The Effect of Thyroxine and Thiouracil on the Composition of Milk

1. THE PARTITION OF PHOSPHORUS IN COW MILK IN RELATION TO PHOSPHATASE AND OTHER CONSTITUENTS

By R. CHANDA AND E. C. OWEN

The Hannah Dairy Research Institute, Kirkhill, Ayr

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It has been shown (Owen, 1948*a, b*) that thyroxine increases the percentage of phosphorus in cow milk without affecting the percentages of nitrogen or calcium. Experiments were therefore planned (for preliminary reports see Chanda & Owen, 1949*a, b*) with the object of partitioning the phosphorus in the milk of cows treated with thyroxine to find out whether any particular type of phosphorus compound was responsible for the increase. In the earlier experiments (Owen, 1948*a, b*) thyroxine decreased the positivity of the balance of nitrogen. It also increased the negativity of the balance of calcium so that more calcium was lost in the faeces than was present in the food and some of the calcium in the faeces must have come from skeletal stores. Under these conditions the paradoxical observation was made that thyroxine caused the lactating cow to retain phosphorus more avidly so that metabolism of phosphorus followed neither metabolism of nitrogen nor that of calcium. The results of the present experiments help to explain this.

To throw into greater relief any chemical differences between the milk of control cows and of those receiving treatment with thyroxine, a third group of cows was treated with the antithyroid drug, thiouracil.

EXPERIMENTAL

Two experiments were made, the first with three cows and the second with five. In each experiment cows in mid-lactation and with comparable milk yields were chosen.

Lactating cows treated with thyroxine show a progressive and readily reproducible decrease in the concentration of phosphatase in the milk (Folley & White, 1936; Owen, 1948*a*). Phosphatase concentration in the milk increases with advancing lactation. In the present experiments, therefore, cows were chosen with dates of calving as near as possible to one another so as to give approximately equal initial concentrations of phosphatase in their milks.

When these experiments were envisaged we had no knowledge of the effects of injecting thiouracil into lactating animals of any species. It was known, however (Owen, 1948*a*), that 10 mg. approached the maximum safe dose of thyroxine for a lactating cow. By comparison with this injected amount, the amounts of thiouracil 100–500 mg. (British Pharmaceutical Codex, 1949) given by mouth to human patients suffering from toxic goitre appeared huge. It was therefore decided to use the phosphatase titre of the

milk to compare the relative potencies of thiouracil and thyroxine. In both experiments the phosphatase was determined routinely on the milk. In Exp. 1 the effect of 10 mg. thiouracil/day was compared with the effect of 10 mg. thyroxine. In each experiment the design was the same. A period of 3 weeks, during which hormonal treatment was given, was preceded by a control period and succeeded by a recovery period. The composition of the milk during treatment could therefore be compared with the composition of the milk of the same cow before and after treatment, and simultaneously with the milk from an untreated cow. All the cows were identically managed. Milking was done by machine in the milking parlour attached to the dairy, and at every second evening milking a sample of milk (1 l.) was taken from the recorder vessel for analysis. Partitions of N and of P were determined throughout the experiments. The amounts of fat, lactose, total solids and certain other constituents in the milk were also determined and will be discussed in a subsequent communication.

Methods of analysis

P partitions were determined by a slight modification of the method of Graham & Kay (1934). P was estimated by the method of Fiske & Subbarow (1925), using a Spekker photoelectric absorptiometer. The blue colour was allowed 20 min. to develop.

Total P. An accurately weighed sample of milk was diluted with water to 10 vol. and 3 ml. of the resulting mixture were digested as described by Horecker, Ma & Haas (1940). The digest was neutralized and then diluted to 100 ml. P was determined in the resulting solution by the method of Fiske & Subbarow as described by Hawk, Oser & Summerson (1947).

Total acid-soluble P. Into a 50 ml. volumetric flask 5 ml. milk were weighed and diluted to 25 ml. with distilled water. The contents were made to the mark with 25% (w/v) trichloroacetic acid previously cooled to 4–5°. After 5 min. the contents were filtered. P was determined in 3 ml. of the filtrate after digestion as described under 'Total P'.

Inorganic phosphate. Inorganic phosphate was precipitated from 15 ml. of the filtrate from the preceding estimation contained in a 50 ml. centrifuge tube and neutralized to phenolphthalein (pH 8–9) with saturated Ba(OH)₂. After addition of 2 ml. 20% barium acetate the tube was left in the ice box for 20 min. prior to being centrifuged. The supernatant liquid was discarded and after the precipitate had been washed once with 10 ml. 1% barium acetate the phosphate in it was dissolved by addition of 1 drop of conc. H₂SO₄ and the contents of the tube were made to volume in a 50 ml. measuring flask. P was determined in the resulting solution as before. As a technical check inorganic P was

determined directly in the trichloroacetic acid filtrate. Table 1 demonstrates the agreement of the two methods.

