+	5+	-	6.5	12	15 (slight)
BAgi	Neg.	Neg.	-	6.0	10	normal
BAg2*	Neg.	Neg.	-	6.5	8	normal
RMbi	5+	5+	+	6.0	45	12 (doubtful)
RMb2*	5+	5+	+	7.0	40	12 (doubtful)
BAii	I+	3+	neg.	6.0	35	14 (doubtful)
BAi2*	I+	2+	neg.	6.0	32	18 (slight)

* After venous compression as in the test of Hess

(See page

Table 15:- Showing the results of the PFF and PFG reactions without constriction and after five minutes venous constriction in five cases.

altered to positive reactions by venous constriction of this order. Less prolonged constriction may suffice to make the reactions positive in some such cases. In this respect the formol reactions differ from the ESR; it was observed herein that the ESR was slower after venous constriction although the corrected ESR showed no constant difference.

In the five tests concerned the coagulation zones (plasma) were generally longer after constriction and the dilution tests were generally more strongly positive.

Icterus. The thirteen samples from icteric subjects are classified overleaf. The classification is clinical and pathological rather than biochemical, e.g. NH and NKa died shortly after the tests here described and at post-mortem the entire liver appeared to be disorganised by tumour, etc.; case XH was diagnosed two years earlier by biopsy.

No conclusions are possible regarding the PF reactions in obstructive and haemolytic icterus. Similarly in two samples (BSa and BSb2) hepatic cirrhosis was complicated by a recent transfusion. In a third transfused case with evidence of hepatic insufficiency the PF reactions were also strongly positive.

Since the series here reported was completed I have examined the blood of a child with an aplastic anaemia before and immediately after a transfusion of stored whole blood. Immediately before the transfusion the child's blood and the transfused blood were both PF negative but after the transfusion the child's blood gave strongly positive PFG and PFF reactions.

PFF and PFG Results in the Icteric Cases of the Series.

A. Icteric Cases Transfused within 3 weeks of the test.

Sample	PFF	PFG	Notes
BSa	5+	5+	Splenectomy and transfusion 3 weeks before. Advanced cirrhosis at operation.
XT	5+	5+	Post-transfusion icterus. No evidence hepatic disease.
BSb2	4+	2+	Banti's syndrome. Transfused and paracentesis 48 hours earlier. Ascites re-accumulated.

B. Obstructive and Haemolytic Icterus.

NP	neg	2+	Carcinoma head of pancreas. Obstructive jaundice.
BPa	neg	neg	Haemolytic, normocytic anaemia of pregnancy.
NGb	2+	2+	Gastric carcinoma with liver involvement and persistently positive faecal occult blood.

C. Hepatic Insufficiency.

i Advanced Carcinoma Cases

NKa	2+	neg	Advanced hypernephroma. Liver +++. Haematuria.
NM	neg	neg	Subacute hepatitis with nodular hyperplasia of liver tissue and primary hepatoma.
NX	neg	neg	Advanced carcinoma. Primary tumour not detected. Faecal occult blood negative.

ii Infective Cases.

IH	neg	neg	Post-penicillin hepatitis. Homologous serum jaundice.
IP	3+	neg	Chronic cirrhosis and acute pyogenic septicaemia.

iii Primary Hepato-cellular Insufficiency.

BSbi	2+	neg	Banti's syndrome. Before transfusion and paracentesis.
XH	2+	1+	Subacute hepatitis. Diagnosed by biopsy 2 years earlier.

Table 16.- PFF and PFG results in the icteric cases of the series.

There was no clinical evidence of any incompatibility in this case. It may be, therefore, that the transfusion is responsible for the positive PF reactions in the three cases referred to above.

Six of the remaining seven samples came from cases which were known or were later confirmed to be suffering from gross hepato-cellular insufficiency. The seventh patient, IH, developed jaundice three months after penicillin treatment for gonorrhoea; this patient was acutely ill at the time of the test and clinical icterus persisted for about four weeks.

The PFG reactions were negative in all seven cases but in four cases the PFF reactions were positive. It is important that four of the seven cases also suffered from carcinoma or an acute infection, either of which alone usually gave positive PFG reaction. The plasma specific gravity was low in all seven cases, but positive gels were seen in plasma with lower specific gravities than any of the seven concerned; hypoproteinaemia alone cannot therefore account for the negative PFG tests in these cases.

Splenomegaly and Lymphadenopathy. Positive PF reactions were significantly frequent in cases with palpable enlargement of the spleen, 14 (75%) PF positive out of 19 cases, and in cases with hyperplasia of the lympho-reticular tissues, 8 (80%) positive out of 10 cases.

Wassermann Reaction was negative in all but one case...BIa. The PF reactions were negative in samples BIai and BIa2.

PFF.	PFG 5+	4+	3+	2+	1+	0	Totals.
5+	I7	1	0	1 (BAd)	0	0	19
4+	7	0	1	1 (BAe)	0	0	9
3+	2	6	8	4	3	2 (NKa, BSbi)	25
2+	1 (XN)	3	1	5	4	3 (XVb, IP, NGa)	17
1+	0	0	0	0	3	1	4
0	0	0	0	1 (NP)	3	52	56
Totals	27	1 0	10	12	13	56	130

Table 17 :-Absolute frequencies of the PFF results in the samples of the present series arranged according to the PFG results. Reference letters are given in bracquets for the samples showing a striking difference between the PFF & PFG results.

OTHER SIDE ROOM TESTS.

PFF and PFG Reactions.

See Table 17, facing this page.

Generally the intensity of the 24 hour plasma-formol mixture was proportional to the time taken to form a solid gel; where no gel formed the 24 hour opacity was usually less than 1+ on the PFF scale in this work. Thus PFG 5+ and 4+ usually occurred in PFF 5+ to 3+ samples, and PFG 1+ and 0 in PFF 1+ and 0 samples. The relationship is less clearly defined in PFF 3+ and 2+ samples, but the average PFG reactions in these tests were 3+ and 2+ respectively.

There are several exceptions to this general principle, thus .....

(a) Rapid gel formation relative to the 24 hour PFF reaction was clearly present in two tests, NP and XN.

(b) PFF reaction was unusually intense relative to the PFG reaction in seven samples; in five of the seven the PFG reaction was negative (0) although the PFF reaction was 2+ or 3+. The results in these nine cases are shown in Table 17, facing this page.

The most significant feature shared by cases NP and XN is that they both suffered from conditions which are reported (see page 96) to give low serum albumin, low or normal gamma-globulin, and high alpha and beta-globulin on electrophoretic analysis of the serum (see page 119). The cases in group (b) above share several features, e.g. the plasma specific gravity was low in all but one of the seven cases. Interpreting low plasma/

H Group.	PFG			Totals per H group.
	5+ to 3+ (24 hrs)	5+ to 2+ (48 hrs)	5+ to 1+ (96 hrs)	
45-49%	4..44%	5..55%	7..78%	9(100%)
40-44%	7..28%	8..32%	11..44%	25(100%)
35-39%	11..32%	16..46%	21..60%	35(100%)
(Less than 35%	25..42%	30..49%	33..55%	61(100%))
30-34%	10..53%	12..63%	14..74%	19(100%
25-29%	12..59%	14..67%	14..67%	21(100%)
20-24%	2..15%	2..19%	2..19%	11(100%)
Less than 20%	1..10%	2..20%	3..20%	10(100%)
Totals	47..36%	59..46%	72..55%	130..100%

H Group.	PFT.		Totals per H group.
	5+ to 2+	5+ to 1+.	
45-49%	5..55%	6..67%	9(100%)
40-44%	8..32%	10..40%	25(100%)
35-39%	21..60%	21..60%	35(100%)
(Less than 35%	36..55%	37..55%	61(100%))
30-34%	15..79%	15..79%	19(100%)
25-29%	15..71%	15..71%	21(100%)
20-24%	3..27%	3..27%	11(100%)
Less than 20%	3..30%	4..40%	10(100%)
Totals	70..54%	74..59%	130(100%)

Table 18 :- Showing the frequencies of positive PFG and PFF reactions in the samples of the series arranged in groups according to the packed red cell volumes. The frequencies are expressed as absolute numbers and per cent of samples in each H group.

plasma specific gravity as hypoproteinaemia, the more common causes of low Gp are recent acute haemorrhage, chronic blood loss, chronic protein loss, e.g. in the urine or into the serous cavities of the body, malnutrition and hepatic insufficiency. In BSbi, Banti's syndrome, almost all of these causes may have operated; NGa and NKa were advanced carcinoma cases with extensive hepatic involvement, chronic blood loss and wasting. IP was known to have chronic hepatic cirrhosis and had recently developed an acute pyogenic septicaemia. XVb suffered from malnutrition. In the five cases discussed so far the PFG reaction was negative but in cases BAd and BAe the PFG reactions were positive. They both suffered from pernicious anaemia and were undergoing intensive treatment with liver because of the very recent evidence of cord involvement. In BAd there was also an acute cystitis.

One observation in the five PFF positive, PFG negative tests may be important although I can find no mention of the phenomenon in the literature to date. In most of these cases the plasma-formol mixture after 24 hours appeared to be more viscous than in other PFG negative tests and in each case I fully expected to find a solid gel after 48 hours. Instead there was an increase in the opacity and a fall in the viscosity of the plasma-formol mixtures. In these cases, therefore, gelation was replaced by coacervation

Packed Red Cell Volume (H): See Table 18, facing this page.

When the samples of this series are arranged in groups according to their packed cell volume per cent blood volume (H) the/

the maximum incidence of positive PF reactions occurs when H is between 25 and 35%. For the PFG reaction this range is from 25-29% and for the PFF reaction it is from 30-34%. Positive reactions become less common as H increases or decreases from these ranges as far as H=20% and H=44%. There is a slight rise in the incidence of PFG and PFF reactions in the group H = 44-49% and a rise in the incidence of PFF positive reactions when H is less than 20%.

In rheumatic cases Gibson and Pitt (1946) reported a rising incidence of positive plasma formol gel tests as H decreased from 49% to less than 35%. The results in the present series are compared with their results below .....

	H.			
	45-49%	40-44%	35-39%	Less than 35%
Gibson & Pitt (1946)	27% °I	63%	90%	94%
Herein (PFG positive = 3+ - 5+, i.e. 24 hour gels as above)	44%	25%	32%	42%
	*(57%	40%	35%	42%)
Herein (PFG positive = 2+ - 5+ as in this work)	55%	32%	46%	49%
	*(70%	47%	55%	49%)

* .. as for line above but normal samples excluded.

°I .. frequency positive PF reactions % samples in H group.

The higher incidence in the group 45-49% is probably due to the cases with venous congestion in this series; the lower incidence when H is less than 40% is similarly due to the primary anaemia cases in this work.

Square-contingency tests (Fisher, 1946) show that it is most unlikely that the distribution of the PF results in the/

	PFG			PFF		Total Samples in Group.
	5+-3+	5+-2+	5+-1+	5+-2+	5+-1+	
A. ESR(Wintrobe-uncorrected)						
i.High	36 (56%)	44 (69%)	47 (73%)	51 (80%)	53 (83%)	64 (100%)
2.Normal	11 (17%)	15 (23%)	25 (38%)	19 (29%)	21 (32%)	66 (100%)
B. ESR(Wintrobe-corrected)						
i.High	32 (63%)	39 (77%)	43 (85%)	46 (90%)	47 (92%)	51 (100%)
2.Normal	15 (19%)	20 (25%)	29 (37%)	24 (30%)	27 (34%)	79 (100%)
Totals A or B	47	59	72	70	74	130
C. DILUTION TEST (Plasma)						
i.Positive	36 (77%)	37 (84%)	37 (91%)	40 (95%)	40 (95%)	41 (100%)
2.Negative	2 (7%)	8 (14%)	15 (25%)	12 (21%)	14 (24%)	64 (100%)
D. COAGULATION ZONES(Weltmann/plasma)						
i.Less than 5.5	4 (57%)	4 (57%)	5 (57%)	5 (71%)	5 (71%)	7 (100%)
2.5.5-7.0	24 (28%)	31 (37%)	40 (46%)	40 (46%)	42 (50%)	85 (100%)
3.7.5-9.0	10 (91%)	10 (91%)	10 (91%)	7 (64%)	10 (91%)	11 (100%)
Totals C or D	38	45	55	55	57	103

Table 19 Showing the frequency of the PFF and PFG results in relation to other side-room tests in the present series. Absolute frequencies are given as well as the frequency per cent of all samples in the groups concerned.

the H groups of this series is due to coincidence. Contingency tables were prepared (see Table 20, page 107) showing the incidence of positive and negative PF reactions in the H groups as in Table 19. To compare different criteria for positive reactions one table was prepared grouping all samples giving PFG 5+ - 3+ as PF positive; similar tables showed PFG 5+ - 2+, PFG 5+ - 1+, PFF 5+ - 2+ and PFF 5+ - 1+ as positive. X^2 was calculated for each table and P, the probability that the association was due to coincidence, was obtained from X^2. In each table, n, the number of degrees of freedom = 6. Results were obtained as follows ....

i. PF positive = PFG 3+ - 5+ ... X^2 = 11.76; P = 0.10

2. PF positive = PFG 2+ - 5+ ... X^2 = 14.33; P = 0.02

3. PF positive = PFG 1+ - 5+ ... X^2 = 15.6; P = 0.01

4. PF positive = PFG 2+ - 5+ ... X^2 = 16.45; P = 0.01

5. PF positive = PFG 1+ - 5+ ... X^2 = 13.40; P = 0.04

Hence the probability of coincidental association is minimal when samples giving PFF 2+ - 5+ are classed as PF positive; statistically the other associations are significant except where only 24 hour gels (PFF 5+ - 3+) are accepted as PF positive.

Erythrocyte Sedimentation Rate (ESR)

See Table 19, facing this page, and Table 20, overleaf.

As in the case of the packed cell volume the probability of a coincidental association between the PF reactions and the crude or corrected ESR is least if bloods giving PFF 5+ - 2+ are regarded as PF positive; furthermore the association with the corrected/

Table 20: Analysis of the association between the PF reactions and the ESR in the present series.

Method : Square contingency test-Fisher, 1946.
Twox two contingency tables constructed thus.....

PF reactions	ESR Normal	ESR Accelerated.	Totals.
No. pos.	a	b	a+b
No. neg.	c	d	d+c.
Totals	a+c	b+d	a+b+c+d (=130)

Degrees of freedom =1; X^2 calculated; P from tables. (Fisher, 1946)

Results: When a+c is number of tests with normal crude ESR and b+d is the number with accelerated crude ESR, and...

1. a+b is number giving PFG 5+-3+(24 hour gels)... X^2=23.7
2. " " " " " 5+-2+(48 " ")... " 28.4
3. " " " " " 5+-1+(96 " ")... " 22.6
4. " " " " PFF 5+-1+ ..... " 33.8
5. " " " " " 5+-2+ ..... " 34.0

When a+c is number with normal corrected ESR and b+d is number with accelerated corrected ESR, and...

1. a+b is number giving PFG 5+-3+..... X^2=26.5
2. " " " " " 5+-2+..... X^2=32.7
3. " " " " " 5+-1+..... X^2=29.4
4. " " " " PFF 5+-2+..... X^2=42.5
5. " " " " " 5+-1+..... X^2=44.6

In all the above tests P is less than 0.01, i.e. the chances of such an association occuring by coincidence are less than one in a hundred. The probability of coincidental association is, however, lowest when PFF 5+-2+ is related to the corrected ESR.

corrected ESR is less likely to be due to coincidence than the association with the crude ESR.

The PFF reactions were therefore positive (5+ - 2+) in 80% of cases with accelerated ESR and in 90% of cases with accelerated corrected ESR. The best association with the PFG results is seen when PFG 5+ - 2+, i.e. 48-hour gels, are classed as PFG positive. This occurred in 69% of cases with accelerated ESR (uncorrected) and 77% of cases with accelerated corrected ESR. The 24-hour gel criterion for positive reactions gave only 56% and 63% ;ositives in cases with accelerated crude and corrected ESR respectively. For this comparison the ESR was corrected by Wintrobe's chart (Wintrobe, 1946). In four of the PF negative, accelerated corrected ESR samples, the corrected rate by Whitby and Britton's chart (1946) was normal. No one line on either chart gave a clear division between PF positive and PF negative bloods. The ESR corrected by one method was no better related to the PF reactions than by the other method, except that by Whitby and Britton's chart many samples were left 'doubtful' on correction.

There are altogether six samples with accelerated corrected ESR (Wintrobe's chart) and negative PFF reactions. In four of the six the corrected ESR by Whitby's chart was normal; there was no clinical evidence suggesting acceleration of the ESR in five of the six cases. The sixth case, NP, was PFG positive as described on page .

Conversely in 24 samples the corrected ESR was normal although the PF reactions were positive; in 19 of these samples the crude ESR was also normal but in ten of the cases concerned, there was clinical evidence to suggest that the sedimentation rate should/

Plasma Specific Gravity (Gp)								
a. 1.018 to 1.020	b. 1.021 to 1.023	* a+b	c. 1.023 to 1.024	d. 1.025 to 1.026	e. 1.027 to 1.028	* c+d+e	f* 1.029 to 1.031	Total. (a+b+..-f)
PFF pos. (2- to 5-)								
4	3	7 (35%)	13	11	10	34 (43%)	4 (100%)	45
PFF pos. (2- to 5-)								
5	6	11 (55%)	19	11	10	40 (50%)	4 (100%)	55
ESR accelerated (Wintrobe)								
7	11	18 (90%)	13	10	5	28 (35%)	2 (50%)	48
Corrected ESR accelerated (Wintrobe)								
4	6	10 (50%)	12	9	4	25 (32%)	2 (50%)	37
Positive dilution tests (plasma)								
1	6	7 (35%)	13	7	8	28 (36%)	4 (100%	39
Coagulation zone (plasma Weltmann)								
i. From 4-5, i.e. short.								
2	1	3 (15%)	3	0	1	4 (5%)	0 (100%	7
ii. From 5.5 to 7.0, i.e. probably normal.								
4	11	15	34	29	7	70	0	85
iii. From 7.5 to 9.0, i.e. prolonged.								
2	0	2 (10%)	0	2	3	5 (6%)	4 (100%)	11
Totals/Gp group.								
8	12	20 (100%)	37	31	11	79 (100%	4 (100%	103

*'a+b'..hypoproteinaemic samples.
'c+d+e'...samples with total plasma proteins within range of normal.
'f'... hyperproteinaemic samples.

Table 21:- The absolute frequency of positive PF and other reactions in the samples of the present series arranged in groups according to the estimated plasma specific gravities. Figures in bracquets give the frequencies per cent of the samples in the Gp group.

should have been accelerated; e.g. one case of pulmonary tuberculosis with active lesions on X-ray, positive sputum, and pyrexia; some neoplastic cases where there was extensive tissue destruction; chronic reticulo-endothelioses with accelerated ESR on previous occasions when the condition was no more severe.

Thus there are fourteen samples where the PF reactions were positive and the ESR was normal on correction. These cases may be grouped as ...

Acute allergic states ... Samples XAi, XA2, XB.

Treated Addisonian anaemia ... BAc2, BAc4, BAe.

Hepatic insufficiency ... BSa, BSbi & 2, XH, (?XVa), XP.

Muscle breakdown + + ... XM, XVa, XCb2.

