Osmotic Pressures of Experimental and Human Lipaemic Sera. Evaluation of Albumin-Globulin Ratios with the Aid of Electrophoresis

By G. POPJÁK (Beit Memorial Fellow for Medical Research) AND E. F. MCCARTHY
*Department of Pathology, St Thomas's Hospital Medical School, London, S.E. 1,
and The Lister Institute, London, S.W. 1*

(Received 15 June 1946)

It was reported in a previous article (Popják & McCarthy, 1943) that extraction of lipids from normal human serum does not alter the colloid-osmotic properties of serum-proteins. This result was not in agreement with the claims of Macheboeuf (1929), Macheboeuf & Sandor (1931), Fishberg (1929) and Rabinowitch (1930) that the lipids exert an osmotic pressure. Macheboeuf reported that lipaemic sera of patients suffering from lipoid nephrosis (i.e. subacute nephritis of the nephrotic type) exerted a higher osmotic pressure/g. protein than sera of normal subjects, and concluded that this was caused by the high concentration of lipids in these sera. He suggested that the lipaemia of nephrosis is a compensating mechanism for the diminution of osmotic pressure due to the loss of serum-albumin. Rabinowitch suggested that cholesterol in the serum of diabetics exerts an osmotic pressure.

Although our own results showed that the lipids of normal serum have no osmotic effect, there was the possibility that in lipaemic sera osmotic pressures due to lipids might be detectable. We decided therefore to study this question further both in human and animal material. The results reported here were obtained with (a) normal and lipaemic rabbit sera; (b) lipaemic rabbit serum before and after extraction of lipids; (c) normal human serum and a lipaemic serum obtained from a case of subacute nephritis of the nephrotic type.

MATERIAL AND METHODS

Production of lipaemia in rabbits. This was done by feeding amorphous cholesterol (1 g./day) in a watery suspension by the method of Dömösi & Egyed (1939). It was shown by Popják (1945, 1946) that the oral administration of amorphous cholesterol to rabbits, without the addition of fat to the diet, causes a marked increase in all the lipid fractions of the blood-plasma. The cholesterol administration was usually continued for 1-2 months. The rabbits, under nembutal anaesthesia, were bled from the aorta. The sera, when in sufficient quantity, were investigated individually; otherwise the sera of two or more rabbits were pooled.

Normal rabbit serum was obtained in a similar way.

Human serum. Serum was obtained from the blood of a patient (W.C.) aged 54, who suffered from subacute

nephritis of the nephrotic type. He had massive oedema and albuminuria, losing 10-20 g. of protein/day in his urine. There was no haematuria, nor nitrogen retention (serum non-protein-N 39 mg./100 ml.) at the time and no high blood pressure. Approximately a year after our sample was obtained the patient progressed to chronic nephritis with N retention and hypertension.

Normal human serum was obtained from 10 healthy male medical students, equal volumes from each being pooled.

Osmotic pressure measurements. These were made by the method of Adair (1925), using collodion sacs of such permeability that at a pressure of 480 mm. of Hg the rate of filtration of water through the membranes was 0.0015-0.0030 ml./sq.cm./min. Membranes with 30-35 sq.cm. total surface area and a volume of 8-10 ml. were used. These large membranes proved of particular value with low protein concentrations by improving the accuracy of osmotic pressure measurements.

The sera were dialyzed for 48-72 hr. at 0° against several changes of $M/15 PO_4^{3-}$ buffer ($M/30 KH_2PO_4 + M/30 Na_2HPO_4$), pH 6.8. The same buffer was used for the outer fluid in the osmometers. The osmometers were equilibrated for 10-14 days at 0°. The osmotic pressures of several dilutions of the sera were measured in mm. of the protein solution and were calculated as mm. of Hg after determination of the densities of the solutions by pycnometry at 0°. Corrections for capillarity forces were made.

Protein concentration was measured by determination of N by a micro-Kjeldahl method. When the digestion mixtures became clear after being heated with a 1% solution of selenium dioxide in 50% (v/v) H_2SO_4 , the flasks were cooled, a few drops of N-free water and 10 drops of H_2O_2 (B.D.H. micro-analytical reagent, 100 vol.) were added and the digestion was continued for a further 8 hr.

Albumin-globulin ratios. The globulins were precipitated by saturating with $MgSO_4$: to 1 ml. of serum were added 1.2 g. of finely powdered $MgSO_4$ and 7 ml. of a saturated watery solution of $MgSO_4$. The mixture, with occasional shaking, was left at room temperature for 24 hr., after which it was filtered through a Whatman no. 42 filter paper until a crystal-clear filtrate was obtained. Usually two, occasionally three, filtrations (through the same filter paper) were necessary. Albumin-N was determined on a sample of the filtrate.

In some cases the methanol precipitation method of Pillemer & Hutchinson (1945) was also used for the determination of albumin-globulin ratios. According to these authors this method gives albumin-globulin ratios agreeing within 5-10% with values obtained by electrophoretic analysis. We found that this method could not be used on

protein solutions dialyzed against $\text{PO}_4^{=}$ buffer and taken from the osmometers, on account of the incomplete precipitation of globulins. It was employed therefore on undialyzed sera only.

In calculation of protein concentration from N content, the figures for horse serum albumin (15.6% N) and horse serum globulin (15.13% N) were used (Adair & Robinson, 1930a). Thus total protein concentration (C) in g./100 ml. of solution was obtained by the formula

$$C = \left(6.41 + 0.2 \times \frac{\text{g. of globulin-N}}{\text{g. of total N}} \right) \times \text{g. of total protein-N.}$$

Corrections for phospholipid-N were made, 1.8% being taken as the mean N content of the phospholipids (lecithin + cephalin) (cf. Maclean & Smedley Maclean, 1927, pp. 17, 49). In the determinations of albumin-globulin ratios it was assumed that all the phospholipids were precipitated with the globulins.

Lipid analyses. The sera were extracted by boiling with at least 20 vol. of an ethanol-ether mixture (3:1, v/v) (Bloor, 1928). The filtered extracts were evaporated to dryness under reduced pressure (20–40 mm. of Hg) at 30–40° external temperature while a slow current of N₂ gas was passing through the extract. The dry residue was extracted with light petroleum (b.p. 40–60°) and the various lipid fractions were isolated from this light petroleum extract. The phospholipids were precipitated by acetone and MgCl_2 and were determined by the dichromate-oxidation method of Bloor (1929). For the determination of non-phospholipid fatty acids (derived from neutral fats and cholesteryl esters) a sample of the supernatant liquid obtained after precipitation of the phospholipids was evaporated to dryness and the residue saponified with ethanolic KOH (0.2–0.3 ml. of a 40% (w/v) aqueous KOH solution added to 5 ml. of absolute ethanol) at 75° for 2 hr. The ethanol was then evaporated off and the mixture acidified with 5% (w/v) HCl, and was extracted with light petroleum. The fatty acids, redissolved in hot absolute ethanol, were titrated at 75° with 0.02 N-aqueous NaOH. Their average molecular weight was assumed to be 275. Free and total cholesterol were determined by the digitonin method of Popják (1943); ester cholesterol was calculated by difference. Neutral fats were calculated by the formula:

$$\text{Neutral fat} = (\text{non-phospholipid fatty acids} \\ - \text{ester cholesterol} \times 0.72) \times 1.05.$$

The lipid contents of the sera are shown in the tables as mg./g. total protein; this method of presentation was chosen instead of the customary mg./100 ml. because it allows the calculation of the lipid content, from the protein concentration, of all the serum-dilutions used in the osmometry.

RESULTS

I. Evaluation of albumin-globulin ratios with the aid of electrophoresis

It will be seen later that for the interpretation of osmotic pressure measurements it was important to know how far the albumin-globulin ratios obtained by the MgSO_4 method approximate the 'true' values. The ratio obtained by salt precipitation methods can give only approximate values on account of the solubility characteristics of proteins.

