more powerful and more prolonged narcotic effect than the unsubstituted compound. However, conjugation with glucuronic acid is also an inactivating reaction, but it is localized mainly in the liver. It is also possible that chlorine substitution also retards the rate of glucuronic acid conjugation, so that the narcotic effectiveness of the substituted alcohol may be partly due to inhibition of oxidation and retardation of conjugation.

At the end of Table 1, values for the glucuronic acid conjugation of the three related tertiary alcohols, 3-methylpentan-3-ol, 3-methylpent-1-en-3-ol and 3-methylpent-1-yn-3-ol, are included to show the effect of unsaturation upon conjugation. The total conjugations of these three alcohols are not very different and it appears that the conversion of ethyl into vinyl or to ethynyl has little effect on conjugation. 3-Methylpentyn-3-ol (Oblivon, Dormison, Methylparafynol) has mild narcotic properties but nothing appears to be known about the narcotic properties of the other two alcohols.

SUMMARY

1. The glucuronic acid conjugation of a number of chlorinated and other aliphatic alcohols has been studied in the rabbit, and the glucosiduronic acids have been isolated as triacetyl methyl esters in each case.

2. Conjugation with glucuronic acid becomes a significant metabolic path when two or more chlorine atoms are present on the carbon atom next to that carrying the hydroxyl group. Thus 2-chloroethanol is not conjugated, whereas 2:2-di- and 2:2:2-tri-chloroethanols are highly conjugated (more than 50%).

3. It is suggested that chlorine substitution retards the oxidation of the alcohol and thereby increases its narcotic activity.

4. 3-Methylpentan-3-ol, 3-methylpent-1-en-3-ol and 3-methylpent-1-yn-3-ol are conjugated with glucuronic acid to about the same extent (50%). Diethylaminoethanol does not cause the excretion of extra glucuronic acid in rabbits.

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A Note on the Estimation of Sphingomyelin in Nervous Tissue

By R. M. C. DAWSON*

Department of Biochemistry, University of Oxford

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The estimation of sphingomyelin in animal tissues is almost exclusively carried out by measuring the lipid phosphorus which is stable to mild alkaline and acid hydrolysis. Apart from sphingomyelin, all the known phospholipids are decomposed by incubating with N potassium hydroxide at 37° followed by standing at room temperature at an acid pH (Schmidt, Benotti, Hershman & Thannhauser, 1946). However, the work of Brante (1949) has suggested that certain kephalin fractions isolated from cerebral tissue contain some lipid phosphorus which is stable to mild alkaline and acid hydrolysis, although the fractions themselves contain no choline.

* Betty Brookes Research Fellow.

It was found in the present experiments that when sphingomyelin was estimated in brain tissue by the method of Schmidt *et al.* (1946) lower values were obtained when the lipids in the tissue were initially precipitated with trichloroacetic acid (TCA) before they were extracted with solvents. This has been shown to be due to a loss during the precipitation of non-sphingomyelin lipid phosphorus which becomes insoluble in TCA after alkaline hydrolysis and consequently is estimated as sphingomyelin. Even after the precipitation of brain lipids with TCA, the molar choline/phosphorus ratio of the alkali-stable 'sphingomyelin' is less than unity, indicating that the method of Schmidt *et al.* (1946) is unsuitable for the accurate estimation of this phospholipid.

