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BIOCHEMICAL STUDIES ON A LYSOSOMAL STORAGE DISEASE IN ABYSSINIAN CATS

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ABSTRACT

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Blood lipid analysis was performed on the serum of 2 normal kittens and 1 adult cat and on serum from 3 affected kittens.

Thin layer chromatography was done on tissue extracts of various organs from clinically affected kittens and unaffected unrelated kittens of a similar age, and on serum from carrier cats, affected kittens, related unaffected kittens and unrelated kittens. Spleen and lymph node cell cultures were prepared from 1 affected kitten and the growth medium and cell cultures were analysed for lipids. A lecithin-like phospholipid was identified in the serum of an affected kitten, a carrier cat and a related unaffected kitten. This substance was produced by the liver of affected kittens and also by macrophage-like cells in spleen cell cultures prepared from the spleen of a kitten with signs of the disease.

INTRODUCTION

Lange, Bland van den Berg & Baker (1977) described a suspected lysosomal storage disease in Abyssinian cats which was characterized by inco-ordination, body tremors and sometimes seizures. The disease was of genetic origin, with an autosomal recessive mode of inheritance (Bland van den Berg, Baker & Lange, 1977). Histological lesions included vacuolization of neurones and macrophages, and ultra-structural examination of these cells showed lamellated membranous cytoplasmic bodies (Lange *et al.*, 1977). Subsequent histochemical and ultrastructural studies on cell cultures of the spleen and mesenteric lymph nodes of affected kittens indicated that the accumulated substance in lysosomes was probably a phospholipid (Lange, 1980). In an attempt to identify the substance, biochemical studies were done on serum, various organs and cell cultures of the spleen and lymph nodes of pure and cross-bred Abyssinian kittens that exhibited clinical signs of the disease.

MATERIALS AND METHODS

Experimental animals

Affected kittens. At various times 9 affected kittens (No. 13, 43, 44, 45, 47, 48, 49, 50 and 51) were anaesthetized by the intravenous administration of pentobarbitone sodium. At least 10 mℓ of blood was collected from the hearts of each of these kittens while anaesthetized. The blood was centrifuged at 3 000 rpm for 5 min and the serum collected and frozen for later analysis. The anaesthetized kittens were then killed with an overdose of pentobarbitone sodium. Within minutes of their death, various organs were aseptically removed from kittens 13, 43–45, 47–49 and frozen for biochemical analysis. The organs involved were portions of the live, kidneys, lungs and spleen, half of the cerebrum, cerebellum and spinal cord and one of the mesenteric lymph nodes.

Carrier cats. Blood was collected from 3 known carrier cats (No. 54, 55 & 56) and the serum so obtained was frozen for later use.

Related unknown cats. Unaffected cats and kittens bred from known carrier cats were bled and serum specimens from these animals were frozen for later examination. Eleven cats (No. 58, 63–72) were subjected to this procedure.

Unrelated cats. Two unrelated kittens (No. 59 & 60) of similar age to the affected kittens were anaesthetized with pentobarbitone sodium, and blood was collected from the heart for preparation of serum. They were subsequently killed, and from them as well as from 2 other kittens (No. 61 & 62) the organs listed above were collected and frozen for biochemical analysis. In addition, 6 more kittens (No. 73–78) were anaesthetized, and blood was collected from the heart. Serum was prepared from this blood and frozen. Serum was also collected from an adult female cat (No. 57).

Cell cultures

Spleen and mesenteric lymph nodes were collected from kitten 51 and cell cultures were prepared as described by Lange (1980). The cultures were maintained for 7 days with daily replacement of the culture medium, i.e. 50% Hank's balanced salt solution and 50% Eagle's minimal essential medium to which 10% inactivated bovine serum and 1,5 g/ℓ sodium bicarbonate had been added. The decanted medium was frozen immediately for later biochemical analysis. The 7-day-old cell cultures together with the medium of that day were frozen for later examination. Unused medium was frozen separately for control analysis.

METHODS

Refer to Table 1 for the tests performed on the various experimental animals.

Blood lipid analysis

1. Total blood lipids

   This was done according to the method described by Woodman & Price (1972).

2. Total esterified fatty acids (TEFA)

   The method described by Galletti (1961) was used for determination of total esterified fatty acids.