These and other methods are known to yield conflicting results. Taylor & Keys (1943) concluded from their experiments and a review of earlier work that the ratios in human serum estimated by sodium sulphate are about 5% higher than those given by electrophoresis and that this discrepancy is increased in abnormal sera. Ultracentrifugal analysis yields a considerably higher ratio than electrophoresis (Kekwick, 1939). Pillemer & Hutchinson (1945) reported a closer agreement, within 5%, between the electrophoretic and methanol methods on normal sera, although a greater divergence occurs in the case of pathological sera. It seemed probable that the magnesium sulphate method, which according to our experience gives lower values than the sodium sulphate method, might yield results in agreement with those from electrophoresis. This point was therefore tested.

Electrophoresis. The serum samples were dialyzed in cellophan bags against $\text{PO}_4^{=}$ buffer, pH 8, $\mu=0.2$. The difference in refraction between the inner fluid (n_1) and the outer (n_0) was measured with a dipping refractometer ($\lambda=546 \text{ m}\mu$). The choice of ($n_1 - n_0$) values was determined in each case by the turbidity of the solution. All samples were centrifuged before electrophoresis to remove the slight precipitate which formed during dialysis.

The electrophoretic examination was made in a Tiselius (1937) apparatus at 0° with a potential gradient of 5 V./cm. The diagonal schlieren method and monochromatic light ($546 \text{ m}\mu$) were used for the optical observations. The relative concentration of components was measured by optical projection and tracing of the photographs on graph-paper followed by extrapolation and planimetry. Anode and cathode limb pictures were used in the analyses.

The high degree of lipaemia in some of our sera caused considerable experimental and analytical difficulties. Slowly separating δ or ϵ and γ boundaries also rendered the estimation of γ -globulin inaccurate in certain experiments. Discrepant results in our early attempts to measure the albumin-globulin ratios of lipaemic sera by electrophoresis led to the adoption of various technical modifications.

The results of electrophoretic analyses of the sera are shown in Table 1 and Fig. 1. Exps. 1 and 2 (Fig. 1 (a) and (b)) record results obtained with two samples of pooled normal rabbit sera. The agreement found between the albumin-globulin ratios based on exposures obtained simultaneously from the anode and cathode limbs appears to be reasonably close in both experiments. The pattern of the descending boundaries in Exp. 1 (Fig. 1 (a)) showed the ' β -globulin disturbance' (Longworth, Shedlovsky & MacInnes, 1939); the area due to this effect was included as globulin. The percentage albumin content of this serum found by electrophoresis seems in agreement with that obtained by MgSO_4 precipitation but differs widely from the result determined by the methanol method.

The data of Exp. 2 were measured on a more representative sample of rabbit serum composed of

Table 1. *Percentage albumin content of normal and lipaemic sera as determined by electrophoresis and chemical methods*

Exp. no.	Material	Albumin content determined by				Duration of electro- phoresis (sec.)	(n ₁ - n ₀)
		Electrophoresis		MgSO ₄	Methanol		
		Cathode	Anode				
		(% of total protein)					
1	Pooled serum of three normal rabbits	64.2	63.6	64.2	74.3	14,410	0.00388
2	Pooled serum of ten normal rabbits	71.4	70.0	68.8	74.1	13,680	0.00400
3	Normal human serum	66.0	64.6	68.5	—	16,620	0.00394
4	Lipaemic rabbit serum, pooled from three cholesterol-fed animals	56.0	39.9	58.0	42.8	14,700	0.00179
5	<i>Idem</i> , Seitz-filtered	55.1	40.2			15,480	0.00342
6	Lipaemic rabbit serum no. 3; pooled from five animals, Seitz-filtered (1)	55.3	—	55.55	46.0	20,220	0.00180
7	<i>Idem</i> , Seitz-filtered (2)	63.6	51.8			—	—
8	<i>Idem</i> , 'defatted', dried and reconstituted	62.2	59.6	55.55	—	19,620	0.00288
9	<i>Idem</i> , 'defatted' but not dried	60.9	58.7			17,820	0.00301

Fig. 1. Electrophoretic patterns of normal rabbit and human sera, and of lipaemic rabbit sera. (a) and (b) normal rabbit serum; (c) normal human serum; (d) lipaemic rabbit serum; (e) same as (d) after Seitz-filtration; (f) lipaemic rabbit serum no. 3 Seitz-filtered (1); (g) same as (f) Seitz-filtered separately (2); (h) same as (f) after 'defatting', the serum was dried and reconstituted; (i) same as (f) after 'defatting', the serum was not dried.

equal volumes of sera from 10 animals. The result is higher than that of Exp. 1; the latter agrees with the latest findings of Svensson (1946). The values yielded by the magnesium sulphate and electrophoretic methods are in substantial agreement, although the former gave slightly lower albumin content in Exp. 2 than the latter.

The values given in Exp. 3 (Fig. 1 (c)) were obtained on the normal human serum used for measuring the osmotic pressures (see Tables 8, 9; Figs. 7, 8). The average albumin-globulin ratio computed from both limb patterns is slightly lower than that estimated by MgSO_4 precipitation.

Data for a lipaemic rabbit serum are shown under Exp. 4, Table 1, and in Fig. 1 (d). The serum was very milky and had to be diluted until photographic recording was possible. Analysis showed a relative increase in the globulin area, mainly in the α - and β -components. Blix, Tiselius & Svensson (1941) observed that human β -globulin was characterized by a high lipid content and Zeldis, Alling, McCoord & Kulka (1945) reported a similar attribute of the α -globulin of dog plasma. The enlargement of the globulin area in our experiments was much more marked on the anode pattern and caused a great discrepancy in the albumin-globulin ratio computed from the exposures obtained simultaneously from both limbs. This finding may indicate the presence of some interaction of components which is more strongly marked in one limb of the U-tube. Seitz-filtration of the same serum and subsequent dilution and electrophoresis yielded the data of Exp. 5, Table 1 (Fig. 1 (e)). It is obvious that either one or both of the chemical methods is subject to great error.

The albumin concentrations recorded under Exps. 6 and 7 (Fig. 1 (f) and (g)) were measured on two separately Seitz-filtered samples of lipaemic rabbit serum no. 3, which was used for osmotic pressure measurements (see Tables 5, 6, 7; Figs. 5, 6). The discrepancy in the results seemed to indicate a loss of protein in the filtration process. Since the turbidity of the unfiltered solution made complete photographic recording impossible and since facilities for supercentrifugation at 30,000 r.p.m. (cf. Longworth *et al.* 1939) were not available, an attempt was made to measure the albumin-globulin ratio indirectly by the following procedure. The serum (6 ml.) was centrifuged at 3600 r.p.m. for 1 hr. until a thick supernatant pad of fat had separated. The solution was then dialyzed and diluted to a $(n_1 - n_0)$ value of 0.00096, at which concentration the separated albumin boundary was visible in advance of the opaque globulin region during electrophoresis. The albumin concentration was calculated from the following equation (Svensson, 1939):

$$C = \frac{U}{F \cdot G \cdot k \cdot l \cdot a \cdot \tan \theta},$$

in which C = protein concentration; U = the area of the component on the photographic plate; F = magnification factor between the plate and the inclined edge (horizontal magnification); G = magnification factor between the plate and the cell (vertical magnification); k = refractive index increment per unit concentration; l = the optical distance between the cell and the slanting edge; a = cell thickness; and θ = the angle of the inclined slit with the vertical.

The mean value of the albumin concentration calculated from five exposures was $0.48\% \pm 0.02$. The corresponding albumin-N, calculated on the basis of 15.6% N content, was 0.749 mg./ml. of solution. The solution contained 0.923 mg. total protein-N/ml., yielding an albumin-globulin ratio of 4.3. This improbable finding led to the discontinuance of the method.

The difficulties encountered in our attempts to measure the albumin-globulin ratio in lipaemic sera by electrophoresis suggested the extraction of lipids ('defatting') as a preliminary step. The alteration caused by this procedure in the electrophoretic patterns of lipaemic human and dog sera described by Zeldis *et al.* (1945) seems to demonstrate, moreover, that electrophoretic analysis of untreated lipaemic sera does not assess accurately the distribution of the protein fractions.