METHODS

Extraction procedure. Brain tissue was removed from adult guinea pigs soon after death and kept frozen at $-20°$ until required. It was then finely minced and two representative samples were weighed. One sample was ground in a mortar with 5 or 10% (w/v) TCA solution, the precipitate centrifuged off, and washed once with water. The lipids were then extracted from the precipitate and the untreated sample by various solvent-extraction procedures; the initial extraction being always carried out with cold solvents to eliminate the possibility of residual TCA causing phospholipid hydrolysis. The combined extracts of the two samples were each filtered and made up to 500 ml. Occasionally a fine precipitate separated from the direct extract, and was removed. For the estimation of 'alkali-stable' lipid P duplicate samples were taken to dryness *in vacuo* at room temp. and then either directly, or after a further re-extraction with solvents, the lipid residue was incubated for 18–24 hr. with N-NaOH or KOH at 37°. The hydrolysates were cooled in ice, and neutralized with 6N-HCl. An equal volume of 10% (w/v) TCA was then added and after standing 2 hr. at room temp., the precipitated 'alkali-stable' phospholipids were filtered off and copiously washed on the filter paper with large quantities of 5% (w/v) TCA. The paper and the lipids were then completely digested and assayed for phosphorus.

Estimations. Phosphorus was determined by the Fiske & Subbarow method (1925). Choline estimations were performed in duplicate by the procedure of Entenman, Taurog & Chaikoff (1944) after the 'alkali-stable' phospholipids had been filtered off through an asbestos pad and hydrolysed with baryta. If any of the precipitates of choline reineckate was amorphous, all the precipitates, including those of the standards, were crystallized by the procedure of Schmidt, Hecht, Fallot, Greenbaum & Thannhauser (1952). The term 'alkali-stable' P used throughout this paper denotes, for convenience, that phosphorus which remains insoluble after incubation with N-KOH at 37° (24 hr.) followed by standing in 5% TCA for 2 hr. at room temp.

RESULTS

When brain tissue is precipitated with TCA at room temperature before the lipids are extracted with solvents, the 'alkali-stable' lipid P found in the extracts is appreciably lower than that recovered from a direct extract of the tissue (Table 1). This apparent lowering of the sphingomyelin content of the lipid extract was observed with three different procedures for lipid extraction, and also when magnesium chloride was added to the TCA solution to improve the precipitation of the lipid material (Johnson & Dutch, 1951). The decrease appeared to be no greater when dissected white matter was used rather than whole brain tissue. Similar, if somewhat smaller, decreases were observed with samples of lung and liver tissue. The decrease in 'alkali-stable' lipid P accounted for a considerable part of the loss of total lipid P found after the TCA precipitation of brain tissue (Table 1). This loss of total lipid P after TCA precipitation has been observed in other tissues (e.g. Swanson & Artom, 1950), and was

Table 1. *The recovery of total and 'alkali-stable' lipid P from guinea pig tissues by direct lipid extraction and by extraction of lipids after TCA precipitation*

Results are given as mg. P/100 g. fresh tissue.

			Total lipid P		'Alkali-stable' lipid P		
Tissue	Method of lipid extraction	Lipid precipitant	Direct extraction	Extracted after TCA precipitation	Direct extraction	Extracted after TCA precipitation	Recovery of 'alkali-stable' lipid P after TCA precipitation (%)
Brain	Johnson, McNabb & Rossiter (1948)	10% (w/v) TCA	249	195	34·9	20·4	58
			188	176	28·0	18·6	66
Brain	McKibbin & Taylor (1949)	10% (w/v) TCA $+0·4$M-$MgCl_2$	—	—	30·6	22·0	72
Brain	Schmidt, Benotti, Hershman & Thannhauser (1946)	10% (w/v) TCA $+0·4$M-$MgCl_2$	224	209	30·6	21·6	71
Brain		10% (w/v) TCA	245	214	32·7	24·8	76
Brain		5% (w/v) TCA	228	208	33·5	22·8	68
Brain white matter		10% (w/v) TCA	286	266	49·1	36·5	74
Lung		10% (w/v) TCA $+0·4$M-$MgCl_2$	104	98	15·7	13·2	84
Liver			150	131	14·8	12·9	87

generally considered to be due to the removal of acid-soluble phosphorus which contaminates lipid extracts (McKibbin & Taylor, 1949), and also the partial hydrolysis of plasmalogens (Feulgen & Bersin, 1939).