Plasma Dilution and Weltmann Tests Related to the PF Reactions and to Plasma Specific Gravity. See Table 21 facing this page.

All samples with Gp greater than 1.028 gave lengthened plasma coagulation zones (between 7.5 and 9.0) and PFG and PFF reactions between 3+ and 5+. Similar lengthening of the coagulation zone was present in 5 - 10% of all samples with normal Gp. The PF reactions were positive in each case. The coagulation zone was also lengthened in two samples with low Gp, one of which gave PFG4+, PFF 2+; in the other the PF reactions were negative.

Short coagulation zones were seen in 15% samples with

Gp/

Gp less than 1.023 and in 5% samples with normal Gp. The PF reactions were negative in two of the samples with low Gp and shortened coagulation zones.

Statistically the association between the coagulation zones and Gp, and the PF reactions are significant. (PFF - coagulation zones ... X^2=6.87, n=2, P=0.02; Gp and coagulation zones .. X^2=24.9, n=4, P less than 0.01.)

In this series therefore, the PF reactions were usually positive when the coagulation zone was abnormally long and when it was abnormally short. Hyperproteinaemia as indicated by Gp 1.028 or more was always associated with positive PF reactions and with extended coagulation zones. Hypoproteinaemia, i.e. Gp less than 1.023 gave both short and long coagulation zones and in either case the PF reactions were usually positive.

Plasma Dilution Test Related to Gp and PF Reactions. See Table 19 facing page 106.

The plasma dilution test was positive in all samples with Gp over 1.028 thus associating with positive PF reactions and extended coagulation zones in this Gp group. Although the dilution test was positive in 35% of all samples with low Gp and 38% of all samples with normal Gp, it was only once positive in eight samples in which Gp was less than 1.022. Generally the PF reactions were positive when the plasma dilution test was positive. Statistically the results of the dilution test are significantly related to the estimated Gp - X^2=18.36, n=4, P= less than 0.01.

Summary of the Clinico-Pathological Associations.

The PFF and PFG reactions were usually positive in infective states, in neoplastic conditions and in the reticulo-endothelioses, but negative reactions were seen when there was coincident hepatic insufficiency and in acute and subacute forms of the reticulo-endothelioses. In these complicated cases of hepatic insufficiency the plasma specific gravity was low and the PFF reactions were occasionally positive although the PFG reactions were negative; coacervation was seen to occur instead of gelation. The reactions were strongly positive in all three cases examined after blood transfusion. Positive PF reactions were also seen in pregnancy (7 - 8 month), allergic conditions, treated and complicated Addisonian anaemia. The reactions were usually negative in iron deficiency anaemia and in untreated uncomplicated Addisonian anaemia, in post-haemorrhagic states, lead absorption and in peptic ulceration. Positive reactions occurred in isolated cases of amyotrophic lateral sclerosis, clinical nephrosis, sarcoidosis and diffuse lupus erythematosus.

There was no significant variation in the incidence of positive reactions in different age and sex groups , but positive reactions were significantly frequent in febrile patients, in patients who were losing weight, and in chronic established conditions. The Wassermann reaction was positive in one case; in this case the PF reactions were negative. Venous congestion and stasis accelerated and accentuated the reactions. The PF reactions were most commonly positive when the/

the packed red cell volume was between 25 and 35% blood volume. The erythrocyte sedimentation rate (ESR) was closely related to the PF results, i.e. high ESR usually found in PF positive samples. The corrected sedimentation rate followed the PF reactions more closely than the crude ESR. In several cases the PF reactions were positive when the corrected ESR gave a false normal result. Positive PF reactions and normal ESR were seen in association with muscle breakdown, hepatic insufficiency (PFF positive but not necessarily PFG positive) and allergic states.

When Gp was more than 1.027 the PF reactions were strongly positive the plasma dilution test was positive, and the plasma Weltmann coagulation zone was prolonged. In plasma of normal specific gravity the dilution test was rarely positive unless the PFF reactions were positive. Usually bloods giving abnormally short or abnormally long coagulation zones gave positive PF reactions but PF positive reaction were seen with normal coagulation zones. When Gp was low, positive dilution tests were less common, but positive PF reactions were seen when the plasma gravity was as low as 1.018 - 1.019. The coagulation zone was variable in bloods of low Gp, but PF reactions were positive when the coagulation zone was shortened as well as when it was prolonged.

DISCUSSION.

The discussion which follows begins with some general observations on the reactions between formol and proteins. Succeeding sections deal with the plasma and serum proteins in health and in disease, the manifestations of protein instability in plasma and serum, and the relationship between protein instability and positive formol reactions in this series and in the literature. This part of the work establishes a relationship between positive formol reactions and serum or plasma protein instability. A possible mechanism for positive formol reactions is then outlined and it is postulated that the serum formol reactions and the tests known as 'non-specific liver function tests' are actually 'serum protein stability tests': similarly, the plasma formol reactions and the ESR are grouped together as 'plasma protein stability tests.' Finally some applications for the plasma formol reactions in clinical work are suggested.

Formol and Proteins in General.

Despite the importance of the physico-chemical reactions between formol and proteins, the exact nature of the reactions is not yet clear. To the pathologist formol is the tissue fixative of choice for most routine purposes; it is essential for the preparation of certain toxoids and vaccines; in industry the reactions between formol and collagen are extensively employed in the tanning of leather, and in recent years industrial chemists have found many new uses for formol in the manufacture of plastics, one variety of which is prepared from casein and formol. This particular compound has many different uses, e.g. artificial tortoiseshell spectacle frames are usually made of casein plastics. Other materials of a similar nature are prepared from phenol, urea, and amino-acids by means of formol, e.g. bakelite is a phenol-formaldehyde compound. Yet in spite of every effort by biochemists and industrial chemists even the nature of formaldehyde in aequeous solution is not known for certain. The most popular opinion, at present, is that of Cohn and Edsall (1943) who consider that in aequeous solution formaldehyde takes the form of methylene glycol ... $CH_2(OH)_2$.

The polymerising properties of formol are responsible for many of its varied uses. In aequeous solution 40% formaldehyde, i.e. formol, readily forms compounds made up of, it may be, as many as a hundred formaldehyde molecules. Commercial formol and liquor formaldehyde, B.P. both contain traces of methanol which, it is believed, minimise polymerisation. On standing, formol

becomes/

becomes increasingly acid owing to the formation of formic acid but, apart altogether from the presence of formic acid, formol appears to have acidic properties (Levy, 1934). While formol probably reacts with several radicles in complex amino-acids, the characteristic reaction is with the amino radicles. It seems to be generally agreed that formol forms additive compounds with amino groups and by so doing increases the acidity of the mixture concerned. There is no doubt that the addition of formol to solutions of amino-acids and of proteins increases the acidity of the solutions but there is considerable doubt as to the true nature of their reactions. French and Edsall, 1945, reviewing the literature on the reaction between glycine, one of the simplest amino-acids, and formol, record eight different reactions described by different workers and state that their list is by no means exhaustive. Considering the complexity of the amino-acid make-up of the plasma protein in health and in disease, it is manifestly impossible, at present, to anticipate the clinical associations of the formol reactions from chemical considerations alone.

Formol reacts with many substances in plasma besides protein e.g. with ammonium salts (page 88), creatinine, urea, simple carbohydrates and polysaccharides. The literature on the serum formol gel test however, clearly establishes a relationship between gel formation and the serum proteins; it seems unlikely that any of the other reactions are ever primarily responsible for gel formation in serum formol mixtures.

Normal and Abnormal Plasma and Serum Proteins. The literature summarised herein shows that the serum formol reactions are positive when there is an increase in the amount of globulin and of euglobulin in the serum. In the investigations quoted the serum proteins were fractionised by salt-precipitation. Within recent years other methods of fractionisation have been developed; two of these methods are referred to in this work, i.e. electrophoresis and Cohn's method (Cohn et al, 1944 and 1947).

Although protein fractions separated by different methods may have the same name it cannot be assumed that they are the same substance, e.g. electrophoretic albumin is now known to differ from the albumin left in solution after precipitation of the globulins by salts (see below). Similar confusion is liable to result from the use of the term "euglobulin" which may have several meanings. Enders (1944) and other electrophoretic workers define a euglobulin as any protein which is insoluble in low concentrations of salts at, or near, its isoelectric point. According to Lloyd and Shore (1938) a euglobulin is a water insoluble protein containing phosphorus. The protein which is precipitated from serum by 33% sat. ammon. sulph. or 14.5% sat. sod. sulph. is generally called euglobulin. The "salt-precipitated" fractions are separated from serum and plasma by varying concentrations of saturated ammonium or sodium sulphate. The original method of Howe (1921) separates albumin from globulin by means of 22.5% saturated sod. sulphate solution; the protein left in solution is called albumin and the protein precipitated from serum is called globulin. The globulin fraction may be

further/

further split into 'euglobulin', precipitated by 14.5% saturated sod. sulph. and 'pseudoglobulin', precipitated by 22.5% but not by 14.5% saturated sod. sulph.. Relatively low concentrations of sodium chloride or calcium chloride, or both, are used to precipitate the clot-forming fibrinogen from plasma. The accepted concentrations of saturated ammonium sulphate which precipitate similar fractions are 20% (fibrinogen), 33% (euglobulin) and 50% (pseudoglobulin).

Cohn's fractions (Cohn et al, 1944 and 1947) are separated from plasma by means of alcohol-water mixtures at various pH levels and at low temperatures. These fractions have been numbered I - VI rather than named.

The electrophoretic analysis of serum, or plasma, groups the protein molecules according to their mobolity in an electrical field. The fastest moving protein is called albumin; subsequent fractions in order of diminishing mobility are called, alpha, beta and gamma globulin; the clot-forming protein of plasma has a mobility between that of beta-globulin and that of gamma-globulin. The original alpha, beta and gamma globulins have each been further split into alpha one, alpha two, beta one, etc.

None of these fractions has so far been shown to be homogenous in molecular make-up. The salt separated fractions are made up of mixed electrophoretic fractions. The electrophoretic fractions contain variable amounts of carbohydrate, lipoid and protein. The proportion of carbohydrate is highest in the alpha-globulins; the beta-globulins contain more lipoid than the other fractions; it would appear therefore, that gamma-globulin/

o1 Cohn et al,1946.
Cohn et al,1944.

o2 Hawk,Oser and Summerson,1947.

o3 Stacey,1947.

04 Chow,1947.

globulin contains more nitrogen than the other globulins.

The relationship between the fractions separated by different methods is still uncertain even in normal blood. Cohn's fraction I consists mainly of clot-forming protein and therefore corresponds to fibrinogen by the 'salting out methods' and on electrophoretic analysis. By electrophoretic methods the amount of fibrinogen in normal blood is 0.6 g.% plasma whereas the amount by salt precipitation is 0.25 - 0.30 g.%.[o2] This difference is as yet unexplained. Gamma-globulin appears to be the main protein in Cohn's fraction II[o1] and in the protein precipitated from serum by 33% sat. ammon. sulph. or 14.5% sat. sodium sulph. solution[o2]. Most of the alpha and beta globulins and some of the gamma-globulins are distributed throughout Cohn's fractions III and IV and in the protein precipitated by 22.5% sat. sod. sulph. or 50% sat. ammon. sulph..[o1, o2] It seems probable, however, that much of the alpha-globulin remains in solution with the albumin after precipitation by the 22.5 or 50% solutions is complete,[o3, o4] particularly in acute infections with increased alpha-globulin.

Kagan (1943a and b) reported that hyperproteinaemia was invariably associated with hyperglobulinaemia, i.e. in his series the amount of protein precipitated from the serum by 22.5% sat. sod. sulph. was always more than 3.0g.% serum when the total serum proteins exceeded 8.0g.%. When the total proteins were between 7.5 and 8.0g.% serum a similar result was usually obtained with the 22.5% sat. solution. Kagan's findings and the various conditions in which hyperglobulinaemia occurred are summarised/

Conditions Associated with Hyperglobulinaemia from Kagan (1943a and b).

Globulins "salted out".

Definition. Hyperglobulinaemia ... serum globulin more than 3.0g.%.

Associated Findings I. Hyperglobulinaemia invariably present when total serum proteins exceed 8.0g.%, and usually when serum proteins exceed 7.5g.%..

II. High total serum proteins in all hyperglobinaemic bloods except ... a) all forms of hepatic disease

b) acute glomerulo-nephritis

c) simultaneous occurrence of one cause of hyperglobulinaemia and of hypoalbuminaemia.

Pathological Processes Associated with Hyperglobulinaemia.

Dehydration.
Acute glomerulonephritis.
Hepatic insufficiency.
Malnutrition.
Secondary carcinoma in bone marrow or liver ... quoting Peters and Eisenman, 1933.
Myelomatosis.
Lymphatic leukaemia.
Monocytic leukaemia.
Chronic infections ... Tuberculosis ... quoting Eichelberger and McCluskey, 1927.
Syphilis.
Osteomyelitis.
Lymphogranuloma venereum.
Bacterial endocarditis ... quoting Kurten, 1937.
Periarteritis nodosa ... quoting Kurten 1937.
Pyonephrosis.
Rheumatoid arthritis.
Still's disease ... quoting Taussig, 1938.
Sarcoids.
Lupus erythematosis ... quoting Kurten, 1937.

Table 22:- Hyperglobulinaemia as described by Kagan (1943a and b).

summarised in Table 22, facing this page. The literature on the serum formol gel test confirms these observations in that serum gels have been reported whenever the serum globulins exceed 3.0g.%, when the total serum proteins exceed 8.0g.% serum, and in most of the conditions listed by Kagan. In terms of plasma specific gravity the normal range of Gp allowing 0.3g. fibrinogen % plasma, was seen in Part One of this work (page) to be from 1.023 - 1.028 inclusive but Gp = 1.028 corresponds to total plasma proteins of 7.85g.% or total serum proteins over 7.5g.%. Kagan found that sera with this amount of protein were usually hyperglobulinaemic. Gp = 1.029 corresponds to total plasma proteins of 8.55g.%. Thus all bloods with Gp = 1.029 or more and most bloods of Gp = 1.028 should show the features associated below with hyperglobulinaemia.

Bing (1937) considers that an excess of 'euglobulin' is more directly related to positive serum gels than is hyperglobulinaemia. Similarly Taussig (1938) attributes positive SFG tests to an excess of 'euglobulin'. As used by Bing and Taussig 'euglobulin' refers to the 33% sat. ammon. sulph. precipitable serum proteins; Taussig also identifies his fraction of the serum proteins with the water insoluble protein precipitating when certain abnormal sera are diluted with distilled water. From these papers which between them cover most of the common and many of the rare conditions seen in general medical wards, it seems that hyperglobulinaemia, by salt precipitation, is usually/

Electrophoretic Pattern of Serum Proteins in Disease.

Normal pattern

.....Albumin diminished.....

Beta-globulin
.....low,normal,or raised .....

Alpha-globulin increased

Acute inflammation[1,2]
Tissue destruction,[3]
(acute or chronic infection or carcinoma)
Primary nephrosis 4,5
Early acute rheumatism6.

Gamma-globulin increased

Late stages acute infection with antibody and healing6,7.
Chronic fibroid infections3.
(tubercle and syphilis)
Lymphogran. -inguinale8
Later stages acute rheumatism6.
Rheumatoid arthritis10.
Sarcoidosis11,3.
Diffuse lupus erythematosus12
Kala-azar[13] malaria,14
Typhus 15
Myelomatosis* 16
Hepatic insufficiency,17,18,3 .
Recovery from severe haemorrhage.19
New-born 20
Pregnancy 20

*Cases of myelomatosus have been reported with increased beta-glob. and normal gamma-globulin.

1. Stacey,1947.
2. Shedlovsky & Scudder,1942.
3. Siebert et al,1942 & 1947.
4. Longsworth,1940.
5. Luetscher,1940.
6. Dole,Watson & Rothbard,1945.
7. Enders,1944.
8. Kabat et al,1942.
9. Fisher et al,1942.
0. Dole & Rothbard,1947.
11. Siebert et al ,1947.
12. Coburn & Moore,1943.
13. Cooper et al,1943.
14. Dole & Emerson,1945.
15 Dole et al,1947.
16. Moore et al,1943.
17. Martin,1946.
18. Grey & Barron,1943.
19. Zeldis et al,1945.
20. Longsworth et al,1941.

According to Siebert et al ,1947,fibronecrotic chronic infections are associated with increased alpha & gamma-globulin as is tissue destruction and hepatic insufficiency,e.g. in carcinoma with secondary hepatic insufficiency. Apparently, therefore, the two patterns shown above may be superimposed on one another.

Table 23:- Summarising the reported electrophoretic patterns of the serum proteins in disease. Note the ressemblance to Table 11, page 81 , where the coagulation zones (Weltmann serum) in disease are illustrated.

usually associated with an excessive protein precipitation in 33% sat. ammon. sulph. and with positive dilution (serum) and serum formol gel tests. Most bloods with Gp over 1.027 should give the same result. It is known that the 33% sat. ammon. sulph. precipitated serum protein includes most of the gamma-globulins in normal bloods; the conditions listed (Table 22 facing page 118) as associated with hyperglobulinaemia are nearly all associated with increased gamma-globulins (Table 23, facing this page). Hyperglobulinaemic sera usually give an increased protein precipitation in 33% sat. ammon. sulph.. Hence it seems likely that the additional gamma-globulin in hyperglobulinaemic bloods is precipitated by 33% sat. ammon. sulph.. I can find only one exception to the general rule that hyperglobulinaemia by salt precipitation indicates increased gamma-globulins: Moore et al (1943) describe one type of protein pattern seen in a few cases of myelomatosis; hyperproteinaemia, hyperglobulinaemia, increased beta-globulin and normal gamma-globulin.

Hyperglobulinaemia, as defined by Kagan, also occurs in some bloods with low total serum proteins. The commonly recognised causes of hypoproteinaemia are ...

i. Hepato-cellular insufficiency.

ii. Malnutrition.

iii. Chronic protein loss from the circulation into the urine or into the body cavities.

iv. Chronic whole blood loss of haemodilution after an acute loss of blood.

Not infrequently more than one of these factors combine to reduce the serum proteins, e.g. ... in Banti's syndrome there may/

may be hepato-cellular insufficiency, malnutrition consequent on prolonged anorexia, chronic protein loss into the peritoneal cavity, chronic blood loss from oesophageal or rectal varices or acute massive haemorrhage from oesophageal varices.

In almost all the conditions mentioned above there is hypoproteinaemia, hyperglobulinaemia (see Table 22) with all the manifestations of hyperglobulinaemia outlined above. The main exceptions to this rule are ...

(a) "primary nephrosis"

(b) early acute infections

(c) tissue destruction

(see Table 23 facing page 119). In primary nephrosis the total serum proteins are greatly reduced; there is gross hypo-albuminaemia and the electrophoretic pattern of the serum proteins shows an increase in alpha-globulin and possibly also in beta-globulin.

In the early stages of an acute infection and in the presence of tissue destruction there may be a slight fall in the total serum proteins but the characteristic change is again an increase in alpha-globulin with or without an increase in beta-globulin. According to Stacey (1947) and Chow (1947) this increase in alpha-globulin is accompanied by a corresponding fall in electrophoretic albumin. They believe that the increased alpha-globulin (sometimes called C-substance) is not precipitated by 50% sat. ammon. sulph. It follows, therefore, that this fall in electrophoretic albumin and the corresponding rise/

Serum Protein Patterns in Disease.