The analysis listed in Table 1 as Exp. 8 (Fig. 1 (h)) was made on lipaemic rabbit serum no. 3 used in Exps. 6 and 7 and in measurement of osmotic pressures (lipaemic rabbit serum no. 3 after 'defatting'; see Tables 5, 6, 7; Figs. 5, 6). 'Defatting' was effected by the method of McFarlane (1942) and loss of protein was prevented by the precautions described in section III of the present article. After ether extraction a portion of the serum was dried from the frozen state *in vacuo* over P_2O_5 and reconstituted subsequently. After the usual preliminary dialysis, an electrophoretic analysis was made. The originally clear solution developed a considerable turbidity during temperature equilibration (0°), and photographic recording and tracing were rendered difficult. The experiment was repeated therefore on the same extracted serum with the omission of the drying process. As an additional precaution, prior to electrophoresis, the dialyzed serum was poured into an inclined test-tube and left at 0° overnight. The supernatant fluid was pipetted off from a very slight sediment and yielded on analysis the data of Exp. 9 (Fig. 1 (i)). The experimental error could account for the difference in the results of Exps. 8 and 9. The average albumin content computed from anode and cathode limb patterns of Exps. 8 and 9 is 61.55%. By the MgSO_4 method an albumin content of 55.55% was found.

Finally the accuracy of the MgSO_4 method for the estimation of the albumin-globulin ratio was investigated by the following procedure. Two large

volumes of rabbit serum, normal and lipaemic, were fully saturated with MgSO_4 and filtered three times through a Whatman no. 42 paper. The clear 'albumin' filtrates were dialyzed against distilled water at 0° until the dialysis fluid gave a negative BaCl_2 test for $\text{SO}_4^{=}$. The protein solutions were then equilibrated against $\text{PO}_4^{=}$ buffer, pH 8, $\mu = 0.2$. The precipitate, collected on the filter-paper, was washed three times with a saturated solution of MgSO_4 and then redissolved. The solution was dialyzed against distilled water and $\text{PO}_4^{=}$ buffer. The 'globulin' solution of the normal serum was slightly cloudy, while that of the lipaemic serum was intensely milky. Both were 'defatted' by the method of McFarlane (1942). The clear solutions which resulted were again dialyzed against $\text{PO}_4^{=}$ buffer. The protein solutions prepared from the precipitates and filtrates were then subjected to electrophoresis.

of doubtful significance, since the experimental error was increased by turbidity of the solutions during electrophoresis. Since mobility measurements were not made, the identity of the components in the 'albumin' (Fig. 2 (a), (b)) and 'globulin' (Fig. 2 (c), (d)) preparations remains open to conjecture. Inspection alone indicates, however, that the filtrates contained α -globulin as well as albumin and that the β - and γ -globulins are retained in the precipitates, which show no evidence of presence of albumin.

The amounts of α -globulin remaining in solution were small and approximately equal in the normal and lipaemic serum. Since we have no data regarding the molecular weight of this globulin fraction, we decided to use the albumin-globulin ratios obtained by the MgSO_4 method, without any corrections, in the theoretical interpretation of the osmotic pressure data. It can be inferred that the

Table 2. *Percentage distribution of electrophoretic components in the 'albumin' and 'globulin' fractions prepared from normal and lipaemic rabbit sera by saturation with MgSO_4*

Fig. 2. Electrophoretic patterns of 'albumin' and 'globulin' fractions prepared from normal and lipaemic rabbit sera by saturation with MgSO_4 . (a) 'albumin' fraction from normal and (b) from lipaemic serum; (c) 'globulin' fraction from normal and (d) from lipaemic serum.

The results are shown in Table 2, Fig. 2. The quantitative data on the 'albumin' solutions were obtained by extrapolation of the small component only, which was then expressed as a percentage of the total area. The slight difference found in the percentage of the smaller component in both filtrates is probably not significant in view of a marked discrepancy between the anode and cathode limb measurements made on the filtrate of the lipaemic serum. The small differences observed in the proportions of globulins in the two preparations are also

albumin-globulin ratios estimated by MgSO_4 precipitation are very nearly the 'true' ratios and that even the presence of very large amounts of lipids does not influence the results. The latter point was further emphasized by the observation that this method gives identical results for lipaemic sera before and after extraction of lipids. It is of interest to note also that, according to Dr M. E. Adair (1945), of several procedures tested, the MgSO_4 method gives the closest agreement with albumin-globulin ratios estimated serologically.

II. Results of osmotic pressure measurements on normal and lipaemic rabbit sera

Table 3 shows the lipid content of the normal and of the lipaemic rabbit sera. It can be seen that administration of amorphous cholesterol alone, without the addition of fat to the diet, produced a very

Table 3. Lipid content of normal rabbit serum and lipaemic rabbit sera nos. 1 and 2 used for measurement of osmotic pressures

	Normal serum*	Lipaemic serum no. 1†	Lipaemic serum no. 2‡
	(mg./g. total serum protein)		
Neutral fat	8.5	43.5	119.0
Phospholipids	12.9	65.0	78.5
Cholesterol {free	2.4	46.0	58.5
ester	8.9	118.0	129.0
Fatty acids of cholesterol esters	6.4	85.0	93.0
Total lipid	39.0	346.0	479.0

* Pooled serum of three normal rabbits.

† Pooled serum of two cholesterol-fed rabbits (Ch 5 and 6).

‡ Serum of cholesterol-fed rabbit (Ch 3).

marked increase in all the lipid fractions of the serum (cf. Popják, 1945, 1946). It would seem, therefore, that if any of the serum-lipid fractions exert an osmotic pressure or influence the colloid-osmotic properties of the proteins such effects should be detectable with these sera.

In Fig. 3 osmotic pressures are plotted against protein concentrations. An inspection of the curves reveals that the lipaemic sera exerted a lower osmotic pressure/g. of total protein than did the normal serum.

The observed osmotic pressure (p) of unfractionated serum proteins is the sum of a number of factors and by applying Dalton's law of partial pressures (Adair & Robinson, 1930b) it can be expressed as

$$p = p_a + p_g + p_i, \quad (1)$$

where p_a and p_g = the partial pressures of serum albumin and globulins respectively; and p_i = the ion pressure difference due to the excess of ions within the membrane. Since the unequal distribution of ions between the inner and outer fluids in the osmometers may affect considerably the observed osmotic pressures at high protein concentrations (cf. Adair & Robinson, 1930b), the comparison of the osmotic effects of different sera has to be made at infinite dilutions of the proteins, when the term p_i in equation (1) becomes zero. Such comparison can be effected by the determination of π_0 , i.e. the limiting value of the ratio osmotic pressure/protein concentration (p/C) = π in an infinitely dilute solution, by extrapolation. Further, since the osmotic pressure of serum at infinite dilution is equal to the sum of the partial pressures of serum albumin and of serum globulins, in analyzing our data the relative proportions of these proteins in the sera have to be taken into account also.

