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Note

Silica gel-impregnated paper chromatographic determination, by differential staining, of N-acyl lipids

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The increasing interest in N-acyl phosphatidyl ethanolamine (NAPE)[1] from various biological sources has required techniques which can suitably differentiate these substances from other N-acyl lipids and from amino lipids, notably phosphatidyl ethanolamine (PE). We report here our observations derived from chromatography on Whatman SG-81 silica gel-impregnated filter paper and modifications of the N-chlorination *o*-tolidine–KI procedure representing, largely, the cumulative experience of others[2–4]. To facilitate this examination a convenient chlorination apparatus has been assembled from commonly available laboratory glassware.

EXPERIMENTAL

The chlorination apparatus

As shown in Fig. 1, this consists of a 500-ml, three-neck, round-bottomed flask with a 21-cm 45/50 standard-taper tube, inserted into the center neck, to contain the rolled chromatogram. This tube serves as the chlorination chamber and is capped by a PTFE plate through which extends a short stainless-steel rod. The 24/40 side-arm at the left is for introduction of the pulverized $NaCl:KMnO_4$ stoichiometric mixture (500 mg) and the separatory funnel in the right side-arm contains the 9 *M* sulfuric acid necessary for the chlorine generation. To ensure that the chlorine promptly reaches the top of the tube a brief (20 sec) gentle flow of nitrogen is introduced via a connection with the left side-arm. Evidence that chlorination has been adequate is provided by inspection of the inner end of the stainless-steel rod, which acts as a cold-finger: 10 min exposure to the chlorine appears to be sufficient.

Spot-tests

The actual spot-testing occurs after (1) aeration of the chlorinated chromatogram for 10 min, (2) three 20-sec washes in water, (3) blotting with paper towels and air drying (complete dryness is not required). Both sides of the chromatogram can be briefly sprayed with the *o*-tolidine–KI (2:1) mixture for maximal visualization of the

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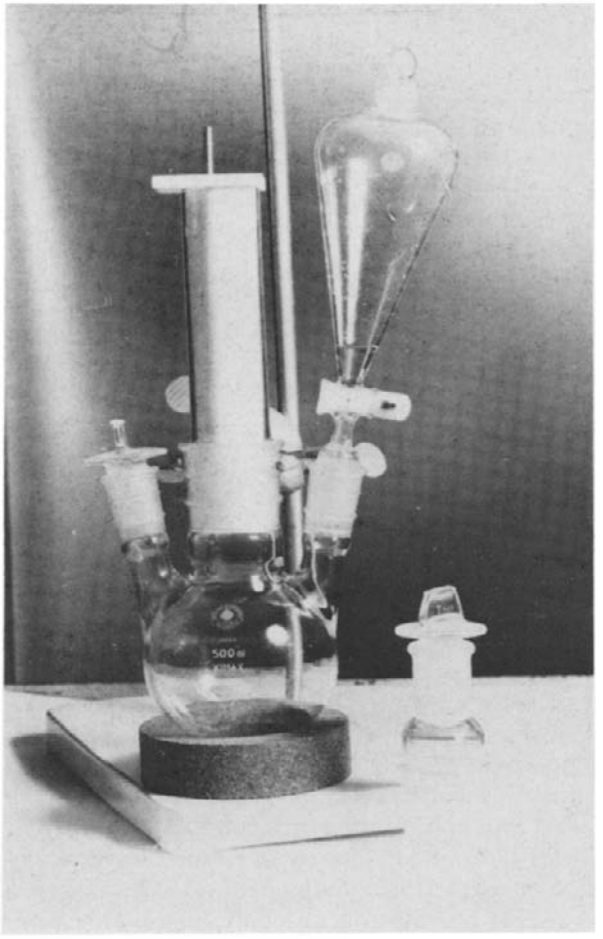


Fig. 1. Chlorination apparatus with rolled chromatogram in place.