It was clear that the lower recovery of 'alkali-stable' lipid phosphorus found after TCA precipitation was caused either by the precipitant rendering some of this phosphorus non-extractable by lipid solvents or by its removal in the acid-soluble fraction. The former alternative was tested by measuring the 'alkali-stable' P both in the lipid extract and also in the residual P fraction obtained after the solvent extraction of the lipids (Table 2). The 'alkali-stable' P found in this residual fraction was slightly higher when the tissues were precipitated with TCA prior to solvent extraction of the lipids. However, it is apparent from Table 2 that this small increase in 'alkali-stable' residual P could not nearly account for the loss of 'alkali-stable' P from the lipid extract. When measurements were made of the total 'alkali-stable' P present in brain tissue before and after TCA precipitation, it was found that the precipitation resulted in a considerable loss of phosphorus (Table 3). When the precipitation of the total 'alkali-stable' P was carried out at 0°, a similar, although smaller, loss of phosphorus occurred. If, therefore, it is assumed that the cerebral deoxyribonucleic acid P is completely recoverable in the TCA precipitate (Schmidt & Thannhauser, 1945) this result would suggest that most of the 'alkali-stable' lipid P lost during the precipitation becomes soluble in the precipitant.

Table 2. *Estimation of 'alkali-stable' lipid and residual* P *in guinea pig brain after direct lipid extraction and extraction of lipids after TCA precipitation*

Results are given as mg. P/100 g. fresh tissue.

Tissue	Lipid precipitant	'Alkali-stable' lipid P: Direct extraction	'Alkali-stable' lipid P: Lipids extracted after TCA precipitation	'Alkali-stable' residual P: Direct extraction	'Alkali-stable' residual P: Lipids extracted after TCA precipitation
Brain	7% (w/v) TCA	38·4	24·0	7·7	11·3
	10% (w/v) TCA	34·6	23·5	7·8	10·4
Brain white matter	10% (w/v) TCA	—	—	6·0	7·4
	10% (w/v) TCA	49·1	36·5	7·1	8·2

Table 3. *The loss of total 'alkali-stable'* P *during precipitation of guinea pig brain tissue with TCA*

Expt. no.	Conditions of precipitation	Total 'alkali-stable' P (mg. P/100 g. fresh tissue)	'Alkali-stable' P lost by precipitation (mg. P/100 g. fresh tissue)
1	No precipitation, direct lipid extraction	49·4	—
	Precipitation with 10% (w/v) TCA at room temp.	37·8	11·6
2	No precipitation, direct lipid extraction	48·1	—
	Precipitation with 10% (w/v) TCA at room temp.	34·0	14·1
	Precipitation with 10% (w/v) TCA at 0°	39·1	9·0
3	No precipitation, direct lipid extraction	52·1	—
	Precipitation with 5% (w/v) TCA at room temp.	38·9	13·2
	Precipitation with 5% (w/v) TCA at 0°	49·5	2·6

Table 4. *The effect of TCA precipitation on the choline content of the 'alkali-stable' lipids of brain*

Expt. no.	Method of preparation of 'alkali-stable' lipids	Choline (mg./100 g. fresh tissue)	Molar ratio choline/phosphorus
1	Direct lipid extraction	70	0·69
	Extraction of lipids after precipitation of brain with 5% (w/v) TCA (room temp.)	69	0·91
	Extraction of lipids after precipitation of brain with 5% (w/v) TCA (0°)	68	0·76
2	Direct lipid extraction	78	0·61
	Extraction of lipids after precipitation of brain with 5% (w/v) TCA (room temp.)	81	0·89
3	Direct lipid extraction	39	0·39
	Extraction of lipids after precipitation of brain with 10% (w/v) TCA (room temp.)	44	0·58

It therefore became important to know whether the loss of 'alkali-stable' lipid P occurring during TCA precipitation of the tissue was associated with an equivalent loss of choline. It was found, however, that the precipitation resulted in no appreciable loss of choline from the 'alkali-stable' phospholipids (Table 4). When the lipids were directly extracted from the tissue the 'alkali-stable' lipids had a molar choline/phosphorus ratio which was considerably less than unity. Although the loss of 'alkali-stable' lipid P during TCA precipitation raised this ratio somewhat, it was still less than unity, suggesting that the 'alkali-stable' phospholipids still did not consist entirely of sphingomyelins.

