Journal of Chromatography, 154 (1978) 3–11

CHROM. 10,862

GEL FILTRATION OF PHTHALATE ESTERS

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(Received November 2nd, 1977)

SUMMARY

Calibration of the gel-filtration system polystyrene beads–benzene is effected in terms of the molecular weights of solutes without using a void volume indicator. Correction for dead volumes is made by incorporating in the loads a dye producing a peak of accurately known elution volume to serve as reference point on a strip-chart absorption diagram of column effluent. The calibrated column separates small amounts of plasticizer phthalate esters from excess of lipids extracted from biological materials, providing evidence of identity in samples sufficiently pure for estimation by gas chromatography. Further purification can, where necessary, be achieved quantitatively by high-performance liquid chromatography on silicic acid with 2% (v/v) diisopropyl ether in *n*-hexane as mobile phase.

INTRODUCTION

Diesters of *o*-phthalic acid are used in enormous quantities as components of plastic materials in common use which includes food packaging and the fabrication of medical equipment. Because of interest in potential hazards[1], there is a requirement for methods by which these phthalates may be estimated in a diversity of samples, with emphasis on those of biological origin. The ester most frequently employed as a plasticizer[2] is di-2-ethylhexyl phthalate (DEHP), known to be present in, for example, blood that has been stored in plastic containers[3].

Quantitation of DEHP from all samples may in principle be readily achieved by gas chromatography[4,5] following various processes which have been used[5–7] for extracting it from tissues, foods, etc. Difficulties arise, however, since DEHP thus obtained is often accompanied by a disproportionately large amount of other materials of lipid nature. Purification of the extract before final estimation is a virtual necessity in many cases, and hitherto this has been effected, for example, by preparative thin-layer chromatography (TLC)[8] or by partition between carefully selected solvents[9]. However, existing methods are subject to drawbacks which appear to have been obviated by the alternative use of gel filtration for fractionating extracts according to molecular dimensions. Such an application, suggested as early as 1970 by Mulder and Buytenhuys[10], who adumbrated the separative properties of polystyrene beads used in conjunction with organic solvents, was put into practice by Pfunderer

et al.[11] who used Sephadex LH-20 as the gel. The present communication describes a procedure developed to provide a calibrated system of polystyrene beads with benzene which allows the expeditious and usually sufficiently effective isolation of microgram amounts of phthalic esters from milligram quantities of unwanted lipid.

THEORY

In the decade following the pioneering work of Porath[12] published in 1963, numerous reports were made on relationships between the elution volume (V_e) of a solute in gel filtration and its molecular weight or its Stoke's radius (r_S). Interest throughout was centered almost entirely on aqueous solutions of macromolecules, with results that were summarized and extended by Determan and Michel[13] and by Siegel and Monty[14]. These results should, *a priori*, apply also to organic solvents and solutes of lower molecular weights. From the literature and from unpublished work in this laboratory, it is reasonable to base present considerations on the general equation

$$r_S = k_G \sqrt{-\ln \frac{V_e - V_0}{V_T - V_0}} \tag{1}$$

while the molecular weight (M) is related with r_S as

$$M = k r_S^3 \tag{2}$$

where V_T is the total volume of liquid in the chromatography column and V_0 is the void volume, *i.e.* the volume of liquid external to the particles of gel; k_G is a "gel constant" and $k = \frac{4}{3}\pi F d$, F being a factor[15,16] to correct for departure from true spherical shape of the molecule, with d expressing the relative specific mass. From the two equations above, r_S may be eliminated, giving

$$2 \ln \frac{M}{k} = 3 \ln \left(-\ln \frac{V_e - V_0}{V_T - V_0}\right) + 6 \ln k_G$$

If three solutes with molecular weights M_1, M_2 and M_3 elute at volumes V_1, V_2 and V_3, and all three have for F and d approximately common respective values, it then follows that

$$\left(\frac{M_1}{M_2}\right)^{2/3} = \frac{\ln \dfrac{V_1 - V_0}{V_T - V_0}}{\ln \dfrac{V_2 - V_0}{V_T - V_0}} \qquad \text{etc.} \tag{3}$$

