STUDIES ON EXPERIMENTALLY PRODUCED FRACTURE IN DOGS

Part III - Changes in calcium, total phosphorus and alkaline phosphatase content.

By

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Several groups of investigators have observed marked loss of nitrogen, sulphur, phosphorus and calcium in the urine of human subjects and experimental animals, exposed to moderate or drastic traumatic injury (Cuthbertson, 1930, 1931, 1932 and 1936; Cuthbertson *et al.*, 1939; Howard *et al.*, 1945). Out of these elements, calcium and phosphorus are known to play important roles in the process of calcification during healing of fracture. The enzyme, phosphatase is also believed to be concerned with the deposition of calcium salts in bone (Robison, 1923 and 1932).

As early as 1922, Telfer reported that the amount of calcium and phosphorus eliminated, depends upon the amount ingested. Roche and Mourgue (1940) have shown that during the early stages of fracture repair, these minerals are deposited in the callus tissue at the very site of fracture. Hence, it appears that the substances required for the ossification of the callus are to be transported to the site of injury by the tissue fluids, which in turn probably receive their mineral supply from the blood.

In view of the above, it was deemed necessary to study the progressive changes in the concentration of calcium, total phosphorus and alkaline phosphatase at the site of experimental fracture, beginning from the very onset of such alterations, and to correlate them with the changes in the level of these substances in circulation. By this approach, it is expected that some indication of the major biochemical changes occuring during the early stages of fracture healing would be obtained.

METHODS

Fractures of the middle third of the femur were produced under anaesthesia in male mongrel dogs weighing between 11 to 15 kg. (Ghatak *et al.*, 1957). The animals were kept on standardised dietary conditions throughout the period of these experiments and determinations of the calcium, total phosphorus and alkaline phosphatase were made in the venous blood and exudate at the site of fracture, withdrawn at regular intervals.

Calcium was estimated by a modification of Clark and Collip's procedure (1925).

Total phosphorus was determined by digesting 0.2 ml. of serum or exudate with 0.5 ml. concentrated sulphuric acid (A.R.) and two drops of hydrogen peroxide in a graduated pyrex glass test tube and developing the blue colour of phosphomolybdic acid by Fiske and Subbarow's method (1925). The spectrophotometric measurements were made in a Klett-Summerson photoelectric colorimeter at 660 mμ.

The alkaline phosphatase activity was determined by a slight modification of the method proposed by Bodansky (1932, 1933). 0.1 ml. aliquot of serum or exudate was incubated with buffered solution of sodium β-glycerophosphate (0.016M, pH 9.4) for one hour at 37°C; and the inorganic phosphate liberated by the enzyme reaction was estimated by the colorimetric method of Fiske and Subbarow (1925).

All the data obtained were statistically analysed.

RESULTS

Effect of ether anæsthesia on the calcium, the total phosphorus and the alkaline phosphatase contents :

Blood was withdrawn from the dogs before and soon after administering ether anaesthesia, and calcium, total phosphorus and alkaline phosphatase contents were determined in the serum. Statistical analysis of the data given in Table I would indicate that the differences from the normal were not significant at 5 per cent level.

Effect of fracture of the femur on the calcium, total phosphorus and alkaline phosphatase conteuts :

TABLE I

Statistical analysis of the data on the effect of ether anæsthesia on the calcium, total phosphorus and alkaline phosphatase of serum

	Difference in (after anæsthesia-normal)		
	Calcium	Total P	Alkaline phosphatase
Mean difference of 8 dogs	− 0.0125	+ 2.275	+ 0.0428
S.E. of mean differenee	0.02004	1.395	0.1850
t	− 0.624	+ 1.631	+ 0.230
P	0.50∠P∠0.60	0.10∠P∠0.20	0.80∠P∠0.90

Blood—Venous blood was withdrawn ½, 24, 48, 72, 96 and 120 hours after the fracture and the calcium, total phosphorus and alkaline phosphatase contents of the sera of 8 dogs at different time intervals are recorded in Table II and III. It may be seen that soon after the fracture, the total

TABLE II

Effect of fracture on the calcium and the total phosphorus contents of serum (mg/100 ml.)

