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The nitrogenous constituents of the lipids of several dog tissues*

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SUMMARY

A technique is described for the resolution and quantitative determination of the nitrogenous constituents of dog tissue lipids. Results of representative analyses are presented for seven different tissues. All but a few per cent of the total lipid nitrogen has been specifically accounted for as choline, ethanolamine, serine, other amino acids, ammonia, sphingosine, and hexosamine. Serine-containing lipids comprise a maximum of 2.6% to 7.7% of total lipid nitrogen of the tissues studied. Despite extensive purification of the lipid extracts, other amino acids and peptides are present in quantities comparable to serine. The ammonia content of the lipid hydrolyzates ranges from 4.3% to 5.9% of lipid nitrogen. From 1.0% to 9.4% of lipid nitrogen becomes water soluble after hydrolysis, and is neither choline, ammonia, nor primary amine. This is considered to be substituted amine, although no important fraction of this material has been identified.

A number of analytical procedures have been used for the determination of the nitrogenous components of lipids (1, 2, 3). However, a complete analysis of the lipids of animal tissues for all the components is beset with several difficulties. First, lipids may combine with amino acids and other nitrogenous substances to form rather stable, solvent-soluble adducts as an artifact of extraction (4, 5). The amount of protein or peptide in the lipid extracts depends on the extraction procedure (6, 7) and the method of purification. Some of this peptide may be split from the lipids by further treatment with neutral solvents, and is therefore not a true component of the lipids. Second, amino acids and peptides interfere with most of the primary amine reactions which have been used to measure ethanolamine and serine. Much of the existing data on the distribution of these substances is open to question. Third, the stability of certain lipids to hydrolytic agents (8) necessitates drastic conditions of hydrolysis which may destroy the liberated bases or produce artifacts. Finally, purified animal tissue lipids contain minor amounts of unidentified nitrogenous substances (9).

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This paper describes the fractionation of the hydrolysis products and a reasonably complete analysis of the fractions. The procedure includes (*a*) removal of most of the interfering peptide, (*b*) a two-stage hydrolysis which permits removal of the free bases released by mild hydrolytic conditions before the use of more drastic conditions, (*c*) a chromatographic resolution of the base mixture for a more accurate determination of the minor components, and (*d*) the use of periodate oxidation, methylation, and paper chromatography for a specific analysis of the primary amines. The results of analyses of a variety of dog tissues are presented and compared with those from other laboratories.

EXPERIMENTAL

Extraction and Purification. Frozen tissue powder was refluxed with ethanol-ether 3/1 (v/v) for $1^1/_2$ hours, as described previously (10). The dried tissue residue was then extracted with chloroform, in a Soxhlet extractor, and the two extracts were combined. The extracts were purified by re-extraction with chloroform and emulsification into 0.25 M $MgCl_2$, as described (10). However, the final emulsion interphase was not combined with the clear chloroform infranatant. Instead, it was dissolved in a minimal volume of

chloroform-methanol 2/1 (v/v), and the resulting upper aqueous-alcohol phase was discarded. The upper phase contained insignificant amounts of phospholipid and sphingolipid but much peptide material, as evidenced by its nitrogen content (14% to 15%) and the release of 70% to 90% of its nitrogen as free amino nitrogen by acid hydrolysis. The lower chloroform phase was combined with the original washed chloroform infranatant. The lipid extracts gave somewhat lower nitrogen to phosphorus ratios than we have obtained previously for dog tissues and for the same tissues extracted and purified by the method of Folch *et al.* (11). Minimal amounts of "phosphatido-peptides" (7) were present, since neutral solvents were used in the extraction. The extraction of these animal tissues was essentially complete, and the losses of lipid incurred in purification were measured precisely and found to be small (10).

Hydrolysis of the Lipids and Fractionation of the Bases. The over-all procedure is shown diagrammatically in Figure 1. An aliquot of the purified lipids, equivalent to about 3 mmoles of lipid nitrogen, was freed of solvents by vacuum distillation. Eighty milliliters of 1 N NaOH was added, and saponification was allowed to continue at room temperature for 3 days under an acid trap. Fifty-six milliliters of concentrated HCl and then 100 ml of chloroform were added, and the mixture thoroughly shaken. The emulsion was broken by refrigeration and centrifugation, yielding an aqueous phase, fraction A, and a chloroform phase, fraction B.