DISCUSSION

The finding that the molar choline/phosphorus ratio of the 'alkali-stable' brain lipids is less than unity suggests that other phospholipids apart from sphingomyelins are present in this fraction. The estimation of choline by reineckate precipitation is notoriously affected by interfering substances and therefore, though good recoveries of added choline were obtained from hydrolysates of the fraction, such evidence on its own cannot be regarded as conclusive. However, the observation that TCA precipitation of brain tissue removes considerable amounts of lipid P from the subsequently isolated 'alkali-stable' lipid fraction without removing choline, confirms that this fraction, isolated from direct lipid extracts, cannot consist entirely of sphingomyelin. The results suggest that this conclusion may also apply to other tissues, and in this connexion Mallov, McKibbin & Robb (1953) have recently reported that in fresh lipid extracts of heart muscle, the sphingomyelin P measured by the procedure of Schmidt *et al.* (1946) was much greater than could be accounted for by the sphingosine present in the extract.

The identity of this 'alkali-stable' lipid phosphorus which is not sphingomyelin is not clear. None of the known phospholipids existing in a free state are stable to mild alkaline and acid hydrolysis, except sphingomyelin. The possibility must be considered that the phosphorus is contained in some compound which is not a lipid as the only evidence indicating it is combined in a lipid is its solubility in fat-solvents, and its insolubility in TCA solution. It seems more likely, however, that the phosphorus is derived from a new phospholipid or from some known phospholipid present in a modified or combined form. Present attempts to identify any base in the 'alkali-stable' phospholipids, apart from choline and sphingosine, have been foiled by the presence of protein material derived from proteolipids in the fraction. No more than traces of glycerophosphoric acid could be found in total hydrolysates of the 'alkali-stable' lipids. It is not likely that the substance is produced by a rapid post-mortem enzymic action as a similar effect of TCA precipitation has been observed with rat brain tissue frozen *in situ* with liquid oxygen.

The solubility of the non-sphingomyelin 'alkali-stable' lipid phosphorus during an initial TCA precipitation of the tissue is somewhat puzzling, as after alkaline incubation of the directly extracted lipids it estimates as 'alkali-stable' lipid P, even though it stands for a considerably longer period in contact with TCA solution. The stability of the plasmalogens seems to be the reverse of this behaviour; in crude lipid extracts they are relatively stable to acid conditions, while after alkaline incubation or in the free state they are quickly decomposed (Schmidt *et al.* 1946; Schmidt, Ottenstein & Bessman, 1953; Ansell & Norman, 1953). The possibility of incomplete precipitation by the TCA is unlikely as the resulting TCA solution contains only a negligible quantity of phosphorus which estimates as sphingomyelin.

The estimation of sphingomyelin P in nervous tissue by alkaline hydrolysis therefore only gives an approximate result. Although the accuracy can be improved somewhat by initially precipitating the tissue with TCA, it is probable that for a more precise determination it is preferable to estimate the choline in the 'alkali-stable' lipids or to measure the difference between lecithin and total choline (Brante, 1949).

SUMMARY

1. The molar choline/phosphorus ratio of the cerebral lipids which are stable to mild alkaline and acid hydrolysis is less than unity, indicating that sphingomyelins are not the only phospholipids present.

2. Part of this non-sphingomyelin lipid phosphorus is removed by initially precipitating the tissue lipids with trichloroacetic acid at room temperature, a procedure which does not affect the recovery of sphingomyelin.