3. Triglycerides

   The colorimetric method described by Fletcher (1968) was used for the determination of triglyceride values.

4. Total cholesterol

   Total cholesterol values were determined according to the method of Connerty, Briggs & Eaton (1961).

5. Phospholipids

   The first eluate obtained from the column by the method described by Van Handel & Zilversmit (1957) was used for determination of triglyceride values as a check control on the method described in (3). The eluate obtained from the elution of the column residues with methanol was used for the determination of phospholipid values according to the method of Noël, Marcel & Davignon (1972).

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TABLE 1 Experimental design: tests done on various experimental animals

Methods	Affected kittens									Carrier cats			Related unaffected cats											Unrelated kittens										
	13	43	44	45	47	48	49	50	51	54	55	56	58	63	64	65	66	67	68	69	70	71	72	57	59	60	61	62	73	74	75	76	77	78
Blood lipid analysis																																		
1. Total blood lipid	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−
2. TEFA[1]	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−
3. Triglycerides	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−
4. Total cholesterol	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−
5. Phospholipids	+	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	−
Thin layer chromatography																																		
1. Serum	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−	−	−	−	+	+	+	+	+	+
2. Tissues	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−
3. Cell cultures	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[1] Total esterified fatty acids

TABLE 2 Serum lipid analysis

Cat No.	Total blood lipids g/ℓ	Total esterified fatty acids g/ℓ	Triglycerides mmol/ℓ	Total cholesterol mmol/ℓ	Phospholipids g/ℓ
(a) Unaffected cat and kittens					
57	5,61	1,04	0,47	2,51	0,94
59	13,10	1,64	4,67	3,95	2,93
60	14,03	2,07	4,06	3,70	2,83
(b) Affected kittens					
13	4,68	1,36	0,38	2,97	0,83
43	5,30	1,42	0,63	3,95	0,81
45	13,72	1,91	2,51	5,35	1,89

Thin layer chromatographic procedures

Thin layer chromatography was performed on both plastic-backed[1] and glass-backed[2] plates. All reagents used for the biochemical procedures were of analytical grade[3].

The various spray reagents that were used to determine the nature of the lipids involved included ninhydrin for the demonstration of aminogroups, Rhodamine 6G for the location of lipids in general, the Hanes-Isherwood spray for the location of phospholipids, ammonium reineckate for the location of lecithins and iodine vapours for the location of unsaturated lipids. Apart from the Hanes-Isherwood reagent, which was made up as described in the original publication by these authors (Hanes & Isherwood, 1949), the spray reagents were prepared as described by Smith & Stevens (1969).

Thin layer chromatography was performed on the following:

Serum

Serum specimens obtained from the affected kittens (No. 47–51), the breeding cats (No. 54–56 & 58), the related unknown cats (No. 63–72) and the unrelated kittens (No. 73–78) were extracted according to the column chromatography method of Van Handel & Zilversmit (1957). The eluate obtained from the column residue was then used for thin layer chromatography.

Tissues

Extraction of lipids from the tissues obtained from the affected kittens and from the unrelated kittens (No. 59–62) was done according to the method described by Sperry (1955). The eluates obtained were used for thin layer chromatography.

Cell cultures

The medium that was decanted daily from the spleen and lymph node cell cultures was processed separately, and lipids were extracted according to the method of Sperry (1955). The unused culture-medium was treated in the same manner for control purposes.

RESULTS

Blood lipid analysis

A marked decrease in total blood lipids, total esterified fatty acids, triglycerides and phospholipids was found in 2 of the 3 affected kittens (No. 13 & 43). The total cholesterol value of 1 of them (No. 13) was significantly less than the values for the other 2 affected kittens (No. 43 & 45) and for the 2 unaffected kittens (No. 59 & 60). However, it was similar to the total cholesterol value for the adult cat (No. 57). The serum of this cat (No. 57) yielded equivocal results for the other substances determined, and the values were different from those found for Kittens 13 & 14. The total serum cholesterol value of Kitten 45 was much higher than the values found for either the unaffected or other affected cats. The values for total blood lipids, total esterified fatty acids and triglycerides in this kitten were similar to the values in unaffected Kittens 59 & 60, and the phospholipid value was lower than for these 2 kittens but still higher than the values found for the other 2 affected kittens (Table 2).