The aqueous phase, fraction A, was evaporated to dryness under reduced pressure with several additions of water to remove the excess HCl completely. Seventy-five milliliters of absolute ethanol was added, and the mixture was allowed to stand for 24 hours. The alcoholic solution was decanted and the salt residue washed with an additional 35 ml of ethanol, which was then dissolved in the least volume of water (usually about 10 to 15 ml) and precipitated with $1^1/_2$ volumes of alcohol. The salt precipitate was rinsed with alcohol, and the supernatant and washings evaporated to dryness *in vacuo*. The alcohol precipitation from the aqueous solution was repeated twice, and the final alcohol supernatant was added to the original alcohol extracts. The combined salt residues, fraction Al, contained about 1% of the total original lipid nitrogen. The aqueous alcohol extract was evaporated to dryness *in vacuo* and the residue was taken up in 40 ml of water. The solution was adjusted to pH 2 with 1 N NaOH, and any insoluble matter, fraction A2, was centrifuged down. The latter usually contained less than 1% of the original lipid nitrogen and is included in the "Unassigned N" in Table 1. The aqueous solution was then chromatographed on a Dowex 50 Na column prepared according to the method of Moore and Stein (12). The column had an internal diameter of 10 mm and contained 33 cm Dowex. After passage of the solution, the column was washed with 30 ml of 0.01 N HCl, which was combined with the original effluent and designated fraction A3. The column was eluted with 95 ml of 0.05 M sodium acetate buffer, pH 5.0 (fraction A4), 60 ml of 0.05 M $NaHCO_3$—

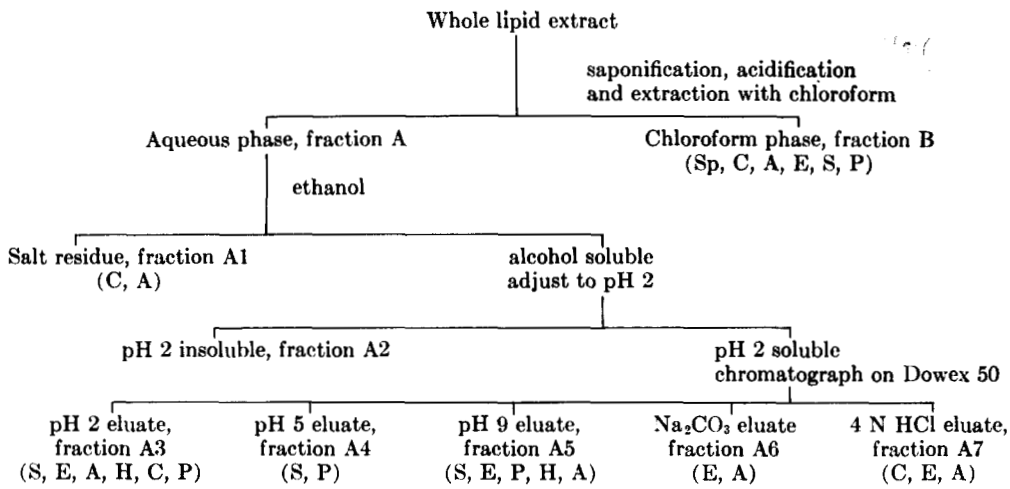

FIG. 1. Fractionation scheme for the analysis of lipid extracts. Nitrogenous components determined directly are shown with each fraction in which they occur. *S* = serine, *E* = ethanolamine, *P* = other primary amine, *C* = choline, *Sp* = sphingosine, *H* = hexosamine, *A* = ammonia.

