may well be some additional steric and ionization effects to be considered which would account for these differences in equivalence of acid groups and albumin molecules.

SUMMARY

1. Polyacids interact with albumin in salt-free solutions to prevent coagulation of the protein by heat. Polyphosphates (nucleic acids), polysulphates (heparin and dextran sulphate) and polycarboxylates (alginic acid and bacterial polyglutamic acid) were all effective. Effects on optical rotation of the albumin were also demonstrated.

2. The protecting agent acts by preventing the coagulation of the albumin, although denaturation occurs to some extent. The molecular size of the protecting agent seems of less importance than the number of acid groups in determining the degree of protection.

This work was carried out in the laboratories of the Birmingham branch of the British Empire Cancer Campaign.

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Phospholipids

2. ESTIMATION OF AMINO NITROGEN IN INTACT PHOSPHOLIPIDS

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During the course of work on the separation of phospholipids by chromatographic procedures the need became apparent for a rapid micromethod of detecting and estimating amino-containing phospholipids, particularly phosphatidylethanolamine, in the presence of much larger quantities of lecithin (phosphatidylcholine)—if possible without prior hydrolysis of the phospholipid.

The procedure previously employed involves a lengthy hydrolysis with acid or alkali followed by removal of the liberated fatty acids and estimation of amino nitrogen in the water-soluble portion of the hydrolysate, usually by the Van Slyke method. Direct application of the Van Slyke method to the unhydrolysed phospholipid has been attempted but is subject to serious errors and requires a comparatively large quantity of material. Ninhydrin has been used qualitatively for the detection of free amino-containing phospholipids on paper chromatograms by Hecht & Mink (1952), Hack (1953) and others.

It has been suggested (Lea & Rhodes, 1953) that ninhydrin in the buffered methyl cellosolve reagent used by Moore & Stein (1948) for the estimation of amino acids, and by Levine & Chargaff (1951) for the estimation of nitrogenous constituents in lipid hydrolysates, might be applicable to the determination of unhydrolysed amino-containing phospholipids. The present paper describes this application and records some observations on the behaviour of phospholipids and of simpler fatty esters in the direct Van Slyke determination.

EXPERIMENTAL

Materials

Egg phospholipid. Crude hen's egg phospholipid was prepared by extraction of the freeze-dried yolk with methanol:chloroform (1:1, v/v) followed by evaporation, solution in chloroform, filtration and precipitation several times from ether at 0–3° with acetone (4 vol.). Contaminating, non-phospholipid substances containing amino N (particularly amino acids) were removed by passage in chloroform:ethanol solution through a cellulose column (Lea & Rhodes, 1953).

'Pure' lecithin. Phosphatidylcholine, prepared from egg phospholipid by the chromatographic (alumina column) method of Hanahan, Turner & Jayko (1951), contained no amino N detectable by the Van Slyke method after hydrolysis, and possessed an atomic N:P ratio of 1·01. As shown

below, it was still contaminated by a trace of amino-containing material which could be estimated by the more sensitive ninhydrin method.

The ninhydrin method

Procedure. The solvent, usually ethanol or chloroform, is rapidly removed at a low temperature from the phospholipid samples in 5 × ⅝ in. test tubes, conveniently by use of a simple, rotary-film, vacuum evaporator (Lea, Hannan & Rhodes, 1951), and the residue taken up in 1 ml. methyl cellosolve, in which it readily dissolves. 1 ml. of the ninhydrin:citrate:stannous chloride in 50% (v/v) methyl cellosolve reagent of Moore & Stein (1948) is added and, on shaking, a cloudy solution is obtained. The tubes are fitted with slip-on aluminium caps and heated in a boiling-water bath for 20 min. After being cooled, 5 ml. methyl cellosolve are added and the optical density at 570 mμ. measured in a 1 cm. cell, using a Unicam Grating Spectrophotometer or other suitable instrument.

