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MODIFICATION OF AORTIC ATHEROMA AND FATTY LIVER IN CHOLESTEROL-FED RABBITS BY INTRAVENOUS INJECTION OF SATURATED AND POLYUNSATURATED LECITHINS

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PLATES XIX–XXI

ADAMS *et al.* (1963*b*) and Adams and Morgan (1967) have recently suggested that phospholipids may play a dual anti-atherogenic role by mobilising cholesterol from arteries and by preventing the sclerogenic action of this sterol on connective tissues. The arterial wall and certain other organs react to the presence of cholesterol by synthesising phospholipid *in situ* (Zilversmit and McCandless, 1959–60; Zilversmit *et al.*, 1961; Christensen, 1961, 1962). Such endogenous synthesis of phospholipid may be a local defence mechanism against sterol deposition (Adams, 1964).

The present investigation concerns the effect of intravenous administration of either egg lecithin or polyunsaturated lecithin (Lipostabil) on the development of cholesterol-induced fatty liver and atheroma in rabbits.

MATERIALS AND METHODS

Effect of lecithins on cholesterol feeding. Eleven young male New Zealand White rabbits (*c.* 2·0–2·5 kg.) were fed on a diet of SG1 pellets enriched with 1 per cent. cholesterol and 6 per cent. sunflower-seed oil (McMillan, Horlick and Duff, 1955). Seven animals received intravenously under light Nembutal anaesthesia an ultrasonically cleared 10 per cent. suspension of ovolecithin (BDH) in water (Saunders, Perrin and Gammack, 1962). The 7 animals received injections of 1·0 g. of ovolecithin on Mondays and Thursdays of each week: in 3 of these animals injections were started 2 wk before feeding the cholesterol-enriched diet, and continued for 3–4 wk; in the other 4 animals ovolecithin injections were started 2 wk after the beginning of the diet and continued for 3–7 wk. The remaining 4 animals received an injection under light Nembutal anaesthesia of 10 ml. of clear 5 per cent. aqueous solution of polyunsaturated phosphatidyl choline (Lipostabil—without pyridoxine: from Natterman, Köln); the dose was divided into equal parts for intravenous and subcutaneous injection and injected on Mondays, Tuesdays, Thursdays and Fridays every week, starting 2 wk after feeding the high cholesterol diet, and continuing for 1½–4 wk. Thus, all 11 animals were given, during the experimental period, a weekly injection of 2·0 g. of phospholipid as either ovolecithin or Lipostabil.

Control experiments. Three young male New Zealand White rabbits on stock SG1 diet were given injections, under light Nembutal anaesthesia, of either ovolecithin or Lipostabil for 2 wk as described above. These animals were bled daily on weekdays and their plasma was used for estimation of phospholipid and

cholesterol. A further 11 rabbits were fed on the cholesterol-enriched diet, but did not receive injections of phospholipid; these animals were given injections of Nembutal on the same days and in the same dose as used previously for the intravenous administration of phospholipid.

Collection of samples. Blood was collected into oxalate tubes from a punctured ear vein at the beginning of the experiment and at weekly intervals thereafter for the estimation of plasma cholesterol and phospholipid levels.

At suitable time-intervals animals were killed with an overdose of Nembutal, blood was collected by cardiac puncture and the organs were removed for examination. The aorta was incised longitudinally from its origin to its upper abdominal part and the severity of atheroma was noted. Exactly 2·0 cm. of thoracic aorta immediately distal to the origin of the left subclavian artery was removed and used for the estimation of cholesterol and phospholipid. The tunica adventitia was scrupulously dissected away from this sample, which was then washed in distilled water and weighed to the nearest 0·1 mg. The arch of the aorta and the lower thoracic aorta were fixed in Lillie's (1954) 1 per cent. calcium acetate-4 per cent. formaldehyde for histological and histochemical examination.

A sample from the right lobe of the liver was removed for chemical estimation of cholesterol and phospholipid. Blocks were taken from the liver, lung, kidney, adrenal, spleen and testis for histological and histochemical examination.

Chemical methods. Cholesterol was estimated by Leffler and McDougald's (1962) modification of the Zlatkis method. Phospholipid was estimated as lipid phosphorus by Marinetti's (1962) modification of Bartlett's method.

Histochemical methods. Calcium-formol-fixed tissues were cut on a De La Rue Frigistor thermo-electric freezing microtome. The sections were stained by the osmium tetroxide-α-naphthylamine (OTAN) method for phospholipid (Adams, 1959), NaOH-OTAN method for sphingomyelin (Adams and Bayliss, 1963), gold-hydroxamic acid method for phosphoglycerides (Adams, Bayliss and Ibrahim, 1963*a*), perchloric acid-naphthoquinone (PAN) method for cholesterol (Adams, 1961) and the lipase-lead sulphide method for triglycerides (Adams *et al.*, 1966).

Gas chromatography. Gas-liquid chromatography was carried out on the methyl esters of the component fatty acids of our phospholipids by Dr R. W. R. Baker (Department of Chemical Pathology, Guy's Hospital Medical School). The ovolecithin contains 34·9 per cent. saturated fatty acids, 44·9 per cent. oleic acid (18:1), 10·8 per cent. linoleic acid (18:2) and small traces of linolenic (18:3) and arachidonic (20:4) acids; its saturated/monounsaturated/polyunsaturated fatty acid ratio is about 1·0/1·3/0·37. The polyunsaturated phosphatidyl choline (Lipostabil) contains 15·0 per cent. saturated fatty acid, 9·5 per cent. oleic acid, 69·3 per cent. linoleic acid, 5 per cent. linolenic acid and 1·8 per cent. of other triene polyunsaturated fatty acids; its saturated/monounsaturated/polyunsaturated fatty acid ratio is, therefore, about 1·0/0·6/5·1.

Results

Gross appearances. The control rabbits fed with the cholesterol-enriched diet without phospholipid injection showed fatty streaks and small atheromatous plaques in the arch and descending thoracic aorta from 4½ wk on diet onwards (table I); their livers often appeared grossly fatty from the 5th wk. Cholesterol-fed rabbits given injections of ovolecithin showed more aortic atheroma than the control group (table I), but their livers appeared less fatty. Cholesterol-fed animals given Lipostabil showed no macroscopic evidence of either aortic atheroma (table I) or fatty liver. The blood plasma in animals receiving injections of Lipostabil appeared relatively clear and translucent.

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whereas that of both cholesterol-fed controls and ovolecithin-injected animals was always opaque as a result of excess chylomicrons.

Histochemical results. The descending aorta and aortic arch of rabbits given injections of ovolecithin usually show greater intimal thickening and lipid deposition (table I, fig. 1) than the corresponding control animals (table I, fig. 2). In contrast, the aorta of only one animal given injections of Lipostabil shows a small focus of atheroma (table I, figs. 3–6), in which lipid stains red with OTAN (fig. 3) and blue with PAN (fig. 5), indicating that it contains phospholipid and free cholesterol.

TABLE I

Severity of aortic atheroma in lecithin-injected cholesterol-fed rabbits

Duration of expt (wk)	Gross appearance * of aorta in			Histological grade † of aorta in		
	cholesterol-fed control rabbits	ovo-lecithin-injected cholesterol-fed rabbits	Lipostabil-injected cholesterol-fed rabbits	cholesterol-fed control rabbits	ovo-lecithin-injected cholesterol-fed rabbits	Lipostabil-injected cholesterol-fed rabbits
2	0, 0	0, 0	...	0, 0	0, 0	...
3	0	1	...	0	2	...
3½	0	...	0	0	...	0
4½	1	...	0	1	...	0
5	1, 1	2	0	1, 2	5	1
6	2	...	0	2	...	0
7	1	2	...	2	1	...
8	2	2	...	2	3	...
9	2	2	...	2	5	...
Mean score	0·9	1·3	0	1·1	2·3	0·3

* Fatty streaks and spots = 1; plaques = 2.

† Sum of scores for intimal thickness and amount of lipid: intimal thickness < media, 1, or = media, 2, or > media, 3; lipid scanty, 1, or numerous discrete droplets, 2, or confluent lipid, 3. Maximal score therefore = 6.

The livers of the controls (tables II and III, figs. 7 and 8) develop more severe fatty change than in ovolecithin-injected rabbits (tables II and III, figs. 9 and 10). There is less phosphoglyceride in the livers of the ovolecithin-injected animals (fig. 11) than in the controls (fig. 12). Lipostabil injection has a markedly protective effect against the development of fatty liver (tables II and IV, figs. 13–16), even more so than does ovolecithin. Triglyceride is not demonstrated with the lipase-lead sulphide method in any of these fatty livers, so it is concluded that the patchy black reaction of the hepatic lipid with OTAN must be due to deposition of unsaturated cholesterol esters.

Lipid-laden macrophages that are stained black by OTAN are seen in the pulmonary alveolar walls and in the spleens of control cholesterol-fed rabbits. Lipid is less obvious in these organs in ovolecithin-injected animals and is absent when Lipostabil is administered.

Chemical results. On stock diet, control rabbits given intravenous injections of ovolecithin developed marked hyperphospholipidaemia

TABLE II

Severity of fatty liver in lecithin-injected cholesterol-fed rabbits

Duration of expt (wk)	Rank of fatty liver in		
	cholesterol-fed control rabbits	ovolecithin-injected cholesterol-fed rabbits	Lipostabil-injected cholesterol-fed rabbits
2	5, 7	3, 4	...
3	11	6	...
3½	12	...	1
4½	13	...	2
5	19, 14	9	8
6	15	...	10
7	20	16	...
8	21	18	...
9	22	17	...
Mean rank	14·5	10·4	5·3

The results have been ranked in order of staining intensity with the OTAN method: 1 = weakest; 22 = strongest.

TABLE III

Aortic and liver lipids in ovolecithin-injected cholesterol-fed rabbits

Duration of experiment (wk)	Cholesterol and phospholipid content of			
	aorta in		liver in	
	cholesterol-fed control rabbits	ovolecithin-injected rabbits	cholesterol-fed control rabbits	ovolecithin-injected rabbits
5	CH * 1·55 PH † ...	0·63 ...	6·05 0·086	3·86 0·075
7	CH 0·38 PH 0·007	0·62 0·021	8·85 0·092	5·91 0·078
8	CH 2·38 PH 0·046	3·67 0·047	10·66 0·100	3·41 0·090
9	CH 0·87 PH 0·051	3·37 0·054	9·83 0·098	9·2 0·091
Significance of differences	CH: not significant PH: not significant		CH: $P \simeq 0·03$ PH: $P \simeq 0·001$	

* CH = mg. cholesterol per 100 ml.
† PH = mg. lipid phosphorus per 100 ml.

(up to 26·4 mg. lipid phosphorus per 100 ml.) for 1–4 hr after each injection; after 2 wk of repeated injections the blood lipid phosphorus

TABLE IV

Aortic and liver lipids in Lipostabil-injected cholesterol-fed rabbits

Duration of experiment (wk)	Cholesterol and phospholipid content in			
	aorta of		liver of	
	cholesterol-fed control rabbits	Lipostabil-injected rabbits	cholesterol-fed control rabbits	Lipostabil-injected rabbits
3½	CH * 0·20 PH † 0·021	0·19 0·031	1·00 0·109	0·60 0·136
4½	CH 0·27 PH 0·023	0·16 0·025	2·08 0·112	0·66 0·104
5	CH 0·39 PH 0·026	0·33 0·031	4·91 0·091	0·99 0·101
6	CH 0·39 PH 0·027	0·19 0·032	2·08 0·135	1·61 0·091
Significance of differences	CH: $P \simeq 0·04$ PH: not significant		CH: $P \simeq 0·003$ PH: not significant	

* CH = mg. cholesterol per 100 ml.
† PH = mg. lipid phosphorus per 100 ml.

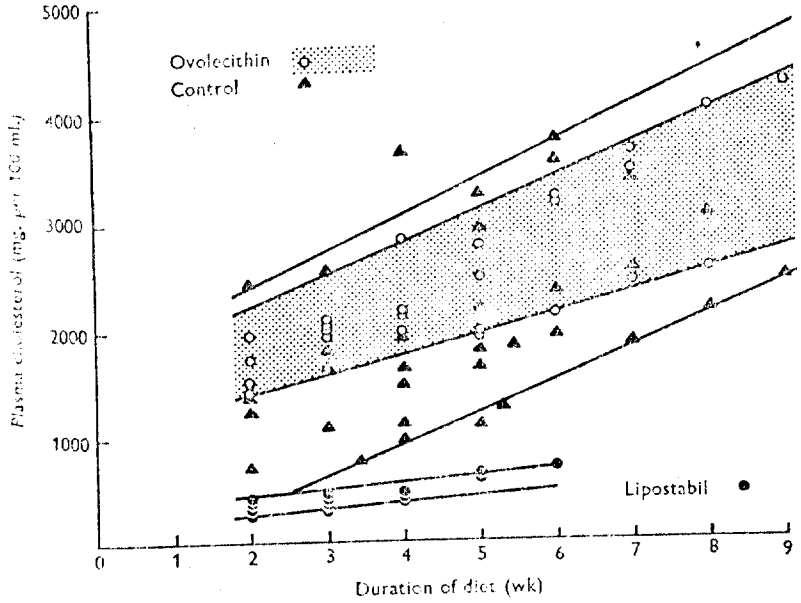

FIG. 17.—Plasma cholesterol levels in ovolecithin-injected, Lipostabil-injected and control cholesterol-fed rabbits.

level, estimated 72 hr after the last injection, rose to 12·1 mg. per 100 ml. from the initial value of 6·2 mg. per 100 ml. Hyperphospho-

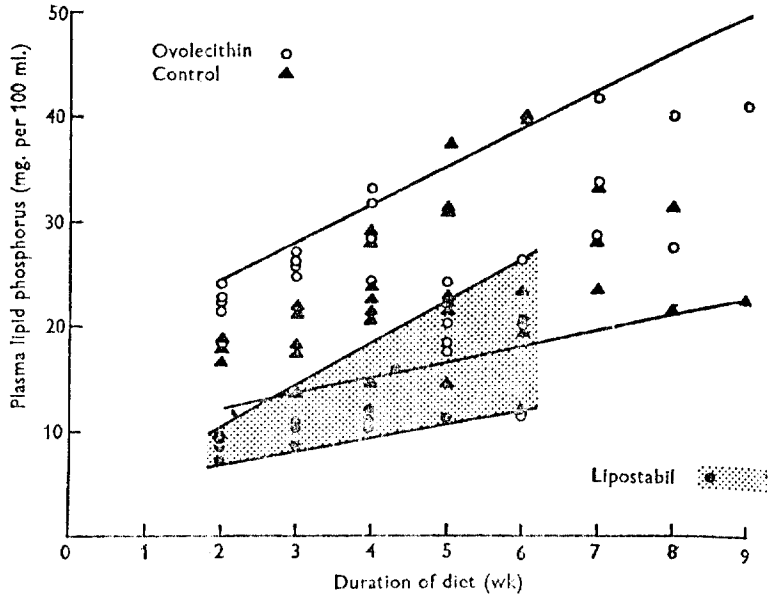

FIG. 18.—Plasma phospholipid levels in ovolecithin-injected, Lipostabil-injected and control cholesterol-fed rabbits.

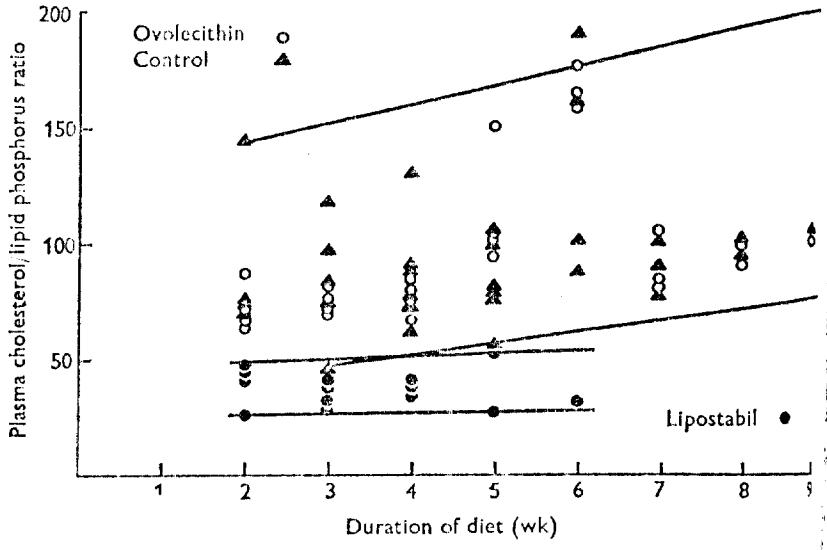

FIG. 19.—Plasma cholesterol/lipid-phosphorus ratios in ovolecithin-injected, Lipostabil-injected and cholesterol-fed rabbits. The ratio is expressed on a weight basis.

lipidaemia occurred immediately after Lipostabil injection (up to 15·2 mg. per 100 ml.), but to a lesser degree than after injection of

ovolecithin. After 2 wk of repeated Lipostabil injections, the blood lipid phosphorus level, estimated 72 hr after the last injection, rose to only 8·4 mg. per 100 ml.

Ovolecithin-injected rabbits fed on the cholesterol-enriched diet showed greater aortic cholesterol and lower hepatic cholesterol than did control cholesterol-fed animals (table III), whereas both aortic and hepatic cholesterol were reduced in Lipostabil-injected cholesterol-fed animals (table IV). The plasma cholesterol level was substantially lower in the Lipostabil-injected than in ovolecithin-injected and control animals (fig. 17). The plasma phospholipid levels and plasma cholesterol/phospholipid ratios in all 3 groups of animals are illustrated in figs. 18 and 19 respectively; there is again a pronounced reduction in the Lipostabil-injected animals.

Discussion

Effect of lecithin injections on plasma phospholipid. From our studies on stock-fed rabbits, it is clear that intravenous injection of ovolecithin causes hyperphospholipidaemia. A high peak of plasma phospholipid is obtained soon after injection, but it falls to lower levels within 8 hr afterwards. Nevertheless, the plasma phospholipid level in the " steady state " (i.e., 72 hr after injection) is increased two-fold at the completion of 2 weeks' repeated ovolecithin injections. A peak of hyperphospholipidaemia also occurs soon after injecting poly-unsaturated phosphatidyl choline (Lipostabil), but the " steady state " plasma phospholipid level is not significantly elevated at the end of 2 weeks' injections. The failure of Lipostabil to sustain an elevated plasma phospholipid level may well be due to the presumably fast metabolism of this *unsaturated* lecithin, when compared with the probably slow turnover of *saturated* lecithins.

Cholesterol-fed animals given injections of ovolecithin develop approximately the same plasma phospholipid levels and plasma cholesterol/phospholipid ratios as do control cholesterol-fed animals (figs. 18 and 19). In this connexion, Byers, Friedman and Sugiyama (1962) have pointed out that elevation of serum cholesterol is usually accompanied by an increase in plasma phospholipids. The failure of our cholesterol-fed rabbits to develop augmented plasma phospholipid levels after injection of ovolecithin cannot be explained with certainty. However, it is possible that the endogenous hepatic synthesis of phospholipid is suppressed by injecting ovolecithin. This explanation is supported by the observation that less phospholipid was detected biochemically (table III) and histochemically (figs. 11 and 12) in the livers of ovolecithin-injected rabbits than in the livers of control animals. Nevertheless, diminished hepatic content of phospholipid does not necessarily imply that synthesis is correspondingly reduced. The above explanation is supported by the observation that feeding phosphatidyl ethanolamine to rats suppresses endogenous hepatic synthesis of phospholipid

(Billimoria, Howell and Maclagan, 1965; MacLagan, Billimoria and Howell, 1966).

Injection of Lipostabil into cholesterol-fed rabbits causes less elevation of the plasma phospholipid level than occurs in either ovolecithin-injected or control animals (fig. 18). However, the plasma cholesterol phospholipid ratio is considerably lower in animals given Lipostabil than in the other two groups (fig. 19). Therefore, the amount of plasma phospholipid attributable to exogenous (i.e., injected) phospholipid, and not to cholesterol-stimulated endogenous phospholipid synthesis, is apparently greater in the Lipostabil-injected rabbits than in the animals that were given ovolecithin.

Effect of lecithin injections on cholesterol deposition in tissues. Our results indicate that intravenous injection of polyunsaturated phosphatidyl choline (Lipostabil) into cholesterol-fed rabbits reduces the severity of both aortic atheroma and fatty liver. On the other hand, injection of a crude commercial preparation of ovolecithin alleviates the development of fatty liver but accentuates atheroma formation. Other organs, in particular the lung and spleen, are also partly protected from cholesterol deposition by administration of both types of lecithin, but this effect is not so marked as with the liver.

Previous studies indicate that oral or intravenous administration of polyunsaturated phosphatidyl choline (Lipostabil) prevents the development of fatty liver, adrenal cholesterosis and aortic atheroma in rats fed on a diet enriched with cholesterol, vitamin D and an anti-thyroid compound (Dornbusch and Zielke, 1960); Konecki, Pietkiewicz and Samochowiec, 1962; Kozubska-Niebrój, Niebrój and Samochowiec, 1962. Likewise injection of Lipostabil is reported to improve the peripheral circulation in patients with arteriosclerosis (Pabst, Strohm and Klemm, 1961), to clear human alimentary hyperlipaemia (Meardi, Taverna and Airò, 1960; Schön, 1960) and to be effective prophylaxis against fat embolism (Bross, Orlowski and Badura, 1961; Lechner and Schuster, 1963).

Intravenous infusion of crude lecithins of animal or plant origin has not been found to alleviate aortic atheroma in the rabbit during the course of a cholesterol feeding experiment (Byers and Friedman, 1959–60). However, infusion of such crude lecithin does lead to more rapid resolution of atheromatous lesions during the regressive phase of a cholesterol feeding experiment, i.e., when the rabbit has been returned to a normal diet for 1–3 mth (Friedman, Byers and Rosenman, 1957; Byers and Friedman).

Mechanism of cholesterol resorption by lecithins. The resolution of fatty liver with all forms of lecithin injection is consistent with the view that phospholipid has a lipotrophic action (Ahrens and Kunkel, 1949; Best, 1956; Dixon, 1958; Rossiter and Strickland, 1960; Adams, 1964). Such lipotrophic action is probably due to the "detergent" and dispersing action of phospholipids on hydrophobic lipids. The "detergent power" of lecithins depends on their amphophilic properties (Bangham, 1963). Thus, one end of the lecithin molecule is composed of hydrophobic paraffin chains that are miscible with hydrophobic lipid (i.e., cholesterol and triglycerides), and the other end is hydrophilic and is miscible with water on account of its highly polarised choline and phosphoryl groups.

However, this " detergent " action of lecithin does not protect the aortic wall against lipid infiltration, for our injections of ovolecithin during cholesterol feeding, and Byers and Friedman's lecithin infusions, do not lead to less aortic lipid deposition. Conversely, injection of Lipostabil during cholesterol feeding does ameliorate aortic lipid infiltration. Because ovolecithin and Lipostabil have the same polar " head " groups (i.e., both are phosphatidyl cholines), the anti-atherogenic effect of the latter must be ascribed to its high content of polyunsaturated " essential " fatty acids. The failure of ovolecithin to protect the aorta against lipid deposition would then be due to the relative saturation of its constituent fatty acids.

The mechanism whereby polyunsaturated phosphatidyl choline (Lipostabil) protects rabbits against atheroma has not been elucidated in this present investigation. Lipostabil is markedly more active in mobilising and dispersing cholesterol than is ovolecithin (Adams and Morgan, 1967), and it could be argued that the presence of a high density of diene and triene bonds in the former lipid increases its surface-active properties. Alternatively, Lipostabil may exert its anti-atherogenic action merely by lowering the serum cholesterol level (see fig. 17). This hypocholesterolaemic effect is a well-known characteristic of polyunsaturated fatty acids; the weight of evidence in the literature suggests that it results from increased biliary excretion of sterols and cholic acids.

Although intravenous administration of Lipostabil has a marked anti-atherogenic action in the cholesterol-fed rabbit, such repeated intravenous injections are not well tolerated. Moreover, the technique is too difficult to allow more than a few animals to be treated experimentally at any one time. For these reasons, we next propose to investigate the effects of Lipostabil on cholesterol-induced atheroma after subcutaneous and oral administration of the phospholipid.

Summary

Repeated intravenous injection of a relatively saturated lecithin derived from eggs (ovolecithin) diminishes fatty liver in cholesterol-fed rabbits, but does not protect the aorta against atheroma. Similar injection of a polyunsaturated lecithin (Lipostabil) protects cholesterol-fed rabbits from both fatty liver and aortic atheroma. It is suggested that the surface-active property of highly ionised phosphoryl and choline groups in lecithin inhibits the development of fatty liver, while the amount of polyunsaturated fatty acids in these lipids determines their anti-atherogenic activity.

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EFFECT OF PROLONGED LECITHIN FEEDING ON HYPERCHOLESTEROLEMIA

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The significance of hypercholesterolemia is not well established. It may lead in some instances to severe organic changes, particularly in the cardio-vascular apparatus. Therapeutic attempts with diets, free of cholesterol and low in fats, were limited in success.

The interdependence of cholesterol and lecithin in many biological processes, and the lipotropic effect of lecithin and choline suggested therapeutic trials with lecithin in hypercholesterolemia. Lecithin has recently become commercially available in large amounts and at a price permitting prolonged clinical administration. Amounts from 10 to 70 grams of soy bean lecithin may be taken in palatable form (cookies) and produce but rarely, when given in excessive amounts, gastric discomfort in sensitive individuals. We have studied the effect of lecithin feeding on several blood constituents and on the intestinal adsorption of fat and vitamin A in man.[1] Whereas the cholesterol level of the serum in normals shows an ephemeral downward trend, prolonged feeding does not affect it.

In five cases of hypercholesterolemia, associated with various clinical conditions, a striking decrease of the serum cholesterol level was achieved by addition of commercial lecithin to the diet (see accompanying table).

The serum cholesterol decreased considerably in all cases, apparently as a result of the lecithin feeding. In Case 1, a cirrhosis of the liver and xanthomatosis, the total lipids of the serum dropped parallel to the cholesterol, and remarkable clinical improvement was observed. In Case 2, a generalized xanthomatosis, the decrease of the serum cholesterol followed the administration of lecithin, but was associated with no striking clinical changes. Six months after the feeding of lecithin was stopped, the serum cholesterol had returned to its former high level. In Case 3, a case of diabetes and obesity, the serum cholesterol dropped by about one-third during lecithin feeding, but was found back at the elevated level 7 months after the feeding of lecithin had been stopped. In Case 4, one of localized xanthomatosis, the feeding of lecithin caused a diminution of the total and particularly, of the esterified serum cholesterol. Simultaneously xanthelasmata of the lids diminished in thickness, but failed to disappear. A similar observation was made in Case 5, whose xanthomatosis was complicated by psoriasis.

Commercial soy bean lecithin represents a mixture of the following composition: lecithin, 20 per cent; cephalin, 20 per cent; soy bean oil, 30 per cent; carbohydrates, 10 per cent; inositol and allied compounds, 15 per cent; phytosterols, 2 per cent. The described effects[1] cannot be ascribed to soy bean oil,

[1] Adlersberg, D., and Sobotka, H.: Effect of lecithin feeding on fat and vitamin A absorption in man. To appear in J. Nutrition, Vol. 25, 1943.

since they could also be produced with defatted products. The role of cephalin and inositol remains to be studied.

TABLE I

Effect of lecithin feeding on serum cholesterol level

CASE	DATE	SERUM CHOLESTEROL (MG./100 CC.)
Case 1. Female, 38 years, biliary cirrhosis, cholangitis, pericholangitis, xanthomatosis. 15 grams defatted lecithin *per diem* during period indicated.	5/28/1941	1420
	7/28	1370 (Total lipoids 3850 mg./100 cc.)
	8/ 1	Lecithin started
	8/ 2	1050 (Total lipoids 3400 mg./100 cc.)
	8/11	Lecithin stopped
	8/15	740
	8/28	875
	8/28	Lecithin resumed
	9/ 2	770 (340)*
	9/10	630 (360) (Total lipoids 1575 mg./100 cc.)
	9/11	625 (330)
	9/15	540 (340)
	9/19	590 (340)
	11/ 3	445 (315)
Case 2. Female, 48 years, xanthomatosis of palms and lids. 15 grams soy bean lecithin *per diem*.	1/21/1942	875, Lecithin started
	2/12	550, Lecithin stopped
	4/ 6	530
	6/ 1	560, Lecithin resumed
	6/15	430
	7/16	295, Lecithin stopped
	1/12/1943	570 (400)
Case 3. Female, 55 years, diabetes, obesity. 15 grams soy bean lecithin *per diem*.	9/20/1941	360
	9/24	Lecithin started
	10/18	365
	11/ 8	235, Lecithin stopped
	6/27/1942	500
Case 4. Female, 41 years, xanthomatosis of lids and palms. 12 grams soy bean lecithin *per diem*.	11/10/1942	620 (550) Lecithin started
	12/ 8	460 (220)
	12/22	470 (330)
	1/ 5/1943	420 (270)
	2/ 9	300 (205) Lecithin continued
Case 5. Male, 35 years, xanthomatosis of lids, extensive psoriasis. 12 grams soy bean lecithin *per diem*.	11/14/1942	440 (345) Lecithin started
	1/12/1943	260 (180) Lecithin continued

* Esterified cholesterol values in parentheses.

Summary: Five cases of xanthomatosis and hypercholesterolemia showed a striking decrease of the serum cholesterol level upon prolonged feeding of lecithin. A few months after the feeding of lecithin was interrupted, the serum cholesterol returned to the original high figures. Further therapeutic trials are suggested.

G. ROENTEROL 10(5): 822-830 '48.

VITAMIN A METABOLISM IN LIVER DISEASE

II. Effect of Bile Acids and Lecithin*

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INTRODUCTION

The alterations of vitamin A metabolism in liver disease have been analysed in previous studies[1,2]. Severe hepatic damage was characterized by low levels of vitamin A in the serum and lack of elevation of the vitamin A concentration after the oral administration of large standard doses of vitamin A in oil ("flat curves in the vitamin A tolerance test"). These changes were correlated with the clinical course and various liver tests. Clinical improvement was accompanied by the gradual return of the vitamin A level in the serum to normal figures, and the tolerance curves showed a normal or even abnormally high course.

The absence of post-prandial elevation of the vitamin A concentration in the serum in liver disease has been attributed either to increased removal of vitamin A from the blood or to impaired intestinal absorption. The former hypothesis was based on the well established depletion of the hepatic reserves of vitamin A in fatally terminating liver disease, especially cirrhosis of the liver. It was stressed, however, that clinical observation casts doubt on the relationship between low curves and low liver reserves of vitamin A[2]. In early stages of hepatitis considerable reserves are still present in the liver; nevertheless, flat vitamin A curves may be found. Poor vitamin A absorption in liver disease also has been attributed to lack of bile; some authors found improved vitamin A absorption after administration of bile salts[3,4], while others obtained negative results[5]. There exists a discrepancy of opinion in regard to the effects of bile acids in man. However, in animals experimentally produced obstructive jaundice, associated with complete absence of bile in the intestinal canal, interferes only with the absorption of carotene, but not of vitamin A[6,7]. A study of the problem was indicated in both normal individuals and in patients suffering from various forms of liver disease.

Sprue, according to our present knowledge, is a nutritional disorder characterized by inability to absorb fat and fat soluble vitamins. Vitamin A and especially carotene levels in the serum are very low during the active stage of the disease; there is complete lack of elevation of the vitmain A content of the

* Aided by research grants from the New York Foundation, American Lecithin Company and Ames Company, Inc.

serum after the ingestion of a standard dose of vitamin A in oil. During a remission, however, absorption gradually improves[8]. It was then found that addition of lecithin to fat or to vitamin A enhances the intestinal absorption of these substances in active sprue[9]. Therefore, it seemed profitable to include a study of the effect of oral administration of lecithin on the defective absorption of vitamin A in liver disease. On the other hand to complete the circle, oral administration of bile acids had to be investigated in active sprue. We expected from the studies additional information on the obscure mechanism of defective absorption in two completely different conditions: liver disease and sprue.

METHOD

Two methods were used for the estimation of vitamin A and carotene in the serum. The first method closely followed the technique of Dann and Evelyn. The Carr-Price reaction was quantitatively estimated in the photoelectric colorimeter ("macro-method"). Six to eight, but preferably 10 ml. of fresh serum were used for each analysis. Readings of smaller amounts are not reliable, as was emphasized previously; this is particularly important in severe liver disease associated with low concentrations of vitamin A in the serum[2].

The second method used was the micro-technique of Bessey, Lowry, Brock & Lopez[10] the chief advantage of which is the small amount of blood necessary for analyses. We applied the following modifications: first, venous blood was used rather than finger tip blood in order to run duplicate determinations; second, the irradiation time was increased to two hours because the shorter time was not sufficient to destroy the vitamin A before the blank reading. A series of tests proved that the method of Dann and Evelyn and that of Bessey, Lowry et al. give comparable results.

In each individual blood was drawn for the estimation of the vitamin A and carotene level of the "fasting" serum whereupon the vitamin A test dose of 180,000 IU was administered. It consisted, as in our previous work, of 3 ml. of Percomorph oil which was given in 30 to 50 ml. of orange juice with a slice of white toast and a cup of tea. A second blood specimen for the estimation of vitamin A was drawn 4 hours later. Because of the comparatively large amounts of blood necessary for the analyses, no later specimens were obtained when the macro method was used. In later series in which the micro method was employed, blood specimens were drawn at 0, 4, 6, and 8 hours. Since no appreciable elevations were found 2 hours after administration of the test dose, the two hour specimen was eliminated. In all tests no food was given during the first 4 hours, and the individuals remained in bed. After 4 hours, a light luncheon was given, the vitamin A content of which was kept very low by omission of carrots, butter and other vitamin A or carotene carriers from the diet.

After the preliminary vitamin A tolerance test (control test) and after an interval of at least 4 to 7 days, a second test was performed with the same individual with the addition of bile salts to the test meal. We used 0.6 Gm. of chemically pure dehydro-

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cholic acid or dehydrated ox bile or, in most cases, chemically pure desoxycolic acid. In many instances the order of the test was purposely reversed, the vitamin A tolerance test with the addition of bile acids was first done and the control test without bile acids followed after an interval of several days.

TABLE 1

Vitamin A Tolerance Tests in controls without and with administration of bile acids

The figures indicate in all Tables micrograms vitamin A in hundred ml. serum, those in parentheses indicate micrograms carotene in hundred ml. serum

INDIVIDUAL NO.	180,000 IU VIT. A		180,000 IU VIT. A AND 0.6 GM. DEHYDROCHOLIC ACID		180,000 IU VIT. A AND 0.6 GM. DESOXYCHOLIC ACID		180,000 IU VIT. A AND 0.6 GM. DEHYDRATED OX BILE	
	Fasting	4 h. p.c.	Fasting	4 h. p.c.	Fasting	4 h. p.c.	Fasting	4 h. p.c.
Series A								
101	88 (104)	125 (108)	123 (217)	197 (208)				
102			83 (97)	111 (99)				
103	69 (128)	99 (135)	149 (144)	230 (152)				
104	83 (127)	124 (139)	92 (120)	120 (135)				
105			90 (132)	173 (128)				
Average...	80 (120)	116 (127)	107 (142)	166 (144)				
Series B								
106	53 (65)	181 (78)						
107	96 (146)	272 (146)			104 (130)	283 (156)		
108	102 (142)	255 (155)			85 (161)	208 (184)		
109					97 (155)	167 (161)		
110	94 (145)	126 (128)			110 (170)	320 (178)		
111	91 (24)	140 (242)			58 (162)	106 (154)		
Average...	87 (148)	195 (150)			91 (156)	217 (186)		
Series C								
112					58 (155)	68 (140)	66 (156)	120 (130)
113							48 (80)	75 (80)
114					84 (140)	95 (130)	85 (165)	116 (160)
115					75 (175)	165 (185)	85 (165)	153 (150)
116	79 (150)	333 (175)			73 (165)	288 (165)	90 (150)	269 (180)
117	56 (190)	126 (155)			64 (165)	151 (175)	62 (195)	71 (190)
118	95 (265)	111 (215)			80 (260)	113 (250)	84 (250)	120 (238)
Average...	77 (202)	170 (182)			72 (177)	147 (178)	74 (166)	132 (168)

CONTROLS

Physicians, nurses, laboratory personnel, as well as convalescing patients of the medical and surgical wards served as controls. In their selection, absence of any organic disease, and in convalescing patients, normal temperature for at

least ten days were the conditio sine qua non. The results are grouped in 3 series and presented in Table 1.

Series A shows the results of administration of dehydrocholic acid. Although the average 4-hour increase of the vitamin A level is 59 microgram per cent with dehydrocholic acid and 36 microgram per cent without the latter, the resulting difference of 23 microgram per cent in favor of the dehydrocholic acid is certainly insignificant in view of the normally found great variations of the vitamin A tolerance curves in normal individuals. Series B in which the effect of desoxycholic acid on vitamin A absorption was studied shows similar results. Although the average 4-hour elevation in the tests performed with addition of desoxycholic acid is 126 microgram per cent whereas the average of the vitamin A tests without this bile acid is 108 microgram per cent, the resulting difference

TABLE 2

Average figures of all individuals whose data were presented in Table 1

TEST USED	NO. OF INDIVIDUALS	AVERAGE FASTING LEVELS	AVERAGE 4 HRS. P.C. LEVELS	ELEVATION OF VIT. A LEVEL
180,000 IU Vit. A.....	11	82.4 (155)	172 (152)	89.6
180,000 IU Vit. A & 0.6 gm. dehydrocholic acid.....	5	107 (142)	166 (144)	59
180,000 IU Vit A & 0.6 gm. desoxycholic acid.....	12	80.8 (167)	179 (180)	98.2
180,000 IU Vit. A & 0.6 gm. dehydrated ox bile.....	7	74 (166)	132 (168)	58

is hardly significant. In series C, 3 normal individuals were subjected to the control test as well as to the desoxycholic and dehydrocholic acid tests, while the other individuals of this series were only tested in 2 tests with each of the 2 bile preparations but without the control test. The average 4-hour increase of the serum vitamin A is 93 microgram per cent without the bile acids, 75 microgram per cent with the addition of desoxycholic and 58 microgram per cent with the addition of dehydrated bile. The average figures of all 18 controls of the series A to C are presented in Table 2. It is evident that none of the examined bile acids exerted a noticeable effect upon vitamin A absorption in "normal" individuals.

LIVER DISEASE

This group of patients included 4 with hepatitis (2 acute, 2 subsiding), 4 with cirrhosis of the liver, 4 with complete biliary obstruction due to carcinoma and 2 with incomplete biliary obstruction caused by stones. Each of these patients had icterus and bilirubin was present in the urine. The results

are presented in Table 3, which also includes 2 cases of active sprue which will be discussed separately. In 7 of these patients 2 tests were done, the control test and the bile acid test with 0.6 Gm. desoxycholic acid. The remaining 9 patients could be examined only once, 6 had the bile acid test, and 3 the control test only.

TABLE 3

Liver disease and sprue

Effect of desoxycholic acid on Vitamin A Tolerance Test

PATIENT NO.	DIAGNOSIS	180,000 IU VIT. A		180,000 IU VIT. A AND 0.6 GM. DESOXYCHOLIC ACID	
		Fasting	4 hrs. p.c.	Fasting	4 hrs. p.c.
201	Cirrhosis of liver	49 (85)	53 (102)	70 (67)	125 (56)
202	Acute Hepatitis	42 (74)	40 (80)	41 (87)	43 (82)
203	Cirrhosis of liver	84 (88)	123 (87)	30 (69)	143 (71)
204	Partial Biliary Obstruction (stones)	91 (151)	140 (141)	82 (162)	151 (154)
205	Complete Biliary Obstruction (Carcinoma of Pancreas)	42 (12)	39 (12)	42 (8)	41 (10)
206	Sprue	48 (32)	48 (28)	29 (32)	49 (32)
207	Cirrhosis of liver	53 (75)	66 (63)	56 (109)	89 (110)
Average		58 (74)	73 (73)	50 (76)	92 (74)
208	Subsiding Hepatitis	51 (99)	141 (102)		
209	Subsiding Hepatitis	46 (215)	173 (200)		
210	Complete Biliary Obstruction (Carcinoma of Pancreas)	60 (43)	61 (46)		
211	Partial Biliary Obstruction (stones)			73 (55)	215 (59)
212	Complete Biliary Obstruction (Carcinoma of Pancreas)			45 (65)	42 (71)
213	Sprue			61 (31)	60 (33)
214	Acute Hepatitis			46 (49)	49 (56)
215	Cirrhosis of Liver			30 (55)	137 (66)
216	Complete Biliary Obstruction (Carcinoma of Pancreas)			44 (59)	41 (51)

The average 4-hour elevation of the vitamin A concentration of the serum is 15 microgram per cent in the first 7 cases without and 42 microgram per cent with the addition of desoxycholic acid which represents a moderate difference in favor of the bile acid. Analysis of the individual cases reveals that in patients who showed complete lack of absorption as evidenced by absolutely flat curves, the addition of the bile acid was without any effect, e.g. No. 202 and 214 (acute hepatitis), No. 205, 212, 216 (carcinoma of pancreas, complete biliary obstruction). It is important to notice that the underlying pathological

process, parenchymal jaundice versus obstructive jaundice, made no difference. The addition of bile acids failed to affect absorption of vitamin A in both pathological conditions. It must be stated, however, that the 3 cases of carcinoma of the pancreas had clinical evidence of icterus for 6 to 8 weeks and that there was evidence of secondary hepatic damage.

In mild (or subsiding) hepatitis as well as in partial biliary obstruction caused by stones when some absorption of vitamin A was evident in the control tests, a moderate additional elevation was seen when desoxycholic acid was used, e.g. in No. 203, 204, and 207. It must be added that these pathological conditions are rarely static. The clinical course is associated with improvement in some or enhanced impairment in other individuals and that this change may occur during the interval between the 2 tests. Thus, in patient No. 203, the clinical course as well as the function tests were indicative of progressive liver damage which is in good agreement with the drop of the "fasting" vitamin A serum level from 84 to 30 microgram per cent and of the carotene level from 88 to 69 microgram per cent. The 4-hour elevation of the vitamin A serum level to 143 microgram per cent in the bile acid test must be compared with the corresponding elevation of 39 microgram per cent in the control test thus indicating improved absorption in the bile acid test despite progression of the disease. On the other hand, the higher response in the bile acid test of patient No. 201 might have been caused by beginning spontaneous improvement which was evident clinically and chemically and is probably responsible for the gradual elevation of the "fasting" vitamin A serum level from 49 to 70 microgram per cent. We emphasized in a previous publication that "clinical improvement is accompanied by the gradual return to normal levels of vitamin A in the blood and to normal or even abnormally high tolerance curves"[2]. Despite all these limitations of comparability of both tests which were caused by the "natural" dynamic course of the disease, a certain effect of bile acids on the post-prandial vitamin A elevation of the blood was observed but only in patients with mild forms of hepatic damage and in incomplete biliary obstruction.

In 3 patients who were studied with the 8-hour test, in which the "micro method" of vitamin A estimation was employed, similar results were obtained (Table 4A). While patient No. 217 (slowly subsiding hepatitis) shows increased post-prandial elevation of the vitamin A serum level in the bile acid test, the results in the 2 other patients are not convincing. The average elevations are: 11, 15 and 20 microgram per cent for the 4, 6, and 8 hour periods of the control tests and 20, 37, 31 microgram per cent for the corresponding periods of the bile acid tests.

The effect of lecithin administration on the abnormal vitamin A tolerance test in liver disease was studied in 2 patients (Table 4B). Both had moder-

ately severe hepatic damage and the type of vitamin A curves which usually showed moderate elevation with the addition of bile acids. In contrast, the administration of 20 Gm. of defatted soy bean lecithin had no noticeable effect. The average post-prandial elevations were 29, 35, and 51 microgram per cent for the 4, 6, and 8 hour periods of the control test, and 29, 52, and 43 microgram per cent for the corresponding periods of the lecithin tests.

TABLE 4A

Effect of Desoxycholic Acid on Vitamin A Tolerance Test in Liver Disease

NUMBER	DIAGNOSIS	180,000 IU VITAMIN A				180,000 IU VIT. A AND 0.6 GM. DESOXYCHOLIC ACID			
		Fasting	4 h. p.c.	6 h. p.c.	8 h. p.c.	Fasting	4 h. p.c.	6 h. p.c.	8 h. p.c.
217	Subsiding Hepatitis	41 (42)	59	41	49	47 (52)	106	116	86
218	Subsiding Hepatitis	59 (119)	59	84	72	78 (100)	75	89	92
219	Partial Biliary Obstruction	46 (55)	61	66	85	65 (47)	67	95	103
Average		49 (72)	60	64	69	63	83	100	94

TABLE 4B

Effect of defatted lecithin on Vitamin A Tolerance Test in Liver Disease

NUMBER	DIAGNOSIS	180,000 IU VITAMIN A				180,000 IU VIT. A AND 20 GM. DEFATTED LECITHIN			
		Fasting	4 h. p.c.	6 h. p.c.	8 h. p.c.	Fasting	4 h. p.c.	6 h. p.c	8 h. p.c.
220	Cirrhosis of Liver	36 (21)	62	71	108	29 (20)	42	73	58
221	Subsiding Hepatitis	20 (64)	51	54	49	20 (36)	66	81	77
Average		28	57	63	79	25	54	77	68

SPRUE

The addition of bile acids was studied in 2 typical cases of sprue, both characterized by low "fasting" levels of vitamin A and especially low levels of carotene in the serum (patients No. 206 and 213, Table 3). There was no post-prandial elevation after the oral administration of the vitamin A in oil even when the latter was given with 0.6 Gm. of desoxycholic acid. It may be interesting to recall that the addition of lecithin resulted in improved absorption of oil solutions of vitamin A in sprue as was demonstrated in our previous work[9].

DISCUSSION

The results of the study seem to be in agreement with the experimental observation that absence of bile in the intestinal tract does not interfere with the absorption of vitamin A[6, 7]. None of the employed bile acids (dehydrated ox bile, dehydrocholic acid, desoxycholic acid), was able to improve

vitamin A absorption in "normal" individuals. The explanation might be sought in the observation that normal functions are "optimal" per se and cannot be improved by the addition of physiological factors already present, for instance, bile acids. The observation that addition of lecithin enhanced fat and vitamin A absorption in normal individuals[9] as well as fat absorption in animals[11] is not in disagreement with this view since the emulsifying effect of lecithin probably introduced a different mechanism into the process of vitamin A absorption than that operating with bile acids.

From the clinical view-point the observation is important that in severe liver disease, characterized by completely flat vitamin A tolerance curves, the administration of bile acids was without any effect. Only in instances of milder hepatic damage or in incomplete biliary obstruction, when vitamin A absorption was impaired, but present, was a moderate acceleration of vitamin A absorption noticeable. These observations prove that the lack of absorption of vitamin A, as it is found in severe and acute hepatic disease, is not caused by lack of bile acids. In a previous publication we suspected that "certain enzymatic processes essential for intestinal absorption of vitamin A are presumably controlled by the liver and affected by hepatic disease. . . .The sudden inhibition of vitamin A absorption in hepatitis and the rapid improvement with the onset of recovery further supports the assumption of the enzymatic character of the processes involved"[2]. Harris and Moore[12] confirmed the failure of the blood to respond to massive doses of vitamin A in acute stages of hepatitis and found in addition that more than half of the vitamin A dose given was excreted in the feces. In later stages of the disease no vitamin A was found in the feces of the same subject. Nevertheless, the inhibition of intestinal absorption in acute hepatic damage represents only one phase of the disturbed vitamin A metabolism. It does not explain the low "fasting" blood levels in the presence of considerable hepatic reserves. These depots are apparently arrested in the liver and not available to the periphery (blood and other tissues). The failure to release the hepatic stores of vitamin A may be connected with the distribution of vitamin A in the lobules of the liver which changes in hepatic disease as seen with the fluorescence microscope[13].

The moderate acceleration of vitamin A absorption by bile acids in milder instances of hepatic disease and in partial biliary obstruction suggests that another mechanism may be at work in causing the diminished, but not absent, intestinal absorption of vitamin A. The evaluation of milder effects upon vitamin A metabolism such as those produced by bile acid administration, is rendered difficult by the natural dynamic course of the disease with spontaneous improvement or impairment of hepatic function.

That the mechanism of disturbed vitamin A absorption in liver disease is not identical with that encountered in sprue may be deduced from the experi-

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ments performed with lecithin administration. Addition of lecithin failed to influence the defect of vitamin A absorption in severe hepatic damage although it did promote vitamin A absorption in active sprue. On the other hand addition of bile acids had no effect on vitamin A absorption in active sprue, while, at least in a few milder instances, it exerted a moderate influence upon the defect of absorption in hepatic disease. Additional work is necessary further to separate, characterize and elucidate the basic defects of absorption in liver disease and in sprue. In recent studies Kramer, Sobel and Gottfried observed improved intestinal absorption of vitamin A when used in aqueous dispersions, in children with celiac disease[14]. In chicks the use of aqueous emulsions of vitamin A resulted in better growth rates[15].

SUMMARY

(1). In normal individuals oral administration of bile acids, dehydrated ox bile, dehydrocholic acid and desoxycholic acid, exerted no noticeable effect upon the serum levels of vitamin A after the administration of standard doses of vitamin A in oil (Vitamin A tolerance test).

(2). In severe hepatic damage or complete biliary obstruction, characterized by "flat curves" in the vitamin A tolerance test, bile acids failed to alter the shape of the curves. In milder forms of hepatic disease or in incomplete biliary obstruction, slight effects of bile acids on the shape of the curves were observed indicating moderate acceleration of intestinal absorption of vitamin A.

(3). Lecithin failed to affect the "flat curves" of vitamin A encountered in severe hepatic damage, while it did promote intestinal absorption of vitamin A in sprue.

(4). These observations suggest that the mechanism of defective absorption in liver disease is not identical with that encountered in sprue.

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0098

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

Food and Drug Administration

[21 CFR Part 19]

PASTEURIZED PROCESS CHEESE, PASTEURIZED PROCESS CHEESE FOOD, AND PASTEURIZED PROCESS CHEESE SPREAD

Proposed Use of Lecithin as an Optional Anti-Sticking Agent

Notice is given that a petition has been filed by the National Cheese Institute, Inc., 110 North Franklin St., Chicago, IL 60606, proposing an amendment to the standards of identity for pasteurized process cheese (21 CFR 19.750), pasteurized process cheese food (21 CFR 19.765), and pasteurized process cheese spread (21 CFR 19.775) to allow optional addition of lecithin for the functional purpose of aiding in the separation of slices of the product in the same package and in the removal of the wrapper from such products.

Grounds set forth by the petitioner in support of the proposal are as follows:

1. Lecithin, besides being a normal constituent in milk and milk products, has been generally recognized as safe by the Food and Drug Administration under 21 CFR 121.101(d)(8), "Miscellaneous and/or General Purpose Food Additives," without tolerance or limitation.

2. Consumers frequently find it difficult to separate slices of the various processed cheese products; in addition, consumers experience the problem of such products sticking to wrapping material.

3. The addition of 0.03 percent of lecithin to the various pasteurized process cheese products reduces the tendency of the slices to stick together and to the wrapping material.

4. Consumers who prefer nonindividually wrapped slices will benefit by the availability of stacked slices of processed cheese products with improved separation characteristics. Also, a reduction in the use of wrapping material for individual slices may have an ecological benefit.

5. Based on a temporary marketing permit authorized by the Food and Drug Administration, notice of which was published in the FEDERAL REGISTER of October 14, 1971 (36 FR 19995), it was determined by the petitioner that the addition of lecithin to process cheese products was effective and desirable when utilized in the amount considered in this proposed amendment.

Therefore, pursuant to provisions of the Federal Food, Drug, and Cosmetic Act (secs. 401, 701, 52 Stat. 1046, 1055, as amended by 70 Stat. 919 and 72 Stat. 948; 21 U.S.C. 341, 371) and under authority delegated to the Commissioner of Food and Drugs (21 CFR 2.120), it is proposed that Part 19 be amended:

1. In § 19.750(d) by adding a new subparagraph (8) as follows:

§ 19.750 Pasteurized process cheese; identity; label statement of optional ingredients.

* * * * *

(d) * * *

(8) Pasteurized process cheese in the form of slices or cuts in consumer-sized packages may contain lecithin as an optional anti-sticking agent in an amount not to exceed 0.03 percent by weight of the finished product.

* * * * *

2. In § 19.765(e) by adding a new subparagraph (8) as follows:

§ 19.765 Pasteurized process cheese food; identity; label statement of optional ingredients.

* * * * *

(e) * * *

(8) Pasteurized process cheese food in the form of slices or cuts in consumer-sized packages may contain lecithin as an optional anti-sticking agent in an amount not to exceed 0.03 percent by weight of the finished product.

* * * * *

3. In § 19.775(f) by adding a new subparagraph (9) as follows:

§ 19.775 Pasteurized process cheese spread; identity; label statement of optional ingredients.

* * * * *

(f) * * *

(9) Pasteurized process cheese spread in consumer-sized packages may contain lecithin as an optional anti-sticking agent in an amount not exceed 0.03 percent by weight of the finished product.

* * * * *

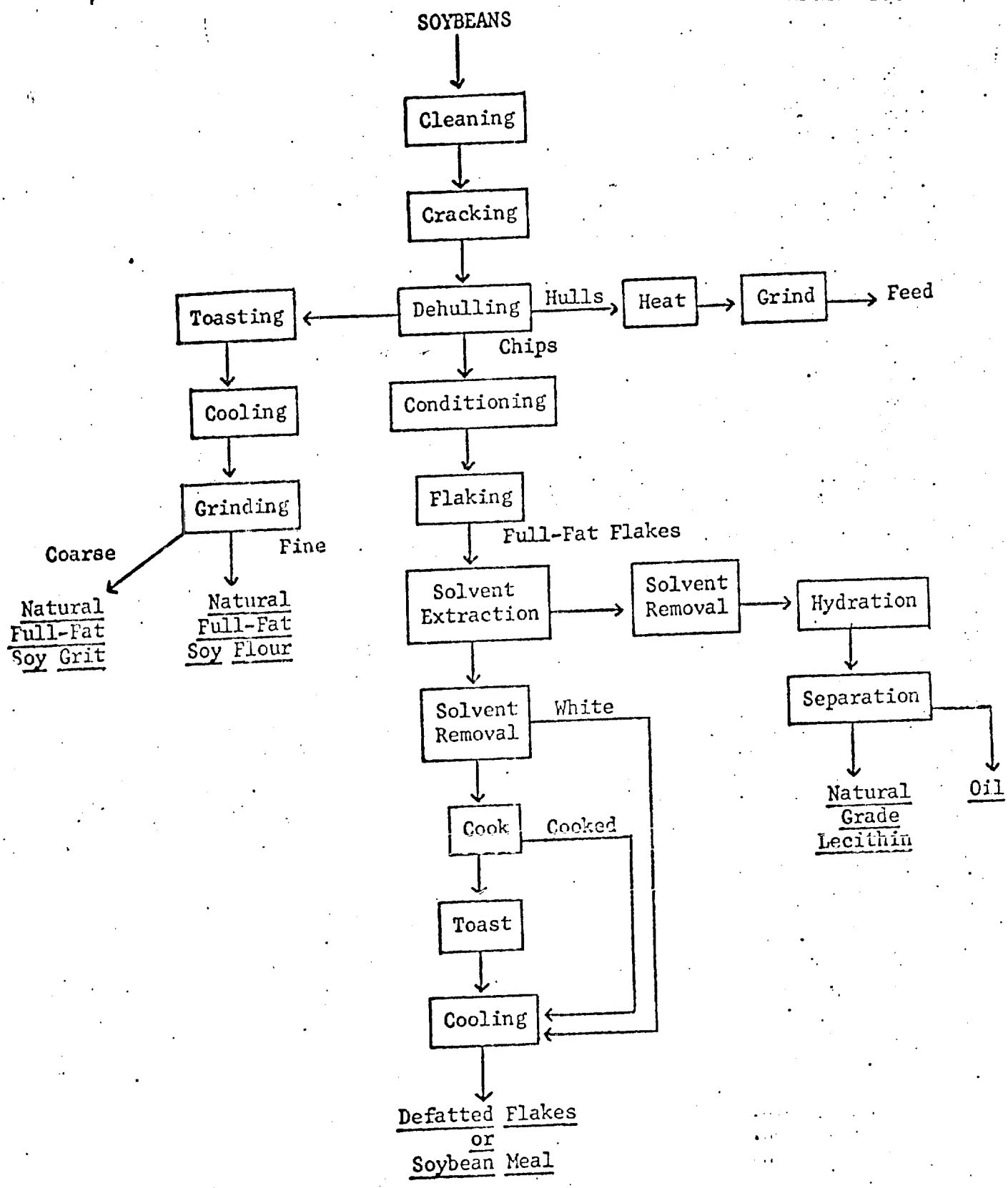
This proposal makes no reference to the label declaration required when lecithin is used; it is not necessary because recent amendments published in the FEDERAL REGISTER of July 7, 1972 (37 FR 13339), and April 23, 1973 (38 FR 9996), require that the common name of each ingredient used shall be declared on the labels of the cheese products covered by this proposed amendment.

Interested persons may, on or before October 19, 1973, file with the Hearing Clerk, Food and Drug Administration, Rm. 6-88, 5600 Fishers Lane, Rockville, MD 20852, written comments (preferably in quintuplicate) regarding this proposal. Comments may be accompanied by a memorandum or brief in support thereof. Received comments may be seen in the above office during working hours, Monday through Friday.

Dated: August 10, 1973.

VIRGIL O. WODICKA,
Director, Bureau of Foods.

[FR Doc.73-17278 Filed 8-17-73;8:45 am]



SOYBEAN PROCESSING

Figure 1

13370

Exhibit 9

HYDROXYLECITHIN SPECIFICATIONS

PROPERTY	LIMITS	METHODS
Acetone Insolubles	60.0% minimum	Meth No Fa 13.0 ref: AOCS No Ja 4-46
Moisture	2.0% maximum	Meth No Fa 1.2 ref: AOCS No Ja 2-46
Benzene Insolubles	1.0% maximum	Meth No Fa 14.0 ref: AOCS No Ja 3-55
Acid Value	25 - 30	Meth No Fa 17.0 ref: AOCS No Ja 6-55
Iodine Value	90 maximum	Meth No Fa 3.0 ref: AOCS No Cd 1-25
Color (Lovibond)	5y/0.5r maximum	Meth No Fa 8.0 ref: AOCS No Cc 13b-45

§ 121.1099 Defoaming agents may be safely used in processing foods, in accordance with the following conditions:

(a) They consist of one or more of the following:

(1) Substances generally recognized by qualified experts as safe in food or covered by prior sanctions for the use prescribed by this section.

(2) Substances listed in this subparagraph, subject to any limitations imposed:

Substances	Limitations
Dimethylpolysiloxane (substantially free from hydrolyzable chloride and alkoxy groups; no more than 18 percent loss in weight after heating 4 hours at 200° C.; viscosity 300-600 centistokes at 25° C.; refractive index 1.400-1.404 at 25° C.).	10 parts per million in food, except zero in milk; 110 parts per million in dry gelatin dessert mixes labeled for use whereby no more than 16 parts per million is present in the ready to serve dessert; 250 parts per million in salt labeled for cooking purposes, whereby no more than 10 parts per million is present in the cooked food.
Formaldehyde	As a preservative in defoaming agents containing dimethylpolysiloxane, in an amount not exceeding 1.0 percent of the dimethylpolysiloxane content.
Polyethylene glycol	As defined in § 121.1185.
Polyoxyethylene 40 monostearate	As defined in U. S. P. XVI.
Polyoxyethylene (20) sorbitan monostearate.	As defined in § 121.1030.
Polyoxyethylene (20) sorbitan tristearate.	As defined in § 121.1008.
Propylene glycol alginate	As defined in § 121.1015.
Silicon dioxide	As defined in § 121.1058.
Sorbitan monostearate	As defined in § 121.1029.

(3) Substances listed in this subparagraph, provided they are components of defoaming agents limited to use in processing beet sugar and yeast, and subject to any limitations imposed:

Substances	Limitations
Aluminum stearate	As defined in § 121.1071.
Butyl stearate	.....
BHA	As an antioxidant, not to exceed 0.1 percent by weight of defoamer.
BHT	As an antioxidant, not to exceed 0.1 percent by weight of defoamer.
Calcium stearate	As defined in § 121.1071.
Fatty acids	As defined in § 121.1070.
Formaldehyde	As a preservative.
Hydroxylated lecithin	As defined in § 121.1027.
Isopropyl alcohol	.....
Magnesium stearate	As defined in § 121.1071.
Mineral oil: Conforming with § 121.1146.	Not more than 150 p.p.m. in yeast, measured as hydrocarbons.
Odorless light petroleum hydrocarbons: Conforming with § 121.1182.	
Petrolatum: Conforming with § 121.1166.	
Petroleum wax: Conforming with § 121.1156.	
Synthetic isoparaffinic petroleum hydrocarbons: Conforming with § 121.1154.	
Oxystearin	As defined in § 121.1016.
Polyoxyethylene (600) dioleate	.....
Polyoxyethylene (600) monoricinoleate	.....
Polypropylene glycol	Molecular weight range, 1,200-2,500.
Polysorbate 80	As defined in § 121.1009.
Potassium stearate	As defined in § 121.1071.
Propylene glycol mono- and di-esters of fats and fatty acids.	As defined in § 121.1113.
Soybean oil fatty acids, hydroxylated	.....
Tallow, hydrogenated, oxidized or sulfated	.....
Tallow alcohol, hydrogenated	.....

[¶ 55,927] Hydroxylated lecithin

§ 121.1027 The food additive hydroxylated lecithin may be safely used as an emulsifier in foods in accordance with the following conditions:

(a) The additive is obtained by the treatment of lecithin in one of the following ways, under controlled conditions whereby the separated fatty acid fraction of the resultant product has an acetyl value of 30 to 38:

(1) With hydrogen peroxide, benzoyl peroxide, lactic acid, and sodium hydroxide.

(2) With hydrogen peroxide, acetic acid, and sodium hydroxide.

(b) It is used or intended for use, in accordance with good manufacturing practice, as an emulsifier in foods, except for those standardized foods that do not provide for such use.

(c) To assure safe use of the additive, the label of the food additive container shall bear, in addition to the other information required by the act:

(1) The name of the additive, "hydroxylated lecithin."

(2) Adequate directions for its use.

Ansell, G.B., J.N. Hawthorne and R.M.C. Dawson
1973
Form and Function of Phospholipids
Elsevier Scientific Publishing Company, New York
pp. 14; 284

Proc Soc. Exp Biol. Med. 61: 13-15; 1946

15210

The Effect of Lecithin Administered Intravenously.

GERALD K. ASHBY. (Introduced by Warren M. Cox, Jr.)

From the Department of Nutritional Research, Mead Johnson & Co., Evansville, Ind.

It has been reported that various lecithins when administered by vein cause hemolysis,[1,2,3] leukocytosis,[4] changes in the fragility of the blood cellular components,[4,5,6] or death.[7] On the other hand, one investigator reports that regardless of the method of administration lecithin alone exerts no toxic action.[8] Because of the current interest in fat emulsions for parenteral use,[9,10,11] a brief study of the effect of lecithin when administered by vein has been made.

Experimental. Each sample of lecithin was prepared as a 7.0% colloidal solution in pyrogen-free distilled water and autoclaved at 10 pounds pressure for 15 minutes. The sterile solutions were rapidly injected into the marginal ear veins of normal rabbits in amounts sufficient to supply 1.75 g of lecithin per kilogram body weight. Table I shows the essential data obtained with 13 samples of lecithin. All but one of the injected rabbits died within 12 hours. These results strongly suggest that none of the tested lecithins is suitable for making fat emulsions for intravenous use.

Various methods of isolating lecithin from eggs were investigated, but the methods generally used for this purpose were not entirely successful in our hands.[12,13] The method finally adopted consisted of exhaustive extraction of powdered egg yolk with acetone to remove the fat, subsequent extraction with alcohol to obtain the crude lecithin, distillation of the alcohol under high vacuum, solution of the residue in ether and final precipitation of the lecithin with acetone. The precipitated lecithin was redissolved in ether, filtered and reprecipitated with acetone several times. Final traces of solvent were removed under high vacuum at low temperature. Yields by this method were 11% of the original weight of the egg powder.

Lecithin prepared in this manner and stored under high vacuum for varying lengths of time up to 228 days was injected into 14 normal rabbits without any apparent ill effects (Table II). The level of 1.75 g per kilo body weight was increased to 3.5 g per kilo and again without any ill effects, but 7.0 g proved to be fatal.

Samples of egg lecithin prepared and tested as above were then exposed to the air at room temperature and at 55°C for varying periods of time to determine if toxic properties would develop. As indicated in Table II, toxic properties did develop in as little as 14 days at 55°C and 47 days at room temperature. The toxicity which developed in 53 days at 55°C was profound, since both rabbits died in exactly 4 minutes after the infusions were begun. It was thought that perhaps this toxicity might be due to lysolecithin but efforts to purify the exposed sample from toxic substances by the differential solubility of lecithin

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TABLE I.
Lecithins from Various Sources.

Type of lecithin	Source*	Label description	Color	Consistency	Color of solution	No. rabbits injected	Lecithin g/kg/BW	Results
Egg	1	about 90%	dk. red	soft wax	dk. cream	2	1.75	Died in 4 and 105 minutes
,,	2	—	,, ,,	,, ,,	,, ,,	1	1.75	Died during night
,,	3	pure	red. bk.	,, ,,	brown	1	1.75	Died in 22 minutes
,,	4	practical	brown	,, ,,	,,	1	1.75	Died in 30 minutes
,,	5	pure	dk. brown	,, ,,	,,	1	1.22	Died during injection
Soybean	6	fat free phosphatides	white	granular	white	5	1.75	Died within 8 hrs†
,,	6	commercial	amber	oily wax	,,	1	1.75	Died during night
,,	6	edible	lt. amber	heavy oil	,,	1	1.75	,, ,, ,,
,,	6	,,	dk. amber	,, ,,	cream	1	1.75	Died in 4 hours
Animal	5	90%	brown	wax	brown	1	1.75	Died during night
Vegetable	5	60% or more	lt. amber	heavy oil	white	1	1.75	,, ,, ,,
,,	5	60% ,, ,,	dk. amber	,, ,,	cream	1	1.75	,, ,, ,,
,,	5	app. 75%	lt. amber	hard wax	white	1	1.75	Complete paralysis recovered in 1 wk

* (1) Merck & Co.; kindly supplied by Dr. L. Emmett Holt, Jr.; (2) Difco Laboratories, Inc.; (3) Coleman and Bell Company; (4) Eastman Kodak Co.; (5) Pfanstiehl Chemical Company; (6) American Lecithin Company; kindly supplied for research by Dr. A. Scharf.
† One died in 7 min.

TABLE II.
Experimental Lecithin Preparations.

Method of preparation or treatment	Color of lecithin	Color of solution	No. rabbits injected	Lecithin g/kg/BW	Results
Acetone ppt'd from ether solution	amber	white	14*	1.75	No apparent ill effects
,, ,, ,, ,, ,,	,,	cream	1	3.5‡	,, ,, ,, ,,
,, ,, ,, ,, ,,	,,	,,	1	7.0‡	Died during night
Exposed to air at room temp. 47 days	dk. red	brown	2	1.75	One died during the night; one no apparent ill effects
,, ,, ,, ,, ,, ,, 86 ,,	brown	,,	2	1.75	,, ,, ,, ,, ,, ,, ,, ,, ,, ,,
,, ,, ,, at 55°C 14 days	dk. red	,,	2	1.75	One no apparent ill effects; one died during week-end, 72-120 hours
,, ,, ,, ,, ,, 53 ,,	black	dk. brown	2	0.625	Both died 4 min. after start of inj.
Cadmium chloride pptn.[12]	amber	white	1	1.75	Died during night
Cadmium chloride pptn.†	,,	,,	1	1.75	No apparent ill effects

* Several of these rabbits were injected 2 or more times.
† Cadmium removed by pptn. with H_2S.
‡ In 14% solution.

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and lysolecithin in ether[14] were unsuccessful.

Chemically speaking, the purest lecithin has been prepared through the cadmium salt.[12] This procedure did give a very light-colored and attractive preparation, but a fat emulsion prepared with it was promptly fatal to a dog and the lecithin itself was fatal to a rabbit (Table II). The toxicity of lecithin prepared in this manner was found to be due to traces of cadmium which could be removed by treatment with hydrogen sulphide.

Summary. Thirteen samples of commercial lecithin were fatal to rabbits when administered intravenously at a level of 1.75 g per kilo body weight.

A method for preparing lecithin from egg yolk is described and it is shown that lecithin so prepared is harmless to rabbits when given by vein even at a level of 3.5 g per kilo body weight. Lecithin so prepared will acquire toxic properties if exposed to the air. Elevated temperatures accelerate this process. If the lecithin is stored under high vacuum, toxic properties will not develop within at least 228 days.

Egg lecithin prepared through the cadmium salt may contain sufficient quantities of cadmium to produce cadmium poisoning.

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J. Nutr. 35(2): 177-186, 1947

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THE EFFECT OF CRUDE LECITHIN ON THE COEFFICIENT OF DIGESTIBILITY AND THE RATE OF ABSORPTION OF FAT [1, 2]

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(Received for publication September 24, 1946)

It has been recognized for years that lecithin plays an important role in fat metabolism. Lecithin is an important component of the lipid fraction of blood. It is believed that when the neutral fat is transferred from the storage depot it is first converted to a water-soluble form by phosphorylation in situ and that it is carried then in the form of lecithin to the liver. Considerable amounts of lecithin are found in the liver. For these reasons it has also been suggested that lecithin may be a form into which the fat must be transformed before it can be oxidized. Verzár and Laszt ('35a, '35b) have suggested that phosphorylation of fats must occur prior to their absorption. On removal of the adrenal cortex, it was suggested that fat absorption is slowed down because of the failure of phosphorylation. The experiments of Bavetta et al. ('41) confirm the results of Verzár and Laszt in demonstrating that a decreased absorption obtains after adrenalectomy in the rat. It was also found that this effect was due to a slowing in the absorption of fatty acids inasmuch as an accumulation of the hydrolysis products of the neutral fat occurred in the

[1] Most of these data are from a thesis presented by Virginia Augur to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Master of Science.

[2] Aided by a grant from the American Lecithin Co.

intestines. However, they gave no indication as to whether the depressing action was due to a failure of phosphorylation.

Frazer ('46) has given much evidence that fats may be absorbed to a considerable extent as neutral fat provided that a fine enough emulsion is produced. If this so-called partition theory of Frazer is correct, then one might expect that any agent such as lecithin which promotes the emulsification might be of aid in speeding up the absorption of fat and in increasing the digestibility of the less readily utilized fats. If, on the other hand, the lipolytic theory is correct, then one might also postulate that the presence of lecithin would increase the speed of absorption by providing the means for producing a greater emulsification which would enable a more rapid action of the lipolytic enzymes.

The results of Adlersberg and Sobotka ('43a) have indicated that both fat and vitamin A are absorbed more rapidly in man when lecithin is added to the diet. The rise in total lipids of the serum on a protein-free diet without lecithin was only 32% after 4 hours, compared with 71% in subjects on a similar diet who received lecithin along with the food. When 120,000 or 180,000 International Units (I.U.) of vitamin A were given as a component of percomorph oil, there was a rise in vitamin A content of blood plasma of only 41% when given without lecithin or an average of 212% increase if given along with lecithin. In addition, lecithin has an effect on the level of blood cholesterol. It was demonstrated by Adlersberg and Sobotka ('43b) that the prolonged administration of lecithin brought about a slight decrease in serum cholesterol, although it returned to high levels after the administration of lecithin was stopped. Similar results are recorded by Steiner and Domanski ('44), although the latter investigators found that the high levels of blood cholesterol returned after 4 or 5 weeks in spite of continued lecithin feeding. Urbach ('46), however, was able to maintain persistently low levels of blood cholesterol when a diet low in carbohydrate and fat was given along with the lecithin. The results of Adlersberg were also confirmed by Gross and Kesten ('43). Slanetz and Scharf

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('43, '44) have reported that some unknown factor is necessary for the utilization of vitamin A in the rat which is present in crude lecithin and which is not vitamin E.

The total availability of the fat (i.e., the coefficient of digestibility) as well as the rate of absorption are both important in evaluating the nutritive value of fat. Although the coefficient of digestibility of poorly utilized fats and their rate of absorption are roughly parallel, differences in rate of absorption may occur in fats which are practically completely digestible. Whereas the coefficient of digestibility may be obtained by calculation from the fat ingested and that excreted in the feces, the rate of absorption can only be determined by short term tests where the actual speed of removal of the lipid from the small intestine is ascertained.

Because most of the work which has been done on the effect of lecithin on the absorption of fat has been indirect rather than direct, the present experiments were undertaken to determine whether an increased rate of absorption occurs with fats when mixed with lecithin and also whether any improved digestibility of fats obtains when additional lecithin is added.

EXPERIMENTAL

The effect of lecithin on digestibility was demonstrated by feeding rats on diets containing approximately 15% fats of high melting point with or without added lecithin. The animals were allowed a 2-day period for orientation and the feces were then collected for the following 8 days. Food consumption was recorded during this period and the diets were allowed ad libitum. At the conclusion of the digestibility experiment, the animals were fasted for 2 days after which the effect of lecithin on the rate of absorption was determined for 3- and 6-hour periods. The same fats fed on the digestibility experiments were used in the absorption tests. The diets employed are recorded in table 1.

The combined feces of the 8-day period were dried under a vacuum to constant weight, ground with a mortar and pestle and the neutral fat extracted on a Soxhlet extractor with

diethyl ether. The residue was again dried, mixed with a minimum amount of 50% sulfuric acid and the acid paste reextracted with ether. The neutral fat and fatty acid obtained were dried under a vacuum at 60°C. to constant weight. It was demonstrated that no acid could be extracted by diethyl ether from sulfuric acid solution and it was also found that the material extracted from dried feces after acidification was acid in reaction and possessed the properties of a fatty acid.

TABLE 1

Composition of diets used in digestibility studies.

COMPONENTS OF DIET	DIET 1	DIET 2	DIET 3
	%	%	%
Commercial casein	18	18	18
Glucose	56	53	71
Hydrogenated cottonseed oil	15	15	0
Salt mixture [1]	7	7	7
Yeast [2]	1	1	1
Liver extract [3]	3	3	3
Crude Soya Lecithin [4]	0	3	0

[1] Osborne-Mendel ('19).

[2] Anheuser-Busch, Strain G.

[3] Wilson and Co., 1 : 20 concentrate.

[4] Prepared by The American Lecithin Co.

The calculations for digestibility were made in the usual way, correction being made for the metabolic fat. The correction for metabolic fat was determined in experiments where the animals were fed on Diet 3 (table 1) in which the fat has been replaced by glucose. The 10 rats used in this determination weighed on an average. 209 gm, consumed a total of 94.5 gm of food, and excreted stools which had an average dried weight of 5.35 gm. The feces contained a total of 181 mg of fat as neutral fat, 87.5 mg as soap which makes a total of 268 mg or 50.5 mg per gm of dried stool. The latter figure was used in the subsequent calculations. The coefficient of digestibility was expressed as the per cent of the ingested fat which had been utilized. The amount of digested fat was

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determined by subtracting the corrected excretion value from the original weight of ingested fat.

The absorption tests were carried out by the usual procedure as described in the paper of Deuel, Hallman and Leonard ('40). The fat was fed at a level of 300 mg per 100 cm² of body surface based on fasted weight. Surface area was estimated by the formula of Lee ('29). In some preliminary experiments where the effect of lecithin on preventing diarrhea was determined after giving large doses of cottonseed oil, the fats were fed at approximately 1.5 or 2 times the usual level. The experiments were carried out on a commercial sample of cottonseed oil [3] and on 3 samples of cottonseed oil hydrogenated to melting points of 46°, 54°, and 65°C.[4] The crude lecithin was added in the absorption and diarrhea tests in proportions of one-sixth or one-fifth, respectively. According to the manufacturer, the crude lecithin contains 30 to 40% of soybean oil and about 60 to 70% of mixed phosphatides of lecithin, cephalin and lipositol. Adult female rats were used throughout the experiment except in one series of tests on susceptibility to diarrhea and were from the University of Southern California colony.

RESULTS

In a series of preliminary experiments, tests were made to determine whether a greater amount of fat could be digested without the onset of diarrhea if lecithin was added to the diet. A summary of these experiments is recorded in table 2.

When either cottonseed oil or hydrogenated cottonseed oil was fed at several levels, the number of rats which developed diarrhea was invariably higher in the group where no lecithin was added. In a grand total of 50 experiments where fat was fed without lecithin, a total number of 20 cases of diarrhea were noted which is an incidence of 40%. Only 4 rats of the 40 which received lecithin with the fat developed a diarrhea.

[3] Wesson Oil.

[4] These fats were kindly prepared for us by Dr. K. Mattil of Swift and Co.

TABLE 2

The incidence of diarrhea in rats fed several fats with or without added lecithin after a previous 48-hour fast period.

FAT FED	LENGTH OF PERIOD	FAT ALONE				FAT PLUS LECITHIN			
		Sex	Dose	No. of tests	No. with diarrhea	Sex	Dose	No. of tests	No. with diarrhea
	hours		*mg/100 cm²*				*mg/100 cm²*		
Cottonseed oil	3	F	328	3	1				
	3	F	410	4	3	F	410	7	0
	2	F	430	23	5	F	410	16	1
	2	M	615	10	4	M	615	10	2
Hydrogenated cottonseed oil	6	F	337	10	7	F	374	7	1

TABLE 3

Summary table of digestibility of 3 samples of cottonseed oil hydrogenated to different degrees when fed in a diet without lecithin (Diet 1) or with lecithin (Diet 2).

DATA SUMMARIZED	FAT 1 (46°C.)		FAT 2 (54°C.)		FAT 3 (65°C.)	
	Diet 1	Diet 2	Diet 1	Diet 2	Diet 1	Diet 2
Number of rats	10	10	9	10	10	10
Average weight of rats, gm	191	157	173	181	241	243
Average gain, gm	6.5	12.3	10.3	8.0	0.9	—1.4
Fat in diet, %	16.6	20.2	15.7	20.7	17.6	18.4
Average food eaten, gm	88	86	82	83	90	91
Average fat ingested, gm	14.9	17.5	12.3	17.2	15.7	16.6
Average weight of dried stools, gm	7.8	6.7	9.1	8.5	17.7	15.2
Average fat excreted						
As neutral fat and fatty acid, gm	0.73	0.62	0.62	0.79	6.31	4.83
As soaps, gm	2.09	1.86	3.80	2.63	6.93	5.33
Total, corrected [1]	2.39	2.09	3.92	2.95	12.25	9.32
Coefficient of digestibility [2]	83.8 ± 1.4	87.9 ± 0.7	68.7 ± 2.7	82.8 ± 1.4	24.0 ± 2.6	44.2 ± 2.0
M.D. : S.E.M.D.[3]		2.66		4.71		6.05

[1] Corrected for metabolic fat by multiplying weight of dried stools by 50.5 mg.

[2] Including the standard error of the mean calculated by the formula $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[3] Mean Difference : Standard Error of Mean Difference of results on Diet 1 compared with those on Diet 2. When this value exceeds 3, the results are considered significant.

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which equals only 10%. In the cottonseed oil tests, the experiments with and without lecithin were carried out simultaneously. In the case of the hydrogenated cottonseed oil, the experiments on lecithin were carried out several days later on the 7 rats which had developed diarrhea when cottonseed oil alone was fed. The data on the digestibility of various samples of cottonseed oil are summarized in table 3.

The addition of lecithin considerably increased the digestibility of the cottonseed oil, particularly in the case of the

TABLE 4

Summary table of absorption rates of cottonseed oil, of 2 samples of hydrogenated cottonseed oil when fed (A) without or (B) with lecithin.

FAT (A) WITHOUT OR (B) WITH LECITHIN	NO. OF TESTS	DURATION	AVERAGE RAT WEIGHT	AVERAGE FAT IN MG				M.D. : S.E.M.D.[3]
				Fed	Recovered[1]	Absorbed		
						Total	Per 100 cm² per hr.[2]	
				Cottonseed oil				
		hrs.	*gm.*					
A	11	2	135	911	693	238	47.8 ± 2.3	
B	14	2	132	908	635	292	61.8 ± 3.7	3.21
A	6	3	112	705	488	237	38.5	
B	8	3	121	835	460	395	59.9	
				Hydrogenated cottonseed oil (m.p. 46°C.)				
A	10	3	200	864	601	293	26.5 ± 1.8	
B	10	3	204	925	476	479	48.9 ± 3.0	3.56
A	11	6	169	775	403	402	24.7 ± 2.0	
B	9	3	207	946	323	643	36.7 ± 2.2	4.12
				Hydrogenated cottonseed oil (m.p. 54°C.)				
A	9	3	197	822	674	178	18.0 ± 1.8	
B	8	3	189	837	587	280	30.1 ± 2.8	3.58
A	9	6	197	836	709	157	8.5 ± 0.9	
B	9	6	198	870	519	381	21.8 ± 1.5	7.65

[1] Does not include the correction for lipid content of fasting rats. The value used for the cottonseed oil tests was 20 mg, and for the hydrogenated cottonseed oil tests, 30 mg.

[2] Including the standard error of the mean calculated by the formula $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[3] Mean Difference : Standard Error of Mean Difference of results on "A" compared with "B." When this value exceeds 3, the results are considered significant.

highest melting sample. The low digestibility of the fat melting at 63°C. is not to be ascribed to a failure of lipolysis but to the inability to absorb the stearate so formed. We have no evidence from our tests on how large a proportion of the material which was ether-soluble before acidification was neutral fat and how much represented the free fatty acid. The total stool weight was slightly lower in all cases with the lecithin-fed rats.

The results of the absorption tests with cottonseed and hydrogenated cottonseed oils are recorded in table 4.

DISCUSSION

There would seem to be little doubt that the addition of a crude lecithin preparation to fat lessens the susceptibility to diarrhea in rats. This effect might be the resultant of a depressing action of some components of the crude lecithin on gastric or intestinal motility or secondly because it causes a more rapid rate of absorption whereby the quantity of fat present in the gut is sufficiently reduced to lessen the chance of an ensuing diarrhea.

The present experiments indicate that the above effect is probably to be traced to an acceleration in the absorption rate. In the case of cottonseed oil the rate of increase in absorption is 29% at 2 hours and 56% at 3 hours. With the hydrogenated fat melting at 46°C., the results are equally striking, being 85 and 49% at 3 and 6 hours, respectively. The increased absorption rates for the higher melting fat (54°C.) containing lecithin are 67 and 156%, respectively, for the corresponding periods.

There are several possible explanations as to why crude lecithin may increase the absorption rate. In the first place, it might be suggested that lecithin is preferentially absorbed from the fat-lecithin mixture at a faster rate than the balance of the fat. It is difficult to see how this could occur from the lecithin-fat mixtures which are entirely homogenous. Moreover, if one assumes a 100% absorption of the lecithin in all 4 series of tests with the hydrogenated cottonseed oil samples,

there still would be an additional absorption of the neutral fat in the lecithin groups over those experiments where lecithin was not administered. In the cottonseed oil tests, the increase is approximately equivalent to the lecithin fed.

Secondly, it may be argued that the lecithin can not be recovered from the gut by the flushing procedure with diethyl ether. In tests where the recovery of known amounts from the gastro-intestinal tract was tested by removing the gut immediately after the fat had been discharged into it, it was found that the average recovery after cottonseed oil was 95.4 and after cottonseed oil-lecithin mixture, 92.9%.

Thirdly, it is possible that lecithin promotes absorption by increasing the speed and degree of emulsification. Such an effect could be explained either by an increased rate of absorption of unhydrolyzed triglyceride due to increased emulsification (according to the Partition Hypothesis of Frazer), or that an increased speed of the action of the lipolytic enzymes is possible because of the greater surface for their action.

The action of lecithin in increasing digestibility must be related to its speeding up of absorption. With the large amount of fat available at one time in the intestine, it is impossible for it to be removed from the gut at the very slow rate that absorption normally obtains before it has passed beyond the absorptive portion of the gastro-intestinal tract. Anything which will increase the rate of absorption during the interval that it is in the area where it can be absorbed will obviously improve the digestibility.

SUMMARY

The addition of lecithin to cottonseed oil or to a hydrogenated cottonseed oil markedly lowers the susceptibility to diarrhea caused by a large dose of these fats to rats.

Fats containing one-sixth or one-fifth crude lecithin are absorbed more rapidly than a similar fat without any added phosphatide.

It was found that hydrogenated cottonseed oil melting at 63°C. had a digestibility of 24 in the rat; that melting at

54°C. was digested to the extent of 69% while that with a melting point of 46°C. had a digestibility coefficient of 84. These were increased by the addition of lecithin to 44, 83 and 88%, respectively.

A considerably larger portion of the lipid in all cases was excreted as soaps than as neutral fat plus fatty acids.

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Lecithin in Food Processing

Part 1

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Until comparatively recent times the complex phosphorus-containing fats grouped together under the term lipins—which include the important group of lecithins—were of interest primarily to those concerned with human physiology and cellular metabolism. Some industrial applications were proposed at an early date but the extensive use of lecithin in the food and other industries is essentially a development of the past 20 years, following the production of lecithin as a by-product of soya bean processing. The earlier (and more expensive) commercial source was egg yolk. In view of the current shortages of fats, lecithin may find important applications in bakery products.

LECITHIN has been known for nearly a hundred years as a major constituent of egg yolk. An egg weighing 60 g. contains approximately 20 g. of yolk of which about 4 g. is neutral fat (tri-glyceride) and 2 g. lecithin and allied substances. Lecithin has been found associated with glycerides in all plants and animals and in micro-organisms, and appears to play an important part in the structure of living cells as well as in their physico-chemical and biochemical activities.

The tri-glycerides are soluble in acetone, in contrast to the phosphorus-containing fats which are insoluble in this solvent and which can therefore be prepared in the laboratory by treatment of the ethereal extract of a tissue with excess of acetone.

