COMPOUNDS OF PHOSPHORUS IN MILK—I

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Although phosphorus compounds of milk have been investigated by many in the field, their classification and general nature are still incomplete and obscure.

Jordan Hart and Patten[1] have estimated the total phosphorus in the whole milk and in its acid-soluble portion; they have also estimated the amount of organic phosphorus in the acid-soluble portion.

Lenstrup[2] states that there are four different forms of phosphorus compounds in milk which can be estimated.

Total phosphorus
- Acid-soluble P
  - Inorganic P
  - Organic P
- Acid-insoluble P
  - Protein P
  - Lipoid P

He separated the acid-soluble phosphorus compounds from the acid-insoluble by picric acid. Inorganic phosphorus is then precipitated from the acid-soluble fraction by adding ammonia-magnesia mixture, leaving organic phosphorus in the solution. He analysed the acid-soluble fraction and the portion containing organic phosphorus.

Lüddecke[3] found that picric acid in cold slowly attacks lecithin on standing. Therefore Graham and Kay[4] used trichloracetic acid as a precipitant instead of picric acid. In their paper (*loc. cit.*) they state that their values for inorganic phosphrous are vitiated by the possibility of the

[1] *Amer. J. Physiol.*, 1906, **16**, 268.
[2] *J. Biol. Chem.*, 1926, **70**, 193.
[3] *Inaugural Diss. Munich*, 1905.
[4] *J. Dairy Res.*, 1933, **5**, 54–62–63–74.

organic phosphorus from compounds of the type phosphagen, decomposing in presence of strong acids.

W. Hochheimer[5] found hexose mono-phosphoric acid, pyrophosphate and adenosine triphosphoric acid in cow's milk.

An attempt to find the nature of phosphorus compounds in the buffalo's milk available in Bombay City, is made in this communication.

Experimental

Compounds of phosphorus in the acid-soluble portion of milk were estimated by employing the methods developed by Eggleton and Eggleton[6] for muscle tissue, with some modifications.

To obtain the acid-soluble portion of milk, 0·5 c.c. of milk was taken in a centrifuge tube, 2·5 c.c. of water and 2 c.c. of 25 per cent. trichloracetic acid were added, and centrifuged. The acid extract was neutralised by the addition of finely ground baryta until neutral to phenolphthalein (*i.e.*, pH = 9). The mixture was centrifuged and decanted. The liquid separated from the precipitate is called Fraction B. The precipitate (Fraction A) was dissolved in a drop of concentrated hydrochloric acid and diluted to 10 c.c. with water.

Fraction A.—The orthophosphate was detected by Brigg's method before any hydrolysis had set in. The pyro- was estimated by Lohman's method.[7] The organic phosphorus was looked for after the solution was oxidised by sulphuric acid and 100 volume hydrogen peroxide, and hydrolysed. In all estimations a quantity of solution expected to contain 0·15 to 0·2 mg. of phosphorus was used.

Fraction B.—This contains hydrolysable and non-hydrolysable organic phosphorus compounds soluble in barium hydroxide at pH 9.

Hydrolysable phosphorus.—To estimate the amount of phosphorus associated with the hydrolysable organic compounds, the solution was hydrolysed with 2 c.c. of 5·5 N. Sulphuric acid for sixty minutes at ordinary temperature before the application of Brigg's method.

For Non-hydrolysable phosphorus, the total amount of phosphorus in this fraction was estimated by the method of oxidation and hydrolysis referred to already. The difference between this amount and that due to the hydrolysable variety gives the amount of phosphorus associated with the non-hydrolysable organic phosphorus compounds.

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[5] *Kinderhelik*, 1932, **54**, 49–64.
[6] *J. Physiol.*, 1929, **68**, No. 2, 193.
[7] *Biochem. Zeit.*, 1928, **203**, 172.

Whole milk and *the acid-insoluble portion of milk* were separately oxidised and after complete hydrolysis, Brigg's method was applied for the estimation of phosphorus. We have been able to check up the values obtained by direct estimations by comparing with those obtained indirectly, *i.e.*, by difference.

The acid-insoluble portion of milk was analysed for *lipoid phosphorus* and *casein phosphorus*.

For the estimation of lipoid phosphorus the method adopted was that of Graham and Kay (*loc. cit.*).

Casein in milk was obtained by Hammerstein method,[8] and the phosphorus content in it was estimated by subjecting it to oxidation and hydrolysis as in the case of whole milk.

