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Lipid accumulation in liver, spleen, lungs and kidneys of miniature-pigs after chloroquine treatment

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Chronic chloroquine treatment of type-Göttingen miniature-pigs induced lipid accumulation in the liver, spleen, lungs and kidneys. The lipid analyses showed marked quantitative and qualitative differences between the organs. In the liver the lipids affected most were cholesteryl esters and glucosylceramides, which were increased at the most 20 times. Cholesterol and ganglioside concentrations were also increased, though less markedly. The concentration of acidic phospholipids was slightly increased but that of the neutral phospholipids was unaffected. There was a considerable inter-individual variation in the lipid changes. Spleen and lung showed significant increases of all the major lipids. Glucosylceramide was increased more than the other lipids, namely 6-fold in the spleen and 10-fold in the lung. The concentration of acidic phospholipids as well as that of gangliosides was increased by 50% in the spleen and by 100% in the lung. The organ affected least was the kidney, in which only the glycolipids, both acidic and neutral, were significantly increased. Common to all the organs of the chloroquine-treated pigs was the large increase of glucosylceramide, ganglioside G_{M2} and bis(monoacylglyceryl)phosphate. The ganglioside increase affected all the individual gangliosides and, except for the increased proportion of ganglioside G_{M2}, there were no remarkable changes in the ganglioside pattern in any of the organs.

Prolonged use of chloroquine in the prophylaxis of malaria and therapy of rheumatoid diseases has several side effects. The clinically most serious effects involve the eyes and the skeletal muscles (Goldman & Preston, 1957; Hobbs *et al.*, 1959; Whisnant *et al.*, 1963). Gleiser *et al.* (1968) demonstrated severe myopathy with loss of the normal histological architecture in high-dose animals groups. In paraffin sections the ganglion cells showed a foamy appearance, and electron microscopy showed them to contain lamellated cytoplasmic bodies (MCB), which were thought to be microscopically identical with the storage granules described in Tay–Sachs disease. Independently of these observations 'a foam cell syndrome' was reported, which was shown to be caused by a drug, a coronary vasodilator, 4,4'-dimethylaminoethoxyhexoesterol (Yamamoto *et al.*, 1971*a*). Yamamoto *et al.* (1971*b*, 1976) demonstrated an increased concentration of phospholipids and of cholesterol in the liver, spleen and kidneys. The concentration of an acidic glycerophospholipid, bis(monoacylglyceryl)-phosphate, was particularly high. Since the same lipid had previously been found to be markedly increased in sphingomyelin lipidosis, Niemann–Pick's disease, they regarded the condition as drug-induced phospholipidosis. Several other drugs have also been shown to induce similar lipidosis; a list of them reported up to 1977 has been constructed by Lüllmann *et al.* (1978).

It is thus evident that several drugs can induce a phospholipid and cholesterol accumulation. However, we recently demonstrated that chronic chloroquine treatment was attended in skeletal muscle by a 3-fold increase in the phospholipid concentration but

Abbreviations used: NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolloylneuraminic acid. Ganglioside abbreviations follow the nomenclature system of Svennerholm (1977): G_{M3}, II^3NeuAc-LacCer; G_{D3}, $II^3(NeuAc)_2$-LacCer; G_{M2}, II^3NeuAc-$GgOse_3Cer$; G_{M1}, II^3NeuAc-$GgOse_4Cer$; Fuc-(NeuAc)G_{M1}, IV^2Fuc,II^3NeuAc-$GgOse_4Cer$; Fuc-(NeuGc)G_{M1}, IV^2Fuc,II^3NeuGc-$GgOse_4Cer$; G_{T1b}, IV^3NeuAc,$II^3(NeuAc)_2$-$GgOse_4Cer$; G_{L1a}, glucosylceramide; G_{L1b}, galactosylceramide; G_{L2}, dihexaosylceramide; G_{L3}, trihexaosylceramide; G_{L4}, tetrahexaosylceramide.