Writing a_1 for $(M_1/M_2)^{2/3}$, a_2 for $(M_2/M_3)^{2/3}$ and a_3 for $(M_1/M_3)^{2/3}$, and putting V_T $(1 - \alpha)$ for $V_T - V_0$, since V_0 is a constant proportion (α) of V_T, the last equation gives rise, *mutatis mutandis*, to the expressions

$$\left[\frac{(V_2 - V_0)^{a_1}}{V_1 - V_0}\right]^{a_2-1} = \left[\frac{(V_3 - V_0)^{a_2}}{V_2 - V_0}\right]^{a_1-1}; \quad \left[\frac{(V_3 - V_0)^{a_2}}{V_2 - V_0}\right]^{a_3-1} = \left[\frac{(V_3 - V_0)^{a_3}}{V_1 - V_0}\right]^{a_1-1}$$

$$\text{and} \quad \left[\frac{(V_2 - V_0)^{a_1}}{V_1 - V_0}\right]^{a_3-1} = \left[\frac{(V_3 - V_0)^{a_3}}{V_1 - V_0}\right]^{a_1-1} \tag{4}$$

Any one of these can be taken to provide a solution for V_0 if the three elution volumes are known; V_0 is found by reiteration with successively approximating estimates until the difference between right- and left-hand sides of the equation is zero, and for the first estimate supplied it is helpful to take 0.25 V_T, with V_T derived provisionally as the product of the column cross-sectional area and its height. The use of 0.25 for α arises from the geometrical consideration of closely packed spheres, in which the void volume is $(6 - \pi\sqrt{2})/6 = 0.2595$ of the total volume.

Now that V_0 is known, $V_T - V_0 = V_T(1 - \alpha)$ is evaluated, since (from eqn. 3)

$$V_T - V_0 = \left(\frac{(V_2 - V_0)^{a_1}}{V_1 - V_0}\right)^{a_1-1} = \left(\frac{(V_3 - V_0)^{a_2}}{V_2 - V_0}\right)^{a_2-1} = \left(\frac{(V_3 - V_0)^{a_3}}{V_1 - V_0}\right)^{a_3-1} \tag{5}$$

giving immediately solutions for V_T and then for α.

By this method, all parameters of the column are determined without recourse to measuring V_0 directly as the elution volume of a solute which is totally excluded from the gel particles.

In eqn. 1, $(V_e - V_0)/(V_T - V_0) = K_{AV}$, the partition coefficient[17] for the solute between the solvent within the gel particles and that external to them, so that K_{AV} has values within the limits of zero to unity: thus $Y = -\ln[(V_e - V_0)/(V_T - V_0)]$ is zero or positive, and from eqns. 1 and 2,

$$Y = (k_G k^3)^{-2} \cdot M^{2/3} = KM^{2/3} \tag{6}$$

Calibration of a gel column may therefore, as is well known, be presented graphically by plotting the dependent function Y against $M^{2/3}$ as independent variable.

An alternative inductive method by which V_0 may be deducted is based on treatment, through comparison of similar triangles (Fig. 1), of the slope (K) of the above regression (eqn. 6) as represented by intervals between Y_1, Y_2 and Y_3 for the calibration solutes (M_1, M_2, M_3) postulated above. The three relationships immediately derived are

$$-\ln\frac{V_1 - V_0}{V_T - V_0} + \ln\frac{V_2 - V_0}{V_T - V_0} = K(M_1^{\frac{2}{3}} - M_2^{\frac{2}{3}}) \quad \text{etc.} \tag{7}$$

and may be written as

$$\ln\frac{V_2 - V_0}{V_1 - V_0} = K(M_1^{\frac{2}{3}} - M_2^{\frac{2}{3}}) \quad \text{etc.} \tag{8}$$

Putting $b_1 = M_1^{\frac{1}{3}} - M_2^{\frac{1}{3}}$, $b_2 = M_1^{\frac{1}{3}} - M_3^{\frac{1}{3}}$, $b_3 = M_2^{\frac{1}{3}} - M_3^{\frac{1}{3}}$ gives

$$b_2 \ln \frac{V_2 - V_0}{V_1 - V_0} = b_1 \ln \frac{V_3 - V_0}{V_2 - V_0} \qquad \text{etc.}$$

so that

$$\left(\frac{V_2 - V_0}{V_1 - V_0}\right)^{b_2} = \left(\frac{V_3 - V_0}{V_1 - V_0}\right)^{b_1}; \qquad \left(\frac{V_3 - V_0}{V_1 - V_0}\right)^{b_3} = \left(\frac{V_3 - V_0}{V_2 - V_0}\right)^{b_2};$$

$$\left(\frac{V_2 - V_0}{V_1 - V_0}\right)^{b_3} = \left(\frac{V_3 - V_0}{V_2 - V_0}\right)^{b_1} \qquad (9)$$