Results and Discussion

TABLE I

Composition of Milk

(In gm. per 100 c.c. of Milk)

Expt.	Sp. gr. at 30°C.	Total Solids	Fat	S.N.F.	Casein	Lactose	Ash	Chloride
1	1·029	17·80	8·50	9·30	3·42	4·92	0·721	0·0648
2	1·028	16·36	8·36	8·00	3·13	4·95	0·703	0·0623
3	1·030	20·44	9·24	11·20	3·92	4·90	0·810	0·0729
4	1·030	21·63	9·63	12·00	4·18	4·55	0·862	0·0766
5	1·030	20·36	9·45	10·91	3·96	4·65	0·803	0·0723
6	1·031	21·66	9·70	11·98	4·26	4·29	0·864	0·0778
7	1·031	21·66	9·70	11·98	4·28	4·23	0·861	0·0775
8	1·030	18·63	7·37	10·93	3·68	4·98	0·765	0·0689
9	1·030	19·20	9·48	11·88	3·83	4·98	0·793	0·0720
10	1·031	21·36	8·91	11·03	4·06	4·94	0·801	0·0723
Average	1·030	19·03	9·03	10·91	3·87	4·74	0·798	0·0717
Standard deviation	0·000	±0·20	±1·8	± 1·015	±1·048	±0·24	±0·048	±0·0025

S.N.F. = Solids not fat.

[8] *Z. Physiol. Chem.*, 1883, **7**, 227 ; 1885, **9**, 273.

TABLE II

Analysis of Ash of Milk

(In gms. per 100 c.c. of Milk)

Expt.	Ash	Calcium	Phosphorus	Ratio CaO/P_2O_5
1	0·721	0·1521	0·0800	1·161
2	0·703	0·1482	0·0710	1·276
3	0·810	0·1717	0·0820	1·091
4	0·862	0·1828	0·0873	1·279
5	0·803	0·1723	0·0803	1·309
6	0·864	0·1830	0·0875	1·278
7	0·861	0·1828	0·0874	1·279
8	0·765	0·1603	0·0775	1·272
9	0·793	0·1673	0·0790	1·290
10	0·801	0·1698	0·0802	1·294
Average ..	0·798	0·2325 (as CaO)	0·1895 (as P_2O_5)	1·253
Standard deviation	± 0·048	± 0·20	± 0·036	± 0·071

TABLE III

Analysis of Compounds of Phosphorus

(Percentage of Total Phosphorus)

	I	II		III		
No.	Acid-soluble	Fraction B		Fraction A		
	P	Easily hyd. P	Non-hyd. P	Non-hyd. P	Ortho-P	Pyro-P
1	78·27	9·22	4·88	9·50	42·39	13·81
2	75·50	7·88	4·68	8·91	39·87	12·59
3	75·90	9·49	4·77	8·64	39·82	14·13
4	78·50	9·90	4·65	8·72	41·09	12·88
5	77·26	9·66	4·90	9·57	40·10	13·06

(Hyd. = Hydrolysable.)

TABLE IV

Concentration of Different Types of Phosphorus

(In mg. per 100 c.c. of Milk)

	1	2	3	4		5		
Expt.	Total phosphorus	Total acid-soluble P	Total acid-insoluble P	FRACTION B		FRACTION A		
				Easily hyd. P	Non-hyd. P	Non-hyd. P	Ortho-P	Pyro-P
1	110·63	86·10	24·20	10·20	5·40	10·52	46·90	15·28
2	128·56	97·10	31·26	10·14	6·02	11·50	51·26	16·18
3	120·10	91·20	27·32	11·40	5·73	10·38	47·83	16·97
4	106·83	83·90	21·98	10·50	7·10	9·32	43·89	13·76
5	124·90	96·50	27·63	12·07	6·02	11·95	50·09	16·32
6	..	98·33	..	12·30	6·14	12·20	51·26	15·20
7	..	78·66	..	9·83	4·91	9·76	40·56	12·15
8	..	76·44	..	9·56	4·77	9·48	39·84	11·81
9	..	83·32	..	10·42	5·19	10·32	42·89	12·86
10	..	74·46	..	9·31	4·65	9·24	38·45	11·15
11	..	78·90	..	9·86	5·01	9·79	41·29	12·20
12	..	79·96	..	9·99	5·99	9·999	41·99	12·36
13	..	86·40	..	10·87	5·39	10·72	45·10	13·36
14	..	96·32	..	12·04	6·01	11·95	50·12	14·89
15	..	95·45	..	12·00	5·96	11·84	49·62	14·75
Average	118·20	86·87	26·48	10·70	5·62	10·60	45·4	13·95
Standard deviation	± 2·65	± 2·98	..	± 0·96	±0·63	± 0·95	± 1·34	± 1·61