TABLE 1. SUMMARY OF NITROGENOUS COMPONENTS OF DOG TISSUE LIPIDS

Tissue	μmole/g Dry Lipid-Free Tissue		Per Cent of Total Lipid N										
	Total N	Total P	Ethanolamine N	Serine N	Other Primary Amine N	Total Ammonia N	Saponification Ammonia N	Hexosamine N	Choline N	Substituted Amine N		Sphingosine N	Unassigned N
										Total	Fractions A3, A4, and A5 only		
Liver	177	180	17.3	3.4	7.0	4.6	3.9	0.5	49.6	9.4	5.1	4.1	4.5
Spleen	166	136	26.5	7.7	4.6	5.9	4.9	0.5	39.0	4.3	2.6	13.1	0.7
Pancreas	169	173	20.4	2.8	4.1	4.4	4.0	0.2	53.9	3.7	2.8	7.9	3.9
Lung	173	146	20.4	6.6	4.4	4.4	3.3	0.7	45.4	3.3	3.0	13.3	2.4
Lung	167	141	22.6	6.3	3.0	5.0	4.0		44.5	2.4	1.4	9.1	7.7
Kidney	208	188	31.6	4.9	4.1	4.4	3.3	0.3	38.7	3.7	3.4	10.8	4.0
Intestine	119	110	25.9	6.3	4.8	4.8	2.8	0.9	43.0	1.0	0.9	13.7	0.5
Heart	131	148	29.0	2.6	2.5	4.3	3.6	0.4	49.5	5.1	3.8	7.9	1.3

Na_2CO_3 buffer, pH 9.0 (fraction A5), 95 ml of 0.1 M Na_2CO_3 (fraction A6), and finally 35 ml of water, followed by 140 ml of 4 N HCl (fraction A7). These fractions were analyzed by the methods described below.

Analytical Methods. Total nitrogen was determined by a modification of the Kjeldahl method (10). Total phosphorus was determined by a modification of the Fiske and SubbaRow method (10). Ammonia nitrogen was determined by distillation and nesslerization or direct nesslerization (10). Hexosamine was determined by the method of Elson and Morgan (13), as modified by Blix (14), on an aliquot hydrolyzed by refluxing $3^1/_3$ hours with 2 N HCl. Choline was determined as the Reineckate salt (10). Hydrolysis of fraction A3 for choline phosphoric acid was carried out in 5.5 N HCl in sealed tubes at 130° for 15 hours. Primary amines were determined by the naphthoquinone sulfonate color reaction (2). A correction for ammonia content based on the 67% molar color yield given by ammonia was applied, and the corrected value designated "True Amino N" in Table 2. Hydrolysis of fractions A3 and A5 for release of amino nitrogen was carried out in 2 N HCl in sealed tubes at 120° for 15 hours.

Total ethanolamine and serine were determined as ammonia liberated by periodate oxidation under the conditions of Frisell and Mackenzie (15). The ammonia was adsorbed on 1 g of washed Permutit (Folin Decalso) in a 25-ml volumetric flask, washed several times with water to remove formaldehyde, and then eluted directly with Nessler's reagent by the procedure of Folin and Bell (16). The difference between true amino nitrogen and ammonia liberated by periodate is designated "Other Primary Amine" (Table 1). This calculation is valid because the naphthoquinone sulfonate reagent gives approximately the same molar color yield for ethanolamine, serine, other primary amines, and amino acids (17).

The separation of ethanolamine from serine in the hydrolyzate of fraction A3 was carried out by Permutit adsorption, as described previously (2). The Permutit eluates were analyzed for primary amine using the naphthoquinone sulfonate procedure. The "ethanol-

TABLE 2. NITROGENOUS COMPONENTS OF THE AQUEOUS PHASE (FRACTION A) AFTER SAPONIFICATION OF INTESTINE LIPIDS

Fraction	Total N	Total P	Choline N	Ammonia N	True Amino N	Substituted Amine N	Serine N	Ethanolamine N	Other Primary Amine N	Unassigned N
A1	22.7		7.2	1.5	9.8	4.2				9.8
A3	199		0.0	14.7	110					
A3 hydrolyzed			15.9	27.9	148	7.2	44.3	38.7	65.0	0.0
A4	131	0.8	0.0	0.0	125	6.0	88.0	0.0	37	0.0
A5	45.3	0.6	2.4	2.9	12.4					
A5 hydrolyzed				11.2	19.0	12.7			13.4	5.6
A6	558	1.6	0.0	39.4	510	0.0	0.0	523	0.0	0.0
A7	1050	0.0	1040	20.8	18.9	0.0	0.0	18.9	0.0	0.0

All values are given in micromoles. Total original lipid N, 2960 μmoles; P, 2740 μmoles; hexosamine, 27 μmoles.