	Pattern A.	Pattern B.
Total serum proteins.....	n or n-	... n,n- or n#.
33% sat. ammon. sulph. precipitate...	n or n-	... n#.
50% sat. ammon. sulph. precipitate...	n or n-	... n#.
Electrophoretic pattern		
Albumin.....	n-	... n-
Alpha-globulin.....	n#	... n,or n-.
Beta-globulin.....	n,or n#	... n,n- or n#.
Gamma-globulin.....	n,or n-	... n#.
"Cryoglobulins".....	Absent	... present.
Serum(& plasma) dilution tests.....	neg..	... pos..
Weltmann coagulation zones.....	short	... long.
Serum(& plasma) formol reactions...	neg.	... pos..
Non-specific liver function tests....	neg.	... pos..

'n'..normal. 'n#'..greater than normal.
'n-'..less than normal.

Associated Pathological Conditions

Pattern A...Early acute infections;tissue destruction in infections ,carcinoma etc.; primary nephrosis.

Pattern B. .Infections,late with antibody formation and fibrosis; protozoal infections:reticulo-endothel hepatic insufficiency;other causes of ~~/~~ ioses; hyperglobulinaemia.

Table 24:- Serum protein patterns in disease as described herein; compiled from various sources referred to in the text.

rise in alpha-globulin will not be detected by any of the usual methods of salt fractionisation. Certainly the literature contains no reports of significant alterations in the salt precipitable protein fractions in early acute infections or in tissue destruction. Minor increases in the amount of protein precipitated from serum by 50% saturated ammon. sulph. may occur but none of the other manifestations of hyperglobulinaemia have been reported to date in primary nephrosis, early acute infections or tissue destruction.

It appears therefore that abnormal serum protein patterns by electrophoretic analysis are of two main types (A) increased alpha-globulin and (B) increased gamma-globulin. Both abnormal patterns show hypoalbuminaemia and either normal or increased beta-globulin. Pattern A is seen in primary nephrosis, acute infections and where there is tissue destruction. Pattern B occurs in many chronic bacterial and protozoal infections, in the reticulo-endothelioses, in hepato-cellular insufficiency and is characterised by an excessive precipitation of protein in 50% and 33% sat. ammon. sulph., by positive serum dilution and formol gel tests. Pattern A may show none of these abnormal reactions. When the total serum proteins exceed 7.5 g. the serum proteins confirm to Pattern B; total serum proteins less than 7.5 g. may give a normal protein pattern (6.0 - 7.5 g.) or Pattern A or B.

Mixtures of Patterns A and B may be encountered, e.g. (i) where antibody products and tissue breakdown occur side

by/

normal coagulation zone irrespective of their direction, since both prolonged and shortened coagulation zones occur in association with hypoalbuminaemia. It must be made clear that it is electrophoretic albumin which is mentioned here. Albumin, by salt precipitation has been shown to be (page 117) a mixture of electrophoretic albumin and alpha-globulin; it should, because of its alpha-globulin content tend to shorten the coagulation zone, but because of its albumin content this reduction should be most marked when the zone is already prolonged. It appears, therefore, that the various conditions in which serum formol gels have been reported, give serum protein pattern B above; the dilution test is positive and the Weltmann coagulation zone (serum) is usually prolonged in these conditions. Since positive serum formol reactions are always associated with positive plasma formol reaction, the main interest here is in pattern B. Some further manifestations of hyperglobulinaemia will therefore be discussed.

Serum Protein Precipitation at Temperatures Between 5 and 37°C.

The spontaneous precipitation of protein seen in some abnormal sera at temperatures below 37°C, is associated with hyperglobulinaemia and increased gamma-globulins according to Lerner and Watson (1947). The phenomenon is important to this thesis as it may account for the discrepancy between the weight of the packed red cells and the haemoglobin content in the eight bloods with Gp = 1.028 or more (see Part One, page 34). In addition the association with hyperglobulinaemia and increased gamma-globulin indicates that spontaneous protein precipitation occurs/

Reported Cases in Which Spontaneous Protein Precipitation Was Observed in Serum at Temperatures Between 5 and 37°C.

Multiple Myelomatosis	Schumacher and Williams (1937) Bing (1940) Von Bonsdorf et al (1938) Lerner and Watson (1947) Shapiro et al (1943) Wintrobe and Buell (1933)
Kala-azar (Humans and dogs)	Stein and Wertheimer (1943)
Chronic Infective Rheumatism	Holmberg et al (1942) (From plasma). Anderson and Samuelson (1944)
Periarteritis nodosa	Shapiro and Wertheimer (1946)
Purpura, Provoked by Cold	Lerner and Watson (1947) Waldenstrom (1944) Hansen & Faber(1947)
"Essential Hyperproteinaemia"	Waldenstrom(1944)
Subacute Bacterial Endocarditis .... (Six cases out of six)	Lerner and Watson (1947)
Cirrhosis of the Liver	" " "
Lymphatic leukaemia	" " "
Polycythaemia vera	" " "
Congenital Hypoprothrombinaemia	" " "
Pneumonia, Emypema, Bronchiectasis ..	" " "
Brucellosis	" " "
Addison's Disease	" " "
Rheumatic Heart Disease	" " "
Ulcerative Colitis	" " "
Cardiovascular-renal disease	" " "

Table 25:- List of conditions in which spontaneous protein precipitation from serum and plasma at temperatures between 5 and 37°C has been reported.

occurs when the serum proteins conform to Patterns A or B above. Thus, the phenomenon should be associated with positive serum formol gels, prolonged coagulation zones (serum), and positive dilution tests. The various conditions in which cold precipitating protein has been described are listed in Table facing this page. It will be seen that the conditions concerned are those previously associated with hyperglobulinaemia (see page 118), increased gamma-globulin (page 119) and positive serum formol gel tests (pages 76 to 80).

Prior to the work of Lerner and Watson the literature on the subject describes isolated cases or small groups of cases. Lerner and Watson examined serum from 121 mixed general medical cases and found naked-eye and chemical evidence of cold-precipitating proteins or "cryoglobulins" as they call them, in 30 sera. In their series cryoglobulins were present if there was protein precipitation at 5°C, but they observe that the precipitate may be visible at room temperature. The serum dilution test was performed in their series by diluting the sera with 99 vols. of water, not with 9 vols. as suggested by Taussig (1947). This serum dilution test was positive in six sample of their series. Cryoglobulins were present in five of the six samples. From this it appears that the cold precipitation test is more sensitive as a test for hyperglobulinaemia etc., than the serum dilution test as performed by Lerner and Watson. They also observe that the gamma-globulin content of the blood may be high in sera with no cryoglobulins: The example cited is a case of cirrhosis with 8.0 g. protein % serum, "most of which

was/

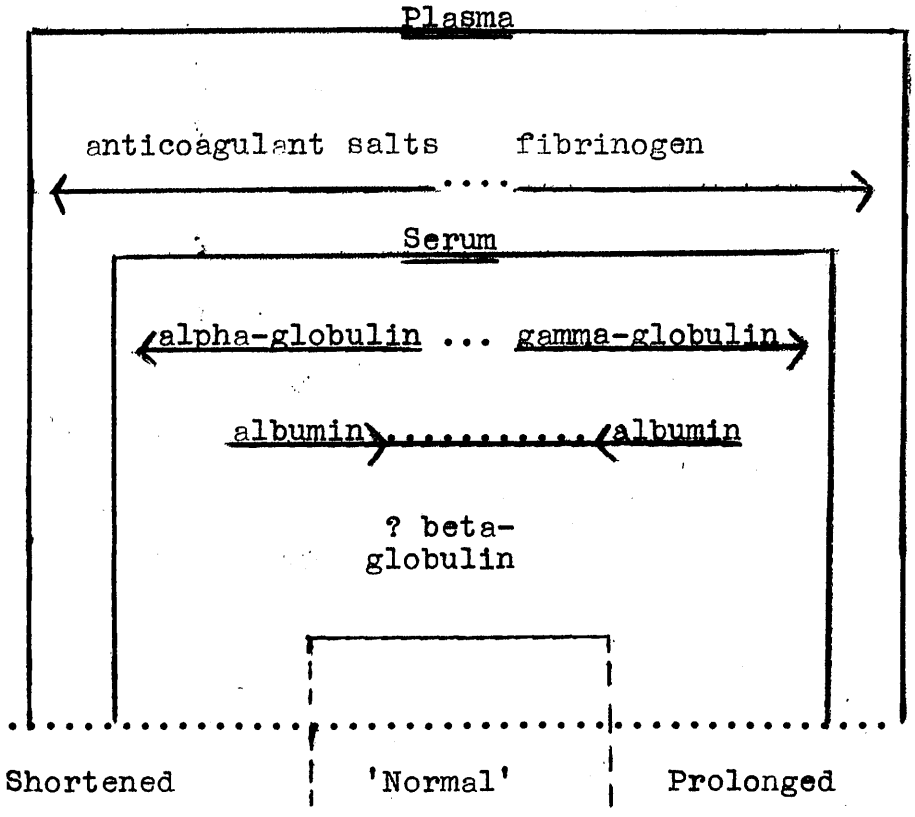
SERUM PROTEIN FRACTIONS IN HEPATO-CELLULAR INSUFFICIENCY.

Hepato-cellular insufficiency, so far as present methods of fractionisation are concerned, gives the same protein pattern as chronic infections and the reticulo-endothelioses except that albumin and total proteins tend to be lower in hepatic insufficiency. Apart from qualitative changes in the protein fractions concerned therefore, one would anticipate that those 'non-specific liver function tests' which depend on the serum proteins would be positive in many of the chronic infections and other conditions giving the same protein pattern. Many references are available to establish that this is in fact, true; a few examples are given here. Maizels (1946) found the Takata-ara tes positive in sarcoidosis (100%), nephritis (70%), most macrocytic anaemias but not in microcytic anaemias. This work is quoted here particularly because of the observed frequency of positive plasma formol reactions in macrocytic anaemia in the present series. Carter and Maclagan (1946) found frequent positive cephalin-cholesterol and thymol turbidity tests in subacute bacterial endocarditis, rheumatoid arthritis. Kissane et al (1947) applied the cephalis cholesterol test to the sera from 136 cases of rheumatic endocarditis and obtained positive reactions in 72% of the sera. Other non-specific tests giving positive reactions in hepatic insufficiency and in other conditions associated with serum protein patterns A and B are the colloidal gold test, cadmium and Scharlach red tests. The serum Weltmann reaction also belongs to this group of non-specific tests; Levison and Macfate (1946) refer to its use in jaundiced patients as/

as a means of distinguishing between obstructive jaundice and jaundice associated with hepato-cellular insufficiency. It seems clear therefore, that those non-specific tests are closely related to the serum formol gel test and hence to, at least, a proportion of positive plasma formol gel tests.

Plasma and the Clot-Forming Proteins:- For the purpose of this discussion plasma will be regarded as serum plus clot-forming proteins and an anticoagulant salt mixture. Plasma thus contains more protein and more salts per unit volume than does serum even though some of the additional ammonium salts are removed with the red cells.

Electrophoretic analysis as well as chemical analysis have clearly indicated that there is an increase in the fibrinogen content of the blood in the early stages of an acute infection and in the presence of tissue breakdown. Schedlowsky and Scudder (1942) related acceleration of the ESR to increased alpha-globulin in acute infections but the subsequent work of Morrison (1946) establishes a close relationship between the clot-forming proteins and the ESR and it seems likely that the association between acceleration of the ESR and increased alpha-globulin is coincidental rather than causative. The accuracy of the observation made by Schedlowsky and Scudder is born out by subsequent works, referred to on pages 117 and 118, which report an increase in alpha-globulin in acute pyogenic infections and in tissue destruction. Thus in early acute pyogenic infections increased fibrinogen occurs along with increased alpha-globulin; in chronic infections, etc. fibrinogen may be increased as well as/



Coagulation Zones (Weltmann Reaction)

Fig. 12 :- Illustrating the influence of the various protein fractions and of the anticoagulant on the Weltmann reaction (serum and plasma) as suggested herein.

as gamma-globulin. Hence serum protein Patterns A and B both associate with an excess of clot-forming protein. Pattern A will nearly always be associated with such an excess of clot-forming protein, but when Pattern B is due to or associated with hepatic insufficiency, the amount of clot-forming protein may be reduced or normal.

In the control tests it was seen that the anticoagulant salts produced shortening of the coagulation zone (Weltmann-plasma) when added to plasma containing the usual amount of anti-coagulant. In normal plasma, however, the coagulation zone was slightly longer than in the normal serum. Hence it is argued that in the Weltmann reaction at least, there is a balance between the tendency of the clot-forming proteins to prolong the coagulation zone and the shortening effect of the anticoagulant. Figure opposite, illustrates the influence of the various protein fractions on the Weltmann reactions; it is developed from the results observed in this work and from several other sources referred to elsewhere in this work.

The ESR and the Clot-Forming Proteins:- Morrison (1946), relates the ESR to the amount of protein precipitated from plasma by 16% sat. ammon. sulph.. Normal fibrinogen is precipitated by 20% sat.; there is "almost no" precipitation of protein in 16% sat., according to Morrison, unless the ESR is abnormally accelerated. In 90 bloods Morrison found the ESR to be directly proportional to the amount of protein precipitated by 16% sat. ammon. sulph. and, using suspensions of red/

red cells in saline, he found the protein so precipitated to be very much more active as an accelerator of red cell sedimentation than any of the protein fractions precipitated by higher concentrations of salts. He calls this 16% precipitable protein "contractinogen" and relates it to clot contraction. Otherwise the physical properties of this protein complex are similar to those of Lerner and Watson's cryoglobulins. Thus Morrison states that it is insoluble in water and precipitates spontaneously on cooling to 5°C, redissolving again at 37°C. At room temperature it precipitates slowly from plasma even in the presence of oxalates or citrates. No doubt some of the "contractinogen" seen to precipitate at low temperatures was, in fact, "cryoglobulin" but Morrison noted that the ESR was always rapid when there was an abundant precipitation from the plasma at 5°C. Unfortunately details of the 90 cases examined are not given; it does, however, seem possible for the ESR to be normal in some cases of hepatic insufficiency with cold-precipitating protein similar to the cryoglobulins of Lerner and Watson.

Red Cell Agglutination and Protein Precipitation in Hayem's Solution.

Support is given to Morrison's observations by the recent work of De Angelis and De Angelis (1947) who have related acceleration of the ESR in infective and neoplastic conditions with an abnormal protein precipitation in whole blood diluted with Hayem's solution.

Gradwohl (1938) mentions the clumping of red cells in blood from cases of lymphadenoma diluted with Hayem's solution.

Chu/

Chu and Forkner (1947) and De Angelis and Huntsinger (1944) observed that the red cells are clumped around precipitated protein particles. Wintrobe (1946) states that the phenomenon occurs in myelomatosis, kala-azar, pneumonia, and hepatic cirrhosis; he relates it to rouleaux formation, cold agglutination and pseudo-agglutination. The clumping occurs at temperatures below 37°C and the red cells become dispersed again if the temperature is raised to 37°C. This reversible effect of cold suggests that either cold-precipitating globulins or contractinogen or both together may be responsible for the phenomenon. No such clumping occurs in Gower's solution and it is generally accepted that small amounts of mercuric chloride in Hayem's solution are the precipitating agents.

Morrison's work is important to this thesis in that it allows positive plasma and serum formol reactions to be related to one common feature in abnormal plasma or serum, i.e. protein instability. It is here postulated that an excess of unstable serum protein may give positive serum and plasma formol reactions. Unstable clot-forming protein may, of itself, cause positive plasma formol reactions but, in some cases at least, the combined instability of the serum and the clot-forming proteins may suffice to give positive plasma formol reactions even though the serum reactions are negative.

The terms 'stable' and 'unstable' as applied to protein solutions in this work must, therefore, be defined.

'Stability' and 'Instability' of the Serum and Plasma Proteins.

In this work the serum and plasma proteins are said to be 'unstable' if the amount of protein precipitated from them by any agent is greater than the amount precipitated from normal serum or plasma by the same agent under similar conditions. Conversely, if the amount of protein so precipitated is equal to or less than the corresponding amount precipitated from normal serum or plasma, the serum or plasma proteins concerned are said to be 'stable'. The precipitating agent may be a salt solution, e.g. 16, 33 or 50% sat. ammon. sulph. or any other concentration of sat. ammon. sulph. or of any other salt. Alternatively the precipitating agent may be a change in temperature or in pH.

Two main properties of any protein influence its stability in solution .....

1) Its solubulity in water and in dilute saline.
2) The isoelectric point of the protein, i.e. the pH level at which the number of positively charged ions in the protein molecule equals the number of negatively charged ions. At the isoelectric point the solubility of any protein in water or weak salt solutions is minimal.

In any given solution the amount of protein precipitated by salts depends on .....

a) the concentration of protein in the protein/salt mixture. In general the amount of protein precipitated by/

by any concentration of salts increases with the protein concentration of the mixture,

b) the salt concentration of the protein/salt mixture. In high salt concentrations the amount of precipitated protein increases as the salt concentration increases. Globulins, however, are essentially insoluble in pure water and in salt solutions of low ionic concentration the solubility of a globulin increases as the salt concentration increases.

c) the pH of the mixture ... Precipitation is maximal in the region of the isoelectric point of the protein. Solubility increases as the pH moves in either direction away from the isoelectric point.

d) temperature of the mixture ... For most serum and plasma proteins the amount of protein precipitated by salts increases as the temperature is lowered from 37°C.

Thus for any given protein its stability in solutions depends on the concentration of protein and of salts in the solution and on the pH and temperature of the solution. Increases in either the protein concentration, e.g. by withdrawal of water, or in the salt concentration will diminish the stability of the protein and may cause some of the protein to precipitate. Alterations in pH and in temperature may increase or decrease the protein stability.

In serum or plasma therefore, increased protein concentration or alterations in temperature or pH, may increase the/

the amount of protein precipitated by a given salt concentration. Lower concentrations of salts may then suffice to precipitate any given amount of protein from the serum or plasma. Thus an alternative measure of serum or protein instability is obtained by comparing the concentration of salts required to precipitate a given amount of protein from the serum etc. with the concentration required to precipitate the same amount of protein from normal serum or plasma.

Hence, in serum, protein instability may manifest itself in any or all of the following ways....

i. By an excessive precipitation of protein in 33% and 50% sat. ammon. sulph., or by a precipitation which is greater than that given by normal serum in any given concentration of any salt. An excessive 50% sat. ammon. sulph. precipitate and an excessive 33% sat. precipitate are seen in Pattern B.

2. By an excess of water-insoluble protein precipitated by dilution with water. This may be really an extension of i. above in that the salt solution concerned may be given any name but the concentration of the salt is 0.0g./100 ml. (approximately).

3. By/

3. By spontaneous protein precipitation on cooling to temperatures between 5 and 37°C.
4. By prolongation of the Weltmann coagulation zone (serum). In such cases concentrations of calcium chloride which prevent heat coagulation of normal serum proteins are insufficient to prevent heat coagulation. Conversely shortening of the coagulation zone may indicate increased stability in that the concentration of salt required to prevent heat coagulation is lower than in normal serum.
5. According to the hypothesis advanced in this work ..... By positive serum formol reactions and positive reactions in a number of other tests grouped here under the title of 'protein stability tests.' This group includes non-specific liver function tests such as the Takata-ara, colloidal gold test (see page 126).