Lipid P. Lipid P was extracted by ether-ethanol mixture (Hawk *et al.* 1947) and then determined, after evaporation of the ether-ethanol, exactly as for total P.

Table 1. *Ester phosphorus in milk determined either as (a) the difference between the total acid-soluble phosphorus and the barium precipitable phosphorus or as (b) the increase of phosphate by acid hydrolysis*

Sample no.	Ester P (mg. P/100 g. milk)	
	(a)	(b)
1	7.8	7.4
2	6.2	6.0
3	8.1	7.5
4	9.2	8.7
5	9.5	9.3
6	6.1	6.4
7	8.1	7.9
8	7.3	7.5
9	7.5	7.8
10	8.3	7.9
Mean	7.81	7.64

$$'t' \text{ (for paired difference)} = \frac{\text{mean difference}}{\text{s.e. of difference}} = \frac{0.17}{0.1044} = 1.628 \text{ (not significant).}$$

Ester P. This was calculated from the difference between the inorganic P and the total acid-soluble P.

Casein P. This was calculated from the equation

$$\text{Casein P} = \text{total P} - (\text{total acid-soluble P} + \text{lipid P}).$$

It was possible to check the reliability of the methods for ester P and casein P by other independent methods. The casein method was checked by direct precipitation of casein

Table 2. *Casein phosphorus in milk determined (a) directly in the casein precipitate or (b) from the difference between the total phosphorus and the total non-casein phosphorus*

Sample no.	Casein P (mg./100 g. milk)	
	(a)	(b)
1	18.4	17.6
2	17.5	18.1
3	15.9	14.5
4	17.3	18.1
5	16.8	17.5
6	16.2	15.4
7	18.9	19.2
8	17.4	15.9
9	18.3	19.1
10	19.5	18.3
Mean	17.62	17.37

$$'t' \text{ (for paired difference)} = \frac{0.25}{0.3077} = 0.8125 \text{ (not significant).}$$

in the presence of an acetate buffer at pH 4 from 10 ml. milk as described by Rowland (1938), but in a 100 ml. centrifuge tube. The tubes were spun in an International centrifuge for 30 min. at 2000 rev./min. The liquid was discarded and the

precipitate was washed three times with 50 ml. ether. The ether-washed casein was treated with a minimal amount of NH_3 and the resulting solution was diluted to 50 ml. with distilled water. After neutralization with $\text{N-H}_2\text{SO}_4$ to phenolphthalein the P content of 5 ml. of this solution was determined as already described for total P. Table 2 shows the agreement of results for casein P obtained by the two methods.

Phosphatase. Phosphatase was determined by Neave's (1939) modification of the Kay & Graham (1935) test. The short test was used in these experiments, the precautions advised by Kay, Aschaffenburg & Neave (1939) being carefully observed. Buffer substrate tablets and the Folin-Ciocalteu phenol reagent were as supplied by British Drug Houses Ltd. (B.D.H.). The amount of phenol liberated during incubation from the diphenyl phosphate present in the B.D.H. tablets was determined quantitatively in the Spekker absorptiometer. The standard graph was drawn from results obtained by using a solution containing 0.02 mg. phenol/ml. The phenol solution was standardized iodometrically (Hawk *et al.* 1947). The results for phosphatase have been recorded as mg. phenol liberated by 100 ml. milk. Such units are reproducible, but empirical, and have been called 'phenol units' in the tables and figures.

Total N. This was determined by the Kjeldahl method, 5 ml. milk being weighed and then digested with 20 ml. H_2SO_4 (sp.gr. 1.84) after addition of K_2SO_4 , CuSO_4 and Se.

Partition of N. To obviate the necessity of washing any precipitate, the various protein fractions were determined from analysis of N in filtrates only, pipettes being calibrated for delivering solutions saturated with salt. Globulin was determined by the difference between casein, precipitated with acetate buffer as described by Rowland (1938), and casein + globulin precipitated as described by Elsdon & Walker (1942). Other protein precipitations were carried out exactly as described by Rowland (1938). The total N in each filtrate was determined by micro-Kjeldahl using the Wagner-Parnas still. When digesting the filtrate with H_2SO_4 a mixed catalyst as mentioned under 'Total N' was used except for the filtrate from the albumin + globulin precipitation, which was so rich in MgSO_4 that a catalyst containing only CuSO_4 and Se was used, the MgSO_4 itself serving to raise the boiling point of the H_2SO_4 . NH_3 was trapped in 2% H_3BO_3 and titrated directly with 0.02N- H_2SO_4 .