Lecithin may be regarded as a complex tri-glyceride in which one of the fatty acids has been replaced by a phosphoric acid and choline compound. The relationship may be summarised as follows:

G—Fatty Acid, Fatty Acid, Fatty Acid *Tri-glyceride*	G—Fatty Acid, Fatty Acid, OH group *Di-glyceride*
G—Fatty Acid, Fatty Acid, Phosphoric Acid *Phosphatidic Acid*	G—Fatty Acid, Fatty Acid, Phosphoric Acid—Choline *Lecithin or Phosphatidyl Choline*

The detailed structure of lecithin is given in Figure 1(a), which shows the four major components of the molecule, namely glycerol, fatty acids, phosphoric acid, and choline (hydroxy-ethyl tri-methyl ammonium hydroxide), all of which can be obtained under suitable conditions by acid hydrolysis. Lecithin is not to be regarded as the name of a single substance, but rather of a whole group of allied compounds, which may differ (i) in the nature of their fatty acids; (ii) in the site of attachment of the phosphoric acid to the glycerol framework (α or β); (iii) in optically active forms of the α compound, and (iv) in the structure of the phosphoryl choline moiety, which may be formulated as shown in Figure 1(a) or alternatively as a *Zwitter or dipolar ion* or as a betaine (internal salt).

Formula 1(b) is similar to 1(a), but emphasises different aspects of the molecule, in particular the presence of the di-glyceride portion which would be expected to be soluble in fat solvents and the phosphoryl-choline portion which would be expected to be water soluble and which, because of the dipolar ion, should be able to react under certain conditions as an ampholyte with either acids or bases. In the third formulation 1(c) the molecule is regarded as an organic derivative of orthophosphoric acid in which two of the hydroxyl groups are esterified.

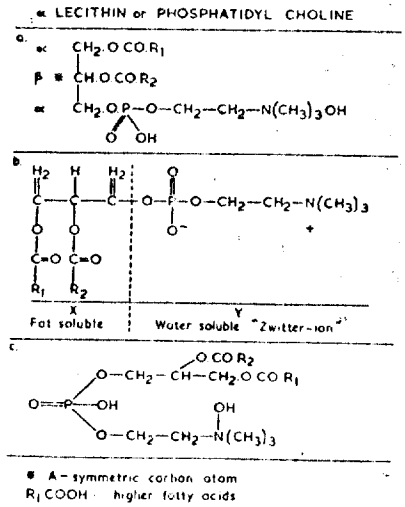

Fig. 1.

All the different methods of formulation, although fundamentally the same, have their uses in the understanding of the properties and in particular the physical and physico-chemical properties of lecithin.

The Phosphatides

Associated with lecithin in soya bean, egg yolk, and plant and animal tissues are many allied substances (grouped with lecithin under the general term phosphatides) in which the choline is replaced by other nitrogenous bases or by amino acids. There may be present also other complex fats of somewhat different structures but all containing nitrogen and phosphorus. These, with the phosphatides, may be described as lipins (or phospho-lipids). The properties of the individual lipins are by no means identical with those of lecithin, thus the properties of a commercial lecithin will vary somewhat on its source and on the percentage of true lecithin present.

Chemical Properties

The chemical properties of lecithin are to some extent a reflection of the properties of the components of the molecule. Thus hydrolytic agents (acids, alkalis,

and enzymes) attack lecithin, and after varying periods of time yield either a mixture of glycerol, fatty acids, phosphoric acid, and choline, or alternatively intermediate products such as glycero-phosphoric acid or phosphoryl-choline.

The fatty acids present in lecithin from different sources have been examined in detail by the methods developed by Hilditch and others in their investigations on tri-glycerides, and a range of higher fatty acids, saturated and unsaturated, containing 16 or more carbon atoms have been isolated. Because of the presence of unsaturated acids, the lecithins can take part in addition reactions: (i) they can be hydrogenated by standard methods to give hydro-lecithins of various degrees of unsaturation; (ii) they readily absorb iodine and the iodine value can be determined in the usual way and used as an index of unsaturation; and (iii) they absorb oxygen.

Stability of Lecithins

Purified lecithins change rapidly when exposed to air, no doubt by a combination of oxidative and hydrolytic processes comparable to, but probably even more complicated than, those responsible for the onset of rancidity in ordinary fats. Iron salts and other reagents catalyse the oxidation processes.

These changes do not occur so rapidly with crude lecithin preparations, especially those from vegetable sources, because of the presence of natural antioxidants, which are associated with the phosphatides, and which play an important protective role in stabilising both the lecithins and the tri-glycerides.

Thus we have the paradoxical situation that in spite of the instability of pure lecithin, commercial lecithin can often be recommended for its antioxidant properties.

Lecithin does not give a true melting point but is decomposed by heat from 110°C. onwards; crude mixtures containing triglycerides may be more stable. Wet combustion methods (concentrated sulphuric acid and hydrogen peroxide) may be used to break down the molecule, as in the Kjeldahl method for the determination of nitrogen and the corresponding technique for the estimation of phosphorus. In pure lecithin the atomic ratio nitrogen/phosphorus is unity, but this is true also for certain other phosphatides; thus the ratio has only limited uses as a criterion of purity.

Physical Properties

Lecithin when freshly prepared is an almost colourless waxy solid, but on exposure to air it rapidly becomes dark brown in colour, following the absorption of oxygen. To obtain pure samples of lecithin, many precautions have to be taken at all stages and in particular low temperatures must be used and air and light excluded.

The typical organic solvents (ether, petroleum ether, chloroform, benzene, alcohol) dissolve lecithin to form either true or colloidal solutions, but, as already noted, ketones—especially acetone—do not dissolve phosphatides and in fact will precipitate them to a greater or lesser extent from ether solutions. The phosphatides are also insoluble in methyl acetate.

Investigations have shown that the solubility of lecithins in a solvent such as ether varies somewhat with the fatty acids present, the highly unsaturated lecithins being most soluble and the fully hydrogenated compounds least soluble. This relationship of physical properties with the degree of saturation is well known in other sections of fat chemistry and forms the basis of the separation of saturated and unsaturated fatty acids by the lead salt (or Twitchell) method.

Colloidal Properties

The behaviour of lecithin with water has been extensively studied, especially in relation to its surface-active properties. Different stages can be distinguished depending on the relative quantities of phosphatide and water present. Lecithin is hygroscopic and rapidly absorbs water vapour from the air. When a small amount of water is placed in contact with lecithin, the solid appears to bud, giving irregular outgrowths—the so-called myelin forms. In the presence of more water the imbibition process continues and the mass swells, until (if sufficient fluid is available) a colloidal solution results.

These and related phenomena have been studied by the usual technique of colloid chemistry, and it has been shown, for example, that lecithin, like fatty acids, will form molecular films on water. Lecithin will lower the surface tension of water and aqueous solutions, and will lower the interfacial tension between water and insoluble substances such as fats or benzene.

It was found at an early date that various neutral salts including cadmium chloride and magnesium chloride would precipitate lecithin from colloidal solutions, and salts are frequently used in combination with acetone to obtain complete precipitation. Pure acetone may precipitate only part of the phosphatide from a dilute ether solution; quantitative results may be obtained with an acetone-magnesium chloride mixture, a fact which is used in the determination of small amounts of phosphatide in tissue extracts. Investigations have shown that different anions and cations vary in their effectiveness as precipitating reagents and with certain exceptions the Hofmeister rule which applies to other colloids is followed.

Lecithin may form loose combinations with sodium chloride, the resulting product being soluble in ether and other fat solvents. Complexes of lecithin with sugars and proteins have also been examined and these—like the salt combinations—may be of importance in physiology, and may help to explain certain results obtained in connexion with the food uses of lecithin.

Emulsifying Powers

Lecithin is a hydrophilic colloid and, because of its effect in lowering the interfacial tension between water and other sub-

stances, serves as a wetting agent and will promote emulsions of the oil-in-water type.

A demonstration of the emulsifying power of lecithin may be obtained by using two stoppered 50 ml. measuring cylinders to each of which is added 5 ml. of olive oil (coloured by the red fat-soluble dyestuff Sudan III) and 20 ml. of water. To the second cylinder 1 ml. of an aqueous lecithin preparation is added. Both cylinders are then shaken and the coloured oil is dispersed throughout the water. On allowing to stand, the dispersion in the first cylinder settles out, with the layer of the red oil above the clear water phase; in the second cylinder a stable emulsion results, because of the presence of the lecithin.

It has already been pointed out [see Figure 1(b)] that the lecithin molecule, like that of other surface-active agents can be considered in terms of two portions, one potentially fat soluble, the other potentially water soluble. When lecithin acts as an emulsifying agent in an oil-water mixture, the molecule is orientated at the surface of the oil (see Figure 2) so that the fat soluble moiety (X) is directed onwards towards the oil, the water-soluble moiety (Y) outwards towards the water. This is the essential basis of the emulsifying role of lecithin which contributes to its successful use in certain food industries.

Lecithin will not only stabilise oil-water emulsions, but will also act as a protective colloid in delaying or preventing the precipitation by salts of hydrophobic colloids (*e.g.* metallic particles) dispersed in water. This observation forms the basis of some of the non-food uses of lecithin.

Physiological Properties

The physiological role of lecithin has been investigated over the last 50 years both by classical methods and by the more modern technique of "labelled molecules" using compounds in which isotopic forms of phosphorus have been incorporated into the phosphatide molecule.

It would appear that lecithin may have one of three distinct but allied functions in the animal body; it may be part of the actual structure of the living cell; it may play a part in cell membrane structure and permeability or in other physico-chemical processes; it may be actively concerned in the metabolism of fats. There is considerable evidence in favour of all these hypotheses, but whatever the exact function may be it is clear that lecithin is a naturally occurring substance of great importance, and that its use as a food adjunct can in no way be harmful.

Considered from the nutritional viewpoint, it can contribute to the body the same components as the tri-glycerides, namely glycerol and fatty acids; it contributes also phosphoric acid (important in mineral metabolism) and the base choline which has certain properties which entitle it to a place among the water-soluble vitamins.

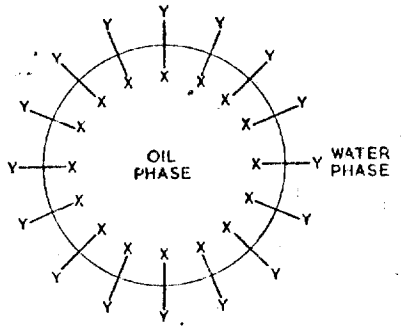

Fig. 2. Oil-in-water emulsion promoted by lecithin (X—Y) distributed at interface.

The nutritional role of lecithin can well be illustrated by reference to the egg of the hen or any other bird. The egg contains all the nutrients necessary for the initial growth of the embryo, and during the incubation process the lecithin of the yolk is utilised partly to supply energy for growth, and partly to supply materials required for the formation of body tissues. Thus phosphorus originally in the lecithin becomes part of the bone structure of the chicken.

Considered in terms of calories, the fatty acids of lecithin will on combustion make the same energy contribution to the body, as will the fatty acids from tri-glycerides or any other source. The total energy contribution of lecithin will be high, but somewhat lower than that of the tri-glycerides because of the presence of the phosphoric acid and choline.

Economic Aspects

At one time regarded almost as a chemical curiosity rarely mentioned in standard chemistry textbooks, lecithin during the past 20 or 30 years has attained a position of some importance, and its increased uses in industry following its greater availability, have led to a gradual decrease in its market price.

American statistics show a change from 75 cents per lb. in 1930 to 15 cents in 1950, and reveal also that 8,000,000 lb. of lecithin were produced in the United States in 1948, but that a further 40,000,000 lb., which could have been obtained from soya and other sources, were not recovered. It is clear, therefore, that in the United States there is a large potential reservoir of lecithin which can be manufactured in response to an increased demand.

In Germany, lecithin from rapeseed oil has been extensively used, and in Britain lecithin is being produced both from groundnut and soya oil. The amounts available at any time will be roughly proportional to the total quantities of oil extracted and will therefore fluctuate with the supplies of edible oils from these sources.

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(*To be continued*)

Lecithin in Food Processing

Part II

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In Part I of this series it was shown that commercial "lecithin" obtained from soya, maize, groundnut and rapeseed oils is a mixture of substances, known collectively as phosphatides, which can be regarded as complex phosphorus and nitrogen-containing fats. The phosphatides are naturally occurring compounds of [nutritional value and because of their surface-active and other properties they are widely used in food processing. In this instalment the author discusses the use of lecithin in bread production.

OVER the past twenty years commercial lecithin has been accepted as an ingredient in bread, biscuits, and flour confectionery. Its use has passed through several stages. In the first instance it was proposed as a substitute for egg yolk in flour confectionery, and although there is no doubt that it contributes to the properties of egg yolk (of which it is a major component) it is an ineffectual substitute.

The second stage was marked by the use of lecithin to replace part of the fat used in cereal products; world shortage of fats from 1939 onwards, in particular during the war years, did much to stimulate its sales as a baking ingredient. The third stage was reached with the realisation that, quite apart from its emergency function in replacing fats, lecithin has certain properties which enable it to claim a place in its own right in many baking formulae. This concept is not new—it was inherent in claims made in patents and in reviews twenty years ago, but the effective establishment of such claims has taken place only in the past decade.

During the "bread hearings" held at Washington to establish Federal standards under the U.S. Food and Drug Act, it became clear that lecithin was regarded by many bakers as a common raw material and it was agreed that it should be considered as a normal but optional constituent of shortening.

Literature on Lecithin

The use of lecithin in the production of bread and other cereal foods is now noted in the standard text-books[4, 10] on phosphatides and on soya bean derivatives, and has been subject of many patent applications, and of some review articles.[2, 8] Detailed investigations have been carried out in a number of industrial laboratories, and the experimental results obtained form the basis of publications by various firms which are engaged in the manufacture and distribution of phosphatides.

Nevertheless there are comparatively few published scientific studies of the function of lecithin in bread, cake, and biscuit making, and this is in part a reflection of the difficulty in obtaining conclusive and definitive results in the processes which are characterised by a very large number of variable factors—a general problem facing the scientific worker in the cereal field.

Variable Factors

The results obtained in American experiments on lecithin in relation to breadmaking must be interpreted with caution before they can be applied in Britain.

In the first place the commercial loaf as produced in the United States invariably includes fat (*e.g.* 3 or 4 lb. for each 100 lb. of flour) and often non-fat milk solids (*e.g.* 4 lb. per 100 lb. of flour); it is thus highly enriched compared to the typical and traditional commercial loaf in the U.K., a loaf composed essentially of four standard ingredients—flour, yeast, salt, and water.

A second reason for caution lies in the judging and scoring methods used. Such methods are still largely subjective in character and depend in great measure on the view-point of the observer—whether a professional judge or a member of a panel of housewives. There is ample evidence that opinions on good bread and cakes differ on opposite sides of the Atlantic, in fact local tastes and preferences vary in different parts of Britain; people in the north of England, for example, like a richer loaf than those in the south.

In investigations on breadmaking many variables have to be considered: (*i*) The source and type of the commercial lecithin, (*ii*) the method of addition, (*iii*) the type of flour used, (*iv*) other constituents of the dough, such as fat, sugar, milk solids, (*v*) the method of breadmaking used, whether short process or sponge process, (*vi*) the technique, whether small scale hand production or large scale plant production, (*vii*) times and temperatures used at the different stages—fermentation, intermediate proofing, final proofing, and baking.

Criteria for Use

From the standpoint of the baker there are two distinct, although allied, preliminary series of questions to be answered in regard to lecithin. (1) In what respect will it affect the breadmaking *process*? Will it help rather than hinder the already difficult problem of controlling this process? (2) What will be the effect on the final product? Will it produce a loaf with better appearance, taste, and flavour, and with better keeping qualities?

If the answers to these questions are positive, then subsidiary, but important, issues remain. In what quantities should lecithin be used, and are these quantities to be modified—if at all—with batches of flour of variable protein content and baking quality? In what form should lecithin be

added? What variations are required in the standard bread formulae and processes? What, if any, are the economic advantages?

A Better Loaf

In Table 1 a summary is given of the effects of fat and lecithin on the characteristics of the loaf, both immediately after baking and during storage.

Most observers agree that shortening of the tri-glyceride type produces a loaf with a more symmetrical shape, better external characteristics—a thinner and less tough crust, a better quality of colour and surface texture. The internal characteristics are also modified to give a better crumb colour, a greater evenness of grain or vesiculation and of general appearance, and what is perhaps most important—a softer crumb.

American evidence suggests that all the above effects can be reproduced or reinforced when the fat is supplemented or partly replaced by lecithin. Thus Pratt[6] in 1946 using a formula 4 per cent. sugar, 4 per cent. milk solids, and 3 per cent. fat examined the action of supplementary lecithin (0, 1, 0·25, 0·5, 0·75, and 1 per cent.—measured in relation to initial weights of flour) and concluded that 0·25 per cent. gave the best results in enhancing the effects of the fat, particularly in respect of crust properties, crumb texture and grain, and external appearance. In comparison with the fat-containing control, he suggested that "there was no effect on crumb *colour*, other than the *illusion* of whiteness brought about by finer cell structure."

Somewhat contradictory results have been reported regarding the effects of lecithin on loaf volume. Pratt's investigations suggested that lecithin produced little or no effect on volume over and above that produced by fat, but others have found that lecithin promoted an increase in volume. It seems likely that the question of final volume is intimately linked with the type of flour employed, and with the ability of the flour to take up water in the dough-making process.

Keeping Qualities

There seems to be general agreement that bread made with lecithin supplements not only has a softer crumb immediately after baking, but also has better keeping qualities as measured by the penetrometer. Thus Pratt made three batches of bread containing the following supplements:

	Fat *Per cent.*	*Lecithin* *Per cent.*
(*a*)	3	0
(*b*)	3	0·25
(*c*)	1	0·25

He found that the keeping qualities of (*b*) were better than (*a*), and that the keeping quality of (*c*) was approximately the same as (*a*); these results, in conformity with those of other workers,[7] provide the basis for suggestions that lecithin can be used for the partial replacement of shortening. Pratt, however, is of the opinion that lecithin should be considered primarily for its effect in improving the value and efficiency of the existing shortening in the bread formula, and that it should only be used to replace shortening when this is unavoidable because of short supplies of fat.

The desire of the public for a loaf with a softer crumb has encouraged the American baker (and in particular the plant baker) to produce a loaf with a crumb that will remain soft over several days. This has been the motive behind the extensive use in breadmaking of various surface-active agents such as mono- and di-glycerides, esters of ethylene glycol, and of sorbitol. In the "bread standards" recently announced in Washington, the glyceride esters are permitted as optional ingredients as shortening; the other esters are banned.

Softening Action

It is not at all clear from published information how far detailed comparisons have been made regarding the efficiency of lecithin, as a "softening" agent, in contrast to the glyceride esters and the glycol and sorbitol compounds, but it is probable that the effects produced by lecithin and by the other substances, all have a common origin, namely the surface-active properties, which can bring about a more even distribution of constituents and a modification of the granular structure of the loaf.

The lecithin may owe its beneficial effects on the keeping qualities of the loaf, in part to its ability to form loose compounds or complexes with other dough constituents (*e.g.* gluten or starch), in part to its ability to form surface layers around these constituents, and in part to its ability (as a lyophilic colloid) to hold water. Because of this characteristic it may help to preserve the structure of the fresh crumb, by retarding the movement of water, and the loss of water vapour from the starch gluten complex.

How far the softening action of the surface-active agents can be

TABLE 1

EFFECTS OF FAT AND LECITHIN SUPPLEMENTS ON THE FINISHED LOAF AND ON STORED PRODUCTS

Characteristics	*Fat Supplement*	*Lecithin Supplement*
A. External Characteristics.		
Volume.	Somewhat larger. Varies with flour.	Little effect on high fat formula, more pronounced in other cases.
Shape.	Improved.	Effect accentuated; more symmetrical loaf.
Crust.	Better bloom (better quality of colour and texture of surface).	Pronounced effect on crust; improved bloom.
B. Internal Characteristics.		
Grain or vesiculation.	Greater evenness of grain.	Accentuated.
Crumb colour.	Better crumb colour and sheen.	Apparent improvement—may be due to finer grain.
Nature of crumb.	Softer crumb.	Pronounced effect on crumb softness.
C. Storage.		
Keeping qualities.	Keeping qualities improved; crumb remains more soft than in control loaves.	Pronounced effect on crumb. Further improvement in keeping quality.

regarded as an "anti-staling" effect is not yet known; there are differences of opinion among cereal chemists on the precise meaning to be attached to the term "staling."

The Breadmaking Process

The action of both tri-glyceride and lecithin supplements can be seen at different stages in the breadmaking process whether this can be carried out by the straight-dough (see Table 2) or by the sponge method.

Perhaps the most valuable contribution of fat and of lecithin is to improve the "handling" properties of the dough. This is important to the family baker engaged in the hand production of bread and whose skill in his craft depends in great measure on his ability to control the treatment of the dough. The modifying action of fat or lecithin is even more welcome to the plant baker who adjusts his machinery to a time schedule and who endeavours to work with doughs of standard types. The greater resilience produced by fat or lecithin supplements enables doughs to stand up to harsher mechanical treatment, without any damage to their structure.

A second significant effect of the supplements is the improvement of the surface or "skin" of the doughs; this results in greater ease of working, in that stickiness is reduced, and it also contributes to the final shape of the loaf, giving a more symmetrical external appearance.

The question of the influence of fat and lecithin on water absorption during the mixing stage, is complicated by the great variations that can occur with flours of different origins. It would appear that with certain flours, the normal water absorption can be increased with a consequent enlargement in the volume of loaf.

TABLE 2

EFFECTS OF FAT AND LECITHIN SUPPLEMENTS IN BREADMAKING
STRAIGHT-DOUGH PROCESS

Stage.	*Fat Supplement, e.g. 3 or 4 per cent.*	*Lecithin Supplement, e.g. 0·25 per cent. In addition to fat.*
A. Mixing of flour and and other ingredients.	Slightly softer but less sticky dough; water absorption may be increased.	Similar but more pronounced effects.
B. Fermentation.	High percentages may produce retarding effect; longer fermentation time required.	Little effect.
C. Dividing and handing-up.	Improves handling properties. Dough better able to withstand "felling" and rough treatment. More resilience, less tendency to tear.	Definite improvement in handling properties, and increase in resilience. Dough less sticky.
D. Intermediate Proof.	Less tendency to stick to pockets. Less tendency to skin at surface.	Effects accentuated.
E. Final Moulding.	Resilience to moulding action. Good skin which gives good shape.	Effects accentuated.
F. Final Proof.	Probably greater tolerance.	—
G. Baking.	Some reduction in baking temperature advisable.	—

The Mixing Process

Detailed studies have been made on the action of supplements on the mixing process; the *subjective* observations made by experienced bakers on the consistency and "feel" of different doughs, have been followed up by *objective* measurements using the standard recording machines that have been available in recent years. It is well known that the behaviour of a flour in a mixer is contingent on the quality of its major protein component—gluten—and the quantity and quality of the gluten varies with the source of the wheat. Both fat and lecithin[5, 9, 11] have favourable effects on the gluten, and the lecithin in particular has a "mellowing" action, increasing the tolerance of the dough to mixing and thus improving the mixing qualities.

The same idea has been expressed by saying that lecithin will produce a "neutralisation of the extremes of weakness and strength" in flours, altering high and low protein flours in converging directions. This action of lecithin appears to be over and above that of the fat and has been explained in various ways, for example as a "lubrication" effect, brought about by the surface-active properties, which may enable the dispersed phosphatide to cover effectively the gluten strands.

Use of Lecithin without Fat

The American conclusions regarding the advantages of lecithin and regarding the optimum amounts are based largely on a comparison of bread made with fat and lecithin supplements and bread made *with fat*, but *without* lecithin. Lecithin when added as a supplement to fat has two main advantages in breadmaking; in the first place it assists in the control of the process, and secondly it produces a better loaf with better keeping qualities.

It is not unreasonable to suppose that the above advantages may, with certain provisos, be all the more obvious in bread of the more traditional type, *i.e.* made without the addition of fat, milk powder, and sugar. Although the published information on this question is limited, there is evidence from commercial practice in Germany and in the Netherlands that from 1930 onwards lecithin has been accepted as an important supplementary ingredient. Investigators in the Netherlands found that lecithin was particularly useful as an adjunct to the very soft flours produced by the incorporation in the grist of home-grown wheat. Comparisons of such flours, with and without lecithin, showed that up to 10 per cent. more water could safely be employed in the presence of the lecithin supplement, and this increase was reflected in the volume of the baked products.

Similar observations were published in 1934 by Bennion,[1] whose preliminary experiments demonstrated the effect of lecithin on water absorption and loaf volume. Hutchinson[3] (1936) in

his report from the Research Association of the British Flour Millers placed lecithin in the category of bread improvers, and noted the improved keeping qualities of the bread produced. Similar results were obtained by Rewald.[7]

Recent investigations (unpublished) carried out by the author and his colleagues, have shown that good results can be obtained by the incorporation of 0.3 per cent. of lecithin in doughs made from present-day 80 per cent. extraction National Flour; improvements in the handling qualities of the dough, in the final loaf volume, and in the colour and softness of the crumb are apparent.

The question of crumb colour is of particular interest at the present time in that 80 per cent. extraction flour gives a crumb which is darker than that from flour of the pre-war extraction rate of 70-72 per cent. It has been demonstrated that the National Flour, when supplemented with fat (*e.g.* 1 per cent.) and subjected to high speed mixing, will often produce a much lighter crumb. This is almost certainly not a bleaching effect, but rather an optical illusion brought about by the finer structure. Similar observations have been made in experimental work on lecithin in relation to National Flour.

Addition of Lecithin

Several methods have been proposed for the addition of lecithin to doughs including: (i) the use of lecithinated flour, (ii) the use of shortening in which lecithin has been incorporated, and (iii) the use of lecithin emulsions. All three methods have their place in bakery practice, but clearly the second method (common in the United States) is primarily designed for fat-containing doughs. Under English conditions the third method is likely to prove most useful, the commercial lecithin in a suitably emulsified form being added at some stage in the mixing process.

The figures given in the literature for the amounts of lecithin to give optimum results, can only be interpreted if information is available regarding the percentage of lecithin and other phosphatides in the commercial preparation used. Thus an emulsion of lecithin-oil suitable for bakery practice may contain only 40 per cent. of phosphatide, the edible oil being in part a diluent to facilitate the addition. However, lecithin, like other flour supplements, is self-limiting in action, and the amounts recommended by manufacturers can usually be taken as a guide to economical as well as to efficient use.

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(To be continued)

Correspondence

To the Editor of

FOOD MANUFACTURE

Copper in Foods

DEAR SIR,—I should like to refer to your editorial on copper in foods on page 264 of the July issue of your journal. In general, it is not customary for members of a Committee to defend their actions, but, before the Food Standards Committee set out its views on the quantities of copper to be permitted in food, one of its members, Dr. G. W. Monier-Williams, had written a small volume on Trace Elements in Foods, and had devoted the first 63 pages to this important metal. Dr. Monier-Williams points out that copper appears to be a natural constituent of many foods, such as tea, cocoa, and crustaceans, and also takes the trouble to inform his readers that no copper is extracted from the tea into its infusion.

In the good old days it was customary to take pride in the large open evaporating pans used in food and pharmaceutical factories and these would be given a delightful polish at least once a week, but it is now realised that not only did the employees give them a polish, but that the pans themselves yielded considerable quantities of copper to the extracts prepared in them. Some years before the last war, it was discovered that tomato purée, yeast extract and similar products derived practically the whole of their copper content from these old fashioned copper pans and that with improved manufacturing plant better extracts, almost free from copper, could be obtained. It was for this reason that Port Medical Officers first decided to restrict the quantity of copper, which they would approve in imported tomato purée, and the Food Standards Committee has been even more lenient than the Port Medical Officers of Health.

While little is known about the quantities of copper that the animal system can absorb with impunity, it would appear that the Committee have been minded to allow copper to be present in foods, where it occurs naturally or where the use of copper-containing fungicides are the most satisfactory methods of dealing with certain conditions, but to prohibit its presence as far as possible where this presence is unnecessary. It was on account of the argument that the copper in tomato purée was caused by copper fungicides and not by copper pans that action to prevent its presence was delayed, but tomato plants, like grape vines, should be sprayed with fungicides at certain times in order to avoid contamination or even spoilage of the quick growing fruit.

Yours faithfully,

THOMAS MCLACHLAN.

London.

TO AUTHORS

FOOD MANUFACTURE is prepared to consider the publication of articles on the subjects covered. Books on food would also be considered.

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Lecithin in Food Processing

Part IV

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The use of commercial lecithin in cereal products has been reviewed in Parts II and III of this series. This concluding article gives an outline of the application of phosphatides in other sections of the food industries, notably in the production of chocolate and sugar confectionery, ice cream, and edible fats.

THE surface-active properties of lecithin, which have led to the use of phosphatides in the preparation and stabilisation of oil-water emulsions, have also been utilised to promote the formation of uniform and stable dispersions of solids in oils.

It is for this reason that lecithin has been extensively used in chocolate manufacture, an important part of chocolate processing being the formation of an intimate mixture of cacao material, sugar, and other solid constituents with cocoa butter and other fats. This mixing process is carried out in several stages depending on the end in view, but the qualities of the final product, whether it is to be sold as chocolate or used as a couverture, are related to the degree of dispersion of the components.

Uniform Dispersions

Some of the effects brought about by phosphatide supplements at different stages in chocolate processing are shown in Table 1. In the mixing process lecithin acts as an "inter-surface modifier," probably through the formation of mono-molecular films coating the solid particles and bringing them into union with the fatty medium. The increase in uniformity of the lecithin-containing products can be shown by microscopic examination, and is evident from micro-photographs.

For the establishment of these surface films a relatively small percentage of lecithin is effective; somewhat larger quantities are required to produce other effects, such as a decrease in viscosity.

The stability of lecithin-containing chocolate is reflected in its resistance to changes during processing (for example in melting, heating, and agitation) and in ageing. Thus the product can be heated over a wider range of temperature without a breakdown of the dispersion and a precipitation of the solid constituents, a matter of special importance in chocolate containing a high level of milk solids.[27] This increased stability to heat treatment is analogous to the "protective action" of lecithin and other surface-active agents in simple solid-water colloidal systems.

Reduction of Viscosity

In the processing of chocolate for various purposes it is necessary to obtain material of the correct degree of fluidity, and this is especially important in the preparation of chocolate to be used as couvertures. Thus considerable attention has been given to viscosity determinations and the ways and means by which the viscosity of a product can be standardised and controlled. Clearly the "covering power" of a given weight of molten chocolate will depend partly on the viscosity of the mixture; the formation of layers of constant thickness will be contingent on the maintenance of constant viscosity of the chocolate fluid.

Within certain limits the viscosity of chocolate may be reduced by the addition of increasing amounts of cocoa butter, and this is the normal method adopted in industrial practice.

The use of commercial lecithin as a supplement to cocoa butter has been accepted by the industry following the investigations of Working,[28] Avent,[29] Morgan,[30] and others who have studied the effects of lecithin on chocolate-cocoa butter systems. Thus Avent and Morgan[31] demonstrated that a progressive lowering of viscosity took place with the addition of phosphatide, the maximum effect being achieved with 0·5 per cent. lecithin. Cook[32, 33] found that 0·3 per cent. lecithin was sufficient to reduce the viscosity by 50 per cent., and Stanley[34] showed that 0·35 per cent. lecithin had the same effect in viscosity reduction as 8 per cent. of cocoa butter.

It was pointed out by Working[28] that lecithin could be used to replace part of the cocoa butter required for chocolate processing in that, with suitable lecithin supplements, a product could be obtained of the same viscosity but with a lower (by 30 to 35 per cent.) cocoa butter content. Stanley[34] came to the conclusion that a saving of 20 per cent. of the cocoa butter content could often be effected, with a consequent re-

TABLE 1

USE OF PHOSPHATIDES IN CHOCOLATE MANUFACTURE

Stage	*Effects of added phosphatides*
Processing.—Grinding of: (*a*) solids — cacao material, sugar — with (*b*) cocoa butter	(1) Ready "wetting" of ingredients to give (2) Decrease in time and effort in grinding and (3) More even distribution of constituents with (4) Consequent increase in stability.
Heat treatment. — Tempering, etc.	(5) Product more resistant to the effects of overheating and can be processed over a wider temperature range.
Preparation of couverture for enrobing, etc.	(6) Changes in physical properties, especially reduction in viscosity of chocolate—cocoa butter mixtures leading to (7) Possibility of reducing cocoa butter content.
Final product.—Stability and keeping qualities	(8) Product more resistant to changes brought about by absorption of moisture (9) Separation of constituents (leading to "fat bloom") retarded.

duction in the cost of production.

The optimum amount of lecithin required will vary with the chocolate being processed and with the end in view.

Resistance to Moisture

An important problem in chocolate processing is that produced by the absorption of water resulting in a thickening which can be measured by an increase in viscosity. Langwill[35] pointed out that lecithin would protect chocolate from the effects of imbibition of water vapour, and Stanley[36] reached the same conclusion by carefully controlled experiments in which he compared two samples of chocolate both of the same initial viscosity (112 degrees MacMichael) and of the same moisture content (0·50 per cent.). As the moisture content was progressively increased (up to 2 per cent.), the first sample (containing 0·3 per cent. lecithin) gave uniformly lower viscosities than the second (containing 6 per cent. of added cocoa butter). In this connexion the observations of Erb and Collins[37] are of interest; they showed that the full effect of phosphatides in chocolate are exerted only when a small percentage of moisture is present.

Stability of Final Product

A well-known difficulty arising in chocolate production is the deterioration known as "blooming" or "greying"—caused by the deposition of fat ("fat bloom") or sugar ("sugar bloom") in the form of scattered patches or thin films of greyish material on the surface leading to the disappearance of the gloss. The fault may extend into the body of the product and give texture of poor appearance.

Various workers have claimed that the presence of lecithin will counteract the tendency to bloom formation although Clayton and his colleagues[38] suggest that the contribution of the phosphatides is small, and recent investigators[39] have recommended the use of some of the newer surface-active agents. It is clear that irrespective of the possible role of lecithin, adequate control of tempering and other stages of processing is very important.

Amounts of Lecithin Supplements

Cook[32] has drawn attention to the necessity for controlling carefully both the amount of lecithin and the method of its addition to chocolate, and has pointed out that if the lecithin is added prior to or during refining and conching, a considerable reduction in the activity of lecithin may occur, particularly because of the heat generated. As already indicated there are advantages in using lecithin at an early stage, but if the aim is to obtain a maximum effect on viscosity, more lecithin must be added later. He recommends the use of about 0·25 to 0·3 per cent. lecithin to achieve minimum viscosity and further quantities if the chocolate is likely to come into contact with moisture—as in the production of chocolate covered ice cream bars.

Confectionery and Ice Cream

The use of commercial lecithin in the production of various types of sugar confectionery has been investigated by Jordan[10] and by others,[35] who have shown that lecithin is particularly effective in the preparation of fat-containing products, such as caramels and nougats. As an emulsifying agent (in amounts of the order of 1 per cent. in relation to fat) it aids in the mixing processes bringing about a rapid distribution of the fat in the water-soluble ingredients, such as sugar, syrup, or milk. It also retards the separation of the components on cooking and the consequent deterioration of the texture (graining) and surface of the product.

Stanley[27] has described further uses of phosphatides in relation to sugar confectionery, namely as a carrier and fixative for volatile flavours, and for water soluble colours.

Ice cream is a complex physico-chemical system consisting of butter or other fats, milk solids, sugar, and other ingredients. It has long been known that certain substances (such as eggs or gelatine) with colloidal properties must be added to the ice cream mix in certain minimum amounts if a stable product of smooth texture is to be obtained (*cf.* Clayton[11] p. 261). In the absence of such stabilisers, the ice cream is gritty, or becomes so on standing because of the formation of lactose crystals, or—in some cases—of ice crystals.

A number of investigations on the use of phosphatides in ice cream manufacture have shown that lecithin will ensure a better distribution of the fat in the mix and will retard the onset of "sandiness" in stored products, even in those prepared with relatively high concentrations of milk solids, *i.e.* with a high lactose content. Thus it is claimed that lecithin will produce a smoother product and that it can in part replace egg yolk.

The advantages of lecithin have been discussed by Stanley[12] and by other workers,[43, 44] and have been the subject of patent claims.[45, 46] Cook[32] has pointed out the importance of using lecithin in the right form—namely in aqueous dispersion—if full benefits are to be obtained. Other writers have considered the contribution of lecithin to sherbets and frozen desserts.

Edible Fats

As already noted commercial lecithin has been used in the manufacture of edible fats as an emulsifying agent to promote blending, and advantages have been claimed for lecithinated shortenings to be used as bakery, chocolate, and sugar confectionery ingredients. There is one important restriction that must be placed on the use of lecithinated oils—they cannot be used in processes which involve high temperatures for long periods, as in deep fat frying.[32] High temperatures bring about a decomposition of the phosphatide with a consequent darkening of the oil.

Margarine, a by-product of the Franco-Prussian War of 1870, gradually became accepted as a butter-substitute in Europe and in the United States. The earlier formulae involved the emulsification of milk and animal fats to give a water-in-oil dispersion.

Later, many different fats of animal or vegetable origin were utilised, cotton-seed oil and soya oil being important ingredients in the United States, and coconut oil, palm oil, and hydrogenated whale oil in Europe.

Emulsifying Agents

At an early stage it was demonstrated that one of the key problems was the production of an emulsion, comparable in stability to that which exists in good quality butter. In the absence of suitable emulsifying agents, syneresis occurs — the margarine "leaks" or "bleeds" during storage with the exudation of water from the emulsion. This separation of the oil and water phases is hastened by heat, and is especially evident when the margarine is used for frying; the steam generated from the liberated water causes the oil to spatter and a residue of milk protein and other solids adheres to the pan. Corresponding changes do not occur when butter is used for frying because of the emulsifying agents present in the natural product.

As early as 1884 egg yolk was introduced as an emulsifying agent in margarine manufacture; later egg yolk lecithin was used and from 1925 onwards[49] soya and other plant lecithins in amounts ranging from 0·1 to 0·5 per cent. fat. It is claimed that phosphatides facilitate the mixing process and ensure a more uniform distribution of the triglycerides and greater stability both during storage at ordinary temperatures and during processes such as frying.

In recent years (*cf.* Bailey,[47] p. 283) investigations have been carried out on the use of lecithin in combination with other emulsifiers, such as mono-glycerides, and it is said that the mono- and diglycerides are effective as "anti-leaking" agents at low concentrations, but that they do not have the same anti spattering action as the phosphatides.

Miscellaneous Products

In addition to the major uses of lecithin so far considered (cereal products; chocolate and sugar confectionery; ice cream and related products; edible fats—shortenings and margarine), a great number of other food uses have been suggested. These have been reviewed by Wittcoff,[18] and include proposals for the incorporation of phosphatides in such products as cocoa powder, dried soup preparations, cheese, sandwich spreads, peanut butter, mayonnaise, and chewing gum. Cook[12] gives helpful information regarding the optimum quantities of lecithin and the method of use in some of these products.

Phosphatides as Antioxidants

Many of the food uses of phosphatides can be forecast from, or traced back to, the surface-active properties of the molecules which enable them to act as emulsifying agents in aqueous-oil systems and as inter-surface modifiers or as protective agents in other colloid systems. Allied to these properties is the ability of phosphatides to form imbibitions with water and complexes with a wide variety of substances. On the basis of such considerations an explanation can be given of the role of phosphatides in such diverse products as bread doughs and chocolate-cocoa butter mixtures.

In a different category must be placed the many claims that commercial phosphatides can serve as antioxidants. This question was reviewed by Bibby[52] in 1945 and has been the subject of extensive investigations in many countries. Three aspects of the problem have received special attention: (i) Do the antioxidant properties arise from some one phosphatide, for example from the phosphatidyl choline (lecithin), or are they the result of non-phosphatide components present in commercial phosphatide preparations? (ii) What are the effects on the stability of different types of fats? (iii) Can the effects be carried through to the final product?

There would appear to be no doubt that under certain conditions phosphatides are effective in retarding the oxidative rancidity of certain animal and vegetable fats and the oxidative destruction of vitamin A and other substances. Thus work has been carried out on soya phosphatides[50] and on cotton-seed and maize,[51] groundnut,[52] and animal preparations.[53] The problem is complicated by the large number of possible substrates (lard and other animal fats and marine oils, vegetable fats of different types, vitamin A preparations) as well as by the complexity of the commercial phosphatide preparations, and hence there are many conflicting reports in the literature.

The effects of phosphatides in improving the colour and shelf life of biscuits have already been considered, and similar effects have been noted in other cereal products. Some of the precautions necessary for the scientific study of stability of cereal products have been outlined by Riemenschneider,[54] who has stressed the pro-oxidant action of traces of metals which may be present in the original ingredients or which may become incorporated during processing.

Recent investigations on antioxidants have shown the importance of synergistic effects, namely the ability of a combination of chemical substances to exercise a more powerful action than would be expected from the summation of their individual effects. Phosphatides have been successfully used as components of such (antioxidant) mixtures.

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(*Concluded on page* 411)

the feed arrangements, but normally an average speed of 30 to 40 can be maintained accurately for long periods by one operator.

The installation of this type of machinery enabled one company producing daily 3,000 tins, each containing 18 bags, by the old hand-weighing and filling methods, to increase its output, from the same quantities of raw materials, to 3,300 tins per day and at the same time reduce its labour force in the packaging section by 40 per cent. This represented an increase in sales revenue of nearly £50 per day, in addition to an appreciable reduction in the costs of production.

The packaging operations start with the crisps entering the line in mass in large bins. The feed to the machines is then made either by a horizontal conveyor with ploughs, each plough diverting part of the flow to a vibrator feed on the machine itself, or by one or a series of "shuffler" feeds leading to the vibrators. The shuffler is, in effect, a large triangular tray sloping down to a narrow discharge at the apex of the triangle, the whole tray being vibrated so as to spread the product out and produce an even discharge.

The crisps are then measured into 1 oz. weights, and delivered *via* discharge spouts to the packets or bags which are fed by hand. The filled bags are then placed on a further conveyor for insertion of the salt twists, and the bags are sealed, either by paste or heat sealing. A further conveyor delivers the sealed bags to the tin packing benches.

The basic unit of the machine is the weighing element which utilises the volumetric "positive displacement" principle, and which can be pre-set to weigh any specified measure in the range ½ oz. to 4 oz. It can also be used for packaging such products as biscuits, cheese straws, and confectionery.

Pest Infestation

THE task of the pest infestation laboratory of the D.S.I.R. is to study the insects which cause the loss of and damage to stored foods and other products, such loss amounting to millions of pounds each year. The progress made last year is described in a report* just published.

The insect population left in warehouses after the stores have been taken out was one of the subjects of study. Some of the foodstuff is left in the cracks of the walls and floors where the insects can live and remain immune from the action of sprays and fumigants. When the warehouse is filled again, the insects emerge and invade the food. In one of the buildings, which had wooden cavity walls, over 7,000 of one of the main grain pests were found in one small area between the walls.

The object of another survey was to identify the mites attacking cheese and to ascertain what conditions favour them and what effect methods of storing and curing have on infestation. It was found that mould-ripened cheese, especially Stilton, were most likely to become infested as they were ripened for a long time in an environment which is perfect for mites. A series of cheese is being kept experimentally, all infested with mites at the same time. This investigation, which is being carried out in collaboration with Reading University, is hoped to provide valuable information about the rate of damage by mites.

The results of the investigations of insects and mites in disused birds' nests show that these are an important source of attack on nearby warehouses and dwelling houses. Over 25 species of insects have been found in the nests of sparrows, pigeons, swallows, and other birds. The kinds of insects found in such nests correspond almost exactly with those that are found in a neglected food warehouse.

Work on blowflies in slaughterhouses has been continued to ascertain the severity of the "blown" carcass problem, the species of fly causing the damage, and the most attractive kinds of meat. It has been found that bluebottles, although they are only five per cent. of the population, cause 90 per cent. of the damage.

A large multiple store collaborated in experiments on the over-wintering of flies. Seven premises were searched during the winter. Only in one store were a few flies found. It seems unlikely that they stay in the stores and reinfest them in the following spring. Thus the problem of how flies spend the winter still remains unsolved.

Methods of fumigation at reduced pressure have been tested. The fumigant used was methyl bromide. The idea is that at pressures lower than those of the atmosphere, penetration is deeper and more rapid. The tests showed that with granular products such as wheat or groundnuts there is no advantage over normal "atmospheric" fumigation. However, penetration into a box containing compressed dates, which is normally extremely slow, was greatly improved. Experiments are continuing to improve the efficiency of this promising method.

Other research described in the report includes work on taint in wheat flour produced by fumigation and the mechanism by which "DDT-resistant" houseflies break down the insecticide into a harmless substance.

* *Pest Infestation Research, 1951.* Pp. 36 + iv. Published for the D.S.I.R. by H.M.S.O. Price 2s.

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Instant Whole Milk Powder

In many countries instant skim milk powder has been a popular consumer product for some years because of the ease of reconstitution and dispersion in cold water. World-wide interest has therefore been generated by the development in New Zealand of an instant whole milk powder with similar properties. A brief outline of the important factors in the production of this powder is presented.

For an instant powder, larger particles than a

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normal spray dried powder are required. Small particles cannot penetrate the water surface and, while retained on the surface, wet to form impervious, low density aggregates which dissolve slowly. In contrast, very large particles sink rapidly but take a long time to dissolve. Increased particle size was normally obtained in the past by rewet-redry procedures. When applied to whole milk powder this process results in increased free fat and the development of flavour defects which limit the acceptability of the product. More recently, straight through agglomeration techniques have been introduced using one or more fluidized beds attached to the base of a conventional conical drying chamber and recirculating fines from the beds to the atomizing zone of the spray drier to increase agglomeration. With whole milk powder, considerable fines recirculation with normal inlet air temperatures can result in powder solubility problems. However, if suitable precautions are taken in the drying process this method can produce powder suitable for instant whole milk powder. The required size distribution for instant products can also be obtained by the production of relatively large primary particles, using a single pressure nozzle, in a "tall form" drier equipped with a secondary drying system. For good dispersibility of instant whole milk powder a limited size range of 100-300 microns appears optimum.

Even with large particles, however, the wetting properties of whole milk powder are poor, because of the hydrophobic nature of the fat on the particle surface. The wetting properties can be improved by the use of certain surfactants. Since the initial powder wetting is a surface phenomenon the additive must be coated on the surface after drying, and results in no significant improvement when mixed with the milk before drying. The most effective surfactant available is lecithin, which dramatically improves the wetting properties of whole milk powder. When applied at a level of only 0.2 per cent it results in a powder with a dispersibility of 85-90 per cent, equivalent to instant skim milk powder. In comparison, normal whole milk powder has a dispersibility of less than 20 per cent.

Lecithin is a natural surface active agent consisting of a mixture of phosphatide compounds which contain a hydrophylic phosphoric acid radical in a fat molecule, and is similar to the membrane material which stabilizes the fat globule in milk. Because of the relatively high cost of lecithin it is important to minimize the level used. A level of 1 per cent or more is required if the lecithin is added in the presence of water, but lower levels are possible if a non-aqueous solvent is used. Anhydrous milkfat appears to be the most suitable carrier. The mixture is sprayed onto the powder while it is in a fluidized state, either in a fluidized bed or in a medium density pneumatic conveying line. In the latter system powder is picked up by a blowline and transported to a small spray chamber where the milkfat/lecithin mixture is injected through a two fluid atomizing nozzle. The chamber is designed to prevent spray impingement on the walls provided the powder to air density, i.e. the ratio of powder throughput to air flow rate, is sufficiently high. The treated powder is then conveyed for a short period at a temperature of 40°C-60°C. The lower viscosity of the milkfat/lecithin mixture at this temperature allows the material to spread over the particle surface and produce a uniform coating. The powder is then cooled and preferably gas packed to maintain flavour.

In addition to whole milk powder, this lecithin instantizing technique can be applied to a range of fat-containing powders and infant formulations, resulting in products with increased consumer acceptability due to their ease of reconstitution in cold water. The coating system described here can also be used for adding small percentages of liquid additives to other dairy products. The inherent flexibility of a pneumatic conveying line means that it is easily adapted to widely different equipment configurations.

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Effect of Lecithin Feeding on Serum Lipoproteins*

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Science is still a long way from explaining the relationship of the lipoproteins to atherosclerosis. Studies reported here and related ones may eventually become a part of the mosaic which gives the final answer.

Introduction

GOFMAN AND ASSOCIATES,[1,2] employing their ultracentrifugal technic, have observed that a certain class of serum lipoproteins appears to be intimately related to the incidence of atherosclerosis. These are present in the so-called low-density lipoproteins of serum, which can be separated by fractional centrifugation after adjusting the serum to a specific gravity of 1.063 with a more dense sodium chloride solution. Flotation of lipoproteins occurs in this process because the final mixture of serum and salt solution has a density greater than that of the lipoproteins. Thus, rather than sedimentation taking place, the lipoprotein molecules actually are displaced in a direction opposite to the centrifugal field. Once isolated, these "low-density lipoproteins" can be separated into classes or groups by means of an analytical ultracentrifuge in which they are subjected to centrifugal forces of 200,000xg in a sodium chloride solution of specific gravity 1.063. With precision photographic data obtained during centrifugation, it is possible to quantitate the levels of these different classes of lipoproteins in serum. Based on their relative flotation rates, the various classes have been characterized in terms of Svedberg units of flotation (S_f) and are indicated by the notations S°_f 0-12, S°_f 12-20, S°_f 20-100 and S°_f 100-400, the ranges in each case referring to the lowest and highest relative flotation rates for the lipoproteins comprising that class. The concentration of each class of lipoproteins is expressed in terms of mg. per 100 cc.

Gofman and associates[1] have demonstrated that in both sexes and in all age groups the S°_f 12-20 serum lipoproteins are present with a much higher frequency and at higher concentrations in patients who have survived a myocardial infarction than in corresponding individuals without known vascular disease. Moreover, in both males and females in all age groups, this lipoprotein class occurs with increased frequency and concentration in other diseases characterized by prematurely intense atherosclerosis (diabetes mellitus, the nephrotic syndrome, hypothyroidism, and hypertension). These observations have led Gofman and co-workers to the thesis that this lipoprotein class supplies the lipid for atheroma formation; and the descriptive term for this class, "atherogenic lipoproteins," has come into more or less common usage.

The relationship between serum lipoproteins and atherosclerosis has been further clarified recently by Hanig and collaborators,[3] who demonstrated the presence of the S°_f 12-100 fraction in excised atheromatous plaques. The S°_f 12-100 fraction was always present in substantial amounts in aortas revealing atherosclerosis, whereas the S°_f 0-12 lipoproteins were almost completely absent regardless of the presence or absence of atheromatous plaques. This observation implies that this latter fraction is unassociated with atherogenesis and is a normal component of serum.

In view of the association of the S°_f 12-20 lipoproteins and atherosclerosis, it is of great practical importance to consider means by

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to Grant's parabolic F trend analysis[7] and was also characteristic of the male and female groups when analyzed separately.

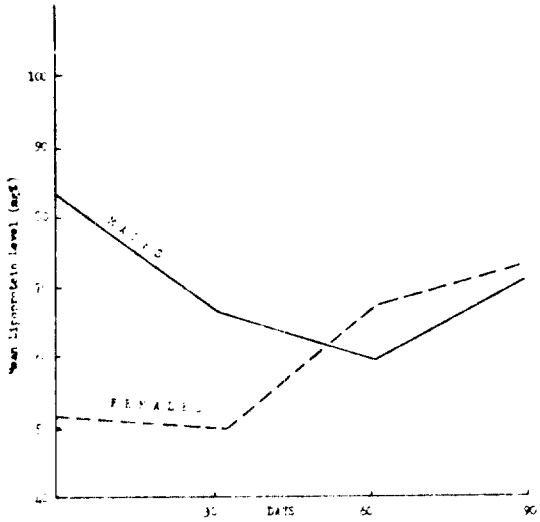
In neither the S°_{f} 0-12 nor the S°_{f} 12-20 lipoprotein fraction was a significant difference in trend between men and women patients observed. However, a sex difference was observed in the case of the S°_{f} 12-100 fraction. In figure 3 it is apparent that the level of the S°_{f} 12-100 fraction in men was considerably higher than in women initially, and decreased significantly in men during the 90 day feeding period. The effect of lecithin feeding was quite the opposite in the female group, causing a significant increase in this fraction. Analysis of these data for both female and male groups revealed a statistically significant linear trend.

Four of the men studied appeared to be more alert toward the end of the study. The systolic and diastolic blood pressures were progressively lowered in 14 of the 18 patients during the 90 days of lecithin feeding.

Discussion

The results of this study reveal that a dietary supplement of lecithin produces a sharp decline in the S°_{f} 12-20 fraction of serum during the first 30 days with an eventual rise to above normal levels by the end of the following 60 days. These data suggest that since lecithin feeding will transiently lower the atherogenic lipoprotein fraction, intermittent administration of lecithin would retard atherogenesis. In this connection it is of interest that Steiner and Domanski[8] have observed a marked lowering of serum cholesterol after 35 days of lecithin feeding. Since Hanig and associates[3] observed the presence of the S°_{f} 12-100 lipoprotein fraction in atheromatous plaques, the potential therapeutic value of lecithin in the male, at any rate, is further indicated by the reduction in the S°_{f} 12-100 fraction produced by lecithin feeding. The opposite effect of lecithin on this fraction in women may be related to their substantially lower initial level of S°_{f} 12-100 lipoproteins.

FIG. 3



Levels of S°_{f} 12-100 Lipoproteins During Lecithin Feedings in Male and Female Patient Groups

In contrast to the S°_{f} 12-20 and S°_{f} 12-100 lipoprotein fractions, the S°_{f} 0-12 fraction, which exhibits the lowest flotation rate, showed no change in concentration following lecithin administration. This suggests that no appreciable conversion of other fractions into this fraction occurred and that it is the most stable fraction of the entire lipoprotein spectrum.

It is important to note that the patients in this study range in age from 70 to 90 years. Lipoprotein levels of individuals in this age bracket have been shown by Chapman and collaborators[9] to be as low as levels observed in the 20 to 29 year age group. The levels reported in this study are in agreement with those of Chapman and his group. It is conceivable that if subjects with higher lipoprotein concentrations, such as normal middle-aged subjects or patients with diabetes mellitus, hypothyroidism, or myocardial infarction, had been employed in this study, the changes following lecithin feeding would have been more striking.

Summary

A group of aged male and female patients with a diagnosis of psychosis with cerebral arteriosclerosis, who were fed a dietary supplement of lecithin for a period of 90 days, demonstrated a definite lowering of serum S°_{f} 12-20 lipoprotein concentration followed by a return to above the initial level at the end of the feeding period. The S°_{f} 12-100 fraction was observed to decrease in concentration in the male patients but increase in female patients. The S°_{f} 0-12 lipoprotein fraction remained unchanged in concentration during the experimental period.

In view of the ability of lecithin to transiently reduce S°_{f} 12-20 lipoprotein concentration, it is suggested that intermittent lecithin feeding may retard atherogenesis.

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Discussion (Abstract)

Dr. Merritt W. Foster, Jr., Richmond, Va. I would like to compliment Dr. Barak and his fellow authors on presenting an interesting paper from the standpoint of their approach. This particular method of studying the management of the aged psychiatric patient indicates a continued trend in the closer relationship of neuropsychiatry and biochemistry. The field of neuropsychiatry has in recent years become closely allied with many other related fields, particularly in the realm of the social sciences, and it is stimulating to see a continuing alliance with the physical sciences as well.

I had hoped that there would be more discussion and correlation of clinically observed behavior with lipoprotein alteration and would be interested if the authors could elaborate on this point beyond the information given in the paper.

I would also be interested in a discussion of the dietary intake of the patients involved in the study. This could be a very pronounced and possibly deciding factor in the values obtained unless the diet were constant throughout the study.

Finally, it is interesting to note, as demonstrated in figure 3, that the lipoprotein in females of this aged group does not closer approach that of the males. This is particularly interesting since blood cholesterol in the two sexes does approach the same figure in older people. It is generally accepted that blood cholesterol levels in females prior to the menopause is definitely lower than that of males but following the menopause approaches the same level. I would be interested in the authors' comments on this apparent disparity in their findings with lipoprotein changes.

Dr. Cortez F. Enloe, Jr., New York, N. Y. The most significant fact revealed by this research is that this combination of phosphatides "RG" Lecithin has, in this instance and under the conditions of this study, demonstrated its ability to alter the lipoprotein pattern of elderly, and presumably, arteriosclerotic patients. In a sense this is not surprising, for our theoretical knowledge of this substance suggests that such results may be expected. If the work is confirmed, we have a new therapeutic or, more accurately stated, nutritional agent for use in the prophylaxis and treatment of this disease.

We would call your attention to the fact that there was an elevation in the mood of these patients. This has been reported previously as a result of lecithin therapy. Unfortunately, there is no biochemical measurement for this phenomenon, but it is significant that a great many investigators using lecithin in elderly patients do notice this subjective response in mood elevation or, better stated, a sense of well-being that ensues.

The substance used in the treatment of these patients was a refined grade of Soya Lecithin. It is the only "RG" type available and is an alcohol soluble RAIG fraction of lecithin, inositol and cephalin phosphatides. It is thus very high in unsaturated fatty acid content which very likely explains its effect we are observing on serum cholesterol.

As we see it, this study demonstrates in a limited manner the fact that "RG" Lecithin can disturb the homeostatic mechanism which is deranged in atherosclerosis. The curious fact here is that these deranged lipoprotein levels tend to shift to more normal patterns, but then return to the more pathologic side on prolonged feeding.

We have asked ourselves why this beneficial effect noted at the thirty day interval does not persist. The only answer we might offer is that the homeostatic mechanism which is deranged in atherosclerosis drifts toward the normal in earlier therapy, then gradually readjusts itself and reverts to the pathologic. It causes us to speculate that in using "RG" Lecithin in the treatment of atherosclerosis, one might feed the phosphatide with interrupted "rest periods." Thus, the inborn defect might not become unresponsive to the therapy.

which the serum lipoprotein spectrum may be altered so as to reduce the atherogenic fraction. Nichols and associates[4] have reported that vegetable fat will effect a decrease in the S°_{f} 12-20 lipoprotein concentration. This effect has been attributed to the unsaturated fatty acids present in the vegetable fat.

Lecithin, one of the group of lipids known as phospholipids, plays an important role in lipid metabolism in liver. Normal lecithin turnover in liver appears in some obscure way to be intimately associated with the ability of liver to oxidize fat and with mobilization of fat from liver to peripheral sites.[5] Since serum lipoproteins are believed to be derived from liver, it is conceivable, in view of this relationship of lecithin to lipid metabolism in liver, that feeding lecithin could favorably alter the serum lipoprotein spectrum. Moreover, since lecithin contains a high percentage of unsaturated fatty acids, primarily linoleic and linolenic acids, it is reasonable to expect, in view of the observation of Nichols and co-workers,[4] that lecithin administration would reduce the S°_{f} 12-20 lipoprotein fraction. Pertinent, also, to a consideration of the possible efficacy of lecithin feeding, is the observation of Ahrens and Kunkel[6] which may be interpreted to mean that serum lipoproteins require a certain complement of lecithin in the lipid prosthetic group for maximum stability.

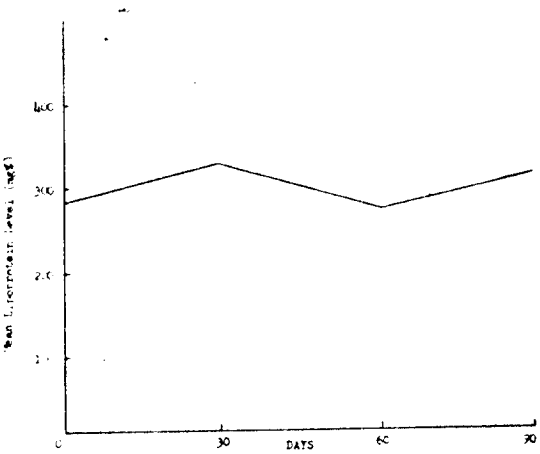
The present study was designed to determine the effect of lecithin feeding on the serum lipoprotein spectrum.

Methods

Nine male patients and 9 female patients ranging in ages from 70 to 90 years at the Norfolk State Hospital in Nebraska were used in this study. All were diagnosed as suffering from psychosis with cerebral arteriosclerosis but were sufficiently cooperative to take oral medication. Blood samples were taken in the fasting state prior to the administration of lecithin,* and then serially every 30 days throughout a feeding period of 90 days. One tablespoon of lecithin three times a day, which amounted to approximately 23 Gm. of lecithin per day, was given as a supplement to the normal ward diet during this period.

*RG Soya Lecithin was kindly supplied by The Glidden Co., Chicago, Ill.

FIG. 1

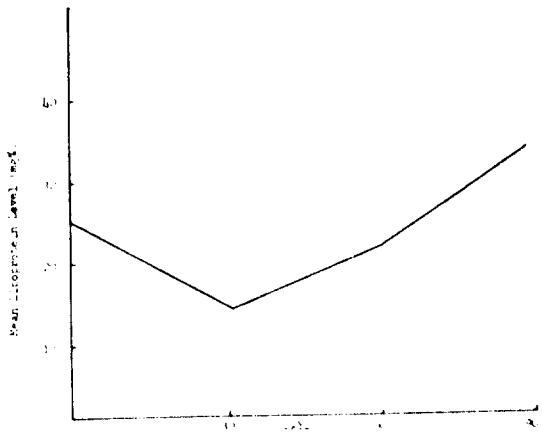


Levels of S°_{f} 0-12 Lipoproteins During Period of Lecithin Feeding in Total Patient Group

Results

The S°_{f} 0-12 serum lipoprotein fraction does not appear to be altered by the feeding of lecithin. Figure 1 shows the average value for this fraction in the entire group of patients at the end of each of the 30 day intervals. Statistical analysis of these data by the method of Grant[7] shows that the changes with time are not significant. Figure 2 presents the corresponding data for the S°_{f} 12-20 fraction. A definite lowering of this fraction during the first 30 days was observed with a return to above the initial level by the end of the 90 day period. This lowering with the subsequent rise has been found to be significant according

FIG. 2



[illegible] Lipoproteins During Period of Lecithin Feeding in Total Patient Group

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THE EFFECT OF LECITHINE ON FAT DEPOSITION IN THE LIVER OF THE NORMAL RAT.

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and M. ELINOR HUNTSMAN.

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The very interesting effects of some component of crude lecithine upon the condition of diabetic animals have been discussed in previous communications from this laboratory [Hershey, 1930; Hershey and Soskin, 1931; Best and Hershey, 1932]. The symptoms exhibited by these animals and the autopsy findings indicate that the characteristic condition is largely attributable to failure of liver function. The results of these studies suggested that an investigation of the effect of lecithine on deposition of fat in the livers of normal animals might be profitable. The literature relevant to this subject has been reviewed in the monographs by Leathes and Raper [1925] and by MacLean and MacLean [1927]. As far as we know, the particular problem in which we are interested has not been investigated by previous workers. The experiments were planned with a view to obtaining deposition of large amounts of fat in the livers of a group of control animals. This was accomplished by feeding a diet high in fairly saturated fats. A second group of animals comparable in every way, as far as we could determine, with the controls, received the same amount of fat as those in the control group and *in addition* varying amounts of crude or purified lecithine. The results demonstrate, among other points, that crude and purified lecithine influence the accumulation of fat in the livers of the test group of animals.

Methods.

White rats of the Wistar strain, weighing between 150 and 230 g., were used as test animals. Previous to the experiment they had been receiving a stock diet which was adequate in all respects. All the animals used in the experiment were apparently healthy, and great care was taken to ensure that the various groups into which they were divided for any one experiment were as similar as possible. Much time was wasted

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in preliminary experiments in which attempts were made to use groups of animals in one cage, but this procedure was not found to be feasible. In all the experiments reported in this paper individual cages were used, and notes on the condition of each animal were made throughout the experiment. Every animal in any experiment ate approximately the same amount of the stock diet, which has the following composition: 32·5 p.c. of each of the following—whole cracked wheat, rolled oats and corn meal, and 2·5 p.c. of bone meal. The amounts of fat and of the lecithine under test are given in each of the tables or summaries. In all cases the fat was provided in the form of beef dripping, which has an iodine number of approximately 40. The food was prepared daily. The dripping was melted and added to the stock diet. The mixture was then heated in a boiling water bath for 2 hours, with intermittent stirring. The stirring was continued during the subsequent cooling to ensure the equal distribution of the added fat. The lecithine was thoroughly mixed with the other food by prolonged grinding. The diet for each rat was carefully weighed each day, and in cases where food was left in the cage at the end of 24 hours the amount was determined by weighing. The food trays were designed so that there was little or no food spilled, and the construction of the cages made it possible to recover any spilled food without difficulty. Fat estimations on the food residue showed that the fat had been thoroughly mixed with the other constituents. At the end of the experiment the animals were stunned and decapitated. The animals were not fed on the day on which they were killed. Blood samples were collected from the severed carotid arteries. The livers were removed as soon as the animals were dead, and the content of fatty acid determined by the Leathes and Raper modification of Liebermann's saponification method. The iodine number of the fatty acid of the liver was determined by Wijs' procedure, as outlined by Leathes and Raper. The phospholipines of the liver and blood were calculated from the phosphate content as determined on the alcohol-ether soluble portion by the Briggs' modification [1922] of the Bell-Doisy method. Glycogen was estimated by the Pflüger technique.

Experimental results.

Egg yolk lecithine. In the first experiment ten rats were used in each of the two groups. The details of the experiments and the results are shown in Tables I and II. The egg yolk lecithine used in these experiments was secured from a commercial firm and was prepared from dried egg yolk, after removal of the fats with benzene, by alcoholic extraction of

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TABLE I. Fat diet. Stock diet with fat added (fat 40 p.c. of total food).

Rat No.	Length of experiment (days)	Total fat eaten (g.)	Fat eaten daily (g.)	Wt. of rat before (g.)	Wt. of rat after (g.)	Wt. of liver (g.)	Fatty acids in liver (p.c.)
1	36	99	2·8	170	188	7·77	20·50
2	36	95	2·6	154	167	5·02	5·88
3	35	91	2·6	152	160	4·71	4·20
4	36	94	2·6	178	175	5·58	4·05
5	35	97	2·8	155	167	4·42	5·52
6	36	94	2·6	135	144	4·78	5·12
7	38	100	2·6	121	135	4·51	3·67
8	38	100	2·6	150	144	5·22	6·87
9	38	100	2·6	149	152	6·44	11·30
							Average = 7·36

TABLE II. Fat diet with crude egg lecithine. Stock diet with fat (fat 40 p.c. of stock diet and fat ration) and with lecithine.

Rat No.	Length of experiment (days)	Total fat eaten (g.)	Fat eaten daily (g.)	Wt. of rat before (g.)	Wt. of rat after (g.)	Lecithine daily (g.)	Wt. of liver (g.)	Fatty acids in liver (p.c.)
11	35	102	2·9	181	185	1·4	7·32	3·20
12	36	89	2·5	149	159	1·2	4·57	3·78
13	36	90	2·5	162	157	1·2	6·33	3·38
14	38	103	2·7	193	189	1·3	7·34	3·09
15	38	93	2·4	154	148	1·2	6·86	3·29
16	35	96	2·7	190	182	1·3	7·61	3·30
17	35	91	2·6	144	135	1·3	6·37	3·75
18	38	96	2·5	140	147	1·2	5·48	3·12
19	38	99	2·6	176	170	1·3	6·59	3·97
20	38	108	3·1	185	192	1·5	6·85	3·50
								Average = 3·38

the phospholipines and subsequent removal of the alcohol *in vacuo*. The results of the experiments in which this material was used appear to demonstrate in a convincing manner that some component of lecithine modifies the deposition of fat in the livers of the test animals. The figures also illustrate the very important fact that there is a tremendous variation in the deposition of fat in the livers of different members of a group of animals, each of which is receiving approximately the same amount of fat in the diet.

In most of the experiments all the rats consumed a satisfactory amount of fat. In certain cases, however, the quantity eaten by some of the test animals was not of the same order as that ingested by the controls. The figures for these animals have not been included in the results.

The effects of smaller amounts of lecithine. To study the effects on fatty acids of the liver when small amounts of lecithine were provided, a group of thirty white rats was used. The animals were divided into three groups of ten each, care being taken that the groups were as similar as possible.

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Ten rats received 6·15 g. of stock diet *plus* fat 3·5 g. (beef dripping, iodine number 40). Fat formed 40 p.c. of the total food. The experiments lasted 26 days. The average amount of fat taken daily was 3·4 g. The average weight of the rats at the beginning of the experiment was 190 g., and at the end 182 g. The percentage of fatty acids in the livers varied from 7·0 to 25·3 p.c. The average was *15·7* p.c. The iodine numbers varied from 78 to 108. The average was 95. One extraordinarily high iodine number was probably due to an error, and that result is not included in this average.

In the second group all rats received the same amount of stock diet and fat, and in addition egg yolk lecithine. The lecithine formed 9·4 p.c. of the total food. The average figure for the fat eaten daily was 2·97 g., while that for the lecithine was 0·85 g. The average weight of the rats at the beginning of the experiment was 191 g., and the average weight at the end was 177 g. The percentage of fatty acids in the liver varied from 3·3 to 5·1. The average was *3·7* p.c. The iodine number varied from 101 to 136, the average was 129.

The diet for the third group was exactly the same as that of the second, except that lecithine formed 4·9 p.c. of the diet instead of 9·4 p.c. The average figure for the amount of food eaten daily was 3·12 g., while the average figure for the lecithine was 0·44 g. The average figure for the weight at the beginning of the experiment was 191 g., and at the end 187 g. The percentage of fatty acid in the liver varied from 3·1 to 8·2 p.c. The average figure was *4·6* p.c. The iodine numbers varied from 83 to 107. The average figure was 98.

These results appear to furnish evidence that as little as 0·44 g. of crude egg yolk lecithine daily is sufficient to prevent the deposition of large amounts of fat in the liver of a rat under the conditions of these experiments. It will be observed that the rats in the test groups in this series did not eat quite as much added fat as the controls. We have no hesitation in reporting these results, however, for the following reasons: (1) In many other experiments rats from the same colony have invariably shown as high an average fat content of the liver as the test animals in this particular experiment. In these other experiments the controls have eaten even less fat than that taken by the test animals of this experiment. (2) The figures for the individual rats show that many of the animals did eat as much fat as the controls. (3) The test animals ate a certain amount of fatty material in the crude lecithine, which can perhaps be placed to their credit. (4) Furthermore, a study of the tables which contain the results of the determinations made on the control rats demonstrates the fact that the extent of the deposition of liver fat is not proportional to the amount of fat eaten when this amount varies between 2 and 3·5 g. per day. For example, in Table IX the rats which ate 2·3 g. of fat daily showed a much higher fat content than several which ate 2·5 g.

The effects of purified lecithine. Crude egg yolk lecithine was purified by the cadmium chloride procedure of Levene and Rolf [1927]. This fractionation was carried out by one of us (M. E. H.) in the Department of Biochemistry under the direction of Prof. H. D. Kay. The purified lecithine (iodine number 65) obtained was tested on a group of animals.

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The results are shown in Tables III and IV. These results establish the fact that purified egg yolk lecithine exerts the same effect as that produced by the crude material.

TABLE III. Fat diet. Stock diet with fat added (fat 40 p.c. of total food).

Rat No.	Length of experiment (days)	Total fat eaten (g.)	Fat eaten daily (g.)	Wt. of rat before (g.)	Wt. of rat after (g.)	Fatty acids in liver (p.c.)	Iodine No.
1	22	54·55	2·48	300	253	6·35	105
2	22	54·55	2·48	202	174	19·2	100
3	22	54·35	2·47	190	194	27·6	—
4	22	54·20	2·46	179	171	19·3	97
5	22	53·60	2·43	189	172	16·2	102
6	20	48·00	2·40	180	177	20·5	95
7	22	54·60	2·48	177	180	15·9	96
8	22	52·90	2·40	169	166	28·2	102
9	22	54·10	2·45	162	160	12·5	104
10	22	53·80	2·44	150	150	17·2	77
					Average =	18·3	

TABLE IV. Fat diet with purified lecithine. Stock diet with fat (fat 40 p.c. of stock diet and fat ration) and with lecithine.

Rat No.	Length of experiment (days)	Total fat eaten (g.)	Fat eaten daily (g.)	Wt. of rat before (g.)	Wt. of rat after (g.)	Lecithine daily (g.)	Fatty acids in liver (p.c.)	Iodine No.
11	22	52·80	2·40	278	257	0·48	3·49	137
12	22	53·60	2·43	203	177	0·49	3·65	107
13	22	49·50	2·35	190	150	0·47	7·4	113
14	22	52·30	2·38	180	171	0·48	3·15	117
15	22	53·00	2·41	199	186	0·48	3·98	118
16	22	46·90	2·13	184	164	0·43	3·58	124
17	22	48·70	2·22	180	166	0·44	3·74	139
18	22	52·80	2·40	170	167	0·48	8·4	76
19	22	51·60	2·34	160	155	0·47	4·05	129
20	22	53·90	2·44	160	151	0·49	4·11	128
						Average =	4·5	

Lecithine from beef liver. In the next series of experiments lecithine, prepared from fresh beef liver, was tested. The lecithine fraction was prepared in the following manner: fresh beef liver was minced and dried in a vacuum oven at low temperature. The dried material was ground and then thoroughly extracted with absolute ethyl alcohol. The material was filtered through paper and the filtrate evaporated to small volume in an efficient vacuum still. The residue was evaporated to a syrup in a large glass flask, and ether was then added until no more material would go into solution. The mixture was allowed to stand overnight in a cold room, and then filtered through paper. The clear ethereal solution was added with stirring to three volumes of acetone. After 48 hours the supernatant fluid was decanted, and the precipitate removed. This material was then

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stored over paraffin in an atmosphere of nitrogen. This was the material which was subsequently purified by the cadmium chloride procedure. We are indebted to the Connaught Laboratories for the fresh beef liver, for the various reagents, and for the use of the large apparatus required in the preparation of the lecithine.

The effect of crude beef liver lecithine. The results (Tables V and VI) demonstrate that 1 g. and 0·5 g. of lecithine from this source daily are capable of preventing the deposition of fat in the liver of normal white rats.

TABLE V. Fat diet. Stock diet with fat added (fat 40 p.c. of total food).

Rat No.	Length of experiment (days)	Total fat eaten (g.)	Fat eaten daily (g.)	Wt. of rat before (g.)	Wt. of rat after (g.)	Fatty acids in liver (p.c.)	Iodine No.
1	21	52·5	2·5	189	181	29·6	110
2	21	52·5	2·5	171	173	14·5	109
3	21	52·5	2·5	170	172	20·3	116
4	21	52·5	2·5	226	206	25·1	102
5	21	52·5	2·5	164	165	9·2	110
6	22	54·0	2·45	175	167	21·2	100
7	22	55·0	2·5	173	169	16·6	115
8	22	55·0	2·5	169	160	7·8	133
9	22	55·0	2·5	158	154	20·7	63
10	22	55·0	2·5	150	142	7·9	138
					Average =	17·3	

TABLE VI. Fat diet with crude liver lecithine. Stock diet with fat added (fat 40 p.c. of stock diet and fat ration) and with lecithine.

Rat No.	Length of experiment (days)	Total fat eaten (g.)	Fat eaten daily (g.)	Wt. of rat before (g.)	Wt. of rat after (g.)	Lecithine daily (g.)	Fatty acids in liver (p.c.)	Iodine No.
32	22	52·4	2·38	209	200	0·95	3·1	120
33	22	54·2	2·47	160	168	0·99	4·6	116
34	22	53·7	2·44	172	188	0·97	4·9	175
35	22	51·8	2·36	192	187	0·94	9·5	172
36	22	51·2	2·33	150	143	0·93	4·4	246
37	22	54·2	2·47	151	123	0·99	4·1	192
38	22	51·6	2·35	202	193	0·94	5·1	194
40	22	53·5	2·43	162	175	0·97	3·6	215
41	22	53·2	2·42	170	172	0·48	4·1	205
42	22	55·0	2·50	181	190	0·50	4·0	212
43	22	53·7	2·44	186	192	0·49	5·7	168
44	22	52·5	2·38	165	167	0·48	3·7	213
45	22	53·2	2·42	181	175	0·48	5·7	175

Average 12·4 p.c. lecithine = 4·7
6·7 p.c. lecithine = 4·8

Purified beef liver lecithine. Some 340 g. of purified lecithine (iodine number 75) were obtained by fractionation of the lecithine according to the Levene and Rolf cadmium chloride procedure. Varying amounts of this lecithine were added to the stock diet in the manner described

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above. The smallest effective daily dose studied was 0·1 g. The results of the experiment in which 0·25 g. was given daily are slightly more consistent, however, and these are given in Tables VII and VIII.

TABLE VII. Fat diet. Stock diet with fat added (fat 40 p.c. of total food).

Rat No.	Length of experiment (days)	Total fat eaten (g.)	Fat eaten daily (g.)	Fat excreted daily (g.)	Wt. of rat before (g.)	Wt. of rat after (g.)	Fatty acids in liver (p.c.)	Iodine No.
1	20	50·0	2·50	0·17	210	200	9·2	97
2	20	50·0	2·50	0·15	190	184	11·0	95
3	20	50·0	2·50	0·16	170	174	16·0	90
4	20	50·0	2·50	0·15	170	166	8·5	94
5	21	52·5	2·50	0·17	180	176	21·1	—
6	21	52·5	2·50	0·16	176	174	8·2	100
7	21	52·5	2·50	0·16	180	170	11·7	95
8	21	52·5	2·50	0·17	182	170	28·2	97
9	21	51·5	2·46	0·18	166	152	13·8	89
10	21	52·5	2·50	0·14	162	158	16·2	91
						Average =	14·4	

TABLE VIII. Fat diet with purified liver lecithine. Stock diet with fat added (fat 40 p.c. of stock diet and fat ration) and with lecithine.

Rat No.	Length of experiment (days)	Total fat eaten (g.)	Fat eaten daily (g.)	Fat excreted daily (g.)	Wt. of rat before (g.)	Wt. of rat after (g.)	Lecithine daily (g.)	Fatty acids in liver (p.c.)	Iodine No.
11	21	52·5	2·50	0·17	160	145	0·25	5·30	107
12	21	52·5	2·50	0·20	160	152	0·25	6·55	111
13	21	52·5	2·50	0·18	180	170	0·25	5·85	112
14	21	51·5	2·45	0·19	230	220	0·24	3·87	117
15	21	51·5	2·45	0·18	160	160	0·24	5·85	115
16	21	52·5	2·50	0·15	180	172	0·25	5·40	109
17	21	52·5	2·50	0·15	164	152	0·25	5·08	110
18	21	52·5	2·50	0·15	198	190	0·25	4·27	118
19	21	51·5	2·45	0·16	188	180	0·24	6·12	110
							Average =	5·76	

Proof that the excretion of fatty acids plays no part in the effects of lecithine. Although these results may be shown subsequently to have little physiological significance, they are of considerable interest provided that the effect of lecithine is not due to some relatively unimportant mechanism. The only obvious pitfall seems to be that the lecithine might increase the excretion of fat. In preliminary experiments in which the food residue and fæces were examined for fat, no evidence could be obtained that more fat was lost in the animals on lecithine than in others. In an attempt to make this point perfectly clear, however, the fat in the fæces has been estimated by the saponification method, for each of the members of control and test groups of animals, in three experiments. The fæces were collected daily and were immediately placed in the alkali.

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The figures in Tables VII and VIII show that there is no difference in the fat excretion of the control and test groups, and they provide very strong evidence that fat excretion is not a significant factor in the interpretation of these results. In the other experiments in which phospholipines prepared from beef liver and egg yolk lecithine prevented deposition of liver fat, the fat excretion of the animals which received the lecithine was of the same order as that of those on the control diet. The figures for the experiment in which egg yolk lecithine was used are given in Tables IX and X. These results, in addition to the data on fat excretion which they provide, furnish further evidence of the effect of phospholipines derived from egg yolk on deposition of liver fat.

TABLE IX. Fat diet. Stock diet with fat added (fat 40 p.c. of total food).

Rat No.	Length of experiment (days)	Total fat eaten (g.)	Fat eaten daily (g.)	Total fat excreted (g.)	Fat excreted daily (g.)	Wt. of rat before (g.)	Wt. of rat after (g.)	Fatty acids in liver (p.c.)	Iodine No.
1	21	52·5	2·50	2·46	0·12	171	150	8·9	110
2	21	51·0	2·43	2·19	0·11	155	145	21·5	86
3	21	52·1	2·48	2·74	0·14	158	148	14·8	106
4	21	52·1	2·48	2·67	0·13	200	195	17·3	100
5	21	52·5	2·50	2·49	0·12	174	170	12·3	89
6	21	51·0	2·43	2·69	0·13	165	150	27·7	84
7	21	52·5	2·50	2·38	0·12	163	160	25·0	97
							Average =	18·2	

TABLE X. Fat diet with crude egg lecithine. Stock diet with fat added (fat 40 p.c. of stock diet and fat ration) and with lecithine.

Rat No.	Length of experiment (days)	Total fat eaten (g.)	Fat eaten daily (g.)	Total fat excreted (g.)	Fat excreted daily (g.)	Wt. of rat before (g.)	Wt. of rat after (g.)	Lecithine daily (g.)	Fatty acids in liver (p.c.)	Iodine No.
11	21	51·5	2·43	2·11	0·10	153	150	0·49	3·75	—
12	21	48·7	2·32	1·65	0·08	177	162	0·46	3·50	133
14	21	52·0	2·47	3·16	0·16	181	175	0·49	3·32	140
16	21	50·0	2·38	2·33	0·12	161	137	0·48	3·15	137
20	21	51·6	2·45	2·27	0·11	150	148	0·49	3·50	114
							Average =		3·44	

Lecithine in liver and blood. Determinations of alcohol-ether soluble phosphorus on samples of liver and blood from control and test animals were very kindly made for us by Dr Kay of the Department of Biochemistry. The average liver value, calculated as lecithine, in four control animals was 2·94 p.c. In four test animals each of which ate approximately 0·85 g. of egg yolk lecithine daily the value was 3·38 p.c., and in four test animals which ate 0·14 g. of the same material 3·20 p.c. We are not prepared to conclude without further experiments that the difference

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between control and test animals is significant, but it is interesting to note that a very large proportion of the total fatty acid in the livers of the test animals must have been derived from the phospholipines. The livers of test animals which contained 3·38 p.c. lecithine showed an average value of 3·85 p.c. fatty acid, those with 3·20 p.c. lecithine 3·80 p.c. fatty acid. The livers of the controls contained 2·94 p.c. lecithine and 11·2 p.c. fatty acids.

The results for the phospholipines, calculated as lecithine, for the blood samples from control animals demonstrate that these values are definitely lower than those obtained from the animals given lecithine. While the animals were not fed on the day on which they were killed, and while we are certain that some of the animals did not eat anything for at least 5 hours previous to their death, we cannot conclude that absorption of lecithine from the intestine did not influence these values obtained for the blood.

Glycogen. Glycogen determinations on samples of the livers of control and test animals have been made for us by our colleague Dr E. T. Waters. Both the fatty livers of the control and the relatively fat-free specimens from the lecithine animals may contain normal quantities of glycogen. The results thus far obtained do not suggest that there is any significant difference in the amounts of liver glycogen in the two groups.

Fatty acid content of whole animal. In several experiments the total fatty acid content of control and test animals has been determined by the saponification procedure. The figures suggest that the animals which had lecithine do not contain as much fatty acid as the controls. The difference is not great, however, and further experiments are required to settle the point. The iodine numbers of the fatty acids obtained from the bodies (livers excluded) of the control and test animals were of the same order.

Discussion.

A review of the literature shows that there is very little information available concerning the effects of diets rich in fat on the deposition of fat in the livers of various species of animals. In the experiments which we carried out during the hot summer weather, difficulties were encountered in securing a high average fat content in the livers of white rats. During the autumn and winter, however, a daily ration containing approximately 2·5 g. of fairly saturated fat has consistently produced a high average fat content in the livers of the control animals (Tables I, III, V, VII and IX). These results are so consistent that it might be considered unnecessary to provide a group of control animals for every new

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test if one could be absolutely certain that the rats were from the same colony and had received the same diet and attention. It is certain, however, that rats from one colony cannot be used as controls on those from another. In one experiment in which a series of rats from a new colony was used the results had to be discarded because we made the mistake of assuming that the control group would exhibit fatty livers on the same diet as that received by the control rats referred to in the above tables. None of the rats from the new colony which were used as controls had a high liver fat. The cause for this great difference between rats from various colonies would be an interesting subject for further study.

The results of the experiments reported in this paper provide evidence that the addition of crude and purified lecithine from egg yolk and from fresh beef liver to the diet of normal white rats prevents the accumulation of liver fat. These results are very clear-cut. The evidence appears satisfactory that the effect is not due to excretion of fat by the animals on the "lecithine" diet. The fæces were carefully examined by one of us each day, and it appeared probable, even before the actual estimation of the fæcal fat in the control and test groups had been made, that fat excretion by the test animals was not a significant factor in the interpretation of the results. It is in the highest degree unlikely that the saponification method would fail to detect fatty material in one case and not in the other. It is also most improbable that the fat in the intestine of the lecithine-fed animals would be decomposed beyond the fatty acid and glycerol stage.

In the determination of the iodine numbers of the fatty acids from various sources the reagents and the technique have been repeatedly checked by determination of the iodine absorption of a sample of Merck's oleic acid. The iodine number of the beef dripping has ranged between 39 and 41. The tables demonstrate that the iodine number of the fatty acids from the livers of the lecithine-fed animals are on the average considerably higher than those of the control groups. While the average figures may have no great significance in this connection, calculation of these values from the experiments which are reported in this paper shows the following result—control rats 100, lecithine-fed rats 132. The iodine number of the fæcal fat varied between 20 and 50, and was usually between 30 and 40. An interesting point arising out of the determination of the iodine numbers is the very high figures obtained for the fatty acids of the liver of certain of the rats which received lecithine prepared from beef liver. These estimations were made with the same reagents and at the same time as those on the control animals. These high

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figures suggest that the livers of certain of the animals receiving crude lecithine may contain a large proportion of highly unsaturated fatty acid, but further study is required on this point. If this result can be confirmed however in experiments on larger animals, a good opportunity might be provided for the isolation and identification of the fatty acid or acids responsible.