Similarly in plasma, protein instability may manifest itself in any of the above ways, or by the additional reactions indicated below all of which have been shown to be related to acceleration of the ESR.

1. An excessive protein precipitation in 16% sat. ammon. sulph..
2. Protein precipitation and red cell aggregation in whole blood diluted with Hayem's solution. This phenomenon is grouped with plasma instability reactions here because it has been related to acceleration of the ESR by/

by de Angelis and De Angelis (1947) but in some cases serum protein may be responsible as well as clot-forming proteins.

3. Positive plasma formol reactions and positive plasma dilution tests as postulated in the present work.

Protein Instability and the Formol Reactions in The Present Series.

The plasma dilution test and the plasma Weltmann reaction in the present series may be regarded as side-room tests for plasma protein instability. It was found that plasma giving positive dilution tests usually gave positive plasma formol reactions but the formol reactions were sometimes positive when the dilution test was negative. This association between positive plasma formol reactions and positive dilution tests appears to correspond with the association noted by Taussig between positive serum dilution tests and positive serum formol reactions.

Prolongation of the Weltmann coagulation zone, serum or plasma, has been shown (page 134) to be a manifestation of protein instability. It was seen most commonly in bloods with high plasma specific gravity but even in bloods of normal or low specific gravity it was usually associated with positive PF reactions. Similarly the PF reactions were positive in five out of seven bloods with shortened plasma coagulation zones. Although/

Although shortening of the serum coagulation zone probably indicates unusual stability in the serum proteins it cannot be so interpreted in plasma. The conditions which give shortening are nearly all associated with acceleration of the ESR and therefore with instability of the plasma proteins. In control tests the difference between the plasma and serum coagulation zones in abnormal bloods was never more than one tube so that it is quite possible for the plasma coagulation zone to be shortened in the presence of an excess of unstable clot-forming protein. Abnormal coagulation zones were seen less frequently than either positive dilution tests or PFG reactions.

The close association observed between the acceleration of the corrected ESR and positive PF reactions may also be regarded as evidence of the relationship between positive PF reactions and protein instability. In this series the plasma formol reactions were more reliable as indicators of 'activity' than either the crude or the corrected ESR. This was particularly so in cases with abnormally low packed cell volumes and in cases with doubtful corrected ESR. Positive plasma formol reactions were more common than was acceleration of the corrected ESR but some of these cases can be attributed to increased gamma-globulin; the best examples of this sort are cases BSa and BSb where the corrected ESR was below the zero line while the plasma formol reactions were strongly positive. Both of these cases had been transfused shortly before the test and both suffered from gross hepatic insufficiency. Other cases giving normal ESR and positive PF reactions (see page 106) were acute allergic states, muscle wasting and Addisonian/

Addisonian anaemia under treatment. In the allergic cases unstable gamma-globulin antibody or antibody-antigen complexes with normal clot-forming protein may well account for the results. It may be significant that Lerner and Watson (page 125) noted a similarity between their cold-precipitating proteins and the muscle protein myosin; similarly they, as well as Waldenstrom (page 125) observed a resemblance between cold-precipitating protein and nucleoprotein. In treated pernicious anaemia the conversion of megaloblastic nuclei to normoblastic nuclei in hyperplastic marrow and the disintegration of nuclear remants in reticulocytes in the peripheral blood may well overload the blood stream with nucleo-protein or derivatives of nucleo-protein. Similarly when muscle is breaking down myosin or some modification of myosin may well find its way into the blood stream. Certainly endogenous muscle catabolism is associated with increased gamma-globulin in the blood in starvation (Zeldis & Alberg, 1945).

Other observations relating positive formol reactions with protein instability arise from the control tests and from the samples with Gp = 1.028 or more in this work. The fact that strongly positive formol reactions were induced in normal serum and plasma by the addition of a relatively large amount of dry salts suggests that the sensitising influence of the salts was responsible for the protein formol reaction. Greater concentrations of salts were required to produce this effect/

	Plasma						Serum			
	Gp	PFG (No.)	PFF)	Dil^n. test	Coag. zone.	ESR*	SFG No.	SFF)	Diln. test	Coag. zone.
RL2.	1.031	5	5	pos.	8.5	35(D-16)	3	3	pos.	8.0
REa2.	"	5	4	"	7.55	15(N)	3	2	"	7.0
RL3.	1.029	5	5	"	9.0	17(N)	3	2	"	8.5
BSa.	"	5	5	"	7.5	14(N)	2	1	"	7.5
RPa.	1.028	4	3	"	7.5	46(S-24)	2	2	"	7.5
RPb.	"	5	5	"	6.0	15(D-10)	2	1	neg.	6.5
RMb2.	"	5	5	"	7.0	40(D-12)	-	-	-	-
IM	"	5	5	"	6.0	12(N)	0	0	neg.	6.5

* ESR expressed in mm. clear plasma /100 mm. blood /hour(Wintrobe's method). The corrected ESR is given in bracquets...'N',,normal;'D'..doubtful;'S'..slight .. ..from the chart of Whitby and Britton(194);where the corrected ESR is not clearly normal the figure from Wintrobe's chart is also given in the bracquets.

Table 26:- Results in the eight samples with plasma specific gravity over 1.027.

effect in alkaline mixtures than in acid mixtures; this observation agrees with the known facts about the isoelectric points of the protein fractions; the solubility of the proteins should be minimal at pH levels between 5 and 7.

The results of the plasma and serum tests in bloods with Gp = 1.028 or more are shown in Table 24, facing this page. Bloods with Gp = 1.029 are probably within the range of total proteins which according to Kagan always gives hyperglobulinaemia. In all four such samples the PFG reactions were very strongly positive and the SFG reactions were invariably positive. The same was true for samples with Gp = 1.028 except that in one case the serum reactions were negative. These results agree with the published accounts of positive serum gels in bloods with hyperproteinaemia and hyperglobulinaemia. It is also interesting to observe that the Weltmann coagulation zones (serum and plasma) were usually prolonged in these cases and the dilution tests were usually positive. The ESR results showed more variation than the other tests but in none of the eight cases was it markedly accelerated after correction. The main interest in these cases lies in the specific gravity results discussed in Part One of this work (page 33). It was observed that in six out of the eight samples concerned that the weight of the packed red cells was distinctly greater than the estimated haemoglobin suggested. This additional weight in the packed red cells was not seen in any other bloods of the series. Unfortunately the discrepancy was only discovered after the experimental work had been completed and the final method for calculating/

calculating the haemoglobin had been worked out. It is probable however that the additional weight packed with the red cells is due to the presence of cold-precipitating protein similar to the cryoglobulins of Lerner and Watson (page 125). They found such proteins in hyperglobulinaemic bloods in a variety of conditions similar to those from which the donors of the eight bloods were suffering in this series; the positive serum formol reactions confirm that the bloods were actually hyperglobulinaemic. Although some cryoglobulins had, according to this suggestion, precipitated from the plasma before the formol was added the temperature was never below 15-16°C during the examination of the plasma samples concerned; maximal cyroglobulin precipitation occurred at 5°C according to Lerner and Watson; it would appear therefore that the plasma examined at room temperature behaved like a saturated solution of cryoglobulins in that any further reduction in temperature would probably have caused further protein to precipitate. In these cases the instability of the plasma and serum proteins is probably greater than in the other cases of the series and it is significant that the PF reactions were strongly positive.

The relationship between the various tests was uniformly as described above in the different pathological groups. No cases of gout were available but it was striking that, though the literature on the serum formol gel tests describes positive results in cases of hepatic cirrhosis, there are seven cases

in/

	Plasma					Serum.				
	Gp	PFG (No. +)	PFF	Dil^{n} test	Coag. zone	SFG (No. +)	SFF	Dil^{n} test	Coag zone	ESR*
BSbi	1.019	1	2	pos.	7.5	neg	neg	neg.	8.0	N(N)
NX	1.020	neg	neg	neg.	7.0	"	"	pos.	6.5	N(N)
NKa	1.022	neg	2	pos.	6.5	"	2	pos.	6.0	19(S) (female)
NH	1.024	neg	neg	neg.	7.0	"	neg	neg.	6.0	11(N) (male)
IP	"	neg	3	pos.	7.0	"	neg	pos.	6.5	10(D) (female)
X H	"	1	2	pos.	7.0	-	-	-	-	N(N)
IH	1.025	neg	neg	neg.	7.0	-	-	-	-	N(N)

*ESR...mm. clear plasma/100 mm. blood/hour(Wintrobe's method).:'N'..normal:'D'..doubtful:'S'..slight. Corrected results are shown in bracquets(chart of Whitby and Britton).

Table 27 :- Results of the serum and plasma tests in the hepatic insufficiency cases of the series.

in the present series where in spite of evidence of gross hepato-cellular damage, e.g., at post-mortem or on operation, the plasma formol gel test was negative. Serum formol get tests on five out of the seven bloods were negative. In one case the SFF and PFF reactions were positive and in four of the cases the PFF reactions were positive. (See Table 27, facing this page.) Coacervation occurred in place of gelation in the four PFG negative, PFF positive samples. The negative PF and SF reactions are at first surprising since five of the patients concerned suffered from either advanced carcinoma or a coincident acute infection, either of which alone usually gave positive PF reactions.

In these seven hepatic insufficiency cases the serum proteins have been seen to be diminished. The serum protein pattern should conform to Pattern B, i.e., hypoproteinaemia, hypoalbuminaemia, hyperglobulinaemia and increased gamma-globulin. Other conditions with this protein pattern give positive serum formol reactions. The absence of positive plasma reactions in hepatic disorders giving negative serum reactions is understandable if the liver is, as is generally believed, the sole organ responsible for the production of fibrinogen. It is not surprising therefore that the ESR was generally normal in these cases. The unexpected finding in the seven cases concerned was therefore the negative serum formol gel tests in the presence of gross hepatic insufficiency and the normal Weltmann coagulation zones in four of the sera and in six of the plasma samples (see Table 27, facing this page. The possible/

possible nature of a mechanism capable of stabilising gamma-globulin is discussed in relation to the formol reactions on pages 149 and 150. For the moment it suffices to point out that any increase in tissue destruction usually shortens the coagulation zone whereas hepatic insufficiency usually prolongs the coagulation zone; tissue destruction and hepatic insufficiency together give a pattern similar to that of fibrocaseous tuberculosis where the coagulation zone is said to be normal. In the four cases supplying the sera concerned there was ample evidence of tissue destruction either from an acute infection or from carcinomatosis. Here then is evidence of an inhibitory action which may account for the negative SF and PF reactions in these cases.

In three out of five sera and in four out of seven plasma samples from these cases the dilution tests were positive. In the face of the other negative reactions mentioned above this finding appears to confirm the suggestion of a stabilising mechanism but it also suggests that the stabilising agent is some substance in the serum which depends on its absolute concentration for its stabilising effect.

Another possible accessory stabilising factor in the plasma, but not in the serum, may be the presence of the anti-coagulant salts. These were seen to shorten the coagulation zone (page 128) and in the seven cases concerned the clot-forming proteins may exert less than their usual prolonging action on the coagulation zone.

Summarising the possible reasons for negative plasma for-

reactions in these cases....

1) Diminished production of clot-forming proteins and
2) negative serum formol reactions which, it is suggested, are due to the action of some stabilising agent on the unstable gamma-globulin.

This main stabilising substance appears to be associated with increased alpha-globulin, acute infections and tissue destruction. A further stabilising factor in the plasma tests may be the presence of unopposed anti-coagulant salts.

In this connection the case mentioned by Lerner and Watson (page), with increased gamma-globulins but no cryoglobulins suffered from hepatic cirrhosis.

It is therefore suggested that the formol reactions are positive when the serum or plasma concerned contains an abnormal amount of unstable protein. In serum such protein is usually a 33% sat. ammon. sulph. precipitable gamma-globulin; in plasma it is probably also normal fibrinogen and unstable fibrinogen such as contractinogen. In normal plasma the amount of unstable protein in the presence of the anticoagulant is unsufficient to give positive reactions. The three phases of rheumatism previously postulated by Gibson and Pitt (see page 80) are thus explained....

1) the 'early fibrinogen' phase...the accelerated ESR is probably due to an excess of contractinogen but at this stage the serum proteins show increased alpha and beta-globulins, i.e.,/

i.e. they are unusually stable. The total instability of the serum and plasma proteins is insufficient to give positive PF reactions.

2) and 3) throughout the late 'fibrinogen' stage and the 'globulin' stage tissue destruction diminishes and antibody production increases, i.e. the alpha increase disappears and there is in its place an increase in the gamma-globulin with a consequent increase in the amount of unstable serum proteins.

In phase two the total plasma and serum protein instability suffices to give positive plasma formol reactions although the total serum instability is not enough to give positive serum formol reactions. In phase three the amount of unstable serum protein is sufficient to give positive serum formol reactions, and also positive plasma formol reactions.

NATURE OF THE FORMOL REACTIONS.

In this work positive formol reactions have been shown to occur when the serum or plasma proteins are unstable. It was also observed that the plasma reactions could have maximal intensity when the serum reactions were negative; hence it is argued that the clot-forming protein can, in some bloods, impart gel-forming and formol-flocculating properties to part of the serum proteins. Bing (1937) reported that the protein precipitated from serum by 33% sat. ammon. sulph. could similarly induce gel formation in normal serum although, by itself, it gave no gel with formol. These observations suggest that formol reacts with the unstable proteins and by so doing, modifies the stable proteins of the serum.

The various protein fractions in normal plasma are here regarded as having the following stability order, i.e. from least stable to most stable ..... fibrinogen, gamma-globulin (or part of the gamma-globulin), other globulins, part of the alpha-globulin and albumin (electrophoretic). This order is based on the concentration of saturated salts required to precipitate the various fractions from plasma or serum, and on the differences between the pH of maximal precipitation of the fractions and the pH of normal plasma and serum.
Morrison's contractinogen is more easily precipitated by salts than normal fibrinogen; it is the first protein to precipitate from the plasma on the addition of serial quantities of sat. ammon. sulph. solution; in bloods with accelerated ESR it precipitates/

precipitates at 16% sat., according to Morrison; normal fibrinogen is precipitated by 20% sat. ammon. sulph.; some gamma-globulin is precipitated by 33% saturation and the remaining globulins except for some alpha-globulin are precipitated by 50% saturation.

Cohn et al (1944) give the following pH levels for maximum precipitation of their protein fractions (see page 117.)

..... fraction I...7.0 (mainly fibrinogen):

fraction II...6.8 (mainly gamma-globulin):

fraction III..5 to
IV..6.0 (other globulins):

fraction V,
VI....4.8 (mainly albumin).

Thus at pH 7.3, e.g., in normal plasma, fibrinogen is probably closest to its isoelectric point with gamma-globulin almost as close, thereafter the more stable proteins follow in the order already given down to the most stable protein, i.e., albumin.

Thus the least stable serum protein is gamma-globulin or part of the gamma-globulin, and in plasma the least stable protein is the clot-forming, ESR accelerating, 'contractinogen'. Obviously, therefore, plasma is less stable than serum since in addition to the unstable serum protein it contains an appreciable amount of still less stable clot-forming protein. The higher total protein content of the plasma may further add to the instability of the existing serum proteins.

The characteristic feature of the state of instability here postulated is an abnormal liability on the part of the protein to precipitate. Before a hydrophile colloid precipitates, it first loses its water of/

of hydration. In this state it is said to be sensitised and is hydrophobe rather than hydrophile. The hydrophile proteins are known to protect the hydrophobe suspensoids from precipitation by the salts in solution. If, however, there is an excess of sensitised protein and a diminution in the amount of protective protein, the sensitised protein will precipitate.

Formol readily combines with the basic amino-radicles of amino-acids and proteins. It is, therefore, suggested that the basic radicles of sensitised, unprotected serum and plasma proteins are unusually accessible to the formol. In normal serum or plasma, according to this hypothesis, there is less tendency for the proteins to precipitate; there are, therefore, fewer sensitised protein molecules. Furthermore, normal serum and plasma contain more protective proteins. Hence, it is possible to account for the negative formol reactions in normal serum and plasma.

One effect of such a reaction between formol and amino-groups is a diminution in the basicity of the plasma or serum formol mixtures. In plasma this may suffice to sensitise any gamma-globulin which may not already be sensitised. Further amino-groups may thus become accessible to the formol. The resulting reaction between formol and these amino groups may serve to sensitise still more serum globulin. In this way, provided the amounts of unstable protein are sufficiently great, formol may react serially with all the serum or plasma proteins.

It is more probable, however, that the acid compounds formed between formol and the unstable proteins unite directly with proteins still on the alkaline side of their isoelectric

points. Such reactions between oppositely charged colloidal molecules are recognised as the reactions underlying coacervation which was seen instead of gelation in several samples in this work. In the samples concerned there was usually hypoproteinaemia and hypoalbuminaemia and the formol reactions were feebly positive. It is possible, therefore, that gelation depends on the concentration of stable proteins and on the intensity of the reactions between formol and the unstable proteins. In this work it was observed that water of condensation usually formed in tubes containing positive formol gel reactions. The stable proteins, e.g., albumin, may therefore be affected by this loss of fluid and by the heat responsible for the evaporation, i.e., by the heat generated by the reaction between formol and unstable protein. Scatchard et al (1944) tested the stability of fraction V albumin in the presence of various salts by heating 25% solutions to 50°C and observing the time taken to form a gel. I have myself observed complete gelation in a solution of fraction V to which formol was added. Subsequent attempts to repeat the experiment with the same solutions did not succeed, however. It may be significant that the albumin solution was prepared just before the original test in which gelation occurred.

Generally the results in this series agree with the above suggestions, i.e., that the formol flocculation reaction depends on the amount of unstable protein present, while the formol gel reaction depends on the concentration of stable protein such as albumin and on the intensity of the reaction between formol and the unstable proteins. It is interesting, but scarcely significant, that the two samples giving strong/

Formol Albumin Reactions.

Method:-'Albumin solution'...10% cystallised bovine albumin (Armour Lab.-fraction V)in sterile saline+1% universal indicator(B.D.H.).

Ten ml. albumin solution was prepared and divided into two portions ; sample A was tested immediately; sample B was tested after standing for 24 hours at room temperature. Both samples were tested as follows.....

The 5 ml. albumin solution was poured into bottle 1: 4 ml. were withdrawn and passed into bottle 2; 3 ml. were then taken from bottle 2 and put into bottle 3 etc..... till finally 1 ml.from bottle 4 was transferred to bottle 5. The bottles were sterile screw cap containers;bottle 1 did not contain any oxalate but the other bottles contained 5 mg. ammon. pot. oxalate mixture as dry crystals. The oxalate was allowed to dissolve in each bottle before the solution was passed onto the next bottle. Calcium formol 0.1 ml. was added to each bottle.

Observations:- i)Sample A became distinctly cloudy in bottle 5 before any formol had been added. No such cloud developed in sample B.

2) pH,judged by colour of universal indicator; pH of original plasma formol mixtures....6.5-7.0.