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a 10-fold increase in that of gangliosides (Nilsson *et al.*, 1981) and that in the nervous tissue (except in retina) there was no increase of phospholipids, but only of the gangliosides (Klinghardt *et al.*, 1981). These findings suggest that certain cationic amphiphilic drugs can induce an accumulation of phospholipids in visceral parenchymatous organs, and of gangliosides in the nervous tissue.

A search of the literature failed to reveal any study of visceral gangliosides or any other glycolipids in drug intoxication. We therefore thought it urgent to determine all the major lipid components of liver, lungs, kidneys and spleen of pigs with chronic chloroquine treatment, in an effort to understand the underlying mechanism of the lipid changes in drug treatment.

Materials and methods

Chloroquine treatment of the miniature-pigs

The material consisted of cross-breeds between the Vietnamese and the Minnesota miniature-pig ('mini-pig') (Haring *et al.*, 1967). The animals were treated with chloroquine diphosphate (Resochin^{R}) at a dose of 2.0 or 3.0 g/kg standard diet. The control animals were given the same diet, but without chloroquine. The symptoms of the chloroquine-treated animals have recently been described by Klinghardt *et al.* (1981). The duration of the experiments and the doses used are summarized in Table 1. The animals were killed by a pistol shot in the medulla oblongata. The organs were immediately dissected, placed in air-tight polyethylene bags, quick-frozen and stored at −20°C until analysed. All the pigs used in the biochemical study were histologically examined to verify the severity of the treatment.

Chemicals

Organic solvents, chromatographic adsorbents and ion-exchange resins were purchased and processed as recently described (Svennerholm & Fredman, 1980; Fredman *et al.*, 1980). *Vibrio cholerae* sialidase (acylneuraminate glucohydrolase; EC 3.2.1.18) was purchased from Behringwerke A.G., Marburg-Lahn, Germany. Dialysis tubing was obtained from Union Carbide, Chicago, IL, U.S.A., and hexamethyldisilazane and trimethylchlorosilane from Pierce, Rockford, IL, U.S.A. Methanol (Uvasol; Merck A.G., Darmstadt, Germany) was used for methanolysis. Chromatographically homogeneous gangliosides, glycosphingolipids and *N*-acetyl- and *N*-glycolloyl-neuraminic acids used as standards were prepared as previously described (Svennerholm, 1956; Svennerholm & Svennerholm, 1963; Fredman *et al.*, 1980). The glucosylceramide was isolated from the spleen of a patient with Gaucher's disease, and lactosyl-, globotriosyl- and globotetraosyl-ceramide were isolated from normal human erythrocytes.

Extraction of the lipids

Fresh or frozen tissue was used for the extraction. From each organ the tissue to be used was freed from vessels and most of the connective tissue; 10 g of the dissected tissue was homogenized in a scissor homogenizer with 3 vol. of water. Extraction was performed twice, each time with 20 vol. of chloroform/methanol/water (4:8:3, by vol.) (Svennerholm & Fredman, 1980). The two extracts were pooled, evaporated and redissolved in 25 ml of chloroform/methanol/water (60:30:4.5, by vol.). Undissolved material was removed by centrifugation and the clear supernatant was made up to exactly 50 ml with the same solvent.

Separation of the lipids

Of the total lipid extract, 5 ml, corresponding to 1 g of tissue, was used for the lipid analyses. Electrolytes and other low-molecular-weight contaminants were removed by gel filtration on 1 g of Sephadex G-25 (Wells & Dittmer, 1963). The extract was then evaporated and redissolved in 5 ml of chloroform/methanol/water (60:30:4.5, by vol.).

Neutral and acidic lipids were separated by anion-exchange chromatography on Spherosil-DEAE-Dextran (Fredman *et al.*, 1980). The resin (2 ml) was packed in a glass column and equilibrated with chloroform/methanol/water (60:30:4.5, by vol.). The desalted extract was applied to the column, and neutral or positively charged lipids were eluted, with 5 ml and then 10 ml of chloroform/methanol/water (60:30:4.5, by vol.); the fraction eluted was termed the neutral fraction. Acidic lipids were thereafter eluted from the resin first with 25 ml of 0.1 M-potassium acetate in methanol (the first acidic fraction) and then with 20 ml of 0.5 M-potassium acetate in methanol (the second acidic fraction) (containing gangliosides with two or more sialic acids). The acidic fractions were desalted by dialysis.