In an investigation of the effects of the components of lecithine on deposition of liver fat the results of which will be published shortly by two of us (C. H. B. and M. E. H.), evidence has been obtained that the active component of lecithine is choline. In view of this finding a discussion of the physiological significance of the results given in this paper will not be included here.

Summary.

When white rats weighing between 150 and 230 g. receive a daily ration containing 2·5 g. of fairly saturated fat (iodine number approximately 40) for 3 weeks, their livers may be found to contain very large amounts of fatty acids. The average value of the iodine number of these fatty acids in our experiments was approximately 100. Comparable groups of animals receiving the same diet as above, and in addition varying amounts of crude or purified lecithine prepared from egg yolk or beef liver, do not exhibit this increase in liver fat. The values obtained may be as low or lower than those of animals fed on a normal mixed diet. The average value of the iodine numbers of the fatty acids of this group was approximately 132. No evidence could be obtained that increased excretion of fat plays any part in the interpretation of the effect of lecithine.

The results of a preliminary investigation of the effects of lecithine upon liver glycogen and phospholipines, and upon the distribution of fat in other parts of the bodies of the test animals, are briefly discussed.

We would like to express here our appreciation of the expert technical assistance given us by Miss G. I. Harpell.

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Effect of Phospholipid Fractions upon the Coagulation Defects in Patients on Long-Term Dicumarol Therapy

By Paul W. Boyles, Ralph Jones, Jr., and E. Sterling Nichol

THE RELATIVELY FREQUENT occurrence of bleeding in patients on a long-term anticoagulant regimen emphasizes the need for further study of the effects of such therapy. In this investigation, the coagulation defects following dicumarol administration over prolonged periods were studied. Correction of some of these defects has been effected in vitro and in vivo by phospholipid fractions of human brain or of soy beans.

Materials and Methods

Selection of patients.—The patients under investigation had been on continuous dicumarol therapy (25 mg. to 100 mg. per day) for a minimum period of six months. This group consisted of 30 patients examined weekly in the Out-Patient Clinic of Jackson Memorial Hospital. The severity of cardiac disease and the nature of its management were not taken into consideration in evaluating the data obtained in this study.

Control specimens were obtained from normal individuals. Serum samples from patients with known PTC deficiency and Stuart Factor* deficiency were used as indicated under Results.

Coagulation studies.—Venous blood was collected from non-fasting patients with siliconed syringes. Blood allowed to clot in glass test tubes at 25 C. was used to determine the clotting time. Aliquots of the serum obtained from the specimen were incubated at 37 C. for one hour and four hours after clotting for the prothrombin consumption test and the thromboplastin generation test, respectively. Plasma was obtained from whole blood to which 0.1 M potassium oxalate solution had been added. Plasma and serum were separated by centrifugation at 3,000 × g at 25 C. for 15 minutes. Platelet-rich plasma was obtained from whole blood to which 3.8 per cent sodium citrate solution had been added in siliconed test tubes. The RBC were allowed to settle at 25 C. for 15 minutes, and the supernatant plasma was removed for use in the thrombin generation test.

Soy bean phospholipid was prepared by suspending "Lecithin"† in saline (50 mg./ml.) with a Waring Blendor and autoclaving the material in 50 ml. vials. This material was stored in the deep freeze (−20 C.) until used. Brain phospholipid (25 mg./ml.) was prepared as described by Bell and Alton[5] and stored at −20 C. until used.

1. The thromboplastin generation test[6] was used with brain phospholipid to replace platelet suspensions according to the modification described by Bell[7] unless otherwise indicated.

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*Courtesy of Dr. John Graham, Department of Pathology, University of North Carolina School of Medicine, Chapel Hill, N. C.

†The Glidden Company, Chicago, Ill.

2. One-stage prothrombin[4] was performed with the use of human brain thromboplastin with 0.025 M calcium chloride.

3. The two-stage prothrombin determination[5] was modified by the use of bovine serum and a preparation of Fraction I* which had been purified by adsorption with barium sulfate and subsequent precipitation with 25 per cent ammonium sulfate.

4. Fibrinogen (fibrin) determinations were carried out by a previously described method.[6]

5. Accelerator Globulin (AcG) was estimated by the one-stage method of Stefanini.[7]

6. Antithrombin was determined by the method of Wilson.[8]

7. Prothrombin consumption[9] was modified by employing bovine Fraction I* to replace deprothrombinized plasma.

8. Thrombin generation was assayed by the method of MacFarlane and Biggs.[10]

9. Clotting time was performed by the Lee and White technic.[11]

10. Anti-thromboplastin was determined by the procedure of Fantl and Nance.[12]

11. Serum prothrombin conversion accelerator (SPCA) was estimated by the one-stage method of Owren.[13]

The effects of human brain or soy bean phospholipid fractions on the dicumarol-induced coagulation defects were studied in vitro. The thrombin generation test was performed with the use of 0.1 ml. of saline suspension of these phospholipid materials added to plasma. In the determination of the clotting time 0.9 ml. of whole blood was added to 0.1 ml. of phospholipid suspension.

Results

The 30 patients studied had been on long-term dicumarol therapy. Each blood specimen from these patients demonstrated multiple factor deficiencies. Phospholipid fractions corrected the clotting time without alteration in the level of the coagulation factors.

Studies on the Coagulation Defects Induced by Long-Term Dicumarol Administration

In addition to the known effects of dicumarol on the prothrombin conversion factors, long-term anticoagulant therapy was found to cause a defect in thromboplastin and thrombin generation (fig. 1). Ac-globulin level, prothrombin consumption, fibrinogen† and antithrombin† levels were normal in the patients studied.

It was confirmed that prothrombin values were higher than those indicated by the one-stage method (fig. 2).

The thromboplastin generation test was abnormal in patients on long-term dicumarol therapy. This abnormality in thromboplastin generation was not caused by an increase in antithromboplastin (fig. 3). Barium sulfate absorbable components (i.e., serum components) completely corrected the thromboplastin generation test (fig. 4). In 70 per cent of patients PTC-deficient serum partially corrected the serum, and in 30 per cent it completely corrected the defect. Mixtures of dicumarol, PTC-deficient and Stuart Factor-deficient sera completely corrected the abnormal thromboplastin generation, while combinations of any two of these sera resulted in partial correction (fig. 5). The relative independence of the levels of prothrombin and SPCA

*The Armour Company, Chicago, Ill.

†Unpublished data.

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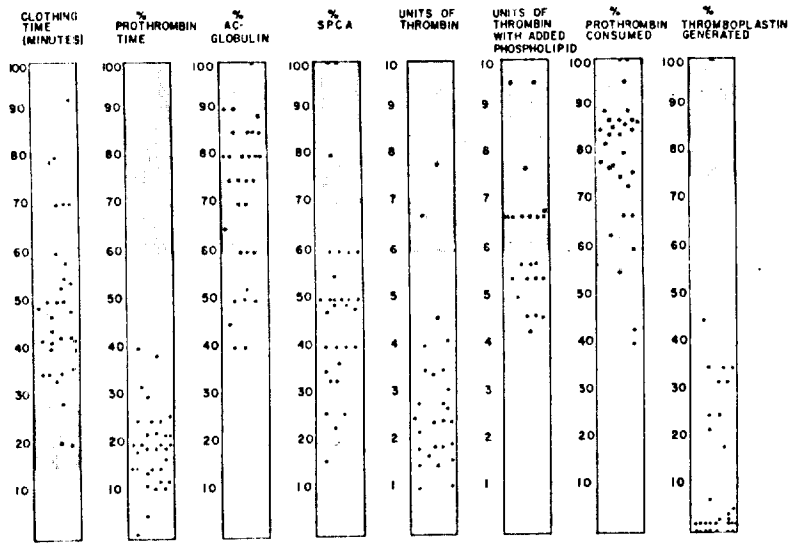

FIG. 1.—Effect of long-term dicumarol on blood coagulation. The dots indicate the actual values obtained in the patients studied. The shaded area represents the normal ranges.

compared to the level of thromboplastin generated from the patient's serum, illustrated by the serial studies in a typical patient, is shown in figure 6.

Effects of a Soy Bean Phospholipid Fraction on the Hypocoagulable State of Long-Term Dicumarol Administration

In vitro studies.—Phospholipid fractions (of human brain or of soy beans) at concentrations of 2.5 mg. per ml. corrected the abnormal clotting times and increased the prothrombin consumption (fig. 7). Acceleration in the rate of prothrombin consumption by phospholipid suspension is illustrated in figure 8. Thrombin generation of blood from patients on dicumarol therapy was uniformly improved by the addition of phospholipid suspension (fig. 9). The corrections are brought about by the phospholipid fractions without alteration in the levels of the prothrombin, SPCA or the serum thromboplastic factors.

In vivo studies.—In these 30 patients on long-term dicumarol administration, six instances of bleeding were encountered during a 12 month period. Hemostasis was generally established within 24 hours after the intravenous administration of vitamin K. Recurrent thrombotic episodes (i.e., the "rebound" phenomenon), due to rapid changes in the concentration of coagulation factors, were not observed.

One patient developed purpura in addition to the other sequelae of dicumarol therapy (i.e., prolonged clotting and prothrombin times and defective generation of thromboplastin and of thrombin.) After the intravenous administration of 2.5 mg. of vitamin K, the patient's hemorrhagic status and defective coagulation remained unchanged for 24 hours. Soybean phospho-

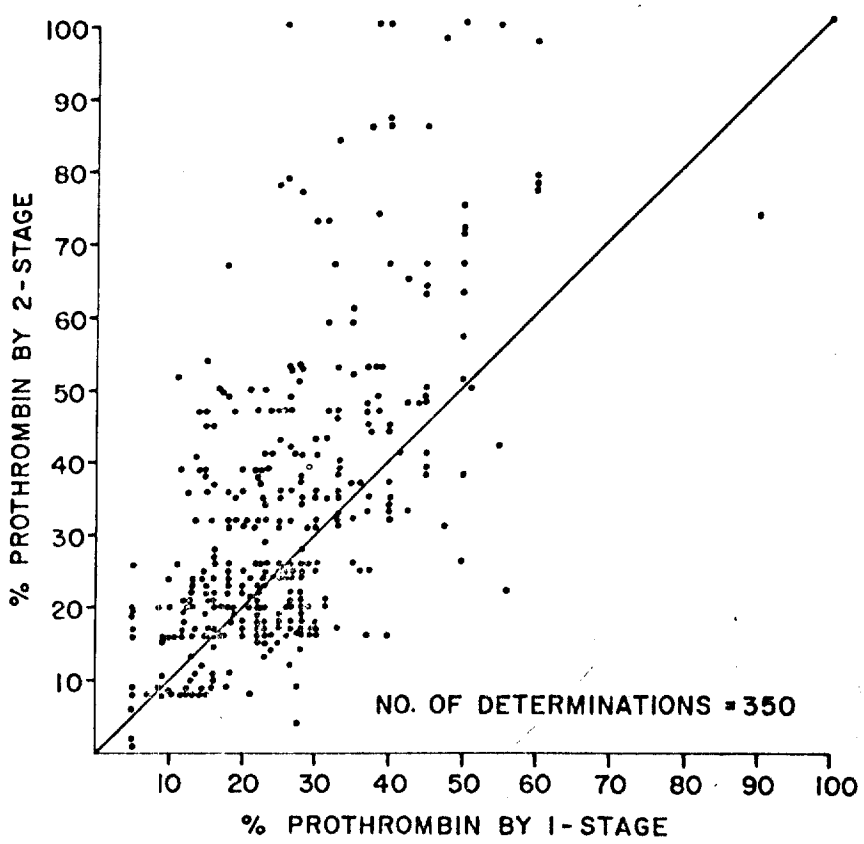

FIG. 2.—Comparison of the one-stage and modified two-stage methods of prothrombin determination. The percentage prothrombin determined in patients on long-term dicumarol therapy by the one-stage and two-stage methods is indicated by dots. The oblique line indicates optimal correlation of the two methods.

lipid, 780 mg. (0.25 per cent suspension in 0.9 percent NaCl), was infused intravenously over a 15 minute period. Immediate correction of the abnormal clotting time and thrombin generation occurred. The prothrombin level and the generation of thromboplastin were changed 24 hours after the administration of the phospholipid suspension. Figure 10 illustrates the data obtained.

One patient with cerebral bleeding from long-term dicumarol therapy was treated with soybean phospholipid, 2,400 mg. (0.25 per cent solution in 0.9 per cent NaCl) intravenously over a two-hour period. The patient's clotting time and thrombin generation improved with the phospholipid therapy but the patient's prothrombin time and thromboplastin generation became progressively more abnormal, and the patient died within four hours after the onset of treatment with phospholipid. Necropsy examination confirmed the presence of massive intracerebral hemorrhage.

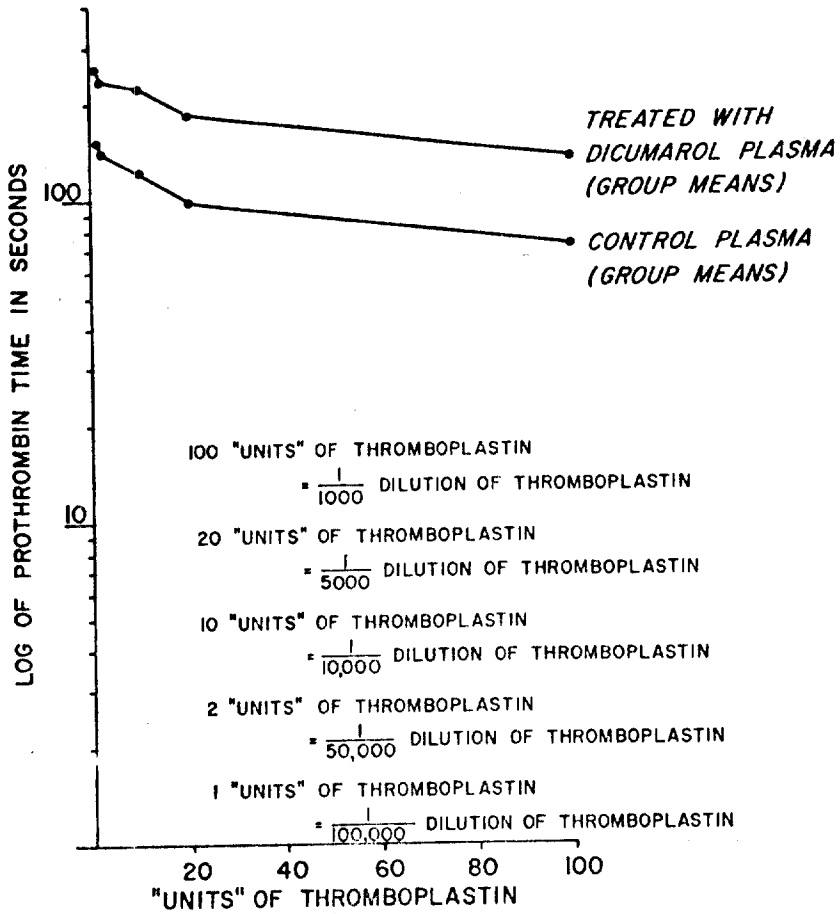

FIG. 3.—Effect of thromboplastin concentration on prothrombin time. The prothrombin times with various dilutions of tissue thromboplastin are plotted for normal plasma and plasma from patients on long-term dicumarol therapy on a logarithmic scale.

DISCUSSION

Our results indicate that prolonged dicumarol therapy depresses the normal plasma prothrombin and SPCA (cf. Owren's report[14]). Recent studies[15-22] have shown that PTC is reduced with long-term anticoagulant therapy. Mixing of PTC-deficient plasma and blood from patients on anticoagulant therapy for more than 30 days has been shown to correct the abnormal prothrombin consumption in many instances.[18,19] Our studies are in agreement with these findings. The ability of the blood to generate thromboplastin is completely independent of the coagulation components measured by the prothrombin time determination, which is commonly used as a guide for anticoagulant therapy. The deficiency of thromboplastin formation can be demonstrated

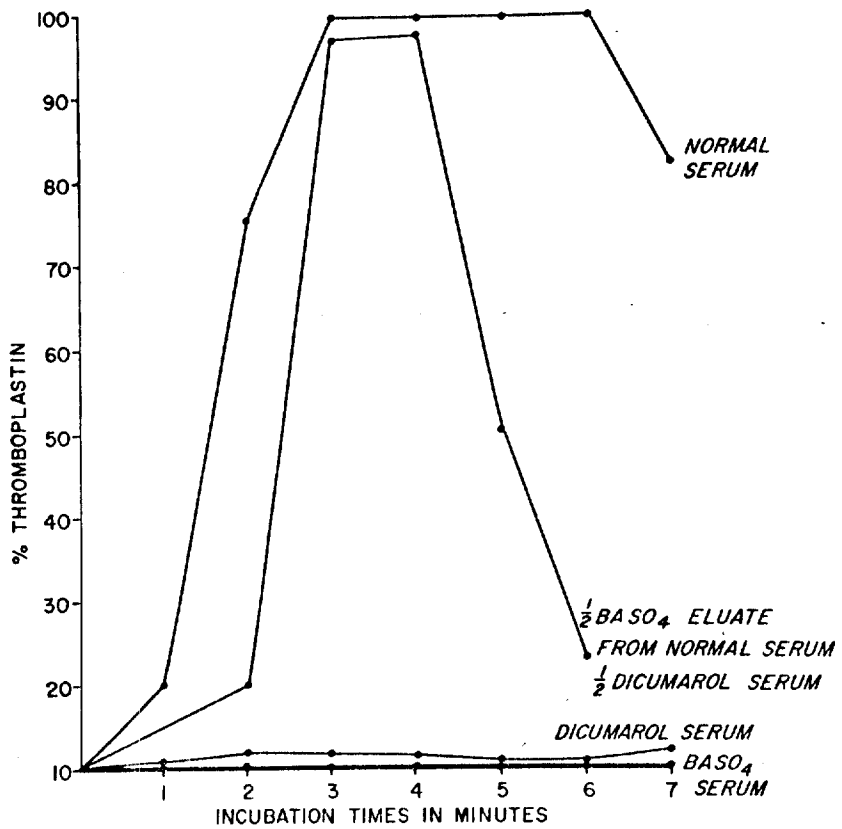

FIG. 4.—Effect of barium sulfate eluate from normal serum on the thromboplastin generation test. The following sera were used with normal plasma and suspension of brain phospholipid in the thromboplastin generation test: Normal serum (control); ½ volume of barium sulfate eluate from normal serum plus ½ volume of dicumarol serum, demonstrating complete correction; dicumarol serum; and barium sulfate-adsorbed normal serum.

most effectively by the thromboplastin generation test. We have found that dicumarol serum when mixed with the serum of a patient congenitally deficient in PTC partially corrects the thromboplastin generation test. This suggests a deficiency in dicumarol serum which may be caused by PTC.

During the past two years we have observed several patients who were bleeding from short-term dicumarol therapy. These patients had, in general, either an excessively prolonged prothrombin time or some complicating pathologic condition associated with the bleeding. In contrast, we have observed bleeding in patients on long-term dicumarol therapy in whom the bleeding occurred with prothrombin times in the range of 30 to 40 seconds, a prolonged coagulation time and poor thromboplastin formation. In addition, some patients on long-term anticoagulants have developed bleeding in association with prolonged prothrombin times, clotting times and poor throm-

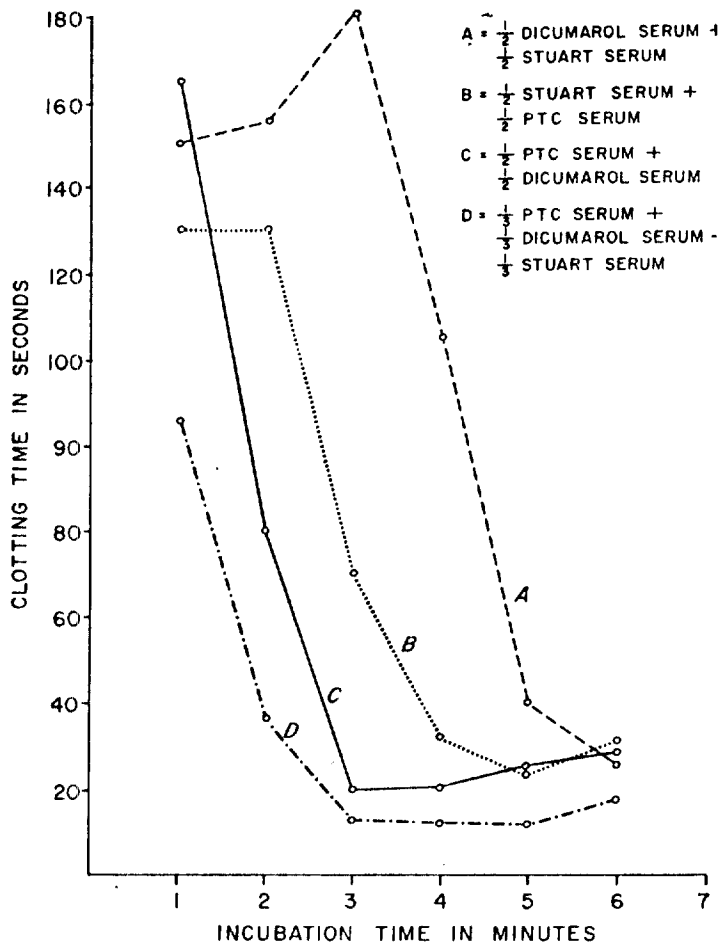

FIG. 5.—Effect of PTC and Stuart Factor-deficient sera on the thromboplastin generation test. The following mixtures of sera were used with normal plasma and suspension of brain phospholipid in the thromboplastin generation test: ½ volume of dicumarol serum and ½ volume Stuart serum; ½ volume Stuart serum and ½ volume PTC serum; ½ volume PTC serum and ½ volume dicumarol serum; and ⅓ volume PTC serum, ⅓ volume dicumarol serum and ⅓ volume Stuart serum (demonstrating complete correction).

boplastin generation. These observations taken together with previous reports suggest that the abnormal thromboplastin formation is an important factor in bleeding of patients on long-term anticoagulant therapy. Sise et al.[23] have recently reported bleeding in 23 patients on long-term anticoagulant therapy in whom 15 of the patients had a Quick prothrombin time between 22 and 39 seconds. These authors consider the level of prothrombin to be the important factor in both recurrent thrombosis or hemorrhage in patients on anticoagulant therapy.

A new clotting factor, the Stuart Factor, was recently reported[24] to be

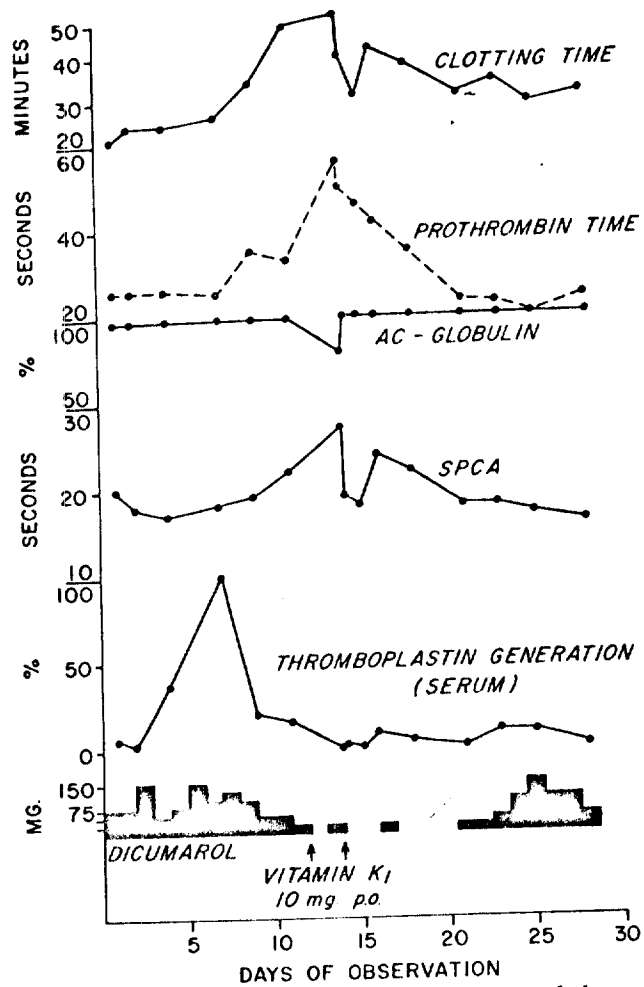

FIG. 6.—Coagulation defect from long-term dicumarol therapy.

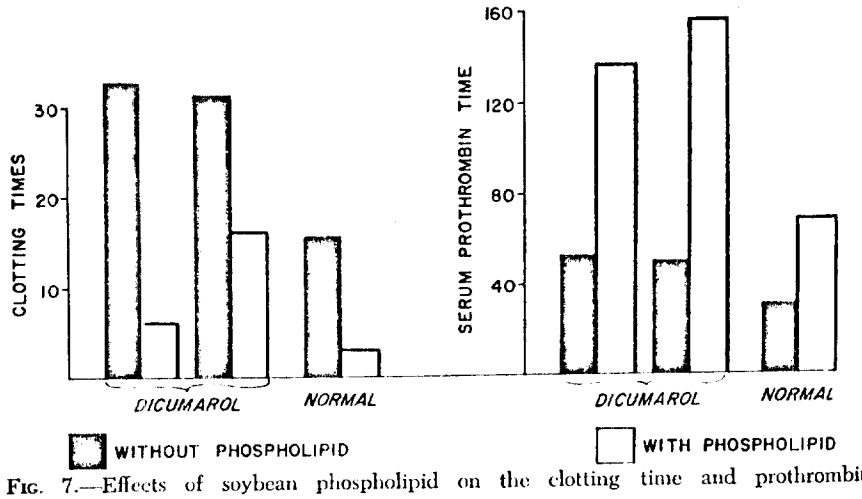

FIG. 7.—Effects of soybean phospholipid on the clotting time and prothrombin consumption.

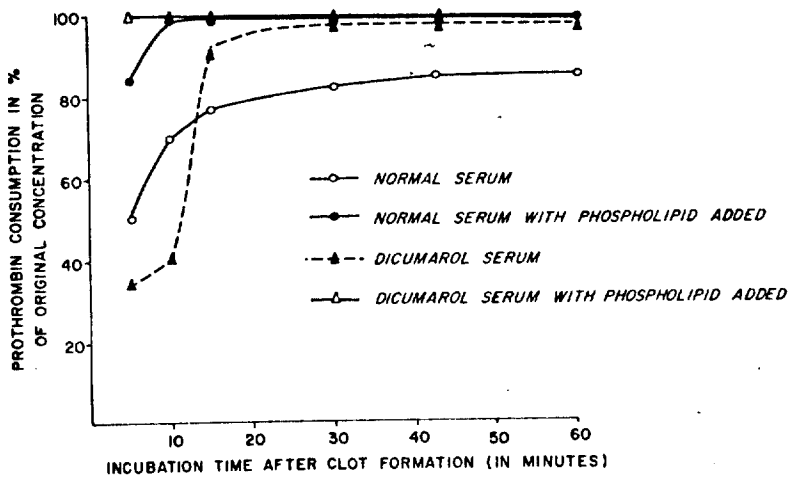

FIG. 8.—Effect of soybean phospholipid on prothrombin consumption.

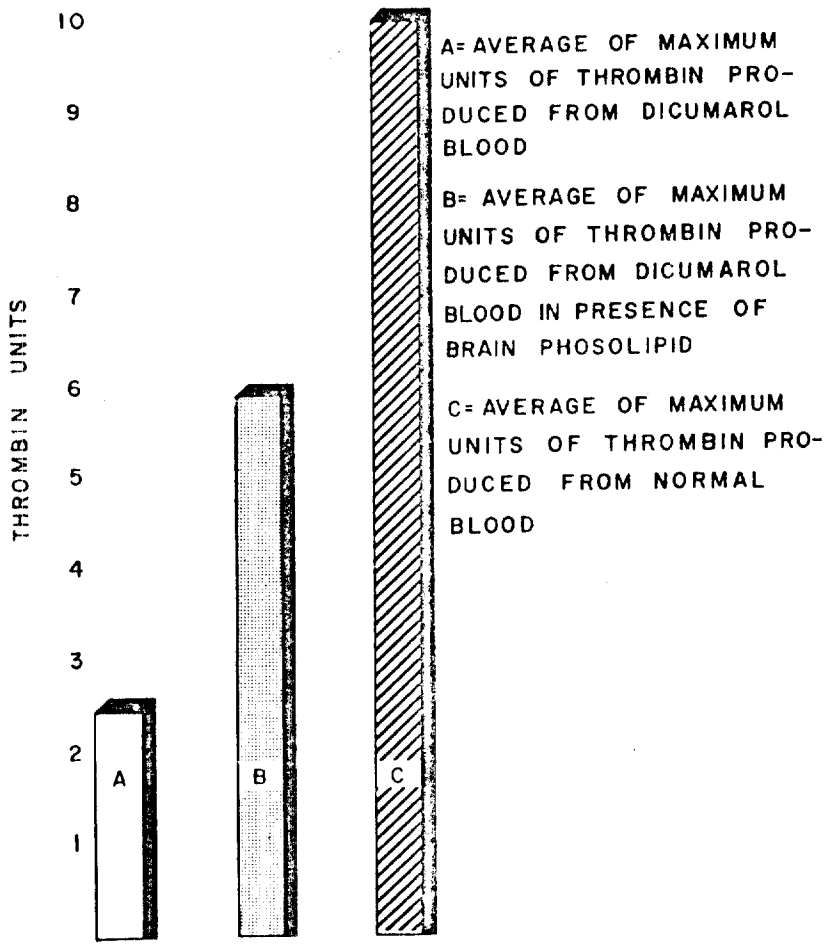

FIG. 9.—Results of thrombin generation test on dicumarol blood.

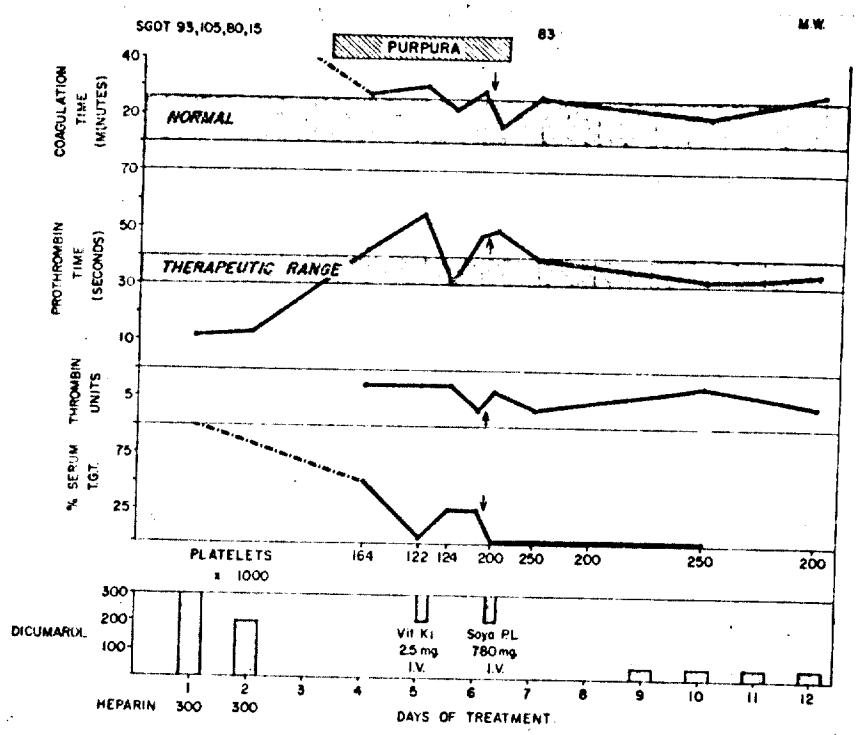

FIG. 10.—Effect of soybean phospholipid in dicumarol bleeding. SGOT is the serum glutamic oxalacetic transaminase determined by the method of Karmen, Wroblewski and La Due.[27]

associated with a serum defect in the thromboplastin generation test which is not PTC. The patient in whom Stuart Factor was described had previously been reported to have congenital hypoproconvertinemia (SPCA deficiency).[25] Mixtures of blood from patients on anticoagulants revealed a deficiency in Stuart Factor. Our findings support the hypothesis that long-term dicumarol administration produces decreased prothrombin, SPCA, Christmas Factor and Stuart Factor concentrations. The results reported indicate that the combined depression of these various factors to a "critical" level is found in patients bleeding from long-term dicumarol therapy. Phospholipid fractions of brain and of soybean reverse the prolonged coagulation time observed in patients on long-term dicumarol therapy, without interference with the specific anticoagulant effect.

The phospholipid preparation accelerates prothrombin conversion. The mechanism of action is not understood at this time. Phospholipid tested both in vitro and in vivo on PTC-deficient patients shows acceleration of the clotting time, improvement in prothrombin consumption and thrombin generation. The phospholipid preparations, however, did not correct abnormal thromboplastin generation.[26] These observations suggest that the mechanism of action of these phospholipids is the same in both PTC-deficient and dicumarol bloods.

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Summary

The coagulation defects of the blood of 30 patients after six months of dicumarol therapy have been studied. A preparation of brain or soybean phospholipids corrected the abnormal coagulation time and the thrombin generation test of patients on long-term dicumarol therapy. The deficiency in thromboplastin generation of these patients was not corrected.

Summario in Interlingua

Le defectos de coagulation in le sanguine de 30 patientes post 6 menses de therapia a dicumarol esseva studiate. Un preparato de phospholipidos ab cerebro o ab soja corrigeva le anormal tempore de coagulation e le test de generation de thrombina in patientes con therapia a dicumarol a longe vista. Le deficientia in le generation de thromboplastina in iste patientes non esseva corrigite.

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The Labeling of Phospholipid with Orthophosphate ^{32}P as Influenced by Procedures Which Alter Plasma Cholesterol Concentration (35611)

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Previous studies (1-4) from this laboratory have indicated that a primary increase in plasma phospholipid concentration induces a secondary increase in plasma free cholesterol concentration. Since an elevation in plasma phospholipid is observed in almost all varieties of clinical hypercholesterolemia, it seems possible that in certain varieties of hypercholesterolemia, an initial dysfunction occurs in phospholipid metabolism, which then in turn effects a sequential rise in the plasma cholesterol. Indeed, from our own studies (5), it would appear that the primary cause of at least one variety of hypercholesterolemia, namely that observed after biliary obstruction, is the initial rise in plasma phospholipid occurring after such obstruction.

In view of this experimentally observed sequential relationship of hyperphospholipidemia and hypercholesterolemia, and in view of the observation (6) that dietary cholesterol depressed ^{32}P uptake by liver phospholipids in rats, we thought it of interest to study labeling of hepatic phospholipid in various states associated with alterations in the plasma level of cholesterol, namely following experimental elevation of plasma phosphatide, biliary obstruction, administration of clofibrate (7, 8) and induction of a hypothyroid state (9-13).

Methods and Materials. A. Infusion of Lecithin. Male rats of the Long-Evans strain, 8 weeks of age and weighing approximately 290 g, were divided into experimental groups, designated I, II, and III together with corresponding groups of control animals designated I-C, II-C, and III-C. Each Group consisted of 5 animals.

All rats were maintained on laboratory chow (Simonsen Farms "Green Diet") until 4:00 p.m. prior to day of use; food was then withdrawn. The morning after food withdrawal each rat was anesthetized with ether and a polyethylene catheter was inserted into the iliolumbar vein. The rat was then placed in a restraining cage (14) and the free end of the catheter was connected to a constant infusion pump. At the start of the infusion period each rat received 2 ml of infusion fluid within approximately 15 sec. The infusion was continued thereafter at the rate of 1 ml/hr until termination of the experiment. The experimental animals received an infusion of 5.2% crude brain lecithin[1] (15) in 0.85% NaCl, adjusted to pH 7.3 with NaOH. Control animals received the same volume of 0.85% NaCl. At indicated times after the start of infusion, each rat received a single injection of carrier-free ^{32}P as neutral orthophosphate in 0.85% NaCl administered through the intravenous catheter. The dose was identical for all rats in a group and their corresponding controls, however, some groups received a dose of 0.21 mCi/rat, others greater amounts up to 0.5 mCi/rat.

Group I rats were infused with lecithin and Group I-C control rats with 0.85% NaCl for 6 hr. Both groups received orthophosphate-^{32}P at the end of the fifth hour of infusion.

[1] Nutritional Biochemical Co., "Animal lecithin 90% pure." The hypercholesterolemic effect of this material is comparable to that of a variety of phospholipids (15). When chromatographed on a thin layer of silica gel H and developed with chloroform-methanol-acetic acid-water, 95:35:3:4, 3 spots were seen after sulfuric acid char. The largest, containing approximately 80% of the total phosphorus was located where phosphatidyl choline was expected; the other two spots were comprised in about equal amounts of material located where sphingomyelin and lysolecithin would be expected. No spots corresponding to phosphatidyl ethanolamine or phosphatidyl serine were observed.

Group II experimental and Group II-C control rats received lecithin or NaCl for 12 hr and received orthophosphate-^{32}P at the end of the sixth hour of infusion. Groups III and III-C rats received lecithin or NaCl for 24 hr and received orthophosphate-^{32}P at the end of the 18th hour.

At the end of this infusion period, each animal was bled from the aorta into a syringe containing heparin. In order to flush residual blood from the liver, 0.85% NaCl solution was injected retrograde through the superior vena cava. The blanched liver was removed, sealed in a polyethylene bag, and preserved frozen until analyzed.

B. Biliary obstruction. Seventeen rats (Group IV) similar to those described above were subjected to double ligation of the common bile duct, ligation thus obstructing the flow of both bile and pancreatic juice. Sixteen control rats (Group IV-C) were subjected to sham operation during which the bile duct was manipulated with forceps. Eighteen hr after operation, the rats of both groups received carrier-free ^{32}P orthophosphate by injection into the penis vein. Six hr later, the rats were bled, killed, and the liver was removed and frozen until analyzed.

C. Oral administration of Clofibrate. Ten male rats (Group V) were fed *ad libitum* the chow diet mixed with clofibrate[2] in proportions of 250 mg of clofibrate for each 100 g of diet. Ten control rats (Group V-C) were fed the same diet without clofibrate. After consuming these respective diets for 4 weeks the experimental rats received 625 mg of clofibrate emulsified in 1 ml of water by stomach tube. The control rats were given 1 ml of 0.85% NaCl. Exactly 1 hr after the oral intubation, each rat received a mixture of carrier-free orthophosphate-^{32}P and 0.3 mCi of tritiated sodium acetate (0.26 mg)[3] by injection via the penis vein. Six hr following this injection, the rats were bled, killed, and the liver was obtained and frozen.

D. Administration of propylthiouracil. Ten male rats (Group VI) were fed *ad libitum* the chow diet containing 150 mg of propylthiouracil/100 g of diet. Ten control rats (Group VI-C) ingested the unsupplemented diet. Three weeks after the beginning of the diet, all rats received carrier-free orthophosphate-^{32}P and tritiated sodium acetate via the penis vein. Six hr after injection, the rats were bled, killed, and the liver was secured and frozen.

E. Chemical procedures. All plasma samples were analyzed quantitatively for cholesterol by the method of Saifer and Kammerer (16), for lipid phosphorus by the method of Zilversmit and Davis (17) and for phosphorus in the form of acid-soluble phosphate by the method of Bartlett (18). The plasma samples obtained from Groups VI and VI-C rats were also analyzed for their thyroxine content by a commercial laboratory according to the method of Pileggi and Kessler (19).

Each liver was weighed wet after light blotting. The livers of rats of Groups I–IV and their respective controls were analyzed only for their labeled and unlabeled phospholipid contents. This was accomplished as follows: A weighed portion of liver was ground in a Potter-Elvejhem homogenizer, suspended in H_2O, and a sample of the suspension was precipitated with 9 vol of 10% trichloroacetic acid. A portion of the supernatant from the trichloroacetic precipitation was analyzed for phosphorus by the method of Bartlett (18) and another portion was counted for ^{32}P activity in a Packard 3003 liquid scintillation counter. Efficiency was checked by counting a commercial standard of known activity.[5] The trichloroacetic acid precipitate was extracted with 20 vol of $CHCl_3$:MeOH (2:1; v/v), and the extract was partitioned with H_2O (20). A sample of the $CHCl_3$ layer was removed for phospholipid analysis by the method of Zilversmit and Davis (17). A factor of 25 was used in calculation to convert lipid P to phospholipid. The remainder of the $CHCl_3$ layer was evaporated to dryness and ^{32}P was counted in the Packard counter.

The livers of rats of Groups V and VI and their respective controls were analyzed for

[2] Atromid-S, Ayerst Laboratories, Inc.

[3] New England Nuclear Co.

[4] Bay Area Pathologists Central Laboratories, San Francisco.

[5] Amersham-Searle, Buckinghamshire, England.

both their labeled and unlabeled phospholipid and cholesterol. The phospholipid analyses and ^{32}P counts of these livers were done exactly as described above except that a window excluding tritium was used in the Packard counter.

For the cholesterol-^{3}H counts, a weighed portion of the liver was cut finely and extracted under reflux with 50 vol of ethanol:acetone(1:1;v/v). The extract was divided, a portion being used for quantitative analysis of cholesterol by the method of Saifer and Kammerer (16) while the remainder was dissolved in chloroform and treated with zeolite[6] to remove phospholipid (21). The phospholipid-free extract was evaporated, taken up in ethanol:acetone (1:1;v/v), saponified with alcoholic KOH, neutralized with acetic acid and precipitated with 1% digitonin in 50% ethanol. The digitonin precipitate was washed twice with ethanol–acetone and once with ether. It was dried and dissolved in glacial acetic acid. A sample of the acetic acid solution was removed for cholesterol determination while ^{3}H radioactivity in the remainder was counted in a Packard model 3003 scintillation counter. Quench was monitored by the channels ratio method and checked by addition of an internal standard of tritiated toluene.

Results. A. Infusion of Lecithin. No significant differences in concentration of liver phospholipid were observed between any group infused with lecithin and its corresponding control group (Table I, Groups I vs I-C, II vs II-C, and III vs III-C). Hence there was no evidence that lecithin infusion resulted in appreciable storage or retention of phospholipid in the liver. The specific activity of liver phospholipid in each experimental group, however, was significantly lower than that of its control group (Table I), showing that lecithin infusion may have depressed the incorporation of inorganic phosphorus into phospholipid. An alternative possibility that lecithin promoted the release of phospholipid-^{32}P into plasma, seems less likely since the total amount of radioactive P in plasma phospholipid did not differ significantly between experimental and control groups (Table I); this also indicates that infusion of lecithin did not suppress entrance of phospholipid from liver to plasma. Plasma cholesterol concentration increased up to 7-fold under the influence of lecithin infusion, the longer the duration of infusion the greater the plasma cholesterol concentration. The plasma ^{32}P radioactivity in Table 1 is expressed in terms of dpm/ml, rather than as specific activity, to avoid distortion by the large amounts of phospholipid infused.

B. Biliary obstruction. This procedure did not alter the concentration of phospholipid in liver, nor did it affect the incorporation of orthophosphate-^{32}P into liver phospholipid (see Table I, Groups IV and IV-C). Bile duct ligation brought about a 70% increase in plasma cholesterol level and an increase of 150% in plasma phospholipid level, as compared with control values. This plasma phospholipid increase was supplied disproportionately from radioactive phospholipid, since plasma from ligated animals contained over 4 times as much phospholipid-^{32}P as did control plasma.

C. Oral administration of clofibrate. As others (22) and we (23) have reported, the average weight (21.0 g) of the livers of the clofibrate-treated rats was significantly greater ($p<0.001$) than that (14.3 g) of the control rats. Despite this disparity in hepatic weight, the hepatic phospholipid concentration (see Table II) was closely similar in the 2 groups. Similarly the average amount of ^{32}P in the total livers also was approximately the same in the 2 groups. However, the specific activity of liver phospholipid (see Table II) was lower in the clofibrate-treated animals. As also found by others (24), clofibrate was seen to depress incorporation of acetate-^{3}H into cholesterol, whether measured by the specific activity of cholesterol or by radioactivity in the total cholesterol content of the whole liver. A similar depression of acetate-^{3}H incorporation was observed in plasma cholesterol. Clofibrate administration also effected (see Table II) a fall in plasma phospholipid concentration. However, it did not cause any change in the specific activity of plasma phospholipid.

D. Administration of propylthiouracil. The

[6] Taylor Chemicals, Inc., Baltimore, Md.

TABLE I. Effect of Lecithin Infusion and of Bile Duct Ligation on Incorporation of Orthophosphate-^{32}P into Phospholipid.

Duration of infusion (hr)	Elapsed time until ^{32}P injection (hr)	Substance infused or procedure performed	No. of rats used	Designation of group in text	Liver phospholipid (mg/g)	p value	Liver phospholipid (dpm/g of liver × 10^5)	p value	Plasma cholesterol (mg/100 ml)	p value	Plasma phospholipid (mg/100 ml)	p value	Plasma phospholipid (dpm/ml × 10^2)	p value
6	5	Lecithin	5	I	20.5[a] ±1.0	NS[b]	0.8 ±0.08	<0.02	110 ±10	<0.001	2000		8.7 ±1.1	NS
		Saline	5	I-C	21.7 ±0.9		1.3 ±0.08		41 ±3.5		95		12 ±1.0	
12	6	Lecithin	5	II	25.9 ±0.6	NS	6.0 ±0.3	<0.001	271 ±11	<0.001	3446		270 ±20	NS
		Saline	5	II-C	28.3 ±0.7		11 ±1		51 ±4		90		280 ±18	
24	18	Lecithin	5	III	28.8 ±1.4	NS	12 ±0.57	<0.01	340 ±49	<0.001	2215		54 ±3.8	NS
		Saline	5	III-C	25.3 ±1.2		17 ±0.48		47 ±8		96		48 ±3.7	
No infusion; bile duct ligation or sham operation	18	Ligation	17	IV	24.3 ±0.8	NS	9.6 ±1.0	NS	75 ±8	<0.01	216 ±31	<0.01	900 ±92	<0.001
		Sham operation	16	IV-C	25.1 ±1.0		8.3 ±0.9		44 ±3		86 ±8		210 ±16	

[a] Values are averages with standard error of the mean and corresponding values of p (probability of occurrence of observed difference from Table of Student's t).

[b] NS = not significant, or $p > 0.05$.

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TABLE II. Effect of Treatment with Clofibrate or Propylthiouracil on Incorporation of Orthophosphate ^{32}P into Phospholipid and of $C^{14}H_3ONa$ into Cholesterol.

Treatment	No. of rats used	Designation of group in text	Liver phospholipid (mg/g)	p value	(dpm/mg of phos. × 10^6)	p value	(dpm/whole liver × 10^7)	p value	Liver cholesterol (mg/g)	p value	(dpm/mg)	p value	(dpm/whole liver)	p value
Clofibrate	10	V	21.3[a] ±1.3	NS[b]	1.9 ±0.14	<0.05	2.9 ±0.24	NS	1.7 ±0.12	NS	1800 ±147	<0.01	47,000 ±4259	<0.02
Control	10	V-C	20.1 ±1.2		2.3 ±0.11		2.6 ±0.13		2.1 ±0.14		4700 ±887		106,000 ±19,553	
Hypothyroid	10	VI	22.6 ±1.4	NS	1.5 ±0.07	NS	4.6	NS	1.8 ±0.10	NS	2200 ±188	<0.01	35,000 ±3817	<0.02
Control	10	VI-C	20.1 ±1.3		1.7 ±0.15		5.1		2.0 ±0.28		6300 ±952		121,000 ±24,783	

Treatment	No. of rats used	Designation of group in text	Plasma phospholipid (mg/100 ml)	p value	(dpm/mg of phos. × 10^6)	p value	Plasma cholesterol (mg/100 ml)	p value	(dpm/mg)	p value	T-4 (μg/100 ml)
Clofibrate	10	V	75 ±4.7	<0.001	1.2 ±0.7	NS	50 ±3.7	<0.001	2400 ±377	<0.01	
Control	10	V-C	105 ±4.9		1.2 ±0.5		73 ±3.5		4400 ±703		
Hypothyroid	10	VI	115 ±9.3	NS	2.2 ±0.53	NS	86 ±5.8	<0.001	2200 ±162	<0.001	0.5
Control	10	VI-C	94 ±11.2		2.9 ±0.58		51 ±5.0		6100 ±1258		2.8

[a] Values are averages with standard error of the mean and corresponding values of p (probability of occurrence from Table of Student's t).
[b] NS = not significant, or $p > 0.05$.

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administration of propylthiouracil led to a hypothyroid state as indicated by the markedly reduced level (see Table II) of plasma thyroxine of the treated rat. However this induced hypothyroidism had no discernible effect upon the rate of incorporation of orthophosphate-^{32}P into liver phospholipid (see Table II) or upon the other aspects of phospholipid metabolism investigated. As expected from our earlier studies (9), the hepatic rate of incorporation of acetate-^{3}H into cholesterol was depressed significantly in the hypothyroid rats.

E. Inorganic phosphate. The concentration and specific activity of acid-soluble phosphorous in the liver and plasma did not differ significantly between any experimental group and its corresponding control. Therefore, the extent of incorporation of ^{32}P into phospholipid may be taken to reflect the rate of the incorporation process itself, rather than to depend upon differences of substrate concentration.

Discussion. The synthesis of liver phosphatide is multicompartmental, of sequential complexity, and yields products showing structural and metabolic heterogeneity (25). The analytical methods we have used have the virtue of simplicity but suffice for only a limited first approach to information about the heterogenous hepatic pool.

The present results indicate that the extent of incorporation of ^{32}P into liver phosphatide was not significantly altered in any of the experimental studies except that one in which the animals were rendered hyperphosphatidemic by the continuous infusion of a phosphatide itself. Thus experimental increase of plasma cholesterol by induced biliary obstruction or hypothyroidism or its decrease by clofibrate administration failed to alter significantly the extent of incorporation of ^{32}P into hepatic phosphatide, even though in the latter two experimental conditions, the extent of incorporation of labeled acetate into hepatic cholesterol was significantly depressed.

The changes in plasma phospholipid in these various conditions were of some interest. Thus the plasma content of labeled phosphatide rose considerably in rats after biliary obstruction despite the fact that no significant change was observed in their extent of incorporation of ^{32}P into hepatic phosphatide. Apparently then, following biliary obstruction not only does retention of phospholipid in plasma occur, but also a good fraction of the latter consists of newly synthesized materials. On the other hand, no change was observed in the plasma content of labeled phospholipid in the rats infused with lecithin despite the fact that a significant fall had occurred in their incorporation of ^{32}P into hepatic phospholipid.

Despite the expected (9) decreased hepatic rate of cholesterol synthesis found in the hypothyroid rats, we were unable to detect, as were also earlier observers (10, 12), any significant change in the plasma content of labeled or unlabeled phospholipid. On the other hand, clofibrate administration significantly depressed the plasma content of both unlabeled and labeled phospholipid as well as that of cholesterol, phenomena also observed (26, 27) in the clofibrate-treated human subject.

The presence of lysolecithin and sphingomyelin in the lecithin preparation was indicated by thin-layer chromatography. It is possible that the effects of this preparation may have been due in part, or entirely, to these substances. Lysolecithin is of particular concern since it has been considered to be toxic because of its hemolytic action (28). It is noteworthy that lysolecithin occurs physiologically in rat plasma, which has been reported to contain on the average 17.5% of its lipid phosphorus as lysolecithin (29). Lysolecithin phosphorus has been reported to constitute 7%, and sphingomyelin phosphorus 19% of the lipid phosphorus of human plasma (30).

Summary. Changes in the rat plasma concentration of cholesterol were effected by lecithin infusion, biliary obstruction, clofibrate administration, and by induction of hypothyroidism. The extent of incorporation of ^{32}P into hepatic phospholipid was altered (*i.e.*, decreased) in only those rats subjected to lecithin infusion. However, changes in the plasma content of labeled phospholipid were observed only in those animals subjected to

biliary obstruction (increase) or clofibrate (decrease) administration.

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EXHIBIT 6

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BRIEF SUMMARY AND GUIDE TO SOME LECITHIN USES

USES	TYPICAL ACTION	SUGGESTED CONCENTRATION	RESULTS AND REMARKS
Animal Feeds (other than pet foods)	Emulsifier, wetting agent	Approx. 1 to 3%	Promotes and stabilizes emulsions. Provides instant wetting of powdered formulations.
Baking	Modify the gluten characteristics of flour, emulsifier, antioxidant, wetting agent.	0.1 to 0.3% based on flour in bread. 1/2 to 1% based on shortening in pies.	Improve the "shortening effect;" reduce emulsifier cost; benefit flavor, shelf-life, texture and moisture retention.
Cake Mixes	See Baking	1 to 3% based on shortening in cakes plus other emulsifiers.	Same as in baking. Also, allows rapid wetting with milk or water. Generally used in combination with monoglycerides.
Candy	Emulsifier	Approx. 1% based on shortening.	Used in fat-containing candy, e.g., caramels, brittle, nougats, and taffies. Aids mixing of sugar, fat, and water to prevent greasiness, graining, and streaking.
Chocolate	Reduces viscosity by wetting and dispersing action.	0.3 to 0.5%	More effective and lower priced than cocoa butter. The higher level may be used to improve moisture tolerance as in ice cream coatings, etc.
Cosmetics	Emulsifier, foam stabilizer, emollient, penetrant, vitamin source, hydrolysis inhibition.	1 to 5%	Normal skin contains about 0.4% lecithin. Natural lecithin contains pantothenic acid, thiamin, folic acid, riboflavin, pyridoxine, biotin and niacin, as well as considerable amounts of inositol and choline.

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USES	TYPICAL ACTION	SUGGESTED CONCENTRATION	RESULTS AND REMARKS
Drugs	Emulsifier, suspending agent		CENTROPHIL S is one of few sources for emulsifiers acceptable for use with oils for intravenous injection. Since unadulterated lecithin is nontoxic, other grades find many uses.
Dyes	Solubilizer, dispersing agent	1/4 to 1% on dry weight of dye.	Water soluble food dyes can be solubilized in oil. Also useful in varnishes and lacquers.
Ice Cream		0.001 to 0.1% of final weight	Improves smoothness, prevents "sandiness" on storage; aids dispersion of chocolate. Reduces use of egg yolk and stabilizes fat.
Instant Foods	Wetting, dispersing, emulsifying	1/2 to 3%	Aids reconstitution by improving wettability and dispersion of solids and fats (i.e., dry beverage mixes, etc.).
Insecticide	Oil soluble emulsifier, dispersant		Used alone or blended with other surfactants.
Inks	Grinding aid, solubilizes dyes	1/2 to 2% (See Remarks)	Allows increased pigmentation and modifies flow properties. Optimum amounts depend on formulation. Usual concentration is 1/2% of pigment weight, although up to 2% may be required for difficult pigments.
Leather	Softening agent, oil penetrant	1% of oil	Added to fat liquor.

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USES	TYPICAL ACTION	SUGGESTED CONCENTRATION	RESULTS AND REMARKS
Latex emulsions	Facilitate pigment dispersion; anti-settling agent; prevent agglomeration; emulsion stabilizer; minimize foaming; easy, uniform application without lap marks; freeze-thaw stability.	1 to 5% of the pigment weight	Also see under "Paints." Water dispersible grades of lecithins give best results.
Paints	Aids pigment grinding and dispersion, retards settling, yields maximum color strength and full hiding power.	1/2 to 2%	Pigmentation has been increased as much as 25%. Vehicles assisted include: alkyds, styrenated alkyds, bodied linseed oils, varnishes, lacquers, and natural oils. Pigments assisted include: lithopones, titanium dioxide, zinc sulfide, basic carbonate, white lead, and a variety of reds, blues, yellows, greens, and carbon black. Generally used at 1/2% of pigment weight but up to 2% may be required in some cases.
Pet Foods	Emulsifier, release agent	Approx. 1%	Aids blending of fats and water. Gives clean release from equipment and can.
	Nutrition supplement	3 to 5%	Improved disposition, activity stimulant, associated with glossy fur, aids digestion, and/or increases the efficiency of nutrient assimilation.
Petroleum products	Emulsifier and wetting agent for marine greases, cutting oils, etc.	1/2 to 3%	Improves wettability and stabilizes emulsions and dispersions.
	Multipurpose additive for motor fuel	50 PPM	Maintains clean carburetor conditions, reducing carburetor icing.

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USES	TYPICAL ACTION	SUGGESTED CONCENTRATION	RESULTS AND REMARKS
Plastics	Slip agent, mold release, pigment dispersant	(See Remarks)	May be used either on mold or included in formula. Where part of formula, suggested concentration is 1%. See "Paints" for pigment dispersion information.
Margarine	W/O emulsifier, antispattering agent, browning agent	0.15 to 0.5% (See Remarks)	Concentration depends on degree of emulsion stability desired and grade of lecithin used.
Rubber	Pigment dispersant, mold release agent		See "Plastics"
Shortening	Emulsifier, dispersant	0.5 to 1%	Produces a more complete and uniform blending of the shortening. Increases the shortening action.
Textiles	Softening agent, lubricant		Concentrations depend upon the application or specific properties desired.

TECHNICAL ASSISTANCE

The foregoing summary of Lecithin uses is meant merely as a guide to the broad scope of utility. Many other uses have been reported in the technical literature.

The following pages review the properties of the different Lecithin grades offered. Each grade provides its own commercial or unique advantages in certain types of applications. Our Lecithin Department welcomes the opportunity of furnishing recommendations for specific uses as well as supplying working samples.

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CENTRAL SOYA 1825 North Laramie, Chicago, Illinois 60639

13393

July 22, 1974

Dr. Humphrey F. Sassoon
Tricor-Jitco
1776 East Jefferson Dr
Rockville, MD 20852

RE: Lecithin Monograph for GRAS review

Dear Dr. Sassoon,

Please find enclosed a copy of Chapter 16 of Markley's book on soybeans and soybean products. I realize that this chapter was written in the early 1950's by Joseph Stanley but much of the information contained is still pertinent to lecithin, it's uses and it's physical properties. There is some new information being generated to bring this chapter on lecithin utilization and production up to date. I foresee this chapter being written within the next two years. The occurance of lecithin is still accurate and the composition has been elaborated on somewhat since that time. The minor or trace components have been isolated and identified by us as well as the researchers at the Hormell Institute under the direction of Dr. Orville Privett.

The uses of lecithin have been expanded somewhat but for the most part the larger volumes are as you see in these products listed. We find more uses in the area of pan release agents and cooking ease agents used by the housewife and institutional kitchens. The health market has also increased in volume the use of lecithin in liquid and granular form. Since these are intended for direct consumption, great care is taken to produce by Holsum and Clean products, both from a chemical as well as the bacteriological standpoint.

The volume of lecithin available today is difficult to pinpoint. It has changed since 1950 where 70-90 million pounds are now available depending upon the soybean crop and the price of soybean meal. Usually the higher the meal prices, the more lecithin will be lost to the meal side. Lecithin constitutes about 3% of the total soybean oil and therefore is less than 1% of the total bean. A rough estimate of lecithin available would be based on the volume of soybean oil produced or projected for the up in coming year.

As to the biological aspects and metabolism of lecithin, I am also enclosing a copy of a review prepared by Dr. E.S. West from Portland, Oregon in 1955. This review, even in its crude form, may provide some information for you. This work does go back to the early days of lecithin but still is considered invaluable. I would have to say that each piece of data would have to be judged on its own merit.

There were a great number of questions that you raised in our phone discussion and I am not able to answer them directly at this time. Mostly because information is lacking. I would like to make this information available to you if and when it is possible. As I indicated to you I will be tied up for the next couple of weeks and do not anticipate finding this information or part of this information until the later part of August.

If I may be of any further assistance to you on this matter of lecithin, please contact me in Chicago.

Thank you for your interest in lecithin.

Bernard F. Szuhaj

Bernard F. Szuhaj, Ph.D.
Fats & Oils Research Director

BFS/cc

cc: W.W. Cravens
L.D. Williams
E.F. Sipos

0488

JOURNAL OF CHROMATOGRAPHY 66(2) 303–310.

CHROM. 5860

ANALYSIS OF PHOSPHOLIPIDS BY THIN-LAYER CHROMATOGRAPHY AND X-RAY EMISSION SPECTROMETRY

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(Received October 20th, 1971)

SUMMARY

The use of X-ray emission spectrometry for *in situ* quantitative analysis of phospholipids after separation by two-dimensional thin-layer chromatography is described. With this procedure, microgram quantities of phospholipids can be analyzed. The precision is comparable to that of other methods for analyses of phospholipids after thin-layer chromatography but time-consuming recovery steps are eliminated.

INTRODUCTION

Chromatographic methods are widely used for the isolation and determination of individual phospholipids. With the limited quantity of sample available from biological tissues, thin-layer chromatography (TLC) has become especially useful. Development with a single solvent system[1,2], multiple development in one dimension[3], and development in two dimensions[1,4–8] have recently been applied in methods for phospholipid analysis. Quantitation of the separated phospholipids is often difficult. The separated components can be recovered from the adsorbent layer and analyzed spectrophotometrically[7,8]. Alternately, the components can be visualized by spraying with an appropriate reagent or with sulfuric acid followed by charring of the organic material and the resultant spots scanned with a densitometer[9,10]. Reviews of the various qualitative and quantitative techniques have been published[11,12].

There are problems involved with both these quantitation steps. Elution from the adsorbent layer and subsequent analysis is arduous. Complete elution and hydrolysis as well as quantitative color development are essential. Densitometry is an excellent technique for quantitation of one-dimensional chromatograms but extremely difficult to apply to two-dimensional separations, which are generally required for the complete separation of phospholipids in biological samples.

The use of X-ray emission spectrometry (XES) to quantitate TLC has been reported[13]. Combined TLC–XES was shown to be applicable to the *in situ* determina-

tion of a variety of materials, including phospholipids. All TLC separations, however, were one-dimensional.

This paper describes a method for the separation and quantitative analysis of phospholipids using two-dimensional TLC and XES analysis (*in situ*) of each component for phosphorus content. The method has been applied to the analysis of rat liver and testis for the following phospholipids: phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol and sphingomyelin.

METHODS

Preparation of phospholipids

Phospholipid standards obtained from Supelco, Inc. were used for calibration of the X-ray spectrometer. The phospholipid fractions of pooled liver and testis lipids from three 2-month old rats (Sprague-Dawley) were isolated by chromatography on Florisil[14].

For chromatography a weighed quantity of phospholipid, either biological sample or standard mixture, is dissolved in sufficient chloroform–methanol (2:1, v/v) to yield a final concentration of 50–100 μg/μl.

All samples were stored under nitrogen at 0°.

Thin-layer chromatography

TLC was carried out on 20 × 20 cm silica gel coated polyethyleneterephthalate sheets (Eastman Chromagram 6061). Reagent grade solvents were used without prior distillation.

Approximately 1 cm of the adsorbent layer was removed from all four sides of the sheet to minimize contamination of the layer during handling. Using a microsyringe (Hamilton No. 701, 10-μl capacity) the sample (200–900 μg for standards, 300–400 μg for samples) was applied in a single small spot in the lower left corner, 3 cm from each edge. The sheet was irrigated in the first dimension for 2 h using a solvent system consisting of chloroform–methanol–ammonium hydroxide (140:50:7). After air-drying for 2½ h, the sheet was irrigated in the second dimension (90° to the first) for 2 h with a solvent system consisting of chloroform–methanol–acetone–acetic acid–water (10:2:4:2:1). After all traces of acetic acid were removed by air-drying, the phospholipid zones were visualized by exposure to iodine vapor for 2–3 min.

Preparation for XES analysis

A clear polyethyleneterephthalate sheet was placed over the chromatogram and the outlines of the phospholipid zones were traced on this overlay. Using this tracing as a guide the portions of the chromatogram containing phospholipid components were cut into 5/8 in. × 5/8 in. sections. Several such sections were required to encompass the larger spots. These sections were then analyzed individually for phosphorus content.

X-ray emission spectrometry

Quantitative analysis for phosphorus was performed with a Norelco PW-1212 spectrometer under a helium atmosphere. Chromium K_a incident radiation (60 kV, 24 mA), a germanium analyzing crystal and a proportional flow counter were used.

Counts were accumulated for 40 sec. The XES analysis was carried out essentially as described by LIBBY[13] with a few modifications. The 5/8 in. square sections of the chromatogram were attached to 25-mm diameter circles of Whatman 40 filter paper with a drop of rubber cement and placed in the spectrometer. As noted, counting of emitted radiation was carried out under a helium atmosphere rather than under vacuum. As no "blank" sections (*i.e.*, areas not exposed to sample during chromatography) of the TLC sheet were available to use for background count correction, the emission at 2.0° on either side of the phosphorus angle (141.06° 2θ) was counted on four sections from each chromatogram. These counts were then averaged and used for background correction.

Calibration curves

Three mixtures of the phospholipid standards containing approximately 50, 100, and 200 μg, respectively, of each phospholipid were chromatographed and analyzed by XES as described above. Curves for μg phospholipid *vs.* net counts (gross counts less average background count) were prepared for each phospholipid of interest.

RESULTS AND DISCUSSION

The TLC separation of approximately 100 μg of each of the five phospholipids is shown in Fig. 1. Several commercially available and laboratory prepared silica gel

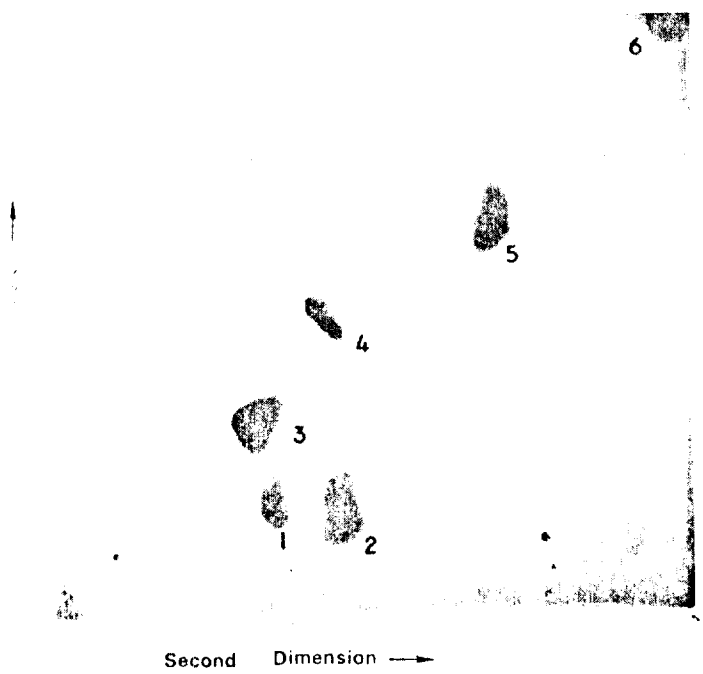

Fig. 1. Separation of phospholipid standard mixture containing approximately 100 μg of (1) phosphatidyl inositol; (2) phosphatidyl serine; (3) sphingomyelin; (4) phosphatidyl choline; (5) phosphatidyl ethanolamine and (6) neutral lipid.

coated polyethyleneterephthalate sheets were investigated for use in TLC. Also, a number of solvent systems were evaluated. The specific combination of adsorbent and solvent systems reported here gave the most consistent performance in terms of reproducible separation and spot size as well as minimal spreading or tailing of the various spots. In an attempt to minimize the adsorption of emitted phosphorus X-rays by the silica matrix, and thus increase sensitivity, the use of other adsorbent layers such as alumina and cellulose was investigated. Unfortunately, satisfactory separation of the phospholipids could not be achieved.

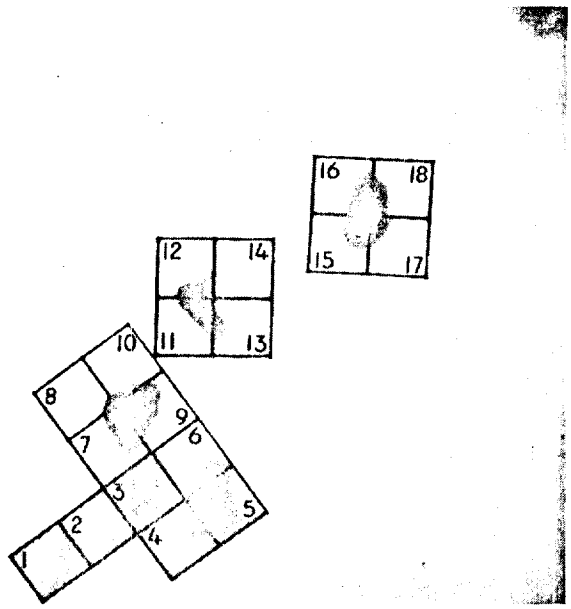

Fig. 2. Sectioning of chromatogram for XES. Each numbered section cut out and analyzed by XES. Numbers correspond to data in Table I.

Fig. 2 demonstrates the sectioning of a chromatogram (see Fig. 1) for XES analysis. The compositing of the XES data resulting from this sectioning is demonstrated in Table I. (These data correspond to the ~100 μg points on the calibration curves.)

The XES analysis of a phosphorus solute in a silica gel matrix is very sensitive to matrix adsorption and the extent of adsorption of emitted phosphorus X-rays by the silica gel layer is different for each phospholipid, perhaps due to differences in penetration[13] or spot shape. Therefore, individual calibration curves were required for each phospholipid (Fig. 3).

Because of variations introduced by XES counting statistics and minor day-to-day changes in the setting up and operating of the XES spectrometer, the analyses of standards and samples were performed the same day. If this was not possible, at least one point of each calibration curve was checked each day and if agreement with the previous data was not satisfactory ($\pm$5% relative), XES analysis of all curve points was repeated.

TABLE I

ANALYSES OF CHROMATOGRAM SECTIONS

See Fig. 2.

Phospholipid	*μg spotted*	*Section No.*	*Net XES counts*	*Total counts*
Phosphatidyl inositol	93.0	1 & 2	—	
		3	740	740
Phosphatidyl serine	94.0	4	81	
		5	423	
		6	260	764
Sphingomyelin	106.6	7	312	
		8	93	
		9	309	
		10	172	886
Phosphatidyl choline	81.4	11	519	
		12	195	
		13	107	
		14	17	838
Phosphatidyl ethanolamine	108.6	15	140	
		16	95	
		17	337	
		18	377	949

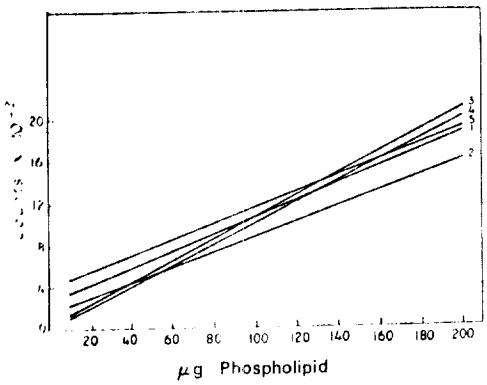

Fig. 3. XES calibration curves. Numbers correspond to Fig. 1.

Figs. 4 and 5 show the separation of phospholipids isolated from rat liver and testis, respectively. No attempt was made to identify any components other than the five phospholipids of interest. Phospholipid compositions determined by XES analyses of these chromatograms are in satisfactory agreement with literature data obtained by TLC and spectrophotometric phosphorus analysis (Tables II and III). The precision on replicate samples is comparable[1,3].

Preparation for XES and the actual analysis required approximately 2 h per chromatogram (5 components). Problems with incomplete recovery from the adsorbent, ashing and color development were eliminated.

A modification of this procedure has been used in this laboratory to monitor Florisil column separations of phospholipids. An aliquot of each eluant fraction is

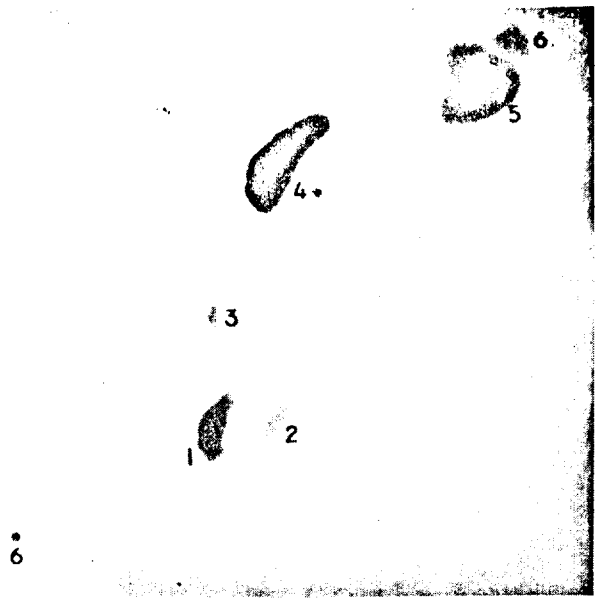

Fig. 4. Separation of 413 μg of rat liver phospholipids. (1) to (5) correspond to Fig. 1; (6) unknown. (XES analysis in Table II.)

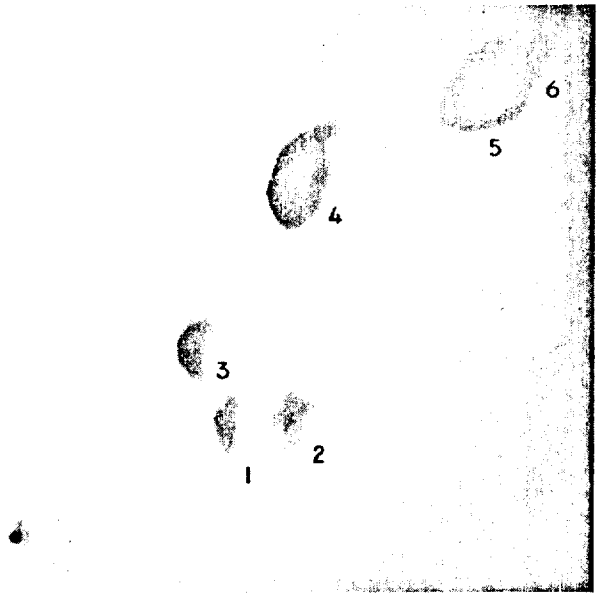

Fig. 5. Separation of 350 μg of rat testis phospholipids. (1) to (5) correspond to Fig. 1; (6) unknown. (XES analysis in Table III.)

spotted on a polyethylene disc and analyzed by XES. The phosphorus content is determined by comparison to a calibration curve prepared in the same manner.

TABLE II

PHOSPHOLIPID COMPOSITION OF RAT LIVER

Phospholipid	*TLC/XES*[a]	*Ref. 1*[b]	*Ref. 3*[b]
Phosphatidyl inositol	10.9 ± 0.9	8.8	6.5
Phosphatidyl serine	3.1 ± 0.3	3.0	4.3
Sphingomyelin	4.0 ± 0.9	1.8	4.1
Phosphatidyl choline	53.3 ± 2.5	55.0	52.9
Phosphatidyl ethanolamine	25.7 ± 2.5	25.3	27.1
Others	4.2[c]	6.0	5.2

[a] Mean of four determinations ± average deviation; expressed as per cent of total phospholipid found.
[b] Expressed as per cent of lipid phosphorus.
[c] Unknown, calculated as phosphatidyl ethanolamine.

TABLE III

PHOSPHOLIPID COMPOSITION OF RAT TESTIS

Phospholipid	*TLC/XES*[a]	*Ref. 15*[b]
Phosphatidyl inositol	7.6 ± 1.4	13.4
Phosphatidyl serine	7.1 ± 0.6	
Sphingomyelin	8.3 ± 1.0	9.6
Phosphatidyl choline	42.3 ± 2.5	44.0
Phosphatidyl ethanolamine	31.0 ± 2.7	28.8
Others	2.6[c]	tr

[a] Mean of four determinations ± average deviation; expressed as per cent of total phospholipid recovered.
[b] Expressed as per cent of lipid phosphorus.
[c] Unknown calculated as phosphatidyl ethanolamine.

ACKNOWLEDGEMENTS

The contributions and assistance of W. B. GEHLING are gratefully acknowledged. The phospholipids from rat liver and testis were isolated by E. J. HOLLENBACH.

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Food Industries 17: 740-741 (1945)

18 MISTAKES IN THE USE OF LECITHIN

Lecithin can be employed to improve the quality of many foods, including confectionery and bakery products, cooking oils, fats, mayonnaise, peanut butter, sherbets and ice cream mixes. These "don'ts" will guide you in using it correctly.—Part I

By L. RUSSELL COOK
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THAT we may better understand the why's and wherefore's of the mistakes made in using and handling lecithin, let us first consider briefly what it does, and how.

Lecithin finds many commercial uses because it is an antioxidant, is surface active and will reduce surface and interfacial tensions. Variable factors govern the extent of antioxidant protection provided by lecithin. These include the extent of unsaturation of the fatty acids in the glyceride, the absence or extent of hydrolysis of the lecithin itself and the quantity of lecithin present compared to the total surface area exposed by the fat requiring protection.

The important factors governing the effect of any given quantity of lecithin upon surface and interfacial tension reduction are as follows:

1. *The facility and speed with which the lecithin will dissolve in the medium in question.* The softer the lecithin, the more quickly and completely it will dissolve. This is important in commercial practice, particularly where unheated products are involved, such as bread doughs, cake batters and uncooked icings.

2. *The efficiency of the lecithin itself.* While concentration of phosphatides is a very rough indicator of efficiency, it is not conclusive nor accurate, because molecular structure and molecular efficiency are both variable. The more accurate means of comparatively evaluating lecithins is to measure their relative ability to reduce interfacial tension. One effective way of doing this is to measure the viscosity drop of a suspension of solids in fat, as caused by the graduated addition of the lecithin in question. The McMichael viscosimeter is a suitable instrument to use.

3. *The percentage of lecithin used compared to the total interfacial area present in the emulsion.* The lecithin molecule reduces the interfacial tension primarily because there is less force of adhesion between itself and an adjacent molecule of the medium than there is force of cohesion between adjacent molecules of the medium itself. It is also the case that the cohesion between two lecithin molecules is greater than the cohesion between two molecules of the medium.

It will therefore follow that the interfacial tension will be progressively reduced with increasing quantities of lecithin until a full monomolecular layer is provided at all interfaces. Further additions will provide a bimolecular or multimolecular layer, which, instead of reducing interfacial tension, will actually increase it. That is the reason, for instance, that the addition of 1 percent of lecithin to an oil will even slightly increase its viscosity, while the same lecithinated oil will produce a solids-in-oil suspension of lower viscosity than would the same oil without the lecithin.

NOW, THE MISTAKES —With Chocolate Products

1. One of the original errors made with lecithin in the manufacture of chocolate coatings and solid chocolate articles was its overuse. The accompanying illustration is a typical viscosity curve showing the rapid initial drop in viscosity with increasing quantities of lecithin; the approach of a "point of diminishing returns"; and the reversal in the viscosity curve when the optimum quantity is exceeded. Maximum quantities are usually about 4 oz. per 100 lb. for dark sweets and vanillas; 5 oz. per 100 lb. for light sweets and milk coatings.

The same error has been made by confectionery manufacturers who use lecithin to correct "mushing" in the enrober pot. Usually 1 to 1½ oz. to 200 lb. of coating in the pot will correct "mushing"; more is likely to aggravate it.

2. The most common error in the use of lecithin in chocolate manufacture is its addition at the wrong time. This statement must be modified by the objective desired, but if maximum viscosity drop per unit weight of lecithin is desired, it should be added after refining and conching are completed, and not before. Both refining and conching, the latter particularly at high temperatures, will destroy anywhere from one-third to one-half of the lecithin potency. The practice of adding part of the lecithin during mixing or at the beginning of conching time is useful in cutting power requirements or reducing—without detrimental effects on quality—the number of pounds requiring processing. This last item refers to the practice of omitting as much cocoa butter as possible until the viscosity is adjusted before molding, and using lecithin instead during the processing stages. Any of these practices are satisfactory for the particular objective in question. But it must be realized that the viscosity of the chocolate will not be stabilized under these conditions unless additional lecithin is used afterwards to compensate for the effects of the processing on the lecithin.

3. The use of 4 or 5 oz. of lecithin to each 100 lb. of ice cream coating is maximum insofar as obtaining minimum viscosity is concerned, just as in the case of confectionery coatings and solid chocolate items. For this reason,

the mistake frequently is made of stopping at this concentration of lecithin. The condition existing during the dipping of ice cream novelties and bars, however, is very conducive to the introduction of excessive quantities of moisture, which causes thickening of the coating to occur, with subsequent decrease in coverage, increase in weight, and tendency of the dipped article to "bleed."

While the 4 or 5 oz. of lecithin per 100 lb. is sufficient to reduce the tension at all interfaces existing in the coating prior to the introduction of the moisture, the latter creates new interfaces adjacent to the fat and also to the hygroscopic solids. When these solids absorb the moisture, they very naturally swell, increasing their own surface area. All of these tendencies are causes of increased viscosity. If an extra quantity of lecithin is present in the fats to emulsify the moisture in the fat alone, and to prevent imbibition by the solids, this viscosity rise can be minimized to that represented by the increased surface area created within the emulsion by the moisture. The amount of extra lecithin required to accomplish this objective is in ratio to the amount of moisture "added," but will usually vary between 2 and 4 oz. per 100 lb. of coating.

Sirups

4. Lecithin has occasionally been labeled as an inefficient stabilizing or dispersing agent by sirup manufacturers who have unsuccessfully attempted to apply it to sirups, particularly chocolate, in which the only source of fat has been that contained in the cocoa powder or milk powder used. They are correct, as far as this application is concerned. It is a mistake to use lecithin in a sirup unless a fair amount of relatively "free fat" is present, either in the form of chocolate liquor, cream powder or actual added fat. In the latter cases, lecithin added to the fat or fat-rich substance will minimize (not eliminate) fat separation and create smoother texture in the sirup by virtue of good fat-globule dispersion. In sirups where there is practically no fat to disperse, the lecithin is wasted.

Lecithin is useful for dispersing the solids of powdered drinks during reconstitution, but in these cases the dispersing effect of the polar group of the lecithin molecule substitutes for the agitation and boiling during sirup manufacture.

5. In fat-bearing sirups, lecithin is usefully employed, as mentioned above, to assist in creating and maintaining adequate fat dispersion. The physical effect of this dispersion is a smoother texture and reduced separation. It must be remembered, however, that to accomplish this objective the lecithin must be permitted to operate at interfaces of which the fat is a part. Instead of adding the lecithin with the fat or fat-rich material itself, the mistake frequently made here is to add the lecithin at the wrong time, as follows:

a. Premixed with sugar, in which case the lecithin will operate at all aqueous interfaces, and not just those including the fat. Fat-solids interfaces would therefore be devoid of lecithin. Likewise, without overuse, there would be insufficient lecithin present to complement fatty interfaces in addition to fat-free interfaces. This is different from the application to ice cream mixes, where action at the aqueous interfaces is desired.

b. Just tossed into the boiling sirup. If all of the fat just happened to be pooled on the surface, this procedure would be successful. More likely, the lecithin would remain partially in little balls undissolved by the sirup, and partially dissolved in the water, which would have the same effect as premixing with the sugar.

Bakery Products

6. The most common error made in the application of lecithin to bakery products is its use to replace or "save" shortening. This it will not do. The fallacy had a natural growth. It is known that in suspensions of insoluble solids in fats (chocolate, nonaqueous coatings and icings, and so forth), fat must be eliminated when lecithin is used if viscosity standards are to be maintained. It is then incorrectly assumed that the same is true in emulsions where moisture and dissolved solids are present as the continuous phase, as in doughs and batters.

Omitted in this deduction was the fact that lecithin operates at the aqueous interfaces just as it does at the fatty interfaces, with the result that the former are extended to the same extent as the batter. Another disregarded fact is that some of the values of shortening in bakery products lie in its actual bulk presence, and not entirely in its lubricating value.

Shortening adds certain definite qualities to bakery products; lecithin adds just as definite a series of qualities. But they are not the same, and neither will replace the other. The only point of overlap is in lubricating effect. The surface tension of fat will cause retraction of the fat from a portion of the surface area covered. Lecithin increases the nonaqueous surface area covered by the fat, and reduces the subsequent retraction from aqueous as well as nonaqueous interfaces. This is the result of the presence of the water-soluble polar group in the lecithin molecule, which serves to effectually lock fatty interfaces with water and water-soluble ones.

The subject of the difference in qualities attributable to shortening

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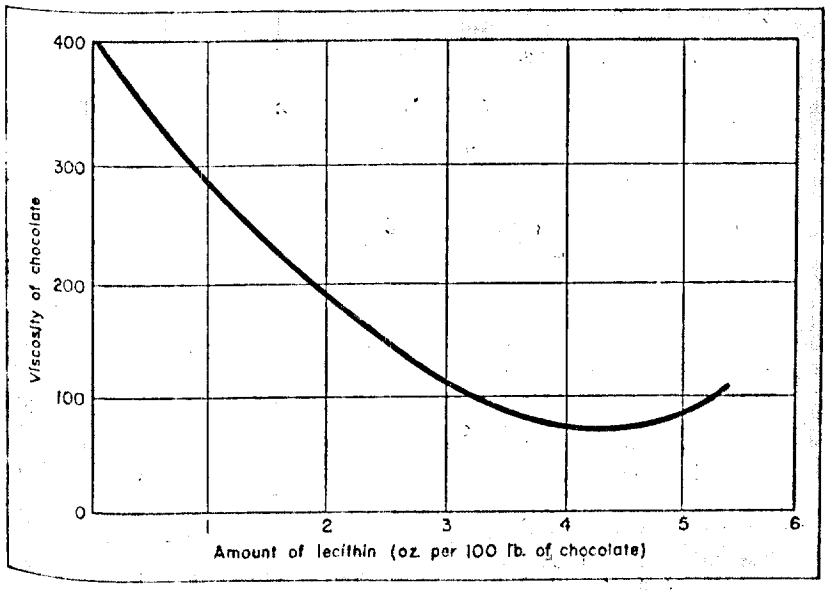

Typical curve showing rapid initial drop in viscosity as quantity of lecithin is increased, the point of "diminishing returns" and the eventual increase in viscosity as the optimum quantity of lecithin is exceeded.

from which the moisture can not as readily infiltrate and evaporate. Therefore, no matter how little or how much shortening may be used, lecithin will improve the quality.

7. It is a mistake to attempt to replace the emulsified types of shortening with regular hydrogenated shortening and lecithin. The principle advantages of the emulsified type of shortenings over regular hydrogenated types is the ability to increase the moisture content of the product. The extent to which moisture content can be increased by the use of lecithin with any type of shortening is negligible. What the lecithin does do with both types of shortening, however, is to reduce the rate of evaporation of whatever moisture is present in the finished baked product. Here again, the attributes of emulsified types of shortenings and lecithin do not conflict with nor replace each other, but are additive.