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Some Enzymic Changes in the Mammary Gland of Rats during Pregnancy, Lactation and Mammary Involution

By A. L. GREENBAUM and F. C. GREENWOOD
Department of Biochemistry, University College, London, W.C. 1

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Recent work has emphasized that the lactating mammary gland is a site of intense synthetic activity (see Folley, 1949). During the past five years, fat synthesis in the gland has received considerable attention, but protein synthesis has been relatively neglected, although the mammary gland is known to synthesize large quantities of specific proteins, and, since these are promptly secreted into the milk, an equilibrium condition is never reached and the high rate of protein synthesis is maintained.

The application of isotopic methods to the study of milk-protein precursors by Campbell & Work (1952) is clearly a most promising line of investigation, but it seemed to us that the study of some of the enzymes involved might also yield interesting information. In view of the present state of knowledge of the mechanism of protein synthesis, it seemed more profitable to make a general survey of some of the enzymes which might be implicated in these processes, rather than an intensive study of a particular enzyme system. We have therefore studied glutamic dehydrogenase, glutamic–aspartic transaminase and cathepsin, which have been suggested as playing some part in protein synthesis. In addition, we have measured the activity of β-glucuronidase, first because this enzyme is also found in the particulate fractions (Walker & Levvy, 1953) and is one which, because it is believed not to be concerned with tissue growth in the rat, would differentiate between a specific enzymic change and a general change in the level of mitochondrial enzymes; and secondly because of its postulated role in oestrogen metabolism (Fishman, 1947).

EXPERIMENTAL

Animals. Virgin rats of the hooded Norway strain of the Medical Research Council, aged 3 months, were mated with males of the same strain and age. Nine groups of rats, each containing five animals, were used; three groups of pregnant animals which were killed on the 10th, 15th and 20th days of pregnancy, four groups which were killed on the 5th, 10th, 15th and 20th days of lactation and two further groups which had their litters removed on the 21st day of lactation and were killed on the 2nd and 4th days of mammary involution. During lactation the litter size was reduced to eight pups, four male and four female, where possible. Rats were killed by dislocation of the cervical vertebrae and the three abdominal mammary glands quickly removed, weighed and disintegrated in water in a Folley & Watson (1948) blender to give a final tissue concentration of 1:5 for glutamic dehydrogenase and transaminase estimations, and 1:10 for cathepsin and β-glucuronidase estimations.

Materials. α-Oxoglutarate was prepared by the method of O'Kane (1949) and was 96% pure by carbonyl-group estimation.

Cytochrome *c* was prepared by the method of Keilin & Hartree (1945) from horse heart. This had a concentration of $3{\cdot}26 \times 10^{-3}$ M as estimated by the method of Potter (1941).

Diphosphopyridine nucleotide (DPN) was prepared by the method of Williamson & Green (1940); various preparations gave assays between 55 and 65% pure.

p-Chlorophenyl glucosiduronic acid was prepared by the method of Spencer & Williams (1951). We found that a better yield could be obtained if the dose of *p*-chlorophenol (0·5 g./kg. of rabbit in 50 ml. of water) was divided into two portions and one given at 10 a.m. and the other at 6 p.m.

Haemoglobin was prepared by the method of Anson (1937) and stored in the freeze-dried state. For use, a 5% (w/v) solution of haemoglobin in water was prepared fresh each day.

Chemical methods. Lactose was estimated by the method of Hinton & Macara (1927) as modified by Folley & Greenbaum (1947). The milk content of the mammary gland suspensions was calculated from the lactose content and all enzyme assays were corrected for the 'milk error' as described by Folley & Greenbaum (1947).

Enzymic methods. Glutamic dehydrogenase was measured by the method described by Copenhaver, McShan & Meyer (1950), except that the reaction was carried out in the presence of 0·1 M nicotinamide, in view of the observation of Moore & Nelson (1952) that mammary gland contains an active nucleotidase and that of Terner (1952), who also