Sample A	Bottle No1	2	3	4	5
Time to form a solid gel(hours).	24	24	3	0.1	0.1
24 hr. opacity(no. +)	1-	1-	1	2	2
pH of gel	6.5	6.5	6	5.5	5.5
Sample B					
Time to form gel(hours)	96	96	48	48	24
24 hr.opacity	0	0	1-	1-	1-
pH of gel	6.5	6.5	6.5	6.5	6.0

Table 28:- Methods and results in experiments discussed opposite this page.

positive PFG reactions and relatively weak PFF reactions, (page 114) came from patients suffering from conditions characterised by an increase in the alpha-globulin content of the blood.

Since the above was written the experiments described on the opposite page were carried out in the Pathology Department, Royal Hospital for Sick Children. The results may be summarised thus..1) Freshly prepared solutions of crystalised bovine plasma albumin readily formed gels with formol but 24-hour old solutions gave gels less readily. 2) Since the amount of oxalate added to the last bottle was sufficient to precipitate some of the protein from the fresh solution, the stability of the albumin solutions probably diminishes from bottle 1 to bottle 5. In both sets of tests gelation was, therefore, most rapid when the protein was most unstable. 3) In the two sets of tests the final pH of the gels was different although the proportions of oxalates, formol, and albumin were similar. Thus the pH of the gel may be determined by the amount of unstable protein rather than by any reaction between the ammonium of the salts and the formol. Rapidly forming gels gave a lower pH than slowly forming gels. 4)ammonium oxalate did not appear to exert any special stabilising action on the albumin; it is likely, therefore, that the stabilising action mentioned on pages 142 and 128 results from a reaction between the salt and the unstable proteins themselves, in serum and plasma. The formol-albumin gels were much less opaque than the usual plasma or serum formol gel forming within the same times.

These observations support the above hypothesis in that they confirm the gel-forming potentialities of albumin and at the same time show that other factors are involved since mature albumin took 4 days to form a relatively clear gel with formol.

Stabilising Influences in Hepatic Insufficiency and in Gout.

Although others have reported positive serum formol reactions in hepatic cirrhosis, seven samples in this series from cases of gross hepatic insufficiency were all PFG negative; in four of the seven, PFF was positive. The seven concerned were advanced cases, six of the patients died within six weeks of the test and in several instances the picture was complicated by metastatic carcinoma or by a complicating acute infection. The stabilising effect of alpha-globulin, and possibly of albumin, beta-globulin and ammon.-pot. oxalate on gamma-globulin has already been discussed. Negative serum reactions in several cases suggest that the main inhibiting factors are probably alpha-globulin and possibly albumin and beta-globulin rather than the anticoagulant. The albumin concentration is low in hepatic insufficiency so that apart from qualitative changes it is unlikely that albumin exerts any more than the normal stabilising or protective influence. It is, therefore, probable that the stability of the gamma-globulin results from a stabilising action exerted by alpha-globulin or related non-protein substances. Alpha-globulin is increased in acute infections and in tissue destruction, both of which were known to be present in several of the seven cases concerned.

The following evidence is also advanced in support of a possible/

possible inhibitory action by certain non-protein substances. Scatchard et al (1946) report that glycine was added to the gamma-globulin preparations issued to the American Armed Forces during World War II. Glycine, they state, has a specific stabilising action on gamma-globulin; even low concentrations of glycine have this property but the stability of the gamma-globulin increases as the concentration of glycine rises. In several of the cases here it is probable that hepatic insufficiency was sufficiently advanced to permit an increase in the free amino-acid content of the blood; other amino-acids besides glycine have a similar stabilising action. Thus it is here postulated that in the late stages of hepato-cellular insufficiency amino-acids and other non-protein nitrogenous substances exert a stabilising action on the gamma-globulins of the serum. This stabilising action may be additional to that suggested for alpha-globulin, or it may be that tissue destruction is associated with an increase in both alpha-globulin and amino-acids in the blood; the apparent stabilising action of alpha-globulin may therefore be due to a coincident increase in the blood amino-acids

It is generally accepted now that the liver is mainly responsible for the production of clot-forming protein. Thus stabilised gamma-globulin, reduced total plasma proteins, and no abnormal increase in the clot-forming proteins, may together be responsible for the negative formol gel reactions in this work.

Positive dilution tests, serum and plasma, were seen in several of the cases concerned. This agrees with the postulated inhibitory mechanism in that the concentration of inhibitory substances/

substances may be so reduced by dilution that gamma-globulin instability reappears.

Gout and the Formol Reactions.

It has been observed (page 80) that the ESR is frequently accelerated in acute gout although the PFG reaction is negative. No cases of gout were available for this series, nor can I find any clear descriptions of a definite protein pattern in the literature on gout. If gouty serum contains increased alpha-globulins, as in pattern A, the difference between the ESR and PFG reactions may be attributed to increased stability of the serum proteins as in early acute infections (page 121). On the other hand it is not improbable that there is increased gamma-globulin in cases of gout. In this event one must look for a possible stabilising factor which may prevent the gamma-globulin from reacting with the formol. Amino-acids, or their salts, may be responsible as suggested in hepatic insufficiency, but it is just as likely that the excess of uric acid, or urates, in the blood, is the stabilising agent.

It may be significant that both of the postulated stabilising substances are weak acids, or the salts of weak acids, and in this respect at least, resemble ammonium oxalate, which is know to stabilise the clot-forming proteins. Bing (page 78), found ammon. oxalate to have an inhibitory action on serum formol gels and in this work it appeared to shorten the Weltmann coagulation zone (page 128). According to the hypothesis advanced herein both of these effects indicate that ammon. oxalate in small concentrations has a stabilising effect on the gamma globulin of the blood as well as on the clot-forming proteins.

If it be/

/be assumed that the clot-forming proteins are stabilised to their maximum by the oxalates, it is understandable that the influence of the additional substances suggested above should be concentrated on the gamma-globulin.

Some of the inconsistency observed in cases of Addisonian anaemia under treatment may well be attributable to an increase in the uric acid content of the blood.

Protein Stability Tests and the Formol Reactions.

The relationship between the formol reactions and the other protein stability tests listed on pages 133 to 135 is discussed in this section.

I have grouped the stability tests as direct and indirect. The direct tests measure the ability of the hydrophile serum proteins to prevent the precipitation of the less stable, possibly hydrophobe, proteins. In the indirect group an extra hydrophobe suspenoid phase is added 'in vitro' and the serum proteins are called upon to protect this additional phase from precipitation. The direct group includes the conventional methods of salt fractionisation of serum or plasma protein, tests for cold precipitating proteins, the dilution tests, the Weltmann and Takata-ara reactions. The indirect group is made up of a number of tests such as the colloidal gold test, the cephalin-cholesterol, and other so-called 'liver function' tests. The Takata-ara reaction is shown as a direct test since it depends on the precipitating action of a mixture of mercuric chloride and sodium carbonate on the serum proteins without the introduction of a further hydrophobe phase.

The respective sensitivities of the various tests will depend on the nature and concentration of precipitant and on such factors as dilution of the serum and temperature and pH of the reaction. Generally, however, the indirect tests should be more sensitive than the direct tests since minor degrees of instability may be characterised by a protective action which, although adequate for the unstable proteins, is inadequate for /

the unstable proteins and the additional hydrophobe phase.

According to the hypothesis advanced herein the formol reactions are essentially direct tests, applied to undiluted serum or plasma. Most of the serum stability tests so dilute the serum that inhibiting substances may well be diluted beyond the minimum concentration necessary for active stabilisation of the gamma-globulin.

The serum and plasma formol reactions have been shown to be positive in hyperglobulinaemia, and the plasma reactions in bloods with acceleration of the ESR. Similarly the Takata-ara reaction is positive in hyperglobulinaemia, and the corresponding plasma test, agglutination of the red cells in Hayem's solution, has been related to acceleration of the ESR. By analogy, therefore, the indirect stability tests should be related to the ESR and to hyperglobulinaemia if they are applied to plasma instead of the conventional serum. I know of no work which establishes this relationship but the red cells in whole blood may well correspond to the hydrophobe suspensoid phase of the indirect tests. At the pH of freshly shed blood the red cells bear negative electrical charges; similarly the suspensoid particles in the indirect tests are negatively charged in the pH range of the indirect tests (7.3-9.0 approx.). Unfortunately normal fibrinogen and gamma-globulin are also negatively charged in this pH range; otherwise erythrocyte sedimentation and the liver function tests could be explained by mutual precipitation of unprotected protein with the respective hydrophobe phase (red

cells in one case and artificially suspended colloidal particles in the other). Considering the weak acidic properties attributed to formol (page 42), it too should be negatively charged when formol is ionised as a weak acid or as a salt. Formol, however, does combine with protein on the alkaline side of its iso-electric point. Lloyd and Shore (1938) on page 314 of their textbook, quote several workers who found the base-binding power of several proteins to be increased by formol "in the lower alkaline range when titrating with alkali". Furthermore, the conception of mutual precipitation appears to fit the known facts about red cell sedimentation and the liver function tests so well that one is tempted to credit the unstable proteins with "zwitterion-like" properties at pH levels not far removed from their isoelectric points.

The Plasma Formol Reactions in Clinical Medicine.

The results in this work suggest that the plasma formol reactions may usefully be employed in general medical conditions as well as in rheumatic conditions as an aid to the interpretation of the ESR in anaemic bloods. The methods at present available for correcting the ESR frequently give 'doubtful' corrected results and in this series cases were encountered in which the corrected ESR was normal although clinically there was evidence of active disease, e.g. Case ITb - pulmonary tuberculosis - active on X-ray; tubercle bacilli in the sputum - fever and tachycardia - corrected ESR normal - PFG and PFF positive.

Wintrobe (1946) describing his technique for estimating the ESR, recommends that five ml. blood should be added to prepared tubes of ammon. pot. oxalate. One ml. of oxalated blood suffices to fill the haematocrit tube for the estimation of the ESR and packed cell volume; with 1.5 ml. of blood a comprehensive examination of the blood can be carried out as described in Part One of this work (page 43). Thus more than sufficient blood for the formol reactions is usually wasted by Wintrobe's technique; furthermore this blood is oxalated as in the present series. Little time is required to mix one ml. plasma with 0.1 ml. formol in a stoppered bottle. One reading of the PFF result after 24 hours should suffice to detect 99% of the positive results. It will of course be necessary to bear in mind the possibility of positive formol reactions due to hyperglobulinaemia, e.g. in allergic states, blood transfusions and in muscle wasting. The occurrence of coacervation between

24 and 48 hours as indicated by a diminution in viscosity and an increase in flocculation was seen only in the presence of hepatic insufficiency in the present series, particularly in cases complicated by some acute infection or associated with tissue breakdown as in advanced carcinomatosis.

It may well be that the plasma formol reactions have many other uses but the general nature of the present series does not permit any evaluation of the reaction in particular diseases. For example, the test may have some value in the diagnosis of allergic states provided the ESR is clearly normal and there is no clinical evidence of any active disease: negative PFG reactions in the later months of pregnancy may indicate hepatic insufficiency: in clinical nephrosis weak PFF reactions may be suggestive of primary lipoid nephrosis rather than of subacute glomerulonephritis where the original infective-allergic nature of the onset is likely to cause an increase in the gamma-globulin of the blood a corresponding increase in the PFF reactions: positive plasma formol reactions in pernicious anaemia after blood transfusion may have some clinical significance: it may even be that the plasma formol reactions offer a simple alternative to the reticulocyte count as an indication of the efficiency of treatment in Addisonian anaemia.

SUMMARY AND CONCLUSIONS.

The plasma-formol gel (PFG) test is studied in a series of general medical cases. Previous work on this test has been largely confined to rheumatic conditions. Since the PFG test has been reported positive when the serum-formol gel (SFG) test is positive, the literature on both tests has been summarised.

In this work the plasma-formol (PF) reactions were differentiated into the plasma-formol gel reaction and a plasma-formol flocculation (PFF) reaction. The intensity of the PFG reaction was recorded as the time taken to form a solid gel. The PFF reactions were recorded after 24 hours, whether a gel was present or not; the intensity of the PFF reactions was expressed in terms of a series of standard opacities with which the opacity of the plasma formol mixtures was compared.

By these separate criteria the formol reactions in plasma prepared with ammonium potassium oxalate mixture, as for the samples examined in this work, were very similar in a series of control normal and abnormal bloods to the formol reactions in plasma from the same bloods containing pot. oxalate alone as used in previous rheumatic investigations. By adopting ammon. pot. oxalate as the anticoagulant the PF reactions were directly comparable with the erythrocyte sedimentation rate (ESR), packed cell volume and the corrected ESR by Wintrobe's methods.

Other side-room tests, introduced to ascertain the physical state of the plasma proteins, were the estimation of plasma specific gravity which gave an approximate estimate of the/

the weight of protein present per 100 ml. plasma, the dilution test and the Weltmann reaction.

Here the dilution and Weltmann tests were applied to plasma instead of serum as in previous work on these tests. The dilution test (plasma) gave a rough estimate of the amount of water-insoluble protein present in the plasma and the Weltmann reaction (plasma) indicated the concentration of calcium chloride required to bring about heat coagulation of the plasma proteins.

Both serum and plasma were examined in a further series of control tests. As in rheumatic conditions the PF reactions were invariably positive when the corresponding SF reactions were positive, the PF reactions were more commonly positive than the SF reactions, and were always stronger than the SF reactions. Similarly the plasma dilution test was always positive when the serum dilution test was positive; plasma dilution tests were more frequently positive than serum dilution tests and the plasma dilution tests were always more strongly positive than the serum tests. Positive serum dilution tests were closely related to positive SF reactions and positive plasma dilution tests to positive PF reactions. The anticoagulant appeared to shorten the Weltmann coagulation zone; the clot-forming protein seemed to prolong the zone. The difference between the serum and plasma coagulation zones was from -0.5 to +1.0 tubes with an average lengthening of +0.35 tubes. This relationship was maintained when the serum zone was abnormally long, or abnormally short.

After the main series of 130 bloods had been examined

criteria were defined for 'positive' (i.e. abnormal) plasma-formol reactions. The PFG test was then positive if a solid gel had formed within 48 hours; the PFF reaction was positive if the opacity of the plasma-formol mixture after 24 hours exceeded a defined standard opacity.

Generally the PFF and PFG results were proportional to one another in each plasma sample but exceptions were noted. In hepatic insufficiency the PFF reactions were relatively stronger than the PFG reactions which were sometimes negative even when the PFF reactions were positive. Positive PFF reactions with negative PFG reactions were associated with coacervation instead of gelation. Rapid gel formation relative to the PFF reaction was less common; the possible significance of this reaction is discussed.

Positive PFF and PFG reactions were seen in all cases of carcinoma, all the reticulo-endothelioses and all the infections of this series with the following exceptions ... i) where these conditions were complicated by hepato-cellular insufficiency; ii) in the acute and subacute reticulo-endothelioses. Positive reactions occurred in smaller groups of patients in the 7 - 8 months of normal pregnancy, and in patients who were suffering from acute allergic conditions or had been transfused within three weeks. Positive reactions also occurred in progressive muscular atrophy and in other conditions associated with muscle breakdown, e.g. emaciation due to malnutrition, subacute combined degeneration of the cord and in coronary thrombosis; the results were variable in these conditions and in pernicious anaemia. In/

In untreated, uncomplicated iron-deficiency and pernicious anaemia cases the reactions were invariably negative; complications may have been responsible for some positive reactions in both groups; treatment with liver was associated with positive formol reactions in pernicious anaemia patients except in one refractory case. In post-haemorrhagic states, lead absorption anaemia and peptic ulceration, the reactions were also negative.

The incidence ofpositive PF reactions did not appear to be related to the age or sex of the patients, but there was a significant association between positive reactions and fever, loss of weight, and the chronicity of the condition present. Venous constriction accelerated and accentuated the reactions. Hepato-cellular insufficiency appeared to prevent positive reactions even in patients suffering from other conditions usually associated with positive reactions; in several such cases the PFG reactions were negative although the PFF reactions were still positive. The Wassermann reaction was invariably negative except in one case where the PF reactions were negative on two occasions.

In relation to other side-room tests, there was a very close association between the PF reactions and acceleration of the corrected ESR; the association between the PFF reactions and the corrected ESR was better than the association between the PFG reactions and the corrected ESR. In some cases the corrected ESR was normal although there was definite clinical evidence of an active disease process which is usually characterised by an accelerated ESR. In these cases the PF reactions/

reactions were positive. Similarly the PFF reactions were more closely related to abnormal packed cell volumes than were the PFG reactions; both reactions were most commonly positive when the volume of packed cells was between 25 and 35% of blood volume. The PF reactions were invariably positive when the plasma specific gravity exceeded 1.027 but strongly positive PF reactions were seen in plasma of gravity 1.018 = less than 4.0g. protein % plasma. In relation to the plasma Weltmann reaction the maximum incidence of positive PF reactions was seen in the bloods with prolonged coagulation zones but they were more common in bloods with short coagulation zones than in bloods with normal coagulation zones.

When Gp was 1.028 or more, the formol reactions were strongly positive, the dilution tests were positive and the coagulation zones for serum and plasma were prolonged. In these cases too the weight of the packed red cells was greater than usual, relative to the amount of haemoglobin present in the cells. <u>High plasma specific gravity, hyperglobulinaemia and spontaneous cold precipitating protein</u>, were shown to be related, and it is suggested that the additional weight and volume packing with the red cells in these cases/

cases is cold-precipitating protein.

From these observations and from the literature positive serum formol reactions were related to an excessive protein precipitation from serum in 33 and 50% sat. ammon. sulph. solution (hyperglobulinaemia and increased 'euglobulins'), to positive dilution tests, prolongation of the Weltmann coagulation zone, spontaneous precipitation of protein below 37°C and to positive reactions with the non-specific liver function tests. These reactions were shown to be manifestations of instability of the serum proteins and were related to increased gamma-globulin in the serum. This type of serum protein pattern has been reported in acute and chronic infections where antibody activity or fibrosis is occuring, in some of the chronic reticulo-endothelioses, in several protozoal infections, and in hepatic insufficiency. In these conditions the serum and plasma formol reactions were frequently positive in this series and in the literature.

Positive plasma formol reactions were also seen in bloods with acceleration of the corrected ESR, in a few cases where there was clinical evidence suggesting that the corrected ESR should have been accelerated, and in some conditions such as allergy, where gamma-globulin instability might reasonably be present. From recent literature it is shown that the ESR is accelerated when there is an abnormal instability of the clot-forming proteins. Hence it is suggested that the plasma formol reactions are positive when there is abnormal instability of the serum and/or clot-forming proteins. In some cases the serum instability alone seemed to suffice to give positive/

serum and plasma formol reactions; in others the serum reactions were negative but the combined serum and clot-forming protein instability sufficed to give positive plasma reactions. Even when there was no abnormal instability of the serum proteins, however, the instability of the clot-forming proteins sometimes gave positive plasma reactions.

The possible mechanism of the formol reactions is discussed. It is suggested that formol flocculation is due to a direct reaction between formol and the unstable proteins, while the gel forms from the stable proteins such as albumin and is dependant on the intensity of the formol/unstable protein reaction. Experimental evidence is produced to support this hypothesis.

Coacervation was seen in place of gelation in several cases of hepatic insufficiency with low plasma specific gravity i.e., hypoalbuminaemia. In these cases the flocculation reactions were not strongly positive.