amine" values may be accepted as ethanolamine, since no basic amino acids are present in the hydrolyzates. In a number of instances, ethanolamine analyses were confirmed by methylation to choline. A suitable aliquot of known choline content was dried *in vacuo* and taken up in 3 to 5 ml of methanol. Methanolic KOH was added until strongly alkaline. Three-tenths of a milliliter of methyl iodide was added, and the mixture was allowed to stand at room temperature overnight. The mixture was acidified with glacial acetic acid, and the solvents removed *in vacuo*. The residue was then taken up in 10 ml of water and choline determined as the reineckate. Ethanolamine is calculated by subtracting the original choline from the total found after methylation. This procedure gives 90% to 95% conversion of ethanolamine, and only 2% to 4% conversion of serine to choline.

Serine is calculated as the difference between total periodate-released ammonia nitrogen and ethanolamine nitrogen. Usually no correction for ammonia is necessary, since this is poorly eluted from Permutit under the conditions of the analysis. There should be no interference from small amounts of N-acylated hexosamine present, since this does not yield ammonia on oxidation with periodate (18).

The "Substituted Amine" in Table 1 is the difference between total Kjeldahl nitrogen of the aqueous phase fractions and the sum of choline, ammonia, and total primary amine.

Paper Chromatography. Three solvent systems were routinely used for ascending chromatograms: (*a*) butanol-glacial acetic acid-water 4:1:5 (v/v), (*b*) butanol-glacial acetic acid-water 2:1:2 (v/v), and (*c*) butanol-ethylene glycol-water 4:1:1 (v/v). In using system (*a*), the paper cylinder was placed in the upper phase, and a beaker containing the lower phase was placed in the middle of the chromatography vessel. Systems (*b*) and (*c*) were monophasic. Fractions A4, A5, A6, and A7 were acidified with HCl, concentrated to dryness under reduced pressure, and extracted with absolute ethanol. After two extractions, the salt residue contained no more than traces of nitrogen. The alcohol extracts were then concentrated and chromatographed. Ninhydrin was used as developer for the primary amines, and Dragendorff's reagent was used for choline.

Analysis of Fraction B. This fraction contained 10.0% to 32.2% of the original lipid nitrogen (Table 3). Only traces of fatty aldehyde were still bound as phospholipid, as revealed by silicic-acid chromatography. Choline and sphingosine were determined on an aqueous $Ba(OH)_2$ hydrolyzate of this fraction. A dried aliquot was refluxed with saturated $Ba(OH)_2$ on a sand bath for 7 hours by the method of McKibbin and Taylor (19). The HCl hydrolysis was omitted to improve recovery of sphingosine (20). For the determination of primary amines, a dried aliquot was refluxed for 12 to 15 hours in 1.6 N ethanolic HCl. The lipids remained in solution throughout the hydrolysis period. The alcohol was evaporated under reduced pressure, and the hydrolyzate was partitioned between chloroform and water Total primary amine, ethanolamine, and serine were determined as described above. The methylation of ethanolamine to choline was especially useful in analyzing for the former in these hydrolyzates. The composition of fraction B from the several tissues is summarized in Table 3. It is seen that ethanolamine contributes a major portion of this nitrogen in all the tissues. The sum of (*a*) the nitrogen of the A fractions, which was not established in the other categories, (*b*) the nitrogen of fraction A2, and (*c*) the uncharacterized nitrogen of fraction B (Table 3) are designated "Unassigned N" in Table 1.

RESULTS

The saponification releases as free base most of the

TABLE 3. NITROGENOUS COMPONENTS OF THE CHLOROFORM PHASE (FRACTION B) AFTER SAPONIFICATION

Tissue	Total P	Total N	Uncharacterized N	Choline N	Ammonia N	Ethanolamine N	Serine N	Other Primary Amine N	Sphingosine N
Liver	1.18	10.0	1.63	1.79	0.69	0.87	0.0	0.98	4.06
Spleen	17.5	25.5	0.0	5.10	0.97	6.83	0.33	0.81	13.1
Pancreas	9.1	18.9	3.57	2.33	0.42	5.03	0.0	0.0	7.87
Lung	18.4	30.1	1.53	6.77	1.11	7.40	0.0	0.0	13.3
Lung	14.3	28.8	5.95	5.13	0.99	6.04	1.57	0.0	9.12
Kidney	20.1	28.6	0.18	6.51	1.01	10.0	0.0	0.0	10.8
Intestine	21.1	32.2	1.45	7.11	2.05	6.35	1.86	0.91	12.5
Heart	13.9	19.6	0.0	3.64	0.64	6.98	0.45	0.0	7.92