As before, any one of these equations (the logarithmic forms being more amenable) can be used to find V_0 by iteration, after which one of the equations (eqn. 8) is used to deduce a value for K to be supplied in the general relationship (eqn. 6) for evaluation of $V_T(1 - \alpha) = V_T - V_0$ from one of the three forms

$$(m_1 - m_2) \ln (V_T - V_0) = \ln \frac{(V_2 - V_0)^{m_1}}{(V_1 - V_0)^{m_2}} \text{ etc.} \qquad (10)$$

where $m_1 = M_1^{\frac{1}{3}}$ etc.

EXPERIMENTAL

Di-2-ethylhexyl, dioctyl, and dinonyl *o*-phthalates (Travenol, Thetford, Great Britain), dimethyl phthalate and cholesterol (BDH, Poole, Great Britain), tristearin, triolein (Sigma, London, Great Britain), trilaurin (Eastman-Kodak, Rochester, N.Y., U.S.A.) and phosphatidylethanolamine (Lipid Products, London, Great Britain) were used as supplied. Sudan Black B (G. T. Gurr, Nutfield, Great Britain) was fractionated by chromatography on a column of silicic acid (Sil R, Sigma) with chloroform as solvent, the fastest running major band being taken. This was now apparently homogeneous when examined by TLC on Eastman Chromatogram Sheet (Eastman-Kodak) either with chloroform or with benzene as developing solvent. All solvents used were of analytical reagent grade and were not purified further except for de-aeration under reduced pressure where appropriate.

A solvent-resistant glass column assembly (Pharmacia), 50 cm long, was silylated with Repelcote (BDH) and its cross-sectional area (5.226 cm^2) measured by filling between marks with a known volume of water. Using 30 g of Biobeads S-X2 (Bio-Rad Labs., Richmond, Calif., U.S.A.) swollen overnight at room temperature in benzene, the column was packed to give a gel height, between flow adaptors, of 30.3 cm. Through connections made with nylon or PTFE tubing (bore 1 mm), liquid entered the column and from there traversed a Uvicord II monitor operating at 281 nm and passed to the syphon (4.90 ml) of a fraction collector. After each delivery of a fraction, an event mark was recorded on the strip chart (6 in./h) of the monitor

by means of a photoelectric device operated by upward movement of the arm supporting the syphon. The passage of all solutes except the triglycerides was adequately detected by absorption. For the latter, fractions were examined gravimetrically and by TLC. The gel column operated with upward flow at 2.4 ml/min from a reservoir of degassed benzene maintained about 130 cm above the final delivery level.

All column loads, in 1–2 ml of benzene, contained a little Sudan B. Corrections for dead volumes were effected by correlating, with the fractions taken, the progress (cm) of the band of dye up the column: as each event mark was made, the position of the centre of the dye band was marked on the column with a crayon. From the regression of progress to fraction number, the absolute elution volume of the dye was ascertained, while its peak on the chart was used as working-zero from which other peaks were measured. Load solutions contained generally 1–10 mg of each solute, together with dye. The single fraction (4.90 ml) was used throughout as the unit of volume, so that geometrically, $V_T = 32.32$ fractions, etc.

Assessments by gas chromatography of the phthalate esters were carried out with a Perkin Elmer F 11 instrument with a flame-ionization detector and fitted with a stainless-steel column 2 m × 1/8 in. O.D. packed with 2.5% (w/w) silicone gum E-301 (Kodak) on acid-washed and silylated Chromosorb (BDH), used at 223°. A standard weight of methyl arachidate was added for internal reference to the sample, which was dissolved in *n*-hexane for injection. Calibration was carried out by further chromatography after adding a known amount of the phthalate ester to the sample.