Methyl cellosolve is used as a diluent in place of the 50% aqueous propanol of the original method, and it has been found that 5 ml. will dissolve a sufficient quantity of egg phospholipid or its fractions to permit the detection of less than 0·1% of amino-containing phospholipid. The absorption spectrum of the colour developed with phosphatidylethanolamine agrees closely with that of dioxohydrindylidene:dioxohydrindamine and with those developed by several amino acids as given by Moore & Stein (1948). It was found that the $SnCl_2$ content of the reagent could be increased 2–3 times without affecting the optical density, but increasing the time of heating to 40 and 60 min. progressively decreased the intensity of the colour.

Egg phospholipid. The amino N content of this material, as determined by the Van Slyke manometric procedure after hydrolysis with 6N-HCl in a sealed tube at 100° for 15 hr. and removal of the fatty acids, was of the order of 18% of the total N (as compared with approx. 20% before purification on cellulose), and extinction coefficients were plotted against amino N (as in Fig. 1). No serine could be detected by paper chromatography of the hydrolysate.

A small sample of synthetic α-palmitoyl-α′-linoleoylphosphatidylethanolamine, made available through the courtesy of Dr W. G. Rose, also showed a linear relationship between extinction coefficient and P content and a molar colour yield close to that of the egg phospholipid. Initial difficulties with reproducibility discussed below, however, caused supplies of the synthetic material to become exhausted before an accurate comparison of the colour yields of the natural and synthetic products could be obtained.

Lecithin. The colour developed by 'pure' lecithin with the ninhydrin reagent was relatively very weak (0·074% of the phosphatidylethanolamine reaction) and was almost certainly due to residual traces of amino-containing impurities in the preparation rather than to any ninhydrin reaction of phosphatidylcholine itself (Fig. 1).

Phosphatidylethanolamine in presence of excess of lecithin. To test for possible interference by lecithin when present in large excess, three dilutions of egg phospholipid in 'pure' lecithin were prepared and examined by the ninhydrin method. When corrected for the small contributions due to the added lecithin the colour measurements showed no evidence of any interference by excess of lecithin in the estimation of phosphatidylethanolamine by this method (Table 1).

Ethanolamine. The ninhydrin method in this form can also be used for the estimation of ethanolamine (plus serine, if present) in a phospholipid hydrolysate and requires much less material than the Van Slyke method (Fig. 1). The recrystallized ethanolamine hydrochloride used gave an amino N:total N ratio of 1·00 by the Van Slyke procedure with a reaction time of 4 or 10 min. Levine & Chargaff (1951) have reported data (in arbitrary units) for ethanolamine and serine by the original Moore & Stein procedure.

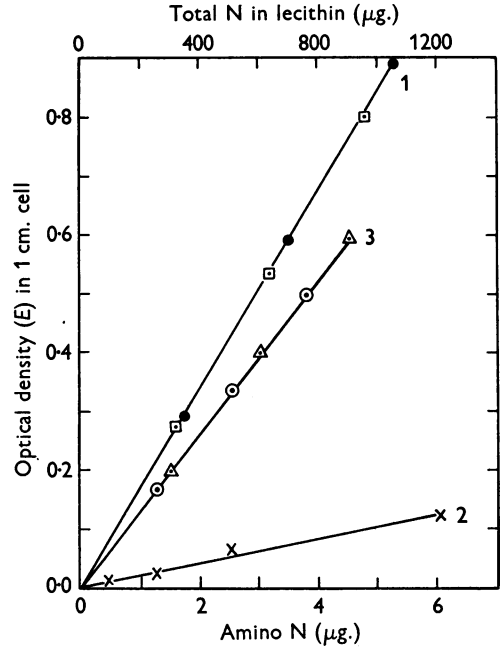

Fig. 1. Ninhydrin reaction of unhydrolysed phospholipids and of ethanolamine. Curve 1: egg phospholipid; curve 2: 'pure' egg lecithin; curve 3: ethanolamine hydrochloride.