8. The practice of basing the optimum quantity of lecithin used in bakery products on the amount of shortening used is usually the correct method, but it falls short in the cases of bread, rolls and any other bakery products with relatively small shortening content. The result is that lecithin is sometimes adjudged ineffectual in these products.

Unfortunately, we do not fully understand precisely just what does happen between lecithin and the gluten in flour when sufficient lecithin is used to make the effect noticeable. The effects are definitely present, however, when from 2 to 3 oz. of lecithin per 100 lb. of flour is used. The mistake here, then, is one of underuse.

9. When applying lecithin to cakes, it must be remembered that more than a strictly oil-in-water type of emulsion is involved. If lecithin is added at the beginning of the mix with the shortening and sugar, the viscosity is reduced to the point where less air can be whipped in, with the result that volume is lost. The lecithin should instead be added after all desired volume is obtained, and just before the last cut-back of water is added. In this way the air already whipped in can be held and no volume lost.

Even when the lecithin is added at the correct time, however, overuse can again cause volume reduction. Depending upon formulation and processing, the correct lecithin range is from 1 to 2 percent of the shortening used. The precise level must be determined for each formula. The first symptom of overuse is loss of volume in the mixing bowl.

10. In biscuits and cookies, both hard and soft, errors of both overuse and underuse are made. The use of more than 2 percent lecithin, based on shortening weight, is eneffectual and wasteful where the doughs are not sticky with that concentration of lecithin.

Where doughs contain exceptionally high quantities of sirups or molasses, however, the use of 2 percent of lecithin, based on shortening weight, is insufficient to remove stickiness. So the lecithin concentration must be increased up to 4 percent of shortening weight.

11. The most frequent mistake made in applying lecithin to pie doughs is the failure to reduce mixing time sufficiently, particularly after the water is added.

Failure to reduce mixing time, short as it already may be, will result in the overdistribution of the shortening in the dough. This, in turn, will cause the production of a very tender mealy dough, but will at least partially destroy flakiness.

Flakiness can be increased by the use of lecithin if dispersion is not forced during mixing, but is allowed to take place in the oven where the lecithin will cause the formation of thinner and more frequent layers of fat.

Part II, which will be published in the August issue, of FOOD INDUSTRIES, *will discuss mistakes made in using lecithin in cooking oils, lard, shortening, margarine, mayonnaise, peanut butter, sherbets and ice cream mixes. There will also be a table showing how lecithin is used, and why, in 32 food products*— THE EDITOR.

—End—

Directions for Using Lecithin and Effects on Product Characteristics and Quality

ITEM	Quantity to use	Time of addition	Dough, batter, batch condition	Machinability or handling quality	Viscosity	Gloss	Texture	Grain	Color	Separation	Dispersion
1. Biscuits, hard cookies, ice box cookies	2% of fat	In mixer with fat or after flour cleared in	Drier, more pliable	Less sticky, clean mold or deposit		Slight	More tender		More uniform		Improved; checking reduced
2. Bread, rolls and sweet goods	2-3 oz. per 100 lb. flour	With shortening	Drier dough, more resilient	Less sticky			More silky crumb; more tender crust	More uniform	Of crust, more uniform		
3. Brittles	1% of fat	Direct to batch following fat					More tender			Retards separation	
4. Cakes (emulsified shortening)	1% of shortening	After obtaining full vol. in batter	More fluid	Cleaner deposit, lays flat in pans			More silky	More uniform	Of crust, more uniform		
5. Cakes (regular fats)	1-1½% of shortening	After obtaining full vol. in batter	More fluid	Cleaner deposit, lays flat in pans			More silky	More uniform	Of crust, more uniform		
6. Caramels, nougats, toffees	1% fat	Direct to batch following fat		Less sticky			More tender, less sticky			Retards fat separation	
7. Chocolate	0.25-0.3%	Prior to tempering			Lowered	Improved					
8. Chocolate flavor for ice cream	0.25%	Prior to molding	Softer	Easier molding	Lowered						More rapid & complete in mix
9. Cookies (molasses)	4% of fat	In mixer with fat or after flour cleared in		Less sticky, clear cut		Slight	More tender	More uniform	More uniform		
10. Cookies (soft)	2% fat	In mixer with fat or after flour cleared in	More pliable	Clear cut, less sticky			More silky	More uniform	More uniform		
11. Doughnuts (cake type)	2% of fat	With fats		Clean deposit			Softer	More uniform	More uniform		
12. Doughnuts (raised)	2% of fat	With fats		Clean deposit			Softer		More uniform		
13. Dry mixes (various, prepared)	*(See individual products)*	With fats									Improved
14. Fat-bearing chocolate sirups and toppings	1% of fat	Premix in fat-containing constituents			Variable	Improved	Smoother			Retards fat separation	
15. Flavors (emulsions)	1-10% of oils	In oils		Rapid dispersion in product reduces flash evap. loss	Raised		Smoother			Reduced	More rapid in product; on consumer's taste buds
16. Flavors (oils)	1%	Unimportant		*(See above)*							*(See above)*
17. Gravies and sauces	1-2% of fats	Variable			Variable		Smoother			Reduced	
18. Hot chocolate and other powdered drinks	0.5-1.0% total dry weight	Before dehydration or before pulverizing or sifting									More rapid & complete in reconstitution
19. Ice creams (low solids) and sherbets	0.15%	Premixed with portion of sugar	Body less watery				Smoother	Less grit		Of air reduced	
20. Ice cream coatings	0.5%	Just prior to tempering			Lowered; maintained	Improved					
21. Icings and fillings of fat and suspended solids	1% of fat	Unimportant	Softer	More pliable	Lowered if no fat omitted	Improved	Smoother				
22. Icings of fat, moisture and solids	1% of fat	After obtaining full vol.	Softer	More pliable	Variable	Improved	Smoother				
23. Lard	0.1-0.25%	When under 150° F. and before Votating	Slightly softer								
24. Meats (canned with gravy)	1-2% of fat	Variable					Gravy smoother			Slight improvement	More rapid and uniform
25. Oleomargarine	0.1-0.75%	In fats		Reduces spattering					Slight tint with high %'s	Of fat reduced	
26. Pan greases and slab grease (straight)	1% of fat	Before use			No change					Reduced weeping	More rapid and more uniform
27. Pan grease with suspended solids	1% of fat	When mixing	More fluid	Easier application	Lowered	Unimportant	Smoother				More efficient coverage
28. Peanut butter for commercial use	0.5%	After grinding	Softer	Easier deposit, brkg. reduced	Lowered	Unimportant	Smoother			Slightly reduced	More efficient coverage
29. Peanut butter for retail sale	0.5%	After grinding	Softer	Improved	Lowered	Slight	Smoother			Unimportant	Improved
30. Pie doughs	2% of fat	With shortening		Mixing time must be reduced			More flaky		More uniform	Greater: correctable with solids	Fat in thinner layers
31. Shortening	0.25%	When under 150° F. and before Votating	Slightly softer								More rapid and uniform
32. Soda crackers	2-3 oz. per 100 lb. flour	With shortening	Drier dough	Less sticky			More tender	More uniform	More uniform		

ADDENDA: (1) Lecithin does not save fat except in items 7, 20 21, and 27. (2) It prolongs shelf life by inhibiting oxidation and by moisture retention. (3) It increases fermentation tolerance of 2 and 12.

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most commonly used in starch manufacture, but liquid screens of the circle-throw vibrator type are coming into the picture for this purpose.

A settling tank is appropriate for the first stage of separation of starch from the crude starch milk, but a conventional settling tank does not perform as well on the coagulum after acidifying and heating. A conical settler operating continuously seems more promising.

The most satisfactory method of thickening or dewatering the partly thickened coagulum is yet to be determined. In the processing-step-flow sheet, a filter or centrifugal is indicated. Conventional vacuum drum filters or filter presses do not work with this material, although filtration with very slight pressure does fairly well on a small scale.

When the coagulum is reduced to a cake in a centrifugal, a vacuum dryer would be recommended to minimize destruction of carotene. Alternatively, a spray dryer might be considered for handling the partly thickened coagulum from the settling unit or the material after further thickening in a high-speed continuous centrifugal.

It must be recognized that the flow sheet as here presented is based on laboratory results. The process has not actually been tried on an integrated plant scale.

References

1. J. F. Villere, D. C. Heinzelman, J. Pominski and H. R. R. Wakeham, *Food Industries*, vol. 16; page 76, 1944.

2. Since completion of this report, U. S. Patent No. 2,348,443 was granted to H. M. Barnett on May 9, 1944. This deals with the preparation of a carotene concentrate from carrots by utilizing a somewhat similar principle.

—End—

18 MISTAKES IN THE USE OF LECITHIN

PART II—Errors made in using lecithin in cooking oils, lard, shortening, margarine, mayonnaise, peanut butter, sherbets and ice cream mixes are discussed. A table shows how it should be employed, and why, in 32 items

By L. RUSSELL COOK, Vice-President,
W. A. Cleary Corp., New Brunswick, N. J.

12. When it is desirable to employ lecithin as an antioxidant in frying and cooking oils for potato chips, doughnuts, french fries, and so forth, no more than 0.2 percent lecithin should be used. The use of 0.1 percent is even better if sufficient antioxidant protection is obtained.

Overuse in this application will result in greatly increased penetration of the cooking fat into the article being treated. Lowered interfacial tension, which is beneficial within the internal structure of the dough, is definitely to be avoided in cooking fats.

Fatty Foods

13. When lecithin is added in the manufacture of lard, shortening, margarine, oleo oils, hard butter, and so forth, the mistake is often made of permitting the fat to be too hot, either at, or subsequent to, the time of lecithin addition. The elevated temperature then causes an undesirable darkening to take place. The cooler the bulk fat at the time of, and subsequent to, the addition of the lecithin, the less danger there will be of darkening. Maximum temperatures should be 150 deg. F.; 130 deg. F. is more desirable. Time is as much of a factor as is temperature, however, so if higher temperatures are necessary for one reason or another, the duration of the high temperature should be shortened as much as possible.

Mayonnaise

14. The use of lecithin in mayonnaise would seem to be a natural application. The fact, however, that the egg yolk used already contains a high percentage of lecithin—and the fact that in this product the egg yolk forms a major portion of the emulsion nucleus—precludes the successful use of additional lecithin. When added, lecithin spreads the nucleus so thin that its membrane strength is reduced to the point of breakage and the emulsion collapses.

Only when the quantity of egg yolk is reduced, and a combination of lecithin and protein substituted, will the use of additional lecithin be successful. Even this will not produce as good a mayonnaise as full employment of egg yolk.

Peanut Butter

15. Lecithin is frequently used in peanut butter to provide antioxidant protection and to soften the consistency of the butter. Lecithin will do both of these things, and where the peanut butter is to be used within a relatively short time for commercial sandwich or confectionery purposes, nothing more needs to be said.

When, however, the application is to peanut butter made for retail distribution, the reduced viscosity of the butter will aggravate oil separation. In this case, therefore, the use of the lecithin should be accompanied by the addition of from 3 percent to 5 percent of flour (soya, wheat, rye). The flour added will prevent separation without destroying either the antioxidant protection or the soft consistency.

Sherbets

16. In sherbets and relatively low-solids ice cream, where functioning at water-solids interfaces is the objective, applications have frequently been unsuccessful because the usual type of lecithin was merely added to the batch in the pasteurizer. The time of addition is correct, but either a water dispersible lecithin must be used, or, if the fat-soluble type is employed, it must be premixed with about ten times its weight of sugar. This admixture with sugar permits absorption of the oil and exposure of the polar group of the lecithin molecule.

Ice Cream Mixes

17. In some products—such as ice cream mixes, gravies and certain types of suspension-emulsion combinations—it is necessary either to have the lecithin operate at water-solids interfaces or to approach water-fat interfaces from the aqueous phase. Where this is the case, it is an error to attempt to use the more usual type of lecithin where the phosphatides are dissolved in or suspended in the oil carrier. In such types, the polar group is not exposed, and the solution in water is difficult.

The type of lecithin required for such applications is a water-dispersible type in which the polar group is exposed and can carry the lecithin into an aqueous medium.

Storing Lecithin

18. The mistakes made in the handling of bulk lecithin usually result from the opinion that special care is needed for preservation. None is needed.

Storing lecithin in refrigerated rooms will cause the lecithin to harden, making it difficult to handle and reducing speed of effectiveness when used. Likewise, reduced temperatures may cause some separation, leaving pockets of oil on the surface and spotted throughout the mass.

Storing lecithin in hot rooms or against radiators is likewise to be avoided. Elevated temperatures, if prolonged, will cause darkening and burning.

Temperatures of anywhere from 70 to 100 deg. F. are quite safe for lecithin-storage purposes.

Food Industries 450: 83-5, April 1949.

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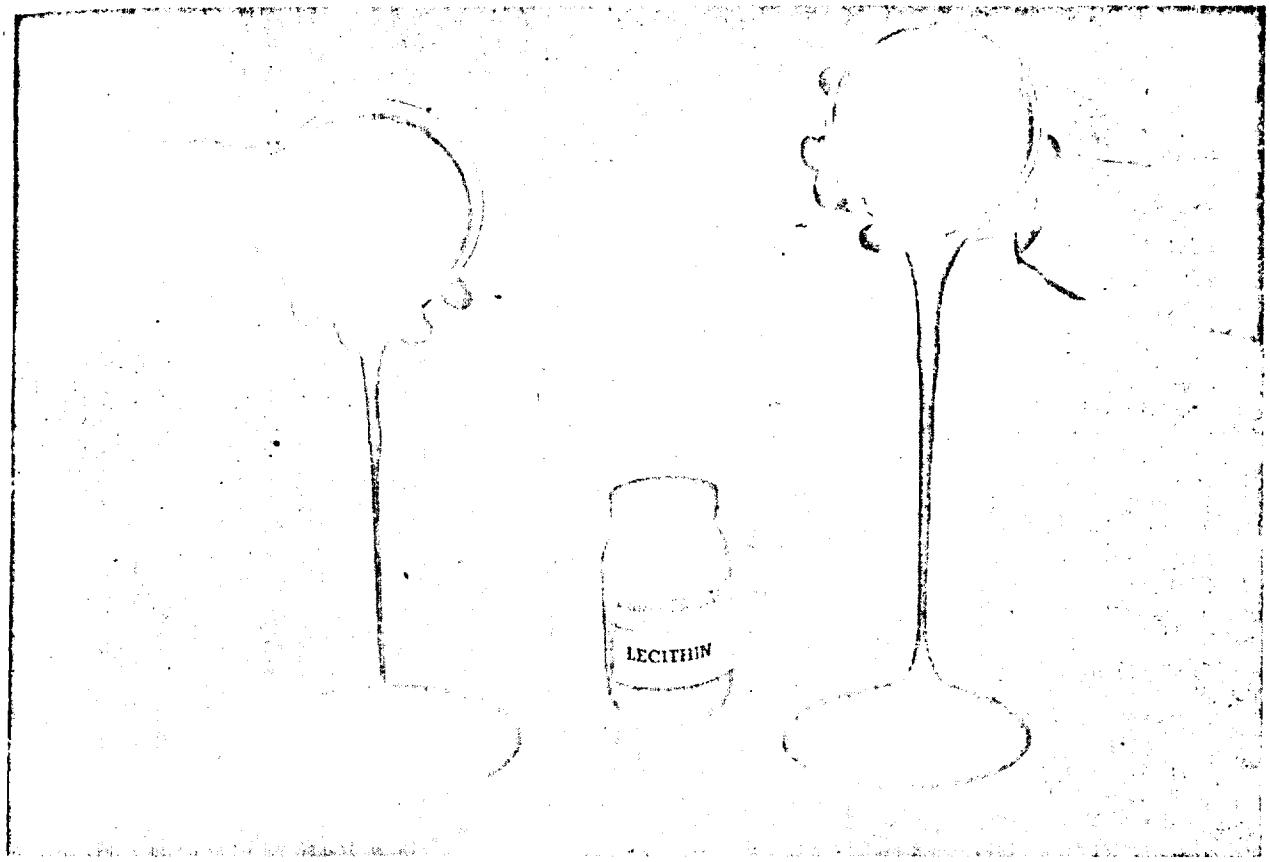

EFFECT of lecithin (0.3 percent) in reducing chocolate viscosity is seen (left). Chocolate (right) has no lecithin.

How Lecithin Affects Cost and Quality of Candy

It increases production, reduces amount of cocoa butter needed in chocolate, and improves quality of fudges, caramels, toffees, and brittles*

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Lecithin, when added to chocolate and other confectionery products, serves the useful purposes of cutting the cost of their manufacture, while at the same time improving their quality. Better quality in candies is the chief advantage in using lecithin for products like caramels, toffees, brittles, and fudges.

But first, a few words about lecithin: This name is given to a group of substances normally present in all living tissues—just as are fats, proteins, and carbohydrates. Commercial lecithin is not a synthetic substitute. Instead, it is a concentrate of the lecithin that naturally occurs in such vegetable materials as corn and soybeans. It is processed in such a way that it performs in food products with as nearly as possible the same efficiency as it does in nature.

All of the phenomena for which lecithin is responsible in chocolate and confectionery products is the result of one or more of the following characteristics: (1) Lecithin is surface active, (2) it reduces surface tension, and (3) it is an antioxidant.

Here is how the economies are achieved in chocolate products: Lecithin reduces the viscosity of chocolate, and hence cuts down on the amount of cocoa butter needed. Before the chocolate is molded, it is invariably brought to a predetermined fluidity or viscosity with cocoa butter.

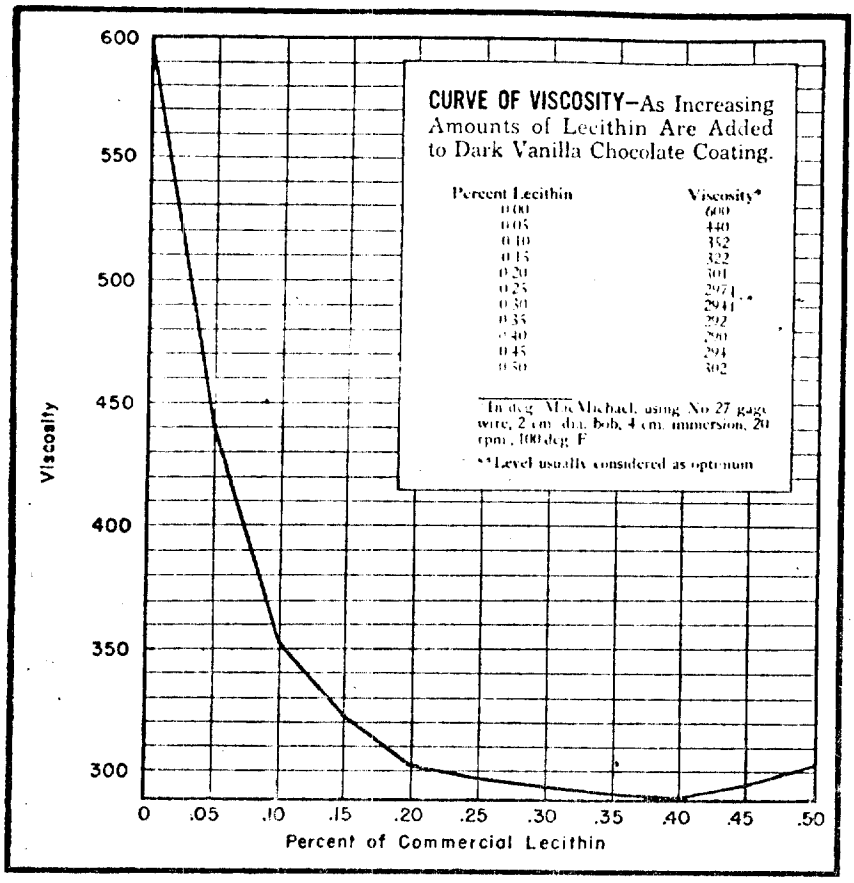
Since lecithin reduces surface tension, the desired viscosity can be obtained with less cocoa butter than would be necessary if lecithin were absent.

There are some limitations to the use of lecithin. Suppose you start out with a chocolate coating containing no lecithin, then gradually add an increasing amount of lecithin. You will find that each portion of lecithin added will reduce the viscosity of the coating to a greater extent than does the next portion added. This curve of diminishing usefulness gradually flattens out until a point is reached where continued additions fail to further reduce the viscosity of the chocolate. As you proceed to add even greater quantities, you will find that the viscosity curve actually begins to rise again. Thus, you finally begin to defeat the purpose for which lecithin is added.

While the savings of cocoa butter that result from the use of lecithin are of vital economic importance, the effect upon quality should not be glossed over too lightly.

Before high-speed equipment was used in the chocolate and confectionery industries, the percentage of cocoa butter was considerably lower in fin-

* This article is based on paper, "Lecithin in Chocolate and Confectionery Products," which was presented by author at annual production conference of Pennsylvania Manufacturing Confectioners' Association.



DOWN goes viscosity of coating as more and more lecithin is added—until 0.25-0.30 level is reached, at which time curve flattens, then rises.

ished chocolate products than is often found today. At that time, chocolate manufacturers used the amount of cocoa butter that gave them the best quality. And they were under no compulsion to use cocoa butter as an assist to rapid depositing or enrobing.

It was only after high-speed equipment was installed that chocolate manufacturers found it necessary to use more cocoa butter. They added more cocoa butter not because the quality of the products was thus enhanced, but because there was thus produced the fluidity or low viscosity required to run high-speed machines.

Actually, they sacrificed a certain amount of quality in the interest of mass production. For, when these excessive amounts of cocoa butter were added, the chocolates became somewhat greasy compared with those made by the older methods. The use of lecithin as a means by which fluidity for high-speed machining can be secured permits the maintenance of percentages of cocoa butter which are once again limited by consideration of quality only.

How Lecithin Steps Up Production

In the manufacture of chocolate, lecithin is usually introduced just prior to final viscosity adjustments. Its use is not entirely limited to this function.

It is possible, for instance, to use 1 or 2 oz. of lecithin per 100 lb. of chocolate in the mixing operation and also during the conching. Judicious use of lecithin at either of these points considerably reduces power requirements as a result of viscosity reduction. Or, if power reduction is neither necessitated nor required, usual mixing and conching viscosities can be maintained by using lecithin, and to a proportionate extent omitting the unnecessary poundage of cocoa butter.

This, in terms of finished chocolate, increases the output of mixers and conches per machine hour. Reason for this is that the omitted cocoa butter need not be added until after conching is completed.

When lecithin is used in either of these two ways, it must be remembered that a good portion of its viscosity-reducing properties is dissipated by the time the coating is finally ready for molding.

Insofar as final viscosity is concerned, 1 oz. of lecithin added at the mixer or at the conch is worth about ½ oz. of lecithin when it is added after conching has been completed.

Several hours of valuable conching time are lost at the start of the process because it takes that long for the conch to break down the coating to the fluid condition necessary for proper conching action. An ounce or two of lecithin added to each 100 lb. of coating at the start of conching will rapidly break down the coating to its proper fluidity. As a result, conching effects will become noticeable several hours earlier.

The amount of lecithin that should be used for chocolate products varies, depending upon circumstances. Ordinarily, if full advantage is to be made of the lecithin, 4 oz. should be used for 100 lb. of dark chocolate coating. For 100 lb. of milk coating or light sweets, approximately 5 oz. of lecithin should be used.

These additions are made on the assumption that no lecithin was used either during mixing or conching. If lecithin has been added at either or both of these points, the total amount should approximate the 4 and 5 oz. mentioned, less about one-half the lecithin added at the mixer and/or the conch. While these amounts are not the absolute maxima, they are considered the "point of diminishing returns" under normal market conditions.

With the price of cocoa butter as high as it has been, quantities of lecithin even greater than those mentioned may be found economical. There is no set rule that can be applied for determining the maximum amount of lecithin that can be used, because of the many variables governing the final viscosity in chocolate products. If it is necessary to take full advantage of lecithin during periods of high cocoa butter prices, viscosity curves should be run on each coating.

Thus, it will be possible to determine the point at which a dollar's worth of lecithin ceases to do more work than a dollar's worth of cocoa butter.

Uniform Coating Coverage

The extent to which lecithin should be used will also be determined by the intended application of the coating. Suppose, for example, a confectioner wants to use the same coating on two or three different centers of varying sizes and shapes, and suppose he wants the same percentage of coverage of coating on all these centers. Obviously, no one coating will do this, unless its viscosity is changed in accordance with the shape and size of the centers.

Under such conditions, the chocolate manufacturer will frequently obtain the standard viscosity of the coating with perhaps only 2 oz. of lecithin per 100 lb. of coating. The coating can then be used "as is" on the center from which the coating drains most easily in the enrober. For smaller and flatter centers, the confectioner can then add more lecithin to the coating

before tempering. Thus, the viscosity of the coating will be reduced so that all centers will have the same percentage of coverage.

It is obvious that the confectioner wishing to use lecithin in coatings should find out from his chocolate manufacturer the amount of lecithin already used in the coating—or he should submit a sample to his lecithin supplier, who in turn will determine the amount of lecithin to add.

If moisture gets into coatings, either through contact with damp atmospheres or by accidental means, additional lecithin is needed. As a good example, consider ice cream coatings, into which moisture invariably finds its way while ice cream bars are being dipped. As a result of this moisture contamination, there is an increase in the viscosity of the coating, which can be minimized by using 8 oz. of lecithin to 100 lb. of ice cream coating, instead of the 4 to 5 oz. normally used.

Where moisture is introduced into confectionery coatings by any means, the original viscosities can be maintained by adding small amounts of lecithin. The amount required will vary with the quantity of moisture.

Frequently the confectioner may need lecithin in the enrober when his coating has thickened because: (1) Starch is washed from the centers into the stream of chocolate, (2) temporary loss of proper temper occurs, or (3) moisture from the centers is introduced to the coatings.

In any of these cases, the coating can be thinned by putting 1 or 2 oz. of lecithin into the enrober pot. After the lecithin is added, the belt carrying the centers to the enrober should be stopped momentarily to permit the lecithin to be dissolved and thoroughly mixed in the coating.

By adding 0.5 to 1.0 percent lecithin to sweetened cocoa powdered milk drinks, balling will be reduced and reconstitution hastened.

Lecithin is added to confectionery products other than chocolate to disperse fats, not to save them as is the case with cocoa butter in chocolate. The fat in certain confectionery centers is rarely, if ever, the main component of the confection. Use of lecithin in such fat-containing confectionery centers can best be illustrated by separately considering its application in caramels, toffees and brittles, and fudge.

Caramel Fats Uniformly Dispersed

If caramels were to be made without fat, while still avoiding stickiness, it would be necessary to cook them to so high a temperature that they would become extremely tough, if not altogether hard. For this reason, as well as for flavor, fats are always added to good caramels so that they will be soft, tender, and free from stickiness.

Since caramels are a water-base product, there is a high interfacial tension between them and any added fats. Good dispersion of the fat in the caramel is, therefore, difficult to obtain. However, when lecithin is added to the extent of 1 percent of the weight of the total fat content in the finished caramel, the interfacial tension between the fats and the caramel is reduced. Consequently, the fats are more uniformly dispersed.

When you cook a batch of caramel, you can see small pools of fat floating on the top. Then, if you add lecithin, in the quantity indicated above, to the batch while it is cooking, the pools of fat will quickly disappear and remain dispersed. Likewise, after the caramel has cooled, its surface is considerably more free of fat than when lecithin is not used, indicating that the fat has been bound inside the caramel.

Furthermore, because lecithin is an anti-oxidant, it will extend the shelf life of caramels by retarding staleness and rancidity.

Toffees and Brittles

In fat-containing toffees and brittles, the same dispersion problems are met as in caramels. Here, too, lecithin assures a complete and permanent dispersion of the fats, thus preventing the formation of a thin greasy layer on the surface of the candies, where it oxidizes and rapidly turns stale.

Good fat dispersion also results in more tender toffees and brittles, with less fragility than is customary. This seems paradoxical, but there is a logical explanation: When toffees or brittles break, it is at their weakest point. When you eat them, however, they are only as tender as their toughest point. These areas of alternating fragility and toughness occur because the fat is not completely dispersed. When the fat is completely homogenized, alternating tender and tough spots are absent, breakage at weak spots is reduced, and the candies are more tender.

As in the case of caramels, lecithin also retards staleness and rancidity in toffees and brittles.

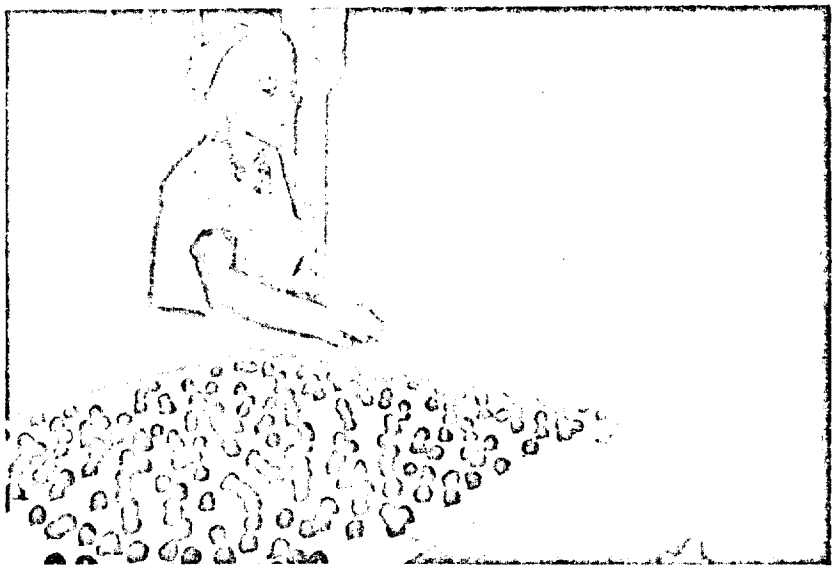
Smoother Fudge

Fudges usually do not present any problems of stickiness or fragility. Nevertheless, adequate fat dispersion does assist in making a smooth fudge. This does not mean that the mere use of lecithin will result in a smooth fudge, since too many other factors enter into the production of a fine crystal structure. All things being equal, the addition of lecithin to the extent of 1 percent of the weight of fat in the fudge will result in a finer crystal structure and a smoother fudge.

Furthermore, since lecithin dissolves partly in the fat and partly in the water present in the fudge, it binds them together and delays drying. Thus, the shelf life of fudge is extended from a standpoint of flavor-freshness and dryness.

In fact, even before the fudge becomes too dry to eat, such losses in moisture cause crystal growth, which turns the fudge sandy. By retarding this moisture loss, lecithin aids in maintaining the same smoothness in fudge that it had originally.

Still another use of lecithin: Addition of 4 oz. to 100 lb. of ice cream mix speeds up and improves the dispersion of the mix when added at the pasteurizer. The lecithin also will reduce the accumulation of chocolate lumps on strainers.



LECITHIN added to coatings in enrober corrects any possible thickening.

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Arch Path 26:456-62 (1938)

EXPERIMENTAL HYPERCHOLESTEREMIA IN DOGS

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Anitschkow and Chalatow,[1] working independently of others, and Wacker and Hueck,[2] also working independently of others, reported almost simultaneously in 1913 that pure cholesterol administered by mouth over a considerable period could produce lesions in the intima of the aorta of the rabbit very similar to those of arteriosclerosis of man. The work of these investigators was referred to by Duff[3] in his excellent review of experimental cholesterol arteriosclerosis. The observation has been confirmed, moreover, by numerous investigators. Bailey[4] in 1915, and several other workers since, have reported the production of arteriosclerosis in guinea pigs by the feeding of cholesterol, but there has been no convincing demonstration of a similar effect in any other species. It is true that lesions have been described as occurring in the arteries of pigeons, chickens, parrots, mice, rats and goats following feeding of cholesterol, but because of the inconstancy of their occurrence they must be regarded with a strong suspicion that they were spontaneous in origin. Up to the present time success has not attended any attempt to produce the condition known as cholesterol arteriosclerosis in cats, dogs, foxes and monkeys.

Duff[3] expressed the opinion that hypercholesteremia of some degree is an essential factor in the development of experimental cholesterol arteriosclerosis of rabbits. If this is true, the failure to produce arteriosclerosis in the dog by feeding a diet rich in cholesterol may be assumed to be owing at least partly to the inability of investigators to produce hypercholesteremia consistently in this animal. Grigaut and L'Huillier[5] found moderate hypercholesteremia in 2 dogs each of which received five daily feedings of cholesterol dissolved in melted butter and mixed with meat. By daily feeding of cholesterol and bile, Anitschkow[6] was able to raise to about twice its normal value the cholesterol content of

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3. Duff, G. L.: Arch. Path. **20**:81 and 259, 1935.
4. Bailey, C. H.: Proc. Soc. Exper. Biol. & Med. **13**:60, 1915-1916.
5. Grigaut, A., and L'Huillier, A.: Compt. rend. Soc. de biol. **73**:304, 1912.
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the blood of a dog which had been subjected to partial ligation of the common bile duct, ovariectomy and splenectomy. Weltmann and Biach[7] found no increase in the concentration of cholesterol in the blood of a puppy following feeding of cholesterol for sixteen days, and Ssokoloff[8] likewise found none in 10 dogs after they had been fed cholesterol for seven days. Muñoz[9] reported that the concentration of cholesterol in the blood of the dog is higher when the diet is of meat than when it is high in fat or carbohydrate.

FIRST SERIES OF EXPERIMENTS

Hueck and Wacker,[10] Versé[11] and Page and Bernhard[12] showed that hypercholesteremia is invariably associated with sufficient increase in neutral fats and in other lipoids of the blood to cause marked lipemia. At the outset of the experimental work to be reported here, therefore, it was deemed desirable to determine what effect on the cholesterol content of the blood might be obtained by the feeding of diets high in fat without added cholesterol, bile salts and lecithin as well as with these substances added in various combinations.

Accordingly, 9 adult dogs, ranging from 12 to 18 Kg. in weight, were chosen, and in preliminary studies normal values for blood cholesterol were set up for dogs fed on the two types of diet most commonly used in this laboratory, namely, the standard diet and the kennel diet. The former diet consists of a mixture of the following foods in the percentages given: horse meat, 38; cracker meal, 35; canned tomatoes, 15; lard, 8, and bone ash, 4. A weighed quantity was given daily. The latter diet consists of dog biscuits, alternating with a mash made of ground horse meat, corn meal, oat meal and bone ash. Determinations of cholesterol were made by the Lieberman-Burchard method as used by Bloor and his associates.[13] The values for the blood cholesterol of 4 dogs maintained on the standard diet for six weeks ranged from 185 to 285 mg., with an average of 225 mg., per hundred cubic centimeters. The concentration of the blood cholesterol of 5 dogs maintained on the kennel diet for the same length of time ranged from 155 to 240 mg., with an average of 195 mg., per hundred cubic centimeters. The values for the blood cholesterol of the same dog varied somewhat from hour to hour, but a characteristic diurnal curve was not obtained. As a rule the highest value was reached from three to six hours after the daily meal, particularly if the dogs were maintained on the standard diet, but this was not observed invariably.

7. Weltmann, O., and Biach, P.: Ztschr. f. exper. Path. u. Therap. **14**:367, 1913.
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12. Page, I. H., and Bernhard, W. G.: Arch. Path. **19**:530, 1935.
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RESULTS IN FIRST SERIES

After the control values had been determined, the animals were placed on the following dietary regimens for eight weeks, with results as described:

Group 1.—This group was made up of 3 dogs which received high fat diet A daily. This diet consisted of 4 Gm. per kilogram of body weight of the standard diet described and 10 Gm. per kilogram of body weight of lard.

This diet was effective in producing moderate hypercholesteremia in 2 of the 3 dogs of this group. Several readings of the cholesterol content of the blood of dog A exceeded 400 mg. per hundred cubic centimeters; the highest reading was 482 mg. per hundred cubic centimeters, while the value for the blood cholesterol of dog B ranged consistently around 350 mg. during the last week of the feeding. Dog C at no time exceeded the upper limit of normal cholesteremia as determined after feeding the standard diet in preliminary experiments.

Group 2.—Dog D was maintained on high fat diet A, and 3 Gm. of solid cholesterol was given by capsule daily. Dog E received high fat diet A and 3 Gm. of sodium cholate daily, while dog F was given high fat diet A, cholesterol and sodium cholate.

The concentration of the blood cholesterol of all of the dogs of this group became moderately augmented. The augmentation, however, did not exceed that which, according to results obtained in group 1, might have been expected from feeding the high fat diet alone. During the last week of the experiment the values for the blood cholesterol of all 3 dogs ranged from 300 to 400 mg. per hundred cubic centimeters.

Group 3.—These 3 dogs each received from 10 to 15 Gm. daily of lecithin of adrenal origin, prepared and furnished by Dr. E. C. Kendall. In addition, dog G received high fat diet A and cholesterol, dog H received high fat diet A and sodium cholate, and dog I received high fat diet A, cholesterol and sodium cholate.

In the 3 dogs of this group hypercholesteremia of considerable proportions developed. The blood cholesterol of each rose to more than 600 mg. per hundred cubic centimeters on more than one occasion during the last two weeks of the experiment. In dog G, which received both cholesterol and lecithin, the value for blood cholesterol on three separate occasions exceeded 800 mg. per hundred cubic centimeters; once the value was 940 mg.

COMMENT ON FIRST SERIES

It is probably safe to conclude from this first series of experiments that diets of which the fat content is very high may produce moderate hypercholesteremia, and that lecithin administered in conjunction with a high fat diet will produce hypercholesteremia of considerable proportions. It should be noted that the fat of the diet employed, lard, is a pure fat and that therefore the effect obtained cannot be attributed to vitamins, phospholipins or other lipoid substances. In all probability neither cholesterol nor sodium cholate has any effect on the blood cholesterol of the dog, at least when administered by mouth in the solid state.

There is no doubt that the feeding of large amounts of fat would be an excellent method of producing hypercholesteremia in dogs if it were not for the fact that most dogs have for such diets a marked intolerance, expressed by discouragingly frequent emesis. Perhaps the relation between ingestion of fat with actual retention and the development of hypercholesteremia may be brought out best by stating that dog C (group 1), which retained practically no food during the last two weeks of the experiment, showed no increase in the concentration of blood cholesterol, whereas dog G (group 3), which vomited not more than two or three times throughout the entire period of eight weeks, presented the most marked hypercholesteremia seen to date in this laboratory.

SECOND SERIES OF EXPERIMENTS

Because the lecithin employed in the previous experiments apparently was efficacious in raising the concentration of the blood cholesterol of dogs, it seemed desirable to investigate its effect still further.

To avoid confusing dogs of the first series of experiments with those of the second series, dogs of the latter series were designated by arabic numerals. Seven dogs were given the standard diet. One dog (dog 8) received the kennel diet for a control period of two weeks. On the seventh and fourteenth days, determinations of cholesterol were made on the blood withdrawn following a fast of twenty-four hours and on blood withdrawn five hours after feeding. The average of the four readings thus obtained was taken as a standard control value of the blood cholesterol for the particular dog in question. The dogs were then given the special diets and medications to be enumerated, and the values for the blood cholesterol were obtained following a fast of twenty-four hours and again five hours after feeding. The values were determined at weekly intervals for an experimental period of six weeks. The administration of the special diets and the medications were then discontinued, and the values for the blood cholesterol were determined at weekly intervals for a recovery period of four weeks.

During the experimental period the 8 dogs were maintained as follows: Dogs 1 and 2 received high fat diet B (fig. 1). This diet consisted of 10 Gm. of the standard diet per kilogram of body weight plus 5 Gm. of lard per kilogram of body weight. This was tolerated much better than high fat diet A because it had a much lower content of fat. High fat diet B contained 33 per cent lard by weight, as against 71.5 per cent in high fat diet A. Dogs 3 and 4 were given high fat diet B plus lecithin (fig. 2). To dogs 5 and 6 the standard diet plus cholesterol and lecithin was administered (fig. 3). Dog 7 was given the standard diet plus lecithin (fig. 4). Dog 8 received the kennel diet plus lecithin (fig. 4).

RESULTS IN SECOND SERIES

From a brief perusal of the accompanying figures it may be concluded that a diet high in fat may or may not effect a rise in the concentration of cholesterol in the blood of the dog (fig. 1). This is in agreement with the findings in the first series of experiments. Lecithin when added to a diet high in fat will produce marked hypercholesteremia (fig. 2). In the opinion of others who observed this work and in my opinion the experiments recorded here were not carried out for a sufficient length of time to determine the true proportions to which

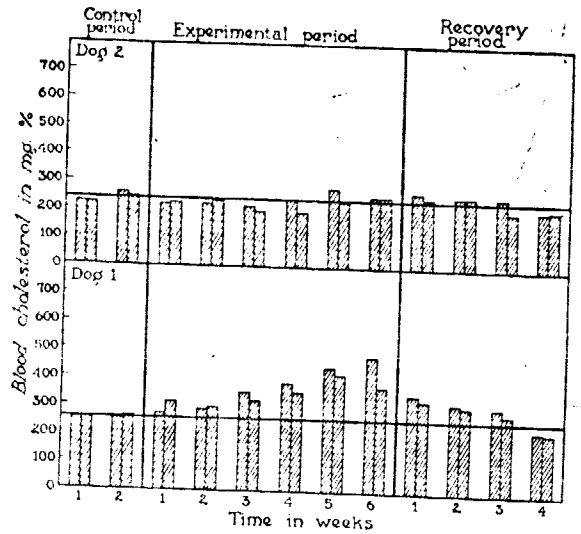

Fig. 1.—Effect of feeding high fat diet B. In this figure and in figures 2, 3 and 4 the first block of each pair represents the cholesterol content of the blood after a fast of twenty-four hours; the second block, the cholesterol content five hours after feeding. The horizontal line represents the average blood cholesterol during the control period.

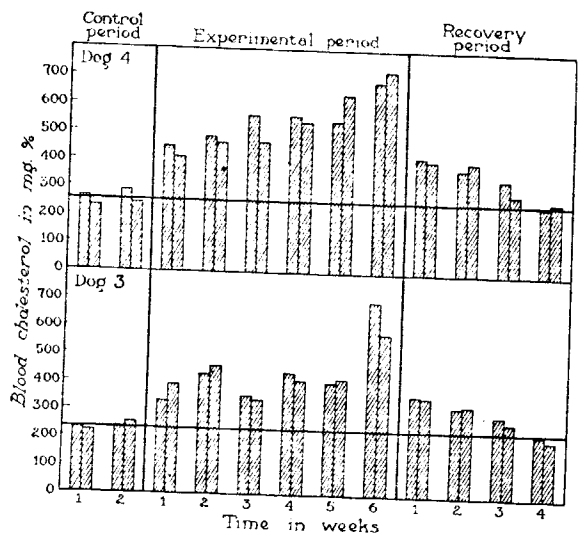

Fig. 2.—Effect of feeding high fat diet B and lecithin.

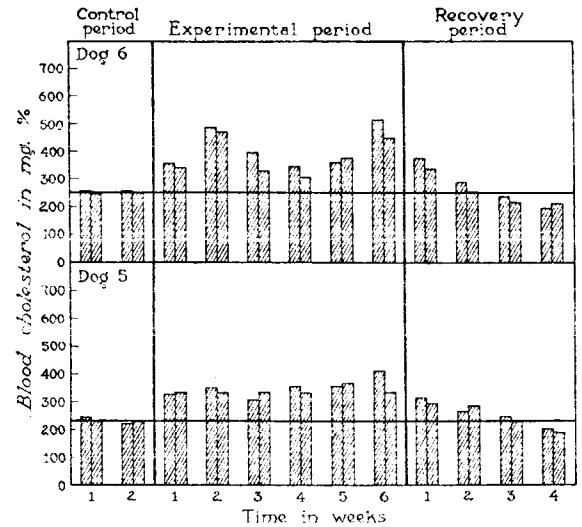

Fig. 3.—Effect of feeding the standard diet, lecithin and cholesterol.

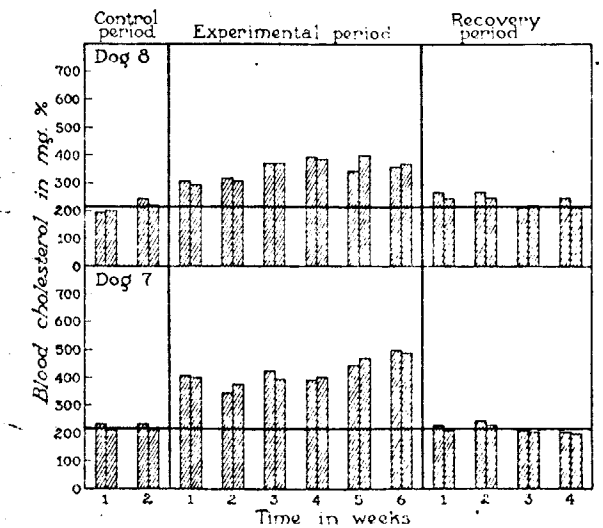

Fig. 4.—Effect of feeding the standard diet and lecithin (dog 7) and the kennel diet and lecithin (dog 8).

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such hypercholesteremia may attain. At the end of the experimental period the values for the blood cholesterol of dogs 3 and 4 were apparently still rising. Obviously, more extended experiments are desirable.

As is indicated in figure 4, a diet high in fat is not essential for the development of hypercholesteremia in the dog, for when lecithin alone was added to either the standard diet or the kennel diet elevation of the level of the blood cholesterol occurred. The addition of solid cholesterol to this regimen did not materially alter the results (fig. 3).

Wacker and Hueck,[2a] and Versé[11] reported that in hypercholesteremia the greater part of the increase is owing to the rise in cholesterol esters, while the augmentation of free cholesterol is not so striking. In my experiments, determinations of cholesterol esters, made by the method of Bloor and Knudson[14] on random samples of blood, indicated that the hypercholesteremia is owing to a rise in concentration of both the cholesterol esters and the free cholesterol. The value for free cholesterol in the majority of instances ranged from 30 to 40 per cent of the value for total serum cholesterol.

COMMENT ON THE SECOND SERIES

If the development of arteriosclerosis in the dog is dependent on the development of hypercholesteremia, as is the case with the rabbit, the studies reported here indicate that perhaps the dog may yet be employed in the study of this disease. At present I am maintaining a small group of dogs on long time feeding with lecithin. Studies of cholesteremia resulting from feeding of lecithin derived from eggs also are being conducted in this laboratory. Since other investigators have demonstrated lipemia in association with hypercholesteremia,[15] the neutral fats and phospholipoids of the blood were followed in my second series of experiments. These are to be reported in a later paper.[16]

SUMMARY

The data obtained from the experiments indicate that a diet high in fat may or may not produce moderate hypercholesteremia in the dog. Lecithin of adrenal origin, when given by mouth daily, will constantly raise the concentration of cholesterol in the blood. Lecithin plus a diet high in fat will effect marked elevation of the level of the blood cholesterol. This elevation is owing to increased concentration both of free cholesterol and of cholesterol esters. Cholesterol and sodium cholate have no effect when administered by capsule in the solid state.

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16. Flock, E. V.; Corwin, W. C., and Bollman, J. L.: Sustained Hyperlipemia of Dietary Origin in the Dog, Am. J. Physiol., to be published.

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Arch. Derm & Syph. 52(4): 252-256, 1945

EFFECT OF FEEDING A LIPOTROPIC SUBSTANCE TO PATIENTS WITH XANTHELASMA

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The association of hyperlipemia with xanthelasma is well known and has been the subject of study by several investigators.[1] The relation which the hyperlipemia has to cardiovascular disease, especially peripheral and coronary arteriosclerosis, is less well recognized but likewise has been found to be rather common.[1] From such observations, it would seem that the presence of these benign fatty lesions within the skin of the eyelids may suggest a disturbance in fat metabolism, in some persons severe enough to warrant further study in regard to the blood lipids, cardiac function and peripheral vascular sufficiency.

If xanthelasma may at times be the herald lesions of disturbed fat metabolism, it then should be determined whether some of the lipotropic substances which have so pronounced an effect on several types of experimental and acquired disturbances in fat storage and metabolism might not be of value in lowering the blood fat levels and possibly, because of these changes, in causing an absorption of the local lesions.

With these theoretic possibilities in mind, we have conducted some experiments, using one related group of lipotropic substances, on a series of patients with xanthelasma.

PROCEDURE AND METHODS

In this study, the total fasting blood lipids of 18 normal hospital employees, the total fasting cholesterol of 10 members and the fasting free cholesterol of 9 members each of this group were used as our controls.

The total fasting blood lipids of 39 patients with xanthelasma were determined and compared with those of the controls. For 26 of these patients, determinations of fasting total and free blood serum cholesterol were made and compared with the normal. For 17 patients, determinations of the fasting total blood phospholipids were also made. When more than one fasting blood specimen was drawn, the average of all determinations was taken.

Eight patients were studied before and during the feeding of the lipotropic substance. Fasting blood was drawn at intervals of seven days throughout the experimental period. This group had 134 separate determinations each of the fasting total lipids and free and total cholesterol and 122 separate determinations of the fasting phospholipids. The experimental interval ranged from 46 to 189 days (46, 63, 69, 98, 126, 150, 160 and 189 days). After preexperimental analyses of fasting blood lipids each patient was advised to ingest soybean lecithin complex in the form of 5 Lexo wafers[2] daily, divided as desired. The diet was not controlled.

Studies and contributions from the Department of Dermatology and Syphilology of the University of Michigan Medical School, service of Dr. Udo J. Wile and Dr. Arthur C. Curtis.

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2. The wafers were supplied by the American Lecithin Company Inc., Elmhurst, Long Island, N. Y. The analysis of the wafers is given as follows:

Average weight per wafer, 16.85 Gm.

Composition	Per Cent	Gm. per Wafer
Moisture	6.88	1.16
Mineral matter	2.44	0.41
Fat	14.92	2.52
Soybean lecithin (lipid P × 47.6)	18.24	3.07
Protein (protein N × 5.7)	5.04	0.85
Carbohydrates (by difference)	52.48	8.82
	100.00	16.85

Calories per wafer (16.85 Gm.) . . 89

The soybean lecithin in the analysis refers to the lecithin complex, which consists of approximately equal parts of lecithin, cephalin and lipositol (inositol phosphatide).

Each patient ingested daily 15.35 Gm. of soybean lecithin, or, according to analysis, approximately 5 Gm. each per day of lecithin, cephalin and inositol. Hereafter, this complex will be called the lipotropic substance.

No definite evidence of intolerance to the lipotropic substance was noted. No more than the usual fluctuation of weight was observed in any patient.

The analysis of the lipids was carried out on aliquot portions of a stock extract prepared by refluxing serum with ether-alcohol, filtering off the precipitate of serum proteins and refluxing the precipitate again, first with absolute alcohol and then with absolute ether. The combined extracts, after concentration by vacuum distillation, were then taken up in purified petroleum benzine, washed with water, dried with anhydrous sodium sulfate and finally made up to volume.

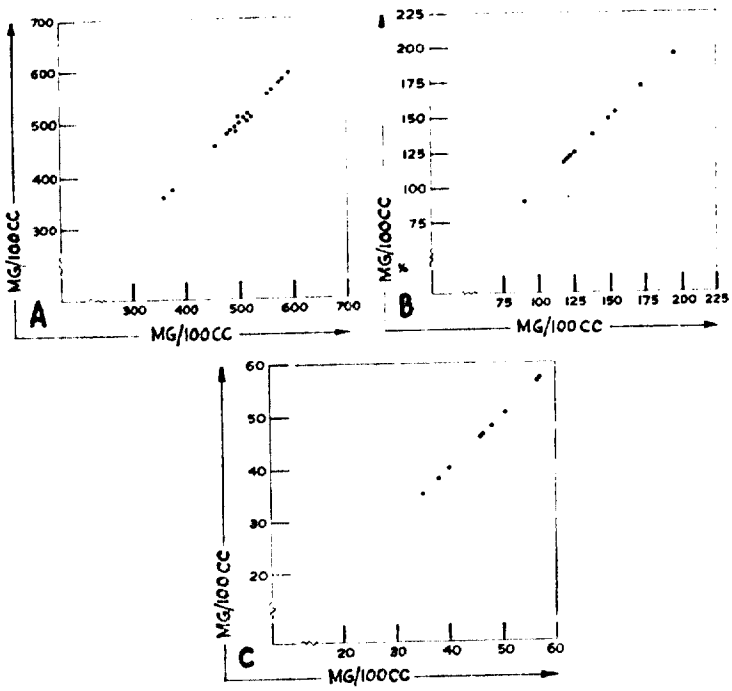

Chart 1.—The range of total fasting blood lipids (*A*), total fasting blood serum cholesterol (*B*) and fasting free blood serum cholesterol (*C*) is shown for 18, 10 and 9 normal persons.

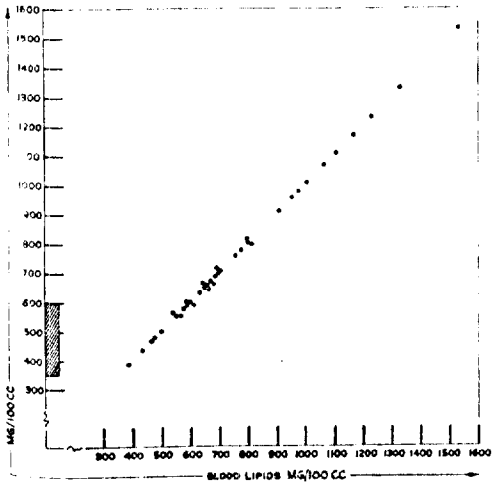

Chart 2.—The total fasting blood serum lipids in 39 patients with xanthelasma are shown, compared with the range of the same blood fats in 18 normal persons (indicated by cross-hatched rectangle). It can be seen that some patients with xanthelasma have severe degrees of lipemia.

Total lipids were determined gravimetrically by the method of Bloor.[3] The simpler alternative oxidative method was not used because (1) oxidation is incomplete in varying degree, (2) other substances present, in addition to lipids, are oxidizable and (3) all such determinations are much magnified by a micromethod.

Determinations of the total and free cholesterol were carried out by the modified colorimetric method of Shoenheimer and Sperry.[4] The usual colorimetric method is not specific because of the presence of interfering substances which also produce color. The digitonin method was used for this study because it yields cholesterol only, and the photoelectric colorimetric reading on the solution of digitonin precipitate is microspecific.

3. Bloor, W. R.: Determination of Small Amounts of Lipid in Blood Plasma, J. Biol. Chem. **77**:53-73 (April) 1938.

4. Shoenheimer, R., and Sperry, W. M.: Micromethod for Determination of Free and Combined Cholesterol, J. Biol. Chem. **106**:745-760 (Sept.) 1934.

Total phospholipids were determined by a combination of Bloor's precipitation method[5] and the phosphorus method of Fiske and Subbarow.[6] The phosphorus method of Fiske and Subbarow is the most accurate for the determination of the phospholipids and lends itself best to photoelectric colorimetry. It may be criticized because with acetone precipitation the yield is only 85 to 90 per cent. However, it is the only method applicable at present for routine analysis of serum phospholipids.

DATA AND COMMENT

Chart 1 shows that the total fasting blood lipids of the normal persons range from 360 to 600 mg., the total cholesterol from 90 to 171 mg. and the free cholesterol from 38 to 57 mg. per hundred cubic centimeters.

Of 39 patients with xanthelasma, the total fasting blood lipids were above these levels in 26, or 66 per cent (chart 2). In 7 patients, more than 1 per cent of the blood was fat. The fasting blood cholesterol was abnormally high in 15, or 57.7 per cent, and of the same 26 patients, 11, or 42.3 per cent, had high fasting free blood cholesterol values (chart 3). Seventeen of these patients had fasting blood phospholipids which were abnormal according to accepted normal levels (chart 4).

Eight patients followed during the ingestion of the lipotropic substance for forty-six to one hundred and eighty-nine days showed relatively little change in the fasting total blood lipid levels for nearly one hundred days (chart 5). The largest number of determinations are in this part of the curve. When the number of the subjects and the number of determinations decrease, the curve begins to fluctuate because of individual variations dominating its character and it loses its statistical value. We believe one can say that for the time these patients were observed the lipotropic substances fed had no apparent effect on the total blood fat values. The curves for total and free cholesterol are almost a straight line (chart 6) and hence were not affected by feeding of the lipotropic substance. Likewise,

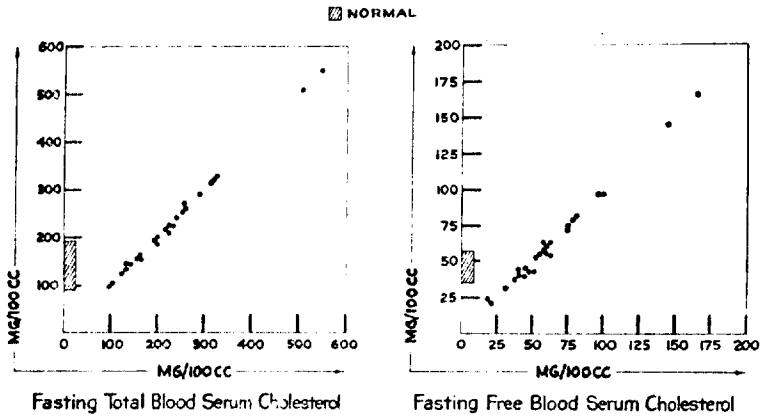

Chart 3.—The fasting total and free blood serum cholesterol of 26 patients with xanthelasma is shown, compared with our normal levels. The increase of both of these substances in many of the patients is apparent.

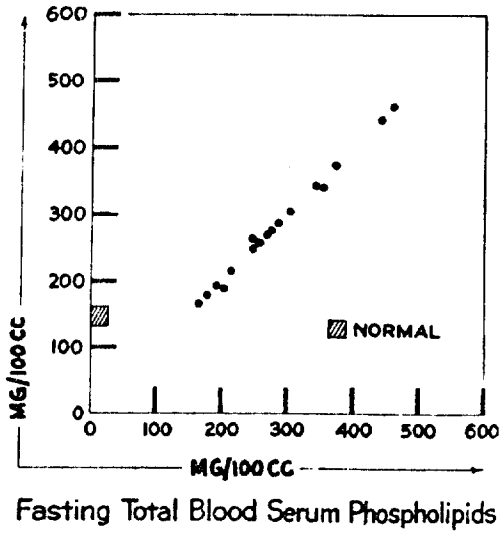

Chart 4.—The total blood serum phospholipid levels of 17 patients having xanthelasma are shown. All determinations are abnormally elevated.

5. Bloor, W. R.: Oxidative Determination of Phospholipid (Lecithin and Cephalin) in Blood and Tissues, J. Biol. Chem. **82**:273-286 (May) 1929.

6. Fiske, C. H., and Subbarow, Y.: Colorimetric Determination of Phosphorus, J. Biol. Chem. **66**:375-400 (Dec.) 1925.

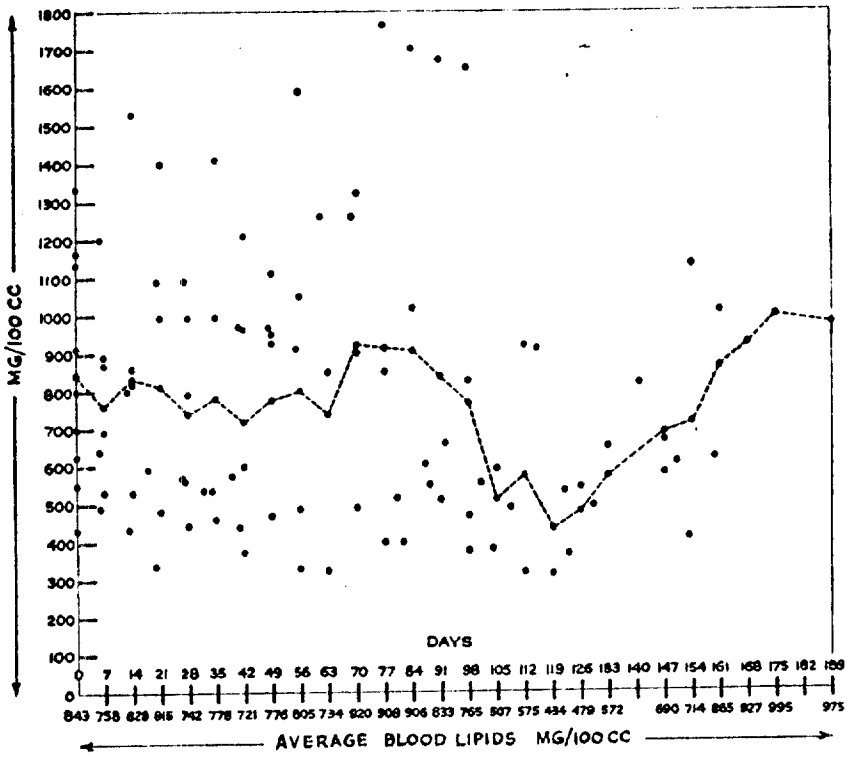

Chart 5.—Individual determinations, plotted in a scatter curve, of the total fasting blood serum lipids of 18 patients followed for as short a period as forty-six days and as long as one hundred and eighty-nine days are shown. There is no appreciable change in the fat levels until the period of one hundred days is reached. After this time, the number of values is too few for prediction of any statistical trend.

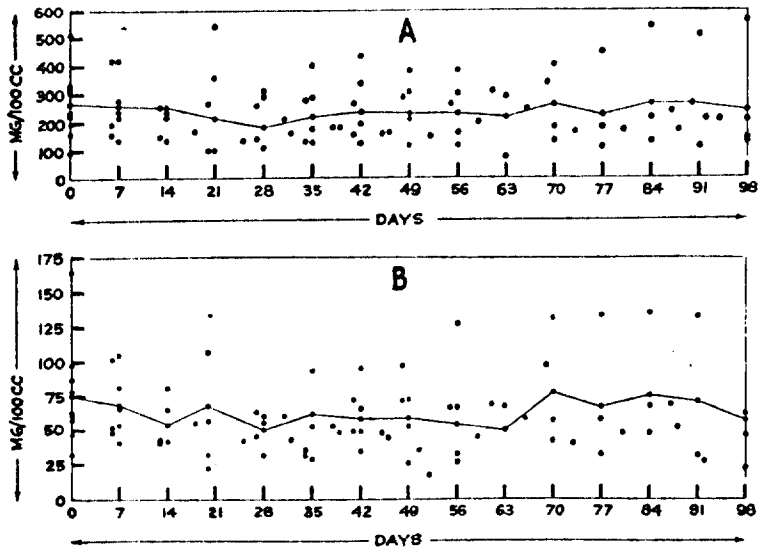

Chart 6.—The total (*A*) and free (*B*) blood serum cholesterol values for 8 patients having xanthelasma who were daily ingesting a lipotropic substance is shown in the two charts. The straight line average in both instances shows that the substance had no effect on either type of blood cholesterol.

there was no consistent change in the total blood phospholipids (chart 7).

From the clinical standpoint, only 1 patient presented changes in the lesions during the period of lipotropic feeding. She had a fasting total blood lipid level of 1,170 mg. per hundred cubic centimeters, a total cholesterol level of 325 mg., a free cholesterol level of 97.5 mg. and a total phospholipid level of 303 mg. After sixty-three days of ingestion of the lipotropic substance, her xanthelasma lesions entirely disappeared, although all blood lipid fractions were not significantly changed. This isolated case may or may not be an example of spontaneous regression of the lesions.

A criticism of our experiments might arise from the fact that we gave only 15 Gm. of lecithin complex daily. This amount was empirically used, but we believe it was enough to show a trend in this disease had the substance been effective.

It is apparent from this study that the lipotropic substance used had no effect on blood lipids and none on the xanthelasma lesions, unless the disappearance of the lesions in the single patient can be attributed to the lecithin complex.

CONCLUSIONS

1. In a series of 39 patients with xanthelasma, 66 per cent were found to have abnormally high fasting total blood fats. Fifty-eight per cent of 26 patients had abnormally high total blood serum cholesterol and 42 per cent of 26 patients had abnormally high free blood serum cholesterol. All phospholipid determinations were above the accepted normal level.

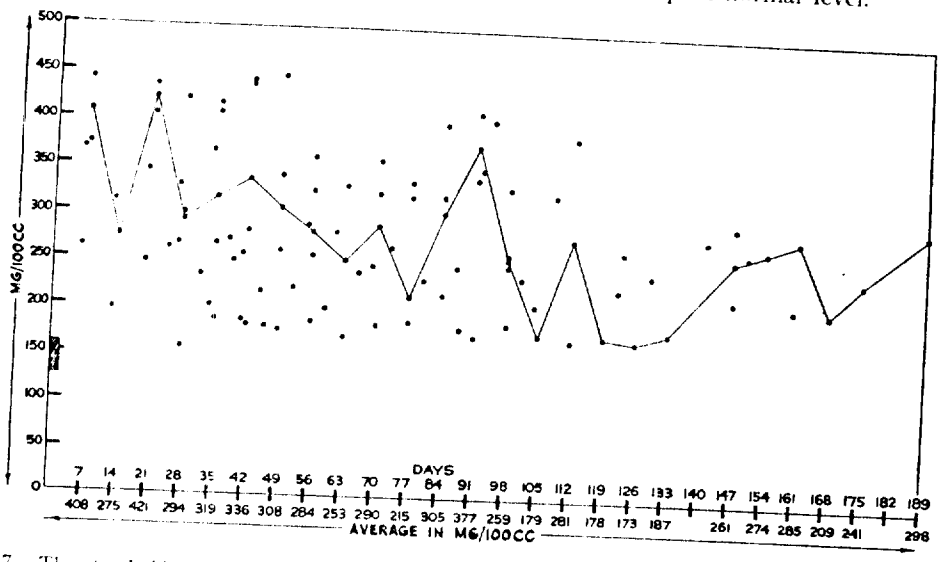

Chart 7.—The total blood serum phospholipids determined for 8 patients having xanthelasma who were ingesting a lipotropic substance daily is shown as a scatter curve. The solid line represents averages. Again, no important deviation in the phospholipids is apparent.

2. Soybean lecithin complex, containing approximately 5 Gm. each of lecithin, cephalin and inositol, fed daily to 8 patients for periods ranging from forty-six to one hundred and eighty-nine days, had no significant effect on the blood lipids or the cutaneous lesions. The disease in 1 patient cleared without change in the blood fats. This is interpreted as a spontaneous resolution.

3. Increased dosages and different lipotropic substances might be a subject for further experimental study on xanthelasma.

Removal of Oxidizing Factors Makes Dry Whole Milk Keep*

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Pennsylvania State College, State College, Pa.

Whole milk powder can be treated in processing so as to remove pro-oxidant constituents which make it subject to oxidized or tallowy flavor. The product then has excellent keeping qualities, should satisfactorily meet present requirements

THAT whole milk powder develops a stale oxidized flavor after some weeks or months of storage is a fact which has hindered this product from gaining wide acceptance. But the present war has focused attention again on dried whole milk because huge amounts are purchased by the military and Lend-Lease authorities. No doubt it will prove to be in great demand, even after actual fighting stops.

Homogenization of the fluid milk, gas packing and the use of higher processing temperatures have been used for years with some success to enhance the keeping quality. Yet dry whole milks still lack sufficient keeping quality. In order to be of the greatest possible use, it should keep for many months under adverse conditions.

The prevalent flavor defect observed is of an oxidative type, and the off-flavor product may be said to be stale, tallowy or oxidized. Oxidized flavor, which has received considerable attention in the past because of its prevalence in fluid milk, ice cream, frozen cream and fluid cream, was formerly thought to be due to oxidation of the butterfat, but it has now been shown definitely[1] to be due mainly to oxidation of the lecithin of the fat globule membrane rather than to oxidation of butterfat alone. The same may hold true for dry whole milk, although no reference has been made in the literature to this conjecture.

Dry whole milk has been shown to have antioxygenic properties when added to whole milk.[2] Yet, antioxidants have been added to whole milk before and after drying for the purpose of enhancing its keeping quality. Jack and Henderson[3] prevented the development of oxidized flavor in dry whole milk for approximately eight months by the addition of "Avenex No. 7," "Avenol" and ascorbic acid. They also found that powders made from milk preheated to 220 deg. F. kept for two years at room temperatures. Hollender and Tracy[4] also showed that antioxidants slightly improved the keeping quality of dried whole milk, and that milk preheated to 170 deg. F. for 30 minutes before drying was less likely to develop oxidized flavor than milk preheated at 150 deg. F. or 190 deg. F. Waite[5] also found that certain antioxidants helped delay oxidized flavor in dry whole milk. He used hydroquinone and oat flour.

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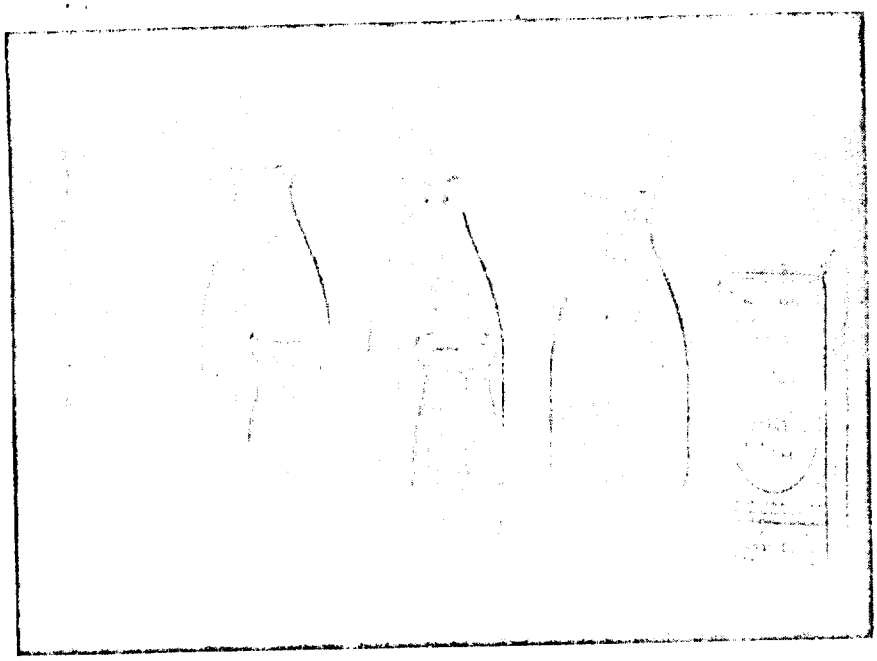

One container of powdered whole milk is the equivalent of 4½ quarts of fluid milk, and it is not highly perishable. Space and weight saving and keeping quality are important economic advantages.

Experimental

Assuming that lecithin may be a factor in the keeping quality, dry whole milk was prepared from milk in which the lecithin content was considerably reduced. Fresh milk was separated into cream and skim milk. The cream was cooled and churned into butter. The butter was melted and the oil separated from the curd and water by filtering. It was then washed and filtered several times. This fat was practically lecithin free. The skim milk resulting from the separation of the milk was divided into two parts. One part was passed through a supercentrifuge at 25,000 r.p.m. to remove part of the lecithin remaining in the skim milk. The following samples were prepared:

1. Fresh milk.
2. Pure butter oil and skim milk.
3. Pure butter oil and supercentrifuged skim milk.

These three samples were pasteurized

TABLE I—Effect of Removing Lecithin from Milk Upon Keeping Quality of Milk Powder.
(Samples stored at approximately 85 deg. F.)

Description of whole milk powder	Sealed in	Flavor after storage period of 1 month	2 months	3 months	5 months
Control	Air	Stale	Oxidized ++	Oxidized +++	Oxidized ++++
Skim milk plus butter oil	Air	Slightly stale	Oxidized +	Oxidized ++	Oxidized ++
Supercentrifuged skim milk plus butter oil	Air	Good	Very slightly stale	Stale	Very stale
Control	Nitrogen	Very slightly stale	Oxidized +	Oxidized ++	Oxidized +++
Skim milk plus butter oil	Nitrogen	Very slightly stale	Slightly stale	Oxidized +	Oxidized +
Supercentrifuged skim milk plus butter oil	Nitrogen	Good	Good	Slightly stale	Slightly stale

TABLE II—Lecithin Content of Whole Milk Powder Expressed as Percent Lecithin in Fatty Extract of Product.

Sample	Percent Lecithin
Control whole milk powder	0.753
Whole milk powder made from skim milk and butter oil	0.480
Whole milk powder made from supercentrifuged skim milk and butter oil	0.366

at 160 deg. F., homogenized at 2,500 lb. pressure, then concentrated in a stainless steel vacuum pan to a concentration of 2 to 1, and dried on a small atmospheric roller dryer. The temperature of the drums corresponds to a steam pressure of 25 lb.

The samples were stored at 85 deg. F. in 4-oz. plastic screw-top brown bottles. One-half of the samples were evacuated in a desiccator and then charged with nitrogen. The bottles were filled to overflowing, capped, and the caps dipped in paraffine to prevent entrance of air. The samples were examined at monthly intervals. They were reconstituted by means of a malted milk mixer and scored by two or three judges. The experiment was repeated on three sets of samples, with the results shown in Table I.

Discussion of Results

In Table I it will be observed that the samples containing the least lecithin had the best keeping quality. Even when the samples were packed under a nitrogen seal the flavors deteriorated considerably after five months' storage at 85 deg. F., except in the one containing the least amount of lecithin. This sample was only slightly stale.

That all of the lecithin was not removed by the treatment is indicated by the figures in Table II. It can be seen that over half the lecithin of the milk may be retained in the skim milk, and that centrifuging the skim milk removed some of it. The supercentrifuging may have removed some other pro-oxidant as well, since a great improvement in keeping quality was obtained with this treatment, although nearly one-half of the original skim milk lecithin still remained in the reconstituted milk. This phase of the work is being investigated further.

The possibility of producing a whole milk powder having excellent keeping qualities is evident from this work.

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J. Dairy Sci 39: 440-7, (1956)

THE EFFECT OF LECITHIN, CHOLINE, AND METHIONINE ON THE VITAMIN A AND CAROTENE PLASMA LEVELS AND LIVER STORES OF YOUNG DAIRY CALVES[1]

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An efficient utilization of carotene and vitamin A is essential for the health of the young dairy calf, especially when the available intakes are equal to or below the actual needs of the calf. It is of fundamental importance, therefore, to determine if various methylating agents might conceivably aid more efficient utilization of carotene and vitamin A by the young dairy calf.

Conflicting data have been published concerning the effect of lecithin on the absorption and utilization of vitamin A in calves and rats. Esh *et al.* *(7, 8)* observed that when lecithin was fed along with vitamin A during the later stages of the gestation period a greater amount of vitamin A was transferred to the colostrum as well as stored in the fetal livers by way of the placenta. These same workers also found that calves fed a skimmilk ration supplemented with lecithin and vitamin A gained in body weight and showed blood plasma levels of vitamin A comparable to a colostrum-fed group of calves. Esh and Sutton *(6)* observed that the feeding of lecithin plus vitamin A or a carotene supplement stimulated greater weight gains and an increased liver shortage of vitamin A in laboratory rats than did vitamin A or carotene fed alone. Choline fed in addition to these supplements did not change the liver stores of vitamin A when compared to a group supplemented with vitamin A alone. Adlersberg and Sabotka *(1)* reported much higher vitamin A blood levels in man after the ingestion of soybean lecithin. Scharf and Slanetz *(12)* observed a marked increase in the liver stores and blood plasma levels of vitamin A in rats after feeding soybean lecithin but reported no increase or decrease of blood plasma levels or liver stores of vitamin A by the presence or absence of choline. Slanetz and Scharf *(13, 14)* later reported that the addition of soybean phosphatides to a ration supplemented with vitamin A or carotene produced better gains in rats. These same workers postulated that soybean lecithin contains an unknown factor essential for the utilization of vitamin A and carotene in the rat. Bently and Morgan *(2)* reported that rats fed a low fat, low protein ration supplemented with choline showed higher vitamin A stores in the liver than when choline was omitted from the ration. The addition of carotene to the same ration produced more vitamin A deposition in the liver, which was not affected by the presence or absence of

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choline. Popper and Chinn *(11)* observed in an experiment with rats that liver stores of vitamin A were dependent upon the intake of choline. A deficiency of choline produced fatty livers that were low in vitamin A. Thorbjarnarson and Drummond *(15)* reported that the addition of choline to a choline-deficient diet fed to rats brought about a decrease in fat and vitamin A content of the livers. Clayton and Baumann *(1)* observed that even during a choline deficiency vitamin A was found to be normally distributed in the livers of rats.

This experiment was conducted to study further the effect of three lipotrophic agents, lecithin, choline, and methionine on the absorption and utilization of vitamin A and carotene by young dairy calves.

EXPERIMENTAL PROCEDURE

Fifty-four 2-day-old Jersey male calves were used in a 42-day experiment which was conducted in three phases during the years 1950, 1951, and 1954. The calves were selected from the Kentucky Agricultural Experiment Station herd and two other privately owned herds located nearby. Two calves each year were randomly assigned to receive one of nine different treatments. The treatments were as follows:

Group 1—basal control ration
Group 2—basal plus 25,000 I.U. vitamin A
Group 3—basal plus 25,000 I.U. vitamin A, plus 3 g. DL-methionine
Group 4—basal plus 25,000 I.U. vitamin A, plus 2 g. choline chloride
Group 5—basal plus 25,000 I.U. vitamin A, plus 10 g. vegetable lecithin
Group 6—basal plus 25,000 I.U. carotene (beta)
Group 7—basal plus 25,000 I.U. carotene, plus 3 g. DL-methionine
Group 8—basal plus 25,000 I.U. carotene, plus 2 g. choline chloride
Group 9—basal plus 25,000 I.U. carotene, plus 10 g. vegetable lecithin

The amounts of the supplements shown were the amounts administered per calf daily. The vitamin A and carotene were administered orally in a gelatin capsule. DL-methionine and choline chloride were dissolved in water and added to the milk. The lecithin was formed into a ball and forced down the calf's throat just prior to milk feeding. All supplements were fed at the time of or just prior to the afternoon milk feeding.

The calves were allowed to remain with their dams until 2 days of age, at which time they were placed in individual calf pens and hand-fed whole milk at a rate of 10 lb. per 100 lb. of body weight. Supplemental feeding was started when the calves were 9 days of age and continued daily until the animals were slaughtered.

The basal grain mixture, which was low in carotene and vitamin A, was fed ad libitum beginning when the calves were 2 weeks of age. The grain mixture was composed of 12 parts ground white corn, 12 parts wheat bran, 24 parts dried skimmilk, and 1 part each of salt and steamed bone meal. Beet pulp constituted

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the roughage portion of the ration, which was fed ad libitum and separate from the grain mixture.

Blood samples were drawn from the jugular vein when the calves were placed on experiment and at weekly intervals thereafter for vitamin A and carotene analysis. The blood was cooled immediately, and plasma vitamin A and carotene values were determined by the method of Kimble *(10)*. An Evelyn Photoelectric Colorimeter was standardized periodically by using crystalline beta carotene dissolved in petroleum ether and crystalline vitamin A acetate in cottonseed oil dissolved in chloroform.

The calves were slaughtered when 42 days of age, and the livers and kidneys were removed, frozen, and stored until analyzed. At the time of analysis the organs were sliced with a meat saw and ground in a food chopper while still frozen to prevent loss of moisture. The entire liver or kidney was mixed thoroughly, and aliquots were removed for analysis of dry matter, ether extract, vitamin A, and carotene. The total solids were determined by drying the samples either under vacuum over sulfuric acid or in a vacuum oven at 60° C. These dried samples were then extracted with anhydrous ethyl ether for approximately 30 hours to determine the per cent ether extract present. Vitamin A and carotene values were determined on the fresh livers and kidneys by the alkali digestion method outlined by Davies *(5)*.

RESULTS AND DISCUSSION

The summarized data showing the effects of the various supplements on the plasma levels of vitamin A, when vitamin A was fed, are presented in Table 1. The control calves, Group 1, exhibited much lower plasma levels of vitamin A than the supplemented calves. This is in agreement with Hibbs *(9)*, who found

TABLE 1

Mean vitamin A and carotene values of blood plasma and livers of calves supplemented with vitamin A plus lecithin, choline, and methionine

Group	Vitamin A /100 ml. plasma	Carotene /100 ml. plasma	Vitamin A /g of fresh liver	Carotene /g of fresh liver
1—Controls	9.6	18.0	12.6	0.5
2—Vitamin A alone	17.6	19.2	102.2	0.3
3—Vitamin A plus methionine	15.0	17.9	123.7	0.5
4—Vitamin A plus choline	16.1	18.0	132.9	0.2
5—Vitamin A plus lecithin	20.1	27.0	113.2	0.5

that the feeding of vitamin A alone in capsule would increase the plasma level of this vitamin. An analysis of variance was used to test for significant differences existing between Groups 2, 3, 4, and 5. These data along with the variance components are presented in Table 2. Group 5, receiving vitamin A plus lecithin, showed significantly higher plasma levels of vitamin A (5% level) than did Groups 3 and 4 receiving vitamin A plus methionine and vitamin A plus choline, respectively, but not significantly higher than Group 2, which was supplemented with vitamin A alone. Since Group 2 served as the control animals for the evalu-

TABLE 2

Analysis of variance

(Vitamin A plasma levels of calves fed vitamin A plus various supplements)

Source of variance	D.F.	Sum of squares	Mean square	F. ratio
Total	119	6020.89		
Age	4	104.95	26.2	
Treatments	3	427.55	142.5	3.68[a]
Years	2	1387.31	693.6	17.9[b]
Age × treatments	12	281.30	23.4	
Age × years	8	199.88	25.0	
Treatment × years	6	371.16	61.9	
Error	84	3248.74	38.7	

[a] Significant at the 5% level.
[b] Significant at the 1% level.
Treatments—Group 5 over 3 or 4 years; 3rd over 1st and 2nd; 2nd over 1st.

ation of the three lipotrophic agents, the differences obtained do not bear any significance. As can be observed from the variance components, there was considerable variation in response between years, which could probably be attributed to the better general health of the calves in the later years. These differences were significantly higher in favor of the last year (1954) as compared to the first and second year, and in the second year (1951) differences were significantly higher than in the first year (1950). In general the greatest response to the various supplements came within the second or third week after supplementation, followed by a downward trend.

Data concerning the plasma levels of carotene when vitamin A was fed are also presented in Table 1. An analysis of variance was used to test for significant differences as similarly outlined in Table 2 for the plasma vitamin A values. There were no significant differences between treatments; however, all groups of supplemented calves showed significantly higher plasma levels of carotene the first and second week after supplementation than was observed at the end of the experiment, or sixth week. These data follow a similar trend as that found for vitamin A, except that the decrease as the calves became older was not significant in the later case. It is of interest to note that the plasma levels of carotene were found to be significantly higher (5% level) the first year, which is the opposite of the plasma level of vitamin A. This would indicate greater conversion of the carotene to vitamin A in the later years. Liver vitamin A values of calves fed the various supplements plus vitamin A are presented in Table 1. There were no significant differences between any of the supplemented groups in the liver stores of vitamin A; however, liver stores in all supplemented groups were significantly higher than in the control calves, Group 1. There was a trend toward higher liver stores of vitamin A when the three lipotrophic agents were fed along with vitamin A. These data were not consistent enough to draw definite conclusions concerning the ability of these supplements to enhance the utilization of vitamin A as measured by liver stores.

These data support findings of Hibbs and Krauss *(9)* and Braun *(3)* that the plasma level of vitamin A is not a good criterion of the liver stores of this vitamin except when liver stores of vitamin A are low. Some calves were found

to exhibit high plasma levels of vitamin A and low liver stores of this vitamin. Calves on experiment during 1954, showed somewhat higher liver stores of vitamin A than the calves receiving the same treatment in the years 1950 and 1951. As was pointed out previously, these calves appeared to be in a better state of health, as reflected by better gains and fewer calf losses than observed in previous years of the study. When vitamin A was fed, the various supplements did not appear to affect the liver stores of carotene.

The effect of the various supplements on the plasma levels of carotene when carotene was fed is presented in Table 3. A statistical analysis of these data showed a significant increase at the 1% level of probability in the absorption of carotene, as measured by plasma levels of carotene, with the feeding of methionine over carotene alone (Group 6) and carotene plus lecithin (Group 9). A significant increase in plasma levels of carotene at the 5% level of probability was found in the group fed choline over Groups 6 and 9. The feeding of lecithin in addition to carotene appeared to have little effect on the plasma levels of carotene. A summary of the analysis along with the variance components is given in Table 4. All of the carotene-supplemented groups showed a significantly higher plasma level of carotene than the nonsupplemented controls (Group 1). This indicates that the basal ration alone was insufficient in carotene to maintain a normal level of plasma carotene. As was true with the vitamin-supplemented calves, the carotene-fed calves showed significantly higher plasma levels of carotene in the later years of the study.

TABLE 3

Mean carotene and vitamin A values of blood plasma and livers of calves supplemented with carotene plus lecithin, choline, and methionine

Group	Carotene /100 ml. plasma	Vitamin A /100 ml. plasma	Carotene /g of fresh liver	Vitamin A /g of fresh liver
1—Controls	18.0	9.6	0.5	12.6
6—Carotene alone	83.5	15.6	1.4	11.6
7—Carotene plus methionine	137.4	19.3	1.2	19.2
8—Carotene plus choline	124.0	18.2	1.4	10.3
9—Carotene plus lecithin	90.8	17.2	0.9	14.8

TABLE 4

Analysis of variance

(Carotene plasma levels of calves fed carotene plus the various supplements)

Source of variance	D.F.	Sum of squares	Mean square	F. ratio
Total	119	685,337.9		
Treatments	3	60,563.7	20,187.9	4.98*
Age	4	25,582.7	6,395.7	1.58
Years	2	147,233.2	73,616.6	18.17*
Age × treatments	12	27,040.7	2,253.4	0.56
Age × years	8	10,389.1	1,298.6	0.32
Years × treatments	6	74,261.7	12,376.9	3.05*
Error	84	340,266.8	4,050.8	

* Significant at 1% level.

Treatments—at 1% level, Group 7 over 6 and 9; at 5% level, Group 8 over 6 and 9.

Years—3rd year over 1st and 2nd (1% level); 2nd year over 1st (1% level).