RESULTS

Estimation of equivalence of responses of phosphatase to thiouracil and thyroxine

There is only speculation about the exact manner in which thiouracil inhibits thyroid activity (Andik, Balogh & Donhoffer, 1949). The toxic dose of thiouracil was not known, but it was assumed in Exp. 1 that 10 mg. thiouracil would produce a measurable response. Of the three cows compared, cow 2 received by subcutaneous injection 10 mg. thyroxine/day just after the afternoon milking, cow 3 received 10 mg. thiouracil, while cow 1 acted as an untreated control. The effects of these treatments on the phosphatase in the milk are shown in Fig. 1. The phosphatase figures for cow 2 diminished rapidly while a slight rise was shown by the

untreated animal. As was expected the phosphatase of the cow receiving thiouracil increased but at only about half the rate at which that of the thyroxine-treated cow decreased. On cessation of the treatments both effects were reversed, so that all three animals came to have comparable titres. As the rate of increase of the phosphatase titre of the milk of cow 3 was about half the rate of decrease shown by the thyroxine animal, the thiouracil dose used in Exp. 2 was doubled (20 mg.). Fig. 2 shows that the effect of this doubling was to produce rates of increase of phosphatase in the two thiouracil animals which were equal to the rates of decrease of phosphatase in the two thyroxine animals. Further-

Fig. 1. Phosphatase in cow milk (Exp. 1: 1948). ■—■, cow 3 (thiouracil, 10 mg./day); —, cow 1. (control); ●—●, cow 2 (thyroxine, 10 mg./day).

more, as in Exp. 1, in all five animals the phosphatase titres approached the titre of the milk of the control cow as the effects of the drugs wore off. On the basis of these experiments it was assumed that, in respect of its power to change the phosphatase titre, 20 mg. thiouracil is equivalent in its effects to 10 mg. thyroxine. These doses have been used in subsequent experiments to be reported in later papers.

The effects of the drugs on phosphatase titre have been numerically analysed in Table 3. The data in the first column were obtained from straight lines fitted by the method of least squares to the phosphatase titres of the treatment periods shown in Figs. 1 and 2. The second column represents the intercept of the line of the first column on the phosphatase axis, i.e. it is a corrected initial titre of phosphatase. The figure for the control animals in the first column of each experiment shows the change of phosphatase which occurred in the untreated animal. The data in Table 3 amplify the conclusion drawn from Figs. 1 and 2.

Fig. 2. Phosphatase in cow milk (Exp. 2). ○—○, cow 7 (thiouracil, 20 mg./day); ●—●, cow 8 (thiouracil, 20 mg./day); —, control; □—□, cow 6 (thyroxine, 20 mg./day); ■—■, cow 5 (thyroxine 10 mg./day).

Variations of phosphatase throughout one day of treatment

Throughout these experiments the time of injection was immediately after the evening milking, and to investigate the phosphatase content of the milk throughout 1 day the five cows in Exp. 2 were milked as usual on the 15th evening, and the four experimental cows were given their usual injections.

Table 3. *Rate of change of phosphatase (phenol units) during the period of treatment*

Cow no.	Treatment	Response per day during the injection period	Intercept of regression line (theoretical initial titre)	Phosphatase on the day of maximal response*	Time required to reach maximal response (days)
Experiment 1					
1	None	+0.37	109.3	—	—
2	Thyroxine (10 mg./day)	-7.82	162.1	39	12
3	Thiouracil (10 mg./day)	+3.25	132.9	198	10
Experiment 2					
4	None	+0.25	117.8	—	—
5	Thyroxine (10 mg./day)	-5.84	121.7	11	18
6		-6.51	149.3	22	18
7	Thiouracil (20 mg./day)	+6.20	130.7	254	18
8		+5.01	143.1	271	16

* Minimum for thyroxine, maximum for thiouracil.