Table 4 gives detailed data for the osmotic pressure measurements illustrated in Fig. 3. Fig. 4 shows the determination of $1/\pi_0$ by extrapolation (Adair & Robinson, 1930b). Within the range of protein concentrations employed, there is a linear relationship

Fig. 3. Relationship between protein concentration and osmotic pressure of (I) normal, (II) and (III) lipaemic rabbit sera nos. 1 and 2 respectively. The continuous curves have been calculated by the equation

$$p = \pi_0 C / (1 - K_b C).$$

○ observed values for normal, △ and ▽ for lipaemic rabbit sera nos. 1 and 2.

between $1/\pi$ ($= C/p$) and C , which can be expressed by the type of equation given below

$$1/\pi = b - aC, \quad (2)$$

where $b = 1/\pi_0$, i.e. the value of $1/\pi$ when $C \rightarrow 0$. The straight lines in Fig. 4 are represented by the following equations:

$$\begin{aligned} \text{I (for normal rabbit serum)} \quad & 1/\pi = 0.467 - 0.0238C \\ \text{II (for lipaemic serum no. 1)} \quad & 1/\pi = 0.507 - 0.0250C \\ \text{III (for lipaemic serum no. 2)} \quad & 1/\pi = 0.550 - 0.0275C \end{aligned}$$

Hence the value of π_0 for the normal serum is 2.14, and for lipaemic sera 1 and 2, 1.97 and 1.82 respectively. The lipaemic sera, therefore, exerted

Table 4. Data for osmotic pressure measurements on normal and lipaemic rabbit sera (see also Figs. 3, 4)

Osmo- meter no.	Protein concentration		Osmotic pressure p (mm. Hg at 0°)	$1/\pi (=C/p)$	$1/\pi_v (=C_v/p)$	$\frac{\text{g. albumin}}{\text{g. total protein}} \\ (X_a)$ (by MgSO_4)	$v_{pi}^\dagger$
	C (g./100 ml. of solution)	C_v^* (g./100 ml. of solvent)					
(a) Normal serum (see also Figs. 3, 4, curve I)							
1	7.13	7.550	24.07	0.296	0.314	0.675	0.767
2	6.21	6.520	19.47	0.319	0.335		
3	4.66	4.838	13.08	0.356	0.370		
4	3.57	3.671	9.36	0.381	0.393		
5	2.59	2.643	6.40	0.405	0.413		
6	2.86	2.925	7.13	0.401	0.411		
7	0.94	0.947	2.15	0.437	0.441		
(b) Lipaemic serum no. 1 (see also Figs. 3, 4, curve II)							
8	6.07	6.495	17.12	0.355	0.379	0.611	1.074
9	4.87	5.140	12.91	0.377	0.398		
10	3.97	4.150	9.75	0.407	0.425		
11	3.00	3.100	6.71	0.447	0.462		
12	1.92	1.960	4.20	0.456	0.467		
13	1.27	1.289	2.69	0.473	0.479		
14	0.82	0.828	1.67	0.491	0.495		
(c) Lipaemic serum no. 2 (see also Figs. 3, 4, curve III)							
15	6.48	7.030	17.58	0.369	0.400	0.520	1.206
16	5.32	5.683	13.15	0.405	0.433		
17	4.23	4.460	9.60	0.440	0.464		
18	3.02	3.136	6.37	0.474	0.493		
19	2.17	2.230	4.56	0.477	0.489		
20	1.13	1.146	2.18	0.519	0.526		

* Calculated by $C_v = C \frac{100}{100 - v_{pi}C}$.

† v_{pi} = vol. of 1 g. dry protein + x g. lipids/g. protein.

definitely a lower osmotic pressure/g. protein than did the normal serum.

Fig. 4. Relationship between protein concentration and $1/\pi (=C/p)$. $\circ$ observed values for normal, $\triangle$ and ∇ for lipaemic rabbit sera nos. 1 and 2 respectively. The straight lines represent the equations: (I) $1/\pi = 0.467 - 0.0238C$; (II) $1/\pi = 0.507 - 0.0250C$; and (III) $1/\pi = 0.550 - 0.0275C$.

Evidence on the question whether the observed differences in the osmotic pressures of the various

sera are due to their different albumin-globulin ratios, or to their different lipid contents, can be obtained by comparing the observed and theoretical values of π_0 .

At infinite dilution the osmotic pressure of the serum can be written (Adair & Robinson, 1930b)

$$p = RT(10C_a)/M_a + RT(10C_g)/M_g, \quad (3)$$

where C_a and C_g denote the concentrations of albumin and globulins in g./100 ml. of solution; M_a and M_g the molecular weights of albumin and globulins respectively. If it be assumed that $M_a = 72,000$ and $M_g = 170,000$ (values known for horse serum albumin and unfractionated horse serum globulins; Adair & Robinson, 1930b), the value of theoretical π_0 at 0° obtained from equation (3) is

$$\pi_0 = 2.36X_a + 1.00(1 - X_a), \quad (4)$$

where X_a = g. of albumin/g. of total protein, and therefore $(1 - X_a)$ = g. of globulin/g. of total protein.

For the normal serum X_a is equal to 0.675, and for lipaemic sera nos. 1 and 2, 0.611 and 0.520 respectively, as determined by the MgSO_4 method. The theoretical values of π_0 for the normal serum and for lipaemic sera nos. 1 and 2 are 1.92, 1.83 and 1.71 respectively. If these figures be compared with the observed values of π_0 , it is evident that, although they are not identical, the divergence from the

normal is of the same order of magnitude in both the observed and theoretical values.

The discrepancy between the observed and theoretical values of π_0 can be explained by three factors: (a) that in the calculation of theoretical π_0 , constants for horse serum proteins were used; (b) that the albumin-globulin ratios determined by the salt precipitation method may not represent the true values; and (c) that in the unfractionated sera osmotically active undialyzable substances of low molecular weight are present besides albumin and globulins.

III. Osmotic pressure measurements on lipaemic rabbit serum before and after 'defatting'

In a further endeavour to obtain a definite answer to our main problem, whether the serum-lipids exert an osmotic pressure, we investigated the colloid-osmotic properties of a lipaemic rabbit serum before and after extraction of lipids ('defatting').

'Defatting' of lipaemic rabbit serum. This was done by the method of McFarlane (1942), i.e. by shaking the serum with ether and freezing the mixture to below -25° , followed by thawing. In our previous work (Popják & McCarthy, 1943), we encountered a loss of protein during the extraction. This loss was caused by the fact that during thawing the vessel was kept in an inverted position, the thawed mixture dripping away, while on the surface of the ice a thick, creamy, yellowish material separated out and was discarded. Analyses of the serum before and after extraction, and of the ethereal extract, indicated that this creamy material must have contained both proteins (mostly globulins) and lipids. As a consequence the albumin-globulin ratio of the serum was altered after extraction. To avoid such a loss of protein and thus alteration of the albumin-globulin ratio, in the present experiments the large centrifuge tube containing the frozen serum-ether mixture was immersed for the thawing in an upright position in cold water. The serum was centrifuged after thawing to obtain a better separation of the ether from the serum. A whitish fluffy material separated out, after centrifuging, between the serum and the ether; only the ethereal layer was siphoned off, the 'fluffy' material being left behind. The originally intensely milky serum became crystal clear after this process had been repeated five times and after the last centrifuging the serum-ether mixture separated into the clear serum and ether without the intermediate zone of 'fluffy' material, the latter apparently becoming redissolved in the serum. The ether remaining in the serum was removed by dialysis against several changes of buffer. After the ether was thus removed, because some dilution had taken place, the serum was concentrated first to its original volume by ultrafiltration and a sample taken for an osmometer and lipid analysis. As ample material was available the rest of the serum was further concentrated.

Table 5 shows the lipid content of lipaemic serum no. 3 before and after 'defatting'. This serum had the highest lipid content (4.17 g./100 ml. undiluted serum) of all the sera investigated. It can be seen that approximately nine-tenths of the total lipids

Table 5. Lipid content of pooled lipaemic rabbit serum no. 3 before and after 'defatting'

	Before 'defatting'	After 'defatting'
	(mg./g. total serum protein)	
Neutral fat	28	0.65
Phospholipids	112	59.00
Cholesterol	85	None
Cholesterol { free ester	253	1.50
Fatty acids of cholesteryl esters	182	0.98
Total lipid	660	62.00

were extracted. Most of the remaining one-tenth is accounted for by the phospholipids, of which only about one-half was removed. The ethereal extract (1.433 g.) was analyzed for the distribution of lipids and N content. From Table 6 it is evident that the

Table 6. Theoretical and observed percentage distribution of lipids in ether extract of lipaemic rabbit serum no. 3

	Theoretical (% of total extract)	Observed
Non-phospholipid fatty acids (fatty acids derived from neutral fats and cholesteryl esters)	34.0	33.8
Neutral fat glycerol	0.5	0.5*
Phospholipids	9.3	9.1
Free cholesterol	14.2	16.2†
Ester cholesterol	42.0	39.0†
	100.0	98.6

* Determined by the periodate method of Voris, Ellis & Maynard (1940).