For high-performance liquid chromatography (HPLC), a stainless-steel column, 50 cm × 2 mm I.D., was packed with the pellicular Perisorb A (Merck, Darmstadt, G.F.R.), in which the active constituent is silicic acid. After cleaning with chloroform (10 ml), the column was equilibrated with 10 ml of 2% (v/v)diisopropyl ether in hexane as mobile phase (0.8 ml/min). Loads (20 μl) of DEHP (20–500 μg) in hexane were admitted from a loop and the DEHP emerged within 3.5 min, whereas cholesterol was retained until swept off later with chloroform. Effluent was monitored with a Uvicord II as used in the gel filtration.

Lipids were examined by TLC with silicic acid using as developing solvents chloroform–methanol–water (70:30:5, v/v/v) and isopropyl ether–hexane (20:80, v/v). Iodine vapour, or reaction with ninhydrin to reveal amino groups, was employed for visualization.

RESULTS

All the solutes used eluted as symmetrical peaks having about 2.5% of each lying more then ± 1.5 fractions from the central (V_e) value. It is seen (Fig. 2) that DEHP is effectively isolated from substances of molecular weights >500 and <300 D, although still accompanied by nearly all of any cholesterol originally present. Data for the solutes are given in Table I.

For any triad of solutes, results for V_0 and for $V_T - V_0$ are the same from eqns. 4 and 5 as from eqns. 9 and 10, thus demonstrating the equivalence of the methods of calculation. Twelve well-spaced triads were selected, and values from these are shown in Table II. As central values for V_0 and for $V_T - V_0$, simple arithmetic means were taken. From these, Y was calculated (eqn. 6) for each substance, as shown in Table I. Least-squares correlation ($r = 0.9988$; $P < 10^{-6}$) of data for the seven

TABLE I

ELUTION VOLUMES AND $Y = -\ln \frac{V_e - V_0}{V_T - V_0}$ FOR GEL FILTRATION ON BIOBEADS S-X2-BENZENE

Solute	*Symbol in figures and Table II*	*Molecular weight M (D)*	$M^{2/3}$	$V_e \pm$ *S.E. (fractions) mean for n values*	*n*	*Y*	*Relative retention**
Tristearin	T/S	890	92.53	12.48 ± 0.21	4	1.542	0.557
Triolein	T/O	884	92.11	12.92 ± 0.17	4	1.473	0.577
Dinonyl phthalate	DNP	419	55.99	17.88	2	0.916	0.798
Diethylhexyl phthalate	DEHP	391	53.47	18.39 ± 0.06	5	0.873	0.821
Dioctyl phthalate	DOP	391	53.47	18.20	2	0.888	0.812
Sudan Black	Sud	214	35.78	22.41 ± 0.04	34	0.586	1.000
Dimethyl phthalate	DMP	194	33.51	22.85 ± 0.21	4	0.559	1.020
Phosphatidyl-ethanolamine	PE	717	80.11	9.04 ± 0.01	4	2.348	0.403
Trilaurin	T/L	638	74.11	14.36 ± 0.09	3	1.277	0.641
Cholesterol	Ch	387	53.11	19.57 ± 0.08	5	0.780	0.873

* Elution volume of Sudan B taken as unity.

TABLE II

CALIBRATION OF THE SYSTEM BIOBEADS S-X2-BENZENE FROM TRIADS OF SOLUTES

*Triad**	V_0 *(fractions)*	$V_T - V_0$ *(fractions)*	V_T *(fractions)*	$100\ V_0/V_T = \alpha\%$
T/S, DEHP, SUD	4.86	29.70	34.56	14.1
T/S, DEHP, DMP	3.96	29.69	33.65	11.8
T/S, DOP, Sud	6.72	29.51	36.23	18.5
T/S, DOP, DMP	5.97	29.00	33.96	17.1
T/S, DNP, Sud	5.06	29.64	34.70	14.6
T/S, DNP, DMP	4.29	29.54	33.82	12.7
T/O, DEHP, Sud	7.18	28.24	35.42	20.3
T/O, DEHP, DNP	6.57	27.85	33.36	19.0
T/O, DOP, Sud	8.52	28.83	37.35	22.8
T/O, DOP, DMP	7.96	27.92	35.87	22.2
T/O, DNP, Sud	7.42	30.17	38.70	22.0
T/O, DNP, DMP	6.86	27.85	34.71	19.8
Means ± S.E. ($n = 12$)	6.27 ± 0.42	28.99 ± 0.24	35.27**	17.8**
Geometrical estimates	8.4	23.9	32.3	26.0

* See Table I.