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As can be observed in Table 3, the various supplements had very little effect on the storage of carotene in the liver when compared with the group fed carotene alone; however, all supplemented groups showed higher liver carotene values than did the control calves. Here again, there appears to be very little correlation between the blood plasma level of carotene and the liver stores of carotene. Considerable variation was observed between calves within groups in this respect. Methionine was the only supplement that appeared to have any appreciable effect on the liver stores of vitamin A when carotene was fed. These data indicate that very little, if any, conversion of carotene to vitamin A occurred, except with the methionine-fed calves, when a vitamin A and carotene-deficient ration was supplemented with carotene. Contrary to these data, carotene supplementation increased the plasma levels of vitamin A, but none of the supplements appeared to have any addictive effect.

TABLE 5

The effect of the various supplements on the average daily gain, per cent dry matter,[a] and ether extract[a] in the liver and kidneys of the calves

Supplements	Av. daily gain	Liver		Kidneys[b]	
		Dry matter	Ether extract	Dry matter	Ether extract
	(lb.)	*(%)*	*(%)*	*(%)*	*(%)*
Group 1—Controls	0.68	27.9	1.97	21.3	1.91
Group 2—Vitamin A alone	0.62	28.3	1.93	20.9	1.81
Group 3—Vitamin A plus methionine	0.59	28.8	1.98	21.2	1.70
Group 4—Vitamin A plus choline	0.70	28.6	1.82	21.0	1.80
Group 5—Vitamin A plus lecithin	0.79	29.1	1.93	20.1	1.61
Group 6—Carotene alone	0.66	28.8	2.04	20.8	1.81
Group 7—Carotene plus methionine	0.48	29.1	2.11	20.3	1.73
Group 8—Carotene plus choline	0.57	28.9	2.30	20.9	1.71
Group 9—Carotene plus lecithin	0.65	28.9	1.97	21.4	2.03

[a] Fresh basis.
[b] Average of 4 calves used in 1950 and 1951.

As can be observed in Table 5, there were no great differences between any of the groups in respect to the per cent dry matter or ether extract in either the liver or the kidneys. The differences that did exist were not statistically significant, although it appears that the carotene supplementation had a tendency to increase the per cent fat in the livers.

The mean per cent fat in the liver and kidneys for each group is given in Table 5. Close observation of the livers revealed no noticeable defects or excessive storage of fat even when choline, methionine, and lecithin were not fed. Calves used in this experiment during 1954, regardless of ration, had less fat in their livers than the calves used during 1950 and 1951. This is difficult to explain, since the treatments were the same for all years; however, it might be partially due to the fact that during 1954 the calves made better gains and were less affected by scours and other complications.

The effect of the various supplements on the growth of the calves is summarized in Table 5. It must be pointed out that considerable variation existed between calves on the same treatment in respect to weight gains during any

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particular year. This was probably due to the general health of the calves, since the basal ration was not formulated to produce fast growth but to be deficient in carotene and vitamin A. Even though only two calves were used per treatment each year of the study, all calves regardless of treatment tended to gain in weight faster with each succeeding year of the study. The average daily gains for all of the calves irrespective of treatment during 1950, 1951, and 1954 were 0.54, 0.61, and 0.80 lb., respectively. This was probably due to fewer cases of scours during the last 2 years. None of the supplements appeared to have any great effect on the weight gains of the calves, although the calves receiving lecithin with carotene or vitamin A tended to make slightly better gains than other supplemented groups.

SUMMARY

Fifty-four 2-day-old male Jersey calves were used to study the effect of three lipotrophic agents, DL-methionine, choline chloride, and vegetable lecithin, on the absorption and utilization of vitamin A and carotene. The feeding of these three agents plus vitamin A did not materially increase the plasma levels or liver stores of vitamin A above those of a group receiving vitamin A alone.

The addition of either choline or methionine plus carotene to the ration of calves caused a significant increase in the absorption of carotene as measured by plasma levels of carotene. All treated groups showed significantly higher plasma carotene levels than did the untreated controls. None of the lipotrophic agents had any significant effect on the liver stores of carotene when carotene was fed, but the feeding of carotene per se increased liver stores of carotene but not of vitamin A, although blood plasma values of vitamin A were increased. None of the supplements appeared to have any appreciable effect on the per cent ether extract or total solids in the livers and kidneys.

The absence of consistent results due to the addition of either methionine, choline, or lecithin to the supplementary vitamin A would indicate, under the conditions of this experiment, that they have little value in increasing the absorption or utilization of vitamin A in the young dairy calf. The results might have been more convincing if lower levels of the vitamin A or its precursor had been fed.

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WM. G. DOWNS, JR. and FAYE JOHNSON BOWMAN, Tennessee Polytechnic Institute. Relationship between cholesterol and lecithin intake to levels of lecithin and free cholesterol in the blood serum of rats and rabbits.

Male rats of a highly-inbred hybrid albino strain, and male and female Angora rabbits were divided into three experimental groups. One group received daily feedings of cholesterol only, one lecithin only, and one both cholesterol and lecithin. Serum determinations were made for lecithin and free cholesterol at specified time intervals.

Rats were found to show a definite and consistent response. In each group it was found that with an increase in lecithin content there was a decrease in cholesterol level. Responses in rabbits were less consistent, although generally in the same direction as in rats. Rabbits receiving only lecithin did not show as high a free cholesterol content as those not fed lecithin, though in some instances both lipids increased or decreased. There were some apparent sex differences in the response of rabbits, although these also were not consistent.

Results indicate that high lecithin intake tends to lower the serum-cholesterol level in experimental animals. (Junior author is currently at Vanderbilt University.)

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THE EFFECT OF CRUDE SOYBEAN LECITHIN ON THE ABSORPTION AND UTILIZATION OF VITAMIN A FED PREPARTUM TO THE EWE AND SOW[1]

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THE importance of optimum nutrition during the prepartum period on the performance of the dam and subsequent development of the newborn has received considerable attention during the past few years. It has been established that, by the addition of supplementary vitamin A fed during the prepartum period, the fetal storage of vitamin A can be materially increased in the calf, the kid, the lamb and the pig (Spielman *et al.*, 1946; Wise *et al.*, 1946; Thomas *et al.*, 1947; Whiting, 1948). In addition numerous reports may be found to show that the vitamin A content of colostrum can be markedly increased by similar means. It has been further reported by Spielman *et al.*, 1947, and 1948, that the feeding of supplementary vitamin A during the prepartum period has resulted in superior performance of Holstein calves as indicated by greater weight gains, higher levels of blood plasma vitamin A and a lower incidence and duration of scours than that observed in calves from dams fed no supplementary vitamin A.

Since the utilization and absorption of supplementary vitamin A is inefficient especially as regards mammary and placental transfer, many investigators have been prompted to find means of increasing the efficiency. In the case of species other than livestock, lecithin has been reported to influence vitamin A absorption and utilization (in man, Aldersberg and Sobotka, 1943; in the rat, Slanetz and Scharf, 1943; Scharf and Slanetz, 1944; Slanetz and Scharf, 1945; and Esh and Sutton, 1948; and in the chick, Patrick and Morgan, 1943). Esh *et al.*, (1947, 1948) have fed soybean lecithin with supplementary vitamin A to dairy cows during the pre- and postpartum periods. They obtained increased absorption and utilization as shown by higher maternal blood values and colostral and fetal storage of vitamin A when soybean lecithin was added to the supplementary vitamin A as compared to supplementary vitamin A alone. Furthermore, the feeding of soybean lecithin plus vitamin A to newborn calves on a skimmilk ration

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resulted in greater absorption and utilization of vitamin A than when no lecithin was added to the vitamin A supplement.

The objective of this study was to determine the effect of crude soybean lecithin on the absorption and utilization of vitamin A fed prepartum to ewes and sows as indicated by maternal blood plasma level, newborn blood plasma and liver storage levels, and 30-day old blood plasma and liver storage levels of vitamin A.

Experimental

Animals

Three groups each of purebred pregnant ewes and sows from the University flock and herd were used in this experiment from January through May 1948. They were allotted to an experimental grouping according to breed, age, and liveweight. All the ewes were started on experiment the first week in January; while the sows were placed on experiment 30 days before the calculated parturition date. All groups within a species received the same basal ration at the same level of intake. In addition each ewe and sow in group II received orally 200,000 I.U. of vitamin A daily until parturition and each ewe and sow in group III received the same daily intake of vitamin A plus 4 grams of crude soybean lecithin added to the vitamin supplement. The vitamin A supplement[4] was in the form of dog fish liver oil which contained by analysis at the time of feeding 2.85 mg. of vitamin A per gram of oil. In the case of the ewes, group II received the vitamin A supplement for an average of 15.2 ± 2.0 days (Mn ± S.E.), while group III received the same level of vitamin A plus soybean lecithin for an average of 13.0 ± 1.8 days. The sows in group II received the vitamin supplement for an average of 33.8 ± 0.9 days and group III for an average of 32.0 ± 0.8 days. The following summary gives the feeds fed and their carotenoid content.

Feed	Carotenoid Content mg./lb.
Ewes	
Yellow corn 2, oats 6 Wheat bran 1, L.O.M. 1	0.17
Corn silage	0.71
Mixed clover and timothy hay	16.41
Sows	
Yellow corn 55, oats 25 Tankage 8, soybean oil meal 6 Alfalfa meal 1	0.69

[4] This oil specially processed to contain 10,000 I.U. of vitamin A per gram was generously supplied by Nopco Chemical Company, Harrison, N. J. The crude soybean lecithin was supplied by Stacey Mfg. Co., Decatur, Illinois.

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Samples

Maternal blood samples were taken from the jugular vein of ewes the first week in January before vitamin A supplementation, within 24 hours after lambing, and 30 days after lambing. Blood was drawn from the anterior vena cava vein or from the tail of sows at 30 days prepartum before vitamin A supplementation, 2 weeks before farrowing, within 48 hours after farrowing, and 30 days after farrowing. Newborn lambs and pigs were bled within 6 hours after birth and were not allowed to nurse. Blood samples from 30-day old lambs and pigs were obtained immediately after removal of the animals from their dams.

Livers were obtained from newborn lambs and pigs which had not been allowed to nurse and which were slaughtered in most cases within 6 hours after birth. Thirty-day old lambs and pigs were removed from their dams and killed immediately. The entire livers were removed, cooled and held at 4° C. The whole liver was homogenized in a Waring blendor, and analyses run on aliquot samples within 48 hours. In several cases the livers were frozen and held at −18° C. until analyses could be made. In the latter case the time of storage did not exceed 2 weeks.

Analyses

Blood plasma vitamin A and carotenoids were determined by the method of Kimble, 1939, and liver vitamin A and carotenoids by the method of Davies, 1933. An Evelyn photoelectric macrocolorimeter that was standardized with crystalline β carotene in petroleum ether and with crystalline vitamin A alcohol in chloroform was used.

The data were treated statistically to determine differences between treatments by the use of the "t" test as described by Love, 1943. Only those differences significant at the 1% level are reported as statistically significant in the results.

Results and Discussion

Maternal blood plasma levels of carotenoids and vitamin A are given in table 1. There were no significant differences or trends between groups in the carotenoid levels in either ewes or sows. The vitamin A plasma levels in those ewes and sows fed supplementary vitamin A with or without soybean lecithin were higher for each observation than those levels for the animals on the basal ration. In the case of the ewes a significant difference occurred only at the time of lambing; and for the sows the only significant difference was for the observations at 30 days after farrowing. Although the addition of soybean lecithin to the vitamin A supplement did increase the blood plasma level of vitamin A to slightly higher levels than when vitamin

TABLE 1. THE EFFECT OF VITAMIN A AND VITAMIN A AND CRUDE SOYBEAN LECITHIN ON BLOOD PLASMA CAROTENOID AND VITAMIN A LEVELS IN THE EWE AND SOW IN μg/100 ML PLASMA*

Prepartum ration and breed	At start of experiment			2 weeks before parturition			At parturition			30 days after parturition		
	Carotenoids	Vit. A	No. of animals	Carotenoids	Vit. A	No. of animals	Carotenoids	Vit. A	No. of animals	Carotenoids	Vit. A	No. of animals
Ewes												
Basal												
Southdowns	—	32.6± 2.2	(10)	—	—	—	10.5± 2.6	26.6± 2.4	(10)	7.8± 0.8	32.6± 4.1	(8)
Shropshires	—	34.3± 2.2	(9)	—	—	—	11.7± 2.3	33.7± 1.9	(9)	7.8± 2.8	37.7± 1.9	(9)
Both breeds	—	33.6± 1.5	(19)	—	—	—	11.1± 1.7	29.9± 1.7	(19)	6.6± 1.5	35.3± 2.2	(17)
Basal+Vit. A												
Southdowns	—	35.7± 2.5	(9)	—	—	—	8.2± 2.8	38.4± 5.6	(9)	4.0± 0.7	35.9± 3.4	(9)
Shropshires	—	36.6± 1.4	(12)	—	—	—	10.4± 2.4	41.7± 4.4	(12)	4.8± 1.4	42.7± 1.9	(12)
Both breeds	—	36.2± 1.3	(21)	—	—	—	9.3± 1.8	40.3± 3.4	(21)	5.4± 0.8	39.8± 1.9	(21)
Basal+Vit. A+lecithin												
Shropshires	—	33.2± 1.9	(10)	—	—	—	9.4± 2.1	41.1± 5.6	(11)	4.8± 0.5	41.6± 2.5	(9)
Sows												
Basal												
Berkshires	3.5± 1.5	16.2± 6.3	(2)	5.0± 2.0	20.5± 5.2	(2)	2.0± 0.0	24.6± 0.6	(1)	1.4± 0.5	18.5± 1.4	(2)
Chester Whites	3.0± 0.0	22.3± 0.9	(2)	3.0± 0.0	20.1± 0.0	(1)	3.5± 1.5	18.3± 2.1	(2)	1.0± 0.0	15.9± 0.0	(1)
Both Breeds	3.3± 0.6	19.2± 3.1	(4)	4.3± 1.3	20.3± 3.0	(3)	3.0± 1.0	20.4± 3.4	(3)	1.3± 0.3	17.6± 1.2	(3)
Basal+Vit. A												
Berkshires	2.7± 0.7	38.1± 10.9	(3)	3.0± 1.0	28.0± 11.4	(2)	1.0± 0.0	23.5± 5.2	(2)	2.1± 0.5	30.4± 0.4	(2)
Chester Whites	4.0± 1.0	18.1± 4.6	(2)	1.5± 1.5	37.2± 4.2	(2)	2.0± 2.0	23.0± 18.3	(2)	6.0± 4.0	37.9± 7.5	(2)
Both Breeds	3.2± 0.6	30.1± 7.9	(5)	3.3± 0.9	33.0± 5.5	(4)	1.5± 0.9	23.7± 7.8	(4)	4.5± 1.9	29.2± 3.2	(4)
Basal+Vit. A+lecithin												
Berkshires	2.7± 0.3	20.7± 2.6	(3)	3.0± 0.0	24.5± 5.8	(2)	1.5± 1.0	23.8± 2.5	(2)	2.0± 1.0	29.4± 0.9	(2)
Chester Whites	2.0± 0.0	35.0± 0.2	(2)	2.0± 0.0	29.2± 4.8	(2)	2.0± 1.0	29.4± 2.5	(2)	1.5± 0.5	28.8± 4.2	(2)
Both breeds	2.5± 0.2	22.4± 1.8	(5)	2.5± 0.5	26.9± 3.3	(4)	1.5± 0.6	26.6± 3.2	(4)	1.8± 0.5	29.1± 1.8	(4)

* Values given are Mean ± Standard Error of Mean.

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A was fed alone, these differences were not significant nor did they approach significance at even the 5% level. Within a group there were no significant breed differences.

The levels of carotenoids and vitamin A in blood plasma for newborn and

TABLE 2. THE EFFECT OF VITAMIN A AND VITAMIN A AND CRUDE SOYBEAN LECITHIN FED PREPARTUM ON BLOOD PLASMA CAROTENOIDS AND VITAMIN A LEVELS IN NEWBORN AND 30 DAY OLD LAMB AND PIG, IN μg/100 ML PLASMA

Dams prepartum ration, and breed	Newborn			30 days of age		
	Carotenoids	Vit. A	No. of animals	Carotenoids	Vit. A	No. of animals
Lambs						
Basal						
Southdown	—	7.4± 1.9	(2)	1.9±0.4	22.7±3.2	(8)
Shropshire	—	—		6.0±1.0	23.1±1.2	(15)
Both breeds	—	7.4± 1.9	(2)	4.6±0.8	22.9±1.3	(23)
Basal+Vit. A						
Southdown	—	6.7± 3.5	(2)	5.6±1.6	25.2±2.1	(12)
Shropshire	—	11.5± 1.3	(2)	5.6±1.1	27.2±1.1	(17)
Both breeds	—	9.2± 2.1	(4)	5.6±0.8	26.4±1.1	(29)
Basal+Vit. A+lecithin						
Shropshire	—	13.6± 8.3	(2)	2.2±0.6	32.2±2.3	(10)
Pigs						
Basal						
Berkshires	2.0±1.0	5.8± 0.1	(2)	1.5±0.3	18.3±1.1	(14)
Chester Whites	0.5±0.5	8.2± 1.1	(2)	2.5±0.4	21.4±1.2	(17)
Both Breeds	1.3±0.6	7.0± 0.4	(4)	2.0±0.2	20.0±0.3	(31)
Basal+Vit. A						
Berkshires	1.5±0.3	9.2± 1.8	(3)	1.3±0.3	24.1±1.1	(12)
Chester Whites	2.0±0.0	6.2± 0.9	(2)	1.4±0.3	23.8±1.0	(17)
Both breeds	1.6±0.2	8.0± 1.3	(5)	1.3±0.2	23.9±0.8	(29)
Basal+Vit. A+lecithin						
Berkshires	1.7±0.3	9.0± 1.6	(3)	1.2±0.2	21.1±1.4	(18)
Chester Whites	1.0±0.0	19.6±11.3	(2)	1.3±0.3	26.2±1.0	(11)
Both breeds	1.4±0.2	13.2± 4.5	(5)	1.2±0.2	23.1±1.3	(29)

30-day old lambs and pigs are listed in table 2. There were no significant differences found between groups for newborn lambs or pigs. However, the levels of blood plasma vitamin A were higher in those newborn whose dams had been fed vitamin A with or without the addition of lecithin. At 30 days of age those lambs and pigs from dams fed supplementary vitamin A

had significantly higher blood plasma levels of vitamin A than those levels found in animals from dams receiving no supplementary vitamin A. The addition of soybean lecithin did not result in a significant elevation in the vitamin A levels in blood plasma. In the case of the blood plasma carotenoid content, those lambs from dams receiving the soybean lecithin had a significantly lower level than those lambs from dams receiving the vitamin A supplement alone. Pigs from dams receiving either vitamin A or vitamin A plus lecithin had lower levels than those pigs from dams receiving no vitamin A. Within a group, the carotenoid level for the 30-day old Southdown lambs from dams receiving only the basal ration was significantly lower than that for the Shropshire lambs and the 30-day old Berkshire pigs from dams receiving vitamin A and soybean lecithin were significantly lower in blood plasma vitamin A than the Chester White pigs.

The liver storage of carotenoids and vitamin A for newborn and 30-day old lambs and pigs is presented in table 3. There were no significant differences in the total carotenoid content of the liver or the carotenoid content per gram of liver. The concentration of vitamin A per gram of liver or the total amount of vitamin A in the liver for both newborn and 30-day old lambs and pigs was significantly higher in those animals from dams fed either vitamin A alone or vitamin A plus soybean lecithin than in those lambs and pigs from dams fed the basal ration only. Soybean lecithin did not significantly increase the liver storage of vitamin A. Within a group there were no significant breed differences.

Discussion

These data are in agreement with previous reports that the fetal storage of vitamin A can be increased by prepartum feeding of supplementary vitamin A. In addition the data show that the storage of vitamin A during the neonatal period is increased by the prepartum feeding of vitamin A. The addition of soybean lecithin to the supplementary vitamin A resulted in increases in the vitamin A levels found in blood and liver, but these increases were not consistent throughout the entire experiment nor were they statistically significant.

The values for blood plasma carotenoids and vitamin A in those ewes and sows receiving the basal ration alone are essentially in agreement with those reported for ewes by Whiting (1948), and for sows by Grummer *et al.* (1948). The absence of a marked drop in the blood plasma levels of carotenoids and vitamin A in ewes and sows at the time of parturition is in contrast to that observed in cows by Sutton, Kaeser and Soldner (1945). Blood plasma levels of vitamin A have been found by Braun (1945), Hibbs and Krauss (1947), Hoefer and Gallup (1947), and Frey, Jensen and Connell (1947)

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not to be a reliable index of the liver storage or dietary level of vitamin A (or carotene) except at very low dietary intakes. The lack of consistent statistically significant increase in the blood plasma levels of vitamin A in those ewes and sows fed supplementary vitamin A with or without the addition of soybean lecithin is therefore understandable, since the basal ration alone resulted in blood plasma levels of vitamin A which appear to be adequate. It should be pointed out that recently Parrish, Wise, and Hughes (1948) have suggested that blood samples containing appreciable quantities of vitamin A in the ester form should be saponified to obtain the true vitamin A values. However, Ross, Knodt, and Guerrant (1948) found no significant differences in blood plasma vitamin A levels in Holstein heifers fed equivalent amounts of vitamin A alcohol and the naturally occurring ester of vitamin A. The plasma was not saponified in the procedure used for vitamin A determination. Therefore it appears that Parrish *et al.* (1948) were able to measure only greater amounts of vitamin A due to saponification and not specifically the ester form of vitamin A.

The blood plasma levels of carotenoids and vitamin A found in lambs and pigs at birth and at 30 days of age are essentially in agreement with those values reported by Thomas *et al.* (1947), Whiting (1948) and Grummer *et al.* (1948). The narrow differences in blood plasma levels of vitamin A as contrasted with liver storage levels supports the findings of those workers cited above in that there is little or no correlation between these two except when the liver storage reaches a very low level. The increase in liver vitamin A storage in the newborn lamb and pig due to prepartum vitamin A supplementation is similar to that obtained by Thomas *et al.* (1947) and Whiting (1948). The maintenance of higher liver levels during the neonatal period as indicated by those values found at 30 days of age is further evidence of the importance of optimum nutrition during the prepartum period.

Slanetz and Scharf (1943), Scharf and Slanetz (1944) and Slanetz and Scharf (1945) have reported that soybean lecithin contains an unidentified substance which increases the utilization of vitamin A as indicated by weight gains and blood and liver storage levels of vitamin A in the rat. Similar results have been found in chickens by Patrick and Morgan (1943), in the cow by Esh *et al.* (1948), and in the rat by Esh and Sutton (1948). In cases of sprue in man, Aldersberg and Sobotka (1943) have shown that the addition of soybean lecithin increased the absorption of vitamin A four-fold as indicated by blood serum levels of vitamin A. Soybean lecithin will increase fat absorption in the rat, as indicated by the work of Augur, Rollman and Deul (1947).

The absence of consistent increases due to the addition of soybean lecithin to the supplementary vitamin A would seem to indicate, under the

TABLE 3. THE EFFECT OF VITAMIN A AND VITAMIN A AND CRUDE SOYBEAN LECITHIN FED PREPARTUM ON LIVER STORAGE OF CAROTENOIDS AND VITAMIN A IN THE NEWBORN AND 30 DAY OLD LAMB AND PIG

Prepartum ration, and breed	Newborn					30 days of age				
	Carotenoids		Vitamin A		No. of animals	Carotenoids		Vitamin A		No. of animals
	μg/g	Total μg	μg/g	Total μg		μg/g	Total μg	μg/g	Total μg	
Lambs										
Basal	.13± .03	8± 6	1.66± 0.85	86± 12	(3)	.18± .31	80± 71	47.90± 9.50	5505± 1314	(2)
Southdowns	.21	12	0.45	24	(1)	.12± .01	74± 23	35.10± 8.50	5337± 2607	(2)
Shropshires	.15± .03	9± 2	1.36± 0.67	71± 18	(4)	.55± .16	81± 31	41.50± 6.38	5431± 1193	(4)
Both breeds										
Basal+Vit. A	.18± .21	31± 11	20.81± 4.11	1168± 328	(3)	.53± .06	64± 4	146.50± 7.00	17613± 57	(2)
Southdowns	.25± .17	16± 12	19.60± 5.04	1155± 457	(2)	.25± .08	35± 13	134.80± 8.03	18200± 2178	(3)
Shropshires	.41± .15	25± 8	20.35± 2.83	1163± 232	(5)	.36± .08	46± 10	139.13± 6.18	17965± 1202	(5)
Both breeds										
Basal+Vit. A+lecithin										
Shropshires	.32± .12	19± 13	31.56± 12.14	2357± 311	(5)	.54± .08	111± 22	126.53± 8.62	25527± 3107	(3)
Pigs										
Basal	.23± .01	3± 0	6.22± 5.05	86± 67	(2)	.23± .00	21± 1	11.30± 9.05	1042± 148	(2)
Berkshires	.30± .23	7± 3	10.25± 1.64	213± 30	(4)	.10± .22	11± 2	20.40± 8.38	2364± 384	(3)
Chester Whites	.28± .24	6± 2	8.91± 1.87	170± 36	(6)	.15± .12	15± 3	16.76± 5.49	1834± 759	(5)
Both breeds										
Basal+Vit. A										
Berkshires	.35± .11	5± 1	52.41± 8.23	966± 257	(2)	.39± .07	63± 27	59.73± 8.37	8348± 1703	(4)
Chester Whites	.29± .15	4± 3	24.40± 20.05	367± 328	(2)	.31± .15	27± 10	43.90± 4.13	4178± 153	(3)
Both breeds	.34± .08	5± 1	46.19± 8.25	833± 215	(6)	.36± .07	48± 16	52.93± 5.72	6160± 1251	(7)
Basal+Vit. A+lecithin										
Berkshires	.32± .09	7± 3	41.97± 3.90	713± 158	(7)	.15± .13	87± 21	67.48± 12.81	10763± 1913	(4)
Chester Whites	.10± .05	2± 0	48.86± 3.40	1390± 405	(2)	.28± .07	30± 2	62.45± 12.65	6613± 193	(2)
Both breeds	.27± .07	6± 2	43.49± 3.25	717± 155	(9)	.46± .10	68± 18	65.86± 8.86	9380± 1124	(6)

conditions of this experiment, that soybean lecithin did not materially increase the absorption or utilization of vitamin A. Therefore, it would appear possible that the levels of vitamin A supplementation used in this experiment were above the optimum level of intakes for ewes and sows, and that any increase or sparing action due to the addition of soybean lecithin could not be demonstrated due to the feeding of an excess of vitamin A.

Summary

The effect of feeding daily 200,000 I.U. of vitamin A with or without the addition of 4 grams of crude soybean lecithin to ewes for an average of 14 days prior to parturition and to sows for an average of 33 days prior to parturition has been studied. The following results were obtained:

1. Both sows and ewes fed supplementary vitamin A with or without the addition of soybean lecithin had higher blood plasma levels of vitamin A at the time of parturition and 30 days after parturition than ewes and sows receiving no supplementary vitamin A. The only statistically significant difference was for the ewes at the time of lambing and for the sows 30 days after farrowing. The addition of soybean lecithin to a vitamin A supplement did not materially increase the blood plasma levels of vitamin A above that of animals receiving vitamin A alone.

2. Both newborn and 30-day old lambs and pigs from dams fed supplementary vitamin A with or without the addition of soybean lecithin were higher in blood plasma vitamin A levels than those animals from dams fed only the basal ration. There was a statistical difference at 30 days of age for both lambs and pigs.

3. The concentration of vitamin A per gram of liver or the total amount of vitamin A in the liver was significantly higher at birth and at 30 days of age in those lambs and pigs from dams fed supplementary vitamin A. The addition of soybean lecithin did not materially increase the liver storage of vitamin A.

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THE EFFECTS OF SOYA LECITHIN ON THE ABSORPTION, UTILIZATION AND STORAGE OF VITAMIN A AND CAROTENE IN THE WHITE RAT

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INTRODUCTION

It has been reported by several investigators (McCoord and Luce-Clausen, '34; Davies and Moore, '34; Bauman et al., '34; LePage and Pett, '41) that the percentage utilization of dietary vitamin A and carotene is small, due to poor absorption and considerable destruction. Hickman et al. ('42), Sherman ('42), and others have demonstrated that the destruction of these constituents can be partially inhibited by the simultaneous ingestion of alpha-tocopherol and other fat soluble antioxidants that are normally present in certain fats and oils. Adlersberg and Sobotka ('43a) reported that in sprue the ingestion of vitamin A did not result in an increase in the blood level, but when lecithin was given at the same time there was a moderate rise in the serum vitamin A. In normal individuals, they (Adlersberg and Sobotka, '43b) found a rise of 41% in the blood 4 hours after a test dose of 18,000 I.U. of vitamin A. When lecithin was fed in addition, the rise in blood vitamin A was of the order of 200%. Slanetz and Scharf ('43, '45) observed a greater growth response when lecithin was fed to vitamin A-depleted rats along with vitamin A and carotene. They demonstrated also that lecithin markedly influenced both liver storage and blood levels of vitamin A. These authors have postulated that lecithin contains an unknown factor which influences carotene and vitamin A utilization in the rat.

[1] Graduate Fellow supported by the Government of India.

In a previous contribution from this laboratory (Esh et al., '47), it was observed that when lecithin was fed to cows along with vitamin A during the later stages of the gestation period, more vitamin A was transferred to the colostral milk as well as to the newborn calves by way of the placenta. Moreover, when a mixture of lecithin and vitamin A was added to a skim milk ration of newborn calves, they grew quite well and the blood vitamin A and liver storage were comparable to those of colostrum-fed calves.

In the investigation reported herein, a study was made in rats of the effect of feeding lecithin during the vitamin A depletion period and during the vitamin A and carotene supplementation periods on the rate of growth, plasma vitamin A level and liver storage of vitamin A.

EXPERIMENTAL

Experiment I. Soya lecithin and vitamin A

Fifty young albino rats reared under comparable conditions in our breeding colony were allotted to this experiment at weaning (24 days of age). Their weights varied from 48 to 52 gm. They were divided into 2 groups of equal sex and litter distribution and depleted of vitamin A on rations (a) and (b) (see table 1).

The rations were freshly prepared each week and stored in a refrigerator. The rats were maintained in individual cages and the rations provided ad libitum for 39 days. Weights were recorded weekly for the first 3 weeks and twice weekly thereafter. The same feeding and management routine was followed in each of the 3 experiments. Four rats were sacrificed from each group at the end of 3 weeks and a second set of 4 from each group at the end of the depletion period (39 days). The blood from each group of 4 animals was pooled

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for serum vitamin A analysis and the livers obtained for individual liver vitamin A storage determination.

At the end of the depletion period the animals were regrouped as shown in table 2, experiment I. This regrouping was done to determine whether there was a carry-over effect of the depletion diet to the period of vitamin A supplementation. At this point vitamin A supplementation began.

TABLE 1

Percentage composition of the basal rations used

INGREDIENT	RATION				
	a	b	c	d	e
Corn starch	50	50	65	65	65
Commercial dextrose (Cerelose)	17	17			
Casein, vitamin free	18	18	18	18	18
Hydrogenated vegetable fat (Crisco)	10	8	12	10	11.93
Crude soya lecithin [1]	..	2	..	2	
Choline chloride	..	..	..	..	0.07
Salt mixture [2]	4	4	4	4	4
Yeast extract [3]	1	1	1	1	1
Distilled water	Ad libitum				

[1] "Centrol II." Lecithin from Central Soya Company, Inc., containing 65% phosphatides suspended in 35% soybean oil. The phosphatides consist of approximately 50% lecithin and 50% cephalin.

[2] U.S.P. XII mixture no. 2 for vitamin A-free ration, to which were added traces of KI and $CuSO_4$.

[3] Yeast extract supplied through the courtesy of Mead Johnson and Co., Evansville, Indiana.

A concentrate of the natural vitamin A ester was diluted with cottonseed oil and 5 μg of the vitamin administered to each rat daily. The vitamin A feeding was continued for 21 days. The rats were then sacrificed, the blood from each group pooled for serum vitamin A determination, and the livers obtained for individual liver vitamin A storage estimation.

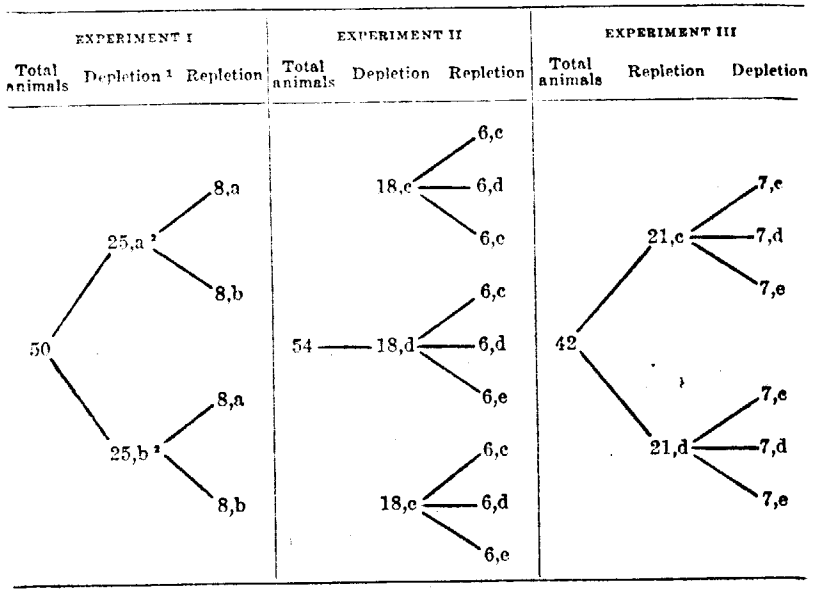
Experiment II. Soya lecithin, choline and vitamin A

Since choline is a physiologically important group in the lecithin molecule, the results obtained in experiment I

prompted an investigation of the effect of choline on the absorption and liver storage of vitamin A. Fifty-four weanling albino rats from the same breeding colony, and raised under similar conditions, were divided into 3 groups of equal sex and litter distribution and depleted of vitamin A on rations (c), (d), and (e) (see table 1). The amount of choline included

TABLE 2

Distribution of the experimental animals in the various dietary groups of the 3 experiments



[1] The letter following the number indicates the ration (see table 1).

[2] Four animals from each of these groups were sacrificed at the end of 3 weeks and 4 more at the end of the depletion period. Two died.

in ration (e) was that calculated to be in the lecithin added to ration (d). This amount is substantially greater than that fed by Slanetz and Scharf ('45).

At the end of the depletion period (39 days) each original group was again divided into 3 groups as indicated in table 2 and 5 μg of vitamin A ester administered daily for the next 21

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days. At the end of the 21-day repletion period the animals were exsanguinated and the livers obtained for vitamin A assay.

Experiment III. Soya lecithin, choline and carotene

This experiment was undertaken in an attempt to verify the unexpected effect of choline feeding and to study the effect of choline, lecithin and carotene feeding on the liver storage of vitamin A.

Forty-two weanling albino rats were divided into 2 comparable groups and depleted of vitamin A on rations (c) and (d) (see table 1). At the end of the depletion period (39 days) the animals were allotted to 6 sub-groups as indicated in table 2, experiment III. For the next 21 days each rat received a daily dose of 20 μg of β-carotene in cottonseed oil solution. Twenty-four hours after the last carotene feeding the animals were exsanguinated and the livers removed for assay.

Assay-procedure

Blood serum vitamin A was determined by the method of Kimble ('39). Vitamin A was determined on the entire liver by the method of Lewis et al. ('42) with minor modifications.

RESULTS

Experiment I

The rats in the lecithin group made more rapid and greater weight gains during the depletion period. Weights of the animals on the basal vitamin A-free ration remained almost constant after 2 weeks, while the lecithin-fed group continued to gain until near the end of the depletion period. During the second week there was a decrease in the weight of both groups. The cause of this unexpected change is not certain. It is thought to have resulted from the extremely hot weather during the week.

Three rats in the lecithin group showed symptoms of vitamin A deficiency after 4 weeks. Toward the end of the depletion period many of the rats of both groups showed the classic symptoms of avitaminosis A. Two of them died after 35 days.

The results of the vitamin A analyses of the blood sera and livers of the rats sacrificed at the end of 3 weeks and at the end of the depletion period are presented in table 3. It will be noted that the liver reserves were low at the end of 3 weeks and essentially exhausted at the end of the depletion period. Although the data are limited, the results indicate that the liver reserves are exhausted more rapidly when lecithin is fed and this is accompanied by lower blood serum vitamin A values.

During the vitamin A supplement feeding period the rats which received lecithin made greater weight gains, as shown in table 4. The liver storage of vitamin A was also greater in the lecithin-fed group. A statistical analysis of the liver storage data showed significance at the 1% level.

There appears to be a slight carry-over effect of lecithin feeding during the depletion period but this effect was not found to be statistically significant. The serum vitamin A at the end of the repletion period was found to be slightly lower when lecithin was fed. These blood data are too few for statistical analysis. Similar results were obtained in experiment III, however, as shown in table 7.

A preliminary inspection of the data indicated a sex difference in liver storage of vitamin A. This was verified by a statistical analysis of the data for the sexes presented in table 8.

Experiment II

The results of this experiment are summarized in table 5. During the depletion period both the lecithin- and choline-fed groups made greater weight gains than the control (basal) group. An analysis of these data showed the difference between the gains of the lecithin group and the control group to be significant at the 1% level. The difference between the choline group and the control group was not significant.

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As in the first experiment, the liver storage of vitamin A was significantly higher (1% level) when lecithin was fed with vitamin A. The liver storage in the choline-fed rats was not significantly different from that in the control group.

TABLE 3

The effect on blood and liver vitamin A of lecithin feeding during vitamin A depletion

DEPLETION RATION	AT THE END OF 3 WEEKS			AT THE END OF THE DEPLETION PERIOD		
	No. of rats	Serum A	Total liver A	No. of rats	Serum A	Total liver A
		µg/100 ml	*µg*		*µg/100 ml*	*µg*
Basal	4	18	1.1	4	15	0.5
Basal + lecithin	4	16	0.6	4	13	0.3

TABLE 4

Effects of lecithin on the rate of growth and liver storage of vitamin A during the feeding of vitamin A (5 µg daily) for 21 days (8 rats in each dietary group)

SUPPLEMENT IN ADDITION TO VITAMIN A	GAIN IN WEIGHT (GM)			VITAMIN A (µG/LIVER)			PERCENTAGE OF VITAMIN A STORED
	Depletion diet			Depletion diet			
	(a)	(b)	Av.	(a)	(b)	Av.	
Lecithin	32.9	33.2	33.0	20.2	22.8	21.5	20.5
None	28.0	32.0	30.0	16.4	18.2	17.3	16.5

TABLE 5

Effects of lecithin and choline on the rate of growth and liver storage of vitamin A during the feeding of vitamin A (5 µg) daily for 21 days (6 rats in each dietary group)

SUPPLEMENT IN ADDITION TO VITAMIN A	GAIN IN WEIGHT (GM)				VITAMIN A (µG/LIVER)				PERCENTAGE OF VITAMIN A STORED
	Depletion diet [1]				Depletion diet [1]				
	(c)	(d)	(e)	Av.	(c)	(d)	(e)	Av.	
Lecithin	33.0	36.0	27.0	32.0	17.0	21.2	18.0	18.7	17.9
Choline	29.0	27.3	22.0	26.1	14.0	12.0	12.7	12.9	12.3
None	26.0	27.0	25.0	26.0	12.2	14.8	13.1	13.3	12.6

[1] See table 1 for description of depletion diets.

Experiment III

The weight gains during the depletion period were so similar to those obtained in experiment I, and in the groups fed the basal ration and the basal ration plus lecithin of experiment II, that the data are not reported. Again the lecithin basal group made significantly greater (1% level) gains. The results of the carotene supplementation are summarized in tables 6 and 7. When either lecithin or choline was fed with the carotene, the weight gains were greater (1% level of significance) than when carotene was fed with the basal diet alone. There was no significant carry-over effect of the depletion diets. The lecithin-supplemented group stored more

TABLE 6

Comparative effects of lecithin and choline on the rate of growth and liver storage of vitamin A and "carotene" during the feeding of carotene (20 µg daily) for 21 days (6 rats in each dietary group)

SUPPLEMENT IN ADDITION TO CAROTENE	GAIN IN WEIGHT (GM)			VITAMIN A (µG/LIVER)			"CAROTENE" (µG/LIVER)			PERCENTAGE OF VITAMIN A STORED
	Depletion diet			Depletion diet			Depletion diet			
	(c)	(d)	Av.	(c)	(d)	Av.	(c)	(d)	Av.	
Lecithin	25.6	26.2	25.9	65.5	67.5	66.5	3.7	2.6	3.1	32.4
Choline	22.0	24.0	23.0	52.9	58.4	55.6	3.7	2.6	3.1	27.2
None	14.3	20.0	17.0	51.7	59.0	55.3	2.7	1.9	2.3	26.8

TABLE 7

Comparative effects of lecithin and choline on blood serum vitamin A following the feeding of vitamin A (5 µg daily) or carotene (20 µg daily) for 21 days

SUPPLEMENT IN ADDITION TO VITAMIN A OR CAROTENE	SERUM VITAMIN A (µG/100 ML)					
	Vitamin A supplemented groups (8 rats in each group)			Carotene supplemented groups (6 rats in each group)		
	Depletion diets			Depletion diets		
	(c)	(d)	Av.	(c)	(d)	Av.
Lecithin	32.9	37.4	35.1	42.5	37.3	39.9
Choline	...	...	...	45.2	42.3	43.7
None	36.5	41.5	39.0	45.2	39.9	42.5

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vitamin A (significant at the 1% level) than either of the other 2 groups, and choline produced no effect in this respect.

It will be noted that small amounts of pigment estimated as "carotene" were found in the livers following carotene feeding. Both "carotene" and vitamin A were converted to International Units (micrograms carotene × 1.66 plus micrograms vitamin A × 3.33) and the percentage storage calculated. While the general pattern of storage on the various diets was similar to that when vitamin A was fed (experiments I and II), a higher percentage storage was obtained when 20 μg of carotene were fed.

TABLE 8

Influence of sex on the liver storage of vitamin A in rats fed 5 μg of vitamin A or 20 μg of carotene daily

SUPPLEMENT IN ADDITION TO VITAMIN A OR CAROTENE	NO. OF RATS	AVERAGE LIVER STORAGE (μG)		DIFFERENCE BETWEEN ♂ AND ♀ OF SAME GROUP	DIFFERENCE BETWEEN ♂ OF LECITHIN AND NON-LECITHIN GROUP	DIFFERENCE BETWEEN ♀ OF LECITHIN AND NON-LECITHIN GROUP
		♂	♀			
				%	%	%
		Vitamin A-fed groups				
None	16	14.6	20.7	41.6 [1]	...	...
Lecithin	16	19.4	24.3	25.1 [2]	32.5 [1]	17.1 [3]
		Carotene-fed groups				
None	12	45.4	65.3	43.8 [1]	...	...
Choline	12	44.6	66.8	49.8 [1]	...	...
Lecithin	12	56.8	76.5	34.7 [1]	25.1 [2]	17.2 [3]

[1] Significant at the 1% level (probability of happening by chance 1 in 100).
[2] Significant at the 5% level (probability of happening by chance 1 in 20).
[3] Not significant.

As in the previous experiments, a sex difference in vitamin A storage was evident. The liver storage data are summarized by sex in table 8. The sex difference in liver vitamin A storage was highly significant, regardless of the supplement fed in addition to carotene.

Again, as in the previous experiments, the blood plasma vitamin A was lower in the group which received both lecithin and carotene.

DISCUSSION

Several investigators (Brenner et al., '42; Davies and Moore, '35; Horton et al., '41; Popper and Greenberg, '41) have observed the rapid loss of vitamin A reserves in rats fed a vitamin A-free diet. The feeding of lecithin appears to hasten the decline in liver stores, but the superior growth response indicates either that the loss of the vitamin by destructive breakdown is retarded, or that greater economy is effected in its functional metabolic processes.

Adiersberg and Sobotka ('43b) and Angur et al ('47) have shown that lecithin enhances the digestion and absorption of fat, and Muelder and Kelly ('42) have produced evidence that a minimum dietary fat level is necessary for the optimum absorption and utilization of vitamin A, which in turn is responsible for greater weight gains. In the experiments reported herein no vitamin A was fed during the depletion period, and the enhanced growth rate accompanied by lower levels of vitamin A in both the blood and liver which resulted from the addition of lecithin to the depletion diet indicates a primary effect on the mobilization and utilization of the vitamin A reserves.

The greater growth response and liver storage of vitamin A in the rats receiving both lecithin and vitamin A indicate clearly both increased absorption and more effective utilization. The slight carry-over effect of lecithin feeding during the depletion period in increasing growth and liver storage during the vitamin A repletion period also supports this view.

Greenberg and Popper ('41) and Brenner et al. ('42) demonstrated the presence of sufficient vitamin A in the retina and blood even in the absence of liver stores. This probably explains the continuation of growth toward the end of the depletion period and indicates that lecithin may have aided in the mobilization and effective utilization of the vitamin from body depots other than the liver. The fact that lower blood levels of vitamin A were observed in the animals being fed lecithin, even though they gained more weight and stored

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more of the vitamin in the liver, is further evidence that lecithin aids in vitamin A utilization.

The results of these experiments support the observations that lecithin feeding increases liver vitamin A storage, as reported by Slanetz and Scharf ('45), and conserves the hepatic reserves of this vitamin, as reported by Polskin ('40).

Crude soya lecithin contains a number of compounds that might be suspected of being responsible for the observations on growth and liver storage of vitamin A. Scharf and Slanetz ('44) and Patrick and Morgan ('44) presented evidence that α-tocopherol was not the active principle responsible for these effects. Since choline plays an important role in fat metabolism, we considered it of interest to compare its effects with those of lecithin under similar experimental conditions.

Choline feeding did not result in greater weight gains during the depletion period or in greater weight gains or increased liver storage of the vitamin during the vitamin A repletion period. The significant carry-over effect of choline feeding during the depletion period in decreasing the growth response to vitamin A feeding is difficult to explain. Thorbjarnarson and Drummond ('38) observed that vitamin A depletion could be accelerated by causing a partial dispersal of fat from the liver by the administration of choline. This effect was not evident from our experiments. Our choline-fed rats actually grew as well as the control group during the depletion period and they did not show deficiency symptoms at an earlier date.

It is possible that the lecithin effect is due to the fatty acids of lecithin, since it has been shown by Sherman ('40, '42) that some unsaturated fatty acids aid in the absorption and metabolism of vitamin A. A more probable explanation is that the lecithin molecule acts as an intact unit, or that the crude lecthin used contains some compound other than lecithin, choline or α-tocopherol which is the active principle.

The greater liver storage of vitamin A observed when carotene was fed in these experiments suggests the possibility

that, up to a certain point, as the level of intake is increased, the percentage stored is increased.

The evidence in the literature regarding sex differences in ability to store vitamin A is conflicting. Lemley et al. ('47) reported that there is no sex difference. The results of our study support the observation of Brenner et al. ('42) that under comparable experimental conditions females store appreciably more vitamin A in the liver than males. The addition of lecithin to the ration enables either sex to store greater amounts of vitamin A in the liver when either 5 μg of vitamin A ester or 20 μg of carotene are fed daily.

SUMMARY

A series of 3 experiments were undertaken to study the effects of crude soya lecithin and choline on the absorption, utilization and storage of vitamin A in laboratory rats. The following pertinent observations were made.

When 2% of the basal vitamin A-free diet consisted of lecithin (⅓ or ⅙ of the fat replaced by lecithin), greater weight gains during the depletion period were obtained than when 0.07% choline or a similar diet without added lecithin was fed. Limited data indicate that liver reserves of vitamin A were exhausted slightly more rapidly when the depletion diet contained added lecithin.

The feeding of lecithin during the depletion period had a slight, although not a statistically significant, carry-over effect in increasing the weight gains during the vitamin A repletion period. On the other hand, choline feeding during depletion resulted in significantly smaller weight gains during the following vitamin A repletion period.

The feeding of lecithin with a vitamin A or carotene supplement resulted in greater gains in weight and increased liver storage of vitamin A. When choline was fed with these supplements, the liver storage of vitamin A was no different from that of the groups which received the vitamin supplement alone.

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At the levels fed, there was a greater percentage storage of vitamin A when β-carotene was the supplement, although the weight gains of the vitamin A-supplemented groups were greater.

Females consistently stored more of the vitamin in the liver under all the dietary conditions studied.

The data indicate that lecithin enhances both the absorption and utilization of vitamin A and carotene. The choline fraction of the lecithin molecule is not responsible for this effect.

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THE EFFECT OF SOYA-PHOSPHATIDES ON THE ABSORPTION AND UTILIZATION OF VITAMIN A IN DAIRY ANIMALS[1]

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It has been shown by various research workers (2, 6, 8, 35) that the liver of the newborn calf contains either little or no vitamin A. Analyses of blood of newborn calves also revealed that the level of vitamin A is exceptionally low (19, 20, 27, 32, 35).

Colostrum, which has been found to be very rich in this vitamin (6, 16, 24, 29, 35), is the first natural material which the calves consume to overcome this deficiency. There is a wide variation in colostral vitamin A among cows of the same breed (29) as well as in different seasons. Attempts have been made to increase the vitamin A potency of colostrum by feeding extra vitamin A during the latter part of the gestation period. Stewart and McCallum (30) failed to find an increase of this vitamin in colostrum following the feeding of carrots or cod-liver oil. Contrary to this finding, Spielman *et al.* (28) were able to demonstrate an increased amount of vitamin A in colostrum and in the blood and livers of calves following the feeding of large doses of the vitamin during the later stages of the gestation period. Wise *et al.* (35) also found higher levels of vitamin A in both the blood and the liver of the newborn calves when the dams were fed supplementary vitamin A during the gestation period. When large doses of vitamin A or carotene were fed during the later stages of gestation, the decrease in the blood plasma vitamin A and carotene of cows at parturition was not prevented (5, 17, 33, 34), although a higher level was maintained than was observed in the controls.

Numerous reports have been published regarding the importance of vitamin A in calfhood nutrition. Attempts were made to raise calves on skim milk supplemented with vitamin A concentrate (18, 19), usually with disappointing results. Wisconsin workers (19) reported that vitamin A, together with ascorbic acid and nicotinic acid, would increase the survival rate of calves on skim milk. Later work (12, 21) showed no beneficial effect of nicotinic acid feeding, especially in conjunction with large doses of vitamin A supplement.

The question is raised as to whether or not other sources of vitamin A or carotene along with skim milk can be utilized in the same way as the

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colostral vitamin A or carotene. Spielman *et al.* (26) have shown that the carotene of commercial concentrates is poorly utilized by the very young calf. They have reported further that the vitamin A and carotene in colostrum are used more effectively than are vitamin A and carotene added to skim milk or similarly fortified reconstituted skim milk. Knowledge of the exact mechanism of absorption and utilization of vitamin A in the animal system still is inadequate.

Considerable work has been done during recent years regarding the synergism of vitamin E and vitamin A (10, 13) as well as of lecithin and vitamin A. It has been reported that in the case of rats (25), chicks (22), and man (1), the utilization of vitamin A and carotene can be influenced by soybean phosphatides.

The purpose of the present investigation was twofold: (a) To determine the extent to which the vitamin A level of the blood and colostrum of cows and the vitamin A reserve of the newborn calves may be affected by feeding large doses of vitamin A along with soybean phosphatides during the later stages of gestation, and (b) to throw some light on the mechanism of absorption and utilization of vitamin A by calves on a skim milk ration from the time of birth.

EXPERIMENTAL PROCEDURE

This experiment was undertaken during the late winter and early spring months of 1947 while all the animals were on a winter herd ration. Twenty-six pregnant dairy cows of the Jersey and Holstein breeds previously maintained under similar conditions of feeding and management were divided into three dietary groups approximately 30 days prior to parturition. Group I, consisting of ten cows, was again subdivided into two groups of five each. Group I–A received only the usual herd ration[3] and Group I–B the same ration plus 10 g. of soybean lecithin[4] daily. Group II, consisting of eight cows, was given one million I.U. (International Units) of vitamin A[5] daily, in addition to the herd ration. Group III, consisting of eight animals, received the herd ration plus one million I.U. of vitamin A and 10 g. of lecithin daily.

These rations were continued to the seventh day following the day of parturition. The vitamin A was given orally in gelatin capsules at approximately the same hour each day. The lecithin was mixed carefully with the vitamin A concentrate (fish-liver oil) and special gelatin cap-

[3] The herd ration consists of ground corn 400, ground oats 300, wheat bran 100, soybean oil meal 100, salt iodized 9, and the silage and hay *ad libitum*.

[4] Soybean lecithin supplied by the American Lecithin Company, Long Island City, New York, contained 70 per cent soya phosphatides (lecithin, cephalin and lipositol) and 30 per cent soybean oil.

[5] Vitamin A capsules were supplied by the Gelatin Products Company, Detroit, Michigan. One gram of this capsule had 25,000 I.U. vitamin A.

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sules each containing 3.33 g. of lecithin and 333,333 I.U. of vitamin A were prepared each week and stored in the refrigerator. Three of these capsules were administered daily to the cows in Group III.

Blood plasma vitamin A and carotene were determined each week before parturition, at parturition, and at 1, 3, 7, 14, and 21 days after parturition. Vitamin A, carotene and lecithin analyses were made on the colostrum and milk samples successively at parturition, and at 1, 3, 7, 14, and 21 days after parturition. Two cows in each of Groups II and III were fed in the same way for approximately 60 days before parturition in order to determine the possible effect of feeding vitamin A for a longer period on the changes of vitamin A and carotene levels in the blood plasma of the cows and their calves and in the liver of the calves as well as in the colostrum and milk.

Blood plasma vitamin A and carotene were determined in the newborn calves before they had access to colostrum or other feed. A few calves from each of the representative groups were slaughtered after birth to determine the liver storage of vitamin A and carotene. All of the calves which were not slaughtered at birth from the above experiment (15 in all) were divided into three groups. There was no predetermined basis for the allotment of the calves to the various groups except that the largest number of calves from cows fed vitamin A and lecithin were allotted to group B, which was assumed to be the group receiving the most rigorous treatment. Preliminary data had shown higher liver storage in the calves from these cows. The calves in Group A were fed colostrum at the rate of 10 lb. per 100 lb. of bodyweight for 7 days after birth. A composite mixture of colostrum was made and stored previously to standardize the feeding in every case. The vitamin A, carotene and lecithin contents of this mixture of colostrum were determined, and the total vitamin A and [illegible] consumed by each calf were calculated. The calves in Group B were fed skim milk at the same rate for 7 days after birth. They were not permitted to receive any colostrum. The same quantity of vitamin A consumed daily by the calves in the colostrum-fed group (Group A) was added to the skim milk every day. The calculated quantity of vitamin A oil (25,000–37,500 I.U.) was homogenized with the skim milk before each feeding. Group C was fed skim milk plus the same quantity of vitamin A and the same quantity of lecithin (3–4.5 g.) as was consumed daily by the calves in Group A. This feeding schedule was continued for 7 days following birth. The appropriate quantities of lecithin and vitamin A oil were homogenized with a small amount of skim milk and then mixed with the skim milk to be fed at each feeding. The surviving calves of all the groups were fed whole milk after the seventh day.

Vitamin A and carotene of blood plasma were determined successively at birth, and at 1, 3, 7, 14, and 21 days after birth. After 21 days, some

of the calves from each of the representative groups were slaughtered to determine the total liver storage of vitamin A and of carotene.

Vitamin A and carotene of blood plasma were determined according to the method of Kimble (15). The vitamin A of the blood samples having carotene concentrations exceeding 300 was determined by the method of Boyer *et al.* (3). The vitamin A and carotene of the colostrum and milk samples were determined by the method of Boyer *et al.* (4), with slight modifications. Instead of cold saponification, a hot saponification procedure was adopted. Five milliliters of colostrum or 25 ml. of milk plus 10 g. of caustic potash plus 50 ml. of methyl alcohol were refluxed in a boiling water bath for 10 minutes in a low actinic flask with a ground glass fitted reflux assembly. After cooling, the mixture was transferred to a separatory funnel, rinsing the flask with 55 ml. of water. The mixture was extracted successively with 50-ml. and with 25-ml. quantities of diethyl ether. The remainder of the procedure was the same as that of Boyer *et al.* (4). Liver vitamin A and carotene were determined by using the extraction procedure of Guilbert and Hart (8), with slight modification. Lecithin was determined according to the procedure adopted by Horrall (14). All of the colorimetric measurements were made in an Evelyn Photoelectric Colorimeter, using the appropriate filters.

RESULTS AND DISCUSSION

Effect of prepartal vitamin A and lecithin feeding on the vitamin A and carotene levels in the blood plasma of cows. The individual animal data are too voluminous to report; therefore the data are summarized in table 1. It will be noted that the feeding of lecithin or of vitamin A, or a combination of lecithin and vitamin A, did not prevent a decrease in blood plasma vitamin A and carotene at the time of parturition and beginning lactation. However, higher vitamin A levels were maintained when vitamin A was fed with or without additional lecithin (34). When both vitamin A and lecithin were fed, the highest blood vitamin A level was maintained; the level following parturition was higher (statistically significant) than that found 4 weeks prior to parturition. These data are considered as presumptive evidence that lecithin facilitates the absorption of vitamin A. There was considerable individual variation both in the time when the maximum decrease was noted after parturition and in the magnitude of the decrease. If samples had been obtained at more frequent intervals, perhaps these differences between groups would have been more clear-cut. Feeding of lecithin apparently has a tendency to delay the time of maximum postpartum decrease in blood plasma vitamin A. Although the vitamin A and lecithin feeding was discontinued on the seventh day postpartum, a carry-over effect was still apparent on the twenty-first day, the greatest carry-over effect being observed when both vitamin A and

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TABLE 1

The effect of feeding vitamin A and vitamin A plus lecithin on the average concentration of vitamin A and carotene in the blood plasma of cows for the period 4 weeks before parturition to 3 weeks following parturition

Groups	No. of cows	Days prepartum				At parturition	Days postpartum				
		28	21	14	7		1	3	7	14	21
Vitamin A in blood plasma (γ/100 ml.)											
I–A (No lecithin)	5	20.5	19.1	17.8	18.0	13.7	9.0	13.8	11.5	17.1	18.8
I–B (With lecithin)	5	17.9	18.8	11.9	10.1	12.4	12.4	6.7	12.1	13.7	12.4
II (Vitamin A)	8	19.5	29.6	25.8	20.1	20.4	18.1	21.3	26.0	23.1	22.1
III (A and lecithin)	8	18.9	38.9	38.6	31.2	25.9	25.1	27.5	24.7	26.8	28.7
Carotene in blood plasma (γ/100 ml.)											
I–A (No lecithin)	5	285	335	320	273	212	157	212	215	242	371
I–B (With lecithin)	5	303	368	340	347	318	273	303	331	297	303
II (Vitamin A)	8	422	351	294	222	181	160	141	145	170	263
III (A and lecithin)	8	390	322	240	216	159	145	128	93	133	172

lecithin were fed. These data provide additional evidence that high levels of vitamin A in the blood plasma of cows can be maintained by high levels of vitamin A feeding (34) and that this can be done more effectively by feeding a combination of vitamin A and lecithin.

TABLE 2

The effects of feeding vitamin A and vitamin A plus lecithin on the changes in the vitamin A content of cows' colostrum and milk up to 21 days postpartum

Cow no.	Vitamin A of colostrum and milk (γ/100 ml.)					
	At parturition	24 hr.	3 d.	7 d.	14 d.	21 d.
Group I–A (control group without lecithin)						
851H	496	67	35	19	17	18
873J	145	79	21	16	15	11
820J	141	52	33	11	14	15
866J	154	69	68	22	13	14
620H	218	113	29	18	.....	...
Mean	226	76	37	17	15	15
Group I–B (control group with lecithin)						
798H	143	46	.....	18	22	25
616H	132	60	58	22	19	17
801H	98	40	21	...		
763J	216	105	43	27	17	18
800J	134	92	22	25	26	16
Mean	145	69	36	23	21	19
Group II (vitamin A)						
755H	229	167	123	31	25	32
692J	420	131	179	71	23	25
812J	163	172	141	97	14	
711J	304	.....	169	201	74	27
649J	216	222	32	144	28	27
752H	705	302	161	191	.....	...
Mean	339	197	134	122	33	28
Group III (vitamin A plus lecithin)						
852H	552	113	175	70	31	24
806H	598	156	195	77	31	39
743H	377	158	112	148	146	22
675J	847	210	125	148	25	40
760J	1119	506	165	162	29	32
Mean	698	229	154	121	52	32

The effect of feeding lecithin alone is not statistically significant although the decrease in blood carotene following parturition appears to be about 10 per cent less when lecithin is fed.

The effects of feeding vitamin A and vitamin A plus lecithin on blood plasma carotene at the time of parturition are difficult to interpret be-

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cause of the depressing effect (7) of vitamin A feeding on blood plasma carotene. This depressing effect was strikingly shown in the case of three cows fed vitamin A and lecithin for 8 weeks prepartum. The average carotene content of the blood of these cows was 329, 53 and 73 γ per 100 ml. at 8 weeks prepartum and at 7 and 21 days postpartum, respectively.

TABLE 3

The effects of feeding vitamin A and vitamin A plus lecithin on the changes in the carotene content of cows' colostrum and milk up to 21 days postpartum

Cow no.	Carotene of colostrum and milk (γ/100 ml.)					
	At parturition	24 hr.	3 d.	7 d.	14 d.	21 d.
Group I–A (control group without lecithin)						
851H	195	42	18	9	14	17
873J	200	157	17	13	20	19
820J	191	74	36	18	22	28
866J	179	105	52	22	22	9
620H	133	71	18	7	.....	.....
Mean	180	90	28	14	19	18
Group I–B (control group with lecithin)						
798H	223	53	.....	11	10	14
616H	200	89	46	16	35	8
801H	105	34	17	.....	.....	.....
763J	218	216	53	16	14	13
800J	172	58	10	8	12	9
Mean	183	90	31	13	18	11
Group II (vitamin A)						
755H	140	108	48	12	8	8
692J	137	36	20	8	8	15
812J	113	62	18	10	7	.....
711J	152	.....	34	27	8	9
649J	103	69	10	18	7	28
752J	115	47	10	6	.....	.....
Mean	126	64	23	14	8	15
Group III (vitamin A plus lecithin)						
852H	171	24	15	6	6	6
806H	195	27	25	9	6	12
743H	168	30	16	6	17	7
675J	243	71	26	6	7	15
760J	200	42	16	6	6	6
Mean	195	39	20	7	8	9

Effect of prepartal vitamin A and lecithin feeding on the vitamin A, carotene and lecithin content of colostrum. The data in table 2 show that when vitamin A was fed, the vitamin A in the colostrum was significantly higher than that produced by the groups receiving no vitamin A. When

both vitamin A and lecithin were fed, the vitamin A in the first milking of colostrum was approximately double that of the colostrum from cows receiving vitamin A alone. This difference became less as the milk approached normal and had vanished by the seventh day postpartum. The effects of vitamin A feeding on the potency of the milk still were evident 2 weeks following the end of the vitamin feeding.

The data on the effect of feeding lecithin and vitamin A on the carotene content of the colostrum and early milk are presented in table 3. The feeding of vitamin A depresses the level of carotene in colostrum and milk, a result which has been reported previously (7). It appears also that the feeding of lecithin with the vitamin A enhances this suppressing effect. Additional data are needed to confirm this point.

As previously noted, three animals were fed vitamin A and lecithin for a period of 8 weeks before freshening. The limited data obtained from these animals indicated that higher levels of vitamin A were maintained in the milk after the third day following parturition and the milk carotene was further depressed. These limited data need further confirmation.

The carotene content of colostrum and milk follows the same trend as in the blood. The apparent antagonistic effect of supplemental vitamin A on blood and milk carotene is not explainable in the light of present knowledge; however, its occurrence seems to be beyond doubt.

The total output of vitamin A in International Units per milking is presented in table 4. Although the level of carotene has been depressed in the vitamin A supplemented groups, the total output of vitamin A is higher in these groups. When lecithin was fed with vitamin A, the total output of vitamin A was highest, especially at the first milking. This difference is so great that there is little doubt of its significance. Following the discontinuation of vitamin A supplementation on the seventh day, a sudden marked drop in vitamin A occurred. In the case of those animals receiving both vitamin A and lecithin, the drop was more gradual. Results in the control groups are interesting. Although the vitamin A concentration in the milk of the control group I–A (without lecithin) was higher than that of the control group I–B (with lecithin, see table 2), the total output of vitamin A is higher in the lecithin-fed group. This is due to the greater milk yield. Whether this increased milk-yield is due to lecithin feeding is to be determined by further experiments with a larger number of cows.

The effects of lecithin feeding on the lecithin content of colostrum and milk are shown in table 5. The lecithin content of colostrum and milk seems to be maintained at a higher level when both vitamin A and lecithin are fed. The carry-over effect is still apparent in the milk on the twenty-first day, 2 weeks after the supplemental feeding was discontinued. When

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lecithin is fed without vitamin A or vitamin A fed without lecithin, the amount of lecithin in the colostrum and milk is little different from that of the control group which received no supplement. It appears from these data that there may be a reciprocal relationship in the absorption and/or metabolism of these two compounds.

TABLE 4

The effects of feeding vitamin A and vitamin A plus lecithin on the changes in total output of vitamin A per milking up to 21 days postpartum

Cow no.	I.U.[a] of vitamin A per milking					
	At parturition	24 hr.	3 d.	7 d.	14 d.	21 d.
Group I–A (control group without lecithin)						
851H	136,140	13,557	13,648	6,717	7,541	8,323
873J	16,565	11,831	3,696	3,769	4,482	3,601
820J	17,998	15,731	8,759	4,839	5,723	6,937
866J	9,456	8,361	8,480	5,133	4,732	3,932
620H	50,592	23,273	6,445	4,147	.....	.....
Mean	46,150	14,550	8,206	4,921	5,620	5,698
Group I–B (control group with lecithin)						
798H	55,600	34,610	.....	9,899	13,075	17,668
616H	51,959	37,710	25,264	11,195	13,978	8,400
801H	87,780	19,854	9,351	.....	.....	.....
763J	72,350	5,304	19,449	8,629	6,353	7,770
800J	20,512	30,058	5,994	8,146	8,781	3,964
Mean	57,638	25,507	15,012	9,467	10,321	8,950
Group II (vitamin A fed)						
755H	58,883	90,360	47,782	16,685	12,693	16,993
692J	112,493	41,939	60,538	25,285	7,645	12,844
812J	45,740	37,675	45,764	32,017	6,353	.....
711J	36,652	.....	52,145	67,821	21,279	8,831
649J	34,285	24,123	6,137	41,377	8,436	13,443
752H	280,100	125,412	60,156	72,996	.....	.....
Mean	94,692	63,902	45,420	42,697	11,282	13,027
Group III (vitamin A plus lecithin)						
852H	102,850	22,056	55,220	19,315	9,649	6,955
806H	455,910	51,539	112,227	33,145	14,169	23,099
743H	295,940	66,164	35,697	59,653	76,830	10,979
675J	146,240	55,198	30,774	42,357	9,092	15,751
760J	141,713	56,997	45,183	51,164	9,417	9,253
Mean	228,530	50,391	55,820	41,127	23,830	13,207

[a] One microgram of vitamin A = 4 I.U. Vitamin A. One microgram of carotene = 1.66 I.U. vitamin A.

Effect of supplementing the maternal diet with vitamin A and lecithin on the blood plasma vitamin A and carotene of the newborn calf. The results of this phase of the study are presented in table 6. The plasma vitamin A of the calves from cows receiving vitamin A was significantly higher than that of those from the control cows. These results are in agreement with those of Wise *et al.* (35) and Spielman *et al.* (27). The

TABLE 5

The effects of feeding vitamin A and vitamin A plus lecithin on the changes in the lecithin content of cows' colostrum and milk

Cow no.	Percentage of lecithin in colostrum and milk					
	At parturition	24 hr.	3 d.	7 d.	14 d.	21 d.
Group I–A (control group without lecithin)						
851H	0.062	0.051	0.049	0.028	0.029	0.024
873J	0.076	.....	0.044	0.039	0.038	0.038
820J	0.058	0.059	0.043	0.038	0.021	0.020
866J	0.068	.....	0.049	0.039	0.029	0.036
620H	0.060	0.080	0.048	0.038	0.034	.....
Mean	0.065	0.063	0.047	0.036	0.030	0.029
Group I–B (control group with lecithin)						
798H	0.076	0.056	.....	.....	0.042	0.026
616H	0.076	.....	0.056	.....	0.021	0.025
801H	0.051	0.043	0.023	.....	0.042	0.026
763J	0.073	.....	0.074	.....	.....	.....
800J	0.055	0.053	0.055	0.048	0.026	0.029
Mean	0.066	0.051	0.052	.....	0.033	0.026
Group II (vitamin A)						
755H	0.068	0.063	0.055	0.046	0.031	0.025
692J	0.064	0.045	0.052	0.047	0.024	0.023
812J	0.056	0.046	0.044	0.039	0.036	.....
711J	0.088	.....	0.074	0.055	0.036	0.040
649J	0.064	0.035	0.026	0.060	0.036	0.038
752J	0.084	0.067	0.059	0.052	.....	.....
Mean	0.071	0.051	0.052	0.049	0.033	0.032
Group III (vitamin A plus lecithin)						
852H	0.094	0.062	0.057	0.058	0.044	0.036
806H	0.094	0.060	0.050	0.051	0.046	0.051
743H	0.069	0.049	0.040	0.040	0.046	0.030
675J	0.093	0.100	0.180	0.081	0.059	0.055
760J	0.093	0.102	0.075	0.056	0.052	0.051
Mean	0.089	0.075	0.069	0.057	0.049	0.045

slightly higher mean value of the blood plasma vitamin A of calves from cows receiving both vitamin A and lecithin is insignificant.

It appears from the data on blood plasma carotene that a combination of lecithin and vitamin A in the maternal diet exerts a suppressing action on the level of carotene in the blood of the newborn calf. These data, however, are within a range where experimental error of determination is

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apt to be rather high, and the difference may not be as significant as the statistic indicates.

The effect of supplementing the maternal diet with vitamin A and lecithin on the liver storage of vitamin A and carotene in the newborn calf. Seven of the calves were sacrificed at birth to determine total liver storage

TABLE 6

The effect of increasing the vitamin A and lecithin content of the maternal diet on the blood plasma and liver storage of vitamin A and carotene in the newborn calf

Dam no.	Calf no.	Blood plasma data: Vitamin A (γ/100 ml.)	Blood plasma data: Carotene (γ/100 ml.)	Liver storage data: Liver wt. (g.)	Liver storage data: Vitamin A (γ/g.)	Liver storage data: Total A (γ)
Calves from Group I (control cows)						
866J	819J	2.4	4.2	.....	.....	.....
873J	820J	3.5	6.3	.....	.....	.....
851H	825H	6.5	11.4	.....	.....	.....
800J[a]	944J	7.7	4.2	.....	.....	.....
820J	821J	5.4	9.2	434	0.59	249
620H	948H	8.9	0.0	1000	0.25	252
801H[a]	828H	8.4	5.6	793	0.08	67
	Mean	6.1	5.8	.....	0.31	190
Calves from Group II (cows fed vitamin A)						
755H	905H	7.2	11.4	.....	.....	.....
812J	946J	9.8	8.5	.....	.....	.....
711J	823J	8.6	8.4	.....	.....	.....
853H	830H	9.3	0.0	.....	.....	.....
752H	829H	10.7	0.0	.....	.....	.....
692J	822J	11.5	4.9	435	12.0	5241
649J	827J	6.2	7.0	500	13.1	6580
	Mean	9.0	5.7	.....	12.5	5910
Calves from Group III (cows fed vitamin A plus lecithin)						
855J	832J	9.2	0.0	.....	.....	.....
760J	947J	6.6	2.8	.....	.....	.....
743H	950H	10.7	0.0	.....	.....	.....
852H	949H	10.2	0.0	.....	.....	.....
857H	951H	13.6	0.0	.....	.....	.....
675J	824J	8.2	5.7	438	22.0	9625
806H	826H	15.1	0.0	945	14.6	13820
	Mean	10.5	1.2	.....	18.3	11722

[a] These cows were in Group I–B; lecithin was fed.

of vitamin A and carotene. Three of these calves were from cows in Group I, two from cows in Group II, and two from cows in Group III. As previously reported by other workers (27, 35), a statistically significant greater liver storage was found in newborn calves from dams receiving massive vitamin A supplements. The cows receiving both vitamin A and

lecithin gave birth to calves with almost double the liver vitamin A storage of those from cows receiving vitamin A alone. As can be noted from table 6, this difference was due in part to higher concentration and in part to greater liver weight. Although the numbers are limited, the magnitude of the difference is so great that there is little doubt of the significance, particularly in the light of the other data presented in this paper. Further evidence of the effect of vitamin A feeding on liver storage is shown in table 9. Calves 832J, 831J, and 830H were from dams that received the vitamin A supplement for 8 weeks prior to parturition. When these calves were sacrificed at 21 days of age (calf 831J died at 11 days of age), the liver storage was significantly higher than that of other comparable calves with similar histories and treatment.

TABLE 7

The effects of feeding colostrum, skim milk plus vitamin A and skim milk plus vitamin A and lecithin on the plasma vitamin A levels of calves

Calf no.	Dam no.	Dam group	Vitamin A fed daily (I.U.)	Vitamin A in plasma (γ/100 ml.): At birth	24 hr.	3 d.	7 d.	14 d.	21 d.
Group A (colostrum-fed calves)									
945H	755H	II	37,500	7.2	4.6	27.9	16.9	9.4	9.3
819J	866J	I–A	25,000	2.4	8.2	24.0	9.9	13.6	8.3
820J	873J	I–A	25,000	3.5	11.9	25.4	23.3	13.5	11.1
944J	800J	I–B	25,000	7.7	13.5	18.2	19.9	14.6	14.1
832J	855J	III	25,000	9.2	11.9	20.2	20.7	18.2	15.9
			Mean	6.2	10.1	23.5	18.2	13.8	11.7
Group B (skim milk plus vitamin A)									
825H	851H	I–A	37,500	6.5	5.3	12.0	Fell sick and died on the 7th day		
946J	812J	II	25,000	9.8	6.6	.....	"	"	"
947J[a]	760J	III	25,000	6.6	6.6	8.7	13.9	11.3	6.6
950H[a]	743H	III	37,500	10.7	10.4	12.2	10.1	9.3	22.0
831J[b]	854J	III	25,000	5.9	9.3	13.2	13.3	died on the 11th day	
			Mean	7.9	7.6	11.4	12.4	.....	.....
Group C (skim plus vitamin A plus lecithin)									
823J	711J	II	25,000	8.6	15.7	15.7	18.7	16.8	13.9
949H	852H	III	37,500	10.2	10.7	30.3	35.0	20.8	16.0
951H	857H	III	37,500	13.6	10.7	19.2	27.8	17.2	10.5
830H	853H	II	37,500	9.3	18.3	14.3	28.1	12.2	15.3
829H	752H	II	37,500	10.7	13.6	16.8	22.8	12.7	12.2
			Mean	10.5	13.8	19.3	26.3	15.9	13.6

[a] Given 2% cocoanut oil per feeding.
[b] Given lecithin from the 6th day.

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Effect of the diet on the blood plasma vitamin A and carotene levels and the liver vitamin A storage of young calves. As previously indicated, the calves from the cows in this experiment were removed from the dams at birth (before nursing) and given special dietary treatments for the first 7 days. These dietary treatments have been described earlier in this paper. The data obtained are presented in tables 7 and 8. The feeding of colostrum resulted in a marked increase in the amount of vitamin A in the blood, as has been noted by others (19, 20, 32). There was an increase in blood plasma carotene following the feeding of colostrum (table 8), but the carotene remained low in both the groups fed vitamin A, as was expected. The carotene increase in the latter groups on the fourteenth and twenty-first days resulted from whole milk feeding following 7 days of age. There was no evidence of scours in the colostrum-fed group. The calves receiving skim milk plus the vitamin A supplement did very poorly.

TABLE 8

The effects of feeding colostrum, skim milk plus vitamin A and skim milk plus vitamin A and lecithin on the plasma carotene levels of calves

Calf no.	Dam no.	Dam group	Vitamin A fed daily (I.U.)	Carotene in plasma (γ/100 ml.) At birth	24 hr.	3 d.	7 d.	14 d.	21 d.
Group A (colostrum-fed calves)									
945H	755H	II	37,500	11.4	5.7	24.7	38.8	20.6	23.2
819J	866J	I–A	25,000	4.2	6.4	4.9	62.1	49.4	37.2
820J	873J	I–A	25,000	6.3	2.8	26.3	58.7	29.0	21.8
944J	800J	I–B	25,000	4.2	21.7	48.1	22.4	51.0	35.6
832J	855J	III	25,000	0.0	5.7	29.4	35.6	57.0	32.4
			Mean	5.2	8.4	26.6	43.5	41.3	30.0
Group B (skim milk plus vitamin A)									
825H	851H	I–A	37,500	11.4	7.8	13.6	died on the 7th day		
946J	812J	II	25,000	8.5	5.6		" " " "		
947J[a]	760J	III	25,000	2.8	5.6	7.7	0.0	12.8	5.9
950H[a]	743H	III	37,500	0.0	0.0	3.5	4.0	17.2	21.8
831J[b]	854J	III	25,000	0.0	0.0	0.0	3.5	died on the 11th day	
			Mean	4.5	4.0	6.2	2.8		
Group C (skim plus vitamin A plus lecithin)									
823J	711J	II	25,000	8.4	15.3	15.3	2.5	15.3	27.9
949H	852H	III	37,500	0.0	0.0	0.0	4.9	32.4	30.2
951H	857H	III	37,500	0.0	0.0	0.0	4.9	9.6	26.3
830H	853H	II	37,500	0.0	2.6	0.0	2.8	28.3	14.3
829H	752H	II	37,500	0.0	0.0	0.0	3.5	12.8	20.9
			Mean	1.7	3.5	3.1	3.7	19.3	24.0

[a] Given 2% cocoanut oil per feeding.
[b] Given lecithin from the 6th day.

Serious scours developed on the third day. Three of the calves died, two on the seventh day and one on the eleventh day. The calf that died on the eleventh day was from a cow that received both vitamin A and lecithin, and at the time of death this calf had an appreciable liver storage of vitamin A (see calf 831J, table 9). Perhaps the liver storage of vitamin A permitted this calf to endure the rigors of the diet longer than those which died on the seventh day. The other two calves, 917J and 950H, were given 2 per cent cocoanut oil and were able to survive. These calves also scoured from the third to the tenth day, and vitamin A absorption, as indicated by the low blood level, was poor.

TABLE 9

The effects of feeding colostrum, skim milk plus vitamin A and skim milk plus vitamin A plus lecithin on the storage of vitamin A in the liver of calves at 21 days of age

Calf no.	Dam no.	Dam group	Wt. of the liver (g.)	Vitamin A (γ/g.)	Total vitamin A (γ)
Calves from Group A (colostrum-fed)					
819J	866J	I–A	519.6	7.2	3,724.0
820J	873J	I–A	526.8	8.7	4,630.0
832J[a]	855J	III	608.0	53.0	32,244.0
Calves from Group B (skim milk plus vitamin A)					
825H[b]	851H	I–A	988.0	0.15	149.0
946J[b]	812J	II	466.5	11.8	5,524.0
831J[c]	854J	III	453.8	56.5	25,570.0
Calves from Group C (skim milk plus vitamin A plus lecithin)					
823J	711J	II	549.0	35.4	19,470.0
830H[a]	853H	II	1124.0	69.0	77,556.0
829H	752H	II	953.0	36.6	34,900.0

[a] The dams got vitamin A or vitamin A plus lecithin for 8 weeks prepartum.
[b] Died on the 7th day.
[c] Died on the 11th day.

The low level of blood vitamin A in the group receiving skim milk plus vitamin A indicates poor vitamin A absorption on this type of diet. When lecithin was added along with the vitamin A, the blood levels were comparable to those of the colostrum-fed group. These results again indicate that soya lecithin enhances the absorption of vitamin A. There were a few mild cases of scours among the calves in this group, but, in general, they did quite well and were comparable to the colostrum-fed calves in rate of growth and general appearance.

It has been suggested that colostral vitamin A may be superior in the nutrition of newborn calves to the vitamin A of fish-liver oil or other concentrated sources (9). The data presented herein provide evidence that the higher concentration of lecithin present in colostrum may be partially responsible for the better absorption and utilization of colostral vitamin A.

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An attempt was made to start calves on a skim milk ration plus coconut oil (two calves) and a skim milk ration plus lecithin (two calves). These attempts failed and all the calves died on the fourth day following birth.

The results of this phase of the investigation show that unless adequate quantities of vitamin A or its precursor are present in the ration and unless favorable circumstances for vitamin A absorption are provided, the animal will quickly succumb to vitamin A deficiency, even when there is considerable liver storage.

Limited data on the liver storage of vitamin A determined on the twenty-first day are presented in table 9. Here again it will be noted that the calves fed the vitamin A plus lecithin had appreciably higher liver storages. While the data cannot provide conclusive proof that vitamin A storage is greater when vitamin A and lecithin are fed, rat data (31) have proved this point conclusively.

Previous workers (12) have shown that the feeding of vitamin A in capsules resulted in increased liver storage. The results of the present investigation provide evidence that the storage will be increased still further if lecithin is fed along with vitamin A.

SUMMARY

Twenty-six healthy pregnant dairy cows of the Jersey and the Holstein breeds were divided into three dietary groups approximately 30 days prior to parturition. Each group received the basic herd ration. Group I, consisting of ten cows, was again subdivided into two groups; Group I–A received no supplement and Group I–B the herd ration plus 10 g. of soya-lecithin daily. Each of the eight cows in Group II was given one million I.U. of vitamin A (fish-liver oil) daily. Each of the eight cows in Group III was fed one million I.U. of vitamin A and 10 g. of lecithin daily. The supplements were continued up to the seventh day following parturition. Assays of blood vitamin A and carotene and of milk vitamin A, carotene and lecithin were made at intervals up to 21 days postpartum. Blood plasma vitamin A and carotene were determined in all calves, and representative animals were sacrificed at birth to determine vitamin A liver storage.

At parturition the plasma vitamin A level in the control cows fell almost to half of the 4 weeks prepartum level. The level in the cows fed vitamin A supplements remained fairly high, especially for the cows fed lecithin plus vitamin A, indicating that lecithin enhanced the absorption of vitamin A. There was no significant effect of feeding lecithin without vitamin A, although the decrease in blood carotene following parturition appears to be about 10 per cent less when lecithin is fed.

Blood plasma carotene was depressed in both vitamin A supplemented groups. However, when vitamin A was fed for a longer period, the carotene level was depressed still further in the cows fed lecithin along with vitamin A. These limited data indicate that lecithin enhanced the action of vitamin A in depressing the carotene level.

The vitamin A in the colostrum of cows fed vitamin A was greater than that of the control group; when both lecithin and vitamin A were fed, the colostral vitamin A at the first milking was approximately double that of the vitamin A supplemented cows. This shows that lecithin, when added to vitamin A, increased the transmission of colostral vitamin A. The transmission of vitamin A and carotene in milk closely followed the trend found in the blood plasma.

The lecithin content of milk was highest when lecithin was fed to the cows along with vitamin A, and a higher level was maintained in the normal milk. Feeding lecithin without vitamin A had no effect on the transfer of lecithin to milk. When both vitamin A and lecithin were fed, the lecithin of the colostrum and milk was increased.

The blood plasma vitamin A level in the newborn calf was highest and the plasma carotene level was the lowest in the calves from dams fed both lecithin and vitamin A.

The total liver storage vitamin A in the newborn calves from the control group was low (190 γ); it was 5,910 γ in the vitamin A supplemented group and 11,722 γ in the vitamin A plus lecithin supplemented group. Thus, the addition of lecithin to the vitamin A supplement remarkedly increased the liver storage.

Three groups of five calves each were fed from birth to 7 days of age as follows: Group A, colostrum; Group B, skim milk plus the same daily quantity of total vitamin A consumed by the calves in the colostrum group (25,000–37,500 I.U. of vitamin A); and Group C, skim milk plus the same quantity of vitamin A and the same quantity of lecithin (3–4.5 g.) available in the colostrum given to Group A.

Every calf in Group B developed serious scours from the third day. Two of them died on the seventh day and a third one on the eleventh day. Their blood plasma vitamin A level was much below that of the colostrum-fed calves. All the calves in Groups A and C grew quite well with slight evidence of digestive disturbance. Blood plasma levels in Group A and C were almost identical, showing the ability of lecithin to increase absorption and utilization of vitamin A.

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LECITHIN:CHOLESTEROL ACYLTRANSFERASE: EFFECTS OF SUBSTRATE COMPOSITION UPON ENZYME ACTIVITY*

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SUMMARY

1. The action of purified lecithin:cholesterol acyltransferase against synthetic dispersions of lecithin and cholesterol in the presence of proteins isolated from high density lipoprotein was investigated. Maximal enzymatic activity was obtained at a lecithin/cholesterol molar ratio of 4, in the presence of a specific protein cofactor.

2. The optimal substrate ratio was not changed by addition of a protein component of high density lipoproteins which inhibited enzyme activity.

3. Cholesterol esters, at proportions similar to those present in high density lipoproteins, reduced enzyme activity to about 20% of that obtained in the presence of only cholesterol and lecithin as lipid components. This lower rate was similar to that found using the native lipoprotein.

4. Triglyceride inhibited the enzyme under the same conditions, but to a lesser extent than did cholesterol ester.

5. Lysolecithin reduced enzyme activity only in the absence of albumin.

INTRODUCTION

Lecithin:cholesterol acyltransferase catalyses the formation of cholesterol esters and lysolecithin from lecithin and cholesterol in serum high density lipoproteins[1]. When highly purified from human plasma, the enzyme acts on high density lipoprotein lipids or synthetic lipid mixtures only in the presence of high density lipoprotein protein[2]. The cofactor activity is associated with only one of the polypeptide species of the lipoprotein[3]. The other major protein species present in high density lipoprotein inhibited enzyme activity. Several other polypeptides which are minor components of high density lipoprotein were inactive as cofactors or inhibitors.