No. of animal & weight (kg.)	Estimations	Normal (before anaesthesia)	After anaesthesia	Interval after fracture (hours)					
				½	24	48	72	96	120
1	Ca	9.7	9.8	9.8	9.5	9.3	9.0	8 8	8.8
15.0	Total P	28.9	28.5	29.1	32.5	34.4	34.8	35.0	36.0
2	Ca	10.1	9.8	9.8	9.9	9.5	8.8	8.6	8.6
11.7	Total P	24.4	25.1	25.9	26.2	26.8	27.6	29.0	32.2
3	Ca	11.0	11.0	10.8	10.4	10.0	8.5	9.0	8.9
13.4	Total P	45.1	45.8	45.6	46.0	46.0	47.2	47.6	48.8
4	Ca	12.0	12.2	12.0	11.5	11.0	10.9	10.7	10.6
11.5	Total P	48.0	47.6	48 5	49.2	50.0	50.8	51.0	54.0
5	Ca	10.4	10.2	10.0	9.6	9.4	9.1	9.0	8.8
12.3	Total P	27.6	27.6	27.6	29.4	30.1	32.6	33.0	35.5
6	Ca	10.2	10.2	9.9	9.8	9.6	9.6	9.4	9.2
13.4	Total P	24.0	24.0	24.4	25.6	27.0	26.8	29.8	31.8
7	Ca	10.9	11.0	10.9	10.5	10.2	10.0	9.8	9.6
12.4	Total P	24.4	24.7	24.2	25.0	25.4	26.8	27.7	31.0
8	Ca	10.5	10.5	10.6	10.2	9.8	9·4	9.0	8.9
11.2	Total P	23.5	23.5	24.4	25.1	26.8	27.9	30.8	33.6

phosphorus content of the serum rose slightly above the normal level. The value increased gradually with time, registering about 25-43 per cent rise in most of the dogs after 120 hours. Dogs No. 3 and 4, however, showed comparatively less increase. The calcium content of the serum on the other hand decreased gradually to a constant level 72 to 96 hours after the fracture. At this time the value was about 10-19 per cent less than the normal level.

TABLE III

Effect of fracture on the alkaline phosphatase of serum (γinorg. P/hr./ml).

No. of animal	Normal (before anaesthesia)	After anaesthesia	Interval after Fracture (hours)					
			½	24	48	72	96	120
1	70.4	70.0	70.8	71.2	72.0	74.3	76.2	79.2
2	56.7	56.7	57.0	55.0	57.5	60.0	61.0	62.5
3	76.5	76.0	75.9	74.8	76.6	81.0	84.4	92.0
4	80.2	80.8	80.2	80.6	81.4	84.2	88.0	92.0
5	63.1	62.6	62.9	62.7	64.0	68.0	69.5	72.0
6	46.4	47.0	47.0	45.2	48.4	51.3	53.1	56.2
7	55.4	56.1	57.6	55.0	57.2	60.5	62.0	63.5
8	34.4	34.0	34.8	34·0	35.5	38.0	39.0	42.6

The alkaline phosphatase level decreased slightly below the normal level, 24 hours after producing the fracture and then it rose progressively reaching its maximum on the last day of the experiment (i.e., after 120 hours).

Statistical analysis of the data (cf. Table IV) shows that the differences in the concentration of total phosphorus, calcium and alkaline phosphatase

TABLE IV

Statistical analysis of the data on the effect of fracture on the total phosphorus, calcium and alkaline phosphatase contents of blood serum

	Difference in (after 3 days of fracture-normal)			Difference in(after 3 days of fracture-anaesthesia)		
	Total P	Ca	Phosphatase	Total P	Ca	Phosphatase
Mean difference of 8 dogs	+35.6375	−1.0625	+4.2857	+33.363	−1.05	+4.2437
S. E. of mean difference	4.7329	0.1101	0.2955	5.881	0.143	0.292
t	+ 7.530	−9.650	+14.503	+ 5.673	−7.358	+14.540
P	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001

before and three days after the fracture were significant at 5 per cent level. The same was true with regard to the differences in the respective values after anaesthesia and three days after the fracture.

Exudate—Half an hour after the fracture, the haematoma at the site of fracture was aspirated, and the determinations of the total phosphorus, calcium and alkaline phosphatase were made in the clear supernatant obtained after centrifugation. The aspiration was repeated after every twenty-four hours upto 5 days when the animal was destroyed. The results obtained are presented in Tables V and VI and Fig. 1. It would be seen

TABLE V

Effect of fracture on calcium and total phosphorus contents of the exudate (mg/100 ml)