** From means for V_0 and $V_T - V_0$.

components selected for the triads yielded the equation $Y = 0.0162\ M^{2/3} + 0.0117$ (Fig. 1) describing the characteristics of the system, and this was used to mark in the ranges for molecular weights shown in Fig. 2.

When lipid (approximately 100 mg) extracted from sausage-meat, to which DEHP (50 ppm) had previously been added, was partitioned as described, the microgram amount of plasticizer present evoked no response from the Uvicord monitor,

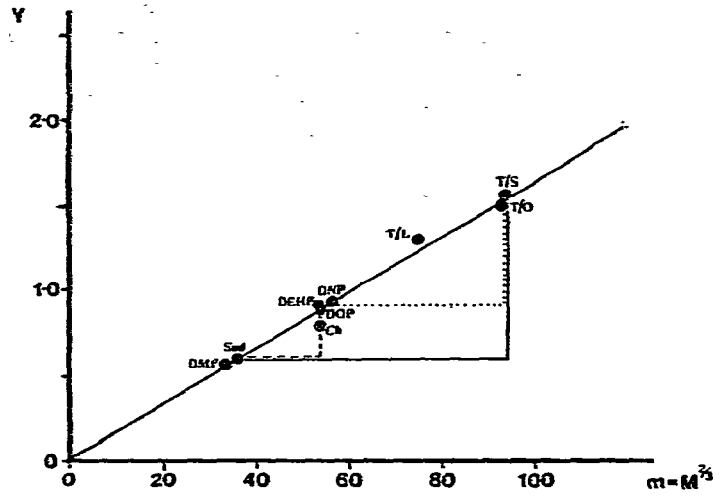

Fig. 1. Calibration for Biobeads S-X2 with benzene at room temperature. Parameters and symbols as in Table II. Ordinates $Y = -\ln[(V_e - V_0)/(V_T - V_0)]$. The regression is $Y = 0.0162M^{2/3} + 0.0117$ ($r = 0.9988$).

but it was recovered and assessed by gas chromatography. When the gel filtration was challenged with loads of 50 mg and then with 100 mg of tristearin in 2 ml of benzene, the weights of residues left after evaporating the pool of fractions that would have contained DEHP were insignificant; when 100 mg of the sausage lipid were chromatographed, these same fractions contained 2.4 mg of lipid "tail", tolerable in subsequent analysis by gas chromatography.

Applications of six loads of DEHP in the range 20–200 μg gave on assay as above a closely ($r = 0.999$) rectilinear response, with mean recovery of 78% (w/w) as estimated from the regression coefficient.

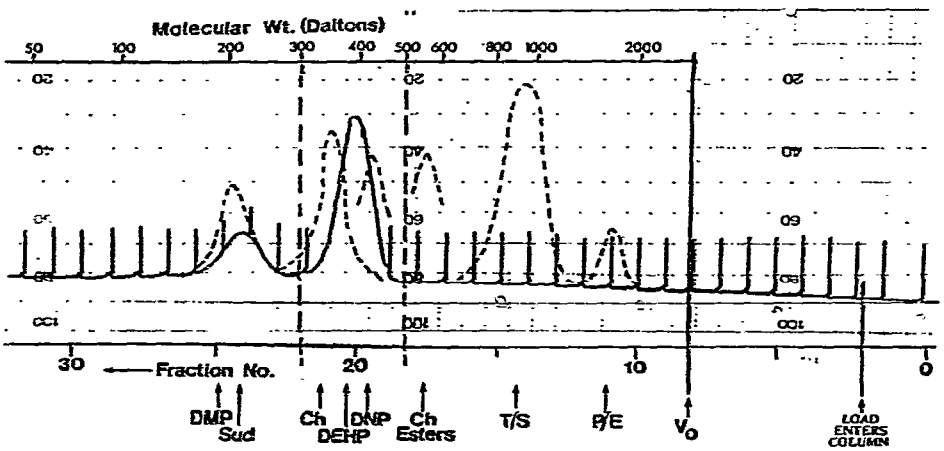

Fig. 2. Elution pattern for mixture T/S, DEHP and Sud with peaks for other solutes superposed from similar charts. Symbols as in Table I.