In addition to the substrate lipids, lecithin and cholesterol, high density lipoprotein lipid contains substantial amounts of cholesterol esters, principally cholesterol

* A preliminary report of this research was given at the American Heart Association Scientific Sessions, Anaheim, Calif., November 9, 1971.

linoleate and oleate[1] together with small amounts of triglyceride. Only small amounts of lysolecithin are present. The bulk of lysolecithin in the plasma exists as a complex with albumin. In the present study, the molecular basis of the preference for high density lipoprotein as the natural substrate for lecithin:cholesterol acyltransferase was considered in terms of the polypeptide and lipid moieties of the substrate. The effects of the several high density lipoprotein constituents on the acyltransferase activity were investigated in systems containing the purified enzyme and synthetic dispersions of different protein and lipid compositions.

MATERIALS AND METHODS

High density lipoprotein was separated from human serum by flotation within the density range 1.085–1.195 g/ml, delipidated with ethanol and diethyl ether, and the component proteins of the lipid-free apoprotein separated by DEAE-cellulose column chromatography, as previously described[4]. Each protein gave a single band on polyacrylamide-gel electrophoresis. Assays of aminoacid composition, and N- and C-terminal amino acids were in agreement with previous results[4,5]. The protein with cofactor activity against lecithin:cholesterol acyltransferase had N-terminal aspartic acid, and contained no isoleucine, cysteine or cystine. The protein inhibitor of the enzyme activity showed a blocked N-terminus, and was devoid of histidine, arginine, tryptophan and cysteine. Several minor high density lipoprotein protein components, with compositions corresponding to those of very low density lipoprotein polypeptides[5], were also identified.

Lipids were dispersed in 5 mM 2-mercaptoethanol in distilled water[3], by sonication of 1 min at 75 W, at 40–45 °C, using the standard probe of a Branson sonifier (Heat Systems, Melville, N.Y.). After addition of high density lipoprotein protein, the mixture was further sonicated under the same conditions for three 1-min periods. Further sonication for up to 5 min neither increased nor decreased the enzyme activity shown against the lipids. Under these conditions, no detectable lysolecithin was produced from the phospholipid component, as shown by thin-layer chromatography after sonication, using Silica gel G plates developed in chloroform–methanol–water (65:35:4, v/v/v). The other sonicated lipid components were also unchanged in chromatographic properties[6]. In all experiments, triolein, cholesterol oleate, cholesterol linoleate, and cholesterol (Sigma Chemical Company, St. Louis) were more than 99% pure as shown by thin-layer chromatography in the laboratory[6]. Egg lecithin (more than 99% pure) was the gift of Dr R. J. Havel.

Lecithin:cholesterol acyltransferase was purified from human plasma by density-gradient ultracentrifugation followed by column chromatography on hydroxyl apatite, as previously described[2].

The product was purified 2500–3000-fold in 10–20% yield. It showed less than 2% of maximal activity against high density lipoprotein lipids in the absence of the high density lipoprotein-protein moiety. The purified enzyme was assayed against high density lipoprotein or synthetic lipid-protein complex, equilibrated with [^{3}H]-cholesterol(Amersham–Searle, Chicago, Ill.)[7].

Production of radioactive cholesterol esters was linear for at least 12 h against the synthetic lipoprotein substrates. Radioactive cholesterol esters were separated by thin-layer chromatography on silicic acid plates[2,7]. Correction was made for in-

complete recovery of labeled cholesterol ester (88–91% in these experiments), and for quenching by use of a [^{3}H]toluene internal standard. Enzyme activity has been expressed in units of nmoles cholesterol esterified per h at 37 °C.

RESULTS

Effects of cholesterol esters on lecithin:cholesterol acyltransferase activity

All references to the composition of substrates refer to the proportions of components initially present in the substrate mixture. Enzymatic esterification of cholesterol was less than 5% initial substrate present.

The acyltransferase activity on dispersions of lecithin and cholesterol, in the presence of the activator protein of high density lipoprotein[3], was investigated over a range of lecithin:cholesterol molar ratios from 0.2 to 30.0. Maximal activity was obtained at about 4:1 (Fig. 1, upper line). Activity was also determined in the presence of cholesterol oleate over the same range of phospholipid: cholesterol proportions, using a cholesterol ester:cholesterol molar ratio of either 2:1 or 8:1 (Fig. 1). At each concentration of cholesterol ester, a lecithin:cholesterol molar ratio of 4 was optimal for esterification of cholesterol by the enzyme. As shown, cholesterol ester greatly reduced the rate of the enzyme activity. Maximal acyltransferase activity, in

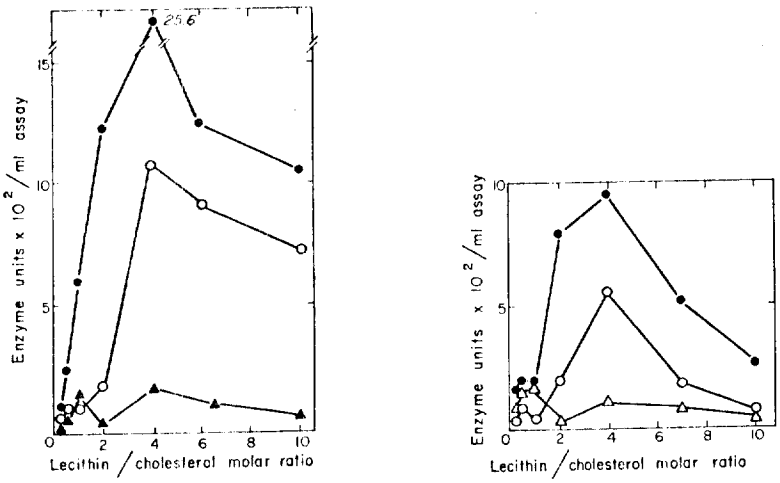


Fig. 1. Effects of added cholesterol oleate on lecithin: cholesterol acyltransferase activity in the presence of cofactor protein. Dispersions of lecithin and cholesterol in 5 mM 2-mercaptoethanol, in the molar proportions shown on the abscissa, together with cholesterol oleate as shown, were sonicated with cofactor protein in the ratio by weight cholesterol/protein 1:1.5. 0.25 ml of lipid–protein mixture (containing 3 μg cholesterol) was mixed with 0.04 ml of 10% (w/v) albumin, pH 7.2, and 0.05 ml of [^{3}H]cholesterol–albumin complex[7] ($9.0 \cdot 10^5$ cpm/ml) + 0.05 ml 0.145 M NaCl. After incubation for 30 min at 37 °C, 10 μl of purified enzyme[2] was added. Incubation was for 8 h at 37 °C. ●—●, lecithin–cholesterol only; ○—○, lecithin–cholesterol + 5 μg cholesterol esters per assay; ▲—▲, lecithin–cholesterol + 20 μg cholesterol esters per assay.

Fig. 2. Effect of added cholesterol ester upon lecithin: cholesterol acyltransferase activity in the presence of cofactor protein + inhibitor protein. Assay conditions were as Fig. 1, except that lipid mixtures were sonicated with cofactor protein + inhibitor protein in the proportions by weight 5:1. Assays were for 8 h at 37 °C. ●—●, lecithin–cholesterol only; ○—○, lecithin–cholesterol + 5 μg cholesterol esters per assay; △—△, lecithin–cholesterol + 20 μg cholesterol esters per assay.

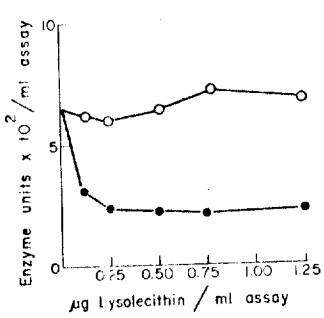
the absence of any cholesterol ester, was considerably greater than with extracted high density lipoprotein lipid mixture with activator protein, or with native high density lipoprotein. Activity against the intact lipoprotein was about 20% (15–40%, 4 experiments) that of maximal activity against phospholipid and cholesterol only. Similar results were obtained using cholesterol linoleate.

Effect of inhibitor protein on lecithin:cholesterol acyltransferase activity

Addition of the inhibitor protein of high density lipoprotein to the lecithin cholesterol acyltransferase reaction mixture as described above, caused a substantia decline in enzyme activity, such that with a mixture of 5 parts activator/1 part inhibitor protein by weight, activity was reduced by about 25–50% over the range c lecithin:cholesterol proportions tested[3]. However, the optimal molar ratio of substrates, and the overall shape of the activation curve, were not greatly differen (Fig. 2). Inhibition by this protein was similar in extent over the whole range of substrate ratios tested. However, at the reduced level of activity obtained at high concentrations of cholesterol ester, peaks were obtained at molar ratios of both 1 and 4

Effects of lysolecithin upon lecithin:cholesterol acyltransferase activity

The effects of the other product of the lecithin:cholesterol acyltransferase reaction, lysolecithin, upon enzyme activity was investigated. It makes up about 2% of high density lipoprotein phospholipid[8]. Lysolecithin in the proportions shown in Fig. 3, was incorporated into lipid dispersions which contained cholesterol together with lecithin and the cofactor protein. This substrate was then incubated with purified enzyme in the presence or absence of albumin. As shown, lysolecithin was significantly inhibitory in the absence of albumin, such that at a concentration of 0.25 μg lysolecithin, 7.5 μg cholesterol, 60 μg lecithin by weight, activity was reduced by about one half. Addition of lysolecithin in this system, up to ten times this amount, did not



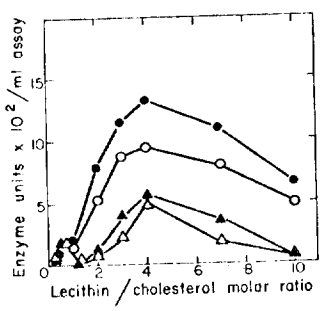


Fig. 3. Effect of lysolecithin upon lecithin:cholesterol acyltransferase activity. Dispersions of lecithin and [^{3}H]cholesterol in the molar proportions 4:1 were mixed with lysolecithin and cofactor protein as in Fig. 1. 0.25 ml of lipid–protein sonicate (containing 3 μg cholesterol, 4.5 μg acyltransferase cofactor protein and lysolecithin as shown on the abscissa) were mixed with 0.145 M NaCl (0.14 ml), or 0.145 M NaCl–albumin (4 mg in 0.14 ml) + 10 μl of purified lecithin:cholesterol acyltransferase. Incubation was for 10 h at 37 °C. ●—●, without albumin; ○—○, albumin (1%, w/v, final concentration).

Fig. 4. Effect of triolein upon lecithin:cholesterol acyltransferase activity. Dispersions of cholesterol + lecithin + cofactor (3 μg cholesterol + 5 μg cofactor) (●—●), cholesterol + lecithin + triolein + cofactor (3 μg cholesterol + 5 μg cofactor + 10 μg triolein) (○—○), cholesterol + lecithin + cofactor as above + 1 μg inhibitor protein (▲—▲) or cholesterol + lecthin + triolein (10 μg) + cofactor (5 μg) + inhibitor protein (1 μg) were prepared (△—△) over the range of lecithin:cholesterol molar ratios shown. Assay conditions were as in Fig. 1.

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further reduce activity. However, the addition of albumin in amounts present in the regular assay medium (1.0%, w/v) completely relieved this inhibition.

Effects of triglyceride upon lecithin:cholesterol acyltransferase activity

Triolein was incorporated into lipid dispersions with cholesterol and lecithin, and sonicated either with activator protein alone, or with a mixture of activator and inhibitor proteins (Fig. 4). Triglyceride was less effective than cholesterol ester as an inhibitor of the enzyme. Activity was decreased by about 10% by triolein in the presence of activator and inhibitor proteins, at a triglyceride:cholesterol molar ratio of 2:1, whilst cholesterol ester under the same conditions inhibited at least 50%. Addition of inhibitor protein reduced activity to a similar extent in the presence or absence of triglyceride.

DISCUSSION

The rate of esterification of cholesterol, in a system containing purified acyltransferase, synthetic mixtures of lipids in proportions present in high density lipoprotein, and high density lipoprotein protein, is similar to that obtained with the native lipoprotein substrate[2,3]. In the present study, the effects of lipid and protein composition upon the rate of enzyme activity have been investigated. The detailed local disposition of lipids on the surface of either high density lipoprotein, or synthetic lipoprotein complexes, remains unknown, as therefore does the effective substrate concentration for enzyme activity. The above results by comparison of the overall lipid composition of both substrates, suggest that the nature of acyltransferase activity against a synthetic substrate containing the major high density lipoprotein lipid and protein components, is quite similar to that obtained with the native lipoprotein of similar overall composition. As shown in Fig. 1, the proportion of the two lipid substrates, markely affected the rate of transesterification. The optimal lecithin:cholesterol molar ratio was about four, under most conditions, which is in accord with previous experiments[9,10] in which lipoprotein-depleted plasma ($d > 1.21$ g/ml infranatant) was the enzyme source. Since this infranatant already contained cofactor protein, (particularly in the density fraction 1.21–1.25 g/ml)[2] the results of these authors correspond to the upper line of Fig. 1. This optimal ratio is similar to the proportions of lecithin and cholesterol in high density lipoprotein[8]. The other phospholipids present in the lipoprotein, principally sphingomyelin and phosphatidylethanolamine are not appreciably attacked by the enzyme[9].

The optimal phospholipid:cholesterol ratio of the enzyme activity may reflect both physical and biochemical requirements for effective enzyme activity. In artificial mixtures of lecithin and cholesterol in which lecithin predominates, studies of the NMR spectrum indicate that the cholesterol molecules are not randomly distributed but may be clustered in regions of locally high concentration in the lecithin film[11]. Such a lipid surface with an overall lecithin:cholesterol ratio well above unity, might contain significant proportions of regions showing local equimolar stoiochiometry, as required for the transesterification reaction. It is an attractive possibility that such clusters of cholesterol and lecithin molecules may be present in the surface of the native lipoprotein. Sonicated equimolar dispersions of cholesterol and lecithin shown impeded movement of the fatty acid side chains of the lecithin moiety[12]. This effect may be involved in the low activity of the enzyme against such substrate mixtures (Fig. 1).

At high cholesterol ester concentrations, especially in the presence of the inhibitor protein, a small peak of activity is obtained at a phospholipid:cholesterol equimolar ratio (Fig. 2). The physical forces active in such complex mixtures are not yet well understood. Addition of cholesterol ester substantially reduced lecithin:cholesterol acyltransferase activity against phospholipid–cholesterol mixtures, irrespective of the molar ratio involved.

As shown in Fig. 3, lysolecithin, a product of the acyltransferase reaction, was inhibitory at low concentrations, but albumin completely removed this inhibition. Lysolecithin forms a stable complex with albumin[13] and is only a minor component of plasma high density lipoproteins[8]. Thus it may not be an important factor in determining the rate of cholesterol esterification in the plasma. Triglyceride comprises about 10% by weight of high density lipoprotein lipid, but, as shown in Fig. 4, when this proportion of triglyceride is present in the synthetic substrate, it is less inhibitory on a molar basis than cholesterol ester. Addition of the acyltransferase inhibitory protein which is present in high density lipoprotein decreased cholesterol esterification whether or not cholesterol ester or triglyceride was present (Fig. 2). The mechanism of such inhibition is not yet known. No evidence at present suggests a common mechanism of inhibition by protein and lipids. The results of studies on the fatty acid composition of high density lipoproteins[8], and the plasma of the acyltransferase-negative individuals[14], suggests that most high density lipoproteins cholesterol ester is derived from transesterification by the acyltransferase of endogenous lecithin and cholesterol. The present results therefore suggest that the major factors in determining the rate of the enzyme activity against its plasma substrate, may be the content of the cholesterol ester product of the reaction, the proportions of lecithin and cholesterol, and the proportions of the activator and inhibitor proteins.

ACKNOWLEDGEMENTS

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THE SPONTANEOUS DECOMPOSITION OF LECITHIN.

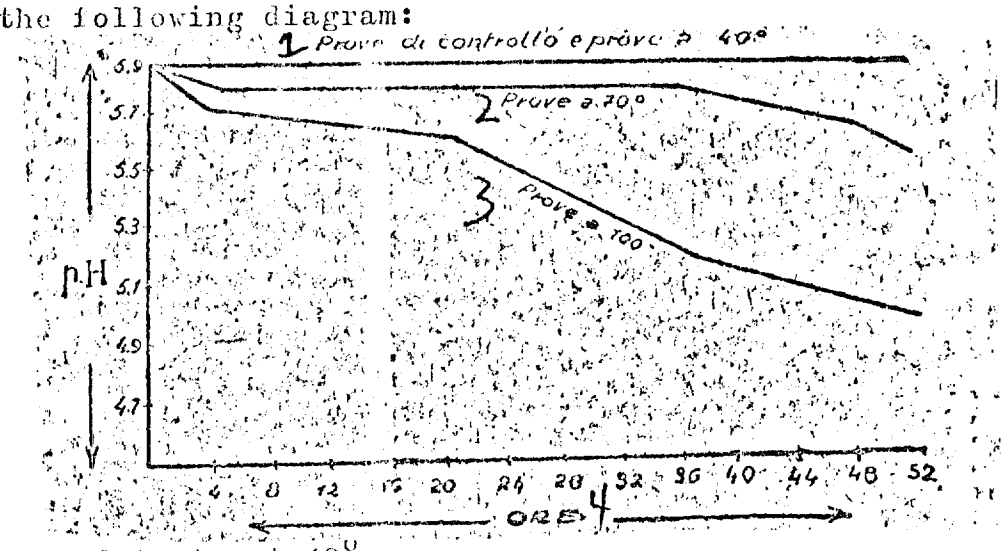
I demonstrated in an earlier comment(1) that lysocithin is obtainable from lecithin by means of prolonged and cautious heating, excluding air contact and under aseptic conditions. The procedure was carried out with lecithin from egg yolk, suspended in water, sealed in vials, and maintained in part at a temperature of 40°, in part at a temperature of 70°, while the rest was kept at room temperature. Periodic pH control with the potentiometer showed the progress of the phenomenon; I refer in this respect to the diagrams published in the original work. When identical experiments were performed with a mixture of lecithin and cholesterin ethers (SERONO Bioplastine), I demonstrated that cholesterin--besides inhibiting the hemolytic action of the forming lysocithin--likewise inhibited the decomposition of lecithin itself.

Recently PIGHINI and DELFINI (2) confirmed my results by other means in an interesting study on the lysocithins of the brain. They express doubts, however, whether the decomposition of lecithin in my experiments should be regarded as simple, spontaneous hydrolysis accelerated by heat, rather than as a result of phosphatase traces (lecithinase) which were detected by them in egg yolk and which could have passed from the latter into my preparations.

While specific ferments for the formation of lysocithin are undoubtedly present in egg yolk as well as in various tissues of the organism, I neverthless believe that the presence of said ferments in my preparations can be definitely excluded on the basis of the experiments and arguments reported below.

When intending to admit the presence and effect of said ferments, test temperatures (40° to 70°) are primarily taken into consideration and it would be necessary to admit a very high temperature optimum for the ferments in question, at least near 70°, in view of the intensified decomposition found in tests at 70°, as compared with those at 40°. Moreover, I wished to repeat my experiments at (temperatures) up to 100° and therefore divided the lecithin suspension vials into four groups: No. 1. at room temperature; No.2. at 40°; No.3. at 70° and No. 4. at 100°.

As customary, I used the electro-ionometer to follow pH variations as a function of time; the results are reproduced on the following diagram:

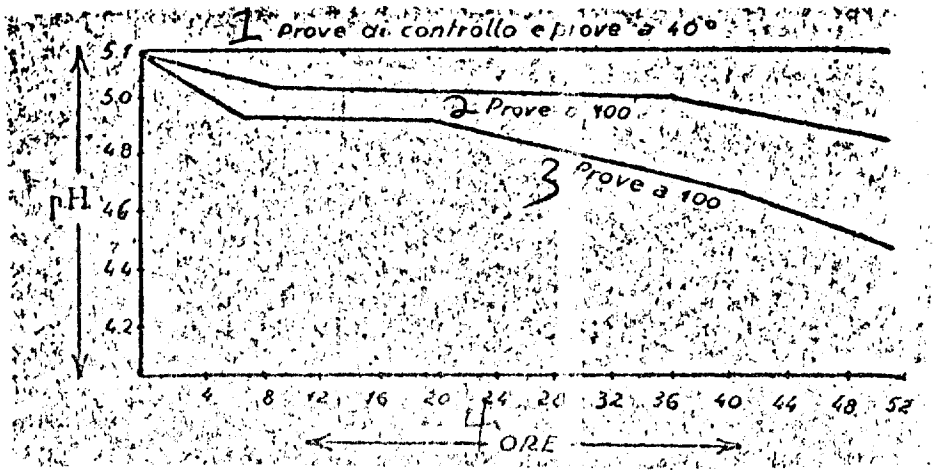


1) Control tests and tests at 40°
2) Tests at 70°
3) Tests at 100°
4) Hours

The examination of the curves showes intensified lecithin decomposition velocity simultaneously with rising temperature, even when the latter is increased to 100°. It would therefore be necessary to admit that the temperature optimum is above 70°, even very near 100°, for the assumed ferment. In my opinion, this is inadmissible. The lecithinase present in snake venoms indicates a slightly higher temperature optimum which however does not exceed 60 to 70°. At 80°, the lecithinase effect is substantially reduced; it is near-zero at 100°, as I was able to determine personally when researching the effect of Lachesis and Crotalus venom in egg yolk at various temperatures.

I applied another method to investigate the causes of the phenomenon; this involved subjecting lecithin to preliminary ultraviolet irradiation. CONTARDI and LATZER (3) did in fact demonstrate that snake and wasp venom ferments lose their effectiveness following u.v. irradiation. Accordingly, in view of the evident analogy between the ferments of said venoms and the ferments supposedly present in the egg yolk and supposedly transferred from the latter into the lecithin, it should result here that irradiated lecithin is no longer adequate for decomposition with lysocithin formation. On the other hand, the experiments reported below indicate that previously irradiated lecithin shows a behaviour approximately similar to the behavior of non-irradiated lecithin when subjected to the prolonged effect of various temperatures.

For this experiment the lecithin, in ether solution, was placed into a quartz sphere and subjected to the effect of a mercury lamp for two hours, while frequent agitation was performed. The effect of atmospheric oxygen had to be carefully avoided, since it decomposes lecithin thoroughly in ultraviolet light. After said treatment the ether was eliminated and the lecithin was suspended in water, then filled into vials. The latter were kept under conditions similar to those in earlier experiments. The attached diagram shows the pH variations as the decomposition proceeds; it is readily comparable with the previous diagram.



1) Control tests and tests at 40°
2) Tests at 100
3) Tests at 100
4) Hours

I intended to convince myself subsequently that the decomposition process in experiments at 100° is identical with the decomposition process in tests at 70°. For this purpose I separated the lysocithin from the vials maintained at 100° with the same method which I had applied on another occasion. This product showed definite evidence of its hemolytic action and when analyzed, a phosphorus content of 5,8 % was found.

These two arguments, i.e. that no temperature optimum exists or at least, that it is too high for an enzyme, and the insensibility to u.v. light, actually demonstrate that lecithin decomposition, under the conditions investigated by me, is not ascribable to the effect of any ferment, but represents merely a natural decomposition process, accelerated by heat. I wish to point out in this context that the decomposition, even though very slowly, takes place at room temperature as well. Moreover: what else is fermentative action but the acceleration of an already existing phenomenon? Said spontaneous tendency of lecithin to dissociate can be a cause of the physiologic lecithin action cycle, as admitted by SERENO (4) and elaborated in one of his recent works.

In conclusion, I refer to a fact which emerged initially in the course of my research work; should these investigations yield any result of interest, they will be the subject of my next publication.

I am analyzing a set of Bioplastine which was stored for more than a year in pasteurizers, at a temperature of 70° maintained during eight hours each day.

The first result determined by me was a remarkable shortage of lysocithine and the abundant presence of phosphoric acid and free choline (phosphate of choline). This seems to indicate that the dissociation process moves towards the complete destruction of the lecithin and that lysocithin merely defines a transition.

The spontaneous decomposition of lecithin would then be comparable to the effect of wasp venom (phosphatase B, according to BELFANTI (5)); nevethless, I am unable as yet to confirm anything regarding this argument with precision.

Summarizing:

I believe that the objection of PIGHINI and DELFINI, i.e. that the decomposition of lecithin under the conditions investigated by me is ascribable to enzymatic action, has been overruled. The reasons for the above are:

No. 1. - The lack of a temperature optimum at which the reaction velocity increases continuously, simultaneously with the increase of the temperature as such.

No.2. - The fact that lecithin irradiated with ultraviolet light decomposes in the same manner as does the non-irradiated lecithin.

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Sulla decomposizione spontanea della lecitina

Dott. A. FIORI

In una mia nota precedente (1) dimostrai che si può ottenere lisocitina dalla lecitina per prolungato e cauto riscaldamento fuori del contatto dell'aria ed in ambiente asettico. L'operazione era eseguita su lecitina da tuorlo d'uovo sospesa in acqua, chiusa in fiale, e mantenuta in parte a 70°, in parte a 40° ed il resto a temperatura ambiente; il periodico controllo potenziometrico del pH mostrava il procedere del fenomeno ed a questo proposito rimando ai grafici pubblicati nel lavoro originale. Eseguendo le stesse prove su di una miscela di lecitina ed etere di colesterina (Bioplastina Serono) dimostrai che la colesterina, oltre che ostacolare l'azione emolitica della lisocitina formatasi, ostacolava anche la stessa decomposizione della lecitina.

Recentemente Pighini e Delfini (2) in un interessante lavoro sulle lisocitine del cervello confermano per altre vie i miei risultati ma espongono il dubbio che la decomposizione della lecitina nelle mie esperienze non debba considerarsi una semplice idrolisi spontanea affrettata dal calore, ma bensì come l'opera di tracce di una fosfatasi (lecitinasi) da loro riscontrata nel tuorlo d'uovo ed eventualmente passata da questo nei miei preparati.

Orbene, senza mettere in dubbio la presenza così nell'uovo come nei vari tessuti dell'organismo di fermenti specifici per la formazione di lisocitina, io ritengo di poter escludere assolutamente la presenza di questi stessi fermenti nei miei preparati e ciò in base alle esperienze e considerazioni quì sotto riportate.

In primo luogo, volendo ammettere la presenza e l'azione di questi fermenti, e considerate le temperature saggiate (40°-70°), bisognerebbe ammettere per i fermenti in questione un optimun di temperatura assai alto, per lo meno assai vicino ai 70° data la maggiore decomposizione rivelatasi nelle prove a 70° in confronto con quelle a 40°. Del resto io ho voluto ripetere le mie esperienze inoltrandomi fino ai 100° e cioè ho diviso le fiale di sospensione di lecitina in quattro gruppi e li ho mantenuti: il 1° a temperatura ambiente, il 2° a 40°, il 3° a 70 ed il 4° a 100°.

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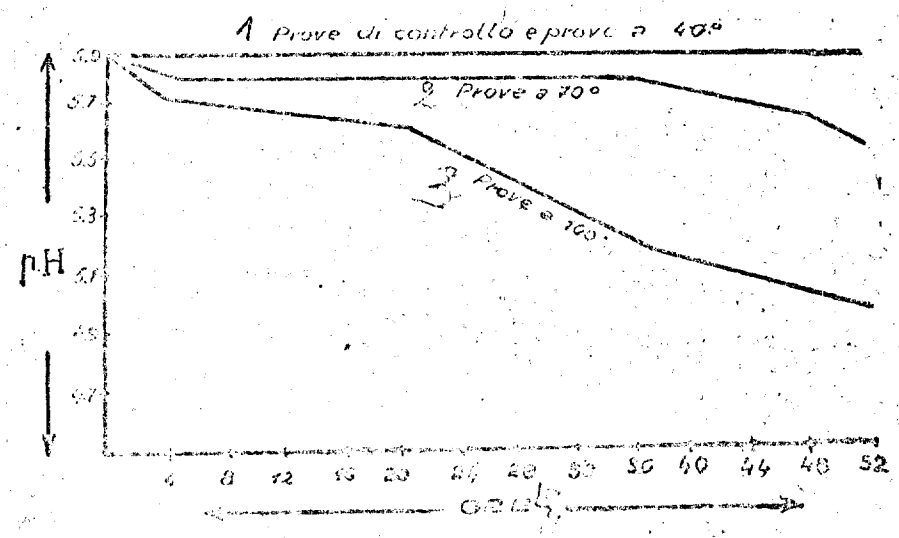
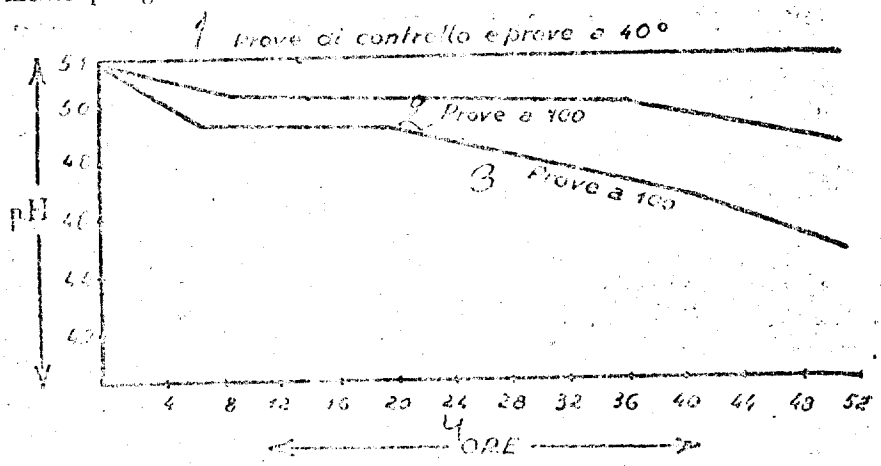
Ho seguito come al solito, coll'elettroionometro le variazioni del pH in funzione del tempo e le ho riprodotte col grafico qui unito.



L'esame delle curve mette in evidenza l'aumento della velocità di decomposizione della lecitina con l'aumento della temperatura anche portando questa a 100° e bisognerebbe perciò ammettere per questo supposto fermento addirittura un optimum di temperatura non solo superiore ai 70° ma bensì assai vicino ai 100°. Ciò io ritengo inammissibile. La lecitinasi esistente nei veleni dei serpenti mostra bensì un optimum di temperatura discretamente elevato ma non superiore ai 60-70° e l'azione lecitinasica a 80° è assai ridotta e a 100° quasi nulla; come ho potuto vedere io stesso ricercando l'azione del veleno di Lachesis e di Crotalus su tuorlo d'uovo a diverse temperature.

Un altra strada che ho battuto per la ricerca delle cause del fenomeno è stata quella di sottoporre la lecitina ad una preliminare irradiazione con raggi ultravioletti. Infatti Contardi e Latzer (3) hanno dimostrato che i fermenti dei veleni di serpenti e di vespe dopo irradiazione con luce ultravioletta perdono affatto la loro azione; orbene, data l'analogia evidente tra i fermenti di questi veleni ed il fermento supposto presente nel tuorlo d'uovo e da questo passato nella lecitina, dovrebbe aversi qui che la lecitina irradiata non è più passibile di decomposizione con formazione di lisocitina. Viceversa le prove qui sotto riportate mostrano che la lecitina preventivamente irradiata si comporta, sotto l'azione prolungata delle varie temperature, all'incirca come la lecitina non irradiata.

Per questa esperienza la lecitina, in sol, eterea fu posta in pallonci-no di quarzo, sotto l'azione della lampada a mercurio per due ore aven-do cura di agitare frequentemente. Dovetti evitare con cura l'azione dell'ossigeno atmosferico che in presenza di luce ultravioletta decom-pone profondamente la lecitina. Dopo questo trattamento l'etere fu eliminato e la lecitina fu sospesa in acqua, messa in fiale e queste pos-nelle stesse condizioni delle prove precedenti. Il grafico qui unito me-stra il variare del pH col procedere della decomposizione ed è perfetta-mente paragonabile col grafico precedente.



Mi sono poi voluto assicurare che nelle prove tenute a 100° tanto per la lecitina irradiata quanto per quella normale il processo di de-composizione era identico di quello delle prove a 70° a questo scopo separai la lisocitina dalle fiale mantenute a 100° con lo stesso metodo adottato l'altra volta. Questo prodotto mostrò netta la sua azione emoli-tica ed alla analisi diede un contenuto in fosforo del 5,8 %.

Questi due argomenti, optimum di temperatura non esistente o al-meno troppo elevato per un enzima, insensibilità alla luce ultravioletta, dimostrano appunto che la decomposizione della lecitina nelle condi-zioni da me esaminate non è da ascriversi all'azione di alcun fermento ma è soltanto una decomposizione naturale accelerata dal calore. A questo proposito faccio notare che la decomposizione avviene, sebbene assai lenta, anche a temperatura ambiente: del resto che cosa è l'azione fermentativa se non che l'accelerazione di un fenomeno già esistente di per sè? Questa tendenza spontanea della lecitina a dissociarsi può es-sere una causa del ciclo d'azione fisiologica della lecitina così come è ammesso dal Serono (4) e svolto in un suo recente lavoro.

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Per ultimo voglio accennare ad un fatto che è un primo frutto di ricerche che sto conducendo e che se porteranno a qualche risultato interessante formeranno argomento di una mia prossima nota.

Sto analizzando una partita di Bioplastina che da più di un anno ho mantenuto nei pastorizzatori a una temperatura di 70° per otto ore al giorno.

Come primo risultato ho osservato una scarsità considerevole di lisocitina ed una abbondante presenza di acido fosforico e di colina libera (fosfato di colina); ciò atti ad indicare che il processo di dissociazione procede verso la completa distruzione della lecitina e che la lisocitina non è che un termine di passaggio.

La decomposizione spontanea della lecitina sarebbe poi da paragonarsi all'azione del veleno di vespa (fosfatasi *B* secondo Belfanti (5) in ogni modo in questo argomento nulla posso ancora affermare con precisione.

Riassumendo:

Ritengo risolta in senso negativo l'obiezione di Pighini e Delfini che la decomposizione della lecitina nelle condizioni da me esaminate possa ascriversi ad un azione enzimatica; le ragioni che conducono a questo risultato sono:

1° — Mancanza di un optimum di temperatura per cui la velocità di reazione cresce in modo continuo col crescere della temperatura stessa.

2° Il fatto che la lecitina irradiata con luce ultravioletta si decompone nello stesso modo di quella non irradiata.

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THE FORMATION OF LYSOCITHIN DURING SPONTANEOUS DECOMPOSITION OF LECITHIN.

in 1911 DELEZENNE and LEBERT (1) obtained a hemolytic phosphatide through the enzymatic action of cobra venom in egg yolk; they named it lysocithin. in 1914 DELEZENNE and FOURNEAUX (2) demonstrated that the lysocithin molecule corresponds to the lecithin molecule, minus one oleic acid molecule. Subsequently, LEVENE and ROLF (3) separated various lysocithins corresponding to various lecithins described earlier by the same authors, and showed that lysocithins deriving from cephalin exist as well. A distinction was therefore made between lysocithins in lysolecithins and in lysocephalins.

The fact the only oleic acid separates from lecithin was finally questioned by CONTARDI (4), BELFANTI and collaborators; at this time, it is believed that lysocithins containing oleic acid might exist.

BELFANTI (5) detected the presence of free lysocithins in the pancreas and in other organs; as a consequence of this finding, lysocithin assumed special importance in biology and physiology, as proven by BELFANTI and collaborators (6).

To date the only artificial source of lysocithin was the enzymatic decomposition of lecithin , using snake venoms. This method resulted in doubts concerning the toxic effect of sub-dura lysocithin injection. While VOLTERRA and SPINELLI (7) questioned whether the violent toxic effect is due to traces of cobra venom left as residue with lysocithin, DESSY and LATZER (8) showed in a series of experiments that the toxic effect at issue is merely due to the combination of lysocithin with cerebral matter.

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Lysocithin prepared without the intervention of the toxin would have been needed to avoid doubts of the above type.

Present work was prompted by attempts to obtain such a preparation. The experiments induced me to study the bahavior of lecithin when subjected to a prolonged heat effect, with the exclusion of air access. I soon convinced myself that excessively high temperatures (100° and above) thoroughly decompose the lecithin; I therefore limited my investigations to lower temperatures, prolonging the duration of the heat effect.

The tests were performed as follows: the lecithin, a pure preparation obtained from egg yolk, was suspended in physiologic serum in a sterile environment, then sealed in vials; the vials were divided into three groups and kept under the following conditions:

Group No. I. Submerged in a water bath at 70°. (Due to laboratory requirements, heating took place in daytime only).

Group No. II. The thermostat was maintained at 40°. (Continuous day and night heating).

Group No. III, (control group). Kept at room temperature and in the dark.

Periodical pH determination was electrometrically performed in the watery liequid of a vial for each group, so as to obtain data on the development of the hydrolysis process.

The diagram below (No. I) shows the progress of said acidification process; its velocity increases simultaneously with rising temperatures, and the process develops--to a minor extent--at room temperatures as well.

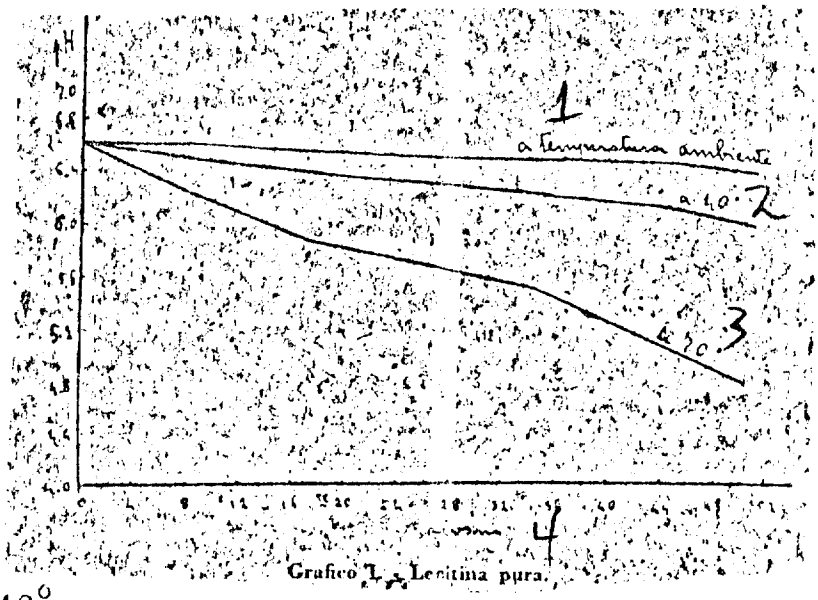

Diagram I. Pure lecithin.

1) at room temperature; 2) at 40°; 3) at 70°; 4) days.

At the start of the experiment the vials showed pH of 6,6. In the vials maintained at 40°, decomposition seemed to be slow and pH dropped to 6 only after two months. On the other hand, the vials maintained at 70° attained pH-4,8 after the same period of time. I then believed it opportune to use hydrolysis products for chemical examination. As a result of heating , the solid phase in these vials remained definitely separated from the liquid phase and formed an infusible block, at 100°, adhering to the walls.

First, I separated the watery liquid in which I immediately detected a slight hemolytic action at the erythrocytes of the rinsed blood; the quantity of the lysocithin dissolved in the watery liquid had to be minimal, since I was unable to separate it with any method.

After dissolving the solid part in alcohol, I partially used this solution to obtain the fatty acids released by lecithin during the formation of lysocithin. For this purpose, I applied treatment with baryte until neutralization occurred; when cooled, I separated the barium soaps which had formed and treated them with hydrochloric acid dissolved in ether; the evaporated ether extract yielded a mixture of fatty acids with:

fusion point	= 44°
saponification count	= 198°
iodine count	= 46,3

The iodine count and the fusion point clearly demonstrate that lecithin did not separate from oleic acid only; this confirms the findings of CONTARDI and of the other authors.

Another part of the alcoholic solution was concentrated to a small volume and then poured into ether; a whitish precipitation separated, which I subjected to centrifugation, rinsed with ether and analyzed.

phosphorus	=	5,2
nitrogen	=	2,3
fatty acids	=	55,7

The theoretical data corresponding to lysocithin are:

phosphorus	=	5,73
nitrogen	=	2,59
fatty acids	=	52,5

Fatty acids separated for saponification of the product with potassium hydrochloride have a

fusion point	=	38 - 40°
saponification count	=	196,6
iodine count	=	75

The well-dried product has the aspect of a yellowish mass and is slightly water-soluble; it is insoluble in alcohol, insoluble in ether and acetone. The watery solution has a very powerful hemolytic capacity.

The above-stated results demonstrate that the obtained substance was a moderately pure lysocithin. I can therefore reach the conclusion that the prolonged heating of lecithin in watery solution, in a sterile environment and excluding air contact results in the formation of lysocithin. I am as yet unable to state whether said genesis is the last, or merely the first step in the complete decomposition process of the lecithin molecule. I believe, however, that the second possibility is more probable, since certain findings seem to confirm my above supposition, i.e. that as the cold-treatment progresses and intensifies, lysocithin can finally be hydrolyzed.

I expect at a later date to be in a position to offer more specific statements on the problem at issue.

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La formazione di lisocitina nella decomposizione spontanea della lecitina

Dott. A. FIORI

Nel 1911 Delezenne e Lebert (1) per azione enzimatica del veleno di cobra sul torlo di uovo ottennero un fosfatide emolitico che chiamarono lisocitina: nel 1914 Delezenne e Fourneaux (2) dimostrarono che la molecola della lisocitina corrisponde a quella della lecitina meno una molecola di acido oleico. In seguito Levene e Rolf (3) separarono varie lisocitine corrispondenti alle varie lecitine che gli A.A. stessi avevano descritto precedentemente e mostrarono che vi sono anche delle lisocitine derivanti dalla cefalina per cui distinsero le lisocitine in lisolecitine ed in lisocefaline.

Il fatto che sia soltanto l'acido oleico a separarsi dalla lecitina è stato ultimamente messo in dubbio da Contardi (4), Belfanti e collaboratori e ormai si ritiene che possano esistere delle lisocitine contenenti l'acido oleico.

Belfanti (5) riscontrò nel pancreas ed in altri organi la presenza di lisocitine libere e con questa scoperta la lisocitina assunse una importanza speciale in biologia e fisiologia posta bene in evidenza da Belfanti e collaboratori. (6).

Finora l'unica sorgente artificiale della lisocitina era la decomposizione enzimatica della lecitina mediante veleni di serpenti; questo metodo diede luogo ad incertezze sull'azione tossica della lisocitina iniettata sotto dura; mentre il Volterra e Spinelli (7) espressero il dubbio che questa forte azione tossica fosse dovuta a tracce di veleno di cobra rimasto insieme alla lisocitina, Dessy e Latzer (8) dimostrarono con una serie di esperienze che detta azione tossica era dovuta soltanto all'unione della lisocitina con la materia cerebrale.

Per evitare dubbi di questo genere, sarebbe stato necessario avere a disposizione della lisocitina preparata senza l'intervento del veleno.

I tentativi di tale preparazione diedero l'inizio a questo lavoro; questi tentativi mi portarono a studiare il comportamento della lecitina sotto l'azione prolungata del calore fuori del contatto dell'aria. Presto mi convinsi che le temperature troppo elevate (100° ed oltre) scompongono

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Since the proportions of Diagram II are similar to those of Diagram I, it becomes immediately evident that the lecithin and cholesterine mixture decomposes much less rapidly under the effect of heat than pure lecithin; in fact, it took 92 days for the mixture to reach pH 4,8 while kept at a temperature of 70°; on the other hand, pure lecithin required 52 days only under identical conditions.

Analoguous reasoning is applicable to the vials maintained at 40° and for those which served for comparison.

Another behavorial difference is the fact that while the serum of decomposing lecithin soon shows hemolytic capacity, the serum of the lecithin-lutein mixture never displays said property. Only when it is dissolved in alcohol and precipitated with ether can a strongly hemolytic precipitate of lysocithin corresponding to the analysis be obtained.

The two behavorial defferences shown by pure lecithin and its mixture with cholesterine under the effect of heat are readily explainable when it is taken into consideration that cholesterine, as known, acts antagonistically to lysocithin and inhibits its toxic effect. In that case, cholesterine could also act as lecithin stabilizer, delaying the decomposition under heat effect, as asserted by SEMONO in 1913.

Summarizing:

1. It was found that, as a result of prolonged heat effect, lecithin in watery suspension, in an aseptic environment and deprived of air contact will decompose , generating fatty acids and lysocithins.

2. The presence of cholesterine ethers delays the dissociating effect.

3. The presence of cholesterine inhibits the hemolytic action of lysocithin forming as a result of lecithin decomposition.

—

The fact detected by me is of special importance regarding the pharmacological properties of preparations based on lecithin.

SERONO (9) who introduced said substance in therapeutics, successfully overcame the obstacle represented by the toxicity of impure lecithin. He neverthless issued repeated warnings against alteration of pure lecithin in a sterile environment without air access (watery suspension in vial). He referred specifically to the formation of acidity evident on litmus paper in preparations which are old or poorly preserved, and showed that the cholesterine ethers act as fixing agents of phosphorated lipoids, preserving the same from the dissociating effect of the mediums containing said phosphorated lipoids (10).

I wished to confirm this by subjecting an emulsion of lecithin and lutein (SERONO Bioplastine) to an examination similar to the reported test. Diagram II indicates accordingly the pH variations of Bioplastine serum maintained under the following three conditions, namely:

1. - heating to 70°;
2. - heating to 40°;
3. - at room temperature and in the dark (control tests).

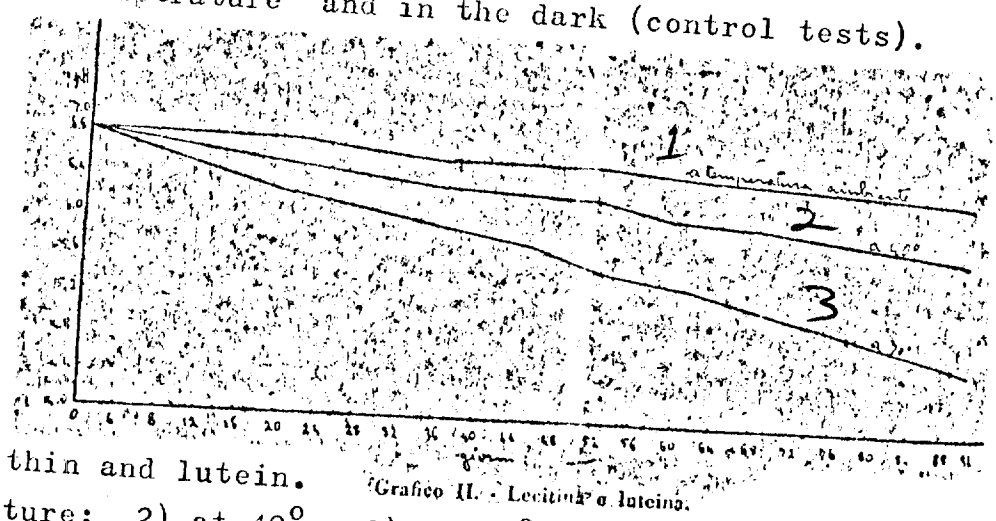

Grafico II. - Lecitina e luteina.

Diagram II. - Lecithin and lutein.
1) at room temperature; 2) at 40°; 3) at 70°; 4) days.

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profondamente la lecitina, e perciò mi limitai all'esame di temperature più basse prolungando più a lungo l'azione del calore.

Le prove furono condotte come segue: la lecitina, preparata pura dal torlo d'uovo, fu sospesa in siero fisiologico in ambiente sterile e chiusa in fiale che vennero divise in tre gruppi mantenuti nelle seguenti condizioni:

I° Gruppo. Immerso in bagno d'acqua a 70°. (Per esigenze di laboratorio il riscaldamento fu solamente diurno).

II° Gruppo. Tenuto in termostato a 40°. (Riscaldamento continuato notte e giorno).

III° Gruppo (di controllo). Tenuto a temperatura ambiente ed al buio.

Di tanto in tanto fu determinato elettrometricamente il pH del liquido acquoso di una fiala per ogni gruppo così da avere un indizio del modo di procedere della idrolisi.

Il grafico che segue (I°) mostra appunto l'andamento di questo processo di inacidimento la cui velocità è maggiore con le temperature più alte e che esiste, sebbene lievissimo, anche a temperature ambiente (prove di controllo).

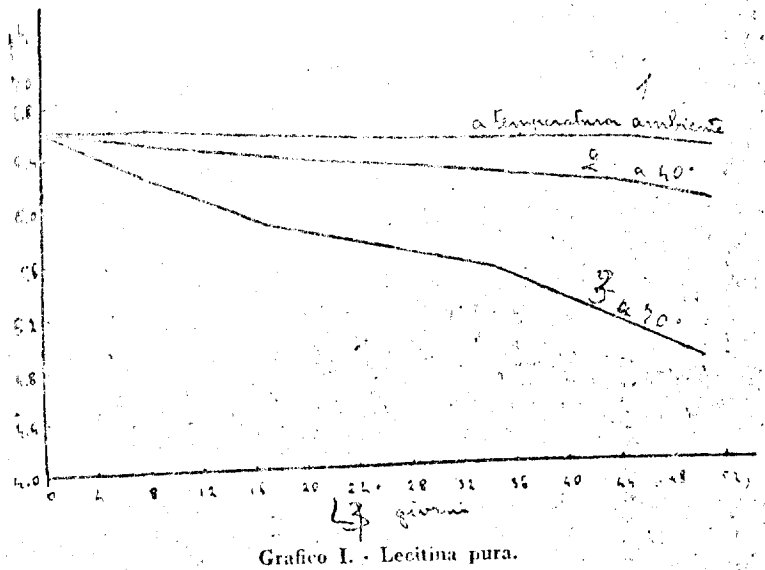

Grafico I. - Lecitina pura.

All'inizio dell'esperienza le fiale avevano pH-6,6; nelle fiale mantenute a 40° la decomposizione apparve lenta, dopo due mesi il pH si era abbassato soltanto fino a 6, invece le fiale mantenute a 70° dopo lo stesso

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tempo, arrivarono al pH-4,8 ed io allora credetti opportuno di utilizzarle per l'esame chimico dei prodotti idrolisi. In queste fiale per opera del riscaldamento la fase solida si era nettamente separata dalla fase liquida ed era rimasta in un blocco infusibile a 100° ed aderente alle pareti.

Prima di tutto separai il liquido acquoso nel quale osservai subito una discreta azione emolitica alle emazie del sangue lavato; la quantità di lisocitina disciolta nel liquido acquoso doveva essere minima, dato che non mi fu possibile separarla in nessun modo.

Disciolta la parte solida in alcool, utilizzai parte di questa soluzione per ottenere gli acidi grassi liberatisi dalla lecitina nella formazione della lisocitina; a tal scopo trattai fino a neutralizzazione con barite, separai a freddo i saponi di bario formatisi, che trattai con acido cloridrico diluito ed etere; l'estratto etereo evaporato mi diede una miscela di acidi grassi aventi:

Punto di fusione	= 44°
Numero di saponificazione	= 198°
Numero di iodo	= 46,3

Il numero di iodo ed il punto di fusione mostrano chiaramente che non si separò dalla lecitina solamente dell'acido oleico e ciò comprova le osservazioni di Contardi e degli altri Autori.

Altra parte della soluzione alcoolica venne concentrata a piccolo volume e poi versata in etere; si separò un precipitato biancastro che separai per centrifugazione, lavai con etere ed analizzai.

Fosforo	= 5,2
Azoto	= 2,3
Acidi grassi	= 55,7

I dati teorici corrispondenti di una lisocitina sono:

Fosforo	= 5,73
Azoto	= 2,59
Acidi grassi	= 52,5

Gli acidi grassi separati per saponificazione del prodotto con potassa caustica hanno:

Punto di fusione	= 38-40°
Numero di saponificazione	= 196,5
Numero di iodo	= 75

Il prodotto ben seccato si presenta come una massa giallognola un po' solubile in acqua, solubile in alcool, insolubile in etere ed in acetone: *la soluzione acquosa presenta un potere emolitico fortissimo.*

Questi risultati dimostrarono che la sostanza ottenuta era lisocitina

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discretamente pura. Dunque posso concludere che il riscaldamento prolungato della lecitina in sospensione in acqua, in ambiente sterile e fuori del contatto dell'aria, porta alla formazione di lisocitine. Cre questa genesi sia l'ultimo oppure solamente il primo gradino di un processo di decomposizione completa della molecola della lecitina non posso ancora dire con precisione, ma ritengo più probabile la seconda possibilità ed anzi alcuni fatti riscontrati mi sembrano confortare questa mia supposizione e cioè che col procedere ed intensificare del trattamento a caldo anche la lisocitina possa venire idrolizzata ulteriormente.

Conto di poter dire in seguito qualche cosa di più preciso su questo argomento.

* * *

Il fatto da me riscontrato assume speciale importanza riguardo alle proprietà farmacologiche dei preparati a base di lecitina.

Il Serono (9) nell'introdurre in terapeutica questa sostanza, superò felicemente l'ostacolo della tossicità della lecitina impura, ma mise tuttavia ripetutamente in guardia contro l'alterarsi della lecitina pura in ambiente sterile e privo di ossigeno (sospensione acquosa in fiale). Egli notò sopratutto il formarsi di una acidità svelabile al tornasole nei preparati di vecchia data e mal conservati e dimostrò che gli eteri di colesterina agiscono come fissatori dei lipoidi fosforati e li proteggono dall'azione dissociante dei mezzi in cui si trovano (10).

A comprova di ciò io ho voluto sottoporre una emulsione di lecitina e luteina (Bioplastina Serono) ad uno studio analogo a quello su riportato. Il grafico II° segna appunto il variare del pH del siero di Bioplastina mantenuto nelle tre condizioni suddette e cioè:

1° - Riscaldamento 70°;

2° - Riscaldamento 40°;

3° - A temperatura ambiente ed all'oscuro (prove di controllo).

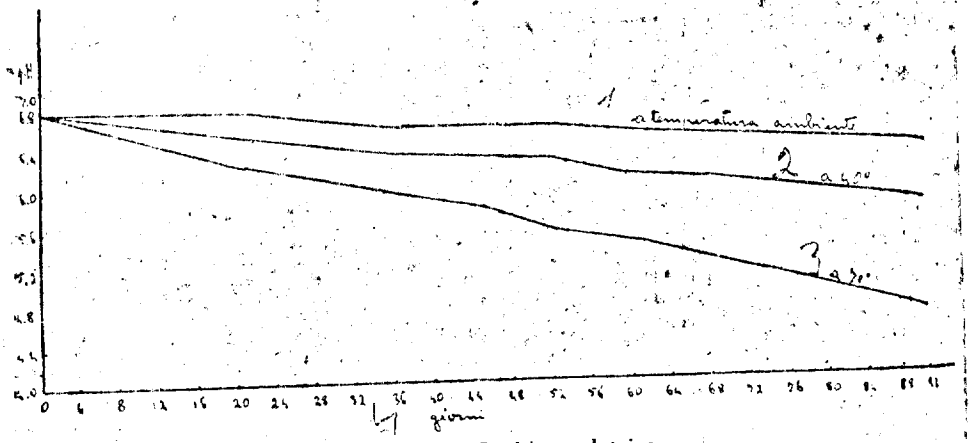

Grafico II. - Lecitina e luteina.

Essendo le proporzioni del grafico (II) uguali a quelle del grafico (I) si può notare subito che la miscela lecitina e colesterina per azione del calore si decompone assai meno rapidamente della lecitina pura infatti per arrivare al pH-4.8 la miscela, tenuta a 70° ha impiegato giorni 92, mentre la lecitina pura nelle stesse condizioni impiegò soltanto giorni 52.

Considerazioni analoghe si possono fare per le fiale mantenute a 40° e per quelle di controllo.

Un'altra differenza di comportamento consiste nel fatto che mentre il siero della lecitina in decomposizione presentò ben presto un potere emolitico, il siero della miscela lecitina-luteina invece non presentò mai questa proprietà: soltanto sciogliendola in alcool e precipitando con etere ottenni un precipitato di lisocitina fortemente emolitica e corrispondente all'analisi.

Le due differenze di comportamento che presentano la lecitina pura e la sua miscela con colesterina sottoposte all'azione del calore, si spiegano facilmente tenendo conto che la colesterina, come è noto, presenta un'azione antagonista a quella della lisocitina e ne impedisce l'azione tossica; in questo caso la colesterina agirebbe anche come stabilizzante della lecitina ritardandone la decomposizione sotto l'azione del calore, appunto come asserì il Serono nel 1913.

Riassumendo:

1° È stato riscontrato che per azione prolungata del calore la lecitina in sospensione acquosa, in ambiente asettico, fuori del contatto dell'aria, si decompone dando origine ad acidi grassi e lisocitine.

2° La presenza di cieri di colesterina ritarda l'azione dissociante.

3° La presenza della colesterina impedisce l'azione emolitica della lisocitina formatasi per decomposizione della lecitina.

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CCLXVIII. THE SPONTANEOUS DECOMPOSITION OF LECITHIN AND ITS BEARING ON THE DETERMINATION OF THE ISOELECTRIC POINT.

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Most of the phosphatides are chemically not very stable. Decomposition can occur by oxidation of the unsaturated fatty acids or by hydrolysis. Investigations on this point have been carried out by numerous workers [Warburg and Meyerhof, 1913; Paal, 1929; Page and Schmidt, 1931; Fiori, 1930, 1, 2; 1931; 1932]. In the present paper some observations on the spontaneous decomposition of lecithin are communicated.

Experimental.

Electrotitrations of lecithin in 90 % alcohol were carried out, using the hydrogen electrode against the saturated calomel electrode. The hydrogen gas was saturated with solvent vapour by passage through 90 % alcoholic solution. The calomel electrode was checked by measuring the potential difference against a hydrogen electrode in $N/10$ hydrochloric acid, prepared by weight from constant boiling-point HCl, as second half cell; the potential difference was 317 $\pm$ 1 mv. at 19°. All titrations were carried out in a paraffin thermostat at 25·7° $\pm$ 0·1°. The potential differences were determined with an accuracy of *ca.* 1 mv. Titrants were $N/10$ NaOH and $N/10$ HCl in 90 % alcohol.

400 mg. lecithin (0·5 millimol) were dissolved in 30 ml. 90 % alcohol and used for every titration. Curve I in Fig. 1 shows the titration curve of a fresh lecithin preparation [Fischgold and Chain, 1934] with $N/10$ NaOH. The curve differs very little from the curve for the solvent alone. On titrating back again immediately with $N/10$ HCl curve II is obtained, indicating a small buffering effect at p_H 7·5. If however the back-titration is made after the lecithin has been standing 66 hours in the presence of a small excess of $N/10$ NaOH at room temperature, curve III is obtained. Obviously a new group has been formed, the p_K of which is about 7·5 in the solvent used (Table I and Fig. 1).

When older lecithin preparations were investigated even the first titration curve showed a shape similar to curves II and III. The usual commercial lecithin, which has a rather acid reaction, has been titrated and corresponds to curve III.

With the titration method described by Fischgold and Chain [1934] the process was investigated further.

The method consists of a titration in a benzene-alcohol (19 : 1) mixture as solvent with $N/50$ $HClO_4$ in the same solvent against dimethyl yellow as indicator and with $N/50$ sodium ethoxide against phenolphthalein. All phosphatides investigated (lecithin, kephalin, lysolecithin, sphingomyelin) bind one equivalent of H^+ ion at acid reaction. The phosphatides of the colamine type (kephalin,

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Table I.

Blank curve		Curve I		Curve II		Curve III	
ml. N/10 NaOH	p_H	ml. N/10 NaOH	p_H	ml. N/10 NaOH*	p_H	ml. N/10 NaOH*	p_H
0	4·68	0	3·63	0	3·96	0	3·07
0·10	12·51	0·10	4·23	0·20	5·48	0·15	3·18
0·20	12·80	0·20	4·91	0·40	6·04	0·45	3·48
0·50	13·33	0·30	5·75	0·60	6·37	0·80	3·87
0·75	13·60	0·40	6·75	0·75	6·68	1·10	4·98
1·00	13·69	0·50	7·85	0·90	7·05	1·45	5·90
2·00	13·88	0·60	10·56	1·10	7·36	1·80	6·31
3·00	14·00	0·70	11·85	1·30	7·71	2·45	6·81
5·00	14·10	0·80	12·32	1·50	8·46	3·10	7·20
		0·90	12·67	1·70	9·63	3·75	7·46
		1·00	12·78	1·75	11·56	4·10	7·58
		1·25	13·05	1·80	12·26	4·40	7·64
		1·50	13·26	1·90	12·51	4·75	7·81
		2·00	13·38	2·20	12·87	5·05	8·05
		3·00	13·67	2·40	12·97	5·40	8·35
		4·00	13·83	2·60	13·08	5·70	8·73
		5·00	13·90	2·80	13·27	6·05	9·83
		6·00	13·94	3·30	13·48	6·40	12·28
				4·20	13·73	7·05	13·06
				5·10	13·81		
				6·00	13·90		

* In the back-titration curves II and III, instead of the amount of titrant used for the back-titration, the corresponding amount of NaOH is given, calculated by deducting the ml. of HCl added from the total amount of NaOH added previously.

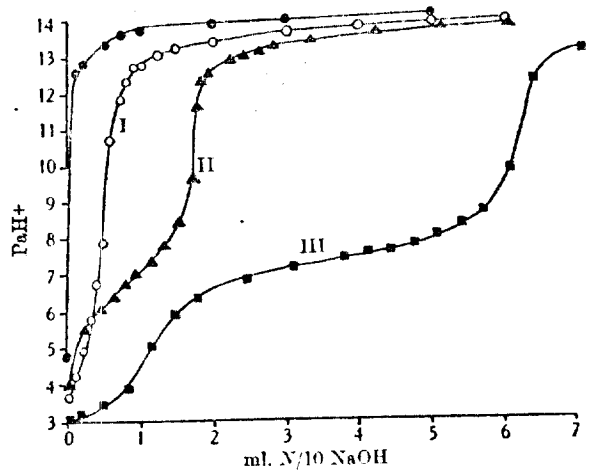

Fig. 1. Titration curve of a fresh lecithin in 90% alcohol. Curve I: o—o titration curve of a fresh lecithin preparation. Curve II: ▲—▲ back-titration curve immediately after the titration. Curve III: ■—■ back-titration after 66 hours. •—• blank curve.

lysokephalin) bind one equivalent of alkali when titrated with sodium ethoxide, while the phosphatides of the choline type (lecithin, lysolecithin, sphingomyelin) have no base-binding power. From these results it was concluded that the phospholipins behave as zwitterions. In the acid titration the dissociated free acid group of the phosphoric acid is converted into the undissociated state. In the titration with sodium ethoxide the NH_3^+ group is capable of giving up an H^+ ion whereas the $N(CH_3)_3^+$ group naturally cannot react in such a manner.

Three different preparations of lecithin were titrated; one while fresh, the second after standing for 6 weeks over $CaCl_2$ in an evacuated desiccator and the third exposed to the air for the same period. The fresh lecithin is white and has a solid consistency; while kept in the desiccator it becomes yellowish, without changing its consistency very much. The preparation kept in the air becomes yellow-brown and acquires the greasy consistency of the commercial preparation.

The results of the titrations were:

Acid side. $N/50$ $HClO_4$. Dimethyl yellow.

Preparation	Titrated ml.	Control ml.	Diff. ml.	Equivalent weight
Lecithin (1): 0·0378 g.	2·34	0·16	2·18	865
,, (2): 0·0360 g.	2·20	0·16	2·04	880
,, (3): 0·0384 g.	2·27	0·16	2·11	910

Alkaline side. $N/50$ Na ethoxide. Phenolphthalein.

Preparation	Titrated ml.	Control ml.	Diff. ml.	Millimols of acid in 100 millimols of lecithin
Lecithin (1): 0·0585 g.	0·24	0·25	—	—
,, (2): 0·0424 g.	0·38	0·20	0·18	6·8
,, (2): 0·0539 g.	0·41	0·20	0·21	6·2
,, (3): 0·0532 g.	2·19	0·21	1·98	59·6
,, (3): 0·0600 g.	2·52	0·22	2·30	61·3

On the acid side no essential change can be found except a small increase of the equivalent weights, the reason of which is probably uptake of oxygen and water. On the alkaline side a great increase of groups titratable with Na ethoxide takes place. Even the preparation kept in the desiccator had a marked acid content (*ca.* 6 mols. %). Lecithin kept exposed to the air contained about 60 % acid valencies. Obviously an acid group was liberated from the molecule. Two possibilities have to be considered with regard to the structure of the lecithin molecule; either choline may be split off and through this process the second phosphoric group liberated, or one or two fatty acids may be split off.

The p_K of the group formed was, according to Table I and Fig. 1, about 7·5. This would indicate rather a liberation of fatty acids than of choline. For the p_K of the second phosphoric group, which is 7·3 in water, should be much higher in 90 % alcohol; on the other hand, fatty acids have p_K *ca.* 5 in water [McBain and Taylor, 1911], so that in 90 % alcohol the p_K might be expected to lie in the range in question. In fact, an electrotitration of stearic acid in 90 % alcohol gave the value of p_K 7·36.

Dissociation constant of stearic acid. 0·1252 g. of stearic acid was dissolved in 30 ml. 90 % alcohol and titrated electrometrically with $N/10$ NaOH in 90 % alcohol at $25·7 \pm 0·1°$ (Table II, Fig. 2).

Further information was obtained by examination for the possible decomposition products choline and fatty acids.

Examination for free choline. A certain amount of lecithin was emulsified with H_2O (fresh lecithin hardly emulsified at all), the emulsion made acid with 2 ml. 19 % HCl and well shaken, and a few ml. of colloidal iron hydroxide were added. After adding a trace of solid sodium sulphate the precipitate was centrifuged off and an aliquot part of the clear solution used for choline determination according to Lintzel [1931]. Neither in fresh nor in old lecithin was free choline found, although the method was sensitive to amounts of choline under 5 mg.

Table II.

N/10 NaOH ml.	p_H	p_K	N/10 NaOH ml.	p_H	p_K	N/10 NaOH ml.	p_H	p_K
0	4·45	—	2·00	7·30	7·33	4·20	12·15	—
0·25	6·15	7·34	2·50	7·52	7·34	4·30	12·46	—
0·50	6·56	7·41	3·00	7·63	—	4·50	12·73	—
0·75	6·67	7·32	3·50	7·86	—	5·00	13·06	—
1·00	6·88	7·39	4·00	8·28	—			
1·50	7·11	7·36	4·10	8·45	—		Average 7·36	

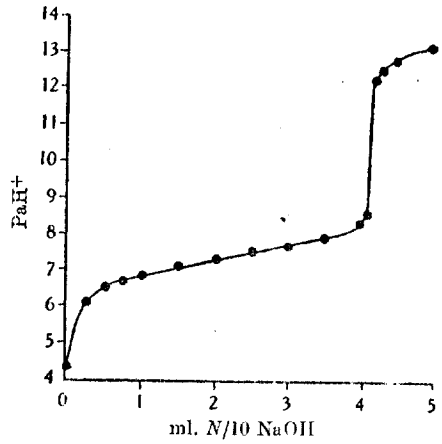

Fig. 2. Titration curve of stearic acid in 90 % alcohol.

Determination of free fatty acids. 0·3–0·4 g. lecithin was dissolved in about 10 ml. alcohol in a 25 ml. measuring flask; to this solution 5 ml. of an alcoholic CdCl solution (1·8 g. $CdCl_2$ dissolved in 1 ml. water, made up to 100 ml. with absolute alcohol) were added, and the solution was made up with alcohol to 25 ml. The precipitate was centrifuged, 20 ml. of the clear solution were evaporated on the water-bath, the residue was dissolved in benzene and titrated with Na ethoxide in alcohol against phenolphthalein as indicator. After this procedure fresh lecithin showed no alkali absorption. Old lecithin on the contrary contained a considerable amount of titratable acid.

Preparation		Acid titrated with 0·05 N Na ethoxide (⅕ of the total amount) ml.	Mols. % acid
Fresh	0·4 g.	—	—
Old	0·320 g.	3·32	50·4
A direct titration of the decomposed preparation gave:			
	0·240 g.	3·10 (total amount)	51·7

The isolated acids must be fatty acids since the greatest part of the lecithin and its decomposition products are precipitated with $CdCl_2$ or are insoluble in benzene.

Of the other phosphatides which were titrated kephalin, lysolecithin and sphingomyelin [Fischgold and Chain, 1934] showed constant titration values while kept in the desiccator. Page and Bülow [1931] observed also in kephalin preparations gradual increase of groups titratable with alkali, although only to a small extent.

The isoelectric point of lecithin.

The easily occurring decomposition of lecithin must cause large errors in the determination of the isoelectric point, an acid being formed during this process. The values that have hitherto been found are unsatisfactory; they are not in agreement with each other and differ from the value which might have been expected from the dissociation constants. Feinschmidt [1912] found for some commercial lecithin preparations the flocculation optimum and the isoelectric point between p_H 2 and 4, Fujii [1924] at p_H 2·7, Remesow [1930] at p_H 2·2. Sueyoshi and Kawai [1932] determined the isoelectric point to be 4·7; on standing for some time the isoelectric point was shifted towards the acid side. The reason for this shift, called by the author "denaturation," has not been investigated. Price and Lewis [1929; 1933] found the isoelectric point from the surface tension values to be 2·6, and from cataphoresis to be 2·7. Freshly prepared lecithin had a lower cataphoretic mobility than a preparation kept for some time. The final determination was carried out after the cataphoretic mobility had reached a constant value. All determinations show an isoelectric point more acid than p_H 4·7 and are in definite disagreement with the value which would be expected from a computation according to the relation

$$\text{I.P.} = \sqrt{K_1 . K_2}$$

(both the constants referred to H^+ ions).

Direct determination of the characteristic constants of lecithin is not possible because of its insolubility in water and the decomposition occurring in alkaline solution. But the probable magnitude can be deduced from compounds containing the same groups. p_{K_1} corresponds to the first phosphoric acid group. In esters of phosphoric acid this group is generally stronger than in phosphoric acid itself.

p_{K_1} of phosphoric acid	= 2·12 [Kolthoff, 1932];
,, glycerophosphoric acid	= 1·40 [Meyerhof and Suranyi, 1926];
,, hexosediphosphoric acid	= 1·55 [Meyerhof and Suranyi, 1926];
,, lysolecithin	= 0·75 [Levene *et al.*, 1923–24].

From this the p_{K_1} of lecithin can be assumed to be about 1. (Levene *et al.* [1923–24] found for lysokephalin p_{K_1} 3·5: but this figure is uncertain because in acid solution a gel was formed immediately.)

p_{K_2} of lecithin corresponds to the choline, which, as a quaternary ammonium base, should be a very strong base (like betaine) and can be expected to be greater than 14. The titrations mentioned above [Fischgold and Chain, 1934] also justify such an expectation. The smaller value of 11·90 found by Levene *et al.* for lysolecithin is possibly due to partial hydrolysis of the substance. Price and Lewis [1933] determined the p_K of choline directly by dissolving a certain amount of choline chloride in water and measuring the p_H of the solution which was fairly acid. From this they calculated p_K 5·06 (referred to OH^- ion concentration) or 9·0 (with reference to H^+ ion concentration), a value indicating a fairly weak base. This result being very surprising the dissociation constant of choline has been reinvestigated by three different methods:

Reaction of a choline chloride solution. Choline chloride was recrystallised from alcohol. The addition of acid was avoided. The crystals were dried over P_2O_5 in the desiccator. 0·7060 g. choline chloride was dissolved in 50 ml. water. The p_H of the solution was determined potentiometrically with the hydrogen electrode against the saturated calomel electrode. At 19° it was found to be 6·98. The solution thus was neutral. This was to be expected with a chloride of a strong base. The different results of Price and Lewis might be explained

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by the fact that the authors added concentrated hydrochloric acid during the purification of the choline chloride. Traces of hydrochloric acid can give rise to very large errors.

Electrotitration of choline chloride. 0·1014 g. choline chloride in 50 ml. water was titrated electrometrically with $N/20$ NaOH (titre 0·972). In order to keep the ionic strength constant the solution was made $N/10$ with respect to sodium chloride. The titration curve was identical with the blank correction curve. Up to an alkalinity of p_H 12·0 no base binding could be detected. From this it follows that the p_K of choline must be at least above 13 (referred to H^+ ion concentration).

Table III.

$N/20$ NaOH ml.	Choline chloride p_H	Blank curve p_H	$N/20$ NaOH ml.	Choline chloride p_H	Blank curve p_H
0	5·42	5·58	3·00	11·56	11·57
0·10	9·37	9·87	4·00	11·68	11·69
0·20	10·23	10·28	5·00	11·77	11·78
0·30	10·43	10·46	6·00	11·83	11·84
0·40	10·60	10·63	7·00	11·88	11·89
0·50	10·70	10·70	8·00	11·94	11·96
0·75	10·88	10·91	9·00	11·98	11·99
1·00	11·04	11·08	10·00	12·02	12·03
2·00	11·38	11·39			

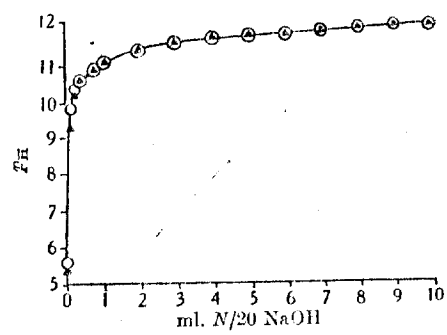

Fig. 3. Titration curve of choline chloride. o—o blank curve; ▲—▲ choline curve.

Base-binding at alkaline reaction. 0·1202 g. choline chloride after having been carefully dried was dissolved in 4·35 ml. $N/10$ sodium hydroxide (titre 0·972); the solution, which was $M/5$ choline chloride, had at 19° p_H 12·89 (H_2-electrode). The sodium hydroxide solution without the choline, but containing an equivalent amount of sodium chloride, had p_H 12·99.

The difference appears to be very small and may be due to a change of the activities or partial decomposition of the choline molecule. But even if the difference in p_H between the choline solution and the control solution is referred to base binding, only a very small value is obtained: assuming for an approximate calculation the activities measured as equal to the concentrations, 16 millimols OH^- ions per litre have disappeared, or in 4·35 ml. of the solution used 0·070 millimols OH^- ions are bound by the choline ion. The solution contains 0·862 millimols choline chloride; 0·792 millimols are choline ions and 0·070 undissociated choline molecules. The p_H of the solution is 12·89. Consequently the dissociation constant is

$$K = \frac{[\text{Free choline}]\,[H^+]}{[\text{Choline}^+]} = \frac{0{\cdot}070}{0{\cdot}792} \cdot 10^{-12{\cdot}89} = 10^{-13{\cdot}94},$$

$p_K = 13{\cdot}94$ (referred to H^+ ion concentrations). This value is compatible with a strong base and is in accordance with values found for betaine [*cf.* Bjerrum, 1923]. The figure naturally is not accurate because in general it is possible to measure such strong bases or acids only approximately.

Discussion.

The first dissociation constant of lecithin is about 10^{-1}, the second about 10^{-14}, as pointed out above. The theoretical value of the isoelectric point therefore should be at $[H^+] = \sqrt{10^{-1}, 10^{-14}} = 10^{-7{\cdot}5}$. The fact that more acid values have always been found may be explained by the liberation of fatty acids which occurs easily and rapidly. Very small traces of fatty acids can bring about a very considerable shift of the isoelectric point. It seemed therefore necessary to determine the isoelectric point again, using carefully purified fresh preparations. In recent investigations [Chain and Kemp, 1934] it has been found to be at p_H $6{\cdot}7 \pm 0{\cdot}2$. This value is in fairly good agreement with the theoretical value deduced from the dissociation constants and is only slightly more acid, which may be ascribed to minute traces of fatty acids. A computation shows the amount of fatty acids necessary to shift the isoelectric point from its theoretical value (7·5) to 6·5.

At p_H 6·5 the quaternary ammonium base is dissociated practically completely. The phosphoric acid group however according to the p_K of about 1 is associated to an extent of $10^{-3{\cdot}5} = 3{\cdot}2 \times 10^{-4}$ %. Therefore a lack of negative charges in the lecithin molecule occurs and a compensation by fatty acid anions is necessary to maintain the isoelectric state of the molecule complex. The dissociation constant of fatty acids is estimated as about 10^{-5} [McBain and Taylor, 1911] so that at p_H 6·5 fatty acids are dissociated to 93 %. To compensate the lack of charges of the phosphoric acid group the presence of $3{\cdot}4 \times 10^{-4}$ mol. % of fatty acids is necessary.

Such a small amount cannot be determined experimentally. Even the freshest preparation may be decomposed to such an extent. It is difficult to explain the still more acid values which have been found; they may be due to other impurities of acid character (oxidation products).

Summary.

1. Electrotitration curves and indicator titrations demonstrate a spontaneous decomposition of lecithin, which occurs even when the substance is kept in a desiccator.

2. The spontaneous decomposition of lecithin consists of a liberation of fatty acids, which have been separated. No choline separation could be detected.

3. The dissociation constant of choline has been determined approximately. Choline is a strong base the p_K of which is about 14.

4. The theoretical isoelectric point of lecithin, calculated from its dissociation constants, is about p_H 7·5. A minute content of acid impurities brings about a considerable shift in the apparent isoelectric point.

We wish to express our gratitude to Prof. J. B. S. Haldane for his generous help during this work, and to Prof. J. C. Drummond and Sir F. G. Hopkins whose kind hospitality and interest have made its completion possible.

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THE EFFECT OF LECITHIN AND LECITHINASE ON DCA HYPERTENSION IN THE RAT

BY

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During the past few years some of the characteristics of the relation between blood pressure regulation on the one hand and sodium metabolism on the other have become clearer. First, we have observed that sodium shifts are regularly linked to acute changes in blood pressure (3). Second, studies of more chronic alterations of blood pressure have led to the conclusion that these too involve sodium shifts (7, 9). Third, the causal nature of the association between sodium and blood pressure has been shown by *in vitro* and *in vivo* studies which have related smooth muscle tone to the extra/intracellular sodium gradient (8).

The basic regulation of sodium transfers between cells and environment seems to be largely governed by the neurohypophysis and mineralocortex and adjusted by the kidney. Our findings, taken in conjunction with the recent observations of others (1, 11) lead to the postulate that adrenalectomy facilitates, while aldosterone hinders sodium extrusion from the cell. In essence, this suggests that the "supporting effect" of adrenal steroids, ably described by INGLE (10), resides in the fact that they build into the membrane and govern its permeability characteristics. That this is a general phenomenon is indicated by the fact that aldosterone and hydrocortisone modify sodium transfers in red cells (suspended in plasma) (5). These steroids thus "support" sodium transfer. It is indeed tempting to view this as a likely method for steroid action in general.

This work was carried out with the aid of a grant from the National Research Council of Canada.

A discussion of these postulates with Professor U.S. VON EULER led him to suggest that lecithin might be expected to compete with the action of mineralocorticoids. In 1945 he and his associates (2) had noted that lecithin modified the response to acetylcholine but they were unable at that time to offer an explanation of the effect. It might be expected that some lecithinases would similarly oppose mineralocorticoid effects.

The present report is concerned with the administration of lecithin and lecithinase to rats under active DCA treatment as well as to rats with permanent post DCA hypertension.

METHODS

Male albino rats of an inbred Wistar strain were used throughout. DCA hypertension was produced by the implantation of five 20 mg pellets subcutaneously, 2 on the first day and the remaining 3 at three-day intervals. In addition, 1 % saline was substituted for drinking water. Permanent post DCA hypertension was produced by prolonging the same treatment for 2 months, then recovering the pellets and removing the saline drinking water. Three months after cessation of treatment, blood pressure was measured in all treated animals and compared with matched controls for the selection of those animals with permanent hypertension. As we have described more fully elsewhere (4, 6) this type of hypertension is similar in many respects to essential hypertension in man.

Extracellular space was measured as the volume distribution of inulin after 2 hours of equilibration in the bilaterally nephrectomized rat according to procedures previously described (3, 9). Na and K were measured by flame photometry. Blood pressures were measured electromanometrically under light ether anesthesia as direct arterial pressures. Lecithin was administered as a saline suspension in 0.1 ml or 0.2 ml. Lecithinase (Lecithinase A, Delta Chemical Co., or Stypven, Russel Viper Venom, Burroughs Wellcome) was given in saline in volumes ranging from 0.1 to 0.5 ml. Further details of procedure will be given with each experiment.

RESULTS

Experiment 1. The Effect of Lecithin Given Concurrently with DCA.

Four groups of weanling rats were used for this experiment. Group 1 served as untreated control, group 2 received 5 mg of lecithin sub-

cutaneously and 5 mg intraperitoneally daily, group 3 received DCA as described and group 4 received both DCA and lecithin treatment. All groups except the first received 1 % saline as drinking water. Blood pressure was measured at the end of the 3rd, 4th and 5th weeks of treatment. The experiment was terminated at the end of the 5th week with the determination of Na, K and extracellular space as described.

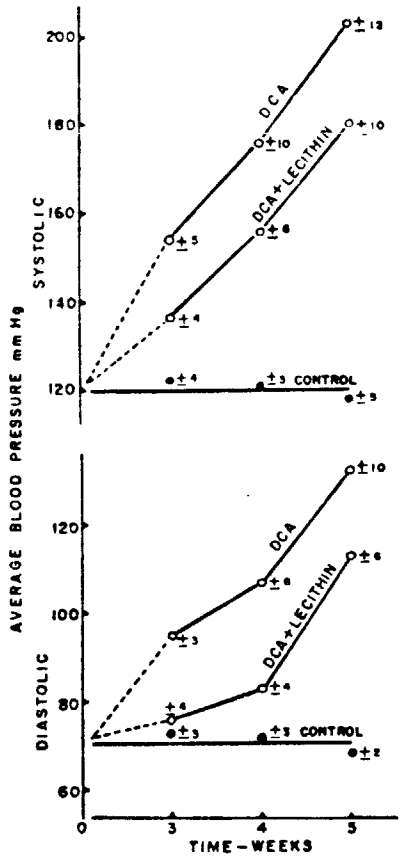

FIG. 1

Direct blood pressure determinations in rats treated with DCA-saline with and without daily lecithin injections compared with those of untreated controls. (Lecithin treated controls are not shown since they do not differ from untreated controls).

The blood pressure findings are shown in Figure 1. Lecithin tended to inhibit the hypertensive effect of DCA. The diastolic pressure records suggest that this inhibition may be more effective in the earlier stages of DCA treatment. Lecithin alone was without effect.

The terminal findings are shown in Table I. The changes in the DCA-

TABLE I

The effect of lecithin on the fluid and electrolyte changes induced by DCA-saline

Group	Control 1	Lecithin Saline 2	DCA Saline 3	DCA Saline Lecithin 4
e.c.f.v., ml/100 gm	23.4 ± 0.4	24.2 ± 0.8	27.4 ± 0.6*	29.7 ± 0.3*
Na concentration, mEq/L	141.2 ± 1.5	142.2 ± 0.9	143.6 ± 1.7	143.4 ± 1.2
K concentration, mEq/L	3.77 ± 0.08	4.00 ± 0.09	3.27 ± 0.20*	2.83 ± 0.06*
e.c. Na, mEq/100 gm	3.31 ± 0.05	3.44 ± 0.10	3.97 ± 0.12*	4.26 ± 0.15*
e.c. K, μEq/100 gm	88 ± 1.7	97 ± 4.2	85 ± 3.9	84 ± 1.5
No. of animals	7	9	6	7
Average body wt., gms	192 ± 10	215 ± 4	190 ± 8	185 ± 9

± = standard error.
e.c.f.v. = extracellular fluid volume = inulin space.
e.c. Na or K = extracellular Na or K.
* = significantly different from group 1 (control).
s = significantly different from group 3 (DCA-saline).

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saline treated group were as expected. Thus, the inulin space was increased, sodium concentration tended to rise and potassium concentration fell. As a corollary, total extracellular sodium was also increased. Lecithin caused a still further increase in the inulin space and a greater fall in potassium concentration. In general, then, it appears that any inhibitory effect which lecithin exerts on a DCA-induced pressure rise is accompanied by an exaggeration of the effects which the steroid ordinarily exerts on fluid and electrolyte patterns.

Experiment 2. The Effect of Lecithin Given by Various Routes Concurrently with DCA.

Five groups of weanling rats were used for this experiment. Group 1 served as untreated control, group 2 received DCA and saline as described, group 3 received DCA and saline like group 2 and in addition 5 mg of lecithin subcutaneously twice daily. Group 4 was treated like group 3 but received the lecithin intraperitoneally while group 5 received it orally. Blood pressure was measured at the end of the 3rd and 4th weeks. The experiment was terminated at the end of the 4th week with measurements as before.

The findings are presented in Table II. Again, lecithin inhibited the DCA hypertensive effect. As usual, DCA increased the inulin space and sodium concentration while dropping pottassium concentration and, in this case, total extracellular potassium as well. Lecithin again consistently exaggerated the increase in extracellular fluid volume and in calculated extracellular sodium.

Experiment 3. The Effect of Lecithin in Post DCA Hypertension.

The experimental groups were made up as usual from a pool of animals in which intensive DCA treatment had stopped at least 3 months previously. Group 1 consisted of normal control, groups 2 and 3 of matched hypertensives. Following selection, the animals of group 3 received lecithin, for the first week 10 mg intraperitoneally and 10 mg subcutaneously twice daily (total 40 mg/day) and for the next 3 weeks double this dose. Blood pressure was measured at the end of the 3rd and 4th weeks. The animals were not killed. The results are presented in Table III.

The groups of hypertensive rats were well matched at the start of the experiment. Further, no rat exhibited a diastolic pressure greater than 90 in the control group or less than 100 in the hypertensive group. Clearly, the hypertension was not affected by the lecithin injections despite the large doses used.