Table 1. *Determination of phosphatidylethanolamine in presence of excess of lecithin*

Phosphatidylethanolamine N in sample (μg.)	*E* values* at various concentrations (%) of phosphatidylethanolamine N/total N				
	19·6	4·67	0·96	0·45	Av.
1·16	0·170	0·158	0·150	0·161	0·160
2·32	0·306	0·288	0·288	0·281	0·291
3·45	0·454	0·453	0·448	0·433	0·448
4·62	0·603	0·582	0·629	0·627	0·610
5·78	0·760	—	0·760	—	0·760

* Corrected for the small absorption of the 'pure' lecithin used in making the dilutions.

Influence of variables in the preparation and storage of the ninhydrin reagents. Moore & Stein (1948) state that, if only a few amino acid analyses are being run at one time, the necessary small quantity of ninhydrin reagent can be prepared for immediate use and run in from a burette. Alternatively, when large numbers of samples are being run the ninhydrin solution can be stored under N_2 in a brown- or red-glass reservoir attached to a pipetting machine.

In the first part of the present work small, freshly prepared batches of ninhydrin reagent were used, and excellent linear calibrations of optical density against amount of phospholipid amino N consistently obtained, but the slope of the curves, i.e. the molar colour yield tended to vary erratically from one run to another. The values recorded in Table 1 were obtained at an early stage of the investigation by the small-batch method and, although consistent within themselves, are approximately 20% lower than those subsequently obtained (Fig. 1) by the method described below using, however, new stock preparations of recrystallized ninhydrin and redistilled methyl cellosolve.

For all later determinations the ninhydrin reagent was prepared in 1 l. batches, de-aerated and stored under N_2 with protection from light. Under these circumstances variability was greatly reduced and the results did not seem to change appreciably with the age of the batch of reagent over a period of a few weeks, nor was there any appreciable difference between several batches of reagent prepared from the same stock of ninhydrin and redistilled methyl cellosolve. One batch of reagent saturated with hydrindantin, the reduced form of ninhydrin, was also prepared according to Fowden (1951) and gave similar results. Data illustrating these points are shown in Table 2 for egg phospholipid and for ethanolamine.

The presence of impurity, presumably peroxide, in the methyl cellosolve used as solvent for the phospholipid could be an important factor in producing erratic results. Moore & Stein (1948) did not describe any purification of the methyl cellosolve used in preparation of their ninhydrin reagent but stated that it gave a negative or very faint positive test with 10% (w/v) aqueous KI. Fowden (1951), in the estimation of amino acids eluted from paper chromatograms, specified redistilled methyl cellosolve shown to be free from oxidizing agents by the starch:iodide test. In our experience, redistilled methyl cellosolve exposed to diffused daylight can regenerate sufficient peroxide during 1 week to give a reduction in colour yield of 15%, but is comparatively stable in the dark (Table 2).

The Van Slyke method

Intact phospholipids. Chargaff, Ziff & Rittenberg (1942) used 5 min. Van Slyke values as a direct measure of the amino-N content of phospholipids and similar methods have been used by others. Unhydrolysed phospholipids, however, do not give satisfactory values in the manometric Van Slyke amino-N determination but continue to evolve N_2 with increasing time of reaction (Fig. 2, curve 1). Further indication of an abnormal reaction was given in this case by the production of a gas (possibly N_2O) which was incompletely absorbed by the alkaline permanganate in the Hempel pipette but which dissolved on further shaking with water or dilute acetic acid in the reaction chamber; reproducible values were then obtained.

