SERUM LIPIDS IN LEPROSY BY SILICIC ACID COLUMN CHROMATOGRAPHY[1]

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Disturbances in lipid metabolism have been observed in the host during the course of pathogenesis in various infections. Liver dysfunctions resulting in fluctuations in the lipid content of the serum in *Mycobacterium tuberculosis* infections have been reported by various workers. Scanty and conflicting reports on the lipid metabolism during *Mycobacterium leprae* infection are found in the literature. Kusuka ([11]) observed an increase in total cholesterol and total phosphatides (especially sphingomyelin) in all tissues studied, whereas neutral fat decreased. He remarked that these changes are more pronounced in peripheral tissues than in the internal organs. Gokhale *et al.* ([8]) found higher amounts of serum lipid constituents in leprosy patients than in normal subjects. Tarabini ([20]) correlated the changes in serum lipid constituents in leprosy with other associated conditions in the patients, e.g., nutrition and kidney and hepatic abnormalities. Kusuka ([12]) observed low serum cholesterol levels in leprosy, in contrast to normal total lipid and phospholipid content in normal subjects. Chekherdemian ([4]) reported low serum cholesterol levels but marked hyperlipemia in leprosy. Ramu and Nagarajan ([18]) found higher serum lipids and low cholesterol in leprosy patients during reactive stages. The serum lipids dropped after a reactive stage, but serum cholesterol showed a slight increase. Recently Nath and Chatterji ([16]) reported no changes in plasma and cell cholesterol in leprosy, while phospholipids showed a reduction as compared to normal values. The conflicting reports of the workers named above could be due partly to inadequacy of methods of lipid analysis.

No detailed and quantitative work has been carried out on changes in the individual serum lipid components in leprosy. The present paper describes an investigation of the serum lipids of patients with the lepromatous type of leprosy. The changes in serum lipid components have been studied through their quantitative separation and characterization by means of chromatographic technics employing a silicic acid column and silicic acid-impregnated paper.

From the large amount of previous experimental work on nutrition, it is quite clear that serum lipids are greatly influenced by the quality and quantity of dietary fats. In a study of this type, therefore, dietary comparability in the experimental control groups is very important. Without such comparability the results obtained could be quite

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misleading. This point was given the utmost consideration in the present study. The diet of the leprosy patients was closely comparable to that of the control group, both in quality and caloric quantity. The individuals used as controls were of apparent good physique.

MATERIALS AND METHODS

Blood collections.—Blood was collected from the antecubital veins of patients with lepromatous leprosy housed in a leprosarium maintained by a Delhi corporation. All the patients were in the nonreactive stage and between 25 and 38 years in age. Their general health was good. Serum was prepared within four hours by centrifuging the clotted blood at 2,000 r.p.m. for 15 minutes. The decanted serum was centrifuged briefly once more and used immediately for lipid extraction. Normally serum of 10 patients was pooled for lipid extraction. Serum from normal subjects in the biochemistry department of this Institute was treated similarly and used as controls.

Lipid extraction.—A measured quantity of serum (20-25 ml.) was added in drops with constant swirling to 10 volumes of a solution containing 2 volumes of chloroform and 1 of methanol, in a 500 ml. Erlenmeyer flask, and the mixture was allowed to stand at room temperature for 4 hours with occasional shaking. The contents were filtered in a Buchner funnel, and the residue was reextracted with 10 more volumes of the solvent described above and filtered. The two filtrates were combined and evaporated to dryness *in vacuo* at 45-50°C in a rotary evaporator. The completely dried residue was freed from proteolipids according to the method of Folch *et al.* ([7]). The dried residue was then dissolved in 100 ml. of the chloroform-methanol solvent and washed with 20 ml. of normal saline. The two phases were allowed to separate overnight at room temperature and the chloroform layer was collected and evaporated to dryness *in vacuo*. The completely dried residue was dissolved in 25 ml. of hexane and stored under nitrogen at −10°C. Normally the analysis for cholesterol and phosphorus, and chromatography of the lipid extract, were carried out immediately. The weight of the lipid was determined on 1.0 ml. aliquot samples on a micro-balance. It is to be noted that all of the solvents, viz., chloroform, methanol, ether, hexane, and acetone (British Drug House), were redistilled and bubbled with nitrogen before use.

