Effect of Haemolysis on Plasma and Serum Immunoglobulin Estimations

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Summary. Plasma IgG, IgM and IgA estimations were carried out in blood samples with varying degrees of haemolysis, produced by controlled ultrasonication. Extent of haemolysis was determined by plasma haemoglobin, plasma potassium and packed cell volume; and plasma IgG, IgM and IgA estimations showed a steady and significant fall with increasing haemolysis. A calculated correction is proposed which compensates for the dilutional effect of haemolysis, during estimations of plasma and serum immunoglobulins.

Variations in serum immunoglobulin estimations, do not depend only on disease[1], age[2] and sex of the individual[3,4] but may also be affected by the degree of haemolysis present in the sample. The effect of haemolysis on determination of plasma electrolytes and other constituents has been investigated[5] but immunoglobulins were not included in that study. This paper describes the results of studies on the effect of haemolysis on plasma and serum estimations of IgG, IgM and IgA and a calculated correction is suggested to compensate for this effect.

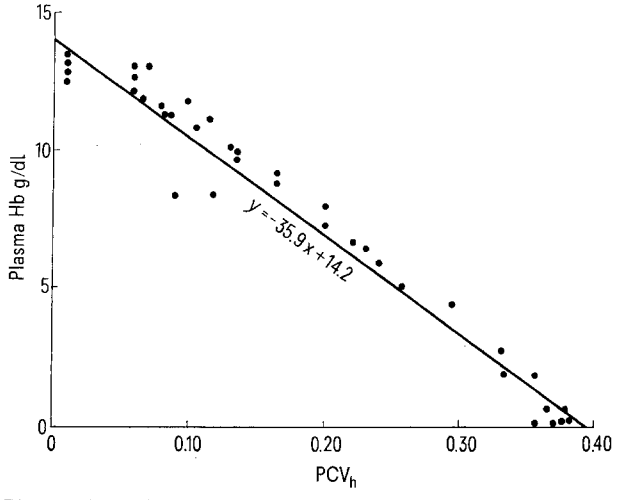

Fig. 1. Plasma haemoglobin measured against packed cell volume following haemolysis.

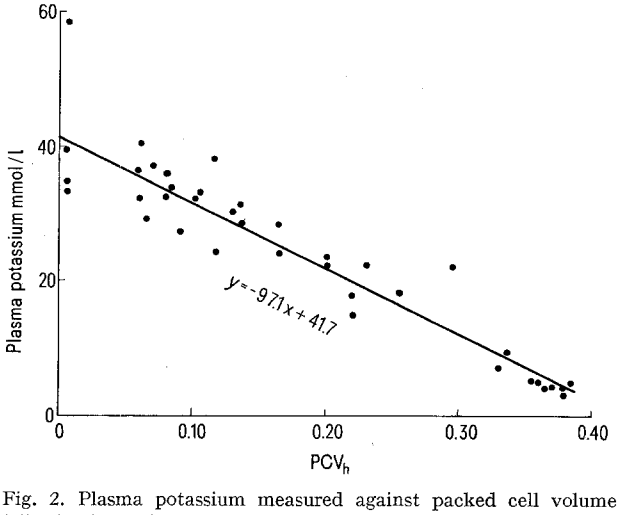

Fig. 2. Plasma potassium measured against packed cell volume following haemolysis.

Materials and methods. Blood samples were obtained from healthy male volunteers, serum separated and stored in 0.2 ml aliquots at −15 °C. Unclotted blood samples were obtained by mixing blood with trisodium citrate and subjected to ultrasonic haemolysis by timed sonication bursts of 1 to 15 sec duration using an MSE 100 Watt Ultrasonic Disintegrator with a titanium vibrator microprobe of end diameter $^1/_8$ inch, having a velocity ratio 7.6/1 at 20 Kc/sec peak probe tip amplitude. Haemolyzed and non-haemolyzed samples were then incubated at room temperature (20 °C ± 2) for 2 h before centrifugation and removal of plasma.