'Pure' lecithin also produced an appreciable evolution of gas in the Van Slyke determination (Fig. 2, curve 2) and the similar shapes of the lecithin- and egg-phospholipid curves suggested that the slowly rising portion at least was due to some factor common to both systems, probably the unsaturated fatty acids. Correction of curve 1 by curve 2 gave a fairly constant value (curve 3) which was only slightly

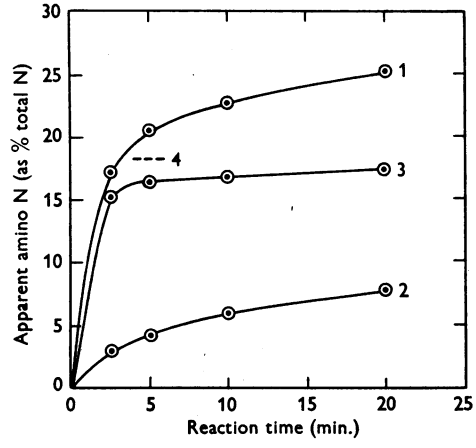

Fig. 2. Behaviour of unhydrolysed phospholipids in the Van Slyke determination. Curve 1: egg phospholipid; curve 2: egg lecithin; curve 3: subtraction of curve 2 from curve 1; curve 4: acid-hydrolysed egg phospholipid after removal of fatty acids.

Table 2. *Effect of variation in reagents on molar colour yield*

Ninhydrin reagent	Cellosolve diluent	$10^{-3}\epsilon$
Egg phospholipid		
Batch *A*, fresh	Freshly distilled	164
Batch *B*, fresh	Freshly distilled	163
Batch *C*, 10 weeks old	Freshly distilled	164
Batch *C*, 11 weeks old	Freshly distilled	163
Batch *C*, 11 weeks old	6 months old (in dark)	162
Batch *C*, 10 weeks old	1 week old (in light)	139
Fresh, with hydrindantin	Freshly distilled	162
Ethanolamine		
Batch *B*, fresh	Freshly distilled	127
Batch *C*, 10 weeks old	Freshly distilled	128
Fresh, with hydrindantin	Freshly distilled	126

lower than the value obtained by Van Slyke determination on the acid hydrolysate after removal of the fatty acids (Fig. 2, curve 4), but such a correction could only be completely effective if the fatty acid compositions of the two systems were identical, which in this case they were not.

A reaction time, in this case 3 min., can be chosen empirically from curve 1 to give the value found after hydrolysis, but such a result will depend in some degree on the composition of the fatty acid mixture present. Kline, Gegg & Sonoda (1951) have made some use, with unhydrolysed egg phospholipid, of a 5 min. reaction period which they found to correspond to the value obtained after hydrolysis for 48 hr. with 2N-H_2SO_4. Kirk, Page & Van Slyke (1934) used three times the 'normal' reaction time of 198 sec. at 23·6° for 'complete' reaction with plasma lipids, but apparently did not calibrate against a hydrolysate. Subsequently, Folch, Schneider & Van Slyke (1940) attributed high values on intact lipids to the presence of unsaturated fatty acids, since hydrogenation greatly reduced the error, but later Folch (1942) reported low and inconsistent results on phosphatidylethanolamine from brain and attributed them to coagulation of the emulsion in the nitrous acid reagent whereby the phospholipid became partly inaccessible to attack. In view of these conflicting observations it seemed advisable to examine the behaviour of a series of simpler fatty esters.

Fatty esters. 'Pure' egg lecithin, the methyl esters prepared from its constituent fatty acids, pure ethyl palmitate (used because it has a lower m.p. than the methyl ester), methyl oleate, linoleate and linolenate and a highly unsaturated methyl ester fraction obtained by crystallization of linseed-oil esters at −60° were all treated with nitrous acid for 5 min. under the conditions of the Van Slyke determination, with the addition of a drop of 'Teepol' (sodium alkyl sulphate, Shell Co. Ltd.) to ensure adequate dispersion of the insoluble esters. The result (Fig. 3) showed an apparent amino-N content which was negligible for the saturated ester but which increased rapidly with increasing unsaturation, especially after the first double bond. Since the value for unhydrolysed lecithin falls on the same curve as those for the simple esters, the high value obtained by direct application of the Van Slyke method to the unhydrolysed phospholipid can be accounted for by its content of unsaturated fatty acids.