N-acyl compounds. Alternatively, spraying can be done first with the ethanolic *o*-tolidine followed (5 min later) by the aqueous KI (or by the *o*-tolidine–KI mixture as discussed in Results. Because of the transience of the resulting blue spots the chromatograms are then photographed prior to any subsequent staining. Visualization of monoglyceride (MG) and ceramide monohexosides is achieved by the PAS reaction and the appropriate use of fluorescamine. Rhodamine 6G, OSPAS and the plasmal reaction, etc. have been described earlier[5]; together they aid in the full characterization of the chromatograms. Commercial preparations of *o*-tolidine are frequently too oxidized to use for the described purpose. However, filtrates prepared from hot saturated solutions of *o*-tolidine in 50 % aqueous ethanol, which have been decolorized by Norite and kept reduced by added ascorbate, produce crystalline material of respectable purity from which the spray reagent is prepared fresh daily (1 mg/ml ethanol); the KI is 1 mg/ml water and is stable.

Chromatography

Extract samples of 10–30 μl are applied to 12 × 19 cm sheets of Whatman SG-81 paper, previously washed with chloroform–methanol (2:1) and by acetone, and run in one of the following developing mixtures in accordance with the nature of the resolution required: chloroform–methanol–14 *M* ammonium hydroxide solution (85:15:1.5), (120:15:1.5) or (180:5:0.5). As indicated in the figures, empiricism remains desirable to achieve the desired optimal resolutions. Repeated use of the developing solvents produces subtle separation effects, as the solvent ratios change, which are not always useful.

The above procedures have been applied to chromatograms prepared from chloroform–methanol (2:1) extracts from freeze-dried samples of (a) normal and infarcted cardiac muscle from dog, rabbit and man, (b) normal hearts of cat, sheep, cow, guinea-pig and monkey, (c) normal brain and optic nerve of the fish *Amia calva* and *Elops saurus*, (d) yolk (hen's egg), yolk-sac and stage-20 chick embryo, (e) NAPE-containing seeds of oat and pea. To assist in the interpretation of the above extracts, co-chromatography with various commercially available lipid standards, of high purity, was resorted to. The palmitoylethanolamide (NAE) was a synthetic product from Calbiochem-Behring (La Jolla, CA, U.S.A.); 10^{-3} μM was readily detectable. The concentration of the extracts was adjusted so that 10–30 μl would produce usable chromatograms, and generally represented 100–500 mg, dry-weight, of tissue per millilitre of solvent. The standards were used at a concentration of 1 mg/ml.

RESULTS

o-Tolidine–KI was found to be a positive spot test for the entire NAE group so far encountered (*e.g.* NAE, NAPE and NA-lyso-PE), for all of the ceramide (Cer) derivatives (*e.g.* n- and h-Cer, CMH, CDH and sphingomyelin), for PE, lyso-PE and MMePE. Neither DiMePE nor PC (TriMePE) stained. The zwitterionic properties of PS probably explain its failure to stain. As expected, the *imine* produced by reaction of PE with acetone was positive but was easily chromatographically resolvable from NAPE. Bis-phosphatidic acids were negative (Figs. 2–4).

Of the above compounds the NAE group was the most refractory to *o*-tolidine staining in the absence of KI (Fig. 5). This behavior therefore provided a useful differential manipulation, particularly under conditions where the ceramides were not clearly resolved from NAE nor CMH from NA-lyso-PE. Cer/NAE resolutions were best in the 180:5:0.5 system, while the 120:15:1.5 system was most useful for extracts containing NAPE, NA-lyso-PE, CMH and PE. More polar NAE/Cer derivatives would require the most polar system.