† These observed values compared with the theoretical ones indicate that a hydrolysis of cholesteryl esters had taken place either during the extraction or while the ether extract was stored before the analysis.

observed percentage distribution of lipids agreed very well, within the error of the methods used for analysis, with the theoretical distribution calculated from the lipid content of the serum before and after 'defatting'. The N content of the ether extract was 0.36 mg./100 mg. dry lipid extract; approximately 50% of this small amount of N is accounted for by the phospholipids. This is in agreement with our previous finding that the amount of protein in such ethereal extracts is negligible.

Fig. 5 (see continuous curves) shows the osmotic pressure-concentration curves of lipaemic serum no. 3 before and after 'defatting', the detailed data pertaining to these measurements being given in Table 7. At high protein concentrations the lipaemic serum had a slightly higher osmotic effect than after 'defatting'. The observed values of π_0 (value of p/C at infinite dilution of protein), however, are the same, 1.923, before and after 'defatting' (see Fig. 6, continuous lines). Because the albumin-globulin

ratio of the serum determined by the MgSO_4 method was the same after the extraction as before ($X_a = 0.556$), the theoretical value of π_0 is identical for this serum both in its lipaemic and extracted state, i.e. 1.76. It is of special interest that the albumin-globulin ratio, determined by the MgSO_4 method, was the same before and after 'defatting', which

Protein concentration: for continuous curves g./100 ml. of solution (C); for interrupted curve g./100 ml. of solvent (C_v)

Fig. 5. Relationship between protein concentration and osmotic pressure of lipaemic rabbit serum no. 3 before ($\odot$ and $\bullet$) and after ($\triangle$ and $\blacktriangle$) 'defatting'. Protein concentration is expressed as g. dry protein/100 ml. of solution (C) for $\odot$ and $\triangle$, and as g. dry protein/100 ml. of solvent (C_v) for $\bullet$ and $\blacktriangle$. The data for the continuous curves have been calculated by the equation

$$p = \pi_0 C / (1 - a\pi_0 C)$$

and for the interrupted curve by

$$p' = \pi_0 C_v / (1 - a'\pi_0 C_v).$$

It can be seen that when protein concentration is expressed as g./100 ml. of solvent the observed osmotic pressures for both the lipaemic and 'defatted' serum fall on a single curve (III).

indicates that this method is not influenced by the presence of even large amounts of lipids. It may be mentioned that, while this serum had a higher lipid content than lipaemic serum no. 1 or 2, its albumin-globulin ratio was intermediate between that of the two sera, and the value of the observed π_0 for lipaemic serum no. 3 similarly occupied an intermediate position between those of lipaemic sera

nos. 1 and 2. These observations confirm our view that the colloid-osmotic effect of the lipaemic sera differed from that of the normal serum because of their different albumin-globulin ratios and not because of their different lipid contents.

From Fig. 5 (continuous curves), however, it is evident that at high protein concentrations lipaemic serum no. 3 exerted a slightly lower osmotic pressure after ether extraction than before. Similarly it can be seen in Fig. 6 (continuous lines) that the values of

Protein concentration: for continuous lines g./100 ml. of solution (C); for interrupted line g./100 ml. of solvent (C_v)

Fig. 6. Relationship between protein concentration, C and C_v , and $1/\pi (=C/p)$ and $1/\pi_v (=C_v/p)$ for lipaemic rabbit serum no. 3 before ($\odot$ and $\bullet$) and after ($\triangle$ and $\blacktriangle$) 'defatting'. $\odot$ and $\triangle$, $1/\pi$ plotted against C ; $\bullet$ and $\blacktriangle$, $1/\pi_v$ plotted against C_v . The straight lines represent the equations:

(I) $1/\pi = 0.520 - 0.0284C$; (II) $1/\pi = 0.520 - 0.0255C$; and (III) $1/\pi_v = 0.520 - 0.0212C_v$.

$1/\pi (=C/p)$ obtained at high protein concentrations of the extracted serum were higher than those of the serum in its lipaemic state, but the straight lines representing the relationships between $1/\pi$ and C converge with decreasing protein concentrations. It is apparent, therefore, that from the point of view of osmotic effect at high protein concentrations there is a difference between the serum in its lipaemic and extracted state.

We sought to explain this difference and, as will be shown, it is due to the fact that in the lipaemic sera the lipids occupy a considerable volume and thus reduce the solvent available to the proteins, with the result that the effective concentration of the proteins is higher in the lipaemic than in the 'defatted' serum. When the corrections for volume occupied by proteins and lipids in the solution are made and the concentrations of proteins expressed as g./100 ml. of solvent, instead of g./100 ml. of solution, the difference between the osmotic pressure-concentration curves of the lipaemic and 'defatted' sera is completely eliminated.

Table 7. *Data for osmotic pressure measurements on pooled lipaemic rabbit serum no. 3 before and after 'defatting' (see also Figs. 5, 6)*

Osmometer no.	Protein concentration		Osmotic pressure p (mm. Hg at 0°)	$1/\pi (=C/p)$	$1/\pi_v (=C_v/p)$	$\frac{\text{g. albumin}}{\text{g. total protein}}$ $\frac{(X_a)}{(\bar{X}_a)}$ (by MgSO_4)	v_{pi}
	C (g./100 ml. of solution)	C_v (g./100 ml. of solvent)					
(a) Serum before 'defatting'							
21	6.32	6.930	18.68	0.339	0.371	0.554*	1.386
22	5.39	5.830	14.57	0.370	0.400		
23	4.09	4.340	10.15	0.403	0.428		
24	3.17	3.315	7.33	0.433	0.452		
25	2.18	2.248	4.75	0.458	0.474		
26	1.10	1.117	2.275	0.484	0.492		
(b) Serum after 'defatting'							
27	7.23	7.670	21.50	0.336	0.357	0.557*	0.791
28	6.10	6.410	16.77	0.364	0.382		
29	4.76	4.950	11.93	0.399	0.415		
30	3.57	3.676	8.28	0.431	0.444		
31	2.35	2.396	5.09	0.462	0.471		
32	1.20	1.212	2.454	0.489	0.494		

* The difference between these two values is within the experimental error of the method, therefore in the calculation of theoretical π_0 for the serum before and after 'defatting' a mean value of 0.556 was taken for X_a .

The specific volumes of unfractionated serum proteins and lipids were determined on highly concentrated sera (16–22% protein) as set out in section VI. We found the specific volume of the unfractionated proteins of rabbit and human serum, without water of hydration, to be 0.729 and 0.731 respectively, and that of the serum lipids 0.996. Thus the volume occupied by 1 g. of dry protein + x g. lipids/g. protein (v_{pi}) in the normal rabbit serum was 0.767, in lipaemic sera nos. 1 and 2, 1.074 and 1.206, and in lipaemic serum no. 3, before and after 'defatting', 1.386 and 0.791 ml. respectively. The corrected concentration of the proteins, C_v (g. dry protein/100 ml. of solvent), was then calculated by the formula:

$$C_v = C \times \frac{100}{100 - v_{pi}C} \quad (5)$$

It follows that at finite protein concentrations $p/C_v < p/C$ and $C_v/p > C/p$. The values of C_v and $1/\pi_v (=C_v/p)$ for the different sera are given in Tables 4 and 7 and the data for lipaemic serum no. 3 before and after 'defatting' are illustrated in Figs. 5 and 6 (interrupted curves). It can be seen from Fig. 5 that when the observed osmotic pressures of lipaemic serum no. 3 before and after 'defatting' are plotted against C_v the points fall on a single curve. Similarly the values of $1/\pi_v$ plotted against C_v are on the same straight line (see Fig. 6, interrupted line), which can be expressed by the equation:

$$1/\pi_v = 0.520 - 0.0213 C_v.$$

It follows that π_0' (the limiting value of π_v when $C_v \rightarrow 0$) is equal to $1.923 = \pi_0$.