Thin layer chromatography

Tissues. Thin layer chromatography on liver extracts of 4 unaffected kittens (No. 59–62) revealed 4 slow-running spots identified as sphingolipid/ganglioside fractions, 2 intermediate spots in the sphingolipid area and 3 fast-running spots, 2 of which were cephalins and 1 a lysolecithin. All these substances were identified by means of the colour reactions with the various spray reagents mentioned in Materials and Methods. The tissues from affected kittens yielded the same chromatograms as above, with an additional fast-running spot (Fig. 1). This substance was identified as a lecithin-like phospholipid by means of the following positive staining reactions: the presence of phosphate groups by the Hanes-Isherwood reagent, amino groups by the ninhydrin spray, the presence of choline with the reineckate reagent and unsaturated fatty acids by the iodine test. It also appeared as if there was a reduction in the sphingolipid/ganglioside fractions in the affected kittens.

The chromatograms of kidney extracts of the unaffected kittens yielded findings similar to those found in the liver specimens. The affected kittens, however, showed a reduction in the sphingolipid/ganglioside fractions and the additional lecithin-like band was absent (Fig. 2).

Chromatographic examination of the spleen, mesenteric lymph nodes, lungs and mixed nervous tissue did not reveal any significant changes between affected and unaffected kittens (Fig. 3).

Serum. The serum of unaffected unrelated kittens showed similar chromatographic features characterized by 1 or 2 slow-running spots in the sphingolipid area, 1 very prominent spot in the sphingolipid area and 2 fast-running spots, 1 a cephalin and the other a lysolecithin. Chromatograms performed on serum extracts from unaffected related kittens revealed a more complex situation. Eight of these kittens (No. 63, 66–72) showed the same chromatographic picture as found in the unrelated kittens, with the addition of a 2nd medium-running spot in the sphingolipid area. Kitten 64 drastically deviated from the above pattern: 2 slow-running spots were found in the sphingolipid/ganglioside area, 1 spot was present in the sphingolipid area and no fast-running spots were encountered. The serum from Kitten 65 produced a chromatogram different from those of all the above-mentioned kittens for, in addition to the 2 spots in the sphingolipid area and a cephalin and lysolecithin spot, a fast-running lecithin-like spot was encountered. A similar variation in the chromatograms of the breeding cats was noticed. Cat 54, a carrier, yielded a chromatogram similar to that of Kitten 63; Cat 58, a non-carrier breeding queen, showed a pattern similar to that of Kitten 64 and Cat 56, the carrier tomcat, showed a pattern similar to that of Kitten 65. Another known carrier cat (No. 55) had a pattern that differed from all the other patterns, namely, 2 spots in the sphingolipid/ganglioside area, 1 in the sphingolipid area and a cephalin spot. Two of the affected kittens (No. 48 & 51) showed chromatograms similar to those of the unaffected kittens, Kitten 50 produced the same pattern as Cat 54 and only 1 kitten (No. 49) showed the lecithin-like substance in the serum. It also had a single spot in the lysolecithin, cephalin, sphingolipid and sphingolipid/ganglioside areas (Fig. 4).

Cell cultures and media

Unused culture medium showed traces of sphingolipid/gangliosides in the slow-running area on the chromatogram, 1 spot in the sphingolipid area and a cephalin and a lysolecithin in the fast-running area. The culture

[1] Merck DC Plastikfolien, Silica gel 60/Kieselguhr F254, 200 × 200 mm

[2] Merck DC Precoated plates, Silica gel 60/Kieselguhr F254, 200 × 200 mm, 0,25 mm