All five cows were then milked 0.5, 1, 2, 8, 12 and 16 hr. after the usual evening milking. Phosphatase in the samples was determined with the results shown in Fig. 3. Since the treatments were well advanced the phosphatase titre of the control animal was always less than that of the thiouracil-treated pair and always greater than that of the thyroxine-treated pair. There was a small initial increase in the phosphatase in the thyroxine and control animals, probably because milk collected at so short an interval after milking is very rich in fat,

Fig. 3. Changes of phosphatase throughout the day from the time of injection. The cows which received hormonal treatment were injected at zero time on the graph.

and cream is known (Kay & Graham, 1934) to be richer in phosphatase than the remainder of the milk. A striking effect in this experiment is the large increase of phosphatase in the milk from the thiouracil animals. This shows that averages for one milking did not fully represent the effect of the thiouracil on phosphatase. This pronounced maximum in the milk phosphatase is probably attributable to ready penetration of the tissues by thiouracil and to rapid elimination of thiouracil by the kidneys (Ely, Olson & Reineke, 1948). An attempt was made to detect thiouracil in the milk by the method of Olson, Ely & Reineke (1947) but, in spite of satisfactory recoveries of thiouracil added to milk *in vitro*, none could be found. However, this was not surprising because the doses of thiouracil used (about 25 or 50 $\mu\text{g.}/\text{kg.}$ body weight) were minute in comparison with the dose of 100 mg./kg. body weight given subcutaneously and 200 mg./kg. given by mouth by Ely *et al.* (1948).

General effects on yields and composition

Thyroxine had its expected effect on milk yield. A graphical comparison of the effect of thyroxine and thiouracil on the milk of two of the cows is shown in Fig. 4, which also shows the percentages of fat, nitrogen and phosphorus in the milk and the

percentage of the nitrogen contributed by casein. Similar data from a control cow are included. The response of the milk yield to thyroxine is typical. A noteworthy feature is the diminution of yield caused by thiouracil. There is a marked contrast between the constancy of the total phosphorus in the milk of the untreated animal and either (a) its increase when thyroxine is given, or (b) its decrease by thiouracil.

Fig. 4. Milk yield, percentages of fat, nitrogen and phosphorus and percentage of casein in total nitrogen in milk, as affected by thyroxine and thiouracil. ●—●, Total P (mg./100 g.); ○—○, casein N as % of total N; —, milk yield; ■—■, fat (%); □—□, % N in fat-free milk.

Partition of nitrogen

Treatment with thyroxine did not affect either the total nitrogen in the milk or the percentage of casein nitrogen in the total nitrogen (Fig. 4). The effects of the drugs on the partition of nitrogen were investigated. The nitrogen partition (Table 4) shows that neither thyroxine nor thiouracil affected the partition of nitrogen between casein, albumin, globulin, proteose-peptone and non-protein nitrogen. There was no trend in the individual figures to suggest any definite effect of the drugs on the partition of nitrogen. This finding agrees with those of Van Landingham, Hyatt & Weakley (1946) and Booth, Elvehjem & Hart (1947), who partitioned the

Table 4. *Partition of nitrogen in milk from three cows given no drug, thyroxine and thiouracil respectively*

Cow no.	Period	Treatment in period 2	Total N (% by wt. of whole milk)	Percentage of total N as				
				Casein	Albumin	Globulin	Proteose-peptone	Non-protein nitrogen
1	1	—	0.604±0.045	74.6±4.5	9.0±2.2	5.4±2.0	6.8±1.6	3.6±1.0
	2	None	0.622±0.061	68.5±3.4	11.9±3.2	9.1±3.0	9.0±5.9	4.7±0.7
	3	—	0.668±0.052	64.4±5.0	3.9±0.7	14.1±3.6	13.9±3.6	4.2±1.1
2	1	—	0.592±0.057	74.0±4.1	10.1±2.8	5.8±3.9	6.4±1.2	3.8±1.0
	2	Thyroxine (10 mg./day)	0.607±0.036	71.5±2.6	12.9±3.9	5.4±3.6	9.9±4.7	4.7±0.5
	3	—	0.769±0.164	63.7±8.6	4.6±0.5	13.7±4.3	13.7±4.3	3.9±1.0
3	1	—	0.518±0.054	78.3±3.3	10.3±3.5	4.1±5.3	5.6±0.9	3.8±1.0
	2	Thiouracil (10 mg./day)	0.519±0.055	76.0±2.4	9.7±2.3	4.3±2.6	7.3±3.1	5.0±0.5
	3	—	0.537±0.051	71.0±4.5	5.4±1.4	9.3±4.1	8.4±3.1	5.8±1.2

Table 5. *Ratio of nitrogen to phosphorus in cow milk at time of maximal response**