The neutral fraction was used for determination of cholesterol (non-esterified and esterified), neutral phospholipids and neutral glycosphingolipids; the first acidic fraction was used for quantification of acidic phospholipids and gangliosides; and the second acidic fraction was used for quantification of higher gangliosides.

Purification of the gangliosides

The gangliosides in the first acidic fraction had to be isolated from the other lipids before analysis. A portion of the fraction was chromatographed on silica gel (230–400 mesh). The extract was evaporated, redissolved in chloroform/methanol (4:1, v/v) and applied to the silica-gel column (1 g, packed in

chloroform). Lipids other than gangliosides were eluted first with 4ml of chloroform/methanol (4:1, v/v) and thereafter with 5ml of chloroform/methanol/water (65:25:4, by vol.). The gangliosides were eluted from the gel with 10ml of chloroform/methanol/water (60:35:8, by vol.).

Sometimes the acidic fraction had to be further purified by removal of acidic phospholipids by saponification. To the sample was added 0.5M-KOH in methanol/water (1:1, v/v), after which it was left overnight at room temperature (20°C). The fraction was then neutralized and desalted on Sephadex G-25 (Wells & Dittmer, 1963) and purified by silica-gel column chromatography as described above.

Isolation of neutral glycosphingolipids

The neutral glycosphingolipids were eluted from the anion-exchange resin in the neutral fraction. The major portion of cholesterol and other simple lipids (triacylglycerols, non-esterified fatty acids, esterified cholesterol) were separated from the glycosphingolipids by chromatography on silica gel (230–400 mesh). The sample was dissolved in chloroform and applied to the column, and cholesterol and simple lipids were eluted with 10 bed vol. of chloroform and 10 bed vol. of chloroform/acetic acid (19:1, v/v). The retained lipids were then eluted with 10 bed vol. of methanol. The glycosphingolipids were separated from the phospholipids by peracetylation and subsequent chromatography on Florisil (Saito & Hakomori, 1971). The peracetylated glycosphingolipids were deacetylated by hydrolysis in 0.2M-NaOH in 90% methanol for 30min at room temperature. After neutralization the salt was removed by gel filtration on Sephadex G-25 (Wells & Dittmer, 1963).

Assay methods

Lipid phosphorus was assayed with a modified Fiske–SubbaRow method (Svennerholm & Vanier, 1972) and cholesterol with an $FeCl_3$ method (Crawford, 1958). Esterified cholesterol was quantified by densitometric scanning. Samples and standards of esterified cholesterol were applied to thin-layer plates, chromatographed with light petroleum/diethyl ether/conc. acetic acid (90:10:1, by vol.) as solvent system and the spots were detected by spraying the plates with cupric acetate (Fewster *et al.*, 1969) and heating the plates for 20min in an oven at 140°C. The plates were scanned with a Zeiss KM3 Chromatogram Scanner at 450nm and the values were integrated with a Varian 110 CDS Computer.

Individual neutral glycolipids were quantified by densitometric scanning after separation on high-performance t.l.c. plates. The mono- and di-glycosylceramides were separated with chloroform/methanol/water (40:10:1, by vol.) as solvent and tri- and tetra-hexaosylceramides with chloroform/methanol/water (65:25:4, by vol.). The spots were visualized with cupric acetate and scanned as described above. Standards of glucosyl-, lactosyl-, globotriosyl- and globotetraosyl-ceramides were chromatographed together with the samples.