[3] All reagents obtained from Merck Chemicals, Darmstadt

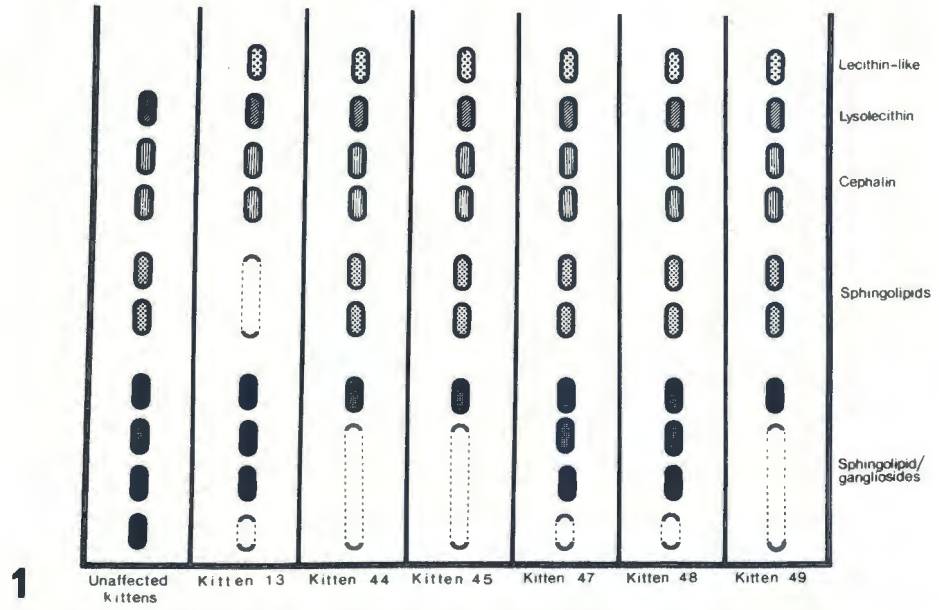
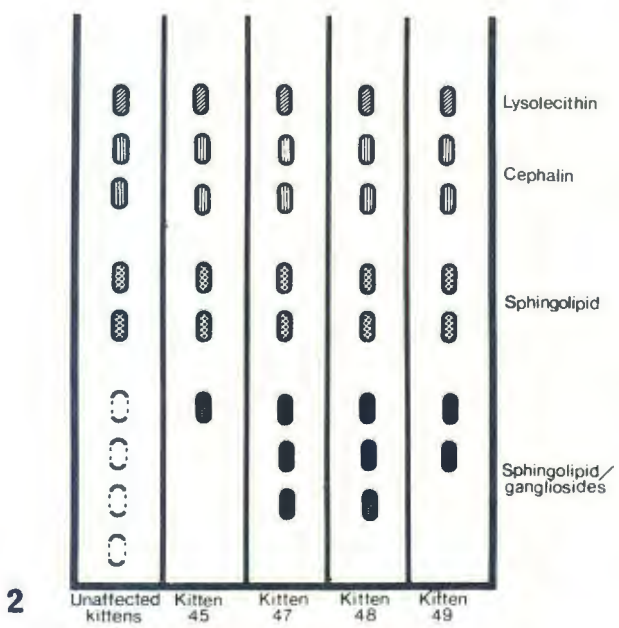


FIG. 1 Schematic representation of a thin layer chromatogram performed on liver extracts of unaffected and affected kittens. Plate—Merck's Kieselgel 60F254 Plastikfolien 200 × 200 mm. Mobile phase—absolute ethanol 90:water 10:concentrated acetic acid 1. Load—8 μl. Spray—ninhydrin