All values are given in per cent of total lipid N or P in the original extract.

nonsphingosine nitrogen from the lipids of these tissues, although the subsequent acidification with HCl is probably responsible for the hydrolysis of plasmalogen. Fractions A4, A5, A6, and A7 comprised 60% to 80% of the total lipid nitrogen, with only traces of phosphorus present. The remaining nitrogen is contained in sphingolipids and other alkali-resistant lipids in fraction B, and in acidic substances (including small amounts of phosphate ester) in fraction A3. The composition of the bases released by saponification is revealed by analysis of the five Dowex eluates, fractions A3 to A7 inclusive. A typical analysis for lipids of the intestine is shown in detail in Table 2. It is apparent that fractions A3, A4, and A5 contain, among them, all of the serine and other amino acids, nearly all of the substituted amines, and a part of the ammonia. Fraction A6 contains only ethanolamine and ammonia, and fraction A7 contains only choline and traces of ethanolamine. Hence, the minor components are entirely segregated into the first three Dowex fractions, which contain only 17% to 30% of the original total nitrogen. This circumstance allows for more accurate measurement of these substances, particularly of the substituted amines, which must be determined by difference. For this reason, the values in Table 1 for substituted amines in fractions A3, A4, and A5 are more valid than those which include fractions A6 and A7. Dowex 50 was used to separate ethanolamine from serine in lipid hydrolyzates by Dittmer *et al.* (21).

The amount of each base in the lipid is given as the sum of all the fractions (Table 1). Recovery of the total original lipid nitrogen is within 0% to 3% in all cases. All but a few per cent of this can be definitely assigned; only the "Unassigned N" remains uncertain.

The ammonia content of the aqueous phase after saponification is surprisingly high ("Saponification Ammonia," Table 1). This has been found consistently in lipid hydrolyzates by others, and has been attributed to decomposition of other bases, including ethanolamine and serine (21). The recovery of ethanolamine from acid hydrolyzates of several tissues may be unsatisfactory (2), but it is doubtful if significant amounts of ammonia arise from these two amines by the hydrolysis methods used in this study. Both ethanolamine and serine appear completely stable to the alkaline hydrolysis. The large amounts of ammonia, in some instances equivalent to over 20% of the ethanolamine found, must arise from other sources. Some may be derived from amide nitrogen in the peptide present, and some may be bound as an artifact of extraction. Ammonia is produced in additional amounts in the more drastic hydrolyses of fractions A3 and A5 (Table 2) and fraction B (Table 3). This is summarized in Table 1 as the difference between "Total Ammonia" and "Saponification Ammonia."

Total lipid hexosamine is also shown in Table 1. This appears largely in fractions A3 and A5. Since the total amount of this substance is small and scattered, not all of the fractions were analyzed. For uniformity, the values given for ethanolamine and serine, in fraction A5, are therefore not corrected for the small amounts of hexosamine present. In fraction A3 the hexosamine is presumably acylated and would be a component of the "Substituted Amines."

The values obtained for total choline are comparable to those published previously from this laboratory (22) and elsewhere. The choline found in lipids of fraction B (Table 3) might be used as a determination of sphingomyelin, since the saponification conditions are similar to those of Schmidt *et al.* (23), and since the plasmalogens are hydrolyzed upon acidification. The sphingomyelin values so obtained are lower than those reported by Schmidt *et al.* (23), who measured phosphorus after selective hydrolysis, and are much lower than those of Marinetti *et al.* (24), who used quantitative paper chromatography. Species and individual variations probably do not account for this magnitude of difference. Our analyses for sphingomyelin depend upon complete release of choline from sphingomyelin and may be low. On the other hand, a total phosphorus analysis might include other unknown alkali-stable phosphatides and give high values. The paper chromatographic values probably include other phosphatides.