In several cases with gross hepatic insufficiency and tissue destruction in this series the plasma and serum formol reactions were negative. Others have reported negative reactions in gout even when the ESR was accelerated. It is suggested that alpha-globulin, free amino-acids and urates, as well as ammon. oxalate, exert a stabilising action on gamma-globulin. Evidence is produced from the literature and from this work to support this hypothesis.

The formol reactions are then grouped along with the liver function tests, the erythrocyte sedimentation rate, and other tests, as 'protein stability tests'. The relationship between these tests is discussed and their mechanisms are shown to be very similar.

Finally, the value of the formol reactions in clinical work is discussed.

APPENDIX A

General Haematological Methods

1. Collection and oxalation of the blood samples.
2. Standardisation tests.
3. The routine examination.

1. Collection and oxalation of the blood samples.

Venupuncture was performed on patients who were rested and comfortably warm. The arm was supported on a pillow and the vein was entered at a point as near to the horizontal plane of the praecordium as possible. Constriction was used only in a few cases where it was otherwise impossible to penetrate a vein; in these cases the constricting band was released before the bulk of the sample was withdrawn.

Five ml. of blood so obtained was mixed with ten mg. of dry ammonium and potassium oxalate (method of Heller and Paul, 1934, described by Wintrobe, 1946). Reserve tubes containing less than ten mg. of the salts were always available for the occasional case where less than five ml. blood was obtained. The amount of oxalate added was always 200mg./100 ml. blood.

2. Standardisation tests.

(a) The estimation of haemoglobin:- In preliminary experiments the modified acid haematin method described below appeared, in the hands of the observer, to offer an end-point which was more localised than that given by other subjective colorimeters. The pipettes and colour standards, the technique and the observer's judgement were simultaneously standardised by estimating the haemoglobin content of 24 blood samples by this method and by the oxyhaemoglobin method described by Bell et al (1945). The photoelectric colorimeter, pipettes etc., used by Bell et al in 1945 were available for these standardisation tests. * Their apparatus had been calibrated against iron estimations by the method of Delory (1943) and Clegg and King (1942).

Further standardisation tests at the end of the series indicated that there had been no significant fading of the colour standards throughout the period of the investigation.

(b)/

* I am indebted to Professor G.H. Bell for the use of the photoelectric colorimeter in the Department of Physiology, Glasgow University.

(b) The centrifuge and the haematocrit tubes:- The haematocrit tubes were of the size specified by Wintrobe (1933), i.e. 110 x 2.5 mm., capacity 0.7 ml..

The centrifuge available in the side-room measured 9 cm. from the axis to the mid-point of the haematocrit tube; it was therefore necessary to compare its efficiency with that of an 18 cm. centrifuge as used by Phillips et al (1945a). Ten blood samples with packed red cell volume (H) ranging from 10 - 50% of blood volume were spun at 3000 r.p.m. till packing was complete in the 9 cm. centrifuge; they were then spun for 60 minutes at 3000 r.p.m. in an 18 cm. centrifuge. The values for the packed red cell volumes by the two centrifuges were compared and a chart was prepared by means of which the reading on the 9 cm. centrifuge could be converted to within ± 1.0 mm./100 mm. blood of H estimated on the 18 cm. centrifuge.

The results confirmed the observations of Phillips et al (1945a) that, given similar conditions of time and speed, a blood packing to 51% in a 9 cm. centrifuge will pack to 47% in an 18 cm. centrifuge.

All H results in this work have been corrected to the 18 cm. figure.

3. The routine examination.

To avoid sampling errors due to sedimentation of the cells, a strict mixing procedure was adopted before any portion of any sample was withdrawn for testing. Mixing was considered adequate when a pipette filled at the same speed with blood from the depths of the sample as with blood from the surface layers; later constancy of the whole blood specific gravity was taken to indicate adequate mixing.

Except where stated otherwise the methods below are as described by Wintrobe (1946) in his text-book "Clinical Hematology".

Within 10 minutes of withdrawing the blood .....

a) films were prepared, two on untreated slides and two on slides prepared with an alcoholic solution of cresyl blue;

b) a white cell counting pipette was filled with a 1/20 dilution of blood in diluting fluid. The pipette was sealed with plasticine and the count was done when convenient within 24 hours.

Within 24 hours .....

c) a haematocrit tube was filled with blood and was left in a perfectly vertical position for one hour at room temperature (15-17°C). The ESR was expressed in mm. clear plasma per 100 mm. blood;

d)/

d) the tube was then centrifuged till packing was complete in the 9 cm. centrifuge as described above. The following information was obtained from the centrifuged blood:-

i) the packed red cell volume and the total packed cell volume by the 9 cm. centrifuge; the red cell volume was then converted to H, 18 cm. centrifuge as above:

ii) the icteric index.

From H and the ESR the corrected ESR was obtained using the charts given by Wintrobe in the 1946 edition of his textbook and by Whitby and Britton (1946) in their textbook "Disorders of the Blood". Correction was made to H=47% for both sexes.

e) Haemoglobin was estimated by a modified acid haematin method. The colorimeter adopted had double identical glass standards; matching was carried out against a diffusing screen built into the colorimeter. I had observed that with this instrument the end-point varied with the distance between the eye of the observer and the colorimeter. Matching was first carried out thirty minutes after the blood and acid were mixed and on this occasion the colours were matched at one foot from the eye. After a further thirty minutes the unknown was a shade darker than the standards at one foot but matched the standards at arm's length. The final end-point thus matched the standards at arm's length 60 minutes after the blood and acid were mixed. According to Bell and MacNaught (1944) the colour change in the last 30 minutes amounts to about 3.0%, i.e. about 0.5g.Hb/100 ml. blood. It was therefore, hoped that by defining the end-point in terms of distance from the eye and time allowed for the acid and blood to react, the error usually associated with the acid haematin methods would be reduced.

f) Red and white cell counts were made.

g) The blood films were stained and examined. Records were made of the appearance of the peripheral erythron; differential white cell counts were made; where necessary, platelet counts, reticulocyte counts and Price-Jones red cell diameter measurements were made.

Chemical estimations of plasma and serum proteins were done by the hospital laboratory as part of the ward routine. A Kjeldhal digest method was used, but I was only responsible for the collection of the blood, one portion of which was transmitted to the laboratory by the usual channels while I examined the other portion.

Appendix B ... The Cases of the Series.

Table A:- The significance of the reference letters and the numbers of cases and samples per group in the present series. Figures in brackets indicate samples examined by the copper sulphate method. Other figures refer to the formol reactions alone.

	No. Cases.	No. Samples.
Group B ... Anaemic states.		
Subgroup BA .. Addisonian anaemia	10	17 (15)
" BI .. Iron deficiency states	10	13 (11)
" BX .. Other anaemias as below		
made up of BS .. Splenic anaemias	2	3 (2)
BL .. Lead absorption	2	3 (3)
BH .. Post-haematemesis	3	4 (3)
BP .. Physiological and pathological anaemias of pregnancy	4	5 (3)
	31	45 (39)
Group R ... Reticulo-endothelioses.		
Subgroup RE .. Lymphadenoma	4	7 (5)
" RM .. Myeloid leukaemia	3	9 (8)
" RL .. Lymphatic "	1	3 (2)
" RP .. Myelomatosis	3	3 (3)
" RX .. Acute thrombocytopenic purpura with splenomegaly	1	2 (1)
	12	24 (19)
Group N ... Neoplastic States.		
NG .. Gastric carcinoma	3	4 (4)
NR .. Bronchial carcinoma	3	3 (2)
NK .. Hypernephroma	2	2 (2)
NP .. Carcinoma head of pancreas	1	1 (1)
NH .. Primary hepatic carcinoma	1	1 (1)
NX .. Widely metastasised carcinoma Primary tumour not localised	1	1 (1)
NF .. Neurofibromatosis with sarcomatous change	1	1 (-)
	12	13 (11)

Group I/

Group I .. Infections.	No. Cases.	No. Samples.
IR .. Acute lobar pneumonia	1	1 (1)
IP .. Pneumococcal septicaemia & hepatic cirrhosis	1	1 (1)
IM .. Malaria	1	1 (1)
IT .. Tuberculosis	3	3 (3)
IS .. Sarcoidosis	1	1 (-)
IH .. Infective hepatitis	1	1 (1)
IL .. Acute diffuse lupus erythematosus	1	1 (1)
IE .. Subacute infective endocarditis	1	1 (-)
	10	10 (8)

Group X ... Miscellaneous Conditions.		
XA .. Chronic bronchitis and asthma	1	2 (2)
XW .. Tapeworm infestation	1	2 (2)
XB .. Pernicious anaemia - allergy to liver	1	1 (1)
XG .. Gastric ulcer (peptic)	1	1 (1)
XG .. Chronic gastritis	1	1 (1)
XD .. Duodenal ulceration	6	6 (-)
XT .. Post-transfusion icterus	1	1 (1)
XP .. Polyserositis	1	1 (1)
XM .. Amyotrophic lateral sclerosis	1	1 (1)
XN .. Nephrotic nephritis (Ellis' type 2)	1	1 (1)
XH .. Subacute hepatitis	1	1 (1)
XC .. Coronary thrombosis	3	4 (2)
XV .. Vitamin deficiency states	2	2 (2)
	21	24 (16)
Group O ... Normal control group	14	14 (10)
Total All Groups	100	130 (103)

Table B:- Summarising the clinical notes on the cases of the series.

Further information is given on cases marked with an asterisk beside the reference letters, in the 'Additional notes' at the end of this appendix.

TABLE B

GROUP	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
SUBGROUP	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	H	H	H	H
CASE	a	b	c	c	c	c	d	e	f	f	g	g	g	h	i	i	j	a'	a	b	c
SAMPLE			1	2	3	4			1	2	1	2	3		1	2		1	2		
SEX	M	F	M				M	F	F		F			F	M		F	M		F	M
AGE (YEARS)																					
OVER 60			+				+				+			+							
50-59	+	+													+		+				
40-49								+												+	+
30-39									+									+			
UNDER 30																					
DURATION OF SYMPTOMS (MONTHS)																					
OVER 12	+		+	+	+	+	+	+			+			+							
1 - 12		+							+	+					+		+				
UNDER 1																		+	+	+	+
TREATMENT & RESPONSE (BEFORE TEST) *1																					
SATISFACTORY	L			L	L	L												M	M	M	M
POOR											L	L	L								
GOOD / RELAPSED UNCERTAIN							L	L													
INADEQUATE TREATMENT		L							L					L							
GENERAL CONDITION (G - GOOD M - MODERATE P - POOR)	G	G	P	M	M	G	P	M	M	M	G	G	G	P	M	M	G	P	P	P	M
FEBRILE			?				+							+							
LOSS OF WEIGHT			+	?			+							+				+			
GAIN IN "					+	+															
PALPAPLE ENLARGEMENT																					
SPLEEN	+		+	+	?		+	+			+			+							
GLANDS																					
LIVER																					
MISCELLANEOUS																					
SUB. COMB DEG	+	+					+	+							+	+					
WASTING MUSCLE		+						+							+	+					
SKIN INFECTION							+														
CYSTITIS							+														
DIABETES MELLITUS														+							
HAEMATEMESIS																		+	+	+	+
PUNCTATE BASOPHILIA.																					
ASCITES																					
HEPATIC CIRRHOSIS																					
PREGNANCY (6-8 MTH)																					

*1 'L' - TREATED WITH PARENTERAL LIVER EXTRACT

'M' - GENERAL MEDICAL TREATMENT

BLa	BLb1	BLb2	BSa	BSb1	BSb2	BPa	BPb1	BPb2	BPc	BPd	BIa1	BIa2	BIb	BIc	BId	BIe	BIf	BIg	BIh	BIi1	BIi2	BIi3	BIj	RL1	RL2	RL3
M	M		F	M		F	F		F	F	F		F	F	F	F	F	F	F	F			F	F		
AGE, CONT																										
+	+		+	+							+		+	+	+	+	+		+	+			+			
DURATION, CONT																										
+	+		+	+		+					+		+	+	+	+	+	+	+	+			+	+	+	+
PREVIOUS TREATMENT, CONT													*1													
			*S								1						1	1				1	1		X	
					T																				X	X
																									(X-X-RAY	
			M										1			1				1					THEPAPY.)	
GENERAL CONDITION, CONT.																										
M	M	M	M	P	P	G	G	G	G	G	M	M	G	G	G	G	G	G	G	M	M	M	G	M	G	M
FEVER																										
WEIGHT. CONT.																										
+	+						+	+	+	+										+			+			
ENLARGEMENT, CONT																										
			*S	+	+																				+ + +	
			+	+	+																				+ + +	
MISCELLANEOUS, CONT																										
+	+	+																								
			+	+	+																					
			+	+	+																					
						+	+		+	+																

*S-SPLENECTOMY AND TRANSFUSION 3 WEEKS EARLIER.

*1 1 - IRON THERAPY. T - TRANSFUSED

TABLE B CONT.

GROUP	RE	RE	RE	RE	RE	RE	RE	RM	RM	RM	RM	RM	RM	RM	RP	RP	RP	RX	RX
SUBGROUP																			
CASE	a	a	b	b	c	d	d	a	a	b	b	b	b	b	c	a	b	c	
SAMPLE	1	2	1	2		1	2	1	2	1	2	3	4	5	6				1 2
SEX	F		F		M	M		F		M						F	M	F	M	M	
AGE (YEARS)																					
OVER 60																	+				
50 - 59						+		+		+								+			
40 - 49	+				+														+		
30 - 39			+													+					
UNDER 30																				+	
DURATION OF SYMPTOMS (MONTHS)																					
OVER 12	+	+			+												+				
1 12			+	+				+	+	+	+	+	+	+	+			+	+		
UNDER 1																+				+	+
TREATMENT AND RESPONSE (BEFORE TEST) *1																					
SATISFACTORY.	X	X																			M
POOR.						X	X		X		A	A	A	X	X			S			
GOOD/RELAPSED			X	X																	
UNCERTAIN																					
INADEQUATE TREATMENT.																					
GENERAL CONDITION (G - GOOD. M - MODERATE. P - POOR)	P	P	G	M	P	G	G	P	P	M	M	M	P	M	M	G	P	P	M	G	G
FEBRILE.	+	+		+	+	+	+	+	+	+	+	+	+				+	+		+	
WEIGHT.																					
LOSS.	+			+	+			+				+	+				+				
GAIN.																					
PALPABLE ENLARGEMENT																					
SPLEEN.	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+				+	+
GLANDS.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+					
LIVER	+	+																			
MISCELLANEOUS																					
VERTEBRAL COLLAPSE																	+	+	+		
PERIOSTITIS & LEUCOBLASTAEMIA																					
MUSCLE WASTING																	+	+	+		
ASCITES.					+																
ERYTHRODERMIA						+	+														
PURPURA.																				+	+
DIAGNOSIS.																					
BLOOD.								+		+					+					+	+
MARROW.								+		+					+	+	+	+			
GLAND BIOPSY.	+		+		+	+		+		+											
OPERATION																					
X-RAY EVIDENCE.				+													+	+	+		
POST-MORTEM.							+														

*1. X.-X-RAY THERAPY A- LIQU. ARSENICALIS. S-STILBAMIDINE.

TABLE B CONT.

	IR	IP*	IH	IE	IL*	IT a	IT b	IT C	IS*	IM	NG a	NG b	NG C 1	NG C 2	NK a	NK b	NP	NR a	NR b	NR C	NH*	NF*	NX	XD a	XD b	XD C	XD D	XD e	XD f
	M	F	M	M	F	M	F	F	F	M	F	M	F		F	M	M	M	M	M	M	F	F	M	M	M	F	M	M
AGE, CONT.		+							+		+									+									
	+											+				+	+	+						+					
		+																	+						+					
			+	+	+	+	+	+		+			+	+	+						+	+			+	+	+	+	+
DURATION, CONT.								+							+								+						
				+	+		+		+				+	+		+	+	+	+	+		+		+	+				
	+	+	+			+				+	+	+									+					+		+	+
TREATMENT, CONT *1	S	P	M	P	PS	M	M	M	M	P														M	M	M	M	M	M
GENERAL CONDTN	G	P	G	M	P	M	G	G	G	G	P	M	G	G	P	M	G	P	P	M	P	G	P	G	G	G	G	G	G
		+	+			+	+				+	+			+			+	+	+			+						
WEIGHT, CONT.	+	+			+	+	+				+	+	+	+	+	+		+	+	+	+		+	+	+			+	+
PALPABLE ENLARGEMENT, CONT				+	+				+						+	+					+								
		+	+								+	+			+						+		+						
MISCELLANEOUS																													
ICTERUS.		+	+									+			+		+				+		+						
HEPATIC INSUFFICIENCY. ("C"-FIBROSIS "I"-INFECTION "T"-TUMOUR)		C	I												T						*2		T						
METASTASES IN BONE											+	+			+					+			?						
LEUCO-ERYTHROBLASTAEMIA.												+																	
DIAGNOSIS, CONT.																													
BLOOD										+																			
MARROW																													
GLAND BIOPSY									+							+													
OPERATION												+	+	+			+												
+ X-RAY					+	+	+				+	+	+	+	+		+	+	+	+		+		+	+	+	+	+	+
P.M					+						+	+	+	+				+		+	+								

*1 X-X-RAY THERAPY. S-SULPHATHIAZOLE, P-PENICILLIN. Q-QUININE. M-GENERAL MEDICAL MEASURES

*2. SUBACUTE HEPATITIS WITH EXTENSIVE FIBROSIS AND NODULAR HYPERPLASIA PLUS PRIMARY HEPATIC CARCINOMA

TABLE B. CONCLUDED

GROUP.	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
SUBGROUP.	A	A	W	W	B	G	U	T	P	M	N	H	C	C	C	C	V	V
CASE.	1	2	1	2									a	b	c		a	b
SAMPLE																		
SEX.	M		F		F	M	M	F	M	M	M	F	M	M		M	F	F
AGE (YEARS)																		
OVER 60	+					+												
50 - 59			+		+					+			+	+		+		+
40 - 49							+		+								+	
30 - 39								+				+						
UNDER 30											+							
DURATION OF SYMPTOMS (MONTHS)																		
OVER 12	+		+							+	+	+						
1 - 12						+	+	+	+								+	+
0 - 1					+								+	+	+	+		
TREATMENT AND RESPONSE BEFORE TEST																		
SATISFACTORY							M	M							M	M	V	V
POOR.	M	M	M						M	M	M	M						
SATIS./RELAPSED.																		
UNCERTAIN																		
INADEQUATE TREATMENT.					L								M	M				
GENERAL CONDITION. (G - GOOD, M - MODERATE, P - POOR)	G	G	G	G	G	G	G	M	P	G	M	G	G	M	G	M	G	G
FEBRILE.								+	+				+	+				
WEIGHT																		
LOSS																		
GAIN.									+	+							+	
PALPABLE ENLARGEMENT																		
SPLEEN.					+							+						
GLANDS																		
LIVER.									+			+						
MISCELLANEOUS.																		
ALLERGIC STATES	+	+	+	+	+													
ADDISONIAN ANAEMIA.					+													
PERIPHERAL NEURITIS.																	+	+
MUSCLE WASTING.										+								
ASCITES & PLEURAL EFFUSION.									+									

*1. L - PROTEOLYSED LIVER BY MOUTH: URTICARIA MARKED FOLLOWING PARENTERAL LIVER ONE WEEK EARLIER

M - GENERAL MEASURES. T - TRANSFUSED. V - DIETETIC + VIT. B COMPLEX.