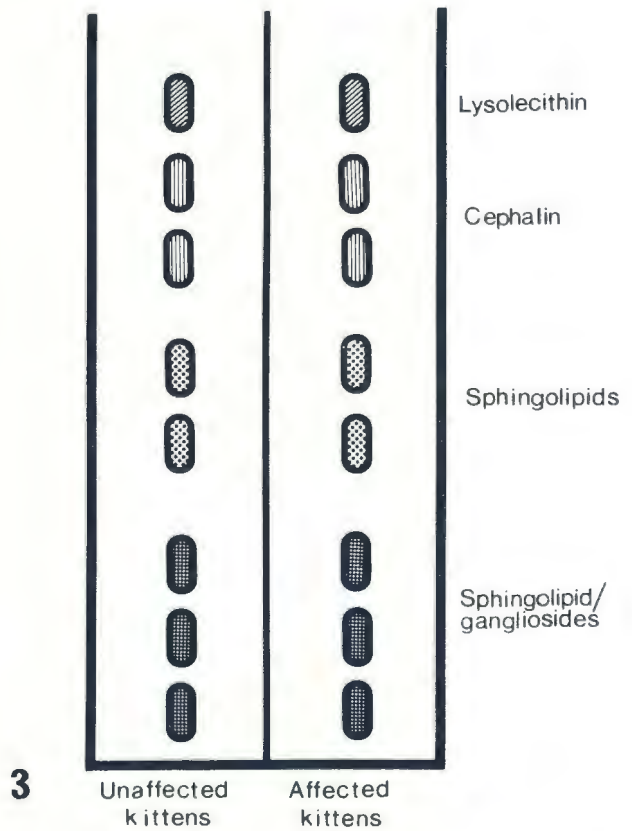


FIG. 2 Schematic representation of a thin layer chromatogram performed on kidney extracts of unaffected and affected kittens. Plate—Merck's Kieselgel 60F254 Plastikfolien 200 × 200 mm. Mobile phase—absolute ethanol 90:water 10:concentrated acetic acid 1. Load—8 μl. Spray—ninhydrin

FIG. 3 Schematic representation of a thin layer chromatogram performed on lung, mixed nervous tissue, spleen and lymph node extracts of unaffected and affected kittens. Plate—Merck's Kieselgel 60F254 Plastikfolien 200 × 200 mm. Mobile phase—absolute ethanol 90:water 10:concentrated acetic acid 1. Load—8 μl. Spray—ninhydrin, rhodamine 6G, reineckate reagent

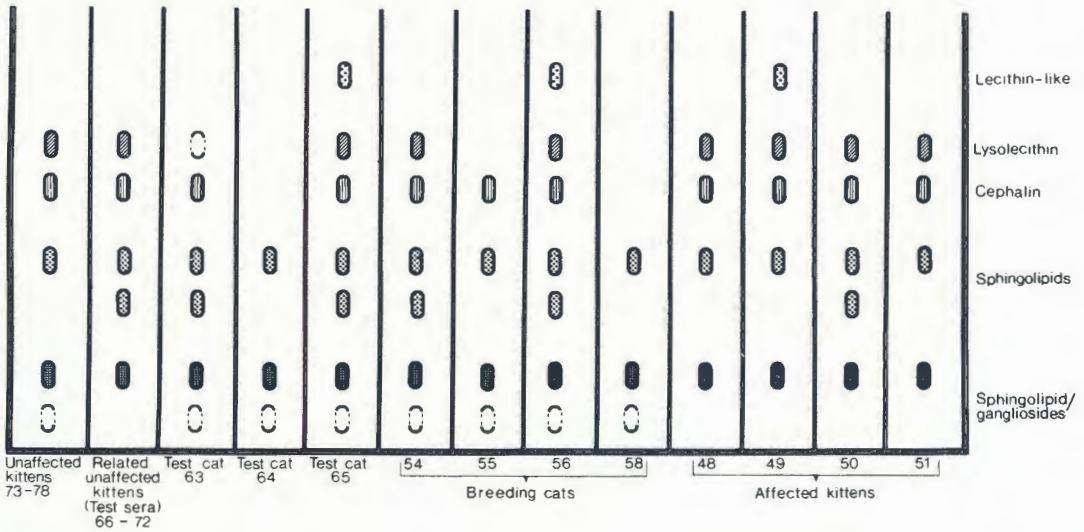


FIG. 4 Schematic representation of a thin layer chromatogram performed on the serum of unrelated kittens,related unaffected kittens. carrier cats and affected kittens. Plate—Merck's Kieselgel 60F254 Plastikfolien 200 × 200 mm. Mobile phase—absolute ethanol 90:water 10:concentrated acetic acid 1. Load—8 μl. Spray—ninhydrin