TABLE V

Acid-insoluble Fraction of Milk

Concentration of Different Types of Phosphorus

(In mg. per 100 c.c. of Milk)

Expt.	Total P	Total acid-insoluble P	Casein P	Lipoid P
1	126·2	25·36	22·98	3·59
2	113·4	22·03	20·20	4·47
3	128·6	30·08	27·26	4·20
4	123·4	25·23	22·96	3·94
Average ..	122·9	25·68	23·35	4·05

TABLE VI

Concentration of Creatine

(In mg. per 100 c.c. of Milk)

Expt.	Creatinine	Creatine	Creatine phosphorus acid
1	0·734	1·287	2·073
2	0·629	1·206	1·942
3	0·593	1·324	2·132
4	0·746	1·543	2·485
5	0·483	1·704	2·744
6	0·599	1·654	2·663

In order to see the average variation in the composition of milk several samples were examined. Analyses of some of the samples are given in Tables I and II. These variations however do not affect our results of the detailed analyses of milk, as the relative amounts of phosphorus compounds do not change (*vide* Table III).

Table IV gives the different types of compounds of phosphorus in mg. per 100 c.c. of milk. It will be seen that 73.6 per cent. of the total phosphorus in the whole milk is soluble in trichloracetic acid, which is in agreement with that obtained by Graham and Kay (*loc. cit.*).

In the acid-soluble portion of milk Graham and Kay have found 65.2 per cent. of the total phosphorus in the inorganic form and 9.8 per cent. as organic, which they call esters. From our method, the results of which are given in Tables III and IV, it is seen that only 50.24 per cent. (ortho + pyro) of phosphorus constitutes inorganic variety and as much as 24.27 per cent. is in the organic form. The lower value for the inorganic variety was expected as the easily hydrolysable organic phosphorus compounds in Fraction B were separated from the inorganic by adjusting the acid extract of milk to a pH 9 with baryta. A study of the nature of this variety accounts for the incorrect values obtained by Graham and Kay.

Fraction B.—The phosphorus in Fraction B exists in two forms: (1) directly estimable by Brigg's method and (2) obtained after oxidation and hydrolysis. The former constitutes 9.1 per cent. and the latter 4.8 per cent. of total phosphorus (*vide* Tables III and IV).

The easily hydrolysable phosphorus changes into ortho condition of Fraction A, in presence of trichloracetic acid, on heating or on dialysing the milk. This organic variety is, therefore, mistaken for the inorganic type.

In order to find the nature of these organic phosphorus compounds various substances were looked for. But, except for creatine and lactose, the indentity of other substances could not be established. Creatine in milk was estimated according to Masayoshi Sato and Kuchi Murata.[9] The amount of creatine found in milk is given in Table VI, column 2. It is likely that creatine might exist as creatine phosphoric acid which is hydrolysed by acid. This creatine phosphorus unlike in the case of muscle (Eggleton, *loc. cit.*) accounts only for a part of the hydrolysable variety. Therefore some other substance may be associated with this organic phosphorus.

It is not clear in what form lactose exists in milk. It has been suggested by Mai Monatsschr[10] that lactose is bound up with phosphorus in loose combination. If so only a very small amount of lactose present in milk will be used in this combination. This point requires further investigation.

Fraction A.—In Tables III and IV are given the amounts of the compounds of phosphorus, insoluble at pH 9 in presence of barium hydroxide. It will be seen that this fraction is divided into three types:

[9] *J. Agri. Chem., Soc. Japan*, 1933, **9**, 1–5.
[10] *Kinderhelik*, 1932, **51**, 391–92.

ortho, pyro and non-hydrolysable phosphorus. From Table IV, column 5, it is seen that 38·42 per cent. of the total phosphorus is in the ortho- and 11·8 per cent. in the pyro- condition. The non-hydrolysable variety forms 9·1 per cent. of the total phosphorus.

The amount of phosphorus in the acid-insoluble portion of milk is given in Table IV, column 3. It may be noted that 22·4 per cent. of the total phosphorus, which is present in the acid-insoluble portion, consists of lipoid phosphorus 3·7 per cent., and casein phosphorus, 19 per cent. (refer Table V).

It will be seen thus from the above discussion that we have been able to establish the existence of five independent types of phosphorus compounds: ortho, pyro, organic phosphorus compounds insoluble in barium hydroxide at pH 9 and hydrolysable and non-hydrolysable organic phosphorus compounds soluble at pH 9; over and above these the acid-insoluble phosphorus consists of casein phosphorus and lipoid phosphorus. The amount of phosphorus present in these seven varieties are estimated directly, as shown below in mg. per 100 c.c. milk :—