The values for sphingosine are substantially lower, and those for ethanolamine correspondingly higher, than we have reported previously for dog tissues (2, 9, 19, 22). The two-stage hydrolysis gives more complete hydrolysis of an ethanolamine-containing lipid. The nature of this lipid is now under investigation. It is apparently not the glyceryl ether ethanolamine phosphatide isolated from egg yolk by Carter *et al.* (8). In liver, some of this ethanolamine is present in the phosphorus-free glycolipid fraction.[1]

The values for lipid serine are lower than we reported previously (2, 9), because of removal of interference from peptides and amino acids by the use of periodate (25). It is apparent that serine phosphatide is a minor component of these dog tissues, and especially of liver, heart, and pancreas. The low values for serine are in agreement with those of Collins and Wheeldon (26), based on hydrolysis of dinitrophenyl phospholipids and separation of the dinitrophenylamines. They are generally lower than those obtained by Nojima and

[1] J. M. McKibbin. To be published.

Utsugi (27) using dinitrophenyl derivatives prepared from lipid hydrolyzates, and by Marinetti *et al.* (24) using quantitative paper chromatography. Again, species and individual differences may account for a part of the variation, but much probably arises from differences in the methods of analysis. Moreover, the values for serine found in this study are maximal, since threonine and hexosamine would be determined as serine, and some of the serine may arise from peptide.

The amount of primary amine other than ethanolamine and serine is designated "Other Primary Amine" (Table 1), and is generally comparable to serine. This fraction is considered to be largely amino acid because it accompanies serine in the Dowex fractions A3, A4, and A5, and reacts with ninhydrin on the paper chromatograms at room temperature. None of the individual amino acids have been determined quantitatively. A number of different amino acids are present in the fractions. The basic amino acids are easily distinguished in these systems by their low R_f values, and have not been found in significant amounts in any of the chromatograms. The valine-leucine-isoleucine group is distinguished by the high R_f values and the characteristic appearance of the ninhydrin spots. This group is prominent in fractions A4 and A5 from all of the tissues. Although the total amount of free primary amine was increased by hydrolysis of fractions A3 and A5, appreciable amounts were free in the aqueous phase following saponification alone. Whether this amino acid and peptide represents a failure in purification, an artifact of extraction, or true lipid-bound amino acid in some stage of metabolism (28, 29) is unknown.

The quantity of "Substituted Amine" is subject to the error of analyses made by difference. The values from fractions A3, A4, and A5 are more reproducible than those from fractions A6 and A7. Material from fraction A3 has been prepared in quantity from dog and horse liver, and attempts have been made to identify the nitrogen-containing components. Small amounts of acylated hexosamine are present, but no sialic acid, as determined by the method of Svennerholm (30). The latter is absent in this same fraction from most of the other tissues, although a trace (0.2% of total lipid nitrogen) is present in lung. O-phosphoethanolamine and O-phosphoserine (21) are not included in this fraction, since they are determined as primary amine with the naphthoquinone sulfonate reagent. Small amounts of material were precipitated with sodium tetraphenylboron, mercuric chloride, picric acid, and reinecke acid. However, no major fraction was obtained in identifiable form.

In fractions A6 and A7, the values for substituted amine may represent artifact of analysis or unidentified lipid base, or both. Since much depends on the specificity of the reineckate determination of choline, this was re-examined in all of the fraction A7. Paper chromatograms showed single spots corresponding to choline in the three solvent systems. In addition, reineckates were prepared from $Ba(OH)_2$ hydrolyzates of whole lipid extracts of all of the tissues reported in this study. The basic material was regenerated from these salts by the method of Kapfhammer and Bischoff (31) and chromatographed on paper. Similarly, only choline spots were found from these salts using five solvent systems, and using Dragendorff's reagent and molybdic acid as developers.[2] It is probable that no quaternary amine other than choline is present, and reineckate choline nitrogen may be identified with Kjeldahl N. Inasmuch as monomethylethanolamine and dimethylethanolamine have been found in hydrolyzates of *Neurospora* (32) and rat liver lipids (33), these might account for a part of the substituted amine fraction. Collins (34) reported unidentified substituted amine in the lipids of sheep brain.

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