Chromatography.—Silicic acid-impregnated paper chromatography: Whatman No. 3 filter papers were impregnated with 100-200 mesh Mallinckrodt silicic acid for chromatography according to the method of Marinetti *et al.* ([14]). Amounts of 10 to 12 μgP in the case of total lipid extract, and 2-4 μgP in the case of individual phospholipid components, were spotted on the paper, and papers were developed in di-isobutylketone-glacial acetic acid-H_2O in the proportions of 40:25:5 ([14]). Rhodamine-6-G (0.001% dissolved in H_2O) was used for detecting all lipid material. Ninhydrin solution (0.4% in acetone-lutidine (9:1, v/v)) was used for amino lipid detection, phosphomolybdic acid and stannous chloride for choline-containing lipids, and iodine vapor for unsaturation.

Silicic acid column chromatography: Mallinckrodt silicic acid for chromatography, of 100-200 mesh size, prepared according to the method of Misra ([15]), was used for column chromatography. Normally 6-8 mgm. lipid (60-70 μgP)/gm. of silicic acid were applied on the glass column (30 cm. × 1.5 cm.). In a typical run 100 mgm. lipid were put on a 15 gm. silicic acid column. The flow rate without any pressure was 10 ml./2-3 minutes. For eluting neutral lipids, increasing concentration of ether in hexane, and for phospholipids, increasing concentrations of methanol in chloroform, were used. Ten ml. fractions were collected automatically and the progress of elution of a particular lipid component from the column was followed by its simultaneous analysis on every alternate tube of the column effluents. The solvent system used for a typical 100 mgm. serum lipid separation is given in Table 1.

Analysis.—Cholesterol was estimated by the method of Hanel and Dam ([9]), phos-

TABLE 1.—*Solvent system employed for eluting 100 mgm. serum lipids from a 15 gm. silicic acid column.*

Number	Lipid component	Solvent composition per cent	Volume in ml.
1	Esterified cholesterol	1.5 diethyl ether in hexane	200
2	Triglycerides	4.0 diethyl ether in hexane	300
3	Free fatty acids	8.0 diethyl ether in hexane	150
4	Free cholesterol	15.0 diethyl ether in hexane	200
5	Diglycerides	30.0 diethyl ether in hexane	100
6	Monoglycerides	100.0 diethyl ether	100
7	Phosphatidyl ethanolamine (+ lysophosphatidyl ethanolamine and inositol phosphatide)	25.0 methanol in chloroform	200
8	Lecithin	40.0 methanol in chloroform	500
9	Sphingomyelin	70.0 methanol in chloroform	250
10	Lysolecithin	100.0 methanol	150

TABLE 2.—*Composition of serum lipids of normal persons and leprosy patients. In each experiment serum from 10 persons was pooled.*

Serum component	No. of experiment	Normal serum (mgm./100 ml.)	Leprosy serum (mgm./100 ml.)
Total lipids	1	550.00	441.25
	2	758.75	485.57
	3	638.40	365.62
		Av = 649.05	Av = 430.81
Total cholesterol	1	173.09	118.25
	2	218.55	128.22
	3	181.72	99.34
		Av = 191.12	Av = 115.27
Phospholipid phosphorus (PLP)	1	4.9535	3.9947
	2	9.3000	4.9973
	3	7.0818	3.0164
		Av = 7.1117	Av = 4.0028

phorus by the method of Bartlett [2], and glycerol by the method of Van Handel and Zilversmit [22]. Amino nitrogen was determined according to the method of Lea and Rhodes [13], and choline according to the procedure of Appleton *et al.* [1]. Free fatty acids were determined titrimetrically according to the method of Dole [6]. Sphingomyelin (alkali-acid stable phosphorus) was determined according to the method of Ren Konen [19].

RESULTS

In Table 2 values are presented for total lipid, total cholesterol and total phospholipid phosphorus in normal and leprosy serum. A decrease in total lipids, total cholesterol and total phospholipid phosphorus is observed in the serum of patients with lepromatous type leprosy, as compared with normal values. This decrease in serum lipid components, however, does not change the percentage composition of