The total sialic acid was measured with the resorcinol method and the ganglioside pattern with thin-layer chromatographic separation and densitometric scanning (Svennerholm & Fredman, 1980). When the plates were scanned they were generally developed in n-propanol/0.25% KCl (3:1, v/v). The different gangliosides were identified by t.l.c. with several solvents and their mobility was compared with that of known human brain gangliosides. The most frequent gangliosides were isolated by preparative t.l.c. (Fredman *et al.*, 1980) and identified by partial enzymic and acidic hydrolysis, chromatographic identification of the products and component assay of sugars and alditol acetates (including mass spectrometry) (Fredman *et al.*, 1980).

The *N*-acetyl-/*N*-glycolloylneuraminic acid ratio was determined by g.l.c. of the trimethylsilyl derivatives of the sialic acids (Yu & Ledeen, 1970). The ratio was used to correct the absorbancy reading in the resorcinol method since the molar absorbancy of *N*-glycolloylneuraminic acid is 30% higher than that of *N*-acetylneuraminic acid (Svennerholm, 1958).

Results

The animals used are summarized in Table 1. Chloroquine treatment had no significant effect on the weight of the organs. The liver was assayed in five controls and five treated minature-pigs; the other organs, kidneys, spleen and lungs were assayed in only three animals in each group. The results are given as means ± S.D. (Table 3–5), but owing to the wide range of inter-individual variation in the liver in the treated pigs the values are given for each animal separately (Table 2).

Liver. Chloroquine treatment led to large changes in the lipid pattern and the inter-individual range of variation was wider than in any of the other organs investigated (Table 2). The increase in the lipids appeared to be largest in the pigs given 3.0g of chloroquine/kg diet, and the highest values were found for cholesterol and neutral glycosphingolipids.

Neither the concentration nor the pattern of the neutral phospholipids was affected in any of the treated pigs. However, the concentration of acidic phospholipids was 13–56% higher than that in the controls. Their pattern was changed, bis(monoacylglyceryl)phosphate constituting up to 15% of the

Table 1. *Survey of the animals investigated*

	Controls					Chloroquine-treated				
Animal no. ...	1	2	3	4	5	6	7	8	9	10
Age at death (days)	223	306	352	359	429	352	360	435	486	640
Duration of intoxication (days)	—	—	—	—	—	223	230	188	184	310
Dose of chloroquine (g/kg diet)	—	—	—	—	—	2.0	2.0	2.0	3.0	3.0
Body wt. at death (kg)	53	57	58	49	47	55	48	55	48	41
Organ weight (g)										
Liver	750	690	770	650	640	752	1100	615	730	1020
Kidney	77	89	134	77	91	143	180	79	77	93
Spleen	67	71	80	56	62	75	75	81	139	129
Lung	191	—	313	195	197	295	266	221	—	227

Table 2. *Lipid composition in liver of chloroquine-treated mini-pigs and controls*

Values for both groups are means ± S.D. The values for sialic acid are corrected for the difference in absorbance coefficient of *N*-acetyl- and *N*-glycolloyl-neuraminic acid. Abbreviations used: tr., trace; n.d., not determined.

		Chloroquine-treated				
Animal no. ...	Control 1–5	6	7	8	9	10
Phospholipids (μmol of phosphorus/g of fresh tissue)						
Neutral	37.8 ± 2.9	38.8	39.9	38.6	41.7	36.2
Acidic	6.68 ± 0.45	7.96	8.33	8.05	10.44	7.57
Cholesterol (non-esterified) (μmol/g of fresh tissue)	9.22 ± 0.93	10.6	19.3	8.17	36.7	39.5
Cholesterol (esterified) (μmol/g of fresh tissue)	0.65 ± 0.17	1.67	10.3	1.15	13.3	10.2
Gangliosides (μmol of sialic acid/g of fresh tissue)	0.43 ± 0.06	0.61	0.86	0.66	0.98	0.97
Neutral glycolipids (μmol/g of fresh tissue)						
G_{L1a+b}	0.07 ± 0.01	0.15	0.20	n.d.	0.68	0.96
G_{L2}	tr.	tr.	tr.	n.d.	0.04	0.06
G_{L3}	0.07 ± 0.01	0.14	0.11	n.d.	0.09	0.19
G_{L4}	0.03 ± 0.01	n.d.	n.d.	n.d.	0.08	0.09

acidic phospholipids in the treated pigs, but not being demonstrable at all in the controls.