Hydrolysis procedures. Many different methods have been used for the hydrolysis of phospholipids prior to estimation of their nitrogenous constituents. In our experience methanolysis by refluxing with 6N-HCl in dry methanol for 3 hr. followed by refluxing with dilute aqueous HCl for 1 hr. (to hydrolyse any serine ester which might be present) and extraction with light petroleum to remove fatty acids gave amino-N values about 9% lower than hydrolysis with aqueous 6N-HCl at 100° for 25 hr. followed by chilling and separation of the fatty acids by filtration. This is in agreement with the observations of Levine & Chargaff (1951) who obtained lower recoveries of N and choline from egg lecithin by methanolysis (79·5%) than by aqueous hydrolysis for 48 hr. (98·2%), although in the same paper (their Table VII) they obtained with a series of brain lipid fractions approximately the same recoveries of N by the two methods.

The fatty-acid residues can readily be split off from glycerophospholipids by acid or (particularly) alkali, and the residual glycerylphosphorylcholine and presumably (by analogy) glycerylphosphorylethanolamine are further decomposed quite readily into glycerophosphoric acid and the nitrogenous base. For example, N-HCl at 97° will completely remove choline from glycerylphosphorylcholine in less than 30 min. (Schmidt, Hershman & Thannhauser, 1945). Phosphorylcholine and phosphorylethanolamine, however, are very much more resistant to hydrolysis. Baer & McArthur (1944) found only 20% splitting of phosphorylcholine by N-HCl at 100° after 12 hr. and our own results, confirming those of Levine & Chargaff (1951), indicate only 95–98% hydrolysis of phosphorylethanolamine (prepared by the method of Outhouse, 1937) by 6N-HCl in 48 hr. at 100°. These latter substances, however, are probably not to any great extent intermediates in the hydrolysis of glycerophospholipids by acid or alkali.

As a check on the rate of hydrolysis of the mixed egg phospholipids, a specimen, purified by rejection of material insoluble in cold ether and in 95% ethanol, by passage through a cellulose column and by dialysis to an atomic N:P ratio of 1·00, was hydrolysed with 6N-HCl in sealed ampoules under nitrogen for 3–24 hr. The fatty acids were then removed by filtration and extraction with light

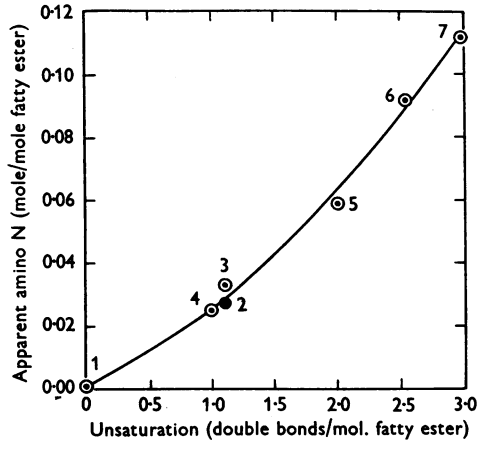

Fig. 3. Apparent amino-N content of fatty esters by the Van Slyke manometric method (5 min. with nitrous acid at 20°) as a function of unsaturation. (1) Ethyl palmitate; (2) egg lecithin; (3) methyl esters of fatty acids of (2), iodine value (I.V.) 94, mean mol.wt. 300; (4) methyl oleate, I.V. 86, mol.wt. 296; (5) methyl linoleate, I.V. 173, mol.wt. 294; (6) methyl ester fraction from linseed oil, I.V. 220, mean mol.wt. 293; (7) methyl linolenate, I.V. 260, mol.wt. 292.