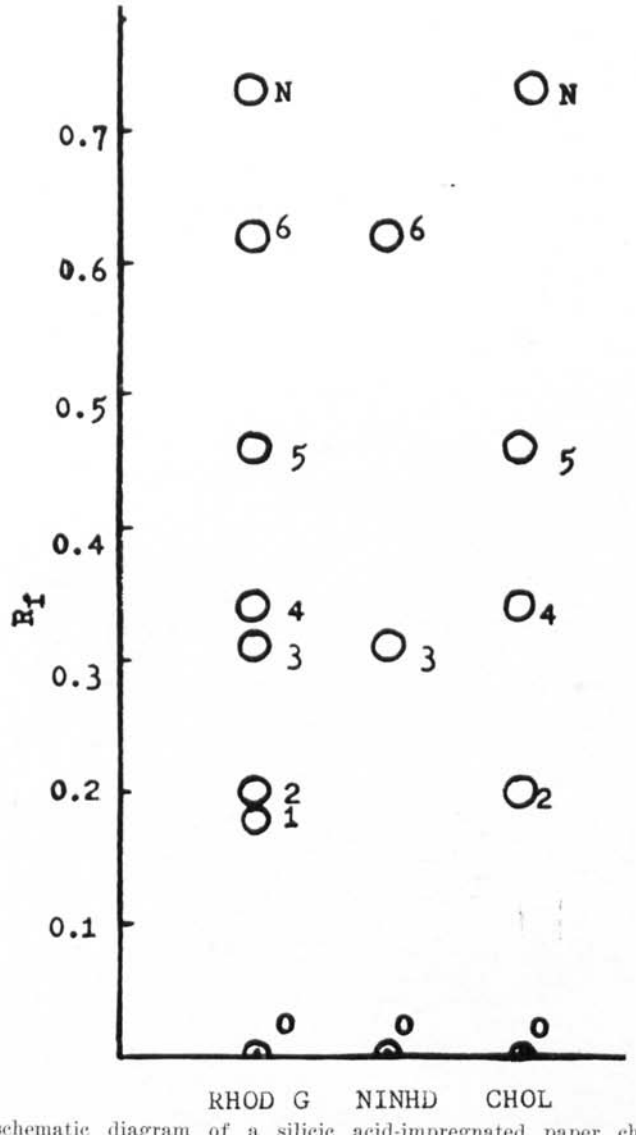

FIG. 1. A schematic diagram of a silicic acid-impregnated paper chromatogram of phospholipids in serum total lipid extract. Twelve μgP was applied on the paper and, after developing, the paper was stained with rhodamine-6-G (Rhod) for all lipids, with ninhydrin (Ninhyd) for amino lipids and phosphomolybdic acid and $SnCl_2$ for choline lipids. The spots are identified as follows: 0 = origin; 1 = inositol phosphatide; 2 = lysolecithin; 3 = lysophosphatidyl ethanolamine; 4 = sphingomyelin; 5 = lecithin; 6 = phosphatidyl ethanolamine, and N = neutral lipids.

cholesterol and phospholipid phosphorus in serum lipids as compared with the normal.

In Figure 1 a schematic diagram is shown of a silicic acid-impregnated paper chromatogram of a total lipid extract of leprosy serum run according to the method of Marinetti *et al.* ([14]). Staining of the paper with rhodamine-6-G revealed six spots, when viewed under ultra-

violet light. Spot No. 1 had the Rf value corresponding to inositol phosphatide of rat liver lipids. Spots Nos. 2, 4, and 5 gave positive choline tests (with phosphomolybdic acid and stannous chloride) and the test for unsaturation (iodine vapors). Spot No. 5 had the Rf value corresponding to an authentic sample of synthetic β-γ-dipalmitoyl-α-DL-

