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 ± 1 mv. at 19° . All titrations were carried out in a paraffin thermostat at $25.7^\circ \pm 0.1^\circ$. 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,

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.

lysokcephalin) 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 $\text{N}(\text{CH}_3)_3^+$ 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. $N/50 \text{ HClO}_4$. 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. $N/50 \text{ Na ethoxide}$. 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 $N/10 \text{ NaOH}$ in 90 % alcohol at $25.7 \pm 0.1^\circ$ (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.

<i>N</i> /10 NaOH ml.	<i>p</i> _H	<i>p</i> _K	<i>N</i> /10 NaOH ml.	<i>p</i> _H	<i>p</i> _K	<i>N</i> /10 NaOH ml.	<i>p</i> _H	<i>p</i> _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. CdCl₂ 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 <i>N</i> Na ethoxide (% of the total amount)	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	51.7
	(total amount)	

The isolated acids must be fatty acids since the greatest part of the lecithin and its decomposition products are precipitated with CdCl₂ 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

$$I.P. = \sqrt{K_1 \cdot K_2}$$

(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 <i>et al.</i> , 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

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. $\circ$ — $\circ$ blank curve; $\blacktriangle$ — $\blacktriangle$ 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

$$K = \frac{[\text{Free choline}][H^+]}{[\text{Choline}^+]} = \frac{0.070}{0.792} \cdot 10^{-12.89} = 10^{-13.94},$$

$p_K = 13.94$ (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 $[H^+] = \sqrt{10^{-1} \cdot 10^{-14}} = 10^{-7.5}$. 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 $p_H 6.7 \pm 0.2$. 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 $10^{-3.5} = 3.2 \times 10^{-4} \%$. 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 3.4×10^{-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.

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