Cow no.	Treatment in period 2	Period	Total N (mg./100 g.)	Total P (mg./100 g.)	N/P
1	—	1	599	88	6.8
	None	2	615	88	7.0
2	—	1	587	92	6.4
	Thyroxine	2	549	99	5.5
3	—	1	525	85	6.2
	Thiouracil	2	547	72	7.6
4	—	1	550	89	6.2
	None	2	564	88	6.4
5	—	1	582	86	6.8
	Thyroxine	2	577	98	5.9
6	—	1	553	90	6.1
	Thyroxine	2	519	98	5.3
7	—	1	499	91	5.5
	Thiouracil	2	496	80	6.2
8	—	1	573	99	5.8
	Thiouracil	2	612	89	6.9

* 'Maximal response' denotes the smallest value of N/P produced by thyroxine and the largest value produced by thiouracil.

nitrogen in the milk of cows receiving iodinated casein. The lack of change of the partition of nitrogen is in marked contrast to the simultaneous effects on phosphorus partition about to be described.

From Fig. 4 it can be concluded that thyroxine and thiouracil affected the ratio of total nitrogen to total phosphorus in the milk. Numerical values recorded in Table 5 show plainly that the N/P ratio which in normal circumstances appeared to average about 6.3:1 can be decreased by thyroxine to 5:1 and increased by thiouracil to 7:1.

Partition of phosphorus

The partition of phosphorus is shown in Table 6 and in Fig. 5. Table 6 shows the content of total phosphorus in the milk and its partition between inorganic phosphate, ester, lipid and casein. Averages only are recorded, and for comparison the average phosphatase titre is also shown. Comparison with Fig. 5, in which individual two-daily data for three typical cows are graphed, serves to

show that the magnitudes of the hormonally induced changes of the partition of phosphorus are very inadequately represented by the averages in Table 6. The results in Fig. 5 and Table 6 confirm the earlier observation made by one of the present authors (Owen, 1948b) that thyroxine notably increases the percentage of phosphorus in the milk. In Figs. 4 and 5 and Tables 5 and 6 the new observation is recorded that thiouracil produces the reverse change, i.e. markedly reduces the percentage of total phosphorus in the milk. The milk of the control animals became richer in phosphorus as lactation advanced. Thyroxine enhanced while thiouracil inhibited this tendency (Table 6).

The most notable change shown in both Fig. 5 and Table 6 is that ester phosphorus was markedly increased by thyroxine and decreased by thiouracil. Lipid phosphorus changed in the same way but not so markedly. In Fig. 5, effects of thyroxine and thiouracil on casein are evident; the casein fraction from thyroxine animals became for a time richer in

phosphorus during treatment, while the casein fraction from thiouracil cows became poorer. These changes in the casein phosphorus were all the more remarkable in view of the lack of change of the casein content of the milk. Table 6 also shows that changes of ester, lipid and casein phosphorus produced by treatment with either thyroxine or thiouracil were in the opposite direction to the changes simultaneously produced in phosphatase. These inverse changes are referred to again later.

Fig. 5. Effect of thyroxine and thiouracil on the partition of phosphorus in cow milk. ●—●, Casein P; ▲—▲, lipid P; ●- - ●, ester P.

Table 6 shows that the increase of total phosphorus by thyroxine hides a decrease of inorganic phosphate, and also that the decrease of total phosphorus by thiouracil hides an increase of inorganic phosphate. Thus inorganic phosphate differs from all the other fractions investigated in changing under either treatment in the same direction as phosphatase. Comparison of Fig. 2 with Fig. 5 indicates how closely phosphatase and ester

phosphorus are correlated. Accordingly coefficients of correlation of phosphatase with various phosphorus fractions have been calculated. In Tables 7 and 8 these coefficients are recorded. They show that, during either treatment, phosphatase was closely correlated negatively with ester and lipid phosphorus and positively with inorganic phosphate. Table 7 also shows that the correlation between ester

Fig. 6. Relation between ester phosphorus and phosphatase in cow no. 5 which received thyroxine.

Fig. 7. Relation between ester phosphorus and phosphatase in cow no. 7 which received thiouracil.

phosphorus and phosphatase is still large during the pretreatment period and in the untreated cows. Nevertheless, the apparently small difference between the coefficients of correlation of ester phosphorus and phosphatase before and after treatment can be shown by Fisher's *Z* test to be significant. Differences between the ester phosphorus phosphatase correlations shown by treated and control animals were similarly shown to be