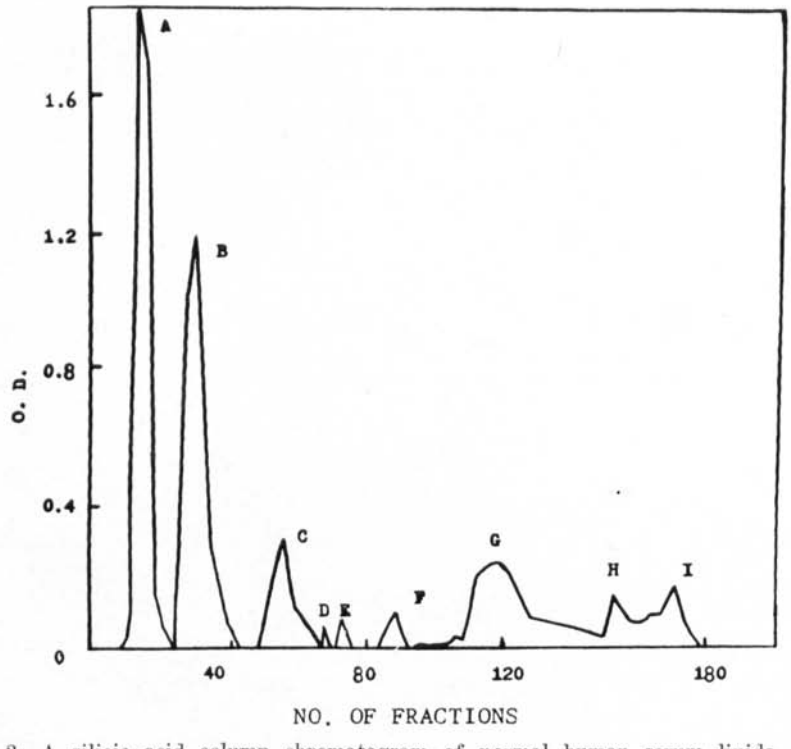

FIG. 2. A silicic acid column chromatogram of normal human serum lipids. Ten ml. fractions/2-3 minutes were collected in an automatic fraction collector and every second tube was analyzed for a particular lipid component. Peaks eluted are in the following order: peak A = esterified cholesterol; peak B = triglycerides; peak C = free cholesterol; peak D = diglycerides; peak E = monoglycerides; peak F = cephalin; peak G = lecithin; peak H = sphingomyelin, and peak I = lysolecithin.

Spectrophotometric estimations of cholesterol, glycerol and phosphorus were made at 528, 570 and 830 millimicrons respectively.

lecithin (obtained from Sigma Chemicals, St. Louis, Missouri, U.S.A.). Spot No. 2 had the Rf value of lysolecithin, present as a contaminant in the standard sample of lecithin. Spot No. 4 had the Rf value corresponding to standard sphingomyelin. Spots Nos. 6 and 3 gave a positive ninhydrin test (0.4% ninhydrin in acetone-lutidine (9:1, v/v), and had the Rf value corresponding to standard samples of synthetic β-γ-dipalmitoyl-DL-α-phosphatidyl ethanolamine (obtained from Sigma Chemicals, St. Louis, Missouri, U.S.A.) and β-acyl lysophosphatidyl ethanolamine[2] respectively. Phosphatidyl serine could not be detected

[2]This sample was kindly provided by Dr. M. M. Rapport of the Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, New York, N.Y.

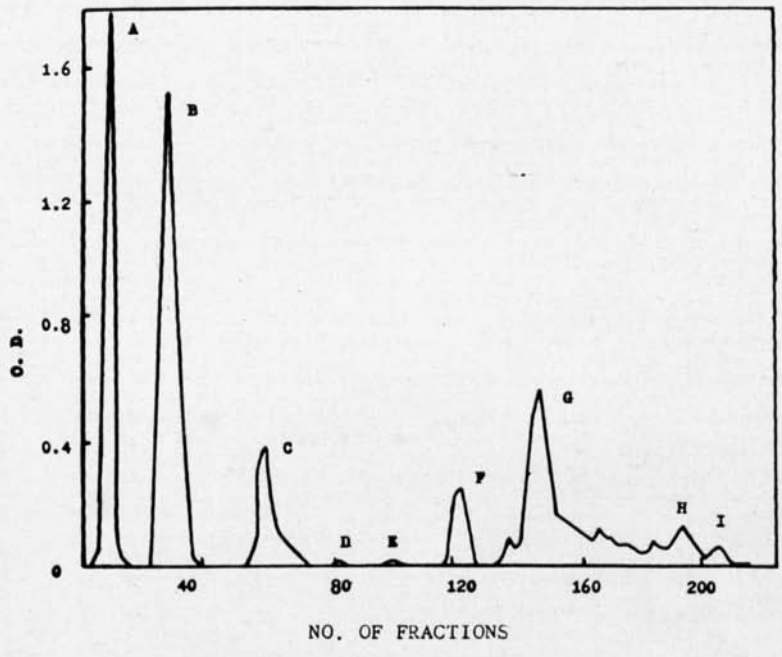

FIG. 3. A silicic acid column chromatogram of serum lipids in leprosy. Explanation of the chromatogram is the same as that for Figure 2.

in either leprosy serum or normal serum.