IV. Osmotic pressure measurements on normal and lipaemic (nephrotic) human serum

The relevant data are presented in Tables 8 and 9 and Figs. 7 and 8. The nephrotic serum, which contained about three times the quantity of lipids as the normal serum (cf. Table 8), exerted a much lower osmotic pressure than the normal serum; the observed π_0 for the former being 1.515 and for the latter 2.020. The albumin content of the nephrotic serum was 32.30% of the total proteins ($X_a = 0.323$), and that of the normal serum 68.50% ($X_a = 0.685$). The theoretical value of π_0 , calculated by formula (4), is 1.439 for the lipaemic and 1.931 for the normal serum.

We could not find, therefore, any evidence to substantiate Macheboeuf's statement that nephrotic sera exert a higher osmotic pressure/g. protein than do normal sera. It might be mentioned that we investigated also another nephrotic serum, with substantially the same results as those presented.

V. Considerations of certain constants (π_0 , K_b) of osmotic pressure measurements. The mean molecular weight of protein complexes of the sera

It has already been stated that, within the range of protein concentrations employed in this investigation, there is a linear relationship between $1/\pi (=C/p)$ and C , or between $1/\pi_v (=C_v/p)$ and C_v , which can be expressed by the empirical equations:

$$1/\pi = 1/\pi_0 - aC, \quad (6)$$

$$1/\pi_v = 1/\pi_0' - a'C_v. \quad (6a)$$

Table 8. *Lipid content of human sera used for osmotic pressure measurements*

	Normal serum (mg./g. total serum protein)	Nephrotic serum
Neutral fat	19.5	77.0
Phospholipids	33.0	70.0
Cholesterol { free	5.3	23.5
ester	20.0	48.5
Fatty acids of cholesteryl esters	14.4	34.9
Total lipid	92.0	244.0

molecular weight of the protein mixture. It was thought, therefore, that an examination of a , as well as that of the product $a\pi_0 = K_b$, might give information regarding differences between normal and lipaemic, and lipaemic and 'defatted' sera.

In Table 10 values of a , π_0 , and K_b are shown when protein concentration is expressed as g./100 ml. of solution and can be compared with the values of the same constants when protein concentration is expressed as g./100 ml. of solvent (a' , π_0' , and K_b'). It can be seen that in the first case the value of a increases with the increasing lipid content of the

Table 9. *Data for osmotic pressure measurements on normal and lipaemic human serum (see also Figs. 7, 8)*

Osmometer no.	Protein concentration		Osmotic pressure p (mm. Hg at 0°)	$1/\pi (=C/p)$	$1/\pi_v (=C_v/p)$	$\frac{\text{g. albumin}}{\text{g. total protein}}$ $(\bar{X}_a)$ (by MgSO_4)	v_{pi}
	C (g./100 ml. of solution)	C_v (g./100 ml. of solvent)					
	(a) Normal human serum						
33	7.237	7.695	24.35	0.297	0.316	0.685	0.823
34	6.088	6.409	18.69	0.326	0.343		
35	5.211	5.444	14.91	0.349	0.365		
36	4.124	4.269	10.75	0.384	0.397		
37	2.675	2.737	6.36	0.421	0.430		
38	1.821	1.849	4.11	0.443	0.450		
39	0.905	0.912	1.96	0.462	0.465		
40	0.906	0.913	1.93	0.469	0.473		
(b) Lipaemic human serum							
41	5.720	6.057	12.09	0.473	0.501	0.323	0.975
42	5.000	5.257	9.84	0.507	0.534		
43	4.700	4.926	8.95	0.525	0.550		
44	3.200	3.302	5.72	0.559	0.577		
45	2.293	2.345	3.88	0.593	0.605		
46	1.450	1.471	2.36	0.615	0.623		
47	0.874	0.881	1.36	0.647	0.648		

The osmotic pressures (p and p'), pertaining to C or C_v protein concentrations, can be obtained from (6) and (6a) by:

$$p = \frac{\pi_0 C}{1 - a\pi_0 C} \quad (7)$$

and
$$p' = \frac{\pi_0' C_v}{1 - a'\pi_0' C_v} \quad (7a)$$

Adair (1928) (cf. Adair & Robinson, 1930b), deriving the same formula from van der Waals' equation, obtained

$$p = \frac{\pi_0 C}{1 - K_b C} \quad (8)$$

It follows that $K_b = a\pi_0$. According to Adair & Robinson (1930b), K_b is a correction factor in the calculation of osmotic pressures which accounts for the ion pressure difference, interionic forces and volume occupied by the proteins (and lipids) in the solution. π_0 is a constant for a particular protein or protein mixtures defined by their molecular weight, its value being equal to $10RT/M_m$, if protein concentration is expressed as g./100 ml., and M_m = mean

serum, while the values for K_b are variable. For lipaemic serum no. 3, K_b had a value of 0.0546 before and 0.0489 after 'defatting' when protein concentration was expressed as g./100 ml. of solution. The difference in the value of this constant for the serum in its two states (although π_0 was the same) explains the differences in osmotic pressures at high protein concentration (cf. formula (8)).

When protein concentration is expressed as g./100 ml. of solvent, a' has about the same value for all sera of the same species (rabbit 0.0203–0.0212; human 0.0236–0.0238). As was expected, $K_b' < K_b$, since K_b is a factor accounting, among other things, for the volume of proteins and lipids. It should be pointed out that the specific volumes of proteins given in section III are those of the dry proteins, i.e. the water of hydration in the solution was not taken into account; the latter was regarded as part of the solvent.

The values of K_b , as already mentioned, were variable and showed no definite trend, while K_b' decreased with decreasing albumin content. We have calculated the values of a' and K_b' for crystalline

horse serum albumin and unfractionated serum globulins from the data of Adair & Robinson (1930b) and obtained $a' = 0.0240$ and $K_b' = 0.0569$ for

Fig. 7. Relationship between protein concentration and osmotic pressure for normal ($\odot$ and $\bullet$) and nephrotic ($\triangle$ and $\blacktriangle$) human sera. $\odot$ and $\triangle$, observed osmotic pressures plotted against C ; $\bullet$ and $\blacktriangle$, observed osmotic pressures plotted against C_v . The data for the continuous curves have been calculated by the equation

$$p = \pi_0 C / (1 - a\pi_0 C)$$

and for the interrupted ones by

$$p' = \pi_0 C_v / (1 - a'\pi_0 C_v).$$

albumin and $a' = 0.022$ and $K_b' = 0.022$ for globulins. It is probable that for a mixture of albumin and globulins a value of K_b' intermediate between that for albumin and globulin would be obtained, as is the case with our sera.

It is noteworthy that the value of π_0' is equal to π_0 within the experimental error, and therefore both methods (determination of either π_0 or π_0') yield similar molecular weights if these are calculated from a modification of Van't Hoff's formula (Adair & Robinson, 1930b)

$$M = 10RT/\pi_0. \quad (9)$$

The mean molecular weights of the protein complexes of our sera are given in Table 11. The determined mean molecular weights of the serum proteins increased parallel with the rise in the percentage of serum globulins.

Fig. 8. Relationship between protein concentration, C and C_v , and $1/\pi (= C/p)$ and $1/\pi_v (= C_v/p)$ for normal ($\odot$ and $\bullet$) and nephrotic ($\triangle$ and $\blacktriangle$) human sera. $\odot$ and $\triangle$, $1/\pi$ plotted against C ; $\bullet$ and $\blacktriangle$, $1/\pi_v$ plotted against C_v . The straight lines represent the equations:

- (I) $1/\pi = 0.495 - 0.0280 C$; (Ia) $1/\pi_v = 0.495 - 0.0236 C_v$;
(II) $1/\pi = 0.660 - 0.0304 C$; and (IIa) $1/\pi_v = 0.660 - 0.0238 C_v$.