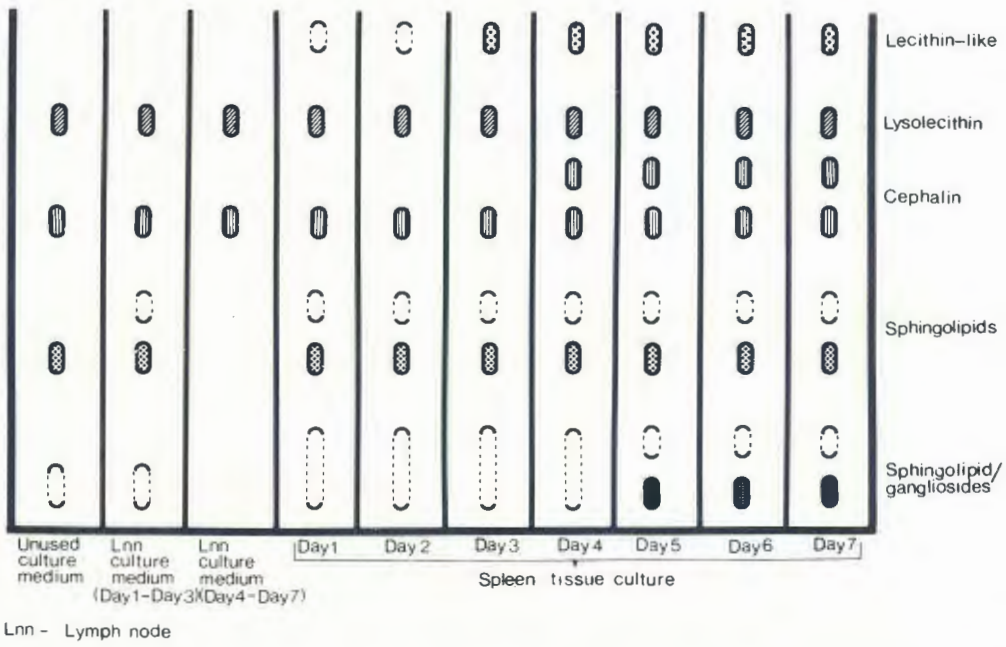


FIG. 5 Schematic representation of a thin layer chromatogram performed on unused culture medium, decanted medium and lumph node cell cultures and decanted medium and spleen cell cultures from an affected kitten. Plate—Merck's Kieselgel 60F254 Plastikfolien 200 × 200 mm. Mobile phase—absolute ethanol 90:water 10:concentrated acetic acid 1. Load—8 μl. Spray—ninhydrin.

medium decanted from the lymph node on Days 1–3 yielded the same chromatogram as the unused culture medium, except that traces of an additional sphingolipid were encountered. Decanted medium for Days 4–7 showed the presence of a cephalin and lysolecithin spot, but no other lipids could be demonstrated. Decanted medium from spleen cell cultures showed traces of a lecithin-like substance on the first 2 days and from the 3rd day onwards this substance was consistently present. Traces of slow-running sphingolipid/ganglioside were found in the medium decanted during the first 4 days, then on Days 5–7 a very prominent slow-running spot as well as traces of other sphingolipids/gangliosides were encountered in this area of the chromatogram. In the sphingolipid area 1 very clear spot and traces of another spot were present from Days 1–7. Two fast-running spots, a cephalin and a lysolecithin were found in medium decanted during the first 3 days, an additional cephalin spot appeared on the 4th day and persisted in the culture medium for the remainder of the period (Fig. 5).

DISCUSSION

The blood lipid analysis used to determine any deviation from the normal values proved to be inconclusive, although 2 of 3 affected kittens showed decreased values in total blood lipid, total esterified fatty acids, triglyceride and phospholipid fractions. It is obvious that too few animals were tested to enable any definite conclusions to be drawn from the above findings.

Thin layer chromatography on liver extracts of affected kittens revealed the production of a lecithin-like phospholipid. This substance was also produced by spleen cell cultures and was present in the serum of a

carrier cat, an affected kitten and an unaffected related kitten. Of all the organs tested, the liver was found to be the only one which produced this lecithin-like substance. In addition to this, it appeared that there was a reduction in the sphingolipid/ganglioside fractions in the livers of affected kittens.

This change might be relative because of the presence of the lecithin-like phospholipid, but the same decrease in sphingolipids/gangliosides was found in the kidneys of affected kittens without the production of the lecithin-like phospholipid. This finding might reflect the reduced phospholipid values in the analysis of blood lipids of affected kittens.

Thin layer chromatography on the serum from various cats revealed a complex situation. The unrelated kittens (No. 73–78), were used to establish the appearance of a normal serum chromatogram. Of the 4 breeding cats tested, 3 were known carrier cats (No. 54, 55 & 56), and No. 58, a breeding queen, did not produce a single affected kitten from 4 litters with a total number of 21 kittens. It was presumed that she was not a carrier cat.