Figures 2 and 3 represent a typical silicic acid column chromatogram of normal and leprosy serum lipids respectively. In each run, values for recovery of total lipids, total cholesterol, and total phospholipid-phosphorus from the silicic acid column were between 94 and 101 per cent. Table 3 shows the percentage compositions of various lipid components of normal and leprosy serum lipids. Reductions in all lipid fractions are observed except for esterified cholesterol, which remains unchanged, and triglycerides, which show a slight increase. A very sharp separation of esterified cholesterol, triglycerides, free fatty acids, free cholesterol, diglycerides, and monoglycerides, has been achieved by means of silicic acid column chromatography.

Identification of phospholipids.—The main cephalin fraction F (Figs. 2 and 3) of phospholipids was eluted with 25 per cent methanol in chloroform. When this fraction was chromatographed on silicic acid-impregnated paper, it gave three rhodamine-6-G-positive spots (spots 1, 3 and 6 of Fig. 1), and two ninhydrin-positive spots (spots 3 and 6 of Fig. 1). Mild alkaline hydrolysis of fraction F (20-25 μgP), carried out according to the method of Dawson ([5]), produced two phosphate ester spots, when the hydrolysate was chromatographed on Whatman No. 3 paper in phenol-H_2O-acetic acid-ethanol (25:2:5:3, v/v), and

sprayed with Hanes-Isherwood reagent ([10]) as modified by Burrows *et al.* ([3]). These corresponded to Rf values for glyceryl phosphoryl ethanolamine (GPE) and glyceryl phosphoryl inositol (GPI) respectively. Fraction F (20-25 μgP) was subjected to 2N HCl hydrolysis in sealed ampules for 48 hours at 110°C. Chromatography of the acid hydrolysate on acid-washed Whatman No. 1 paper, in pyridine-ethyl acetate-H_2O (10:36.5:11.5, v/v) (top layer), produced two spots corresponding to inositol and glycerol when sprayed with Trevelyan reagent ([21]).

Chromatography of the acid hydrolysate for amino acids on Whatman No. 1 paper in methanol-benzene-n-butanol-H_2O (2:1:1:1, v/v) and spraying with ninhydrin solution (0.4% in acetone), revealed ethanolamine and five more faintly ninhydrin-positive spots. One of these five spots had the Rf value corresponding to serine. But as no positive ninhydrin test was obtained for phosphatidyl serine on silicic acid-impregnated paper, therefore the appearance of the serine and other amino acids on acid hydrolysis must be of nonlipid origin. This is in agreement with the findings of Phillips ([17]). Rechromatography of fraction F on silicic acid column did not separate these three phospholipids. Fraction G was sharply eluted with 40 per cent methanol in chloroform (Figs. 2 and 3). This fraction gave only one rhodamine-6-G-positive spot and one choline-positive spot (spot 5 of Fig. 1) on a silicic acid-impregnated paper chromatogram. Mild alkaline hydrolysis ([5]) of this fraction (20-25 μgP) and chromatography of the hydrolysate gave only one spot corresponding to glyceryl phosphoryl choline (GPC). Glycerol was the only reducing sugar revealed on paper chromatography of the 2N HCl hydrolysate of fraction G (20-25 μgP).

Sphingomyelin (peak H, Figs. 2 and 3) was eluted with 70 per cent methanol in chloroform. On a silicic acid-impregnated paper chromatogram, this fraction gave two rhodamine-6-G spots and two choline-positive spots (spots 4 and 5 of Fig. 1). The contamination of sphingomyelin with lecithin was slight. Sphingomyelin phosphorus (alkali-acid stable) was determined according to the method of Ren Konen ([19]). Silicic acid-impregnated paper chromatography of fraction I, which was eluted with 100 per cent methanol, revealed two rhodamine-6-G and two choline-positive spots (spots Nos. 2 and 4 of Fig. 1). Mild alkaline hydrolysis of fraction I (20-25 μgP), and chromatography of the hydrolysate, revealed only one phosphate ester corresponding to glyceryl phosphoryl choline. Acid hydrolysis of fraction I (20-25 μgP) with 2N HCl, and chromatography of the acid hydrolysate for sugars, produced only glycerol.

Table 4 shows the values of phospholipid phosphorus for each phospholipid fraction in normal and leprosy serum. It is noted that a general decrease in all the phospholipids occurs in leprosy, but their respective ratios in serum remain unchanged.