The concentration of gangliosides was increased by 40–130%. The dominating ganglioside in the controls was G_{D3} (55 ± 9% of the total sialic acid), followed by G_{M3}, (23 ± 14% of the total sialic acid). The inter-individual variation was substantial, but the most pronounced change in the ganglioside pattern in the treated pigs was the increase in ganglioside G_{M2}. This gangliosides was not demonstrable in the controls, but was found in a concentration ranging from 5 to 12% of the total sialic acid in the treated pigs. The smallest proportion of G_{M2} was found in the pigs given the higher doses of chloroquine, which had the highest concentration of gangliosides. In these animals G_{M3} was significantly increased and G_{D3} decreased. The amount of fucose-containing gangliosides, determined by analysis of the sugar composition in the total ganglioside extract, was approx. 5%, in both the treated pigs and controls. G.l.c. analysis of the sialic acids showed that the *N*-acetyl-/*N*-glycolloyl-neuraminic acid ratio was 3 : 1.

The increase in neutral glycosphingolipids was largest in the monoglucosylceramide fraction, which consisted of both glucosyl- and galactosyl-ceramide. Glucosylceramide was affected most and increased by up to 20 times. The glucosyl-/galactosyl-ceramide ratio was approx. 0.5 in the controls and increased to approx. 2 in the pigs with the highest dose of chloroquine.

Spleen. Chloroquine treatment induced a statistically significant increase of all major lipids (Table 3). The concentration of the gangliosides was increased by 30–100% in the treated pigs.

Neutral glycosphingolipids were affected most. The largest increase was found for the glucosylceramide fraction, in which the concentration was six times that in the controls ($P < 0.001$). No esterified cholesterol was detected.

Neutral phospholipids showed the same composition in both groups, whereas the pattern of the acidic phospholipid fraction in the treated pigs differed from that in the controls. The phospholipid bis(monoacylglyceryl)phosphate constituted 10–20% of the acidic phospholipids in the treated pigs, but was not detectable in the controls.

The ganglioside pattern in the spleen was very

Table 3. *Lipid composition in spleen in chloroquine-treated mini-pigs and controls*

Values for both groups are means ± S.D. The values for sialic acid are corrected for the difference in absorbance coefficient of *N*-acetyl- and *N*-glycolloyl-neuraminic acid. The statistical significance was calculated with Student's *t* test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Abbreviation used: tr., trace.

Animal no. ...	Control 2, 3, 4	Chloroquine-treated 6, 7, 8
Phospholipid (μmol of phosphorus/g of fresh tissue)		
Neutral	17.2 ± 2.0	21.5 ± 0.30*
Acidic	4.20 ± 0.13	5.87 ± 0.43**
Cholesterol (non-esterified) (μmol/g of fresh tissue)	13.0 ± 0.6	17.53 ± 1.11**
Gangliosides (μmol of sialic acid/g of fresh tissue)	0.42 ± 0.03	0.69 ± 0.17*
Neutral glycolipids (μmol/g of fresh tissue)		
G_{L1a}	0.07 ± 0.01	0.43 ± 0.02***
G_{L2}	0.02†	tr.
G_{L3}	0.14 ± 0.02	0.29 ± 0.04**
G_{L4}	0.18 ± 0.04	0.47 ± 0.04***

† The existence of G_{L2} was detected in only one of the control animals.

Table 4. *Lipid composition in lung of chloroquine-treated mini-pigs and controls*

Values for both groups are means ± S.D. The values for sialic acid are corrected for the difference in absorbance coefficient of *N*-acetyl- and *N*-glycolloyl-neuraminic acid. The statistical significance was calculated with Student's *t* test. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Abbreviation used: tr., trace.