Table 3. *Hydrolysis of egg phospholipid by* 6N-HCl *at* 100°

Time at 100° (hr.)	Amino N*	Choline N*	Amino N + choline N*
3	19·2	80·4	99·6
6	18·7	81·6	100·3
12	18·7	78·5	97·2
24	18·8	82·3	101·1
Average	18·9	80·7	99·6

* As % of the total N recovered.

petroleum and the aqueous phase was analysed for total N, amino N and choline (as reineckate by the method of Entenman, Taurog & Chaikoff, 1944). Approximately 98% of the original N was recovered in the aqueous phase after 3 hr., i.e. a complete recovery within experimental error and all of the choline was already free (Table 3). Apparently the very long periods of hydrolysis often employed are not necessary if the aim is only to estimate fatty acids, amino N and choline N in glycerophospholipids.

DISCUSSION

The ninhydrin method. The results presented in this paper indicate that the ninhydrin procedure of Moore & Stein (1948), modified to maintain the lipids in solution, can be used as a rapid and sensitive method for the estimation of phosphatidylethanolamine in the presence of much larger quantities of non-amino phospholipids without the necessity for previous hydrolysis. The behaviour of phosphatidylserine has not been examined but there is no reason to believe that this substance could not similarly be estimated.

Strict conformity with Beer's law and satisfactory reproducibility were easily obtained within any one run, but considerably different molar colour yields could result in different runs with the same substance unless care was taken with the purification and storage of the reagents and particularly with the elimination of peroxide from the cellosolve used as solvent for the phospholipids. As with any technique involving a ninhydrin reagent, it is desirable to carry out check determinations at intervals with one or more concentrations of a standard substance. The standard chosen should preferably be of the same type as the material under examination in view of the rather surprising observation of Levine & Chargaff (1951) that the molar colour yields from serine and ethanolamine when determined with different ninhydrin solutions by the normal Moore & Stein procedure did not maintain a constant relationship to one another, but fluctuated widely. Using seven different batches of reagent, apparently stored under nitrogen, they found the highest value for serine to be 17·7% greater than the lowest value, while for ethanolamine the corresponding figure was 32·6%. However, determinations on the same batch of reagent carried out a week apart were the same. Moore & Stein (1948) reported no such erratic behaviour and published a table of colour values for amino acids and other compounds based on leucine as 1·00 which has since been widely used.

Data on estimations using ninhydrin reagents are not usually recorded in absolute units, and although we have done so in the present paper it is probable that the molar colour yields obtained will vary appreciably with the purity of the reagents and the experimental conditions under which the estimations are made. It may, however, be noted that the colour yield per amino group of egg phospholipid (Fig. 1) is only 7% higher than that given by Fowden (1951) for alanine, although ethanolamine gives a weaker colour. The sensitivity of the method is excellent and reproducibility is adequate if reasonable precautions are taken.

The Van Slyke method. No explanation of the interference by unsaturated fatty acids with the Van Slyke estimation of amino N in intact phospholipids appears to have been given, although Folch *et al.* (1940) indicated (without quoting any other figures) that the magnitude of such interference was of the order of 0·12 mole nitrogen per unsaturated linkage, presumably for a reaction time of 10 min. at 23·6°. The present observations show a smaller effect (for a reaction time of 5 min. at 20°) which, however, increases with the number of double bonds present in the fatty-acid chain.

Labile methylene groups between two carbonyl groups are known to react with nitrous acid to give oximino compounds which, in turn, can react with nitrous acid to liberate nitrous oxide (Taylor & Baker, 1937) and it may be that in the case of the unsaturated fatty acids the reactive methylene groups adjacent to and particularly between the double bonds are involved. Whatever the precise nature of the side reactions with unsaturated fatty acids there seems little hope of applying the Van Slyke method to the quantitative determination of free amino groups in unhydrolysed phospholipids except in a very empirical and approximate way unless the unsaturated fatty acids are first destroyed, e.g. by hydrogenation.