Table 6. *The effect of thyroxine and thiouracil on the partition of phosphorus*

Cow no.	Treatment in period 2	Period	Total P (mg./100 g. milk)	Percentage of total phosphorus as				Phosphatase (phenol units)
				Inorganic	Ester	Lipid	Casein	
				Experiment 1				
1	—	1	85.3	64.5	10.6	8.8	16.1	99
	None	2	86.9	63.7	10.0	8.8	17.6	113
	—	3	94.8	62.1	9.2	8.5	20.3	116
2	—	1	92.4	62.2	10.0	9.0	18.7	167
	Thyroxine	2	95.9	56.1	14.6	9.9	19.4	79
	—	3	94.5	60.9	9.3	9.0	20.7	129
3	—	1	88.2	63.4	7.6	8.6	20.4	121
	Thiouracil	2	77.7	64.8	6.5	8.3	20.4	167
	—	3	82.4	63.3	7.9	8.0	20.8	132
Experiment 2								
4	—	1	86.7	63.9	8.9	7.2	20.1	112
	None	2	86.0	65.9	8.5	7.2	19.0	121
	—	3	90.6	66.7	6.4	7.4	19.4	129
5	—	1	82.4	56.4	10.2	11.3	22.0	129
	Thyroxine	2	92.9	51.5	17.5	12.1	20.7	43
	—	3	84.6	55.9	11.5	11.5	21.6	120
6	—	1	88.3	64.7	9.8	8.6	17.7	148
	Thyroxine	2	94.5	57.3	14.7	10.3	18.2	64
	—	3	91.8	64.2	9.6	9.4	16.8	125
7	—	1	94.4	63.1	9.5	8.8	18.9	129
	Thiouracil	2	85.1	67.8	6.1	8.1	18.2	213
	—	3	93.1	67.2	6.6	9.0	17.6	165
8	—	1	98.7	61.7	10.0	9.3	18.7	138
	Thiouracil	2	92.0	66.6	5.5	7.7	19.8	209
	—	3	98.4	66.2	7.1	9.2	17.3	170

Table 7. *Correlation of ester phosphorus and inorganic phosphorus with phosphatase*

Cow no.	Treatment in period 2	No. of observations in all periods	Correlation between ester P and phosphatase	Change of phosphatase per unit change of ester P (i.e. the linear regression coefficient)	Correlation between inorganic P expressed as % of the total P and phosphatase
1	None	30	-0.7023***	-16.2	+0.4017*
2	Thyroxine	30	-0.9469***	-17.0	+0.9658***
3	Thiouracil	30	-0.9776***	-24.3	+0.6008***
4	None	27	-0.7637***	-10.0	+0.5189**
5	Thyroxine	27	-0.9265***	-10.4	+0.8267***
6	Thyroxine	27	-0.9600***	-9.5	+0.9347***
7	Thiouracil	27	-0.9529***	-18.3	+0.8817***
8	Thiouracil	27	-0.8656***	-12.2	+0.8183***

* $P < 0.05$.** $P < 0.01$.*** $P < 0.001$.

significant. The same generalization does not, however, apply to the lipid phosphorus (Table 8), which in control cows and in pretreatment periods of treated cows is not significantly correlated with phosphatase at the 5% level of significance. The correlations of the ester phosphorus with phosphatase for a thyroxine and a thiouracil animal are shown in Figs. 6 and 7. Curves have been fitted to the points by the method of least squares. There is thus a curvilinear relationship between ester phosphorus and phosphatase similar to that re-

ported for the relationship between phosphatase and ester phosphorus in human milk by Chanda, Owen & Cramond (1951).

DISCUSSION

The ratio of nitrogen to phosphorus in cow milk shows variations attributable to the thyroid gland. Thyroxine increased the phosphorus content of the milk by 15–20% without affecting the nitrogen, while thiouracil depressed the phosphorus content

Table 8. *Correlation of lipid phosphorus with phosphatase*

Table 8. <i>Correlation of lipid phosphorus with phosphatase</i>					Change of phosphatase per unit change of lipid P (i.e. the linear regression coefficient)
Cow no.	Period	Treatment in period 2	Correlation coefficient	No. of paired observations	
Experiment 1					
1	1, 2 and 3	None	+0.2864	30	—
2	1	—	+0.3592	14	—
	2 and 3	Thyroxine	-0.9625***	16	-70
3	1	—	+0.2957	14	—
	2 and 3	Thiouracil	-0.7182**	16	-31
Experiment 2					
4	1, 2 and 3	None	+0.0470	27	—
5	1	—	+0.1945	8	—
	2 and 3	Thyroxine	-0.9055***	19	-34
6	1	—	+0.0316	8	—
	2 and 3	Thyroxine	-0.8795***	19	-38
7	1	—	+0.0643	8	—
	2 and 3	Thiouracil	-0.7450***	19	-28
8	1	—	-0.0494	8	—
	2 and 3	Thiouracil	-0.8046***	19	-19

Unstarred figures are not significant, i.e. $P > 0.05$.** $P < 0.01$.*** $P < 0.001$.

without any concomitant effect on nitrogen. The ratio of nitrogen to phosphorus (Table 5) was thus altered by either treatment. There is reason to believe that this ratio (N/P) is characteristic of the species. In cow milk it is only 6:1 whilst in human milk it is 20:1 (Chanda, Owen & Cramond, 1951). Even after treatment with thiouracil the N/P ratio of cow milk (7:1) is still much smaller than that of human milk.