When washed total lipid (approximately 15 mg) extracted[18] from 3 ml of normal human blood plasma, dissolved with Sudan B dye in benzene, was submitted to gel filtration, three peaks absorbing at 281 nm were recorded. The first of these (V_e = 8.97 fractions) contained all the phospholipids taken, namely phosphatidylcholine, sphingomyelin and traces of ethanolamine and serine phosphatides, as shown by qualitative TLC. The other peaks were identified similarly as cholesteryl esters (V_e = 15.47 fraction) and as cholesterol (V_e = 19.89 fraction), while the triglycerides were found to elute maximally at 13.04 fractions.

DISCUSSION

In much of the very extensive work on gel filtration that has been described in earlier publications, aqueous solutions were used and have incorporated Blue Dextran to indicate the void volume, but with organic solvents and lipophilic gels such indicators may not be so readily available. For that reason, the present method of calibrating has proved to be very convenient, and should be generally applicable. Further, elimination of uncertainty over correction for dead volumes between loading point and column, column and monitor, and monitor and fraction collector by use of a reference peak of known elution volume as working origin on the elution chart confers considerable practical advantage.

The use of a single fraction as unit volume, although convenient when dealing with results from one particular gel column, is not sufficiently flexible if the performance of other columns is to be considered. It is then advantageous to adopt the convention, common in gas chromatography, of using the concept of relative retention. With Sudan B as the reference solute, elution characteristics can then be expressed as in the last column of Table I. Relative retentions are independent not only of the volume per fraction with any one column, but also of the dimensions of the column, and so are characteristic only of the solute and of the gel–solvent system, although still subject to temperature and to variations from batch to batch of the gel.

For reasons not yet known, the mean values obtained (Table II) for V_0 (6.27 fractions) and for $V_T - V_0$ (28.99 fractions) give V_T as 35.26 fractions differing considerably from the geometrical assessment of 32.3 fractions. Nevertheless, when V_0 and $V_T - V_0$ are taken as so found, a highly significant rectilinear relationship (Fig. 1) is obtained as calibration. On statistical grounds, the estimation of the void volume and the calibration of the column by regression as presented here may be subject to criticism because, whereas errors of mean elution volumes may well be normally distributed, those of the ratios and exponential functions used almost certainly are not. In the present application, however, the procedures appear to be justified. Inevitably, through experimental error, changes in room temperature and non-uniformity of molecular parameters, scatter of derived results is observed. The elution volumes of cholesterol and of phosphatidylethanolamine are seen to be anomalous; the latter behaves as if its molecular weight were about 1700–1800 D, suggesting that this solute is present in di- or trimeric form, but not as the monomer. All the phospholipids from plasma behave similarly, with evidence of very little resolution. Results for cholesterol, for the phospholipids and for trilaurin were not used in calibra-

tion. Cholesterol esters are well separated from the range of fractions in which DEHP is to be found.

As a step in analytical procedure, gel filtration of lipid extracts is rapid and convenient. More perfect separations could undoubtedly be obtained with longer columns and smaller fractions, but in many cases the extra cost in time and solvent would not be justified. All common phospholipids and most triglycerides found in biological samples have molecular weights of more than 650 D and these compounds are well resolved from DEHP in the conditions described. Although benzene was used in this work, it is probable that another solvent might be advantageous, *e.g.* cyclohexane, which is transparent at 281 nm and would allow more sensitive detection by the absorptiometer. It would seem that phthalate esters other than DEHP could also be separated, allowing some evidence on molecular weights to be obtained to corroborate later identification by gas chromatography. When DEHP itself is to be estimated, the use of dioctyl phthalate as an internal standard is not invalidated by gel filtration since the standard and the plasticizer have the same elution volume.

When samples rich in cholesterol are to be handled, the contamination of DEHP recovered from the fractions may not be insignificant, but it was found that final and quantitative (>98%) isolation of the plasticizer from fraction pools were readily accomplished by HPLC as described in the experimental section. Cholesteryl esters were not separated from DEHP by HPLC in the conditions used, but no effort was made to achieve this, since it had been found that the plasticizer eluted from the gel filtration column was not accompanied by any significant proportion of these compounds.

ACKNOWLEDGEMENTS

The author is grateful to Travenol International Services Inc. for generous financial support, and to Miss J. Ferrett for skilled assistance both in the laboratory and in the preparation of the typescript and figures.

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