Animal no. ...	Control 3, 4, 5	Chloroquine-treated 6, 7, 10
Phospholipid (μmol of phosphorus/g of fresh tissue)		
Neutral	17.8 ± 2.7	26.3 ± 3.6*
Acidic	3.93 ± 0.37	6.95 ± 1.27*
Cholesterol (non-esterified) (μmol/g of fresh tissue)	10.6 ± 1.4	14.0 ± 1.21*
Gangliosides (μmol of sialic acid/g of fresh tissue)	0.34 ± 0.03	0.56 ± 0.03***
Neutral glycolipids (μmol/g of fresh tissue)		
G_{L1a}	0.03 ± 0	0.26 ± 0.02***
G_{L2}	tr.	tr.
G_{L3}	0.18 ± 0.02	0.37 ± 0.03***
G_{L4}	0.14 ± 0.01	0.18 ± 0.04

complex, owing to the large proportion of *N*-glycolloylneuraminic acid (approx. 80%). The main gangliosides in the controls were G_{M3} and G_{D3} (37 ± 4 and 23 ± 3% of total sialic acid respectively). Two monosialogangliosides containing fucose were identified as Fuc-(NeuAc)G_{M1} and Fuc-(NeuGc)G_{M1}, and in the controls each of them constituted 5% of the total sialic acid. The proportion of fucogangliosides in the chloroquine-treated pigs was increased from 11 to 17%. The most pronounced change in the ganglioside pattern of the treated pigs was the occurrence of a ganglioside that was not detectable in the controls and that proved to be ganglioside G_{M2} with *N*-glycolloylneuraminic acid. This ganglioside constituted 8–11% of the total sialic acid.

Lung. In this organ, as in the spleen, all the major lipids in the treated pigs were significantly increased (Table 4). Gangliosides and neutral glycosphingolipids showed the most significant increases ($P < 0.001$). The amount of glucosylceramide was increased up to 10 times and that of ganglioside by approx. 60%. Cholesterol was only slightly increased, and none of the esterified form was demonstrable. Neutral phospholipids were slightly, but significantly, increased ($P < 0.05$), and the increase in the acidic phospholipids was more marked. The amount of acidic phospholipids in the treated pigs increased by 50–100% ($P < 0.01$) and t.l.c. showed the appearance of bis(monoacylglyceryl)phosphate in a concentration of 10%. No such substance was visible in the controls.

The major gangliosides in the controls and in the chloroquine-treated pigs was G_{M3} (54 ± 5% of total sialic acids), followed by G_{D3} (approx. 20%). The difference in ganglioside pattern was found in the appearance of ganglioside G_{M2} in the chloroquine-treated pigs (approx. 3% of total sialic acid). Analysis of the sugar components revealed that also the proportion of fucose-containing gangliosides

Table 5. *Lipid composition in kidney (cortex) of chloroquine-treated mini-pigs and controls*

Values for both groups are means ± S.D. The values for sialic acid are corrected for the difference in absorbance coefficient of *N*-acetyl- and *N*-glycolloyl-neuraminic acid. The statistical significance was calculated with Student's *t* test. $*P < 0.05$. Abbreviation used: tr., trace.

Animal no. ...	Control 2, 3, 4	Chloroquine-treated 6, 7, 9
Phospholipid (μmol of phosphorus/g of fresh tissue)		
Neutral	22.7 ± 1.8	24.4 ± 3.4
Acidic	5.47 ± 0.46	6.90 ± 1.06
Cholesterol (unesterified) (μmol/g of fresh tissue)	9.50 ± 0.51	11.2 ± 1.33
Gangliosides (μmol of sialic acid/g of fresh tissue)	0.30 ± 0.03	0.47 ± 0.07*
Neutral glycolipids (μmol/g of fresh tissue)		
G_{L1a}	0.07 ± 0.03	0.18 ± 0.03*
G_{L2}	tr.	tr.
G_{L3}	0.11 ± 0.03	0.11 ± 0.02
G_{L4}	0.12 ± 0.03	0.18 ± 0.02*

increased from 3 to 8%. The *N*-acetyl-/*N*-glycolloyl-neuraminic acid ratio was approx. 1 in both the controls and the chloroquine-treated pigs.