TABLE 3.—*Per cent composition of serum lipids of normal and leprosy patients, as determined by silicic acid column chromatography. In each experiment, serum from 10 persons was pooled.*

Serum lipid fraction	No. of experiment	Normal (mgm. %)	Leprosy (mgm. %)
Esterified cholesterol	1	21.388	18.296
	2	21.472	22.194
	3	18.000	20.364
		Av = 20.286	Av = 20.284
Triglycerides	1	24.417	28.100
	2	25.010	28.800
	3	18.000	34.124
		Av = 22.242	Av = 30.341
Free fatty acids	1	1.226	0.900
	2	1.333	1.500
	3	2.000	1.160
		Av = 1.519	Av = 1.187
Free cholesterol	1	4.738	6.212
	2	4.180	4.452
	3	9.500	4.862
		Av = 6.140	Av = 5.175
Diglycerides	1	1.356	0.200
	2	1.586	2.000
	3	1.500	0.812
		Av = 1.480	Av = 1.004
Monoglycerides	1	1.934	0.600
	2	1.537	2.700
	3	2.000	1.972
		Av = 1.823	Av = 1.757
Main cephalin fraction	1	8.588	6.700
	2	6.893	9.150
	3	7.500	6.368
		Av = 7.660	Av = 7.406
Lecithin	1	23.278	22.200
	2	27.572	27.200
	3	27.000	22.968
		Av = 25.950	Av = 24.122
Sphingomyelin	1	8.136	6.700
	2	5.490	5.750
	3	9.000	5.394
		Av = 7.542	Av = 5.948
Lysolecithin	1	3.277	0.400
	2	4.270	6.600
	3	9.500	1.624
		Av = 4.549	Av = 2.874

Each fraction was pooled, evaporated to dryness *in vacuo* and dissolved in 10 ml. chloroform. One ml. of lipid solution was taken for weight determination.

TABLE 4.—*Composition of individual serum phospholipid phosphorus of normal and leprosy patients, as determined by silicic acid column chromatography. In each experiment serum from 10 persons was pooled.*

Sample	No. of experiment	Main cephalin fraction		Lecithin		Sphingomyelin[a]		Lysolecithin[b]	
		μgP/1.0 ml. serum	%	μgP/1.0 ml. serum	%	μgP/1.0 ml. serum	%	μgP/1.0 ml. serum	%
Normal serum	1	3.008	6.1	30.230	61.0	6.930	14.0	9.174	18.5
	2	9.244	8.0	43.540	46.8	21.838	23.4	18.266	19.6
	3	4.356	6.2	46.505	66.2	12.223	17.4	7.088	10.1
	Mean	= 5.552	6.7	40.091	58.0	13.663	18.2	11.509	16.0
Leprosy serum	1	2.690	6.7	26.740	67.2	8.047	20.2	2.283	5.7
	2	3.861	7.7	28.498	57.0	8.865	17.7	8.717	17.5
	3	1.383	4.5	18.675	61.9	5.535	18.3	4.507	15.6
	Mean	= 2.313	6.3	24.637	62.0	7.482	18.7	5.169	12.9

[a]Contains some lecithin fraction.
[b]Contains some sphingomyelin fraction.

DISCUSSION

By use of combined technics of silicic acid column and paper chromatography, serum lipids from leprosy serum have been fractionated quantitatively, and identified as esterified cholesterol, triglycerides, free fatty acids, free cholesterol, di- and mono-glycerides, phosphatidyl-ethanolamine, lysophosphatidyl-ethanolamine, inositol phosphatide, phosphatidyl choline, sphingomyelin and lysolecithin. Recoveries of the applied lipids, cholesterol and phospholipids from silicic acid columns were quantitative. On comparing the values of total serum lipids, total cholesterol, and total phospholipid-phosphorus of leprosy patients with normal values (Table 2) a general but uniform reduction in their amounts was observed in the lepromatous type of leprosy.

Our results are not in agreement with those of Gokhale and Godbole ([8]), who have reported that serum lipids, cholesterol and phospholipids are higher in leprosy patients than in normal persons. Our observations of lower values for serum cholesterol are in agreement with those of Kusaka ([12]), Chekherdemian ([4]), and Nath and Chatterjie ([16]). The hyperlipemia observed by Gokale and Godbole ([8]) and Chekherdemian ([4]) might have been due to the fact that the patient was in lepra reaction, followed by a drop in lipids to levels below the normal after the reaction. Our observations on the low phospholipid levels in leprosy serum are not in agreement with those of Kusaka ([12]), who did not find any difference in the two groups.