It was consequently observed, in the DI series (six specimens of 17–24-h infarcts), that there was a wide quantitative variation in NAPE and NA-lyso-PE content (mainly as plasmalogen) with only one specimen containing detectable amounts of NAE. The PAS reaction revealed that, in this series, MG was also present and easily co-chromatographed with NAE (although separable when alerted to the problem). None of the plant seeds examined contained NAE, although NAPE and NA-lyso-PE was present. As with the other infarct (and normal) heart specimens Cer was also present: this increased the resolution problem re: NAE, MG and n- and h-Cer, although these, too, could be resolved and differentiated as indicated. None of the

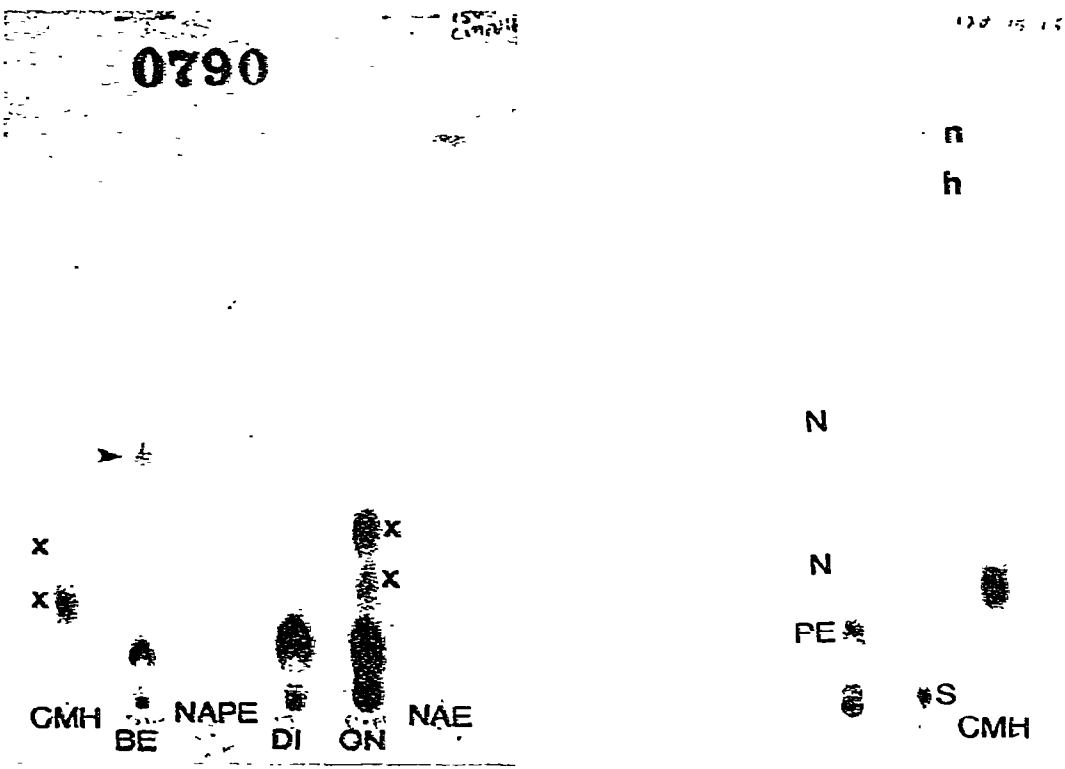


Fig. 2. *o*-Tolidine–KI reaction, chromatogram run in chloroform–methanol–14 *M* ammonium hydroxide solution. From left to rigth, ceramide monohexoside (CMH) with one major and one minor spot (×), black-eye pea, NAPE (silicic acid column isolate from dog-heart infarct), DI, *Elops* optic nerve (ON) and NAE standard. The tissue samples show varying levels of NAPE with pea DI ON; only in the pea (above PE) is an NA-lyso-PE detectable. NAE, right lane, is near the solvent front.