It is known that ester phosphorus in milk acts at a suitable pH as a substrate for milk phosphatase. The present results, therefore, show that in milk the enzyme phosphatase is inversely correlated with its substrate. The part this phenomenon plays in mammary secretion is conjectural. It is noteworthy, however, that, as ester phosphorus increases, both lipid and casein phosphorus increase, partly at the expense of the inorganic phosphorus (Fig. 5 and Table 6). These observations are therefore consistent with current theories of biochemical phosphorylation, in which it is supposed that inorganic phosphate passes by way of ester phosphorus into the more complex phosphate esters found in fat and protein (Zilversmit, Entenman & Chaikoff, 1948; Popják & Muir, 1950).

When the mammary gland is made to work faster, as by thyroxine, more of the intermediate substance (ester phosphorus) is elaborated and less of the raw material (inorganic phosphate) is left. But if, as has been shown in experiments with the livers of rats by Williams-Ashman (1948), thyroxine increases the concentration of enzymes in the mammary epithelium, it is difficult to explain why, when thyroxine

is given, the amount of phosphatase in the milk becomes less. Perhaps the explanation of the lessening output of phosphatase in the milk at a time when the blood contains more than usual (Folley & White, 1936) is to be correlated with the power of the cow body, when treated with thyroxine, to retain phosphorus (Owen, 1948*b*) even while losing calcium and retaining nitrogen less avidly. When the body is made to work faster by thyroxine, this avid retention of phosphorus is probably correlated with an increase in the concentration of enzymes in the body and with an increase of the energy-transferring phosphoric esters which are indispensable to their action. Under such conditions it is not surprising that the phosphorus supplies of the soft tissues are maintained partly at the expense of the bones. Phosphorus passes from the bones to the soft tissues and the equivalent of calcium passing with it from the bones is passed out of the body in the faeces of the cow (Owen, 1948*b*). That thyroxine induces large losses of calcium from the body is well known. Hunter (1930) showed this in man, Parhon & Derevici (1932) in the dog, Blaxter (1948) in the sheep and Owen (1948*b*) in the lactating cow.

It is known from work on dogs and rats (Kleiner, 1948) with phlorrhizin (which inhibits phosphorylation in the kidney) that the retention of phosphate and glucose by the kidney is due to the phosphorylation of glucose in that organ, and the classical division by Lusk (1928) of amino-acids into glucogenic and non-glucogenic was based on experiments with such phlorrhizinized dogs. It may well be that thyroxine increases the concentration of

phosphorylase in the gut thus favouring the retention of phosphorus. Such an explanation makes it necessary, however, to postulate quite different conditions in the mammary gland which secretes phosphoric esters in greater amounts at the same time as its output of phosphatase decreases. It has been shown by Houston, Kon & Thompson (1940) and by Chanda, McNaught & Owen (1949) that phosphorylated aneurin is closely correlated with phosphatase. Chanda *et al.* (1951) found a similar negative correlation between phosphorylated aneurin and phosphatase in human milk. In cows even protein-bound aneurin varies in the opposite direction to phosphatase during treatment with either thyroxine or thiouracil (Chanda *et al.* 1949). With thiouracil, conditions in the mammary gland are such that more phosphatase appears in the milk. This observation makes it unlikely that phosphatase in milk is a 'leakage' product, for with thiouracil the escape of a smaller molecule (phosphorylated aneurin) (Chanda *et al.* 1949) is inhibited, while that of a larger one (phosphatase) is facilitated. Even a molecule so small as acetic acid does not appear in the milk as such in spite of its presence (McClymont, 1949) in ruminant blood and in the glycerides of ruminant milk fat (Achaya & Hilditch, 1950; French, Hunter, Martin & Popják, 1951).

A leakage hypothesis is hardly compatible with the correlations between phosphatase and the various phosphorus fractions reported in the present paper.

The changes produced by thyroxine and thiouracil in the present experiments imply that the phosphorus content of casein is affected by thyroid activity. This may, therefore, account for the difficulty of preparing casein of constant phosphorus content (Elsdon & Walker, 1942; Associates of Rogers, 1935).