ADDITIONAL NOTES ON SOME OF THE CASES OF THE SERIES

RMb. Subleukaemic myeloid leukaemia. This patient had all the clinical features of chronic myeloid leukaemia; the diagnosis was confirmed by lymph-node biopsy and by sternal puncture. Untreated the white cell count was less than 40,000 per c.mm. and the peripheral blood contained very few immature myeloid cells (2% of total white cells). During the three months from RMbi to RMb6 the average white cell count was about 30,000 per c.mm. X-ray therapy reduced the total white cells to 21,000 per c.mm. and there was a coincident rise in the number of myelocytes to 39% i.e. an absolute increase in myelocytes of 7,000 per c.mm..

RPa, RPb, RPc suffered from multiple myelomatosis with spontaneous collapse of the body of one or more dorso-lumbar vertebra. Bence-Jones protein had been found in the urine in all three cases at one time or another, but was present only in case RPa at the time of examination.

REa. Lymphadenoma with periosteal involvement visible on X-ray examination of the right clavicle and the right tibia. Blood films show a marked leucoblastic reaction with leucocytosis. The leucocytosis was between 60,000 and 100,000 per c.mm.. This high white cell count has been maintained for more than a year since REa2. The vast majority of the white cells are polymorphs, and myelocytes. There has been no erythroblastaemia.

RX. Acute thrombocytopenic purpura with spontaneous recovery. The patient, a well developed youth of 18, developed a rash one week before test RXi. He felt well throughout the illness. At the time of test RXi he had a widespread purpuric eruption with grossly impaired capillary permeability, thrombocytopenia and moderate splenomegaly. Ten days later, test RX2, the rash had cleared up, his capillary resistance was normal and his spleen was just palpable. I am informed that he has been perfectly well during the year since this attack. No precipitating cause can be suggested in this case; no drugs had been taken. The only feature of possible significance is a history of 'catarrhal jaundice' three months before test RXi. The Paul-Bunnel reaction was negative and his blood appeared to be normal apart from the thrombocytopenia.

REd had a severe exfoliative dermatitis, enlarged cervical and axillary glands and an enlarged spleen. Lymph-node biopsy between tests REdi and 2 showed non-specific lymphoid hyperplasia. There were however a few immature lymphoid cells in the peripheral blood (REdi) and a provisional diagnosis of aleukaemic lymphatic leukaemia was made. He died six months after REdi but before death a second lymph gland biopsy showed typical/

typical lymphadenomatous tissue.

BId had a moderate iron-deficiency anaemia. The patient however had recently become exceptionally stout and presented the appearance usually associated with dysfunction of the pituitary. The association of hypopituitarism with anaemia is described by Snapper (1937) and has been confirmed by Witts (1942) and Watkinson, (1947).

XB had been treated for Addisonian anaemia for several years. She hsd become sensitive to several brands of liver administered parenterally. Her main symptoms were allergic; she complained of respiratory distress and urticaria after liver injections.

XT was admitted for investigation of menorrhagia. After dilatation and curettage the patient developed intermittent uterine haemorrhage. When first seen she had been given 8 pints of whole blood within 3 days and was distinctly jaundiced. The uterine tissue was reported normal on histological examination. The jaundice was mainly haemolytic in character and cleared up uneventfully.

XP Presented a left sided pleural effusion and ascites. There was no evidence of any active infection. At post-mortem 6 months later a constrictive pericarditis involving the mediastinal tissues was found. Histologically the pericarditis was tuberculous.

IS had painless enlarged glands in the neck and axillae. They remained unaltered in size and consistency for at least six months. Biopsy was reported as 'sarcoidosis'. No other manifestations of sarcoidosis were found and apart from the absence of caseation the histology was identical with a tuberculous infection.

IL. Acute diffuse lupus erythematosus. 6 months before test IL the patient had developed a febrile illness with a transient erythematous rash on her face. The fever continued intermittently and a rheumatoid-like arthritis developed. At the time of test IL she also presented the clinical picture of nephrosis with oedema, polyuria, gross albuminuria and refractile casts. In addition there was extensive necrotic ulceration of the mouth and face. She died ten days later and the diagnosis was confirmed post-mortem.

IP In addition to chronic cirrhosis of the liver this patient developed an acute febrile illness. Pneumococci were grown from a blood culture and she later developed a pneumococcal infection of the superficial tissues of the right leg and of the right cornea and sclera. No primary pneumococcal infection was found but the lesions cleared up uneventfully on penicillin.

NH had subacute hepatitis with nodular hyperplasia and primary/

carcinoma of the liver. He was aged 20, had been well up till 3 weeks before death and gave no history of jaundice before the terminal illness. A sister had jaundice ten years earlier.

NF. Multiple neurofibromatosis with early sarcomatous change.

Appendix C- Results relating to Part One.

Table C .

The Standardisation Tests.

'P'..g. Hb/100 ml. by photoelectric method.
'S'.. " " " Sahli " .
'G'.." " " " copper sulphate method finally suggested in this work (page).

Gc was calculated from Gb, Gp & H in samples 1 and 2 for each blood. Gc was converted to M.C.Hb. Conc. by means of eqn. iii , page 6 , i.e., M.C.Hb. Conc. = 377(Gc-1.007). Where H was not estimated it was calculated from eqn. , page ,i.e.,$H.=\frac{100(Gb-Gp)}{Gc-Gp}$. Hb ('G') is then calculated from these values for H and M.C.Hb.Conc..

	P	S	G	Gb	H
Sample Oa	(Gp.	1.023	Gc	1.095).	
i.	13.7	13.3	13.45	1.052	40.5
2.	16.05	16.0	16.0	"58	48.0
3.	10.9	11.5	11.0	"47	
4.	10.1	10.8	10.25	"45	
5.	8.4	9.0	8.2	"41	
6.	6.5	6.0	6.3	"37	
Sample Ob	(Gp.	1.024.	Gc.	1.094).	
1.	13.1	13.0	13.1	1.052	40.0
2.	16.15	16.5	15.9	"58	49.0
3.	11.6	11.0	11.7	"49	
4.	9.6	9.2	9.4	"44	
5.	6.5	5.9	6.5	"38	
6.	3.6	3.7	3.8	"32	
Sample Oi.	(Gp.	1.026	Gc.	1.096).	
1.	14.6	15.0	14.3	1.056	43.0

Table C. The Standardisation Tests, cont..

Sample Oi, cont.

	P	S	G	Gb	H
2.	16.8	15.9	16.9	1.061	51.0
3.	12.2	13.0	12.4	1.052	
4.	8.4	8.0	8.3	1.043	
5.	7.0	7.0	6.8	1.040	
6.	3.6	3.4	3.45	1.033	
Sample Oj.	(Gpl.026		Gc..1.091.)		
1.	13.2	13.8	13.1	1.053	41.5
2.	15.4	15.0	15.5	1.058	49.0
3.	10.9	11.4	10.8	1.048	
4.	8.4	7.8	8.5	1.043	
5.	7.2	7.5	7.0	1.040	
6.	2.75	3.0	2.9	1.033	
Means	10.277	10.279	10.231		

Table D.

Gb, Gp, H & W and Hb-Sahli in each of 103 bloods of the series. Gc, M.C.Hb.Conc. and Hb are calculated by method D, page .

Sample	H	Gp	Gb	W	Hb Sahli	Gc	M.C.Hb.C. (Gc)	(Sahli)	Hb. (Gc)
1.Oa	40.5	1.023	1.052	N	13.3	1.095	33.2	33.3	13.4
2.Ob	40.0	1.024	"	"	13.0	1.094	32.8	32.8	13.1
3.Oc	39.5	"	"	"	13.3	1.094	32.8	33.5	13.0
4.Od	43.0	"	"	"	13.0	1.089	30.9	30.2	13.5
5.Oe	48.0	1.025	1.058	"	15.0	1.094	32.8	31.3	15.7
6.Of	45.0	"	56	"	14.7	1.095	33.2	32.7	15.0
7.Og	39.5	26	54	"	14.0	1.097	33.9	34.2	14.3
8.Oh	41.5	"	53	"	13.5	1.091	31.7	32.9	13.2
9.Oi	43.0	"	56	"	15.0	1.095	33.2	34.0	14.3
10.Oj	41.5	"	53	"	13.9	1.091	31.7	33.5	13.1
1.XCb1	47.0	1.023	1.055	"	15.7	1.092	32.0	33.0	15.0
2.XCb2	46.0	1.024	1.055	"	15.3	1.092	32.0	32.8	14.7
3.XM	38.5	1.023	1.052	"	13.5	1.099	34.7	35.0	13.4
4.XV	39	1.026	1.054	"	13.0	1.102	35.8	33.3	13.3
5.XT	29.5	1.024	1.046	"	8.9	1.099	34.7	33.5	10.2
6.XH	36.0	1.024	1.051	"	13.0	1.099	34.7	36.1	12.5
7.XG	39.0	1.025	1.046	"	9.7	1.087	30.2	28.5	10.1
8.XP	49.0	1.025	1.054	"	13.5	1.085	29.4	27.0	14.4
9.XU	46.0	1.024	1.056	"	14.7	1.089	30.9	32.0	14.2
10.XM	35	1.018	1.047	"	12.5	1.101	35.4	35.7	12.4
11.XVb	40	1.023	1.052	"	14.0	1.096	33.35	35.0	13.5

Sample	H	Gp	Gb	W	Hb Sahli	Gc	M.C. (Gc)	HbC. (Sahli)	Hb. (Gc)
1. BIii	26	1.018	1.030	N	6.1	1.065	21.1	23.4	8.7
2. i2	17.5	1.020	1.030	N	4.3	1.078	26.7	24.1	4.7
3. i3	26.0	1.020	1.033	N	7.0	1.085	29.4	26.9	7.6
4. b	36.0	1.023	1.045	N	10.1	1.084	29.0	28.0	10.5
5. a2	22.0	1.024	1.035	N	5.5	1.074	25.3	25.0	5.6
6. c	38.0	1.026	1.045	N	10.6	1.076	26.0	27.8	9.9
7. f	32.0	1.025	1.044	N	9.2	1.084	28.7	29.0	9.3
8. j	26.0	1.025	1.040	N	7.5	1.079	27.1	26.8	7.6
9. g	40.0	1.026	1.048	N	12.5	1.081	27.9	31.25	11.2
10. e	24	1.024	1.035	N	5.6	1.070	23.7	23.3	5.7
11. h	38.5	1.027	1.855	N	10.0	1.081	27.9	27.0	10.7
1. BAc1	14.0	1.022	1.033	M	5.2	1.100	35.1	37.1	4.6
2. BAc2	28.0	1.023	1.039	"	7.0	1.080	27.5	25.0	7.7
3. BAc3	43.5	1.024	1.049	"	13.7	1.082	28.3	31.5	12.3
4. BAc4	44.5	1.026	1.054	"	13.3	1.089	30.9	34.0	13.8
5. BAii	36.0	1.024	1.050	"	12.0	1.096	33.5	33.3	12.1
6. BAi2	33.0	1.022	1.049	"	12.1	1.104	36.6	36.4	12.0
7. BAj	17.0	1.022	1.037	"	6.3	1.110	38.9	37.0	6.6
8. BAd	30.5	1.023	1.044	"	9.0	1.092	32.0	29.5	9.8
9. BAgi	21.0	1.024	1.041	"	8.3	1.104	36.6	29.4	7.7
10. BAg2	24.0	1.024	1.042	"	8.3	1.099	34.7	34.5	8.3
11. BAg3	28.0	1.026	1.044	"	9.2	1.090	31.3	32.8	8.8
12. BAb	26.0	1.025	1.045	"	8.8	1.103	36.2	32.8	9.4
13. BAa	35.0	1.025	1.053	"	12.8	1.103	36.2	36.5	12.7
14. BAf1	31.0	1.026	1.050	"	11.5	1.091	31.7	31.4	11.7
15. BAf2	28.0	1.026	1.047	"	10.8	1.101	35.5	38.4	10.0

Sample	H	Gp	Gb	W	Hb Sahli	Gc	M.C.(Gc)	HbC.(Sahli)	Hb.(Gc)
12.XW1	41.5	1.025	1.054	"	13.3	1.098	33.2	32.2	13.8
13.XW2	47.0	1.027	1.056	"	13.5	1.089	31.0	28.8	14.4
14.XA1	35	1.025	1.047	"	11.0	1.088	30.2	31.4	10.5
15.XA2	40.0	1.027	1.049	"	12.8	1.082	28.3	32.0	11.3
16.XB	30.5	1.023	1.044	"	9.5	1.092	32.0	30.2	9.3
1. NRB	34.0	1.025	1.046	N	10.1	1.087	30.2	29.7	10.2
2. C	30.0	1.022	1.044	1.5	11.5	1.094	32.8	37.7	10.1
3. NGa	23.0	1.022	1.037	1.8	7.5	1.087	30.2	29.7	6.9
4. b	15.0	1.022	1.034	1.8	5.6	1.102	35.8	37.3	5.4
5.NGc1	32.0	1.025	1.044	1.0	9.2	1.084	29.0	28.7	9.3
6.NGc2	29.5	1.025	1.040	1.1	8.3	1.080	27.6	28.0	8.2
7.NKa	27.0	1.022	1.039	1.5	8.8	1.086	29.4	32.6	7.9
8.NKb	40	1.022	1.050	1.5	12.5	1.089	30.9	32.2	11.7
9.NP	40.5	1.023	1.049	N	13.7	1.088	30.5	33.1	12.3
10.NH	35.0	1.024	1.050	N	11.5	1.098	34.3	32.8	12.0
11.NX	30.0	1.020	1.049	N	13.3	1.094	32.8	32.1	12.8
1.IR	10.0	1.023	1.050	N	13.7	1.091	31.7	34.2	32.7
2.IP	32.5	1.024	1.046	N	10.0	1.090	31.3	30.8	10.2
3.IL	22	1.019	1.036	N	7.5	1.096	33.5	34.1	7.4
4.IM	30	1.028	1.054	N	11.6	1.100	35.1	21.9	12.6
5.IH	41.5	1.025	1.053	N	13.7	1.092	32.0	33.0	13.3
6.IIa	36	1.026	1.055	N	13.3	1.100	30.1	39.0	13.7
7.IIb	28	1.022	1.039	1.4	7.9	1.082	28.3	28.2	7.9
8.IIc	48	1.027	1.050	N	13.5	1.075	25.6	28.1	12.3

Sample	H	Gp	Gb	W	Hb Sahli	Gc	M.C. (Gc)	HbC. (Sahli)	Hb (Gc)
1.BSa	40.0	1.029	1.050	N	10.1	1.081	27.9	25.0.	11.1 *
2.BSb1	14.0	1.019	1.097	N	3.4	1.076	26.0	24.3	3.6
3.BSb2	18.5	1.019	1.028	N	4.3	1.067	22.6	23.2	4.2
4.BLa	37.0	1.023	1.041	N	11.9	1.097	33.9	32.2	12.6
5.BLbi	30.5	1.025	1.040	N	9.2	1.087	30.2	30.2	9.5
6.BLb2	32.0	1.024	1.035	N	9.7	1.090	31.3	30.3	10.0
7.BPa	14.0	1.025	1.035	N	4.5	1.096	33.5	32.1	4.7
8.BPbi	29.0	1.023	1.040	N	8.3	1.081	27.9	28.5	8.1
9.BPb2	28.0	1.023	1.041	N	8.3	1.083	30.2	29.5	8.4
10.BHai	16.0	1.022	1.033	1.0	5.8	1.091	30.7	34.4	5.0
11.BHa2	18.5	1.021	1.033	1.0	5.6	1.091	31.7	29.2	5.4
12.BHb	27.0	1.022	1.044	1.0	8.2	1.087	30.2	30.2	8.1
13.BHc	24.0	1.024	1.039	1.0	6.6	1.086	29.8	29.8	7.1

* Hb corrected for Gp over 1.027 in Tab.2,page33.

1.REc	26.5	1.023	1.035	N	6.2	1.067	22.1	23.9	5.3
2.REdi	37.5	1.024	1.048	N	11.5	1.090	31.8	30.6	11.7
3.REd2	37.0	1.024	1.043	N	11.5	1.092	32.0	32.6	11.9
4.REb2	32.0	1.024	1.046	N	11.9	1.091	31.7	31.9	11.6
5.RX2	4.35	1.024	1.056	N	15.0	1.097	33.9	34.5	14.7
6.RMc	20.0	1.025	1.046	21%	7.0	1.093	32.5	35.0	6.5
7.RMb1	27.0	1.026	1.044	2%	8.3	1.072	32.0	30.7	8.9
8.RMb2	29.5	1.028	1.046	2%	9.0	1.088	30.5	31.2	9.2
9.RMb3	29.5	1.026	1.044	1.5%	9.2	1.086	29.7	31.2	8.8*
10.RMb4	25.0	1.024	1.039	1.5%	7.9	1.084	29.0	31.6	7.3

*Corrected for Gp over 1.027 in Tab, 2,page 33.

Sample	H	Gp	Gb	W	Hb Sahli	Gc	M.C. (Gc)	HbC. (Sahli)	Hb. (Gc)
11.RMb5	27.0	1.026	1.039	1.1%	7.9	1.075	25.3	29.3	6.8
12.RMb6	30.0	1.027	1.045	2%	8.8	1.087	30.2	29.3	6.8
13.RMa2	32.0	1.027	1.052	1.8%	11.5	1.103	36.2	35.0	7.25
14.RPa	33.5	1.028	1.052	M	10.1	1.099	34.7	30.3	11.6*
15.RPb	36.0	1.028	1.050	M	11.5	1.089	30.9	32.0	11.1*
16.RPc	37.0	1.027	1.050	M	11.9	1.089	30.9	32.2	11.4
17.RL2	35.0	1.031	1.054	3%	9.8	1.093	32.5	28.0	11.3*
18.RL3	34.5	1.029	1.053	2.5%	10.1	1.094	32.8	29.3	11.6
19.REG2	38.0	1.031	1.054	1.2%	11.0	1.091	31.7	28.9	12.0*

* Corrected for Gp over 1.027 in Tab.2,page33.