TABLE II

The effect of lecithin administered by various routes on the fluid and electrolyte changes induced by DCA-saline

Group	Control	DCA Saline	DCA Saline Lecithin subcut.	DCA Saline Lecithin intraperit.	DCA Saline Lecithin per os
	1	2	3	4	5
3rd week Blood pressure mm Hg systolic	122 ± 4	162 ± 10*	147 ± 4*	148 ± 6*	154 ± 10*
				s	
diastolic	75 ± 2	102 ± 4*	90 ± 2*	93 ± 5*	93 ± 4*
				s	
systolic	121 ± 6	170 ± 7*	161 ± 10*	150 ± 8*	156 ± 10*
				s	
4th week diastolic	71 ± 4	107 ± 6*	96 ± 6*	89 ± 6*	92 ± 7*
				s	
e.c.f.v., ml/100 gm	22.9 ± 0.2	24.3 ± 0.9*	25.2 ± 0.5*	26.5 ± 0.6*	25.1 ± 0.6*
				s	
Na concentration, mEq/L	148.4 ± 2.8	151.6 ± 2.4	148.4 ± 2.9	146.4 ± 2.6	151.6 ± 2.8
K concentration, mEq/L	4.22 ± 0.16	3.18 ± 0.09*	2.99 ± 0.13*	3.32 ± 0.14*	2.90 ± 0.11*
e.c.Na, mEq/100 gm	3.40 ± 0.09	3.68 ± 0.13*	3.73 ± 0.09*	3.88 ± 0.11*	3.80 ± 0.05*
				s	
e.c.K, μEq/100 gm	97 ± 3	77 ± 3*	75 ± 3*	88 ± 5	73 ± 3*
No. of animals	8	7	6	7	6
Average body wt., gms	192 ± 9	167 ± 11	165 ± 5	177 ± 7	184 ± 14

* significantly different from group 1 (control).

s significant difference between combined groups 3, 4, 5 and group 2.

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TABLE III

The effect of lecithin on permanent sustained post DCA hypertension

	Normal Control	Permanent Hypertension Control	Permanent Hypertension Lecithin
Preliminary B.P., mm Hg			
systolic	125 ± 3	185 ± 4 (1)	187 ± 10 (1)
diastolic	84 ± 3	120 ± 3 (1)	125 ± 7 (1)
mean	98 ± 3	147 ± 3 (1)	151 ± 9 (1)
No. of animals	8	8	8
Average body wt., gms	285 ± 9	326 ± 11	327 ± 10
B.P. at 3 weeks			
systolic	122 ± 3	180 ± 5 (1)	182 ± 12 (1)
diastolic	85 ± 4	122 ± 4 (1)	120 ± 9 (1)
mean	102 ± 4	149 ± 5 (1)	147 ± 11 (1)
No. of animals	8	8	7
B.P. at 4 weeks			
systolic	122 ± 3	172 ± 2 (1)	180 ± 11 (1)
diastolic	84 ± 2	112 ± 2 (1)	115 ± 9 (1)
mean	100 ± 3	139 ± 2 (1)	143 ± 11 (1)
No. of animals	8	8	6

(1) = significantly different from control.

Experiment 4. The Chronic Effect of Viper Venom (Stypven) in Rats with DCA Hypertension.

These studies with lecithinase were designed to determine whether it could reverse an established hypertension. The doses and procedures of this experiment were selected as a result of preliminary experience with both subcutaneous and intravenous routes of administration. Lecithinase A was first studied, followed by Stypven which was found to be more active.

From a pool of adult male rats with established DCA hypertension and still under active treatment two groups of 8 rats each, matched closely in blood pressure, were selected. Group 1 received saline and group 2 lecithinase daily. For the first 4 days the test group received

5 μg of lecithinase, for the next 5 days 10 μg and finally 50 μg for 4 days. The findings are shown in Figure 2. Blood pressure in the control group was stable. By contrast, although 5 μg of lecithinase was without effect in the test group, 10 μg showed a tendency to lower blood pressure while 50 μg did so definitely. The result is striking in view of the crude nature of the material used and the ample time allowed by our experimental arrangement for the development of resistance. The test animals showed no ill effects and gained weight in the same way as the controls during the experimental period.

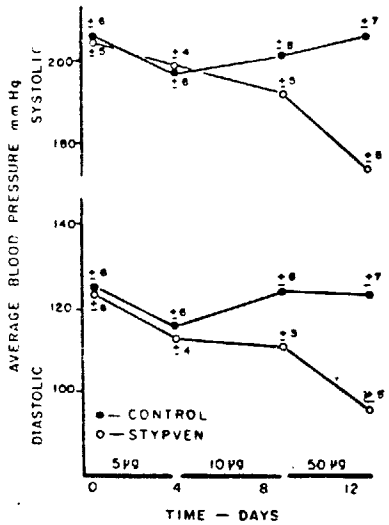

FIG. 2

The effect of viper venom (stypven) in rats with active DCA hypertension.

Experiment 5. The Chronic Effect of Viper Venom (Stypven) in Rats with Permanent Post DCA Hypertension.

Two experimental groups of 8 animals each were made up by selection from a pool of animals in which intensive DCA treatment had stopped at least 3 months previously. Group 1 received saline and group 2 Stypven, 50 μg subcutaneously on the first day followed by 75 μg for 3 days. Blood pressure was measured after the 4 days of treatment and again one week after cessation of treatment. The findings are presented in Table IV. Blood pressure declined under treatment and reverted again after treatment stopped.

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TABLE IV

The effect of viper venom (stypven) given subcutaneously for 4 days to rats with permanent sustained post DCA hypertension. Final blood pressure values were measured 7 days after cessation of treatment

Day of Experiment	Blood Pressure, mm Hg			
	Control		Test	
	Systolic	Diastolic	Systolic	Diastolic
0	162 ± 7	100 ± 5	162 ± 8	100 ± 5
4	156 ± 5	97 ± 4	138 ± 8	83 ± 5
11	159 ± 6	108 ± 5	161 ± 9	112 ± 7

DISCUSSION

There is little doubt that an adequate amount of lecithin administered by any route may interfere with the ability of DCA to cause hypertension during the initial stages of development of this condition. It does not provide a completely effective block but it does as much, in a presumably non-specific way, as is occasionally claimed for specific anti-DCA agents.

Lecithin was completely incapable of altering the established hypertension of the post DCA state. This suggests that it competes only when DCA is active. It further rules out the possibility that the effect of lecithin is due to injury or inflammatory change. The findings with lecithin are consistent with the view that DCA and similar steroids actually build into membranes but, of course, is in no way proof.

An interesting side feature of these experiments is the observation that lecithin inhibits the pressor effect of DCA while actually exaggerating the fluid and electrolyte changes ordinarily caused by the steroid. This is entirely in accord with our view that these changes are not causal to the hypertension but are essentially attempts to preserve homeostasis (8).

Russel viper venom (Stypven) tended to lower blood pressure even when given in a relatively late stage of acute DCA hypertension. More importantly, it was also able to lower pressure in the stable permanent

post DCA hypertensive state. The venom being crude, it is not possible to relate its activity with confidence to its ability to attack lecithin.

These experiments were undertaken as a result of our view that at least certain steroids act on cell membranes to condition sodium flux. This suggested that lecithin might compete with, and certain lecithinases reverse, hormonal hypertension. The results obtained here are consistent with this view.

SUMMARY

Lecithin administered by any route interferes with the ability of DCA to cause hypertension. It is most effective during the initial stages of development of the hypertension and completely ineffective in the sustained post DCA phase. Viper venom, used as a source of lecithinase, given in small subcutaneous doses produced a gradual lowering of blood pressure both in the late stages of acute DCA hypertension and in the stable sustained post DCA phase. These results are consistent with the view that certain steroids act on cell membranes to condition sodium flux.

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THE CALORIGENIC ACTION OF LECITHIN

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The rôle of lecithin in body metabolism is still obscure. Bloor (1915) has observed that the lecithin content of blood may increase 20–30 per cent during the absorption of fat, hence the possibility that lecithin is an intermediate in fat oxidation. If this is the case, lecithin, given orally or intravenously, should be oxidized at least as rapidly as neutral fat and should exert a comparable specific dynamic action. Experiments were therefore planned to test this possibility.

PROCEDURE. Hourly determinations of metabolic rate were carried out on three different dogs whose basal metabolic rate was determined from time to time. The dogs used were trained for 4 to 6 weeks prior to the experiments and were maintained at a practically constant weight on a diet of 75 grams of beef heart, 75 grams of cracker meal, 12 grams of lard and 5 grams of bone ash.

A closed circuit unit of the Benedict-Holmes (1912) type was used and all experiments were conducted in a room maintained at a constant temperature of approximately 25°C. Alcohol checks were repeatedly made. Bodily movements and in some experiments respiratory movements of the animal were recorded by tambour systems on a smoked drum.

After satisfactory basal controls had been established on preceding days or on the same day lecithin was administered either orally or intravenously. The lecithin, approximately 90 per cent pure, was made from beef spinal cord or from egg yolk. (Sixty to 90 grams of lecithin were usually given by mouth.) Three to four hundred cubic centimeters of a water emulsion, made with the aid of a mechanical stirrer and heated to 38°C., were given by stomach tube. Loose stools were generally observed on the day following, but vomiting, diarrhea and restlessness never occurred during the period that the animals were in the metabolism chamber. The metabolic rate in these animals was followed for 7 to 8 hours.

For intravenous administration, emulsions of lecithin, purified according to the method of Bloor (1929) were prepared in 100 cc. of 0.65 per cent sodium chloride solution. This concentration of salt was used since it produced a more stable and finer emulsion than did physiological saline.

Microscopic examination showed the lecithin particles to be all less than 7μ and the majority 1 to 3μ in diameter. The emulsion, kept in a water bath at approximately 50°C., was introduced through a needle into a saphenous or external jugular vein by gravity or under air pressure, the temperature of the entering solution 2 inches from the needle, ranging from 37.5°–40°C., depending on the rate of flow. The usual rate of infusion was 7 to 10 cc. per minute. Introduced in this way as much as 1 gram of lecithin per kilo was tolerated by animals without any obvious effect. Such doses could be repeated every day without apparent harm.

TABLE 1

Oral administration of lecithin

DOG	DATE	CONDITION	NON-PROTEIN R.Q.	URINARY N	CALORIES	PER CENT INCREASE OVER CALORIES GIVEN
				mgm./hr.	*per hour*	
4	4-17-31	Basal	0.83	104	11.73	
	4-17-31	Lecithin 70 gm.	0.83	142	11.58	0
	9-28-31	Basal	0.80	83	14.11	
	9-28-31	50 gm. lard	0.77	92	15.71	2.9
5	5- 1-31	Basal	0.83	62	13.98	
	5- 1-31	Lecithin 90 gm.	0.84	81	15.01	1.9
	9-25-31	Basal	0.91	139	14.05	
	9-25-31	Olive oil 55 gm.	0.79	136	16.45	4.9

Alcohol checks 0.669 ± 1.1. High −0.681. Low −0.657

The experiments in which lecithin was given intravenously were controlled by observing the effects of introducing equivalent volumes of 0.65 and 0.9 per cent saline solution on intervening days.

RESULTS. The data of table 1 illustrate the metabolic changes following oral administration of lecithin in comparison with those after feeding approximately iso-caloric quantities of neutral fat to the same animals. Comparison with basal determinations show that the non-protein R.Q. is unaffected by lecithin. The urinary nitrogen was substantially increased. In dog 4 which had received 70 grams of lecithin by mouth or approximately 150 per cent of the 24 hour basal caloric requirement, the heat production was unaffected. Dog 5, after 90 grams, showed a rise in metabolic rate, averaging through the sixth hour 15.01 calories per hour or an increase of 7.4 per cent. In this dog all doses (60–90 grams) exerted a small specific dynamic action, the average for all being 1.9 per cent of the calories

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given. As shown in figure 1 the peak of the heat production is reached in the sixth hour. This specific dynamic effect is small as compared with the figure of Murlin and Lusk (1915) for neutral fat (4.1 per cent) or with values obtained on the same dogs in this investigation after feeding iso-caloric quantities of neutral fat (table 1 or fig. 1). Furthermore, the latter values were associated with a considerable depression of the respiratory quotient.

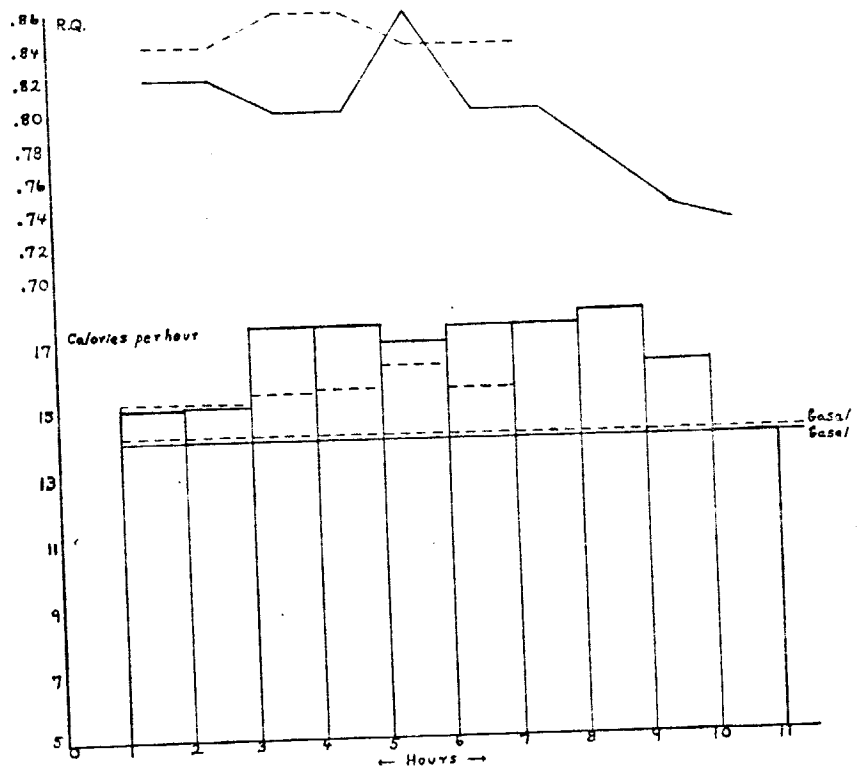

Fig. 1. Dog 5. Hourly changes in heat production and non-protein R.Q. after feeding approximately iso-caloric amounts of neutral fat (heavy line) and lecithin (dotted line).

These and other experiments indicate that lecithin per os has a much less dynamic effect than iso-caloric amounts of neutral fat and in some animals may exert no effect whatsoever. The first possibility that suggests itself for explaining the small or negative effect of lecithin is that it is not readily absorbed. To test this possibility intravenous introduction under controlled conditions was necessary.

Before discussing these results it is necessary to consider briefly the effects of saline infusions themselves. The results on two dogs are included in table 2. The basal determinations, 7 for each dog, show good agreement varying by ±1.3 and ±1.5 per cent respectively. The alcohol checks average 0.665 with a mean variation of ±1.1 per cent. The heat production followed for 5 hours after injection showed interesting

TABLE 2

Intravenous administration of lecithin

Dog	Condition	Non-protein R.Q.	Urinary N	Calories per hour: Average	Calories per hour: High	Calories per hour: Low	Per cent increase over basal
			mgm./hr.				
5	Basals	0.85	89	13.95	14.23	13.63	
	100 cc. 0.65% NaCl	0.92	105	14.87	15.59	14.30	+6.5
	Lecithin 100 cc. emulsion						
	3 gm.	0.94	120	13.96	13.96		0
	5 gm.	0.80	108	14.50	14.54	14.44	+3.8
	9 gm.	0.81	112	15.01	15.23	14.83	+7.5
	Average	0.85	113	14.49	15.23	13.96	+3.9
6	Basals	0.85	91	12.84	13.07	12.49	
	100 cc. 0.65% NaCl	0.90	125	12.59	—	—	−1.9
	Lecithin 100 cc. emulsion						
	3 gm.	0.97	130	13.19	13.53	12.85	0
	5 gm.	0.86	120	12.46	—	—	0
	9 gm.	0.89	124	12.34	—	—	0
	Average	0.91	125	12.67	13.53	12.34	0
	*Basal	0.84	110	12.49			
	5 gm. lecithin	0.86	120	12.46			0
	*Basal	0.86	95	12.81			
	18 gm. lecithin	0.99	—	19.61			+53.0
	Alcohol checks	0.665			0.679	0.651	
			Murlin and Riche				
	Basals	0.80		14.61			
	Lard 3 gm.	0.75		15.33			+7.0

* Basal and experiment immediately below performed on the same day.

results. It varied in different dogs but was constant in the same animal. For instance, in two experiments on dog 6, injection of 100 cc. of 0.65 per cent salt solution did not modify the heat production but in four experiments on dog 5 the heat production was raised an average of 6.5 per cent. The increased heat production following intravenous infusion of saline was associated with the introduction of fluid; mere insertion of a needle and

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conduction of the routine manipulations were found to have no such effect. The dogs displayed no nervousness or movements in the metabolism chamber which could account for the effect observed. The hypotonicity of the solution could also be ruled out since injection of 0.9 per cent saline gave similar results in the same dogs. Saline infusion also increased the nitrogen excretion by 22 per cent and elevated the R.Q. by an average of 0.06.

While the explanation of these facts is not clear, the possibility that intravenous injections of considerable quantities of saline may have a calorigenic effect in some dogs deserves emphasis, because it is so generally disregarded in evaluating metabolic data from experiments in which substances are given intravenously.

A survey of the literature shows that Wilhemj, Bollman and Mann (1931) reported an increase in heat production of approximately 30 per cent following intravenous use of hypertonic solutions of sodium chloride; however, 50 cc. of 0.9 per cent solution of salt had no such effect. An examination of their charts indicates that physiological saline may elevate the basal metabolic rate by 5 per cent; a figure only a trifle less than that found in my experiments.

Observations such as these make it important to determine the degree of susceptibility of each animal to saline infusion before attempting to interpret the effect of intravenous doses of lecithin. Exercising this precaution, the effect of lecithin, given intravenously, is noteworthy in its negativity.

The data of table 2 illustrate typical responses. In dog 5 the caloric output was increased on an average 4.6 per cent (5 experiments) as a result of intravenous doses of 3 to 9 grams of lecithin. The largest increase (7.5 per cent) occurred after giving 9 grams, but was only a trifle larger than the average due to salt solution (6.5 per cent).

In dog 6, three to nine grams of lecithin intravenously caused no discernible change in heat production, the percentage change ranging from 0 to −1.4 per cent. These slight or negative dynamic effects are still more striking when we consider that the larger doses (9 grams) are sufficient to increase the blood lecithin approximately 700 per cent. The non-protein respiratory quotients also gave no indication that lecithin is oxidized when a state of artificial lecithinemia is produced. In both dogs the quotient increased greatly after the injection of 3 grams. In dog 6 it also rose slightly after larger doses; in dog 5 it fell slightly below the basal level. A glance at the figures for hourly nitrogen excretion also shows no greater deviations than those following saline injection alone.

These effects are in striking contrast to the observations of Murlin and Riche (1915) who found approximately a 7 per cent increase in heat production, together with a decrease in the respiratory quotient after intravenous injection of 3 grams of emulsified fat. In view of the observations reported here, the question may be raised whether the increased heat production might not be attributed to an effect of the saline used, but this would not explain the decrease in respiratory quotient found.

Several attempts were made to increase the amount of lecithin injected beyond 1 gram per kilo. For example, dog 6 (table 2), was finally given double this dose (18 grams). The data show that the average heat production was increased 53 per cent over the basal, but the R.Q. increased to 0.99. The maximum heat production occurred during the third and fourth hour when a 75 per cent increase was noted. Such experiments could no longer be considered physiological for the animal later developed hemoglobinemia; the temperature rose to 40°C. for a few hours, paralysis of the legs developed and the animal died. Since lecithin is a hemolytic agent the caloric effect and attendant fever were probably due to a toxic action of lecithin rather than to its oxidation.

On the basis of all these results obtained in an investigation extending over a year it is concluded that lecithin introduced into the gastro-intestinal tract or directly into the blood stream exerts little or no specific dynamic effect, does not reduce the respiratory quotient and when given intravenously has no effect on nitrogen excretion by the kidney. The corollary logically follows that lecithin either plays no part as an intermediary in fat oxidation or that it is burned so slowly that it escapes detection. This also fits in with the observation of Nerking (1909) and Bloor (1914) that injected lecithin remains in the blood stream for many hours, whereas fat similarly injected is quickly removed (15 minutes) by the tissues. The body apparently does not have facilities for the removal of large excess of lecithin, either by storage or combustion.

SUMMARY

1. The possibility that lecithin is an interemdiary in the metabolism of fat was studied on dogs by determining the changes in heat production, in the R.Q. and in urinary nitrogen after feeding lecithin and injecting it intravenously.

2. In addition to control of the apparatus through alcohol checks and of the animal through repeated determinations of its basal metabolism, the effect of saline injections *per se* was taken into account when lecithin emulsions were administered intravenously.

3. The results show that some animals respond to intravenous injections of saline by increased nitrogen excretion and a slight augmentation of heat production which cannot be overlooked in evaluating the effects of lecithin injections.

4. Results indicate that lecithin given *per os* has a specific dynamic effect much less than an iso-caloric amount of neutral fat and that when

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given intravenously in amounts up to 1 gram per kilo has no calorigenic effect at all.

5. These metabolic studies therefore offer but little support for the view that lecithin is concerned in the intermediary metabolism of fat or that it is an available source of material for oxidation.

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THE RELATION OF PHOSPHOLIPIDS TO FAT IN DAIRY PRODUCTS

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It is apparent from a study of the literature on the phospholipid content (as lecithin) of dairy products that there is a wide variation in the per cent "lecithin" between different milk products and also in the per cent "lecithin" in the fat extracted from the different products by the Mojonnier method.

This paper, in a measure substantiates previous work on the "lecithin" content of dairy products and offers an explanation for the results obtained.

REVIEW OF THE LITERATURE

Chapman (4) found that the per cent "lecithin" in the fatty extract obtained by the Mojonnier method was highest in buttermilk (20.25%), next highest in skim milk (10.78%) and milk (1.6%) and lowest in cream (.43%). This agrees with the work of Horrall (8) who reported as follows: buttermilk (19.66%), skim milk (13.91%), raw sweet cream (.428%), milk (.85%), butter from raw sweet cream (.232%). Holm, Wright, and Deysher (6) reported the following amounts as per cent lecithin in the product: whole milk, .0337; skim milk, .0169; 41% cream, .1816; butter, .1819; buttermilk, .187%. When calculated on the basis of per cent "lecithin" in the fat, these figures show close agreement with the results of Horrall and Chapman.

Perlman (13) showed that the phospholipid content of the fatty extract of fresh cream shows a variable decrease with the increase in the fat content of the cream indicating a variable loss of the original milk phospholipids in the skim. The lipide phosphorous per unit of fat was found to decrease between 58 and 80 per cent fat. Close agreement with the foregoing was reported by Jack and Dahle (10).

An extensive review of the literature on the "lecithin" content of dairy products is beyond the purpose of this paper and hence will not be presented; however, should one be desired, see Horrall (8) and Holm, *et al.* (5).

METHODS AND RESULTS

The term phospholipid is used to designate the fat like substances containing phosphorus that are extracted from milk and its products by the Mojonnier method. For convenience of comparison of the results obtained here with other results, the phospholipid was reported as per cent "lecithin" both in the product and in the fat extracted from the product using the for-

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mula given by Horrall (8), which closely approximates those of Bird, *et al.* (1), Holm, *et al.*, (6), and others.

Phospholipid was determined as described by Horrall (8) except that the fat was ashed carefully over a Fisher burner and that Nessler tubes were used instead of a colorimeter. Samples were run in duplicate; on the basis of per cent "lecithin" in the product, the difference between duplicate determinations varied from 0.000 per cent to 0.019 per cent and averaged 0.0046 per cent for all samples. Blank determinations were run frequently to insure freedom from contamination.

The milk and other dairy products used were as delivered to or made in the plant (with the exception of the cream and skim milk which were separated from tank milk in the laboratory) and all were of excellent quality. Milk was separated at 120° F. in a DeLaval S-16 separator. Creams over 60 per cent butterfat were obtained by separating 40 per cent cream. This procedure was found to give a product with the least amount of damaged fat globules. Skim milk from this operation is referred to as skim milk from reseparated cream. The butter and buttermilk were taken from commercial churnings.

All "lecithin" determinations were begun within two hours after the samples were obtained since it was found that the "lecithin" content of cream, skim milk and milk decreases rapidly with age, even when stored at 40° F. This observation is in agreement with the work of Cusick (3) Perlman, (14) and Rimpila and Palmer (15).

In experiments with washed creams, the volume of the original milk was measured (five gallons) and the resulting cream was diluted to this same volume with water at 100° F. in order to approximate the same fat percentage in the milk and maintain the same fat percentage in the cream.

"Lecithin" content of cream

The results of Perlman (13) and Jack and Dahle (10) show that as the fat content of cream increases to about 55 per cent there is a uniform increase in the phospholipid content. But above 60 per cent, they found that there was a decrease. Although they do not so state, it can be assumed that their creams were all separated from whole milk. In the results shown in Table 1 the creams over 60 per cent were separated from 35–40 per cent cream. This difference in method probably accounts for the difference in results since oiling off tests (11), microscopic examination, and general appearance of creams of high fat percentage obtained by reseparating 40 per cent cream indicated no churned or "free" fat.

Table 1 is composed of the results of several separation trials from several batches of milk extending from October to February. The milk to be separated was taken from a tank of about 10,000 pounds of milk after thorough mixing. The "lecithin" content of the milk was determined for each trial and varied from .035 per cent to .036 per cent.

TABLE 1

The "lecithin" content of cream of varying fat percentage

Fat (Mojonnier), per cent	"Lecithin" in product, per cent	"Lecithin" in extracted fat, per cent
14.14	.066	.463
16.84	.077	.459
18.89	.089	.469
21.54	.087	.406
21.90	.081	.367
22.44	.084	.374
32.00	.112	.349
36.23	.124	.344
38.45	137	.358
38.48	126	.327
38.98	.146	.374
39.10	.145	.372
39.51	.138	.349
40.23	.156	.387
40.87	.156	.382
41.00	.154	.376
41.29	.152	.369
41.78	.160	.383
41.81	.151	.359
43.69	.156	.358
43.98	.164	.374
48.11	.153	.318
50.63	.163	.323
53.34	.165	.309
54.30	.162	.300
55.81	.187	.335
56.89	.179	.314
68.43	.211	.308
72.26	.196	.271
75.21	.210	.279
75.79	.194	.256
76.15	.199	.261

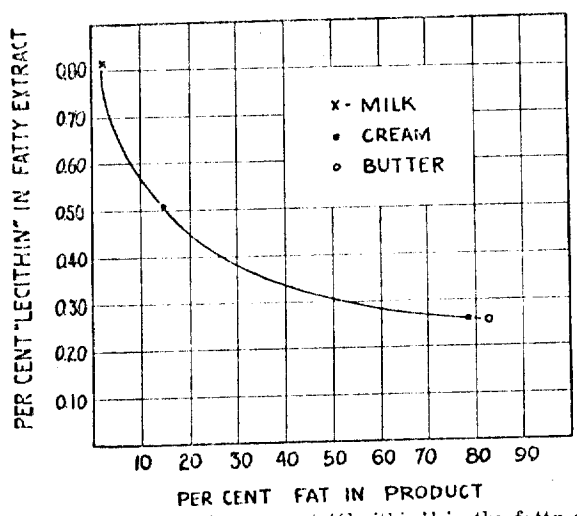

FIG. 1. The relation between the per cent "lecithin" in the fatty extract and the per cent fat in the product. (Buttermilk and skim milk not shown.)

Figure 1 shows the relation of the per cent "lecithin" in the cream (and other dairy products) to the per cent of fat in the cream, while Figure 2 represents the relation of the per cent "lecithin" in the fatty extract to the per cent fat in the cream (and other dairy products).

"Lecithin" content of other dairy products

The results shown in Table 2 for the "lecithin" content of other dairy products are in general agreement with the results of others. The per cent "lecithin" in the fatty extract is not constant but varies considerably among

TABLE 2

The "lecithin" content of dairy products

Product	Fat (Mojonnier), per cent	"Lecithin" in product, per cent	"Lecithin" in extracted fat, per cent
Skim milk	.075	.015	19.65
" "	.077	.018	24.05
" "	.066	.017	25.45
" "	.086	.017	19.79
" "	.080	.017	21.20
" "	.103	.017	16.55
" "	.077	.017	22.00
" "	.080	.016	19.97
" "	.089	.017	18.89
" "	.106	.016	14.78
" "	.096	.016	16.97
" "	.080	.018	21.90
" "	.090	.017	19.10
" "	.126	.018	14.52
" "	.076	.016	20.55
" "	.088	.016	18.42
Whole milk	5.01	.036	.720
" "	4.87	.035	.713
" "	5.50	.036	.655
" "	5.08	.036	.710
" "	5.10	.035	.691
" "	4.66	.035	.752
" "	4.71	.036	.765
" "	4.81	.035	.729
Sep. slime	1.12	.229	20.55
Buttermilk	.672	.114	16.93
"	.764	.128	16.81
"	.644	.126	19.55
Butter (sweet cream)	80.84	.216	.268
" " "	80.25	.209	.259
Butter (9 months in cold storage)	82.93	.165	.199
" " " " " "	82.69	.153	.184
Skim milk from 60–80% cream	.540	.093	17.25
" " " " "	.435	.084	19.36
" " " " "	.460	.080	17.40
" " " " "	1.378	.082	5.918
" " " " "	.196	.035	18.08
" " " " "	.192	.049	25.40

the various dairy products. It is apparently highest in skim milk, followed by buttermilk, milk, light cream, heavy cream, and butter.

The effect of washing on the "lecithin" content

The work of Rimpila and Palmer (15), Thurston, *et al.* (16), and Jack and Dahle (10) was repeated controlling as many factors as possible. Acidities were run to ascertain the amount of plasma present; oiling off tests (11) were run to determine the amount of free fat present after each washing. It was felt that as the oiling off increased, the amount of phospholipids originating from the fat might be increased, and this would give a false idea of the amount removed merely by washing. For this reason only 5 washings were employed.

Table 3 presents the decrease in the "lecithin" content of a cream containing 21.90 per cent fat and a cream containing 38.48 per cent fat. In addition is shown the observed decrease of "lecithin" due to washing and the calculated decrease.

TABLE 3

Effect of washing cream on the "lecithin" content

Sample		Fat, per cent	Acidity, per cent	Oiling off, units	"Lecithin" in product, per cent	Decrease in per cent "lecithin" due to washing	
						Observed	Calculated*
Trial I	Original	21.90	.125	.00	.081	.022	.020
	1st washing	19.46	.040	.02	.062		
	3rd "	21.49	.020	.06	.059		
	5th "	21.79	.015	.14	.059		
Trial II	Original	38.48	.105	.01	.126	.020	.021
	1st washing	36.19	.030	.02	.112		
	3rd "	40.93	.020	.17	.106		
	5th "	44.26	.015	.30	.107		

* Calculated by multiplying the per cent plasma in the product by the per cent "lecithin" in the plasma.

DISCUSSION

Alibo Horrall (8) states that possibly "the lecithin is a separate constituent of milk and is not tied up with the fat," Bird, *et al.* (2), North and Sommer (12) and Jack and Dahle (9) conclude from their work that the phospholipid material is closely associated with the fat globule. Thurston, *et al.* (16) have shown that "agitation of milk caused a reduction in the lecithin content of the cream and a corresponding increase in the lecithin content of the skim milk." That this reaction is not reversible is shown by the work of Rimpila and Palmer (15) who studied the membrane of pure fat globules dispersed in skim milk and found that the normal fat globule membrane is not derived from milk plasma.

Nevertheless, the results presented here indicate that there are some phospholipids in the plasma of milk. Since the method of determining "lecithin" depends upon the determination of ether-alcohol soluble phosphorous, the method may give erroneous results. Indeed, Holwerda (7) states that there is a phosphoric ester found in milk which gives too high a value for phospholipid percentage. This indicates that possibly the plasma contains a substance extracted by the alcohol and ethers which contains phosphorus and which would vary with the amount of plasma in the dairy products. This substance, however, is not inorganic phosphates since Bird, *et al.* (1) and Wiese, Nair, and Fleming (17) state that these are not extracted. Trials have been made here which also substantiate this conclusion.

It seems, therefore, that the material extracted is organic in nature, yet that some of it is present in the plasma of milk. If simultaneous equations be set up as follows:

$$\left(\frac{12.2}{100}\right) x + \left(\frac{87.8}{100}\right) y = .060$$

$$\left(\frac{15.5}{100}\right) x + \left(\frac{84.5}{100}\right) y = .070$$

In which x = the per cent "lecithin" in the fat and y = the per cent "lecithin" in the plasma. The per cent "lecithin" in a cream with 12.2 per cent fat and 87.8 per cent plasma is .060; .070 is the per cent "lecithin" in a cream with 15.5 per cent fat and 84.5 per cent plasma. (This data was obtained from Figure 2.) Solving, x = 0.326% and y = 0.0230%.

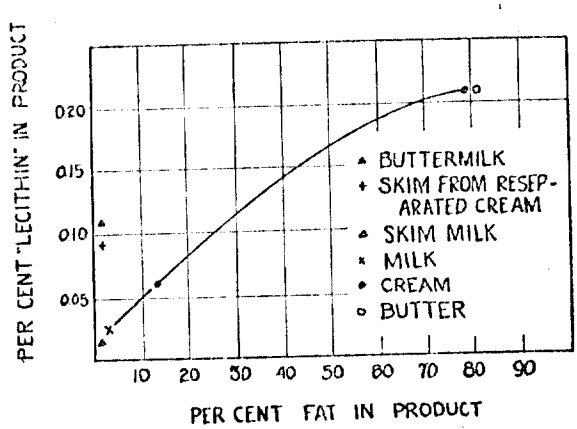

FIG. 2. The relation between the per cent "lecithin" in the product and the per cent fat in the product.

By solving many such equations and taking an average of the various x and y values, results are obtained which approximate the per cent phospholipid in the plasma and in the fat of creams of various fat percentages.

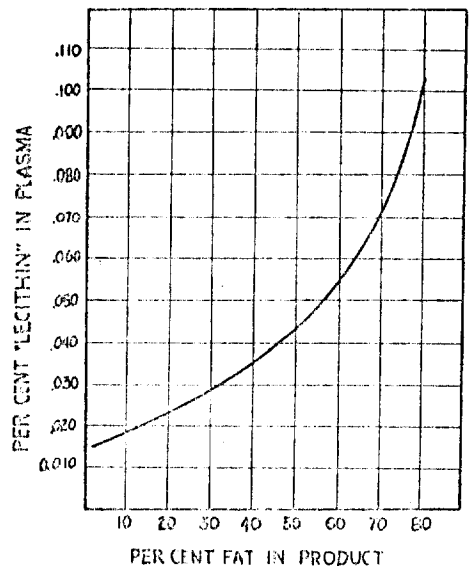

FIG. 3. The relation between the per cent "lecithin" in the plasma and the per cent fat in the product. (Not to be applied to buttermilk and skim milk from reseparated cream.)

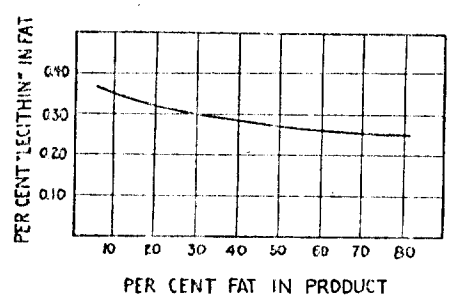

FIG. 4. The relation between the per cent "lecithin" in the fat and the per cent fat in the product.

These values are shown graphically in Figures 3 and 4 which may be further explained as follows:

In the milk products considered here a portion of the "lecithin" is associated with the fat and a portion is in the plasma phase. Figures 3 and 4 give the percentages of lecithin in the plasma and associated with the fat, respectively, as a function of the fat content of the product. The total "lecithin" content of the product can be calculated by dividing the percenage of fat in the product by 100 and multiplying by the corresponding percentage of "lecithin" (Figure 4) and adding to this value the result obtained by dividing the percentage of plasma in the product by 100 and multiplying by the corresponding percentage of "lecithin" in the plasma

(Figure 3). For example, the percentage of "lecithin" in 40 per cent cream would be calculated as follows:

$$\left(\frac{40}{100}\right)(0.29) + \left(\frac{60}{100}\right)(.035) = 0.137\%$$

(It must be pointed out that these figures may be found to apply only to milk from this section of the country and produced during the winter months.)

If the above supposition is true, then by washing cream of a given fat percentage one should be able to calculate the decrease in the per cent "lecithin" in the plasma from the total amount. This result is recorded in Table 3 and the calculated and observed decreases check closely.

The high "lecithin" content of buttermilk and skim milk from reseparated cream, may be explained by this method. According to Figure 3, the plasma of 75 per cent cream contains 0.09 per cent "lecithin." This plasma has the same composition as the skim milk resulting during the separation of 75 per cent cream. Consequently, it would have .09 per cent "lecithin" in its plasma. Since the plasma in such skim milk is about 99.9 per cent, there would be:

$$\left(\frac{99.9}{100}\right).09 + \left(\frac{.1}{100}\right).25 = .090\%$$

This agrees with the measured amount present. The "lecithin" content of buttermilk may be explained on the same basis.

It is possible that the statement of Thurston, *et al.* (16), previously referred to explains the increase in "lecithin" in the plasma as the fat in the product is increased, *i.e.*, there is a greater movement of "lecithin" from the fat to the plasma due to the increased mechanical agitation during the separation of creams of higher fat percentage.

SUMMARY AND CONCLUSIONS

1. The per cent "lecithin" in various dairy products was found to be as follows: skim milk, 0.015–0.018 per cent; raw whole milk, 0.035–0.036 per cent; skim milk from reseparated cream, 0.035–0.093 per cent; pasteurized sweet cream, 0.066–0.199 per cent; buttermilk, 0.114–0.126 per cent; butter, 0.153–0.212 per cent; separator slime, 0.229 per cent.

2. The "lecithin" in the products studied was found to be present partially in the plasma and partially associated with the fat.

3. The results obtained indicate that as the fat content of the product increases, there is an increase in the relative amount of "lecithin" in the plasma and a decrease in the relative amount associated with the fat, except in buttermilk and skim milk from reseparated cream.

4. The "lecithin" content of the fat and plasma in buttermilk and skim milk from reseparated cream is approximately the same as the "lecithin" content in the fat and plasma of the butter and 75–80 per cent cream from which they are derived. This accounts for the abnormally high "lecithin"

content of these two products compared to other products of a like fat content, *i.e.*, skim milk and whole milk.

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A Review of the Technical Applications of Soybean Lecithin*

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SINCE the introduction of lecithin into this country from abroad some twenty-odd years ago, there has developed a new industry devoted solely to the recovery and marketing of this commodity. The lecithin industry in itself is comparatively small but robust, serving today a host of varied industries. It is a tribute to the few enterprising individuals who, recognizing the potentialities of lecithin, introduced it to American industry.

The acceptance of lecithin by industries who use it advantageously was a slow process. Those who introduced it had to begin with only a limited knowledge of its basic properties and functions. Potential users in most cases adapted it to their processes only after prolonged experimentation. Considerable knowledge of the applications of lecithin has accumulated but, aside from patented uses, relatively little information is to be found in published literature. The recovery and purification of lecithin from solvent extracted soybeans is generally known to this group and will not be considered in this paper.

In the interests of clarity and convenience the applications of lecithin will be segregated into two general classifications:

1. Those based on its surface active properties.
2. Those based on its chemical properties.

The applications based on the surface active properties are most numerous and offer unlimited potential new uses. Lecithin, basically, is a surface active substance, and a clear understanding of what is meant by a surface active agent is fundamental. Fischer and Gans (1) define a surface active agent as a compound which in small relative quantity modifies the physical properties of a heterogenous system by adsorption at an interface. The change is considerably greater than any law of mixtures would predict. The term wetting agent is often used instead of surface active agent and in a sense further defines a surface active agent. Bartell (2) states that one of the most commonly occurring phenomena is the wetting of solids by liquids. Processes involving wetting are encountered continually. Life itself is possible only because certain liquids are properly wetting the different solid constituents of the body. Important technical processes are fundamentally dependent upon the degree of wetting of solids by liquids. Surface active agents are not new; soap, the commonest, has been used for centuries; and sulfonated oils have been used for a long time. However, the greatest advance in development of surface active agents has been since 1925 when a number of synthetic products appeared. With these came a clearer understanding of the functions and properties of surface active agents. Most surface active agents have a fairly large molecular structure having a long chain aliphatic group known as the hydrophobic group and a solubilizing group commonly called the hydrophilic group.

LECITHIN has a molecular structure commonly associated with surface active agents. The long chain fatty acid groups constitute the hydrophobic group, and the phosphoric acid-choline group, the hydrophilic group. Lecithin is generally used in non-aqueous systems since it is not soluble in water. It is widely used in systems composed of solids dispersed in fatty vehicles. Specifically, one of the earliest established uses of lecithin was its use in chocolate coatings. Here we have a heterogeneous mixture consisting of cocoa butter, sugar, and cocoa fiber. Sugar, a hydrophilic solid, is not readily wetted by cocoa butter. Since the introduction of a small amount of lecithin in the order of 0.25% produces thorough wetting of the sugar by the cocoa fat, the viscosity of the mixture is reduced sometimes by more than 50%. Chocolate coatings can be produced with stabilized viscosities and reduced cocoa butter. The confectionery industry uses lecithin as an emulsifying agent in fat containing confections. Thorough emulsification of the fat results in improved eating qualities, facilitates cutting and wrapping, and longer shelf life; 1% of the fat content is used. Lecithin has long been used by the margarine industry where it serves as an antispattering agent and a means of keeping the milk solids from sticking when margarine is used for frying. Here we have a water in oil emulsion in which milk solids are dispersed. When margarine is heated as in frying, water is driven off and the

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milk solids are precipitated. Lecithin added in the amount of 0.10% to 0.25% acts at the fat and water interface prevents coalescence of the water with the result that the evolution of water is gradual and small droplets prevent spattering. At the same time wetting the milk solids with the surrounding fat causes them to remain freely movable without sticking.

The development of bread dough is a complex physical and chemical phenomenon. Interface modifiers can and do exert profound changes. Lecithin, when added to doughs to the extent of 3 to 4 ounces per 100 pounds of flour, produces more even dispersement of the shortening and a drier handling dough and retards staling. Pan lubricants are made more effective with the addition of 0.5 to 1.0% lecithin. In the production of icings better flow and higher gloss is possible when lecithin is added, again because it promotes thorough wetting of the sugar by the shortening. The biscuit and cracker industry have used lecithin for many years in short-bread type of cookies. The incorporation of lecithin to the extent of 1%, based on the weight of shortening, gives a more uniform batter and results in cleaner webs and dies.

Emulsions technology, as is well known, is extremely complicated. The use of surface active agents as emulsifiers, stabilizers, etc., are requisites in emulsions. Lecithin finds widespread applications in emulsions for many purposes. It is used as the emulsifying agent or as a stabilizer. The amount of lecithin to use will vary depending upon the type of emulsion, degree of stability required, etc., but most generally lies between 1 to 5%. The cosmetic industry uses considerable quantities of lecithin in creams, lotions, lipstick, and permanent hair wave preparations. In certain creams, particularly the so-called nutrient creams, as much as 5% of lecithin is used, while in others only fractional percentages. The emollient properties of lecithin are also utilized in soap products, particularly liquid and powdered industrial hand soaps.

IN the industrial field lecithin has many applications in protective coatings, leather, textiles, rubber, and plastics. In the production of paints and printing inks considerable time and energy are expended in grinding pigments. The grinding operation is not for the purpose of further reducing the particle size of pigment particles, but to disrupt aggregates of pigment particles into individual particles and to wet the pigment particles with the grinding vehicle. A vehicle with poor wetting properties necessitates longer or repeated grinds. A vehicle with good wetting properties, on the other hand, will require only minimum grinding. Poor wetting vehicles, to which a surface active agent is added, very often become good wetters. Since most paint and printing ink vehicles are oils or varnishes, an oil soluble wetting agent will prove most effective. Lecithin is one of the most effective surface active or wetting agents for paint systems. Paint systems are complicated in that the large number of pigments used each have their peculiar properties. The same is true of vehicles which range from raw linseed oil, through bodied oils and varnishes to synthetic resins which may contain glyceride oils or be only a solvent solution of the resin. The working properties of a given pigment dispersed in raw linseed oil, for example, may be altogether different if dispersed in a short oil alkyd resin. Surface active substances can alter working properties of pigment dispersions markedly. A dispersion having a short buttery consistency may become free flowing by adding a surface active substance such as lecithin. Surface active agents in some dispersions produce less length or give the dispersion a slight puff. Hence the use of lecithin in controlling sagging or curtaining in architectural enamels. Flooding and silking are phenomena peculiar to the paint industry. They occur usually when two or more pigments are used in the formula and shows up as a mottled appearance in the case of flooding and as minute streaks in the case of silking. Silking, however, can occur in single pigment systems. The cause of neither phenomenon is clearly understood. There are many theories. Nevertheless, lecithin often does help in correcting this condition.

It is extremely difficult to lay down specific rules for the use of lecithin in pigment dispersions. Generally the optimum amount will be found to be around 1.0% of lecithin based on the pigment weight. The exact amount, method of addition, and point of addition can best be determined experimentally.

The use of lecithin by the leather industry is chiefly as an ingredient of fat liquors for soft leathers, such as garment leather or shoe uppers. It produces softness and fullness of feel without impairing tensile strength; 1% of lecithin in the fat liquor is commonly employed.

Lecithin is used by the textile industry as a lubricant in spinning woolen yarn, but most particularly as a softening and finishing agent to improve the hand or feel of fabrics, particularly cotton and rayon. The production of softening or finishing agents is a highly specialized art and lecithin is generally only one component of several used. Lecithin by itself will produce a very desirable soft finish on fabrics, but the finish is not as permanent as that obtained with some of the synthetic products now available.

IN the rubber and plastic industries lecithin is being successfully used as a mold lubricant. It is generally milled into the molding stock. While under pressure during molding, the lecithin between the stock and mold interface is an efficient lubricant. The molded pieces are more easily removed and have an exceptionally smooth surface. The molding of intricately shaped pieces in particular is facilitated. Approximately 0.5% lecithin is required. Certain insecticides are improved by the addition of lecithin because of its wetting and spreading properties. The U. S. Dept. of Agriculture [5] has found lecithin an effective deposit builder in nicotine, bentonite, and lead arsenate sprays for control of the codling moth using from 0.20 to 1.5 pounds per 100 gallons of spray mixture.

The applications of lecithin due to its chemical properties include its use as an antioxidant and its use for nutritious and therapeutic products. The applications of lecithin as an antioxidant are well known. There are differences of opinion as to which constituent of soybean lecithin is responsible for its antioxidant properties. It is generally conceded that the cephalin portion contributes more to its antioxidant properties than all others. Be that as it may, lecithin does show marked antioxidant effects. It has long been used in oleo oil, lard, and other edible fats

The work of Riemenschneider and others (3) on the synergistic effects of lecithin with fatty acid esters of d-isoascorbic acid and alpha-tocopherol are also well known. Probably not so well known is its use as an antioxidant in fish liver vitamin oils. Buxton (4) has recently reported excellent results in this field using mixtures of lecithin and alpha-tocopherol. New antioxidants, many of which show greater antioxidant activity on accelerated tests than lecithin, have appeared in recent years. But lecithin in spite of its lower protective properties has certain inherent advantages. It is readily soluble in fats and oils, is a wholesome edible product, has little or no effect on flavor, and is available at low cost. Lecithin will probably always be a considerable factor in the utilization of antioxidants. In the petroleum industry (6, 7) lecithin is used as a stabilizer for gasoline containing tetra ethyl lead and as an additive in lube oils. About 10 pounds of lecithin per 1,000 barrels of gasoline is used to inhibit clouding, also to counteract corrosion of fuel tanks and liners, particularly in aircraft. About 0.5% lecithin is used in lube oils.

As for applications that have to do with nutritional or medicinal uses, soybean lecithin contains approximately 3% of choline, recognized as an important dietary factor. Specifically, clinical tests have shown the effectiveness of lecithin in prevention of fatty livers and in the treatment of psoriasis. The utilization of vitamin A and carotene are also enhanced by lecithin. Research is being continued on this field, but already lecithin is being utilized in considerable quantities for both human and animal nutrition.

The uses outlined are all fairly well established; however, the versatility of lecithin and its present low cost have stimulated research for new uses in entirely new fields. The possibilities for new applications are virtually unlimited.

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CYTOPATHOLOGICAL STUDIES OF MORPHINE POISONING AND CHRONIC MORPHINISM IN THE ALBINO RAT, WITH REFERENCE TO SUBSEQUENT LECITHIN TREATMENT *

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I. Introduction

Recently Wen Chao Ma [1] has described in detail the cytopathology of acute and chronic morphinism in the albino rat, as well as suggested the importance of lecithin administration as a therapeutic agent during recovery from chronic morphine poisoning. Apart from reporting the pathological action of morphine upon the tissues as a whole, this author made a detailed, systematic cytologi-

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cal investigation of the behavior of the cell inclusions during temporary and prolonged treatment. These observations led him to conclude that by studying their consistent and distinctive reactions to the drug during the various phases of poisoning he was able to employ both the Golgi apparatus and the mitochondria as indicators of physiological depression under pathological conditions.

Bearing in mind the recent controversies concerning the functional significance of these cell organoids the contentions of Ma are of importance. Therefore, in view of these significant results and partly because this problem covers certain ground in which there is, apparently, some misconception, it was deemed advisable to reinvestigate this work, not only by applying more recent cytological procedures but also by including additional experiments that might lead to more conclusive results.*

II. Material and Techniques

Albino rats of both sexes, segregated shortly after weaning, belonging to the same strain and weighing approximately 195 to 215 gm., were selected for the study of acute and chronic morphinism, while animals from the same litters were isolated and kept as controls. Subcutaneous injections of morphine hydrochloride administered hypodermically were used throughout the experiments, and in order to induce acute poisoning each rodent received a single dose of 5 cc. of a 2 per cent solution (10 mg. of morphine). The rats set aside to become addicts were first given an inoculation of 3 mg. as a 1 per cent solution in water, the strength of which was increased by 1 mg. every 10 days. After a period of 7 months the daily doses of morphine were discontinued.

Tissues were obtained and fixed from those rats under acute morphinism, at intervals ranging from 1 to 24 hours after injection. In the addicts material was taken at various periods (as indicated in the text), both before and after the daily injections of the drug were discontinued.

The lecithin used was prepared by Merck from eggs; 1 gm. was mixed with 40 gm. of ordinary rat diet of vegetables and bran. A second group of rodents, of precisely the same weight and age as the first, was isolated and fed for a period of 7 months on 4½ gm. of lecithin mixed with 20 gm. of regular food, and given to the animals in the ordinary way.

Glandular Tissues: The glandular tissues studied in this investigation consisted of liver, pancreas, thyroid, submaxillary gland, kidney, stomach and intestine.

Nervous Tissues: The nervous tissues observed were spinal ganglia, Purkinje cells of the cerebellum, motor cells of the spinal cord, as well as the large pyramidal neurones of the cerebral cortex.

* I wish to take this opportunity of thanking Dr. E. V. Cowdry, not only for his kind hospitality, but also for his encouraging interest in this investigation.

Muscle Tissue: The smooth muscular coats of the intestine, skeletal and cardiac muscles were employed.

General Cytological Preparations: For general cytological preparations the tissues were cut into very small pieces in order to ensure rapid penetration of the reagents. Overnight fixation in ice cold Flemming's solution was used, as recommended by Ludford,[2] omitting the 0.5 per cent urea. Sections were stained in iron alum hematoxylin following bleaching with hydrogen peroxide.

Mitochondria: Tissues were treated by Regaud's fixative, in which the sodium sulphate was omitted, and then stained with acid fuchsin and methyl green. This method gave excellent results for all tissues other than brain and spinal cord, for which the Champy-Kull anilin fuchsin technique is recommended. Flemming's solution without acetic acid, followed by staining with Heidenhain's hematoxylin, together with the modifications mentioned by Ma, Lim and Liu[3] was also employed.

Golgi Apparatus: The most successful results were obtained by Ludford's variation of Nassonov's Golgi technique. Instead of staining according to the Volkonsky method, orange G in 95 per cent alcohol was found to give preferable results. In some cases the Nassonov preparations were stained by the Kull method, using toluidin blue and anilin fuchsin. Dehydrating and embedding were always completed in one day, and excellent preparations were obtained by using carbon di-sulphide as a clearing agent. Experiments in times of fixation showed that brain, spinal cord, pancreas and liver render better Golgi bodies after a prolonged fixation of 36 hours instead of the prescribed 24, followed by osmication up to 5½ days at 35° C. Hirschler's modification (1918) was also employed, but the results obtained were more inconsistent than those of the above method. Da Fano's cobalt nitrate (formalin 15 per cent) and Cajal's uranium nitrate techniques were occasionally used. When preparing the reducing fluids for these silver techniques the sodium sulphite was excluded, and the times of fixation for gland tissues were reduced to 3 to 4 hours. Brain and spinal cord, on the other hand, were fixed for 8 to 10 hours.

For studying the mineral organization of the normal and experimental tissues the microincineration procedure, by which the protein compounds of the cells are burned out leaving behind only the mineral residue, was used. The method is similar to that recently described by Policard.[4] Small pieces of tissue were fixed in a solution of 9 volumes of absolute alcohol to 1 volume of neutral formalin. After fixation for 24 hours the material was passed through several stages of absolute alcohol to ensure a complete dehydration and also to remove the formalin. The material was embedded in the usual manner and sectioned at 5 microns. The preparations were then placed on a small quartz slab and transferred to a special electric quartz oven and incinerated at temperatures varying from 625° to 650° C, for periods of 25 to 45 minutes.

The arrangement and deposition of the mineral constituents in the incinerated sections were studied in dark-field illumination obtained by using a Zeiss cardioid condenser. A large sized Spencer Mazda microscope lamp, catalogued as number 394 and equipped with a 500 watt Mazda gas-filled concentrated filament bulb, was used. Directly in front of the bulb was a pair of 4½ inch condensing lenses, which threw a converging beam of light through the cone, illuminating an area about 2 inches in diameter. A pale blue frosted glass, inserted in front of the cone and placed 4½ inches from the substage mirror of the microscope, gave the best illumination for observing the mineral distribution in the incinerated tissues.

The Altmann technique for fixation by drying while freezing, as recently elaborated by Gersh,[5] was also employed for studying the pancreas and liver.* As time would not permit, only normal material and tissue from a rat that had previously received the prescribed single large dose of morphine † were examined by this method 5½ hours after administration of the drug. This procedure consists mainly in freezing the tissue quickly by liquid air prior to dehydration *in vacuo* at −20° C. After embedding in paraffin the material was mounted dry, sectioned at 8 microns and stained by Heidenhain's iron hematoxylin and counterstained in eosin. Tissues thus treated stain extremely rapidly, it being necessary to leave them in the hematoxylin only for 30 to 60 seconds. The advantages of this technique, apart from the rapidity of fixation, are that it should eliminate the possibility of shrinkage, for Gersh contends that when a portion of tissue is maintained at a temperature of −20° C, and dehydrated by means of the Altmann apparatus, the volume of the block of tissue remains unchanged after the process.

III. On the Functional Significance of the Cell Organs and their Behavior During Cell Injury and Disease

Before the effects of morphine poisoning upon the general cytology of the cell can be interpreted it will be well to discuss briefly the possible functional significance of the several cell structures under normal and abnormal conditions.

A review of the literature dealing with the physicochemical nature of mitochondria shows that an enzymatic conception of mitochondrial activity is gradually being realized.[6,7,8] This outlook, from a chemical point of view, is established largely on the investigations of Marston[9] and his co-workers, who conclude, on the basis of their reaction to azine dyestuffs, that mitochondria contain proteolytic enzymes. In addition to this, Robertson,[10] who has carried out an independent study on the function of the lipoid in the mitochondria, has shown that at the phase boundary of the mitochondria and protoplasm synthesis by enzymes may occur. These conclusions derived from the chemical study of the problem receive support when correlated with certain cytological evidence. The assertion of Cowdry,[11] wherein the phase boundary and the surrounding protoplasm of mitochondria are regarded as the seat of processes of

* I wish to express my gratitude to Prof. R. R. Bensley for his hospitality during my visit to his laboratory, and also to Dr. Gersh for kindly fixing the above-mentioned tissues.

† As morphine hydrochloride was not available for this experiment, morphine sulphate was substituted. As far as is known there is no difference in chemical action.

elaboration, commencing with the absorption of molecules of certain solutes, and ending in a series of physical and chemical interactions beween the mitochondria and the incoming substances, is of interest, as this would naturally lead to the production of new compounds of widely different natures. These theories explain, to a large extent, the behavior of mitochondria within the animal and plant cells, where they appear to be closely associated with the production of cellular materials.[12-15]

Another school of thought, however, brings forward evidence to show that the Golgi apparatus alone is associated with secretory processes, and that during this phenomenon the mitochondria play no active rôle. There seems little doubt that the process of fat absorption in the mammalian intestine is correlated directly with the Golgi apparatus.[16] During secretion in the thyroid gland it has been conclusively shown that both the Golgi bodies and the mitochondria are engaged in the process of secretion.[17]

Ma, Lim and Liu,[18] on the other hand, have taken an independent course and conclude that "the Golgi material is the lipoid or fatty component of the mitochondria, which becomes demonstrable on the dissociation of the mitochondrial substance."

Ludford,[19] assuming a broader view of the problem, has taken into consideration the function of the Golgi bodies and correlated it with an enzymatic conception of mitochondrial activity and concludes that synthesis by enzymes may occur at the protoplasmic interface. The resulting products continually diffuse into the cytoplasm, thereby preventing an accumulation at the surface of the mitochondrium, which would inhibit further synthesis. He suggests that these elaborated cell products are concentrated into droplets at the surface of the Golgi apparatus prior to their elimination.

Ludford's theory of a functional interrelation existing between the Golgi apparatus and the mitochondria furnishes an explanation for the behavior of these structures during cellular metabolism, and deserves serious consideration.

Now that the function of these bodies has been considered during normal metabolism it will be necessary to review briefly their response to abnormal conditions and disease. The interesting question arises, and incidentally is still a matter of debate, as to whether or not the reaction of cell inclusions to pathological conditions can be accepted as a diagnostic criterion of disease.

Earlier authors, among whom were Tello,[20] Fananás[21] and Legendre,[22] first reported a definite alteration and behavior of the Golgi apparatus during malignancy, while several others have arrived at contrary conclusions. The general literature in this field is far too conflicting to warrant any detailed discussion, but nevertheless some of the more recent work in relation to the response of these cell organs, following cellular injury and to the administration of chemicals, is of extreme importance when considering the cytopathological action of morphine. In this field experiments of Policard, Garnier and Scott are of interest. The former authors reported that the administration of phlorizin causes a notable decrease in the amount of mitochondrial substance within the tubules of the kidney.[23] Scott,[24] when observing the effect of phosphorous poisoning upon the mitochondria in the acinar cells of the guinea pig, found that these inclusions respond by eventually breaking down into fat granules.

The association of clinical symptoms with functional behavior of the cell organs in severe pathological conditions has been reported. Goetsch[25] has observed a marked increase in mitochondria in many cases of hyperthyroidism, and Homans[26] has correlated mitochondrial changes in the β cells of the islets of Langerhans with diabetic lesions.

The recent work of Ludford[27] on the biological action of X-rays on malignant growths also shows that cytological changes are induced by irradiation. In certain types of transplantable tumors he found that irradiation results in an immediate action on the mitochondria (which become vesicular within 40 minutes), while the Golgi apparatus responds by hypertrophy and fragmentation. Moreover, it is reported that after irradiation enlargement of the cells is accompanied by a definite increase in the mitochondria and an enlargement of the Golgi material.

The physiological investigations of Hall and MacKay,[7] on the effect of administration of glucose on liver cells, show that glucose causes hypertrophy and enspherulation of mitochondria. By these studies they have established a relation between the mitochondria of the hepatic cells and the glucose-glycogen equilibrium. Further evidence is afforded by Weatherford,[28] who, while observing mitochondrial changes in the initial stages of acute inflammation of connective tissue cells, has shown that they give a definite morphological re-

action to this condition and form chainlets, which finally segment into spherules, thus indicating their sensitivity to cellular injury. Nahm [29] has likewise lately shown that the Golgi elements are more readily demonstrated in certain tissues after short autolysis than in the normal.

Although the general literature concerning the changes of these cytological constituents in cell injury and pathological conditions is somewhat conflicting, the evidence as a whole indicates that they are responsive, but at the same time inconsistent in their morphological reactions to such changes. Assuming these cell organs to be the seat of enzymatic action within the living cell, the differences of opinion concerning their behavior under varying conditions might possibly be due to the fact that observations were made during different phases of cellular functioning when their morphological and staining reactions would alter accordingly.

Various authors, when writing of morphine tolerance, point out that there is no substance in the body that will neutralize the poison. Consequently it is natural that one should look for some direct change in the metabolism of the cells, which is quite likely to be morphologically expressed by quantitative and qualitative changes in their cytological components.

IV. General Observations

1. *Acute Morphinism*

Ma,[1] in his dissertation on morphinism, divides the acute reaction to the poison into the stimulated and depressed stages. The former is 1 to 5 hours and the latter 6 to 11 hours after the rodent has been given a single injection. He is able to associate these two periods of the acute condition with accompanying constant cytological phenomena, which are characteristic for each phase. During the first period he reports an increase in the Golgi material, while the mitochondria remain unaltered. In the second, the reverse condition occurs, the Golgi material becoming decreased while the mitochondria respond by a slight increase in numbers.

Detailed examinations of the tissues under acute morphinism, in the present investigation, reveal that, although the cell inclusions in most cases respond morphologically to an acute injection, the results are so variable and inconstant, even in all good cytological

preparations, that it is impossible to associate any alterations with any special phase of the acute condition. In some tissues the cytological demarcation is so slight that it is almost impossible to distinguish between normal and morphinized material. The response, in terms of morphological variations in the cell components during acute morphinism, will now be reviewed.

Liver: An examination of Figures 1–4 demonstrates the general marked differences between the tissue fixed by drying while freezing with the new Altmann technique (Figs. 1 and 2) and the portion of the same liver treated by the ordinary histological procedure (Figs. 3 and 4). The dehydrated tissue shows less affinity for stains and, viewed through the higher powers of the microscope, resembles frozen sections of living tissue. The individual hepatic cells exhibit less shrinkage, and their nuclei, showing less selection for the stain, are larger in proportion to the size of the cell than those seen after histological treatment. The protoplasm presents a granular appearance and the mitochondria, which are preserved, can be faintly distinguished. The endothelium lining the sinusoids of blood vessels is also clearly depicted; the blood cells that stain faintly resemble those observed in the living state, the erythrocytes showing little or no shrinkage.

No gross histological differences could be observed between the acute phase (Fig. 2), 5 hours after injection, and the controls (Fig. 1), beyond a slight hypertrophy of the hepatic cells. The mitochondria and other intracellular components revealed by this technique are too poorly differentiated to observe quantitative or qualitative differences.

Figure 3 depicts a section of liver fixed in Flemming's solution and stained with iron hematoxylin 5 hours after the administration of an acute dose of morphine. A general hypertrophy is likewise observed and is apparent when compared with the normal control (Fig. 4). The hepatic cells following an acute injection invariably show an abnormal number of fat globules (Fig. 3).

Liver fixed from 2 to 10 hours after the last injection, and treated by the general cytological methods, shows a variation from the normal in the number and structure of the mitochondria in the hepatic cells. These changes accompanying the acute condition are found, however, to vary considerably, even in good cytological preparations made from different rats killed at the same time fol-

lowing morphine administration. In certain animals the response to the drug, which involves granulation and hypertrophy of the mitochondria, was so slight that it was difficult to associate any specific alteration with the acute phase. This condition was not observed in all control material, but it is assumed to be a morphological reaction expressing the sensitivity of mitochondria to the poison (Fig. 5).

The Golgi bodies, even in preparations of control liver, exhibit a wide range of structural variation. As the metabolism of this organ is complex, normal and experimental material was obtained at regular intervals after feeding. Even with these precautions it is difficult to establish a definite normal structure in osmic and silver preparations. No fragmentation of the Golgi apparatus is encountered in the morphinized tissue, but a slight hypertrophy, which is inconstant and definitely not specific for any time period following the dose, is occasionally observed.

Examination of nuclear phenomena in controls and acutely affected tissue reveals no deviation from the normal type.

Pancreas: Pancreatic tissue, when treated by the new Altmann freezing method, responds well to this technique. Far less shrinkage is observed, as compared with the ordinary histological controls. The filamentous mitochondria and zymogen granules are detected but are not clearly differentiated from the cytoplasm, thus making quantitative or qualitative estimations impossible. The islets of Langerhans display less affinity for the stain than the acinar tissue. No gross histological differences can be ascertained between normal material and that under acute morphinism.

Control cytological tissue, after fixation in Regaud's fluid and staining with acid fuchsin and methyl green, is represented in Figure 7, where the deeply staining zymogen granules are seen aggregated within the nuclear zone, while the filamentous mitochondria are restricted to the basal regions of the acinar cells. Figure 6 depicts the experimental material, 5½ hours after injection, treated by the above method, and shows clearly an extreme variation in the action of the drug. In most areas the filamentous mitochondria have rounded up into granules which occasionally fuse, forming larger globules. These globules stain poorly and resemble similar structures obtained in the phosphorous poisoning experiments of Scott [24] from the mitochondria of the acinar cells of the guinea pig.

This phenomenon is more marked in some preparations than in others. Examination of a large number of sections demonstrates enormous variations, from a slight abnormality to extreme globule formation. The mitochondria in the islet cells appear to show more resistance to changes. Although the mitochondria in the pancreas of different rats under acute morphinism do not give a constant cytological picture, their general reaction appears to be more specific than in liver cells under similar conditions.

The Golgi apparatus does not show very significant changes when compared with the control material. Slight fragmentation is occasionally met with in the experimental tissue, but no marked increase in the amount of Golgi material, described by Ma during the "stimulated phase," is encountered. Similar variations are observed in tissues 2 hours after an acute administration, at which time the reaction is similar to that produced 5½ hours after the dose. The gross cytological metamorphosis pictured by Ma between these two time periods is not confirmed.

Nervous Tissues: The resistance of mitochondria to pathological conditions in nerve cells, which has been previously commented upon, has been confirmed in this investigation. The cells of the spinal ganglia, anterior horn cells of the spinal cord and the large pyramidal neurones in the cerebral cortex, together with the Purkinje cells of the cerebellum, were examined at various intervals following an acute dose. The mitochondria of these cells of the nervous system display no structural or topographical deviation from the normal. Even in material selected from a rodent that had received a second injection, 3 hours after the first failed, there are no significant alterations. Under these conditions no changes in either the arrangement or staining reactions of the Nissl substance were recorded.

The Golgi apparatus, in contrast with the mitochondria, reveals a variable response to the action of the morphine in the form of a slight hypertrophy and occasional fragmentation. However, the apparatus shows great morphological variation in most nerve cells, even under normal conditions, and varies from rod-shaped bodies to a network formation. This makes it difficult to distinguish any reaction of the neurone to changes in environment of the Golgi apparatus in terms of structural alterations. The hypertrophy and fragmentation is at times most marked (see Figs. 19, 20 and 21) when compared with control material (see Figs. 16, 17 and 18).

These results partly confirm those of Ma, who detected a similar increase in amount during the "stimulated phase" of acute morphinism, but they are contrary to the recent findings of MacEwen and Buchanan,[30] who failed to correlate any deviation from normal in nerve cells under similar conditions. Figures 16 to 18 depict the structure of the Golgi bodies in normal healthy neurones which, in all well controlled preparations, present a broken network of dense anastomosing threads. The apparatus in all larger cells displays a tendency for the network to become dispersed throughout the cytoplasm (see Figs. 17 and 18), while in the smaller cells it appears to be more closely drawn together in a compact mass around the nucleus (Fig. 16). Spinal ganglia, fixed 1 to 5 hours after morphine injection, show that acute poisoning is associated with a slight increase or hypertrophy of the Golgi material. This phenomenon, which is sometimes accompanied by fragmentation of the Golgi bodies, is depicted in Figures 19, 20 and 21. Further observations upon the spinal ganglia during Ma's "depressed period" (10 hours after injection) fail to confirm the contentions of this author. No decrease in the Golgi material is detected in tissues fixed before or after the "depressed period."

Careful studies of a great number of preparations reveal conclusively that acute poisoning is associated with a slight hypertrophy and occasional fragmentation of the Golgi apparatus, the physiological interpretation of which will be a subject of discussion in a later portion of this communication.

Intestine and Stomach: The mitochondria, under normal conditions and during acute morphine poisoning, were extensively studied in the absorbing columnar epithelium of the villi in the small intestine, which lends itself beautifully to cytological investigation. These inclusions within the normal epithelial cells are arranged longitudinally to the axis of the cell, presenting a wavy filamentous appearance (Fig. 8). In the basal or subnuclear region of the cell the mitochondria tend to aggregate and form a capsule-like structure around the nucleus, which is oval in shape and situated in the middle portion of the cell. The accumulation within the subnuclear zone, which consists of granular mitochondria, is apparently a surface tension effect due to their phosphatidal nature.[31] Material fixed after an acute injection reveals small changes in shape and size of the mitochondria. These alterations vary greatly from rod-

shaped structures to globules in preparations taken from different rats at the same time following an acute dose. The most constant feature, in the acute condition, appears to be hypertrophy of the mitochondria. An extreme breaking down and rounding off of these structures is shown in Figures 10 and 11, and contrasts with the normal condition seen in Figure 8. Extensive studies have demonstrated that acute poisoning cannot be definitely associated with any special changes in the form of the mitochondria beyond a tendency for the filaments to break down into polymorphic bodies. It must be pointed out that the mitochondrial structures vary according to the normal functional activities of the cells, and great care must be exercised not to confuse this issue with alterations in shape and size incurred as the result of the drug. It has been shown, for instance, that mitochondria in the epithelial cells of the villi in man alter from the filamentous condition into rod-shaped bodies during fasting. Therefore, it is essential to regulate the diet of the animal as well as the time of collecting the material, before ascertaining any reaction to induced pathological conditions.

The response of the Golgi apparatus to the drug can be observed best in the gastric glands of the stomach, as well as in the glands of Brunner in the submucosa of the small intestine, and can be studied to advantage when sections are cut with a slight obliquity to the long axis of the gland. Under normal conditions the individual cells forming these glands are large, their nuclei being situated in the basal region, while the Golgi apparatus is always seen in the clear area of cytoplasm between the nucleus and the apical region. The mitochondria, when in the filamentous condition, take up a position at the opposite pole of the cell and invariably form a dense mass around the distal region of the nucleus. At the onset of secretory activity the Golgi bodies, which are present in the form of individual polymorphic structures, become reduced in amount and the mitochondrial filaments give rise to granules which migrate with the secretion droplets toward the apical regions of the cell (Fig. 14). This phenomenon has led many workers to believe that the Golgi and mitochondrial substances, by means of chemical interaction, play a dominant rôle in secretory activities, and also stresses the care that should be taken to determine their normal functional variations, before relying on morphological changes as indicators of physiological depression.

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Detailed examinations of the cardiac glands of the stomach, pylorus and those of Brunner in the submucosa of the duodenum, obtained at the same time after feeding and at various intervals following an acute injection, reveal that the action of the drug incurs a slight hypertrophy of the Golgi bodies (Fig. 12). This phenomenon is extensively variable and is more marked in some areas of the same preparation than in others, but is not due to imperfect impregnation. In several instances this process is followed by fragmentation of the Golgi material, as illustrated in Figure 12. Extensive studies of the Golgi apparatus under acute conditions of morphinism demonstrate its inconsistent morphological reaction to the poison.

Muscle Tissue: Preparations of skeletal, cardiac and the involuntary muscle in the walls of the intestine show no cytological deviation from the normal type. The apparent fluctuations in the amounts of Golgi and mitochondrial substance, described by Ma, are not encountered.

Thyroid: The cell components of this gland display no cytological response to acute poisoning, beyond an increase in the amount of fat in the vesicular epithelium following a second injection 8 hours after the first. The secretory polarity of the Golgi apparatus, under these conditions, is maintained.

Kidney: A tendency for the short mitochondrial filaments in the epithelium of the convoluted tubules to break down into globules is a phenomenon generally associated with the acute condition. This reaction is more variable even in tissues obtained from the same rodent, and on the whole is most marked 8 hours after injection. The larger globules stain a bright pink with the acid fuchsin-methyl green technique, and various intermediate colors between the red-staining mitochondria and these structures can be seen. Similar staining gradations are detected with the other methods. The Golgi bodies, as far as could be ascertained, are unaltered. But as structural variations under normal functional conditions cover a wide range, the difficulty in associating specific alterations in the form of the apparatus in this organ with induced poisoning is apparent.

The cytological changes produced by the action of morphine, described above, were not found to be similar in different tissues, as Ma contends, nor could any abnormal nuclear phenomenon be attributed to the morphine.

The behavior of the rats to acute doses of morphine hydrochloride appears to be a controversial point. Ma holds that they show symptoms of slight irritability, while MacEwen and Buchanan[30] report that their rodents, after a similar dose, pass into a deep narcosis for several hours, after 3 to 5 minutes following the injection. My observations, however, entirely confirm those of Ma.

2. *Chronic Morphinism. Cytopathology of Chronically Morphinized Tissues Examined at Intervals Before Discontinuation of the Drug*

When discussing the pathology of morphine tolerance Ma contends that, provided the daily dose is administered regularly, no cytological deviation from the normal occurs, although he describes certain fluctuations in the form of specific rhythmic changes in both amount and form of the cell components during the interval between injections. During what Ma terms the "middle of the craving period satisfied," which is 12 hours after the last injection, a general dispersal and increase in the Golgi and mitochondrial material is reported. It is further contended that microscopic preparations obtained from rats at the end of 24 hours, before they receive their next regular dose of morphine, contrast cytologically with those of 12 hours earlier. In the interim a decrease in the amount of Golgi material occurs and the inclusions gradually return to their normal morphological state.

The evidence derived from this investigation does not confirm that of Ma. Certain slight changes in the morphology of the cell organs, which were by no means specific to all tissues, nor to any period either before or after the regular injection, were observed.

Examination of tissues taken from rats that were under chronic morphinism for 6½ to 7 months revealed the following cytopathological changes.

Stomach and Duodenum: Figure 13 represents a transverse section through a gland of Brunner in the submucosa of the duodenum. In the vast amount of material studied the Golgi bodies in these gland cells possess a depleted appearance and contrast with similar tissues under normal and acute conditions (compare Fig. 13 with 12). This picture reveals an extreme variation of the apparatus in the chronic state, and also shows the loss of power for selective staining

experimental material, are encountered. The mitochondria retain their normal filamentous appearance. This lack of response is not found in the case of acute poisoning (Fig. 6). At the same time they show less absorption for the stains, a phenomenon that appears to be characteristic of the chronic state. Slight fragmentation of the apparatus is occasionally met with, but the marked increase and dispersal of this substance within the apical portion of the acinar cells, in the form of small granules, reported by Ma, 12 hours following the last morphine administration, is not apparent.

Thyroid: Certain interesting pathological changes in this gland appear to be associated with the chronic poisoning. The vesicles, in the majority of cases, contained visibly smaller amounts of colloid, a phenomenon that becomes significant when compared with glands from control rodents. The cytological constituents retain their normal morphology and staining reactions. The accumulation of fat characteristic of the acute condition is not detected, and the lipoid droplets generally seen in the epithelium of the healthy thyroid are entirely absent. The interstitial connective tissue shows no significant variations.

Kidney: The mitochondria present in the renal epithelium of the convoluted tubules are well developed and resemble those seen in the normal organ, except that in certain areas of most preparations the same inability to absorb the stains is met with. Globules, similar to those detected under acute morphinism, are present in a modified degree. The Golgi bodies display an irregular behavior by fragmenting in certain localized areas in the epithelial cells, and come to resemble the structures pictured by Ma during the middle of the "craving period satisfied" of chronic morphinism.

Liver: Preparations of the liver show the mitochondria present in a depleted form, otherwise their morphology and distribution within the hepatic cells appear normal. Variable decreases in the glycogen content of these cells is a conspicuous feature in many regions of chronically morphinized liver.

In view of these results it is of interest to review the observations of several authors in this field. Arnold[33] was one of the earlier workers who found an association of glycogen formation with mitochondrial activity. This work is not in accord with the later contentions of Bang and Sjövall,[34] who found that the distribution of glycogen within the cell protoplasm does not conform to the

distribution of the mitochondria. The previous observations of these authors were later confirmed by Mann [35] in 1928, who likewise discovered no evidence of a morphological relation between the mitochondria and the glycogen of liver cells. Kater [36] in a recent paper has come to the conclusion that there is no evidence for associating the deposition of glycogen in the liver cell with mitochondrial activity. In view of this conflicting evidence it is impossible to determine whether the depletion of the mitochondria, characteristic of liver under chronic morphinism, can be correlated with the decreases in glycogen content. The Golgi material failed to show any reaction to chronic poisoning.

Muscle Tissues: Skeletal and cardiac muscles reveal no significant cytopathological changes that can be attributed to the effect of continuous morphine administration.

3. *Behavior of the Golgi and Mitochondrial Material in Addicts, After Abrupt Withdrawal of the Drug*

The "craving period" of chronic poisoning after withdrawal of the drug is divided by Ma into two phases, the first 1 to 4 days and the second 4 to 6 days after the final injection, and he finds that each period is definitely indicated by some cytological change. The first phase of the "craving period" is marked by such a decrease in the amount of Golgi substance that, in some cells, he reports it as almost absent. The second period is marked by a reappearance of the Golgi material and an increase in the mitochondria, which are enlarged and display better affinity for the stains. At the end of 10 to 12 days these inclusions return to their normal state.

Detailed microscopic examination of the form and fluctuation of the cytological constituents in the tissues of the addicts during these phases reveals that the morphological variations encountered are not necessarily typical of any special state. They are found to exist in different areas of preparations from the same piece of chronically morphinized tissue, and therefore come within the limits of possible variations of the Golgi apparatus and mitochondria in this material. Similar limits of variation may be found occasionally in the cell components in any tissue, even in the normal state.

Observations were made upon the tissues of addicted rats at different intervals from 24 hours until 3 weeks after sudden discon-

tinuation of morphine. Although it is known that abrupt withdrawal leads to severe pathological conditions such as edema, in many tissues, this investigation has shown, contrary to Ma, that it is impossible to correlate any specific cytological state with these phenomena, and no marked cytological demarcation in the form of a sudden decrease of the Golgi lipoid (as Ma terms it), even in preparations 48 hours following discontinuation, was detected. Edema incurred by morphine withdrawal, according to Barbour, Hunter and Richey,[37] is accompanied by rapid loss of lipoid in most tissues. This should correlate theoretically with Ma's findings, when taking into consideration his chemical interpretation of the lipoidal structure of the Golgi apparatus.

Even in tissues such as the gastric glands of the rat of 6½ to 7 months addiction, in which the cell components appear to react to the drug in terms of a variable depletion of the Golgi material, no conspicuous cytological differences can be detected at any interval immediately following withdrawal. The same is found to be the case in tissues that give no significant response to continuous administration of the poison.

Extensive studies have shown that the limits of morphological variation of the cell inclusions seen in the same piece of chronically morphinized tissue are almost identical with those Ma obtained in any particular phase, either during or after the period when the regular morphine injections were withheld.

4. *Microincineration of Normal and Experimental Tissues*

The location of the mineral constituents in normal, acute, and chronically morphinized tissues both before and after withdrawal, as revealed by the method of microincineration of Policard,[38] was extensively investigated. After burning away the protein compounds the distribution and orientation of the inorganic salts were studied in dark-field illumination.

Although thorough examination of the incinerated material shows a specific distribution of the inorganic residues, which are visibly constant in cells forming similar tissues, no marked departures from the normal are observed between the experimental and control material.

Certain abnormal ash distributions are, however, occasionally met with, but repeated experiments demonstrate that this phenome-

non is due, in all probability, to an artificial clumping of the mineral constituents brought about during the process of burning — a factor that appears to be largely dependent upon the temperature and time of incineration.

Nevertheless, definite changes in the amounts and localization of mineral salts in various pathological states have been reported.[39, 40, 41] The most striking results, in view of the negative ones obtained in this investigation, are those of Policard, Noël and Pillet.[42] These authors comment upon the effect of different diets on the mineral distribution in incinerated liver sections from white mice. Variations from the normal were found after feeding the rodents on strict sugar, protein and fat diets, and also in the rate at which these tissues incinerated.

This significant mineral variation, resulting from the changes in diet, observed by these authors, is interesting as no inorganic disturbance, following a lecithin diet of rodents for long periods, was detected by incineration. In the case of lecithin feeding this might be expected, for Glikin,[43] Burow,[44] Altschul,[45] and Stern and Thierfelder [46] concur that iron and calcium are present in lecithin in appreciable amounts.

The administration of lecithin is followed by an increase in the lecithin content of the liver,[47] but incinerated sections of this organ from rats fed for over 4 months on a lecithin diet fail to reveal any differences in mineral localization when compared with liver obtained from control rodents fed on a regular diet.

Appreciable variations in the amounts of inorganic salts in normal and corresponding pathological tissues estimated by chemical analysis have likewise been described. The most noteworthy researches in this sphere are those of Eaves,[48] who gives interesting data concerning the increase in mineral salts during many pathological conditions in the human brain, and that of Adams,[49] who discovered that serum calcium is higher in the blood of rabbits suffering from senile cataract than in normal animals of the same age.

As it has been shown that well advanced morphine addiction not only causes dehydration in certain internal organs, but that sudden withdrawal of the drug results in a remarkable redistribution of water and severe pathological conditions such as edema of the brain, liver, kidney and muscles, as well as a marked increase in blood calcium, one would naturally expect such changes to be indicated by

a disturbance of the mineral organization of the cells. Microincinerated preparations, however, of liver, kidney and brain, which were pathologically affected by sudden morphine withdrawal, show no deviation from the normal controls. Even if the pathological changes incurred by morphine poisoning are inorganically expressed by small specific fluctuations of the mineral constituents, it is unlikely that any such slight variations could be detected by the present microincineration technique, although a recent paper by Scott[50] on quantitative estimations of the ash in incinerated preparations indicates possibilities in this direction.

5. *The Cytological Effect of Lecithin Feeding on Normal Tissues, and on Acute and Chronically Morphinized Tissues, both Before and After Withdrawal of Morphine*

The biological significance of lecithin feeding in normal animals, as well as its effect upon the toxicity of morphine and other alkaloids, has been a subject of much controversy. That lecithin administration results in an increase in the general body weight and acts as an agent stimulating normal growth is a matter of debate.

Ma, when investigating the physiological results of lecithin feeding on the influence of body weight in white male rodents, reported a significant increase. After one month his experimental animals showed in some instances an increase of 9 gm. over the controls. A still greater increase of 27 gm. was found in the lecithin-fed females, as compared with similar females of the same age maintained on the control diet.

Ma attempts to correlate this increase in weight, following yolk-lecithin feeding, with definite cytological phenomena. His preparations showed that the cell components are better developed in tissues from normal lecithin-fed rats, the most marked differences being exhibited by the Golgi material. Ma's physiological interpretation of this phenomenon is based on the supposition that the Golgi substance consists mainly of lipoids, among which lecithin occupies the first place. The fact that lecithin is not absorbed as such will be discussed later.

The growth experiments with lecithin feeding, in this investigation, yielded negative results. Twelve rats (6 males and 6 females) of the same age and weight were segregated and fed for 4 months on

Merck's yolk-lecithin, in proportions as indicated in the text. At the end of this period their weights were compared with corresponding controls fed on the regular diet, and no significant increases in either their growth or weight were recorded. It might be suggested that the vast differences obtained by Ma between his lecithin and control-fed rodents are explicable on the grounds that the control diet was inadequate. Slight variations in body weight, however, were detected between the chronically morphinized rats of 6½ months addiction and those fed upon an ordinary diet. The loss of weight in these addicts was more marked after the first 2 months and on an average never exceeded 4 to 5 gm. Whether this was directly due to the pathological action of the morphine, or secondary effects, associated with a significant loss of appetite, is difficult to ascertain. After 4 months of morphine administration these rodents developed a normal appreciation of food, which was followed by a rapid increase in body weight. It is interesting to record that Barbour, Hunter and Richey,[37] when observing the reaction of dogs to morphine addiction, found that the animals always refused their food during the commencement of a series of injections.

Microscopic examinations of control tissues again demonstrate the enormous limits of cytological variation, particularly expressed by varying amounts of the Golgi substance in normal material. These variations make it exceedingly difficult to determine any relative increase of the cell components in the experimental tissues that might be incurred as the result of lecithin feeding.

Another important factor to be taken into consideration when making quantitative estimation is the metabolic state of the cell at the time of examination. Figure 14 depicts a transverse section through the gastric gland of a lecithin-fed rat killed at the onset of secretory activity, in which an increase in mitochondria and associated granules, together with a decrease in Golgi substance, is involved. Observations on such material during the resting stage of glandular activity and also in other tissues show nothing significantly above normal. The cytological constituents in lecithin-fed tissues are always clearly rendered, and in several instances better developed than in the controls, but as this occasional phenomenon is an irregular one it cannot be attributed solely to the lecithin diet.

In order to restore the Golgi apparatus, which Ma found "almost entirely lacking" during the "craving period," following morphine

withdrawal, he contends that if lecithin is regularly administered together with the usual dose of the drug a few days before discontinuation, the Golgi material is thus prevented from becoming reduced and so retains its normal appearance. He shows cytologically that without lecithin treatment the cell inclusions revert to their normal condition 10 to 12 days after the final injection, but that lecithin feeding, on the other hand, 6 days before and after, prevents this abnormal reduction of the Golgi substance.

As material studied in this investigation reveals that the apparatus, and in some instances the mitochondria, in chronically morphinized tissues undergo a depletion before withdrawal (see Fig. 13), it is difficult to compare the results of subsequent treatment with those obtained by Ma. Experiments, nevertheless, were carried out in this direction, but no significant results were observed. Chronically morphinized tissues from rodents, one-half of which were control-fed and the other lecithin-fed, 8 weeks before morphine discontinuation, were examined 2 days after sudden withdrawal, and no cytological differences beyond the normal morphological variations typical of any healthy cells were observed.

Figure 15 represents the form of the Golgi apparatus, typical of the gastric glands, of a 6½ months addict, lecithin-fed and killed 4 days after withdrawal of morphine. The apparatus has in no way responded to lecithin treatment. It is still reduced in amount, having a slightly depleted appearance, and in no way differs from that of the control-fed material obtained under similar conditions. Extensive examination of various tissues yields no significant results.

The cell inclusions that reacted to the chronic poisoning in the several organs gradually return to their normal state within 2½ weeks after the final injection, which confirms the previous observations of Ma.

The immediate effect of an acute morphine injection upon the Golgi material, of nerve and glandular tissues of a normal lecithin-fed rodent, shows that beyond a slight hypertrophy of the apparatus, frequently observed under such conditions, no cytological deviation from type is detected which would suggest any interaction between the alkaloid and the lecithin.