Fig. 3. *o*-Tolidine–KI reaction, chromatogram run in chloroform–methanol–14 *M* ammonium hydroxide solution (120:15:1.5). The samples are, left to right, oat, n- and h-Cer, CMH (two spots). No NAE is present.

other heart specimens contained detectable amounts of the NAE group of compounds. Our exploratory studies showed an exceedingly small amount of NAPE in the yok of hen's egg, while yolk-sac specimens contained somehwat more; none was detected in stage-20 embryos. Although the *o*-tolidine–KI reaction had the desired selectivity, the sensitivity (as a detector of NAPE) was less than that provided by rhodamine 6G and, for the alk-1-enyl species, by the plasmal $HgCl_2$–Schiff reaction (Fig. 6), neither of which stained the NAE. Olefinic NAE species, however, were appropriately positively spot-tested by the OSPAS reaction.

Other biphenyl diamines (*e.g.* benzidine and *o*-dianisidine) were also examined as visualizing reagents but were found to be no more sensitive than was *o*-tolidine and are not further described.

DISCUSSION

On the basis of gas–liquid chromatographic analysis of the amide-linked fatty acids of NAE, isolated from dog heart infarct by thin-layer chromatography, Epps *et al.*[6] have calculated the NAE content and established the FA profile. The presence of

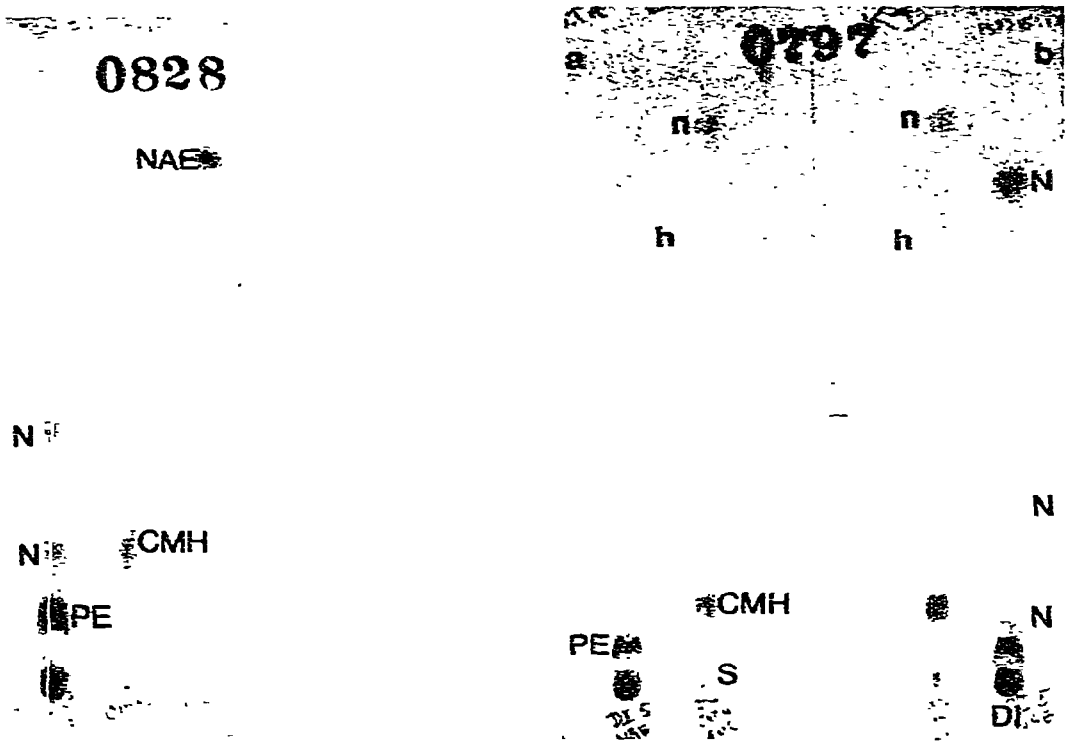


Fig. 4. *o*-Tolidine–KI reaction, chromatogram run in chloroform–methanol–14 *M* ammonium hydroxide solution (120:15:1.5). Left lane, oat; middle lane, CMH (two); right lane, NAE.