According to the chromatographic analyses that were done, 4 distinct groups of chromatograms can be identified:

Type 1—One or 2 sphingolipid/ganglioside spots, 2 sphingolipid spots, a cephalin and lysolecithin spot and a lecithin-like spot. This pattern was seen in a known carrier cat, in an affected kitten and in a related unaffected kitten.

Type 2—One or 2 sphingolipid/ganglioside spots, 2 sphingolipid spots and a cephalin and lysolecithin spot. A known carrier cat, an affected kitten and the majority of the related unaffected kittens revealed this pattern.

Type 3—Two sphingolipid/ganglioside spots, 1 sphingolipid spot and the presence or absence of a cephalin spot. This pattern was encountered in a known carrier cat, in the non-carrier breeding queen and in one unaffected related kitten.

Type 4—The normal chromatogram with 1 or 2 sphingolipid/ganglioside spots, 1 sphingolipid spot and a cephalin and lysolecithin spot. The unrelated kittens and 2 affected kittens had this pattern.

From the above it is clear that the serum of cats can be used for partial screening purposes and that a small number of carrier and/or affected cats may be identified. This method is not reliable, however, since some affected and carrier cats show no deviation from the chromatograms obtained from normal cats.

An interesting finding was the production of the lecithin-like phospholipid by spleen cell cultures, a substance which could not be detected in the spleen of affected or normal kittens. The cells proliferating in the spleen cultures of affected kittens appeared to be macrophage-like cells (Lange, 1980) and it is possible that it is these cells that produce this substance in the spleen cell cultures. If a screening test should be developed to identify affected kittens, it would appear that cell cultures of the spleen offer the most profitable line of approach. At this stage, however, cell culture is not a very practical method. A possible reason for the lack of production of the lecithin-like substance by lymph node cell cultures could be that the cell growth in these cultures was not as prolific as in the spleen cultures, and very few macrophage-like cells were present in the lymph node cultures (Lange, 1980).

Owing to the lack of sufficient material and more sophisticated apparatus it was not possible to identify the lecithin-like substance more precisely. It is difficult therefore to hypothesize where the substance is produced and whether there is a specific enzyme deficiency that can be identified. An accumulation of a lipid was found in neurones and macrophage-like cells in the spleen and lymph nodes of affected kittens (Lange *et al.* 1977). This lipid also accumulated in macrophage-like cells in spleen and lymph node cell cultures and it had staining characteristics and the appearance of phospholipids (Lange, 1980). In the present study it was found that a lecithin-like phospholipid is present in the livers of affected kittens and that this substance is produced by spleen cell cultures. It is feasible therefore to assume that the accumulated substance in the neurones and lymphoid tissue is this lecithin-like phospholipid.

The absence of this phospholipid in the sera of 3 of the affected kittens cannot be explained. If this condition is due to an enzyme deficiency that tends to the accumulation of a phospholipid in certain cells, and if this substance is produced in the liver of affected and carrier cats, it would be logical to expect elevated serum levels in these animals. It is possible that unknown factors may play a role in the occurrence of the lecithin-like substance in the serum of affected and carrier cats and that animals with serum levels of this substance need not necessarily show signs of clinical disease. Both the carrier and related cats that had the lecithin-like substance in the serum are still alive and show no signs of this condition. It may be pointed out that no quantitative assays were done in this study and that such procedures might cast some light on some of the above questions.

Another inexplicable finding at this stage is the failure to demonstrate the lecithin-like phospholipid biochemically in the central nervous system and in the spleen and lymph nodes where its presence is apparent under the light microscope. It may be possible that the lipid is altered or bound to such an extent that the extraction methods used for lipids from these organs were inadequate or that chemical alteration of the lipid during these procedures resulted in an inability to demonstrate the lecithin-like phospholipid with thin layer chromatography.

A new lysosomal storage disease has been identified in Abyssinian cats, and the substance that accumulates in affected cells appears to be a lecithin-like phospholipid. It is difficult, however, to define the condition more accurately because of our inability at this stage to identify the accumulated substance.

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