SUMMARY

1. The ninhydrin procedure of Moore & Stein (1948), modified to maintain the lipids in solution, has been applied to the estimation of phosphatidylethanolamine (1–5 μg. N) in the presence of phosphatidylcholine (up to 1000 μg. N) without preliminary hydrolysis of the phospholipid.

2. Phosphatidylcholine as well as phosphatidylethanolamine liberates gas in the Van Slyke manometric procedure for the estimation of amino N owing to reaction of its component unsaturated fatty acids with nitrous acid. This method can therefore be applied only empirically to unhydrolysed phospholipids using a reaction time chosen for the particular material to agree with a determination on the fatty acid-free hydrolysate. It is much less sensitive than the ninhydrin method.

This work has been carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research. Mr L. J. Parr contributed to the experimental work.

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Studies in Detoxication

58. THE METABOLISM OF ALIPHATIC ALCOHOLS. THE GLUCURONIC ACID CONJUGATION OF CHLORINATED AND SOME UNSATURATED ALCOHOLS

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Kamil, Smith & Williams (1953*a*) have shown that secondary and tertiary aliphatic alcohols are excreted by rabbits largely conjugated with glucuronic acid, and within the group of compounds studied, the extent of this conjugation increases with the number of carbon atoms. With primary alcohols, on the other hand, the conjugation is low. In general, the extent of conjugation increases in the order primary, secondary, tertiary. The reverse order appears to hold for the extent of oxidation of the alcohols *in vivo*. Although the pharmacological activity of alcohols depends on a number of factors, it does appear that, within an isomeric series, hypnotic activity increases as one passes from primary through secondary to tertiary alcohols (cf. Burger, 1951). In fact, the only simple alcohol which has found therapeutic use besides ethanol is tertiary pentanol (amylene hydrate). There thus appears to be a possible correlation between extent of glucuronic acid conjugation and hypnotic activity.

The introduction of halogens into organic compounds by substitution is often looked upon as a reasonable means of increasing pharmacological activity and, in the alcohol series, 2:2:2-trichloroethanol, 1:1:1-trichloro*iso*propanol (Isopral) and 1:1:1-trichloro-*tert.*-butanol (chloretone, chlorbutol) are much more powerful in their anaesthetic and hypnotic effects than the corresponding unsubstituted alcohols. It would therefore be expected that these substituted alcohols would be largely conjugated with glucuronic acid. The glucuronic acid conjugation of a number of chlorinated alcohols has therefore been measured and compared with that of the corresponding unsubstituted alcohols. Trichloroethanol is an active narcotic (Lehmann & Knoefel, 1938; 1939; Butler, 1948), and Butler (1949) has already shown that it is highly conjugated with glucuronic acid in the dog.

Primary alcohols are usually rapidly oxidized *in vivo* and their glucuronic acid conjugation is very low (Kamil *et al.* 1953*a*, *b*). Owing to the reported high conjugation of trichloroethanol, it was therefore of interest to study the effect upon glucuronic acid conjugation of progressive substitution of the β-carbon of ethanol with chlorine.

Recently the acetylenic tertiary alcohol, 3-methylpent-1-yn-3-ol (Oblivon, Dormison) has been recommended as a mild hypnotic, and it was of interest to include this in our studies. Acetylene itself was once used as a surgical anaesthetic, and Bock (1930) reported both diethylethynylcarbinol and *tert.*-butylethynylmethylcarbinol to produce sleep in dogs in doses of 0·2–0·25 g./kg. body wt. In order to assess the effect of the triple bond on the conjugation, the corresponding saturated alcohol, 3-methylpentan-3-ol (diethylmethylcarbinol) and the unsaturated 3-methylpent-1-en-3-ol were also studied. Propargyl alcohol (prop-2-yn-1-ol) was also fed but proved to be lethal to rabbits in doses of