Kidney (cortex). Of all the organs studied the lipid composition was affected least in the kidney, where the only significant changes were increases in gangliosides G_{L1a} and G_{L4} ($P < 0.05$; Table 5). Acidic phospholipids were increased more than neutral phospholipids, and bis(monoacylglyceryl)-phosphate constituted 1–2% of the acidic phospholipids.

Gangliosides G_{M3} (31 ± 5%), G_{D3} (22 ± 3%) and G_{M1} (17 ± 1% of total sialic acid) were the major gangliosides in the control pigs. A remarkable finding was that trisialoganglioside G_{T1b} constituted a relatively large proportion of the gangliosides and also constituted 11 ± 2% of the total sialic acid. The chloroquine treatment did not lead to any increase in ganglioside G_{M2}, which constituted approx. 8% of the total sialic acid in each group. The chloroquine-treated pigs showed a slight increase in the proportion of G_{M3} and G_{M1} and a decrease of ganglioside G_{T1b}. The *N*-acetyl-/*N*-glycolloyl-neuraminic acid ratio was 4 : 1 in the controls as well as in the treated pigs.

Discussion

Drug treatment produces various biochemical and morphological manifestations in different organs of the same species and in the same organ of different species (Seiler & Wassermann, 1975; Yamamoto *et al.*, 1976; Matsuzawa *et al.*, 1977). These differences were ascribed to differences in lipid turnover rates between various organs and to differences in drug-metabolizing capacity between species (Burnstock *et al.*, 1971; Adachi *et al.*, 1972). However, these assumptions were based on a limited number of determinations of certain lipid components. It has been demonstrated that 4,4'-diethylaminoethoxyhexoestrol caused an increase in non-esterified cholesterol and phospholipids, particularly of bis(monoacylglyceryl)phosphate and phosphatidylinositol, in human liver (Yamamoto *et al.*, 1971*a*). The proportion of bis(monoacylglyceryl)phosphate was shown to be increased also in the spleen, lymph nodes and muscles. Administration of the same drug to rats caused similar, but less marked, changes in the liver and spleen (Adachi *et al.*, 1972). The increase of cholesterol and phospholipids, especially bis(monoacylglycerol)-phosphate, was dose-dependent (Yamamoto *et al.*, 1976). The effect of the drug on hepatic lipid metabolism differed markedly between the three species man, monkey and rat; man showed the slowest metabolism of the drug and the largest lipid accumulation, whereas the rat had a high turnover of the drug and only a small lipid accumulation (Matsuzawa *et al.*, 1977). Yamamoto *et al.* (1976) showed that chloroquine also led to an increase in hepatic bis(monoacylglyceryl)phosphate, which was confirmed by Matsuzawa & Hostetler (1980), who also showed that chloroquine led to an increase in hepatic total phospholipids and cholesteryl esters.

A common previous finding is an increased concentration of hepatic total phospholipids, which we have not been able to confirm, in the liver of chloroquine-treated miniature-pigs. Our results (an insignificant increase of phospholipids and a very marked increase of cholesteryl esters in the liver) are more compatible with the results reported by Florén *et al.* (1977) and Stein *et al.* (1977). When working with labelled lipids Florén *et al.* (1977) showed that chloroquine delayed the hydrolysis of the chyle cholesteryl esters taken up by the hepatocytes, whereas no significant proportion of the chylomicron phospholipids was metabolized by the liver. Stein *et al.* (1977) found that chloroquine interfered

with the hydrolysis of the lipoprotein cholesteryl esters in the liver, whereas the metabolism of the low-density-lipoprotein phospholipids occurred mainly in extra-hepatic tissues.