The change in plasma volume following haemolysis was determined by comparison of packed cell volume (PCV) in haemolyzed and unhaemolyzed samples, by filling 75 mm long glass capillaries with anticoagulated blood, sealing and then centrifuging for 10 min in a Hawksley Micro Haematocrit Centrifuge. Using a Hawksley Reader, the PCV value was obtained as a percentage of the total blood volume, and plasma volume obtained by subtraction. After PCV determination, samples were centrifuged at 3000 rpm for 10 min, supernatant plasma collected and stored at −15 °C. Each plasma and serum sample was frozen only once to avoid possible denaturation of immunoglobulins by repeated freezing and thawing[6].

Plasma potassium estimations were carried out by flame photometry in an EEL Mk 11 Flame Photometer (Evans Electroselenium Ltd) using an air/coal gas mixture. Atomic emission of samples at 766.5 nm was compared with that of a series of standards, varying from 2 to 10 mmol/l prepared from a stock solution of 100 mmol/l of 7.46 g KCl (A.R.)/l in deionized water[7]. Haemoglobin concentration (g/dl plasma) was determined by direct reading from the Cyanmethaemoglobin scale of an EEL Haemoglobin Meter (Evans Electroselenium Ltd), which was calibrated using standard cyanmethaemoglobin solutions (Diagnostic Reagents Ltd). Serum and plasma immunoglobulin levels were assayed by the single radial immunodiffusion method[8] as modified by FAHEY and MCKELVEY[9]. Immunodiffusion plates and reference sera were obtained from Hyland Laboratories (California, USA).

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Table I. Comparison of correlation coefficients between uncorrected observed and corrected estimations of immunoglobulins and packed cell volume in haemolyzed plasma

	Uncorrected observed estimations		Corrected estimations	
Immunoglobulin type	Correlation coefficient	Statistical significance	Correlation coefficient	Statistical significance
IgG	+ 0.702	$p < 0.001$	+ 0.2608	N.S.
IgM	+ 0.857	$p < 0.001$	− 0.1460	N.S.
IgA	+ 0.577	$p < 0.001$	− 0.1820	N.S.

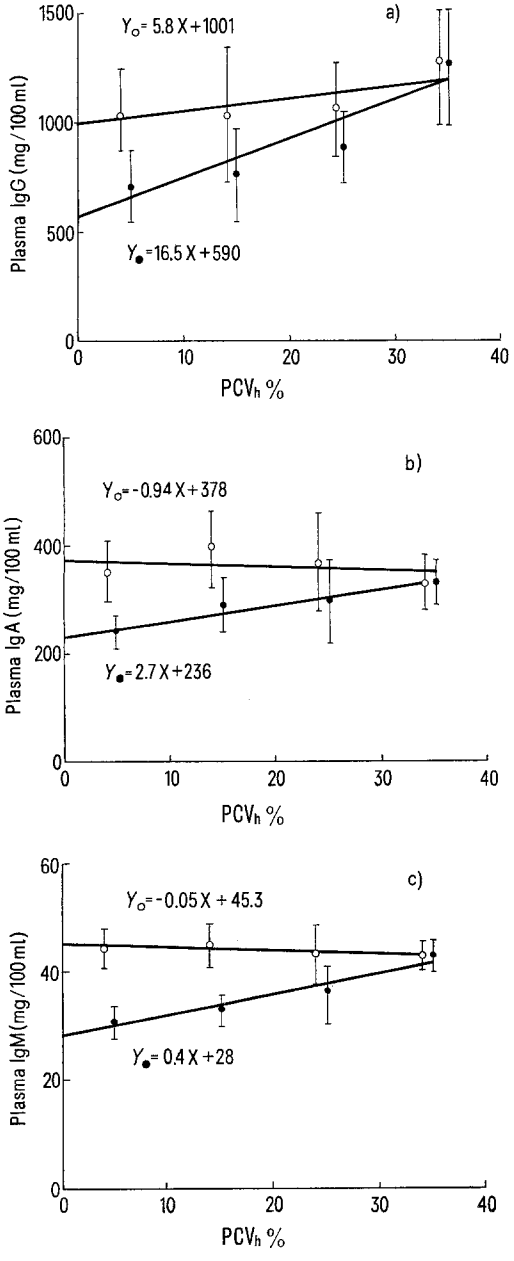

Fig. 3. Regression lines of observed (●) and corrected (○) IgG(a), IgA(b) and IgM(c) (mean ± SE) measured against packed cell volume.