It is well understood that the levels of lipids in the blood at any given time are the resultant of processes removing them from it. The sources of blood lipids, include absorption from the intestine, mobilization from the fat depots, and synthesis, particularly by the liver. As the role of the liver is quite important in the levels of blood lipids, any impairment in liver function will influence the blood lipid levels. Although a general decrease in total lipids, cholesterol and phospholipids is observed in leprosy patients, the respective ratios of these lipids do not seem to be affected. This could be because of decreased synthesis of lipids, due to liver malfunctions, to malabsorption from the intestine, or to decreased mobilization from the liver. Separation of serum lipids into individual fractons by means of silicic acid column chromatography reveals the changes in the respective ratios.

All lipid components in leprosy serum show slight decrease except esterified cholesterol, which remains unchanged, and triglycerides, which show a slight increase when compared to the normal values (Table 3). All phospholipids, viz., cephalin, lecithin, sphingomyelin and lysolecithin, decrease in leprosy (Table 4), but their respective ratios in leprosy serum do not appear affected. The liver is considered to be the major site for the biosynthesis of phospholipids. The decrease in the levels of cephalin, lecithin, sphingomyelin and lysolecithin points

toward a decreased capacity of the liver for the synthesis of phospholipids in leprosy patients.

SUMMARY

Serum lipids of leprosy patients have been separated and measured quantitatively by means of silicic acid column and paper chromatography. A general decrease in total serum lipids, cholesterol, and phospholipids, is observed in leprosy. Values found for serum total lipids, total cholesterol, and total phospholipid phosphorus in patients with the lepromatous type of leprosy were respectively: 430.8 mgm., 115.2 mgm. and 100.0 mgm. per 100 ml., as compared to 649.0 mgm., 191.1 mgm. and 177.8 mgm. per 100 ml. respectively in normal persons. A slight increase in triglycerides is observed in leprosy serum lipids, whereas other serum neutral lipid components are only slightly affected. A general decrease in all serum phospholipids, viz., cephalin, lecithin, sphingomyelin, and lysolecithin, is observed in leprosy.

RESUMEN

Los lípidos séricos de pacientes leprosos han sido separados y medidos cuantitativamente por la cromatografía en papel y las columnas de acido silícico. Se ha observado una disminución en la lepra de los lípidos séricos totales, colesterol y fosfolípidos. Los valores encontrados para los lípidos séricos totales, colesterol total, y fosfolípidos fosforosos totales en pacientes con el tipo lepromatoso de lepra fueron respectivamente : 430.8 mgm., 115.2 mgm., y 100.0 mgm. por 100 ml., comparados respectivamente con las personas normales, que son : 649.0 mgm., 191.1 mgm. y 177.8 mgm. por 100 ml. Un ligero aumento se observa en los triglicéridos de los lípidos séricos de los leprosos, mientras que los otros componentes séricos lipídicos neutros estan solamente muy ligeramente afectados. Se observó en la lepra una disminución general en todos los fosfolípidos séricos, i.e., cefalina, lecitina, esfingomielina y lisolecitina.

RÉSUMÉ

Les lipides sériques des malades de la lèpre ont été fractionnés et mesurés quantitativement par colonnes de silicium et par chromatographie sur papier. Un abaissement général des lipides totaux du sérum, du cholestérol et des phospholipides est observé dans la lèpre. Les taux trouvés pour les lipides totaux du sérum, pour le cholestérol total et pour le phosphore de l'ensemble des phospholipides ont été respectivement: 430.8 mg., 115.2 mg. et 100.0 mg. par 100 ml. chez les malades lépromateux contre des valeurs correspondantes de 649.0 mg., 191.1 mg. et 177.8 mg. par 100 ml. chez les personnes normales. Une légère augmentation des triglycérides a été observée dans les lipides du sérum chez les lépreux, alors que les autres constituants lipidiques neutres du sérum ne sont que légèrement modifiés. Une diminution générale de tous les phospholipides, qu'il s'agisse de la céphaline, de la lécithine, de la sphingomyéline ou de la lysolecithine, est observée dans la lèpre.

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