V. Discussion

A review of the behavior of the several cell organs during acute and chronic poisoning, as well as their reactions to cell injury and disease, shows that they are responsive to gross metabolic and pathological changes, but their use as indicators of fine degrees of physiological depression is not based on sufficiently reliable evidence. When taking into consideration the wide range in the limits of morphological variation of the inclusions, found even in normal healthy cells, it is impossible to correlate any slight structural alteration with any specific degree of morphine tolerance, and on these grounds this investigation fails to concur with the results obtained by Ma. The Golgi elements, in particular, have been shown to be inconstant morphological units, their structure depending largely upon their reaction to the type of technique employed. The apparatus, as seen even in well controlled preparations, in the same piece of tissue treated by either the osmium and silver methods, invariably shows structural diversity.

These experiments also demonstrate that the appearance of the Golgi elements is not only contingent upon the impregnation, but also the period of fixation which had to be regulated according to the type of tissue. Some of the variable factors controlling the impregnation of the Golgi material have recently been discussed in detail by Nahm.[29]

The mitochondria appear to be more consistent in their reactions to different cytological procedures and to induced abnormal conditions. Their extraordinary resistance to acute and chronic poisoning in nerve cells is difficult to interpret, especially when variable morphological changes are associated with this phenomenon in other tissues. These results confirm those of Strongman[51] and McCann,[52] the latter finding that mitochondria in experimental poliomyelitis remained unchanged even after the nerve cells had undergone partial degeneration, while the former demonstrated their immutability in functional exhaustion.

The hypertrophy of the cell organs following administration of an acute dose of morphine, which is very noticeable in the Golgi elements of the nerve cells of the spinal ganglia and in the mitochondria in the acinar cells of the pancreas, is a difficult phenomenon to explain in view of the uncertain chemical composition of these

inclusions. Bancroft and Richter,[53] when inquiring into the physiological action of anesthesia on tissues, suggested that narcotics such as morphine caused an aggregation of the cell colloids. This is worthy of consideration and the possibility that an absorption of the alkaloid might lead to an increase in the surface area of these cell constituents should not be overlooked.

The depletion of the Golgi and mitochondrial material, expressed variably in terms of partial loss of staining reaction combined with a slight decrease in their bulk, typical of most chronically morphinized tissues, is an interesting gross cytological response that might possibly be correlated, in some respects, with the physiological observations of Barbour, Russell, Flowers, Dunham and Hunter.[54] These investigators find that well advanced morphine addiction incurs dehydration of certain of the internal organs, and also that sudden withdrawal of the morphine causes edema, with loss of lipoids, in some parts of the brain, liver and kidney, together with a remarkable redistribution of water.

Assuming that the generally accepted idea of the lipoid structure of the Golgi substance is correct, this might account for the depletion of the apparatus after morphine discontinuation, but as it seems to take place in certain tissues of well advanced addicts, before the drug is withheld, it is difficult to interpret.

Barbour, Hunter and Richey [37] report an interesting observation in this connection. They discovered that when fat-fed dogs were suddenly deprived of morphine a rapid loss of liver fat (in 4 days diminished by 36 per cent in some cases) occurred, while the livers of dogs on a non-fat diet showed an increase. This observation is of interest in view of the recent work of Smith [15] and Kater and Smith,[13] who show that mitochondria in liver cells probably act as catalysts, stimulating a synthesis of fat from the fatty constituents of the cell. It is therefore possible that the reserve fats of adipose tissues in the dogs fed on a non-fat diet are conveyed by the blood stream to the liver cells and here resynthesized by mitochondrial activity.

Overton [55] finds that the power to produce narcosis is entirely dependent upon the power of the substance to dissolve the lipin membrane of the given cell. As it is seen that the cytopathological response to morphine varies according to the tissue acted upon, it might be suggested that this difference in effect is produced by the

fact that the chemical structure of the cell membrane varies with certain types of tissues and gives a different response to morphine in each case.

The results presented in this paper on the negative effect of lecithin administration in normal, and in chronically morphinized rodents, both before and after morphine withdrawal, become more explicable after reviewing the evidence of several workers in this field.

Lecithin, which belongs to the group of phospholipoids, was first isolated by Gabey in 1846, and on hydrolysis yields glycerophosphoric acid, fatty acids and choline.[56] Following its discovery it was used clinically, and later abandoned, as a cure for diseases ranging from beriberi and scurvy [47] to anemia and debility,[57] and its influence as a growth-stimulating factor is still a matter of controversy. Ma,[58] however, reports success in applying this substance to suppress symptoms during treatment of human opium addicts, and also finds that on microscopic examination of their blood the Golgi apparatus in leukocytes, during the "craving period," decreases considerably in amount, and increases provided the patient is treated with lecithin. In a later investigation [59] he finds that the erythrocytes of chronically morphinized rats "received great benefit from the treatment with lecithin after withdrawal."

This last work of Ma is of interest in view of the recent observations of Masasue,[60] and Grönberg and Lundberg.[61] Masasue finds that blood taken from human morphine addicts, when compared with that of normal persons, shows a lower red count. Grönberg and Lundberg have found independently that lecithin administration *in vivo* brings about a rise in the number of red blood cells. From this evidence it might be assumed that morphine has a depressing effect while lecithin stimulates erythropoiesis in bone marrow. It has been shown further that, in the case of advanced human morphine addicts, a single injection of the drug results in a leukocytosis within 30 to 60 minutes. As this change of leukocytosis, however, is only a transient phenomenon (the blood becomes normal in 90 to 120 minutes following the injection), it is difficult to correlate this physiological change with the cytological changes described by Ma,[59] as occurring in the blood of chronically morphinized rats for extended periods after withdrawal.

MacLean,[47] in his monograph, "Lecithin and Allied Substances," admits that the results of feeding experiments with lecithin are in-

conclusive, and that its administration does not appear to be followed by very significant results. Other authors point out that owing to the unsatisfactory state of knowledge concerning the lipins it follows that their exact function in animal economy must necessarily be obscure.

Ever since Danilewsky in 1895 published his startling results in which he found that tadpoles placed in water containing 0.07 per cent lecithin gained 300 per cent more weight than controls kept in ordinary water,[62] many workers have come forward with most conflicting evidence concerning the growth-stimulating powers of this substance. Ma's results entirely confirm those of Hatai,[63] who discovered that white rats, which received lecithin either by injection or mouth, gained in body weight more rapidly than the control-fed animals; the gain in the experimental rodents being on an average 60 per cent. Likewise, Desgrey and Zakey [64] describe an increase in the body weight, especially in the nervous and skeletal systems, following injections of lecithin. It is interesting to note that these conclusions were not entirely confirmed by Hatai, who further found that administration by mouth incurred greater gain in body weight than by inoculations. Then Goldfarb [65] reported that there was no clear evidence of growth stimulation as a result of administration of this substance. He entirely failed to confirm Danilewsky's work on tadpoles, and after extending these experiments to both carnivorous and herbivorous mammals failed to concur with the previous results of Hatai, Desgrey and Zakey.

A more recent publication on this subject by Brachiesi [66] gives an independent view on the nature of lecithin stimulation of growth, which differs from the findings of Ma and other authors. Brachiesi treated guinea pigs with subcutaneous injections of lecithin for 15 successive days. The animals were killed after 18 days. The lecithin-fed animals showed a decided increase in weight over the controls up to the 12th day. Their weight then became constant for the next 6 days. From these experiments this author assumed that lecithin modified the metabolism up to the 12th day and that afterwards an equilibrium was reached. Other groups of animals were similarly treated over longer periods and the same phenomenon was encountered.

There is, in fact, evidence that suggests the nature of the influence of lecithin on growth is rather inhibitory. Robertson [67] found that

large doses of lecithin given to mice produced a slight retardation of growth. Similar retardation in suckling mice, following the administration of 100 mg. of egg-lecithin per day, given by mouth to the mother, was recorded by Robertson and Cutler.[68]

This result is supported by the evidence of Fingerling [69] who contended that the addition of lecithin to the diet is without effect on the secretion of milk.

The specific nature of the fatty acids of lecithin was first doubted by McCollum, Halpin and Drescher,[70] who demonstrated that the degree of unsaturation of the lipin fatty acids of egg yolk is accidental and can be easily influenced by diet. If this is so it might help to explain many of the conflicting results on the growth phenomena in relation to this substance obtained from eggs.

In view of the results of these workers and those obtained during this investigation the following experiments of MacArthur and Luckett [71] are of interest. These investigators succeeded in eliminating not only lecithin but cephalin, cholesterol and fat from the indispensable constituents of foods for mice, and Osborne and Mendel [72] found that the body weights in rats could be maintained on a fat-free diet.

The recent cytological findings of Ikeda [73] on the effect of lecithin on the Golgi and mitochondrial elements in chicken material do not agree with those of Ma, and the present investigation. This author reports that lecithin inhibits the development of the Golgi apparatus, but has the opposite influence on the mitochondria. The results of Okada,[74] on the effects of lecithin upon the mitochondria in the neurones of rabbits, confirm those of Ikeda.

When reviewing the several conflicting observations of these investigators the fact that lecithin is not absorbed as such raises a question which Ma admits is difficult to answer. MacLean [47] showed that it is acted upon by lipase within the intestine and broken down into fatty acids, glyceryl-phosphoric acid and choline. This may also have a bearing in some degree on the negative results obtained by lecithin administration, both as a growth-stimulating substance and a therapeutic agent for the treatment of morphine addiction in rats.

The histological changes in the thyroid gland under acute and chronic morphinism, recorded in this study, do not coincide with those of Scarborough.[75] This worker finds that chronic poisoning

is not associated with any change in the structure of this gland and, moreover, that the combination of chronic morphine poisoning with thyroid feeding inhibits the action of the drug upon the nervous system and checks the edema of the internal organs, generally associated with this condition.

When correlating cytological changes with pathological depression the fact that the cell inclusions are morphologically conditioned according to the chemical state of the cell must first be considered. As has been shown, the reduction of the Golgi material, enspherulation and increase of the mitochondria, are all part of a normal phenomenon associated with the general functional behavior of the gland cells during secretory activity. Even in healthy nonglandular cells, in both the animal and plant kingdoms, fluctuations in the amount of the inclusions, which are probably morphological indications of a synthesis involving condensation of material within them or at their surfaces, are frequently encountered. As physicochemical processes within the cell appear to be largely responsible for the enormous limits of structural variation of cytoplasmic constituents, seen in normal tissues, these experiments have emphasized the danger of relying on these variations for gauging fine degrees of physiological change in cells undergoing diverse chemical influences.

VI. Summary

1. An investigation has been made of the reactions of cytoplasmic structures in different cell types to varying degrees of morphine poisoning, during the periods of regular injections of the drug and at stages following its abrupt withdrawal.

2. Under conditions of acute morphine poisoning the cell inclusiens show variable morphological changes, which are described in detail, but specific changes, indicative of special phases of the acute condition, could not be recognized consistently.

3. More significant, but still inconstant, alterations in the cytoplasm follow addiction for periods of 6 months or more and before the injections are discontinued. In the glands of the stomach and duodenum the Golgi apparatus is depleted and the mitochondria are frequently fragmented and without regular polarity. Minor changes are recorded in the pancreas, thyroid gland and liver.

4. Abrupt withdrawal of morphine, after a prolonged period of addiction, has been shown by other investigators to cause patho-

logical conditions in various tissues. There is no evidence, however, of cytoplasmic alteration which would indicate the effects of withdrawal of the drug.

5. Morphine poisoning does not cause alterations in the mineral constituents of tissues that can be detected by microincineration, although this technique has been successful in the investigation of various common pathological conditions.

6. The growth-stimulating action of lecithin fed to normal rodents is not confirmed, nor does this substance appear consistently to alter the cytoplasmic structures in normal tissues. Lecithin administered therapeutically to acutely and chronically morphinized animals, both before and after withdrawal of the drug, has no significant cytological effect which would suggest that there is any interaction between itself and the morphine.

7. The pathological action of morphine on cells in various tissues appears to occasion changes in the cytoplasm that are so delicate and variable that morphological alterations in cell structures, such as the Golgi apparatus and mitochondria, are not reliable indicators of the acute, chronic and withdrawal phases of morphinism.

8. Morphological changes in the cell organs are met with in special instances following morphine poisoning, but when the limits of variation of the cell organs, which alter even in different functional states of the normal healthy cell, are recognized, these changes are found to range within such limits and therefore lose significance as indicators of the different phases of morphine poisoning.

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ATHEROSCLEROSIS INDUCED IN HYPERCHOLESTEROLAEMIC BABOONS BY IMMUNOLOGICAL INJURY; AND THE EFFECTS OF INTRAVENOUS POLYUNSATURATED PHOSPHATIDYL CHOLINE

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SUMMARY

Groups of 5–8 baboons were fed either a control or hypercholesterolaemic diet for 6 months. During the last 90 days, each group was given 5 i.v. injections of bovine serum albumin (BSA) at 16 day intervals or control injections of saline. Only those animals which were both hypercholesterolaemic and injected with BSA developed aortic and coronary atherosclerosis.

An intravenous injection of 1 g polyunsaturated soya phosphatidyl choline (Lipostabil) thrice weekly into animals receiving the atherogenic diet and BSA, reduced the incidence and severity of aortic atherosclerosis but had no effect on plasma cholesterol, phospholipids or the fatty acid composition of the cholesterol esters and lecithin.

Compared with controls, animals given the hypercholesterolaemic diet had increased aortic lipase and normal cholesterol esterase activity. Those given the same diet and Lipostabil had a normal aortic lipase and over 50% increase in cholesterol esterase activity.

It is concluded that immunological injury hastens the onset of atherosclerosis produced by feeding a hypercholesterolaemic diet and that changes in aortic lipolytic enzymes may be the mechanism by which Lipostabil reduces atherosclerosis.

Key words: *Cholesterol ester hydrolase — Experimental atherosclerosis — Immediate hypersensitivity — Immunological injury — Lipase — Polyunsaturated lecithin*

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INTRODUCTION

Aortic atherosclerosis can be produced in the baboon by feeding cholesterol-rich diets. However, a relatively long period of 1½–2 years feeding is required, and the extent of the disease is slight[1]. For continued experimental work, the time lapse between commencing study and the acquisition of results is too long and costly to make the system of value for the routine production of atherosclerosis.

Several groups have reported that aortic atherosclerosis can be hastened in cholesterol-fed rabbits by the injection of protein antigens such as horse serum or bovine serum albumin[2–6]. Not only is severe disease produced more quickly but the majority of the lesions are of the fibro-fatty type. An attempt therefore has been made to hasten the disease in hypercholesterolaemic baboons by similar means. The antigen chosen was bovine serum albumin (BSA) and was given intravenously on five occasions 16 days apart.

Previous work has demonstrated that i.v. injections of polyunsaturated soya phosphatidyl choline (Lipostabil) lessens the severity of aortic atherosclerosis in rabbits given a cholesterol-containing[7] or a semi-synthetic diet[8]. The most likely mechanism by which the compound acts is by stimulating aortic cholesterol esterase activity. In a continuation of these studies, the effect of Lipostabil has been investigated in baboons made atherosclerotic by means of immunological injury.

METHODS

Experimental design

Groups of male juvenile baboons (*Papio cyanocephalus*; 1–2 years old) weighing approximately 4 kg were placed on either control laboratory pellets or a hypercholesterolaemic diet containing egg yolk and butter (Table 1); ascorbic acid (500 mg) was given twice weekly. Fourteen weeks after starting the experiment, animals were injected intravenously through the femoral vein with saline or bovine serum albumin (BSA 250 mg/kg as a 5% w/v solution in saline) and/or polyunsaturated soya phosphatidyl choline (10 ml, containing 1 g EPL, Lipostabil, Nattermann, Cologne, with no added AMP or nicotinic acid as in the commercial product) according to the following scheme:

Group 1: atherogenic diet, BSA every 16 days, saline thrice weekly.
Group 2: atherogenic diet, BSA every 16 days, Lipostabil thrice weekly.
Group 3: atherogenic diet, saline thrice weekly.
Group 4: control diet, BSA every 16 days, saline thrice weekly.
Group 5: control diet, no injections.

Before the second and subsequent doses of BSA (250 mg/kg), of which there were 5 in total, a smaller dose of BSA (25 mg/kg) was given 48 h previously to remove large quantities of circulating antibody which might otherwise have caused severe anaphylactic shock when the larger dose of BSA was given.

TABLE 1

COMPOSITION OF ATHEROGENIC SEMI-SYNTHETIC DIET

	g/kg
Egg yolk	150
Butter	150
Casein	250
Starch	222
Salt Mix[a]	40
Vitamin Mix[b]	8
Cellophane	150
Choline chloride	4
Potassium acetate	25
Vitamin oil[c] (containing vitamin E)	1

[a] Salt mixture contained in parts by weight: 50 sodium chloride, 400 tricalcium phosphate, 35 ferric citrate, 1 potassium iodide, 80 magnesium sulphate, 136.5 sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$), 250 potassium chloride, 0.2 manganese sulphate, 0.34 copper sulphate, 0.34 zinc carbonate.

[b] Vitamin mixture contained in parts by weight: 60 mg biotin, 100 mg menaphthone, 1.6 g pyridoxine hydrochloride, 1.6 g thiamine hydrochloride, 1.6 g riboflavin, 1.0 g folic acid, 4 g calcium pantothenate, 20 g nicotinamide, 50 g inositol, 5 mg cyanocobalamin, 4.8×10^6 I.U. stabilised vitamin A, 650 g magnesium oxide.

[c] Vitamin oil contained 10 g α-tocopherol acetate in 100 ml arachis oil.

Samples of plasma and serum were taken for biochemical analysis at frequent intervals. After 6 months of the experiment the animals were killed and the heart and other organs were fixed in formol saline for histological study. The aorta was opened longitudinally from the arch to the bifurcation, and was cut lengthways into 2 approximately equal and comparable portions. One portion was fixed in formol saline for histological study, the other was examined for aortic lipolytic enzymes as described below.

Pathological study of aorta and organs

The aorta was stained macroscopically with Sudan IV according to the method of HOLMAN *et al.*[9]; the lesions, if any, were chartered and the extent assessed by direct measurement. Atherosclerotic lesions were examined histologically by a variety of conventional stains. Specimens of lung, kidney, liver, spleen, heart, brain and other organs and vessels were also examined.

Biochemical estimations

Plasma cholesterol. Total plasma cholesterol was estimated by precipitation of the cholesterol digitonide and estimated by the ferric chloride-sulphuric acid method as described previously[10].

Plasma phospholipids. Plasma was extracted with methanol-chloroform (1:2, v/v) and washed with saline according to the method of FOLCH *et al.*[11]; lipid phosphorous was then assayed according to the method of ALLEN[12].

Fatty acid composition. Plasma was extracted and the lipids separated by thin layer chromatography; the fatty acid composition of the cholesterol esters and lecithin determined by the method of BOWYER *et al.*[13].

Aortic lipolytic enzymes. The adventitia and loose connective tissue were carefully stripped off and the specimen was rinsed with ice-cold distilled water, dried with blotting paper and frozen in dry ice. Frozen aortas were pulverised in a freeze press; acetone–butanol powders were then prepared at —20°C according to MORTON[14]. Analyses were carried out on aortas from groups 1, 2 and 5 only. For the assay of lipase* and cholesterol esterase* activities, the 10 mg powder was extracted with 1 ml glycerol in water (1 : 1, v/v) for 10 seconds using an Ultra-Turrax blender (Janke and Kunkel). The extracts were decanted and filtered through Whatman No. 1 paper. Protein was determined according to Lowry *et al.*, as described by LEGGET BAILEY[15].

Substrates were checked for purity and prepared as hydrosols; enzyme activities of the extracts were estimated as described previously[8,16,17]. The reaction mixtures contained 1 m*M* concentrations of substrates in glass distilled water: — glyceryl trioleate at pH 8.3 and reduced glutathione (0.1 m*M*) for lipase; cholesteryl oleate at pH 8.6, reduced glutathione (0.1 m*M*) and sodium taurocholate (4 m*M*) for cholesterol esterase. The enzyme extract (0.06–0.12 mg protein/0.2–0.4 ml) was added to make a total volume of 1 ml. Reactions were carried out by continuous titration under nitrogen in a pH-Stat (Radiometer, Copenhagen) at 30°C, using 0.002 *N* potassium hydroxide solution as the titrating reagent.

TABLE 2

PLASMA LIPIDS AND AORTIC ATHEROSCLEROSIS (mean ± S.E.M.)

Group	*No. examined*	*Treatment*[a]			*Fatty acid (%)*			
		diet	*BSA*	*Lipostabil*	*plasma cholesterol (mg/100 ml)*	*plasma phospholipids (mg/100 ml)*	*cholesterol phospholipid ratio*	*aortic atherosclerosis (% area)*
1	8	A	+	—	245 ± 15.6	281 ± 11.3	0.96 ± 0.09	46.3 ± 12.5
2	8	A	+	+	226 ± 23.3	236 ± 20.6	0.98 ± 0.09	9.5 ± 4.4
3	5	A	—	—	286 ± 17.3	272 ± 23.4	1.08 ± 0.10	0
4	5	C	+	—	118 ± 13.1	125 ± 20.9	1.08 ± 0.26	0
5	5	C	—	—	116 ± 7.5	119 ± 9.8	1.00 ± 0.09	0

[a] A = atherogenic, C = control, BSA = bovine serum albumin.

Statistical comparison				
Groups				
1 *v* 2	NS	NS	NS	$P \leq 0.05$
1 *v* 4	$P \leq 0.001$	$P \leq 0.001$	NS	$P \leq 0.001$
2 *v* 4	$P \leq 0.01$	$P \leq 0.01$	NS	$P \leq 0.001$

NS = not significant.

* Lipase = glycerol-ester hydrolase (EC 3.1.1.3), cholesterol esterase = sterol-ester hydrolase (EC 3.1.1.13).

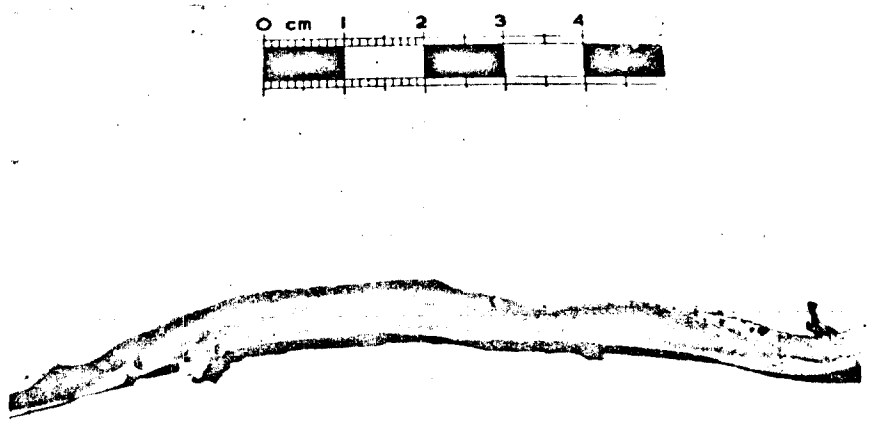

Fig. 1. Aorta showing macroscopic sudanophilic lesions (Sudan IV; × 1).

RESULTS

Plasma lipids and aortic atherosclerosis

Table 2 shows total plasma cholesterol, phospholipids, cholesterol/phospholipid ratios and the degree of aortic atherosclerosis seen macroscopically at the end of the experiment. Those groups given the atherogenic diet (groups 1–3) all showed hypercholesterolaemia, with an elevation of plasma cholesterol 2–2½-fold above control levels. However, aortic atherosclerosis (Fig. 1) occurred only in those hypercholesterolaemic animals injected with BSA. Injections of Lipostabil caused a significant reduction in aortic atherosclerosis but not in plasma cholesterol. Only 3 out of 8 animals had aortic disease in the Lipostabil group, and the extent was slight. Furthermore, the lesions when present were seen by microscopy to contain much less lipid. No atherosclerosis was seen in animals fed the control diet even when injected with BSA.

Increased plasma phospholipids were seen in all groups where plasma cholesterol was elevated, such that the cholesterol/phospholipid ratio remained constant and the same as in the controls. Injections of Lipostabil had no effect on total plasma phospholipids.

Fatty acid composition of lipids

Tables 3 and 4 show the fatty acid composition of the plasma cholesterol esters and lecithin. Compared with controls, those animals fed the atherogenic diet showed elevated oleic and arachidonic acids and decreased linoleic acid in the cholesterol esters, and increased arachidonic acid and decreased linoleic acid in lecithin. Injection of BSA or Lipostabil had no effect on the fatty acid compositions.

TABLE 3

FATTY ACID COMPOSITION OF PLASMA CHOLESTEROL ESTERS (mean ± S.E.M.)

Group	No. examined	Treatment[a]			Fatty acid (%)					
		diet	BSA	Lipostabil	16 : 0	16 : 1	18 : 0	18 : 1	18 : 2	20 : 4
1	5	A	+	—	14.7 ± 2.6	2.2 ± 0.2	3.8 ± 0.4	26.7 ± 2.0	34.4 ± 1.7	15.0 ± 2.5
2	5	A	+	+	14.8 ± 2.4	2.3 ± 0.5	3.0 ± 0.2	25.0 ± 1.1	37.1 ± 1.8	13.7 ± 1.0
3	3	A	—	—	16.0 ± 2.7	2.1 ± 0.2	3.3 ± 0.8	26.1 ± 1.7	34.6 ± 4.2	12.3 ± 1.5
4	5	C	+	—	13.3 ± 3.3	3.0 ± 0.6	2.0 ± 0.5	15.9 ± 1.9	52.7 ± 4.1	6.8 ± 0.7
5	5	C	—	—	10.4 ± 0.7	2.9 ± 0.4	2.1 ± 0.4	18.9 ± 0.8	51.7 ± 2.3	5.6 ± 0.6

[a] A = atherogenic, C = control, BSA = bovine serum albumin.

Statistical comparison						
Groups						
1 *v* 2	NS	NS	NS	NS	NS	NS
1 *v* 4	NS	NS	$P \leqslant 0.01$	$P \leqslant 0.001$	$P \leqslant 0.001$	$P \leqslant 0.01$
2 *v* 4	NS	NS	NS	$P \leqslant 0.01$	$P \leqslant 0.01$	$P \leqslant 0.05$

NS = not significant.

1222

TABLE 4

FATTY ACID COMPOSITION OF PLASMA LECITHIN (mean ± S.E.M.)

Group	*No. examined*	*Treatment*[a]			*Fatty acid (%)*				
		diet	*BSA*	*Lipostabil*	*16 : 0*	*18 : 0*	*18 : 1*	*18 : 2*	*20 : 4*
1	5	A	+	—	23.3 ± 2.1	23.1 ± 0.4	10.8 ± 0.5	14.5 ± 1.4	21.2 ± 2.2
2	5	A	+	+	23.1 ± 2.2	21.1 ± 0.3	10.9 ± 0.5	15.9 ± 1.5	17.6 ± 2.0
3	3	A	—	—	22.2 ± 2.4	22.5 ± 0.3	10.9 ± 0.2	16.0 ± 0.3	18.9 ± 2.9
4	5	C	+	—	26.5 ± 1.1	22.2 ± 1.7	9.0 ± 0.8	22.4 ± 1.5	9.7 ± 0.8
5	4	C	—	—	27.4 ± 2.9	21.3 ± 1.8	13.7 ± 2.3	22.4 ± 1.4	10.0 ± 1.2

[a] A = atherogenic, C = control, BSA = bovine serum albumin.

Statistical comparison Groups					
1 *v* 2	NS	NS	NS	NS	NS
1 *v* 4	NS	NS	NS	$P \leqslant 0.01$	$P \leqslant 0.001$
2 *v* 4	NS	NS	NS	$P \leqslant 0.05$	$P \leqslant 0.05$

NS = not significant.

1222

TABLE 5

ESTERASE ACTIVITIES OF AORTA (mean ± S.E.M.)

Group	No. examined	Treatment[a]			Extracted protein (% w/w)	Lipase (nequiv/min/mg protein)	Cholesterol esterase (nequiv/min/mg protein)
		diet	BSA	Lipostabil			
1	5	A	+		2.8 ± 0.5	67 ± 5.1	19 ± 3.5
2	5	A	+	+	2.9 ± 0.3	54 ± 5.5	36 ± 4.0
5	5	C	–	–	3.7 ± 0.5	38 ± 5.3	22 ± 2.1

[a] A = atherogenic, C = control, BSA = bovine serum albumin.

Statistical comparison			
Groups			
1 v 5	NS	$P \leq 0.001$	NS
2 v 5	NS	NS	$P \leq 0.05$

NS = not significant.

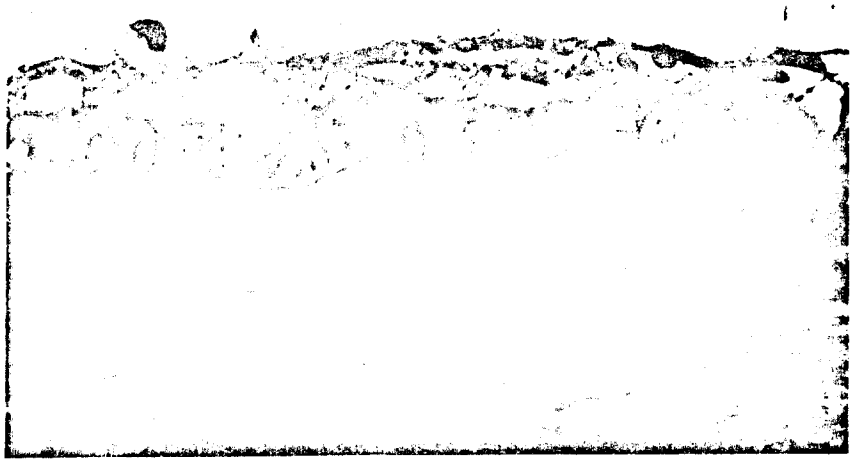

Fig. 2. Aortic intimal thickening containing vacuolated cells (H.-E.; × 750).

Aortic lipolytic enzymes

Specific activities of aortic lipase and cholesterol esterase in control and experimental animals are presented in Table 5 for groups 1, 2 and 5 only. The mean values of protein extracted from the acetone–butanol powders were not significantly different from each other. Compared with the controls, animals given the atherogenic diet and

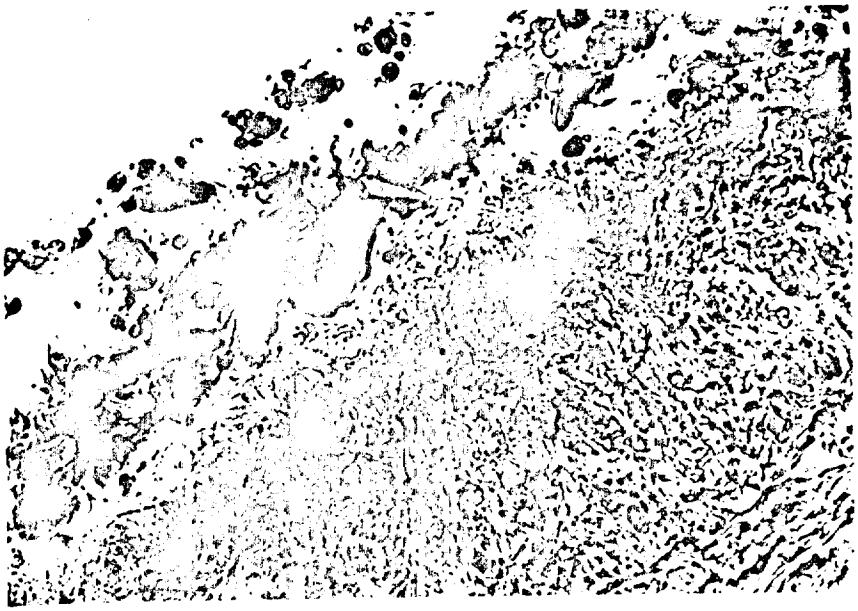

Fig. 3. Sudanophil lipid in aortic intima (Sudan III/IV; × 750).

injected with BSA showed increased lipase and unchanged cholesterol esterase activities. However, in those given the atherogenic diet, BSA and Lipostabil, the lipase activity was unchanged and the cholesterol esterase activity was 50% greater than the controls.

Histological examination

Aortic intimal thickening was found only in those animals in group 1 and 2 which were found to have lesions macroscopically. These lesions were fibro-elastic and in group 1 (saline-treated animals) consisted mainly of vacuolated cells containing lipid (Figs. 2 and 3). Those in group 2 were similar but they contained much less lipid.

Muscular-elastic thickenings of the coronary vessels (Fig. 4) were found in 3 animals in group 1 and 4 animals in group 2. These were small and it was impossible to estimate if there was any difference in extent or severity between these two groups. No lipid was demonstrable with Sudan IV.

There were no renal lesions or evidence of vasculitis in any organ.

DISCUSSION

Injury to the arterial wall is of primary importance in the aetiology of atherosclerosis[18], since it favours the deposition of cholesterol and other lipids at the site of the reaction. The work presented here deals with a specific and easily reproducible type of injury, namely the immunological injury induced by the intravenous adminis-

Fig. 4. Elastic and muscular thickening of coronary artery intima (Hart's modification of Weigert's resorcin-fuchsin; × 750).

tration of a large quantity of protein antigen. Results obtained are a confirmation of the general theory of atherogenesis which has evolved over the past few decades, namely that atherosclerosis (leaving aside the importance of thrombosis) is the product of the effect of elevated blood lipid levels and the severity of the injury.

Under our experimental conditions, no aortic or coronary atherosclerosis was seen with immunological injury or hypercholesterolaemia alone but only when the two factors were present together. These findings are even more striking than those obtained in the cholesterol-fed rabbit in which similar protein antigens were used[2–6]. The chief value of the present results has been to establish that the same phenomenon can be reproduced in a non-human primate and strengthens the case that a similar situation could occur in man. This is important because other authors have suggested that allergic reactions play a role in the causation of a human coronary atherosclerosis and its sequelae[19].

Our previous work has shown that atherosclerosis [illegible] in baboons by the feeding of a hypercholesterolaemic diet alone over a long period[1,20], and that the lesion bears a striking similarity to the human fatty streak. However, the usefulness of the baboon as a research animal for biochemical and pharmacological studies is very limited because of the time factor. The aortic lesions obtained in the present experiments were histologically very similar to those obtained previously[1] and the technique described here of combining immunological injury with hypercholesterolaemia is an advantage because it now means that lesions can be produced in a much shorter time.

A further point of interest was the production of muscular-elastic coronary lesions in animals fed the hypercholesterolaemic diet and injected with BSA. These were essentially lipid free and similar to those described by other authors[21]. In view of the experimental difficulty of inducing coronary lesions in the baboon[22], this technique could be of considerable value in the future.

The present system was used to study the activity of the aortic enzymes in normal and atherosclerotic animals and the effect of Lipostabil therapy. Previous work in the rabbit[8] has shown that a hypercholesterolaemic semisynthetic diet causes increased lipase activity and decreased cholesterol esterase activity in the aorta. In the hypercholesterolaemic baboon, lipase was found to be elevated but cholesterol esterase was unchanged compared with normal animals. Species differences may explain the conflicting result, but it is noted that in our rabbit experiments the plasma cholesterol in the experimental group was almost double that in the hypercholesterolaemic baboons. Thus substrate inhibition may be of importance. It is of interest that an increase in lipase would facilitate the release of free fatty acids from the triglycerides and favour the esterification of cholesterol and the deposition of cholesterol esters in the arterial wall.

Injections of Lipostabil in the hypercholesterolaemic rabbit causes a decrease in aortic atherosclerosis[7,8] and normalisation of the aortic enzymes[8]. In the baboon experiments, a similar result has been achieved with respect to lipase, but in contrast the activity of cholesterol esterase was increased by over 50% above normal. The more pronounced effect in the baboon compared with the rabbit may be due to the use of a much higher dose of Lipostabil (5 times greater/kg body wt.). As for the rabbit given an atherogenic semi-synthetic diet, plasma cholesterol was unaffected by the administration of Lipostabil and its effect on atherosclerosis was not mediated by a lowering of plasma cholesterol.

As found previously[23,24] in baboons given an atherogenic diet containing egg yolk and butter, there were changes in the fatty acid compositions of the cholesterol esters and phosphatidyl choline. Injections of Lipostabil and BSA caused no further changes. The linoleic acid injected weekly in Lipostabil was only 1/10th of that consumed in the diet, an amount presumably insufficient to have any demonstrable effect.

The mechanism by which cholesterol esters accumulate in the arterial wall has not so far been elucidated. Increased esterification of cholesterol or decreased hydroly-

sis of the esters may both be important. Certainly an increase in cholesterol esterase (hydrolase) activity would disfavour their accumulation. It has been argued that the deposition of esterified rather than free cholesterol is more damaging to the arterial wall[25] because free, but not esterified, cholesterol is easily transported across cell membranes[26]. If this view is correct, an increase in cholesterol esterase activity should lead to less arterial lipid deposition. The fact that Lipostabil, which elevates cholesterol esterase activity, also decreases the incidence and severity of aortic sudanophilia is consistent with this view.

However, this hypothesis has been questioned by ADAMS and his co-workers[27] who consider that cholesterol esterification activity is a defence mechanism enabling cholesterol to be mobilised from the tissue[7,27-29]. Whilst this removal mechanism might apply to the esters of polyunsaturated fatty acids, cholesterol oleate is more sclerogenic[28] and less easily removed[29] than cholesterol. In the rabbit[25] and man[30] it is cholesterol oleate which accumulates in the fatty streak, the lesion studied in our experiments. Thus we believe that lipolysis is an important mechanism for the removal of cholesterol esters in the early atherosclerotic lesion. An alternative explanation for the activity of Lipostabil is that it may act by virtue of its lipotropic and surface activity and render cholesterol or its esters more easily transportable[7]. It is also possible, but unlikely, that Lipostabil may affect the immune reaction in such a way as to decrease the injury to the vessel wall.

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The Analysis of Commercial Lecithin

By H. H. HUTT, A.I.C., and H. WEATHERALL, F.I.C.

The analytical examination of the commercial lecithin used in the food and other industries has not received much attention in the literature, apart from the description by Rewald[1] of a method for the determination of phosphatides in the organic raw materials from which they are obtained.

Before the war, the bulk of the lecithin used was produced from the soya bean, either on the Continent or in the United States. Since 1940, however, the Empire-grown groundnut (*Arachis hypogaea*) has been the main source of phosphatides for industrial use in this country and the production of groundnut lecithin has been developed.

NATURE OF THE PHOSPHATIDES—Commercial lecithin is a mixture of lecithin and kephalin which occurs in plant tissue. As prepared industrially it contains *ca.* 30–40% of free oil, which may have varying proportions of neutral glycerides and free fatty acids. It also contains minor amounts of carbohydrates and proteins, either free or as prosthetic groups, and small quantities of phytin may be present in some samples.

The formula of lecithin has been recorded by McLean and McLean[2] as

$$(R.COO)_2C_3H_5O.P{:}O(OH)O.C_2H_4.N(CH_3)_3OH,$$

where (R.COO) represents a fatty acid radicle.

It should be noted that Fischgold and Chain[3] consider lecithin to be a zwitterion. The formula for this may be written as follows:

$$(R.COO)_2C_3H_5.O{-}\underset{\underset{\displaystyle O}{\|}}{\overset{\overset{\displaystyle O-}{|}}{P}}{-}OC_2H_4N(CH_3)_3{+}$$

The constitution of kephalin is not regarded as established beyond doubt,[4] but for present purposes the following formula may be ascribed to it:

$$(R.COO)_2.C_3H_5O.P{:}O(OH).OC_2H_4.NH_2.$$

The most important characteristics of lecithin and kephalin from the analytical point of view are their phosphorus contents and their behaviour with organic solvents. Theoretically, dioleyl-lecithin contains 3·86%, the corresponding kephalin 4·17% of phosphorus.

Both kephalin and lecithin are soluble in light petroleum, diethyl ether, benzene, chloroform and carbon tetrachloride but insoluble in acetone and are pptd. by acetone from solution in other organic solvents. Kephalin is distinguished from lecithin by its insolubility in ethyl alcohol.[5]

The total phosphatide content of a commercial sample may be estimated by either of two methods: (1) by determining the total phosphorus and multiplying by 26·31* or, (2), by determining the % of acetone-insoluble matter. Owing to the presence of small quantities of petroleum-insol. matter and other substances associated with the phosphatides, the % of acetone-insoluble matter is usually higher than the phosphatide content as estimated from the phosphorus determination. The matter insol. in light petroleum consists of finely divided meal, and the presence of protein renders the nitrogen content a less reliable guide to the amount of total phosphatides than the two methods mentioned above.

In assessing the quality of the product, the proportions of free fatty acid, moisture and petroleum-insol. matter should also be taken into account. It should be noticed that, since kephalin behaves as an acid in titration with alkalis,[3] the free fatty acid should be determined in the acetone-sol. fraction, *i.e.*, in the oil after separation from the phosphatides. Crude lecithin is sometimes refined by washing out the glycerides and free fatty acids with acetone and replacing them with a high grade edible fat. These products should be reasonably free from acetone. Methods of analysis are described below.

(1) TOTAL PHOSPHORUS—The organic matter is destroyed by wet ashing, and the phosphorus is pptd. as ammonium phosphomolybdate, re-pptd. as magnesium ammonium phosphate, ignited and weighed as magnesium pyrophosphate. Weigh 3 g of the sample on an 11-cm ashless filter and warm it in a 1-litre Kjeldahl flask with 20 ml of conc. nitric acid for 2–3 min. Cautiously add 20 ml of conc. sulphuric acid in successive small portions and heat strongly for *ca.* 1½ hr., with occasional shaking. Cool, add 10 ml of conc. nitric acid and boil until brown fumes are no longer evolved; then add a further 10 ml of nitric acid and boil off again. Repeat this process until no darkening occurs after evaporation of the nitric acid. Cool, add 100 ml of water, transfer the liquid to a 1-litre squat beaker with a further 100 ml of water, boil for 10 min., make the soln. alkaline with ammonia soln. (0·880), adding 30 ml in excess, re-acidify with nitric acid and ppt. the phosphoric acid with 10 g of ammonium molybdate A.R. in 300 ml of water, in the usual way.

* 26·31 is an arbitrary factor in general use.

There may be a slight colour, due to organic matter, in the supernatant liquid, but if the colour after pptn. is no greater than before, it may be ignored. Dissolve the washed ppt. in ammonia soln. and ppt. the phosphorus with magnesia mixture by the ordinary method.

The above method, checked against pure anhydrous sodium glycerophosphate B.P. gave the following results—(a) 14·27; (b) 14·29; (c) 14·43; (d) 14·25% of phosphorus (theory, 14·35%). In (a) the soln. was not heated after addition of magnesia. This made little or no difference to the result, but there is a greater tendency for the magnesium ammonium phosphate to adhere to the beaker in cold pptn. In (d) 2 g of edible groundnut oil were added to the glycerophosphate.

An alternative method for the destruction of organic matter, using hydrogen peroxide, has been published.[6]

(2) Acetone-insoluble Matter and Free Fatty Acids—In method (*a*), used in this laboratory, the acetone-insol. matter is determined by direct elutriation of the soluble fraction. The determination can also be made by pptng. the phosphatides with acetone from another solvent as described in method (*b*). The pptn. method (*b*), which was communicated to us by Mr. A. C. Francis, probably gives more accurate and concordant results than (*a*), but is somewhat lengthy. We have adopted the elutriation process, as it is quite reliable when used in conjunction with the phosphorus figure and is more suitable for works control analysis where many samples are examined in the course of a week.

(*a*) *Elutriation Method*—Weigh 5–10 g of the sample into a 150-ml CO_2 flask provided with a glass stirring rod, the lower end of which is widened out to facilitate kneading of the lecithin. Add about 100 ml of pure acetone and transfer the mixture to a steam-heated plate. When the solvent has reached b.p. knead the lecithin thoroughly, stirring the solvent into it. Cool to room temp. (20° C.) and allow to settle. Pour off the acetone-oil soln. into a 250-ml beaker. Re-extract the lecithin with two further 100-ml portions of acetone. Transfer *ca.* 50 ml of the acetone-oil soln. to a weighed 150-ml CO_2 flask, evaporate the solvent and re-weigh. Determine the free fatty acid by titration in boiling neutral alcohol (phenolphthalein as indicator). Calculate as oleic acid % in the acetone-sol. matter and convert to free acid % in the original sample by means of the formula at the end of this section.

Remove the solvent from the insol. residue in a current of air on a steam-heated plate and weigh. Express as acetone-insol. matter %.

(*b*) *Precipitation Method*—Weigh 5 g of the sample into a 250-ml beaker. Pour on to it 6 ml of ether and leave with occasional stirring until solution is complete. Add 100 ml of pure acetone gradually, stirring continually. Leave for 10 min. or until the supernatant liquid is clear or nearly clear. Decant the soln. through a No. 4 Whatman filter paper into a 400 ml flask. Evaporate the residual acetone from the insol. matter. Re-dissolve the latter in 6 ml of ether and reppt. with 100 ml of acetone as before. Decant the supernatant soln. through the same filter into the same flask as before. Treat for a third time by repptn. and decantation. Distil the acetone-ether mixture from the soluble matter, removing the last traces in a steam-oven. Dissolve the residual oil in 50 ml of acetone and filter through a No. 4 Whatman filter-paper into a weighed flask. Wash the filter with acetone until free from oil. Evaporate the solvent from the oil and dry to constant weight at 100° C.

$$\text{Acetone-insol. matter, \%} = 100 - \text{moisture \%} - \text{sol. in acetone, \%}.$$

Determine the moisture of the sample as described under (3). Calculate the free fatty acid content of the original lecithin from the above data as follows.

F.F.A. % (as oleic acid) in the original sample =

$$\frac{\text{F.F.A. \% in the acetone-sol. matter} \times (100 - \text{acetone insol. matter \%} - \text{moisture \%})}{100}$$

(3) Moisture—Weigh 3 g of the sample into a glass dish (60 mm in diam.), preferably provided with a ground-in stopper and dry in an oven at 100° C. for 3 hr. Take the loss of weight % as the moisture content.

The loss of volatile matter under these conditions is mainly water but a small increase in weight of the actual phosphatides, due to oxidation, takes place concurrently. This, of course, can be avoided by conducting the heating in a stream of nitrogen, but, as the moisture determination is normally only used to ensure that no excessive amount, such

as would lead to mould formation, is present, small inaccuracies may be overlooked. The upper limit for permissible moisture content is *ca.* 2·5%. In samples which have been dried to much less than 1% a small fraction of the loss at 100° C. may be due to alkaline decomposition products.

We have examined several specimens of commercial lecithin by the common methods for the determination of water and have found that in distillation methods using heptane, toluene and xylene decomposition occurs, and an alkaline substance (probably cholamine), appears in the aqueous distillate. This leads to emulsification of the aqueous and solvent layers. Constant figures were obtained in some expts. by heating *in vacuo*, but fatty acids were observed condensing on the upper parts fo the oven.

The following instances exemplify these points.

Specimen A—Loss in 3 hr. at 100° C. 0·78%.

Moisture determined by distillation with heptane							
No. of hr. under reflux.. ..	18	30	42	52	60	72	80
Moisture, %	0·85	0·89	0·94	1·05	1·10	1·12	—
Moisture determined by distillation with toluene							
No. of hr. under reflux.. ..	18	30	42	54	60		
Moisture, %	0·78	0·89	0·94	1·06	1·06		
Moisture determined by distillation with xylene							
No. of hr. under reflux.. ..	18	30	42	54	60	72	80
Moisture, %	1·25	1·50	1·90	2·00	2·10	2·12	2·35
Moisture determined at 100° C. in 28 in. vacuum*							
Time, hr.	2	4	6	8	10		
Moisture, %	1·18	1·48	1·97	1·97	1·97		

* Fatty acids were observed on the upper parts of the vacuum chamber.

Loss of weight by the vacuum method on three further specimens failed to reach constancy in twelve hours.

Specimen B.

Moisture determined by heating in nitrogen at 100° C.							
Time, hr.	1	2	3	4	5	6	7
Moisture, %	4·25	4·60	4·80	4·84	4·84	4·97	—
Moisture determined by heating in air at 100° C.							
Time, hr.	1	2	3	4	5	6	7
Moisture, %	2·88	3·72	4·30	4·52	4·71	4·66	4·73

A 3-g portion of B was placed in a flask on a boiling water-bath and a stream of warm dry nitrogen was passed over it for 3 hr. The effluent gas was led through 25 ml of $N/10$ sulphuric acid. Back titration showed that no alkaline decomposition products were removed.

Although the data given above lend support to the use of oven drying for the moisture determination, the possibility of oxidation of lecithin from more unsaturated oils should not be overlooked, and with such samples nitrogen should be used.

(4) Determination of Petrol-insoluble Matter—Weigh, accurately, about 5 g of the sample and dissolve it in 30 ml of petroleum spirit (B.P. 40–60° C.) in a weighed centrifuge tube by warming on a water-bath. Centrifuge at 3,500 r.p.m. for 10–15 min. Pour off the supernatant liquid and repeat the washing twice with 30-ml portions of petroleum spirit. Dry on a water-bath. Re-weigh and express the results as petrol-insoluble matter %.

(5) Determination of Residual Acetone in Refined Lecithin—The following method has been adapted from that described by D. D. Van Slyke.[7]

Solutions required—10% mercuric sulphate: 73 g of pure red mercuric oxide dissolved in 1 litre of 4 N sulphuric acid; 50% (by vol.) sulphuric acid adjusted to 17·0 N H_2SO_4; 5% sulphuric acid.

Method—Weigh, accurately, *ca.* 25 g of the sample and dissolve it in 150 ml of light petroleum (b.p. 40–60° C.) and extract with four 50-ml portions of 5% sulphuric acid. Run the first two extracts into a second separating funnel containing about 75 ml of light petroleum and shake lightly. Filter the combined aqueous layer through a fluted Whatman No. 4 filter paper into a 600-ml conical flask. Use the third and fourth extracts from the lecithin-petrol solution to wash the petrol in the second separator and the filter. Add 20 ml of 50% sulphuric acid and 70 ml of 10% mercuric sulphate soln. to the combined

extracts and boil under a reflux condenser for 45 min. Cool and filter off the ppt. of mercuric sulphate and acetone on a weighed Gooch crucible, wash with 200 ml of cold water, dry for 1 hr. at 110° C. and re-weigh (20 mg of ppt. ≡ 1 mg of acetone).

Care is needed in shaking the petrol-lecithin soln. with the sulphuric acid, as too much shaking causes emulsions and too little leads to incomplete extraction.

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DISCUSSION

Mr. K. A. WILLIAMS congratulated the authors on having prepared the first comprehensive account of the analysis of commercial lecithins. Referring to the determination of phosphorus, he asked whether the authors had any comparative figures for the simpler and quicker modifications of the method in which partial combustion of the product was carried out in presence of carbonates in place of wet oxidation, and in which the Pemberton method was used for the actual determination of the phosphorus. The latter, described by E. R. Bolton (*Fatty Foods*, 1st Ed., p. 341), which involved the solution of the phosphomolybdate precipitate in a known excess of standard alkali and titration of the excess by means of standard acid, was in his experience very satisfactory.

He confirmed the difference noted by the authors between the figures calculated for lecithin content and matter insoluble in acetone, and mentioned that the difference had at times been even greater in Japanese and Manchurian lecithins than in those now being produced in this country. He agreed that most of the difference could rightly be ascribed to the presence of sugary matter. It was possible to obtain a good check on the amount of oil present in a sample by determining the total fatty acids yielded by saponification, acidification and extraction with ether, subtracting from this figure 70·39 % of the calculated percentage of lecithin and dividing the result by 0·957; the factor, 70·39%, was based on the composition used for lecithin by Lewkowitsch and corresponded to a factor of 26·02 for the conversion of phosphorus to lecithin.

He asked whether the authors had had any success in attempting to determine acetone-insoluble matter in crude oils known to contain lecithin, as his experience was that no satisfactory precipitation could be obtained; he ascribed this chiefly to the solvent effect of the accompanying oil—an effect that was well-known in determinations of impurities in sulphur olive oils and black greases. For crude oils it appeared necessary to rely on the phosphorus determination as a guide to the lecithin content.

Dr. H. C. LOCKWOOD asked if the authors had considered the determination of free choline, as this substance was stated by some authorities to be the cause of bitterness in lecithin which had been stored for long periods. The method he had in mind was that of Beattie, who used ammonium reineckate.

Dr. B. REWALD said that lecithin, especially plant lecithin, was used in increasing quantities, not only in the food industry but in many technical processes. He asked how plant lecithin could be estimated if other sources of lecithin were present, such as whole egg or egg lecithin. Acetone would dissolve a certain proportion of phosphatides, especially if the amount of free fatty acids was high. This had to be taken into consideration.

Mr. HUTT, replying to Dr. Lockwood, said that he had examined a number of samples (both fresh and stored) for free choline, using an adaptation of Beattie's method (*Biochem. J.*, 1936, 30, 1554; ANALYST, 1937, 62, 57). Very low figures were obtained, and these were possibly due to lecithin passing through the filter during the extraction. Free choline did not appear to be present.

Mr. Bacharach had asked if possible variation in the composition of the complex of mercuric sulphate and acetone might offset the analytical advantage gained by its high molecular weight. The reply was that Van Slyke (*J. Biol. Chem.*, 1917, 32, 456) had found the composition of the complex to be reasonably constant for a given ratio of H_2SO_4 to $HgSO_4$.

Replying to Mr. Williams, who had found very considerable quantities of carbohydrates in Manchurian soya lecithin, Mr. Hutt said that he had found up to 18% of sugars (mainly non-reducing) present in combination with the phosphatides. With regard to the difference between the figures obtained for lecithin content by calculation from the amount of phosphorus and by determination of acetone-insoluble matter he said that other methods of ashing had been tried, but that wet destruction had been found the most satisfactory.

The elutriation method was not applicable to crude oils. Phosphorus was usually determined gravimetrically on 50 to 100 g portions of the samples.

Indian Sugar 21(5): 393-395, 1971

Sugar Confectionery

Commercial Lecithin—its Use in Confectionery and Chocolate Manufacture

By John F. Ingleton

The early patents proposing the use of lecithin in chocolate as an anti-greying agent were taken out by Hermann Bollmann and Dr. Bruno Rewald and dated 8th July 1929 (British Patent 330,450) and 17th October 1930 (British Patent 347,088). The later patent claimed that additions of 0.25 to one per cent quantities of lecithin would give savings of between 10 to 25 per cent in the amount of cocoa butter needed for chocolate manufacture.

Since 1930 a considerable number of patents for lecithin substitutes have been taken out by both European and American producers.

Examples of early British patents for these substitutes are :—

407,248—dated 13th March 1934 : Aigemaine Belegginge :—condensed polyvalent alcohol which contains phosphorus and a fatty acid.

514,721—dated 10the February 1938 : Emulsol Crop—phosphorus containing organic material.

514,848—dated 17th May 1938 : Ross and Rowe—fat condensation product.

528,377—dated 13th February 1939 : American Lecithin Co.—salt of carboxylic acid.

However it is only in recent years that satisfactory synthetic substitutes for commercial lecithins have been prepared and the use of these will be considered later. These synthetic products generally have improved reduc- and stabilising properties and contribute little or no ur to the confection.

The action of phosphatides, the active components in commercial lecithin is not clearly understood but the presence of a substituted phosphoric acid grouping is an important facture in their structure. It has been suggested by L. V. Czernenko (Kolloid Zhur, 1951,13,379) that the phosphatides become absorbed on the fine sugar particles present in chocolate. Results I have obtained suggest that this is indeed the case with either absorbtion of part of the phosphatide molecule occurring on the crystal or a simple coating mechanism. Both systems effectively increase the total surface area of phosphatides that are exposed to the other components in the chocolate mix. The resultant film of phosphatides on the sugar is very thin—less than one micron in thickness. When lecithin is first added to chocolate there is a considerable reduction in viscosity. This continues up to a 0.2 to a 0.3 per cent level of added lecithin. The rate of viscosity reduction then falls away and when the lecithin content exceeds between 0.5 to 0.6 per cent, the mix commences to thicken. The fall off in the rate of viscosity reducing power corresponds approximately to concentration of lecithin needed for the formation of a nearly complete layer of phosphatide material around the sugar. The level of addition of commercial lecithin at which this occurs is not constant due to variations in the sugar content and in the moisture content of chocolate. Water associates with the sugar in chocolate, probably to form a weak syrup which during processing is dispersed in fine droplets throughout the batch. Added lecithin associates with this syrup in a similar way to that described for sugar crystals. Varying moisture contents in chocolate will therefore necessitate varying additions of lecithin. Similarly changes in lecithin content are also necessitated by differing sugar usage in chocolate recipes. Changes in the amount of fat present in chocolate affects viscosity but the amount of lecithin required in a recipe does not appear to be directly linked to the fact contnt.

The viscosity reducing properties arise because the

coating of the sugar fragments with lecithin causes a reduction in the frictional rubbing occurring between the neighbouring crystals present in fluid chocolate. On addition of lecithin the phosphorus part of the molecule becomes associated with the sugar leaving the fatty acid portion facing outwards from the layer on the crystals. It is the fatty acidgroupings of lecithin which have lubricating properties and cause the reduction in frictional darg.

The normal analytical procedure used for the recovery of lecithin is extraction with chloroform. This procedure never recovers all the commercial lecithin which it is known to have been added to the batch. The discrepancy can be partly explained by breakdown occuring through the raised temperatures used in processing and possibly because some of the lecithin is tightly associated with the sugar.

Commercial lecithin is sensitive to heat and therefore only part of the recipe weight should be added at the early stages of processing. A suitable procedure for chocolate manufacture is to make the following staggered additions of commercial lecithin.

1. prior to refining—one fifth of the recipe weight
2. prior to conching—one fifth
3. halfway stage in conching—one fifth
4. one hour prior to completion of conching — two fifths.

The addition of lecithin at too early a stage in processing will lead to

a. loss of viscosity reducing power.

b. development of undesirable flavours in the chocolate

c. excessive retention moisture in the bateh

For most chocolate recipes, commercial lecithin should be incorporated at a 0.3 per cent level, chocolate which is to be used for coating high water content centres such as ice cream. should contain a higher level of lecithin—up to 0.6 per cent. The use of commercial lecithin cloes not prevent fat migration in the chocolate as has sometimes been suggested.

The phosphatides contained in commercial lecithin are white when pure but on separation from the carrier oil become brown due to oxidation. They are all optically active and have high molecular weights of 800 and upwards.

Three commercial lecithins are commonly available to the confectioner. These are in order of availability soya bean, lecithin, groundnut lecithin and cottonseed lecithin. Although these materials tend to be sold and used under the general description of "lecithin," there are considerable differences in phosphatide content which depends on the choice of oilseed. The phosphatide levels normally found in these lecithins are

groundnut lecithin 65-70 per cent

bleached or unbleached soyabean lecithin 60-65 per cent

cottonseed lecithin 50-55 per cent

Commercial lecithins vary in colour from deep yellow to dark brown. Deep colours in lecithins that have been produced from the same source may indicate that some breakdown has occurred but the cause of colour differences is much more commonly due to the colour pigments present in the original oil. Colour is therefore no guide to the probable effectiveness of a sample of commercial lecithin. Lecithin can be purchased either bleached or unbleached. Bleached lecithin is primarily of value when the sweet being produced is pale in colour. A typical confection of this type to Devon Cream Caramels.

Fluidity is also no guide to the effectiveness of a sample of commercial lecithin which is being offered for use as an ingredient in sweet and chocolate manufacture. The fluid nature of lecithin mixes can easily be varied through changes in :—

1. the amount of seed oil carrier
2. the amount of contained water
3. the extent to which oxidation has been allowed to occur.

Contained water has a particularly significant effect on commercial lecithin and if present at too high a level may result in deterioration of the raw material while on

store No more than one or, at the most, two months stock of lecithin should be held at the user factory Commercial lecithin is affected by exposure to are and is slowly oxidised. The presence of small traces of iron considerably accelerates this oxidation process.

Analytical examinations of samples of commercial lecithin should include determinations of :—

Phosphorus content :— a guide to the phosphatide content can be obtained by multiplying the phosphorus content by 26, This results obtained only approximate to the actual phosphatide figure. An examination of 50 samples of lecithin indicated various multiplying factors from 23 to 28

Acetone insolubles content :— This only approximates to the phosphatide content as not all phosphorus containing material is precipitated from in the acetone.

Moisture

Colour

Analytical results are only indicators to the quality of the commercial lecithin and predictions of behaviour made solely on such determinations are liable to error. Sample of commercial lecithins should be compared by testing under the conditions of their use. Thus the viscosity reducing power determinations should be made on standard recipe addition of lecithin added to a 'standard' prepared chocolate mix and tested under Specified conditions on the same intrument. The conditions must conditions on the same instrument. The coditions must be carefully stated and many reported conflicting results obtained using the similar commercial lecithin samples can be traced to minor variations in the conditions used during measurement. Even small changes greatly affect the results obtained using most methods for the viscosity determination of chocolate

In general the fluid commercial lecithins should be purchased for use in chocolate and sugar confectionery manufacture. These lecithins are more adaptable to handling on the continuous manufacturing units now widely used by the industry. Viscous lecithins can be diluted by the addition of refined soyabean oil or refined corn oil.

Phosphatides are sensitive to heat and breakdown occurs once the temperature has exceeded 40 degrees C (104 degrees F). One commercial lecithin has been added, both processing times and temperature should be kept to the minimum commensurate with satisfactory working.

Another effect of high temperature is the develoment of undesirable flavours in the lecithin. These flavours are carried forword and can adversely affect the overall "taste" of the batch of confectionery. It is mainy for this reason that a number of chocolate manufacturers are now using synthetic lecithins in the manufacture of chocolate.

(Confectionery Production June 1971)

279-280

2001

Commercial Lecithin—its Use and Composition

by John F. Ingleton

AMONG the various lesser components used in the manufacture of chocolate and sugar confectionery is commercial lecithin and its synthetic substitutes. This group of substances are particularly interesting in that they have properties which significantly influence the behaviour of the confections out of all relation to the amount of the ingredient being added to the batch. They are added to chocolate mainly because of their action as thinners or viscosity reducing agents and to fat containing confections as emulsifiers.

The use of the word lecithin is confused and has led to apparently conflicting statements being made in the technical literature. Material added to confectionery should not be called lecithin, which infers a pure substance, but best expressed as "commercial lecithin". Within the commercial product there is a component known chemically as phosphatidyl choline and called lecithin and another which chemically is phosphatidyl ethanolamine called cephalin. Some authors refer to the alcohol soluble and alcohol insoluble fractions obtained from commercial lecithin as lecithin and cephalin. Although these fractions are enriched with these materials they are by no means the pure product. The genera-group of compounds to which lecithin belongs is desl cribed by some authors as phosphatides and others as phospholipids.

Commercial lecithin contains a number of different phosphatides the most important of these being

phosphatidyl choline
phosphatidyl ethenolamine
phosphatidyl serine
phosphatidyl inositide

In addition a number of the above materials are present in a partially broken down form and have the suffix lyso e.g. lysophosphatidyl choline. There are also other complex compounds containing phosphorus and normally some vegetable oil as carrier.

Lecithin is present in a wide range of animal tissues and vegetable seeds and beans. That used by the chocolate and sugar confectionery manufacturer are normally extracted from the soyabean, groundnut, cotton seed and rapeseed. Most of the commercial lecithin used in the United Kingdom is derived from soyabeans.

Commercial lecithin has a sixfold effect when used in chocolate manufacture:—

1) it reduces viscosity and eases working.

2) it permits a reduction in the usage of the expensive cocoa butter.

3) it minimises the thickening effect when traces of water are present (an important feature in the production of chocolate for coating ice-cream).

4) it widens the range of processing temperatures that can be used.

5) it tends to improve the snap of chocolate.

6) to a limited extent it contains the development of fat bloom.

The reason why an addition of commercial lecithin behaves in a particular way is by no means clear. The effectiveness of the product cannot be precisely linked to the presence of particular chemical components. There are indications that the components become associated with the sugar that is present in the chocolate and that the part of the phosphatides which are fatty in character reduce viscosity by lowering the frictional drag of the sugar crystals. Basically chocolate is a mixture of solid particles interspersed with fatty layers.

It is difficult to draw conclusions from published works on the effect of commercial lecithin because com-

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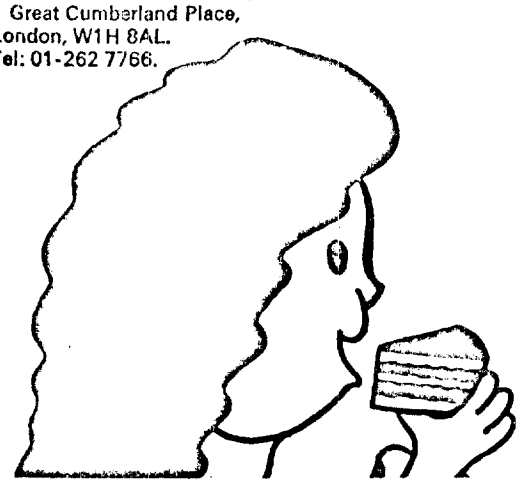


parisons must be made under the same test conditions using portions from the same bulk sample of chocolate. The most significant effect from the addition of commercial lecithin to chocolate comes from the first 0·1 to 0·2 per cent of material used. Further additional of commercial lecithin up to 0·5 per cent will cause falls in viscosity but over this figure the chocolate will show a slight but perceptable thickening.

Phosphatides are made up of

triglycerides which are related to natural fats
phosphatidic acid
mixed fatty acid end groupings
end groupings particular to the phosphatide.

The formula of the phosphatides can be represented as

$$\begin{array}{lll} CH_2.0. & CO. & F_1 \\ | & & \\ CH.0. & CO. & F_2 \\ | & O & \\ CH_2 0. & PO & E \end{array}$$

where F_1 and F_2 are the fatty acid endings and E is the appropriate phosphatide end groupings.

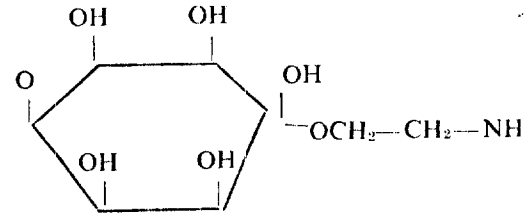
Typical end groupings are

choline $CH_2.\ CH_2.\ N\ (CH_3)_3$ OH

ethunolamine $CH_2.\ CH_2.\ NH_2$

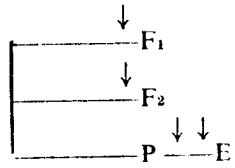
serine $CH_2.\ CH.\ COOH$ | OH

inositol



Nitrogen and phosphorus are significant features in the structure of phosphatides.

If the structure is represented diagramatically as follows:-



Breakages can be brought about by enzymic action at all the points indicated by arrows. When some of the fatty acid groupings have been removed, the phosphatide is of the 'lyso' type. The structure of the phosphatides is even more complicated than appears. The fatty acid groupings are not single acids and can contain six or more different members of this group. The amount of the most important of the fatty acids present in the three most commonly available commercial lecithins are given below

Fatty Acid	Soyabean	Groundnut	Cottonseed
Palmitic	25	21	27
Oleic	9	57	16
Linoleic	55	19	50

From a consideration of the above composition it could be postulated that cottonseed lecithin will be the most stable. However stability does not relate to effectiveness as a viscosity reducing agent and comparisons based on total phosphatide content indicate that the product extracted from cottonseed is by no means the most effective for use in chocolate manufacture.

The composition of a commercial lecithin derived from soyabeans is given below:—

phosphatidyl choline and lysophophatidyl choline, per cent	20
phosphatidyl ethanolamine and lysophosphatidyl ethen lamine, per cent	18
phosphornositol, per cent	10
phosphatidylserine and the lyso phosphatidyl serine, per cent	3
other phospherus containing compounds and phosphatides, per cent	10
soyabean oil, per cent	39

As indicated earlier, phosphatides are present in a wide range of naturally occuring materials. These include mammals and fishes, eggs (up to 10 per cent) and butter (up to one per cent). Phosphatides are present in cocoa beans though the cocoa lecithin that can be extracted is not an effective viscosity reducing agent. Egg lecithin is expensive to extract and relatively ineffective as a means of lowering viscosity. This may however be due to storage changes taking place within the purified lecithin. Commercial lecithin used in chocolate manufacture is primarily that extracted from the soyabean which is also the most effective. Groundnut lecithin is also available but this tends to be more difficult to handle.

Cottonseed lecithin is from time to time produced as is lecithin from the rape seed and from maize. Vegetable lecithins are prepared either by pressure expelling the oil from the seed and then purifying by solvent extraction or by hydration, separating in high speed centrifuges and vacuum drying. The type of processing can affect the properties of the commercial lecithin. This particularly applies to the amount of heat processing that the product receives during extraction.

In the next article, the use of handling and storage of commercial lecithin will be considered particularly with reference to its properties.

Joint FAO/WHO Expert Committee on Food Additives. 1970
Specifications for the identity and purity of food additives and their toxicological evaluation: some food colors, emulsifiers, stabilizers, anti-caking agents and other substances
World Health Organization, Geneva, WHO Tech. Rep. Ser. No. 445, page annex 5

LXVIII. THE ENZYMIC HYDROLYSIS OF PHOSPHATIDES.

III. THE HYDROLYSIS OF NATURAL AND SYNTHETIC PHOSPHATIDES.

By EARL JUDSON KING.

From the Department of Medical Research, Banting Institute, University of Toronto.

(*Received January 30th, 1934.*)

It has previously been shown [King, 1931] that an enzyme is contained in the kidney and intestinal mucosa which is capable of hydrolysing lecithin to liberate inorganic phosphate at an optimum p_H of 7·5. The enzyme appears to be present in other tissues to only a slight extent. Bone, which is very rich in an enzyme hydrolysing primary esters of phosphoric acid, is relatively feeble in its action against lecithin. Lecithinase appears to act most rapidly at body temperature (Fig. 1). It is fairly stable at a neutral reaction but is rapidly destroyed in an alkaline or acid medium. The concentration of substrate (Fig. 2) appears to

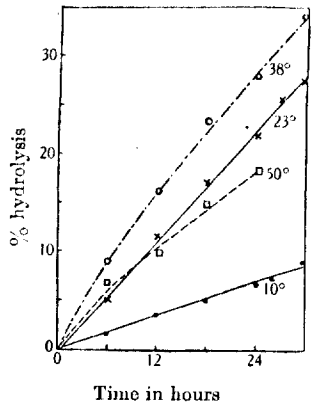


Fig. 1. Effect of temperature on the hydrolysis of lecithin:

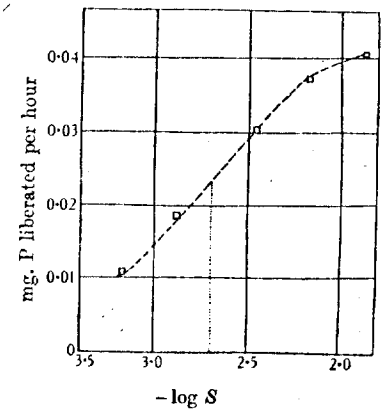


Fig. 2. Effect of substrate concentration on lecithinase hydrolysis:

10 ml. of lecithin emulsion at p_H 7·5 + 2 ml. of dialysed extract of intestinal mucosa.

affect the hydrolysis in a manner similar to that indicated by Michaelis and Menten [1913]. Owing to the slowness with which lecithin is broken down by the enzyme, it has been impossible to obtain any accurate measure of the initial velocity of hydrolysis. The results appear to indicate, however, that the substrate concentration giving half the maximum velocity corresponds to about 0·002 M ($-\log S$ 2·7), which is nearly the same as that found by Martland and

Biochem. Jour. 28(2): 476-481, 1934

Robison [1927] for phosphatase, *i.e.* $<0{\cdot}003\,M$ [cited by Haldane, 1930]. It is the purpose of the present paper to record the results of a study of the action of this enzyme on other phosphatides, both natural and synthetic.

Lecithin, kephalin and phosphatidic acid.

Liver-lecithin behaved much like egg-lecithin, while kephalin showed a lower rate of hydrolysis with not so marked a dependence on hydrogen ion concentration. A sample of phosphatidic acid from cabbage leaf [Chibnall and Channon, 1927] was kindly supplied to us by Prof. A. C. Chibnall. The formula of phosphatidic acid is represented as corresponding with that of lecithin with the choline absent. It showed a fairly high rate of hydrolysis, but the relation of the amount of hydrolysis to the p_H was not definite as in the case of lecithin (see Table I).

Table I. *Hydrolysis of natural phosphatides by extract of the small intestine.*

(mg. inorganic phosphorus.)

5 ml. borax-boric acid or borax-succinic acid buffer, 5 ml. 0·25 % emulsion of phosphatide and 2 ml. enzyme solution. The hydrolysis was carried out in the presence of chloroform and toluene for 12 hours at 37·5° and was stopped by the addition of 2 ml. 25 % trichloroacetic acid. The inorganic phosphate in the filtrates was estimated by the colorimetric method.

	p_H	Control (1); buffer + phosphatide	Control (2); buffer + enzyme	Test; buffer + phosphatide + enzyme	Phosphate hydrolysed
Egg-lecithin	5·5	0·000	0·041	0·058	0·017
	6·5	0·000	0·037	0·082	0·045
	7·1	0·000	0·038	0·109	0·071
	7·5	0·000	0·039	0·119	0·080
	8·1	0·000	0·039	0·112	0·073
	8·9	0·000	0·038	0·091	0·053
	9·2	0·000	0·037	0·068	0·031
Liver-lecithin	5·6	0·000	0·040	0·056	0·016
	6·5	0·000	0·037	0·067	0·030
	7·1	0·000	0·038	0·091	0·053
	7·6	0·000	0·039	0·096	0·057
	8·0	0·000	0·039	0·095	0·056
	8·9	0·000	0·038	0·066	0·028
	9·2	0·000	0·037	0·056	0·019
Kephalin	4·3	0·020	0·035	0·069	0·014
	4·8	0·020	0·034	0·070	0·016
	5·0	0·021	0·034	0·083	0·028
	5·5	0·020	0·041	0·103	0·042
	6·5	0·021	0·037	0·103	0·045
	7·1	0·020	0·038	0·101	0·043
	7·6	0·020	0·039	0·101	0·042
	8·3	0·019	0·039	0·107	0·049
	8·9	0·020	0·038	0·090	0·032
	9·2	0·021	0·036	0·089	0·032
Phosphatidic acid	5·6	0·068	0·040	0·191	0·083
	6·6	0·069	0·037	0·193	0·087
	7·0	0·067	0·038	0·187	0·082
	7·5	0·067	0·039	0·185	0·079
	8·0	0·070	0·038	0·185	0·077
	8·9	0·073	0·038	0·185	0·074
	9·2	0·069	0·037	0·180	0·074

Lysolecithin.

Lysolecithin was prepared by the action of the venom of the fer de lance on the lecithin contained in egg-yolk [King and Dolan, 1933]. The material so prepared has marked haemolytic properties. The behaviour of lysolecithin with

both the intestinal and bone enzymes was investigated. The amounts of hydrolysis at different hydrogen ion concentrations and at different times, when lysolecithin was subjected to the action of an aqueous extract of rabbit intestinal mucosa, indicated that the maximum hydrolysis occurred at about p_H 7·8, which is a little higher than the corresponding value for lecithin, and that this relatively simpler phosphatide was hydrolysed about twice as fast as the parent lecithin.

Bromolecithin.

Bromolecithin was prepared by passing bromine vapour in a stream of carbon dioxide into a mixed ether and light petroleum solution of egg-lecithin and liver-lecithin [Levene and Rolf, 1926]. The precipitated material was purified by solution in ether and reprecipitation by light petroleum.

Analysis for phosphorus was made by the perchloric acid colorimetric procedure [King, 1932], for nitrogen by micro-Kjeldahl, and for bromine by the potash fusion method of Fendler and Stüber [1914], the silver bromide being collected by means of a porcelain filter stick. (Found: P, 2·02; N, 1·04; Br, 41·3 %. Calculated: for octabromoarachidyl-stearyl-lecithin: P, 2·12; N, 0·96; Br, 43·7 %; for octabromoarachidyl-palmityl-lecithin: P, 2·16; N, 0·97; Br, 44·6 %; for hexabromostearyl-stearyl-lecithin: P, 2·42; N, 1·09; Br, 37·5 %.)

The behaviour of bromolecithin in the presence of phosphate-liberating enzymes was in marked contrast to that of the lecithin from which it had been made. With a variety of enzyme preparations from several sources, bromolecithin was practically completely hydrolysed in 24 hours, while lecithin was attacked only to the extent of 20 % in the most favourable instance. This peculiar property of greatly increased susceptibility to enzymic hydrolysis resulting from bromination of lecithin is probably related to an increased affinity of the enzyme for the phosphatide, due to the change in the fatty acid side-chain. The effect of p_H on the enzymic hydrolysis of bromolecithin is shown in Fig. 3.

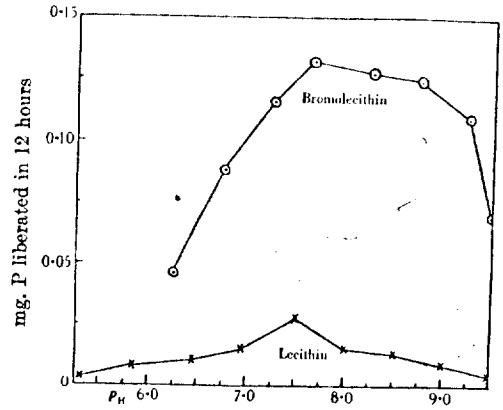


Fig. 3. Effect of p_H on hydrolysis of bromolecithin by cat-kidney extract. Each test contained 0·16 mg. of phosphatide-P, and 0·5 ml. of enzyme. Veronal-acetate buffer; hydrolysis at 38° for 12 hours.

The rates of hydrolysis of bromolecithin, lysolecithin and the parent lecithin are given in Table II, the hydrolysis constant being calculated from $k = \frac{1}{t} \log \frac{a}{a-x}$. The velocity of hydrolysis for bromolecithin was about 10 times as great as for

lecithin in the case of the intestinal enzyme, and 100 times as great with bone-phosphatase. The bone-enzyme, moreover, gave with bromolecithin about twice the velocity of hydrolysis that was obtained with the intestinal enzyme. This is in marked contrast to the action of the two enzyme preparations on lecithin. The velocities for bromolecithin were in about the same ratio as the phosphatase activities of the two preparations measured against glycerophosphate.

Table II. *Comparative rates of enzymic hydrolysis of phosphatides.*

	Lecithin		Lysolecithin		Bromolecithin	
Hours	% hydrolysis	k	% hydrolysis	k	% hydrolysis	k
			(1) Rabbit intestinal extract.			
½	1·0	0·0072	1·6	0·0138	9·1	0·0834
1	1·2	0·0054	2·0	0·0090	17·9	0·0860
2	2·4	0·0054	2·4	0·0054	29·0	0·0744
4	3·2	0·0036	4·8	0·0054	44·8	0·0645
7	4·8	0·0031	7·9	0·0052	59·9	0·0567
	Average	**0·0049**		**0·0078**		**0·0750**
			(2) Rat bone extract (phosphatase).			
½	—	—	0·7	0·0070	15·1	0·1424
1	0·4	0·0018	0·8	0·0036	28·6	0·1461
2	0·8	0·0018	1·1	0·0025	50·0	0·1505
5	1·2	0·0010	1·3	0·0012	65·9	0·0934
7	1·5	0·0009	1·6	0·0010	70·2	0·0751
	Average	**0·0014**		**0·0031**		**0·1215**

Synthetic phosphatides.

Through the kindness of Dr Adolf Grün, of the Hoffman-La Roche Company, Grenzach, Germany, we were able to compare samples of the synthetic phosphatides described by Grün and Limpächer [1927]. Synthetic distearyllecithin did not show the same general effect on enzymic hydrolysis as did the natural lecithin (Table III). The rate of hydrolysis was not so great and the p_H effect

Table III. *Influence of* p_H *on the enzymic hydrolysis of natural and synthetic phosphatides.*

(1) Natural egg-lecithin		(2) Synthetic lecithin (Grün)		(3) Distearyl phosphate (Grün)	
p_H	mg. P liberated	p_H	mg. P liberated	p_H	mg. P liberated
3·6	0·145	5·7	0·121	4·5	0·183
4·3	0·158	6·2	0·115	5·9	0·193
5·9	0·186	6·9	0·119	6·6	0·176
6·9	0·221	7·3	0·121	6·9	0·178
7·1	0·220	7·5	0·118	7·4	0·174
7·3	0·222	8·0	0·111	7·8	0·176
7·5	0·205	8·8	0·127	8·2	0·170
7·8	0·192			9·5	0·145
8·1	0·170				

was absent. It was thought that this difference might be due to the unsaturation and greater solubility of the natural product. This explanation was found to be untenable, however, by the discovery that hydrogenated lecithin has almost the same rate of enzymic hydrolysis as has the lecithin from which it is made (Table IV).

Synthetic distearyl phosphate showed very little relation between the p_H and the amount of hydrolysis. Since it is a primary ester of phosphoric acid,

Table IV. *Enzymic hydrolysis of hydrolecithin and lecithin.* (*mg. P liberated.*)

Tests consisted of 2 ml. of enzyme (rat-intestinal extract), 5 ml. of veronal buffer and 5 ml. of emulsified phosphatide containing 0·086 mg. of hydrolecithin-P per ml. and 0·088 mg. of lecithin-P per ml. The hydrolysis took place at 38° for 48 hours.

p_H	Hydrolecithin*	Lecithin	p_H	Hydrolecithin*	Lecithin
5·8	0·114	0·114	8·0	0·203	0·194
6·5	0·151	0·158	8·5	0·187	0·188
7·1	0·208	0·206	8·9	0·161	0·170
7·5	0·201	0·202	9·4	0·052	0·082

* This sample of hydrolecithin was kindly given to us by Dr P. A. Levene. Other samples prepared in this laboratory by hydrogenation of egg- and liver-lecithins in the presence of palladium gave very similar results.

it might have been expected to behave like glycerophosphoric acid or acetonylglycerophosphoric acid, which are hydrolysed at an optimum p_H of 8·9, whereas lecithin, a secondary ester of phosphoric acid, is hydrolysed at an optimum p_H of 7·4 (diphenyl phosphate and diethyl phosphate are hydrolysed at a slightly lower p_H optimum). Comparative figures for the hydrolysis of natural and synthetic lecithin and distearylglycerophosphoric acid were obtained by incubation of 5 ml. of 0·25 % emulsion of the phosphatide mixed with an equal volume of buffer at several p_H values and 2 ml. of the enzyme solution for 48 hours. The amount of phosphate liberated from the egg-lecithin was considerably greater than that from the distearyl phosphoric acid and at the optimum p_H was almost double that liberated from the synthetic lecithin.

Discussion.

The extraordinary change induced in lecithin by bromination is not easily explained. It is a more common experience to find that a large increase in molecular weight (that of bromolecithin is almost double that of lecithin) leads to a decrease rather than an increase in reactivity. A possible explanation of this anomalous result of greatly increased susceptibility to enzymic hydrolysis may lie in a greater negativity of the alcohol part of the ester due to introduction of several bromine atoms into the side-chain. Among the simple primary esters of phosphoric acid those containing the more negative alcohol groups are most readily attacked by phosphatase; thus phenyl phosphate is much more rapidly hydrolysed than tolyl phosphate, and tolyl phosphate very much more rapidly than ethyl phosphate.

In the case of phosphatidic acid, the p_H effect was almost absent, and since phosphatidic acid is a primary phosphoric ester, it— as also distearyl phosphate— might have been expected to be hydrolysed optimally at p_H 8·9. Since, however, the phosphate-liberating enzyme is relatively unstable above p_H 8, while it is quite stable in more neutral solutions, the discrepancy may be explicable on the basis of destruction of the enzyme during the course of hydrolysis. If this be so, the length of the fatty acid chains may be the determining factor, since it probably conditions the solubility of the phosphatide and its consequent availability for enzymic hydrolysis. The shorter the carbon chains of the substituent groups, the more soluble the substance should be and the more it should behave like a simple primary phosphoric ester with a p_H optimum of 8·9. It is also possible that the nature of the fatty acid radicals may affect, through some mechanism akin to steric hindrance, the union of substrate and enzyme.

Thus the presence of the fatty acids in phosphatidic acid may hinder its union with the enzyme, giving a lower rate than would be obtained with an unsubstituted glycerophosphate, or with a glycerophosphate substituted with less complex groups than stearic and oleic. This, combined with destruction of the enzyme, might account for the failure of this product to be hydrolysed at the optimum p_H of 8·9.

If the difference between the behaviours of natural and synthetic phosphatides on enzymic hydrolysis were due to the unsaturation of the natural product, then phosphatidic acid, which is more unsaturated than egg-lecithin, might have been expected to show a higher rate of hydrolysis. Also, hydrolecithin, which ought to be very similar in chemical behaviour to the synthetic product if existing theory as to the structure of these substances be correct, might have been expected to behave like synthetic lecithin, instead of which it showed an enzymic hydrolysis almost identical with that of its parent lecithin. These facts would appear to cast some doubt on the similarity of the chemical configuration of the synthetic lecithin of Grün and Limpächer with the naturally occurring phosphatide; and further, if the chemical formula assigned by Grün and Limpächer to the synthetic product be correct, then the formula commonly used to represent the chemical structure of lecithin may be in error. The asymmetric formula which appears in most of the textbooks and represents lecithin as always being a derivative of α-glycerophosphoric acid, has been shown to be incorrect [Rae, 1934], and it is possible that a more drastic revision of the manner of representing the formula for lecithin may yet be necessary.

Summary.

An enzyme is contained in kidney and intestinal mucosa which hydrolyses lecithin from several sources with the liberation of phosphoric acid. Hydrolecithin is attacked as fast as its parent lecithin, kephalin and phosphatidic acid somewhat more slowly, the latter without showing any marked dependence on p_H. Synthetic lecithin and distearyl phosphate are attacked by the enzyme at a lower rate and without showing any p_H optima. Lysolecithin is hydrolysed about twice as fast as lecithin. Brominated lecithin is hydrolysed at a very much greater rate than is lecithin.

The thanks of the author are due to Miss M. E. Dolan and Miss M. M. Shaw for valuable assistance during the course of these experiments.

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