Results and discussion. Ultrasonic treatment of blood samples for increasing periods of time led to haemolysis, the extent of which was determined by measuring plasma haemoglobin, plasma potassium and PCV. A mean control PCV of 0.373 (SEM = 0.5, $n = 5$) was obtained in the unhaemolyzed blood samples. The maximum ultrasonic treatment for 15 sec at 7.5 peak to peak amplitude, caused the PCV to fall to a minimum level of 0.01 representing complete haemolysis.

Haemolysis was absent from control plasma samples but with increasing ultrasonication, plasma haemoglobin concentration increased (Figure 1) as PCV decreased and the correlation was statistically significant ($r = -0.9836$, $p < 0.001$). Plasma potassium concentration in unhaemolyzed samples was 4.4 mmol/l and with ultrasonication rose, as PCV decreased (Figure 2) and the correlation was statistically significant ($r = -0.9362$, $p < 0.001$). Following the determination of PCV and plasma haemoglobin, the plasma IgG, IgM and IgA concentrations were determined and the results are shown in Figure 3. Plasma IgG, IgM and IgA estimations showed a steady fall with increasing haemolysis as measured by decreasing PCV and this correlation is statistically significant (Table II) for all three different types of immunoglobulins measured.

The ratio of plasma volume of haemolyzed blood to plasma volume of unhaemolyzed blood can be used as a correction factor for the dilution effect of haemolysis. Plasma volumes were calculated from the PCV after correction made for plasma trapped in the red cell column of haematocrit. The true PCV has been estimated to be smaller by a factor of 0.96 from the observed PCV[10].

True PCV = 0.96 × Observed PCV
Plasma volume = 100–0.96 × Observed PCV
Packed Cell Volume of control, unhaemolyzed blood = PCV_c
Packed Cell Volume of haemolyzed blood = PCV_h
Immunoglobulin concentration at PCV_h = 'Ig observed'

Then, the immunoglobulin concentration corrected for the dilution effect of haemolysis is given by the expression:

$$\text{Corrected immunoglobulin} = \frac{100-0.96 \times PCV_h}{100-0.96 \times PCV_c} \times \text{'Ig observed' mg/dl}$$

Immunoglobulin estimations of plasma IgG, IgM and IgA, when corrected in this way, and plotted against PCV_h (Figure 3, a, b and c) show no apparent drop in immunoglobulin concentration with haemolysis (Table I). When using this correction on an actual haemolyzed serum sample, the PCV_h is estimated from the plasma haemoglobin and plasma potassium concentrations (Figures 1 and 2).

The present study indicates that serum and plasma IgG, IgA and IgM estimations are affected by haemolysis, in that rupture of red blood cells produces an increased extracellular fluid volume which leads to dilution of immunoglobulins. This dilution of immunoglobulins is quantitatively related to the degree of haemolysis which can be assessed by measuring serum haemoglobin and serum potassium levels.

In clinical laboratories, serum rather than plasma is used for the determination of immunoglobulins and therefore an analysis of serum and plasma immunoglobulin estimations on the same blood samples was carried out in this study. No significant difference between serum and

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Table II. Comparison of the actual error due to haemolysis in IgG, IgM and IgA determinations with the calculated CARAWAY percentage error estimation for different values of plasma haemoglobin concentration

Haemoglobin (g/dl)	PCV	% Error of IgG	IgM	IgA	CARAWAY
0	39.5				
1	36.7	3.9	2.3	3.5	2.9
2	34.0	7.5	5.5	6.0	5.7
3	31.2	11.6	7.7	8.3	8.3
4	28.3	14.9	10.3	10.4	10.7
5	25.6	19.0	13.0	12.4	13.1
6	22.8	22.3	15.3	14.4	15.7
7	20.0	25.8	18.0	17.3	17.4
8	17.3	29.3	20.3	18.7	19.4
9	14.4	33.5	23.0	21.3	21.3
10	11.5	37.7	25.8	23.6	23.1

plasma immunoglobulin levels was found, indicating that trisodium citrate has no significant effect upon the single radial immunodiffusion method and that both serum and plasma immunoglobulin estimations give comparable results.