SUMMARY

1. The main results on cow milk of thyroxine and thiouracil injections were: (i) thiouracil increased the phosphatase titre; (ii) comparison of the rates of change of phosphatase after thyroxine or thiour-

acil treatments showed that 20 mg. thiouracil gave an increase of the same magnitude as the decrease caused by 10 mg. thyroxine; (iii) milking at frequent intervals during 24 hr. showed that 2 hr. after a normal evening milking there is a pronounced maximum in the phosphatase titre of cows treated with thiouracil; (iv) neither thyroxine nor thiouracil affected the partition of nitrogen between casein, albumin, globulin, proteose-peptone and non-protein nitrogen; (v) thyroxine increased while thiouracil decreased the content of phosphorus of the milk; (vi) thyroxine decreased while thiouracil increased the ratio of total nitrogen to total phosphorus in the milk; (vii) thyroxine increased ester phosphorus markedly and also increased lipid phosphorus and caused a transient increase in casein phosphorus, partly at the expense of inorganic phosphorus; (viii) thiouracil affected the partition of phosphorus in the milk in exactly the opposite way to thyroxine.

2. (i) As a result of changes in phosphatase and phosphorus partition there were negative correlations between phosphatase on the one hand and ester phosphorus and lipid phosphorus on the other. Inorganic phosphate was positively correlated with phosphatase; (ii) large negative coefficients of correlation between ester phosphorus and phosphatase were found in untreated cows and in the experimental cows before treatment. These coefficients became larger under the influence of either drug.

3. It is suggested in the light of this and earlier experiments that the effect of thyroxine in increasing the metabolic rate may be due to an increase of the phosphorylating enzymes in the tissues.

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Non-peptide Linkages in Gramicidin

By A. T. JAMES* (Beit Memorial Fellow) AND R. L. M. SYNGE†
The Lister Institute of Preventive Medicine, London

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Gregory & Craig (1948; cf. Craig, Gregory & Barry, 1950) showed by counter-current distribution that crystalline gramicidin as usually prepared is a mixture of at least three closely related substances. That present in largest amount (*A*) contained the residues previously reported, whereas *B* contained in addition phenylalanine and *C* tyrosine. The work described in the present paper was done with samples of gramicidin obtained from tyrothricin by simple solvent extraction and crystallization, and hence contained at least these three components. Despite this complication it is felt that the information gained should cast some light on structural features that may be common to members of the group of closely related substances known as gramicidin.

We undertook this work in the hope of gaining information as to how gramicidin, apparently made up entirely from residues of monoamino-monocarboxylic acids and ethanolamine, is nevertheless devoid of basic properties and ionizing groups. A general review of previous data on the structure of gramicidin has been given by Synge (1950; cf. Hodgkin, 1950), and this includes a brief statement of the problem with a preliminary account of some of our present data. Some of the information given in that review needs modification in view of the discovery by Hinman, Caron & Christensen (1950)

that the racemic material obtained on partial acid hydrolysis which was thought by Christensen (1943, 1944) to be an equimolecular mixture of the peptides D-valyl-D-valine and L-valyl-L-valine is more probably a mixture of D-valyl-L-valine and L-valyl-D-valine. While the experiments now described do not lead to any rigorous conclusions as to structure they at least rule out a number of possible solutions of the problem.

It has been established by a number of methods that the gramicidins are neutral substances possessing no free amino or carboxyl groups. No free ionizing groups were found on electrometric titration in 60% (v/v) aqueous ethanol by Christensen, Edwards & Piersma (1941; confirmed in present paper, cf. Fig. 1), on titration in ethanol and in acetone by Hotchkiss (1941) or on titration in glacial acetic acid with perchloric acid (present paper). No amino groups were detected by Hotchkiss (1941) either by reaction with nitrous acid according to Van Slyke or by the colour reaction with ninhydrin. Similarly, we could obtain no stable yellow *N*-dinitrophenyl (*N*-DNP) derivative by reaction with 1-fluoro-2:4-dinitrobenzene (FDNB), and the migration behaviour of gramicidin in an electric field at pH 6 was no different from that of the neutral substance *N*-DNP-ethanolamine under the same conditions.

The only free functional groups of gramicidin other than indole groups seem to be hydroxyl groups possessing an alcoholic function. Gramicidin

* Present address: National Institute for Medical Research, The Ridgeway, Mill Hill, London, N.W. 7.

† Present address: Rowett Research Institute, Bucksburn, Aberdeenshire.