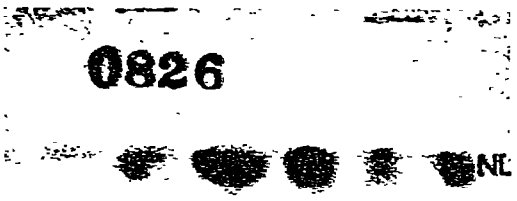
Fig. 5. (a) Sprayed only with ethanolic *o*-tolidine: left lane, DI with added NAE; right lane, Cer (normal and hydroxy standards), CMH and sphingomyelin (S). (b) Sprayed first with *o*-tolidine and then with KI (both sides); same samples as (a), but lanes reversed as shown. From top to bottom (N) refers to NAE, NAPE and NA-lyso-PE, respectively. The solvent (chloroform–methanol–14 *M* ammonium hydroxide solution) ratio is 150:15:1.5.

MG was detected and removed prior to the analysis. From our experience we would expect that any n-Cer present could have contributed to the data.

There have been relatively few reports dealing with the free Cer of tissues[7]. The technique described here may more readily permit the demonstration that the distribution of ceramides is more widespread than currently appreciated and could facilitate its more direct metabolic correlation with the commonly observed sphingomyelin and CMH.

The presence of both NAPE and NA-lyso-PE in various seeds certainly suggests the presence of a phospholipase A as well.

It seems quite remarkable that the two mammalian representatives (cat and dog) for which cardiac infarction results in the appearance of lipids of the NAE group are the same ones whose kidneys are characterized by normal histochemical distribution of neutral lipids in the kidney cortex[8–11]. Does this represent a generalized phenomenon, for these two animals, in the way they handle their fatty acid metabolism?



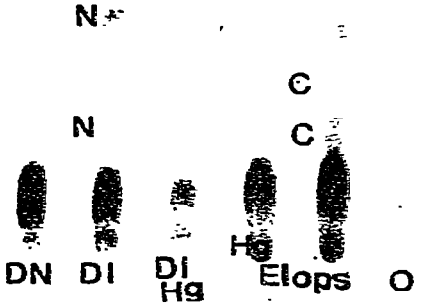


Fig. 6. Plasmal reaction followed by rhodamine 6G; chloroform–methanol–14 *M* ammonium hydroxide solution (120:15:1.5). Left to right, normal dog heart (DN), dog-heart infarct (DI), DI with *in situ* application of aqueous $HgCl_2$ to release free aldehyde from the plasmalogen prior to the chromatographic run, *Elops* optic nerve with and without added $HgCl_2$, oat. The free aldehydes appear at the solvent front with other neutral lipids (NL). Two CMH spots (c) are in the *Elops* samples. The $HgCl_2$ produces Na-lyso-PE (the lower N spot) in both the DI and *Elops* samples.

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REFERENCES

1 M. H. Hack and F. M. Helmy, *Comp. Biochem. Physiol.*, (1982) in press.
2 F. Reindel and W. Hoppe, *Chem. Ber.*, 87 (1954) 1103.
3 A. E. Bresler, *Biochim. Biophys. Acta*, 39 (1960) 375.
4 M. D. Bischel and J. H. Austin, *Biochim. Biophys. Acta*, 70 (1963) 598.
5 M. Helmy and M. H. Hack, *Comp. Biochem. Physiol.*, 71B (1982) 101.
6 D. E. Epps, P. C. Schmid, V. Natarajan and H. H. O. Schmid, *Biochem. Biophys. Res. Comm.*, 90 (1979) 628.
7 M. Sugita, P. Connolly, J. T. Dulaney and H. W. Moser, *Lipids*, 8 (1973) 401.
8 F. M. Helmy and J. B. Longley, *Acta Histochem.*, 25 (1966) 300.
9 F. M. Helmy and M. H. Hack, *Comp. Biochem. Physiol.*, 20 (1967) 55.
10 M. H. Hack and F. M. Helmy, *Comp. Biochem. Physiol.*, 20 (1967) 65.
11 M. H. Hack and F. M. Helmy, *Acta Histochem.*, 27 (1967) 74.