VI. Determination of specific volumes of unfractionated serum proteins and lipids

We wish to record our indebtedness to Mr G. S. Adair, F.R.S., for his advice in connexion with these determinations. He has kindly communicated to us formulae from which the specific volumes of proteins in a buffered solution can be calculated. The derivation of these will be published by him.

The following formulae were employed (Adair, 1946):

$$V = v_w w_w + v_s w_s + v_p w_p, \quad (10)$$

where V represents the volume of solution (in ml.); v_w , v_s and v_p the specific volumes of water, salts and proteins respectively; and w_w , w_s and w_p the weight (in g.) of water, salts and proteins in volume V of solution.

The specific volume of salts can be obtained by

$$v_s = (1 - k_s)/\rho_{\text{water}}, \quad (11)$$

Table 10. *Values of constants in osmotic pressure measurements (a , π_0 , and K_b) at pH 6.8 and 0° when protein concentrations are expressed as g./100 ml. of solution and g./100 ml. of solvent*

Serum	Values of constants when protein conc. expressed as g./100 ml. of solution			Values of constants when protein conc. expressed as g./100 ml. of solvent			Theoretical value of π_0	g. albumin g. total protein ($\bar{X}_a$) (by MgSO_4)
	a	π_0	K_b	a'	π_0'	K_b'		
Normal rabbit serum	0.0238	2.14	0.0510	0.0206	2.13	0.0439	1.92	0.675
Lipaemic serum no. 1	0.0250	1.97	0.0493	0.0203	1.97	0.0400	1.83	0.612
Lipaemic serum no. 2	0.0274	1.82	0.0499	0.0208	1.82	0.0379	1.71	0.522
Lipaemic serum no. 3 before 'defatting'	0.0284	1.92	0.0546	0.0212	1.92	0.0408	1.76	0.556
Lipaemic serum no. 3 after 'defatting'	0.0255	1.92	0.0489	0.0212	1.92	0.0408	1.76	0.556
Normal human serum	0.0280	2.02	0.0566	0.0236	2.02	0.0477	1.93	0.685
Nephrotic human serum	0.0304	1.52	0.0461	0.0238	1.52	0.0361	1.44	0.323

Table 11. *Determined mean molecular weights of unfractionated rabbit and human serum proteins, and percentage albumin and globulin contents of the sera*

	Mean molecular weight	Albumin (% of total proteins)	Globulin (% of total proteins)
Normal rabbit serum	79,700	67.5	32.5
Lipaemic rabbit sera	86,500	61.2	38.8
no. 1	93,600	52.2	47.8
no. 2	88,600	55.6	44.4
no. 3 (before and after 'defatting')			
Normal human serum	84,400	68.5	31.5
Nephrotic human serum	112,400	32.3	67.7

where k_s is the density increment of salts and is equal to $(\rho_{\text{buffer}} - \rho_{\text{water}})/w_s$.

We had to modify slightly formula (10) because of the presence of lipids in the sera:

$$V = v_w w_w + v_s w_s + v_p w_p, \quad (12)$$

where $v_{pl} = v_p + \frac{w_l}{w_p} v_l$; v_l and w_l being the specific volume and weight of lipids in volume V of solution. With the aid of formula (12) only v_{pl} can be determined in one serum; for the calculation of both v_p and v_l two sera of different lipid content are necessary.

The data required for such calculations are shown in Table 12. The sera were concentrated by ultrafiltration and then dialyzed at 0° against $\text{m}/15 \text{ PO}_4^-$ buffer for 72 hr. Sufficient pressure was applied to the membranes to maintain a constant volume of the protein solutions. The lipids were determined gravimetrically. Sodium and potassium were estimated after incineration of a sample of the protein solutions in a muffle furnace at 500°, the former by the method of Butler & Tuthill (1931) and the latter by the method of Salit (1940). All samples were measured by weight for the determinations and their volumes were calculated by dividing the weight (corr. as weight *in vacuo*) by the density of the solutions.

With the aid of the data given in Table 12 we obtained for the normal rabbit serum:

$$v_p + 0.0127v_l = 0.7412, \quad (13)$$

and for the lipaemic rabbit serum:

$$v_p + 0.3873v_l = 1.1143. \quad (14)$$

By solving (13) and (14) for v_p and v_l , we found $v_p = 0.729$ and $v_l = 0.996$. From the data of the concentrated human serum

$$v_p + 0.0608v_l = 0.7923 \quad (15)$$

was obtained. Assuming that the specific volume of lipids in the human serum is equal to those in the rabbit sera, the specific volume of unfractionated human serum proteins is equal to 0.732. Pedersen (1945) reported specific volumes for human serum albumin 0.73₈, for γ -globulin 0.71₈, for a pseudo-globulin 0.73₂, and for euglobulin 0.733.

Table 12. *Composition of concentrated sera dialyzed against $\text{m}/15 \text{ PO}_4^-$ buffer ($\text{m}/30 \text{ KH}_2\text{PO}_4 + \text{m}/30 \text{ Na}_2\text{HPO}_4$), pH 6.8*

(Constituents expressed as g./100 ml. of solution at the temperature of the density determinations.)

	Normal rabbit serum	Lipaemic rabbit serum no. 4	Normal human serum	$\text{m}/15 \text{ PO}_4^-$ buffer
Density (ρ)	1.0668 (at +1.0°)	1.0514 (at +0.5°)	1.0610 (at +0.6°)	1.0081 (at +1.0°)
H_2O (w_w)	83.565	81.849	83.937	99.881
Total solids	23.115	23.301	22.163	0.929
Total N	3.421	2.604	3.138	—
Total protein (w_p)	22.030	16.185	20.134	—
Total lipid (w_l)	0.279	6.268	1.223	—
Phospholipids	0.090	1.839	0.408	—
Sodium	0.149	0.144	0.151	—
Potassium	0.134	0.123	0.126	—
H_2PO_4	0.523	0.581	0.529	—
Salts (w_s)	0.806	0.848	0.806	0.929
w_l/w_p	0.0127	0.3873	0.0608	—

DISCUSSION

We believe that the results presented prove conclusively that the lipids do not exert an osmotic pressure. The experimental lipaemic sera contained such large quantities of lipids (3–4 g./100 ml.) as are rarely met with in man under pathological conditions. If any of the lipid fractions, especially cholesterol, as suggested by Rabinowitch (1930), exert an osmotic effect, it would have been detected with the technique employed.

It is difficult to explain the discrepancy between our results and those of Macheboeuf (1929), Macheboeuf & Sandor (1931), Fishberg (1929) and Rabinowitch (1930). The differences may be due to the technical difficulties of accurate osmotic pressure measurements on protein solutions and to difficulties of interpretation of results. Macheboeuf and Macheboeuf & Sandor did not measure osmotic pressures, but only the degree of dilution of sera which took place during dialysis. Such a method may be influenced by a number of factors other than true osmotic effects. Albumin-globulin ratios were not taken into account by the authors quoted. The comparisons of the osmotic pressures between normal and lipaemic sera were made by Fishberg (1929) and Rabinowitch (1930) at high protein concentrations only. It was pointed out by Adair & Robinson (1930*b*) that such comparisons might be very misleading owing to the ion pressure difference.

It is possible, however, that the higher osmotic effects of lipaemic sera reported by Fishberg and by Rabinowitch were due to the 'volume effect' of the lipids which we observed with lipaemic rabbit serum no. 3 before and after 'defatting'. It is evident, however, from Fig. 5 that the difference between the osmotic pressure-concentration curves of the lipaemic and 'defatted' serum is very slight and was produced with a very high concentration of lipids (4.17 g./100 ml. of the undiluted serum). Whether such an effect could be detected with the methods used by Fishberg, or by those of Rabinowitch cannot be judged. If the lipids *per se* were active osmotically, from their molecular weights very high pressures would be expected as compared with proteins, but no such effects were observed.