When a correction factor, derived from the ratio of haemolyzed to unhaemolyzed PCV was applied to the observed immunoglobulin value, a corrected immunoglobulin estimation was obtained which did not vary significantly in plasma throughout the whole range of possible haemolysis.

An expression based upon the relative intracellular and extracellular concentrations of a substance has been formulated by CARAWAY[11] which gives an indication of the percentage error due to haemolysis when measuring the concentration of that substance. The percentage error of extracellular fluid contamination using CARAWAY's formula is determined from the plasma haemoglobin concentration, and is given by:

$$\text{Percentage error} = \frac{3\,h\,(c/s - 1)}{1 + 0.03\,h}$$

where c = concentration of substance in red blood cell, s = concentration of substance in plasma, h = haemoglobin concentration in plasma (g/dl).

The estimation of plasma constituents using CARAWAY's formula gives calculated percentage errors which are in close agreement with actual percentage errors, determined experimentally for many plasma constituents[6], and extending the use of this formula to our study, it shows good agreement between calculated and actual percentage errors produced by haemolysis (Table II).

The results of the present work have a direct relevance to the determination of plasma and serum immunoglobulins in clinical diagnosis and research. Frequently blood specimens sent to a laboratory for analysis, arrive with varying degrees of haemolysis. It is not always possible to reject such specimens because of the clinical state of the subject especially in debilitated patients or neonatal infants. The extra error introduced into the results when using such haemolyzed samples should be acknowledged and wherever possible corrected. The present work indicates some of the limitations of measuring immunoglobulins in haemolyzed sera and provides a method for possible correction of such estimations.

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Native Resistance of *Peromyscus maniculatus* to *Nematospiroides dubius* Infection

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Summary. Because of its native resistance, *Peromyscus maniculatus* cannot be a natural host for *Nematospiroides dubius*.

Nematospiroides dubius, a trichostrongyloid parasite of mice, was first described by BAYLIS[2] when he isolated it as a parasite of the woodmouse, *Apodemus sylvaticus*. EHRENFORD[3] rediscovered and isolated this nematode from the deer mouse, *Peromyscus maniculatus* in 1954 and investigators have maintained *N. dubius* in laboratory mice since that time. Other reports of natural infections of *P. maniculatus* with *N. dubius* are in the literature[4,5]. However, BABERO and MATTHIAS[4] found only 4 adult worms in 3 infected *Peromyscus* mice. Indeed, FORRESTER[6] was unable to detect any *N. dubius* infections in 231 *P. maniculatus* mice that were examined. Also FORRESTER and McL. NEILSON[7] reported no success in attempts to establish patent infections of *N. dubius* in *P. maniculatus* with conventional methods or with 350 rads of γ-radiation to immunosuppress the mice prior to parasitic exposure.

Attempts to infect *P. maniculatus* with *N. dubius* utilizing both laboratory-reared and wild-trapped mice of varying ages were also unsuccessful in our laboratory even when larvae with enhanced virulence were used[8]. However we now report that infections of *N. dubius* can be established when *P. maniculatus* mice are immunologically suppressed with a steroid hormone.

Methods and materials. Peromyscus maniculatus mice were trapped in the area surrounding Fort Collins, Colorado. Young *Peromyscus* mice were obtained by breeding from the trapped *Peromyscus* stock in our own laboratory; outbreeding was carefully maintained. Mice were housed in disposable plastic mouse cages and bedding consisted

[1] We acknowledge the Western Regional No. 102 Project for support of this study.

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