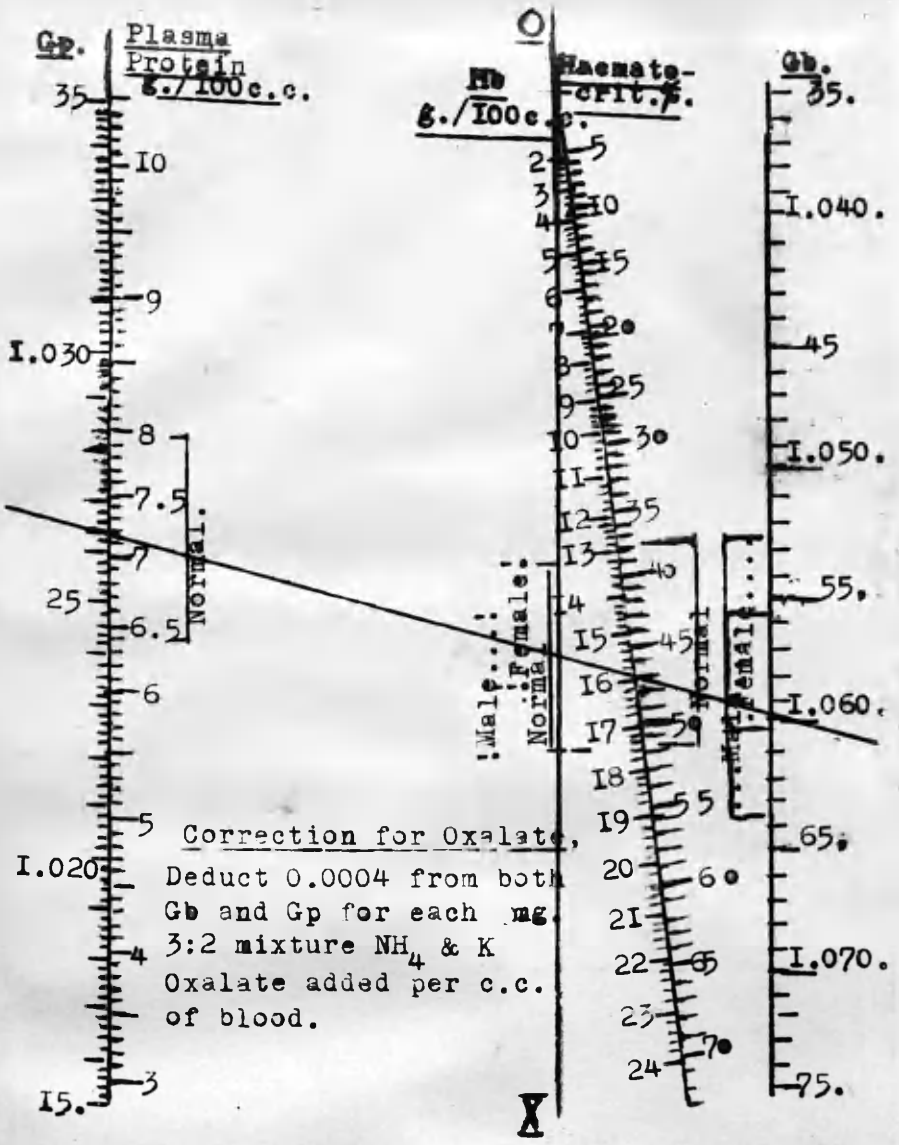


Fig. A:- Nomographic chart similar to that of Phillips et al(1945 b). I have added the line OX which allows the chart to be used to calculate Gc as described on the opposite page.

The Nomogram of Phillips et al (1945b). Figure A, facing this page is similar to the nomogram of Phillips et al (1945b). It differs only in scale and in the additional line, OX.

Method of Use i) Correct Gb and Gp for the anticoagulant, i.e. subtract 0.0004 from Gb and Gp for each mg. oxalate mixture per ml. blood.

Note ... All figures given for Gb and Gp in this work have already been corrected.

ii) The line joining Gb to Gp intersects the oblique line at the required value for H and for Hb.

To Obtain Gc. The H and Hb results assume that Gc is invariably 1.097 and the M.C.Hb.Conc. is 33.9g./100 ml. packed cells. I have added the line OX to the original nomogram; it may thus be used to calculate Gc from Gb, Gp and H .....

Note the intersection of the line joining Gb to Gp with line OX. Join this point on the line OX to the estimated H and extend to meet the scale Gp at Gp". The true value for Gc equals ... 1.097-(Gp"-Gp).

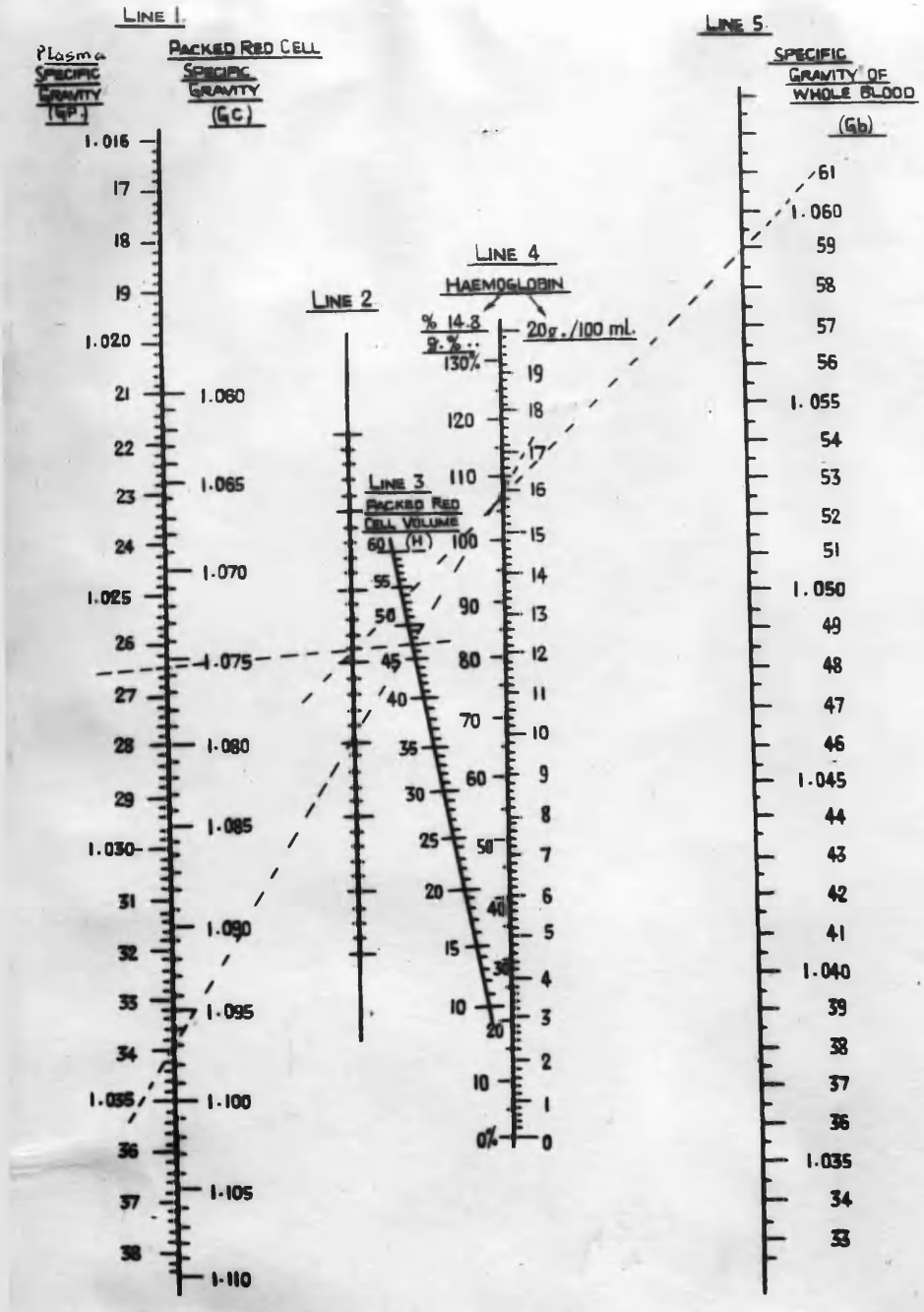


Fig. B:- Chart for calculating Hb,Gc & M.C.Hb.Conc. from Gb,Gp,H & W by the method finally suggested herein(page 43). The example shown is the normal blood of Phillips et al(1945a),i.e. Gb..1.0595,Gp= 1.0264,H = 47%; Hb=15.9g.%, Gc =1.097, & M.C.Hb.Conc.= 33.9 g./100 ml. packed cells.

Nomographic Chart Developed in This Work. See figure B, facing this page.

Method of Use A. To calculate Hb from Gb, Gp, H and W as developed in this work.

Correct Gb and Gp for the anticoagulant as for the nomogram of Phillips et al on the previous page.

Join Gp (line I) to H (line 3). Let this line intersect line 2 at the point Z. The line joining Z to Gb (line 5) intersects line 4 at the required Hb figure, unless ..... when W exceeds 2 mm./100 mm. blood in which case the point Z should be raised by one space on the scale on line 2 for each 2 mm. of W/100 mm. blood. The elevated point Z is then joined to Gb as before.

The correction found necessary in the cases with Gp=1.028 or more in this series may be applied by raising the point Z a further space on the line 2 scale for each 0.001 by which Gp exceeds 1.026.

B. To calculate Gc and the M.C.Hb.Conc.

Join Hb (line 4) to H (line 3). This line will intersect line I at the required value for Gc. The M.C.Hb.Conc. is given by the last two figures of the Gp value opposite to Gc on line I ((by the formula M.C.Hb.Conc.= 350(Gc - 1.000))).

APPENDIX D.

Results Relating to Part Two.

Table E. Sedimentation rates and Plasma tests (PF reactions, plasma dilution and plasma Weltmann reactions) other than plasma specific gravities in Table D, Appendix C.

Table F. Serum tests (SF reactions, serum dilutions and Weltmann reactions.)

Table E:- Results of the whole blood and plasma tests in the 130 bloods of the series.

Group	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Subgroup	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	I	I	I	I	I	I
Case	a	b	c	c	c	c	d	e	f	f	g	g	h	i	i	j	a	a	b	c	a	a	b	c	a	a
Sample	1	1	1	2	3	4			1	2	1	2	3	1	2	1	1	2	1	1	1	2	1	1	2	2
PFG...	3	3	1	5	0	3	2	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0
(No.+s)																										
PFF...	3	2	0	4	1	3	5	4	0	2	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0
(No.+s)																										
Diln. test...	+	—	—	+	—	—	+	?	—	—	—	—	—	—	—	—	—	—	—	—	?	—	+	?	?	?
Coag. zone...	7	7	5.5	6.5	6	7.5	6	?	7.5	7.5	.6	6.5	6.5	?	6	6	6.5	?	6	6.5	?	?	?	?	?	?
ESR.																										
Crude...36	50	17	15	3	4	56	3	3	44	10	11	8	19	35	32	38	16	15	8	2	2	2	2	2	2	2
Corrected																										
1.Wintrobe18	18	n	n	n	n	25	n	n	13	n	n	n	n	14	18	n	n	n	n	n	n	n	n	n	n	n
2.Whitby.. s		s	n	n	n	m	n	n	d	n	n	n	n	n	s	n	n	n	n	n	n	n	n	n	n	n
H...35	26	14	28	43	44	31	40	37	28	21	24	28	33	36	33	17	19	22	36	38	38	38	38	38	38	38

'n'... normal by Whitby & Britton's chart; less than 7 on correction to H=47 % on Wintrobe's chart.
'd'...doubtful " " " " " " "
's'...slight " " " " " " "
'm'...moderate " " " " " " "

Table E.cont..

Group...	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B
Subgroup...	I	I	I	I	I	I	I	I	I	L	L	L	H	H	H	H	P	P	P	P
Case...	d	e	f	g	h	i	i	i	j	a	b	b	a	a	b	c	a	b	b	c	d
Sample...						1	2	3			1	2	1	2				1	2		
PFG....	1	o	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	2	2	2
PFF....	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	3	2
Dil$^{n.}$ test...	?	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	?
Coag. zone...	?	6.5	6	6	6	5.5	5.5	5.5	6	6	6.5	7	5.5	6	5.5	5	?	7	7.5	6.5	?
ESR																					
Crude...	28	12	19	10	2	26	30	13	10	6	15	10	46	34	44	2	10	35	30	31	29
Corrected.																					
1.Wintrobe.	11	n	n	n	n	9	n	n	n	n	n	n	n	n	13	n	n	21	17	12	19
2.Whitby...	d	n	n	n	n	n	n	n	n	n	n	n	n	n	d	n	n	s	s	s	s
H.....	32	38	32	40	38	26	18	26	26	37	31	32	16	19	27	24	14	29	28	35	33

Table E, cont.

Group...	BS	BS	BS	IE	IH	IL	IM	IP	IR	IS	ITa	ITb	ITc	NH	NKa	NKb	NRa	NRb	NRc	NX	NP	NF
Subgroup...		bl	b																			
Case...		1	2																			
Sample...																						
PFG....	5	1	4	4	0	5	5	0	5	2	0	3	4	0	0	5	5	3	2	0	2	2
PFF....	5	2	2	3	0	5	5	3	5	2	0	3	3	0	2	4	5	3	3	0	0	2
Coag. zone...	7.5	7	8.5	?	7	5	6	7	5	2	0	3	3	0	2	4	5	3	3	0	0	2
Dil$^{n.}$ test...	+	–	+	?	–	–	+	+	+	?	–	+	+	±	+	+	?	+	+	–	–	?
ESR.																						
Crude...	14	15	10	11	10	64	12	30	45	15	2	7	2	28	37	44	35	42	44	4	20	54
Corrected.																						
Wintrobe.	n	n	n	n	n	15	n	10	32	n	n	n	n	11	19	32	21	27	32	n	15	13
Whitby...	n	n	n	n	n	s	n	d	m	n	n	n	n	d	s	m	s	s	s	n	d	d
H....	40	14	19	36	42	22	36	32	40	38	36	28	48	35	27	40	36	34	30	30	40	23

Table E.cont..

Group...	NG	NG	NG	NG	RE	RE	RE	RE	RE	RE	RE	RX	RX	RL	RL	RL	RM	RM	RM	RM	RM	R
Subgroup...	G	G	G	G	E	E	E	E	E	E	E	X	X	L	L	L	M	M	M	M	M	M
Case...	a	b	c	c	a	a	b	b	c	d	d						a	a	b	b	b	b
Sample...			1	2	1	2	1	2		1	2	1	2	1	2	3	1	2	1	2	3	4
PFG.....	0	2	5	5	5	5	2	4	4	0	0	5	0	5	5	5	4	4	5	5	5	5
PFF.....	2	2	5	5	5	4	2	3	2	0	0	3	0	5	5	5	3	2	5	5	4	3
Dil$^{n.}$ test....	+	–	+	+	?	+	?	+	+	–	–	?	–	?	+	+	?	+	+	+	+	+
Coag. zone....	7	6.5	6.5	6	?	7.5	?	7.5	5.5	6.5	6.5	?	6.5	?	8.5	9	?	8	6	7	7	7
ESR																						
Crude	65	28	30	28	36	15	15	20	60	12	10	12	3	36	35	17	30	35	45	40	50	60
Corrected.																						
Wintrobe,.	n	8	7	11	7	n	n	8	15	n	n	n	n	17	16	n	11	12	12	12	19	18
Whitby...	n	n	n	d	n	n	n	n	n	n	n	n	n	d	d	n	d	d	d	d	s	s
H.....	15	32	30	35	30	36	35	32	27	27	32	43	47	36	35	35	30	32	27	30	30	25

Table E.cont.

Group...	RM	RM	RM	RP	RP	RP	XA	XA	XB	XC	XC	XC	XC	XD	XD	XD	XD	XD	XH	XM	XN
Subgroup...	b	b	c	a	b	c				a	b	b	c	a	b	c	d	e	f		
Case...	5	6					1	2			1	2									
Sample...																					
PFG....	5	5	0	4	5	3	3	5	4	5	1	4	3	0	0	0	1	0	1	1	2	5
PFF....	4	5	0	3	5	4	3	4	5	5	2	3	3	0	0	0	0	0	0	2	2	5
Diln. test...	+	+	–	+	+	+	+	+	+	–	–	+	–	–	–	–	–	–	–	+	+	+
Coag. zone...	7	6.5	5	7.5	6.5	7	6	6	8	?	6	5	6.5	6.5	6	6.5	7	6.5	6.5	7	7	4
ESR.																						
Crude....	40	47	8	46	18	8	4	3	11	12	2	12	12	5	7	6	2	2	5	3	3	55
Corrected.																						
Wintrobe,.	12	17	n	24	10	n	n	n	n	n	n	d	n	n	n	n	n	n	n	n	n	30
Whitby....	d	s	n	s	d	n	n	n	n	n	n	d	d	n	n	n	n	n	n	n	n	s
H....	27	30	20	33	36	47	35	40	31	42	47	46	48	39	38	40	35	36	38	36	33	35

Group...	X	X	X	X	X	X	X	X	O	O	O	O	O	O	O	O	O	O	O	O	O
Subgroup...	P	V	V	W	W	G	U	T													
Case...		a	b						a	b	c	d	e	f	g	h	i	j	k	l	m	n
Sample..				1	2																	
PFG	1	1	0	3	5	1	0	5	0	0	0	0	1	0	0	1	0	0	0	0	0	1
PFF.....	1	2	3	3	5	0	0	5	0	0	0	0	1	0	0	1	0	0	0	0	0	1
Dil$^{n.}$ test....	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	?	?	?	?
Coag. zone.....	7	6	6	5.5	6.5	6.5	6	6	6.5	6	6.5	6.5	6.5	5.5	6.5	6	6	7	?	?	?	?
ESR. Crude....	6	4	3	12	9	9	6	32	5	3	6	4	4	4	2	2	7	9	10	5	8	9
Corrected. Wintrobe,,	n	n	n	9	9	n	n	8	n	n	n	n	n	n	n	n	n	n	n	n	n	n
Whitby....	n	n	n	d	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n	n
H.....	49	39	40	42	47	39	46	30	47	40	40	39	43	48	45	39	41	43	41	39	38	40

Table E.cont..

Table F:- Serum tests in 53 samples of the series.

Sample	SFG.	SFF.	Dilution test.	Coagulation zone.
REa2	3	2	pos.	7
REb2	o	o	neg.	7.5
REc	o	o	pos.	5.5
REd1	o	o	neg.	6
REd2	o	o	neg.	5.5
RL2	3	3	pos.	8
RL3	3	2	pos.	8.5
RMb4	0	o	neg.	7
RPa	2	2	pos.	7.5
RPb	2	1	neg	6.5
RX2	o	o	?	?
NGb	o	o	pos.	6.5
NGc1	2	3	pos.	8
NGc2	3	3	pos.	8
NKa	o	2	pos	6.5
NH	o	o	neg.	6
NRc	o	o	neg.	6
NX	o	o	neg.	6.5
IE	2	2	?	?
IM	o	o	neg.	6.5
IR	o	o	neg.	6
ITa	o	o	neg.	5.5
ITb	o	o	neg.	5.5
IP	o	o	pos.	6.5
BSa	2	1	pos.	7.5
BSb1	o	o	neg.	8

Table F.cont..

Sample	SFG.	SFF.	Dilution test.	Coagulation zone.
BPa	0	0	neg.	?.
BPb2	0	0	neg.	6.5
BAc4	0	0	neg.	6.5
BAd	0	0	neg.	5
BAh	0	0	neg..	?
BAj	0	0	neg.	7
BLa	0	0	neg.	7
BHa	0	0	neg.	5.5
BIa	0	0	neg.	5.5
BIc	0	0	neg.	5.5
XVa	0	0	pos.	6
XP	0	0	neg.	7
XM	0	0	neg.	6
XCa	0	0	?	?
XG	0	0	neg.	5.5
XDa	0	0	neg.	6.5
XDb	0	0	neg.	6.5
XDc	0	0	neg.	5.5
XDd	0	0	neg.	6
XDe	0	0	neg.	6
XDf	0	0	neg.	5.5
Oa	0	0	neg.	6,5
Ob	0	0	neg.	5.5
Of	0	0	neg.	6

APPENDIX E.

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