It is concluded, therefore, that any of the real differences (differences in values of π_0) found among the osmotic pressures of the various sera were due to differences in their albumin-globulin ratios and that the lipids do not exert an osmotic pressure. In lipaemic sera, however, the lipids reduce the volume of solvent available to the proteins and thus increase their effective concentration. In such a case the correct way of expressing protein concentration is g./100 ml. of solvent.

It seems justifiable to conclude also that the lipids in the serum are in only a loose combination

(physical adsorption?) with the proteins. It would be expected in the experiment with lipaemic serum no. 3 that the mean molecular weight, as determined by osmotic pressure measurements, should be lower after extraction of lipids, if the protein-lipid combination had been of a firmer, chemical nature. It can be seen from Table 11 that the mean molecular weight of the serum proteins was the same before and after 'defatting'.

The MgSO_4 method for the determination of albumin-globulin ratios of sera appears to be very reliable and it is not influenced by the presence of even pathological amounts of lipids. The electrophoretic examination of this method showed that after saturation of the sera with MgSO_4 only the (?) α -globulin remained in solution together with albumin and that there was no precipitation of albumin with the globulins. Recently Moore, Levin & Smelser (1945) have shown that with the sodium sulphite method for the determination of albumin-globulin ratios considerable amounts of globulins remain in solution with albumin and large amounts of albumin are precipitated with the globulins. The albumin could not be removed from the globulins even after several reprecipitations of the latter.

The albumin-globulin ratios obtained on normal rabbit sera by the MgSO_4 method agreed very closely with those obtained electrophoretically, while there was a wider discrepancy between the values obtained by the two methods on lipaemic sera. The indications are that the MgSO_4 method gives the more correct results for lipaemic sera.

SUMMARY

1. Electrophoretic examinations and osmotic pressure measurements were carried out on normal and lipaemic rabbit and human sera.

2. Assessment of the MgSO_4 method, with the aid of electrophoresis, showed that the technique is reliable for the determination of albumin-globulin ratios in both normal and lipaemic sera. While the electrophoretic technique is seriously influenced by the presence of large amounts of lipids, saturation of normal and lipaemic sera with MgSO_4 effects an almost perfect partition of the proteins into albumin and globulins.

3. The osmotic pressure measurements on normal and lipaemic rabbit and human sera proved that the lipids do not exert an osmotic pressure. The differences found in the osmotic effects of the various sera could be accounted for by their different albumin-globulin ratios.

4. The determined mean molecular weights of the serum protein complexes increased parallel with the rising globulin content of the sera.

5. In lipaemic sera the lipids reduce the volume of solvent available to the proteins with the result that the effective concentration of the latter is increased.

In such a case the correct way of expressing protein concentration is g./100 ml. of solvent.

6. The specific volumes of unfractionated rabbit and human serum proteins were found to be 0.729 and 0.732 respectively, and that of the lipids 0.996.

7. It is inferred that the lipids in the serum are at the most in only a loose combination (physical adsorption ?) with the proteins.

It is a pleasure to record our indebtedness to Mr G. S. Adair, F.R.S., for his advice and help given generously in the course of this work; we are also grateful to him and Mrs Adair for the determination of phosphates in the concentrated sera, which were used in the estimation of specific volumes of proteins and lipids. We wish to thank also Dr Evan Jones for the sample of the nephrotic human serum.

REFERENCES

- Adair, G. S. (1925). *Proc. roy. Soc. A*, **108**, 627.
 Adair, G. S. (1928). *Proc. roy. Soc. A*, **120**, 573.
 Adair, G. S. (1946). Private communication.
 Adair, G. S. & Robinson, M. E. (1930a). *Biochem. J.* **24**, 993.
 Adair, G. S. & Robinson, M. E. (1930b). *Biochem. J.* **24**, 1864.
 Adair, M. E. (1945). Private communication.
 Blix, G., Tiselius, A. & Svensson, H. (1941). *J. biol. Chem.* **137**, 485.
 Bloor, W. R. (1928). *J. biol. Chem.* **77**, 53.
 Bloor, W. R. (1929). *J. biol. Chem.* **82**, 273.
 Butler, A. M. & Tuthill, E. (1931). *J. biol. Chem.* **93**, 171.
 Dömösi, P. & Egyed, M. (1939). *Magy. orv. Arch.* **40**, 242.
 Fishberg, E. H. (1929). *J. biol. Chem.* **81**, 205.
 Kekwick, R. A. (1939). *Biochem. J.* **33**, 1122.
 Longworth, L. G., Shedlovsky, T. & MacInnes, D. A. (1939). *J. exp. Med.* **70**, 399.
 McFarlane, A. S. (1942). *Nature, Lond.*, **149**, 439.
 Macheboeuf, M. A. (1929). *Bull. Soc. Chim. biol.* **11**, 485.
 Macheboeuf, M. A. & Sandor, G. (1931). *Bull. Soc. Chim. biol.* **13**, 745.
 Maclean, H. & Smedley Maclean, I. (1927). *Lecithin and Allied Substances*, 2nd ed., pp. 17 and 49. London: Longmans, Green & Co. Ltd.
 Moore, D. H., Levin, L. & Smelser, G. K. (1945). *J. biol. Chem.* **157**, 723.
 Pedersen, K. O. (1945). *Ultracentrifugal Studies on Serum and Serum Fractions*. Uppsala: Almqvist & Wiksells Boktryckeri AB.
 Pillemer, L. & Hutchinson, M. C. (1945). *J. biol. Chem.* **158**, 299.
 Popják, G. (1943). *Biochem. J.* **37**, 468.
 Popják, G. (1945). *J. Path. Bact.* **57**, 280.
 Popják, G. (1946). *Biochem. J.* **40**, 608.
 Popják, G. & McCarthy, E. F. (1943). *Biochem. J.* **37**, 702.
 Rabinowitch, I. M. (1930). *Arch. intern. Med.* **46**, 752.
 Salit, P. W. (1940). *J. biol. Chem.* **136**, 191.
 Svensson, H. (1939). *Kolloidzshr.* **87**, 181.
 Svensson, H. (1946). *Ark. Kem. Min. Geol.* **22 A**, 1.
 Taylor, H. L. & Keys, A. (1943). *J. biol. Chem.* **148**, 379.
 Tiselius, A. (1937). *Biochem. J.* **31**, 1464.
 Voris, L., Ellis, G. & Maynard, L. A. (1940). *J. biol. Chem.* **133**, 491.
 Zeldis, L. J., Alling, E. L., McCoord, A. B. & Kulka, J. P. (1945). *J. exp. Med.* **82**, 411.

Biochemistry of Nitrification in Soil

1. KINETICS OF, AND THE EFFECTS OF POISONS ON, SOIL NITRIFICATION, AS STUDIED BY A SOIL PERFUSION TECHNIQUE

BY H. LEES AND J. H. QUASTEL (with an Addendum by H. LEES)
Agricultural Research Council Unit of Soil Metabolism, at Rothamsted Experimental Station, Harpenden, Herts*

(Received 14 February 1946)

Nitrification is the process whereby nitrogen, in the form of the ammonium cation or in organic combination, is converted into the nitrate anion. Nitrification in soil has long been established as a biological process (Schloessing & Muntz, 1877, 1879), but some evidence that, under tropical conditions, a non-biological nitrification in soil may occur has recently accumulated.

The pioneer work of Warington (1878, 1879, 1884) on culture studies of the nitrifying bacteria of soil culminated in the isolation of the nitrate-forming

bacteria by Frankland & Frankland (1890) and Warington (1891), and the isolation of both nitrite-forming bacteria and nitrite-oxidizing bacteria by Winogradsky (1890). Winogradsky suggested that the two forms of bacteria which he isolated co-operated in soil to produce the overall effect of nitrate formation from ammonium cation. All these workers found that inorganic media were suitable for culture of the organisms, and there is a fairly general agreement that the nitrifying organisms, while nitrifying, are autotrophic. Whether various forms of organic matter stimulate, inhibit, or leave unaffected, the organisms is a question that now has a vast literature

* Now at University College, Cathays Park, Cardiff.