There are very few previous quantitative studies of lipids in organs other than the liver. Seiler & Wassermann (1975) showed that chlorphentermine induced an accumulation of phospholipids and cholesterol in the lungs and adrenals of the rat, mouse and guinea-pig, a finding similar to that made by us in the miniature-pig lung. Chloroquine did not induced any significant increase of total phospholipids or cholesterol in the kidney, which is at variance with the increase found by Yamamoto *et al.* (1976) in rat kidney. To our knowledge no determinations have hitherto been made of cholesterol and total phospholipids in the spleen after drug treatment. This is remarkable, since the first description of the syndrome 'drug-induced lipidoses' has already stressed its resemblance to Niemann–Pick disease, and the spleen is the organ that often has the largest accumulation of cholesterol and phospholipids in this disease.

As for the phospholipid pattern, Yamamoto *et al.* (1970) drew attention to the large increase of an acidic phospholipid, which was not seen in the control material. This was found to be bis(monoacylglyceryl)phosphate, which had previously been demonstrated by Rouser *et al.* (1968) in organs from cases of Niemann–Pick disease. They therefore suggested the name 'Niemann–Pick-like syndrome' for drug-induced lipidoses (Yamamoto *et al.*, 1971*b*). Further studies have also shown increased proportions of phosphatidylinositol (Yamamoto *et al.*, 1971*b*, 1976; Matsuzawa & Hostetler, 1980) and phosphatidylglycerol (Matsuzawa & Hostetler, 1980). In miniature-pigs we found a constant and large increase of bis(monoacylglycerol)phosphate, but no significant increase in the proportion of phosphatidylinositol. There was a marked decrease of cardiolipin in the organs from the chloroquine-treated minature-pigs, an observation made previously by Adachi *et al.* (1972) and Yamamoto *et al.* (1976).

Bis(monoacylglyceryl)phosphate has been shown to be a specific marker for lysosomal membranes (Wherett & Heuterer, 1972) and Matsuzawa & Hostetler (1980) found that among the different subcellular fractions from drug-treated rat liver, the lysosomes showed the most marked increase of bis(monoacylglyceryl)phosphate as well as of phosphatidylinositol. Yamamoto *et al.* (1976) believed these two lipids were necessary for neutralization of the cationic drugs. Bis(monoacylglyceryl)phosphate seems very suitable for this function, since Matsuzawa & Hostetler (1979) showed that the degradation rate of this phospholipid is only one-tenth of that of phosphatidylcholine. Matsuzawa *et al.* (1978) also found that the synthesis of bis(monoacylglyceryl)phosphate from phosphatidylglycerol was stimulated by an increased concentration of phosphatidylinositol.

The increase of acidic lipids was not limited to the phospholipids but we have for the first time also shown that the concentration of gangliosides was significantly increased in all the four organs. The increase involved all major gangliosides and can be regarded as a marker for an increased number of residual bodies, since we have shown that the gangliosides are enriched in the plasma membranes and the lysosomal fraction (P. Fredman, G. W. Klinghardt & L. Svennerholm, unpublished work). Besides, there was an increased proportion of ganglioside G_{M2}, previously also found in nervous tissue (Klinghardt *et al.*, 1981) and skeletal muscle (Nilsson *et al.*, 1981) from chloroquine-treated miniature-pigs. It is also a common feature in many inherited diseases (Svennerholm, 1966). Chloroquine is known to inhibit the lysosomal enzymes, and the enzyme that degrades ganglioside G_{M2} to G_{M3}, which is the rate-limiting enzyme in the ganglioside degradation, is probably more susceptible to the inhibition than the other enzymes.

A particularly remarkable result of chloroquine treatment was the increase of neutral glycosphingolipids, especially glucosylceramide (5–20-fold), in all the four organs. We demonstrated the increase of neutral glycolipids, particularly glucosylceramide, in the spleen and liver in a variant form of Niemann–Pick disease, type C (Ivemark *et al.*, 1963), a finding extended by Philippart (1972), who emphasized the close relation between Gaucher and Niemann–Pick disease, type C. It is difficult to find any common denominator for the glucosylceramide accumulation in Niemann–Pick disease and in chloroquine treatment unless one assumes that the cationic substances choline in sphingomyelin and chloroquine are inhibitors of cerebroside β-glucosidase (glucosylceramidase; EC 3.2.1.21).

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