No. of animls and weigh (kg.)	Estimations	Interval after fracture (hours)					
		½ (initial)	24	48	72	96	120
1	Ca	9.2	9.4	9.6	10.0	9.9	9.6
15.0	Total P	32.2	27.6	26.9	25.8	24.0	23.7
2	Ca	8.5	8.8	9.0	9.0	8.8	8.6
11.7	Total P	32.0	27.6	26.2	26.7	24.0	22.0
3	Ca	9.8	9.9	9.6	10.1	10.2	9.6
13.4	Total P	46.4	42.8	42.0	41.9	40.0	39.0
4	Ca	10.0	10.4	10.2	10.8	10.3	10.2
11.5	Total P	49.5	45.1	41.6	33.5	32.1	31.0
5	Ca	9.8	9.9	10.2	10.6	11.0	10.7
12.3	Total P	30.1	27.6	27.0	27.0	26.2	25.1
6	Ca	9.0	9.0	9.5	9.8	9.8	9.6
13.4	Total P	28.0	26.0	21.0	20.0	19.6	18.2
7	Ca	9.8	10.0	10.3	10.1	11.2	10.4
12.4	Total P	30.7	27.6	25.9	25.9	25.1	24.8
8	Ca	9.4	9.5	9.8	10.2	9.8	9.6
11.2	Total P	27.6	21.0	19.4	18.5	18.2	17.6

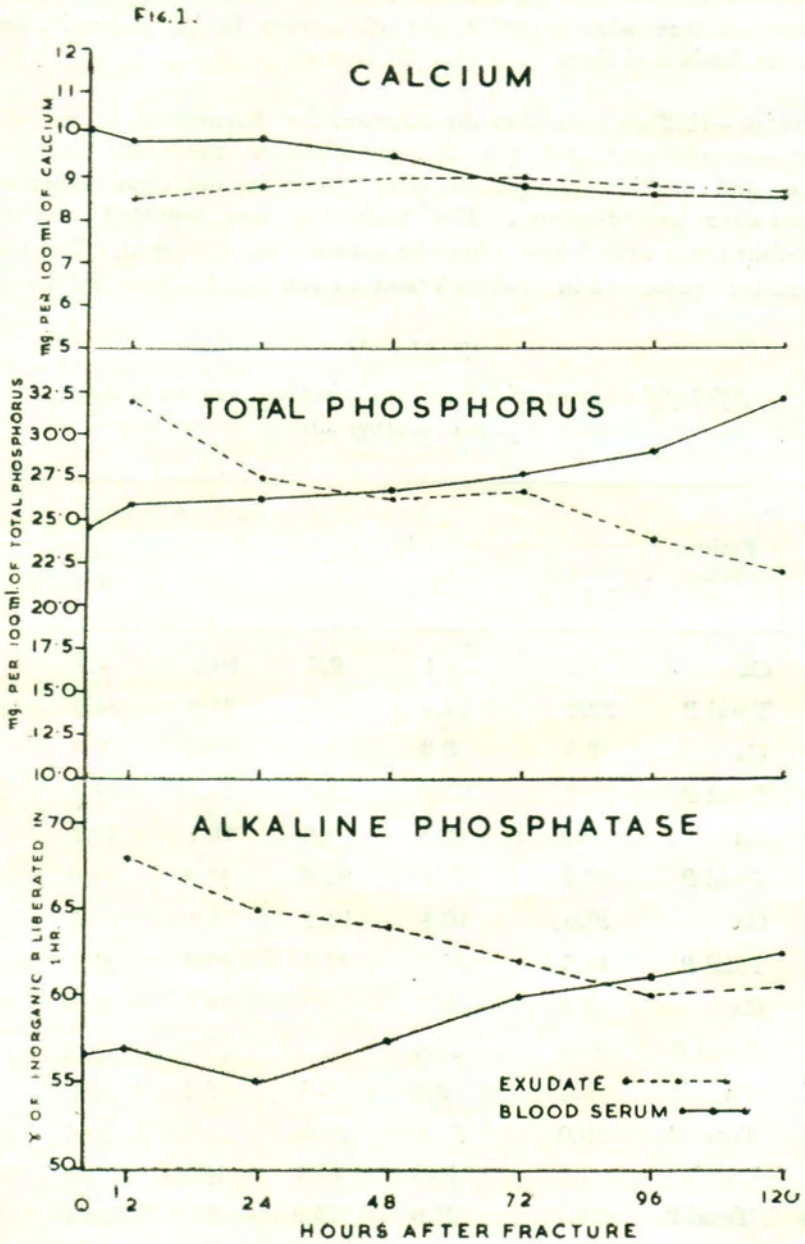

Fig. 1. Calcium, total phosphorus and alkaline phosphatase contents of serum and the exudate after femur fracture. (Dog No. 2)

TABLE VI

Effect of fracture on the alkaline phosphatase of the exudate (γ*inorg P/hr./ml*)

Number of animal	Interval after fracture (hours)					
	$\frac{1}{2}$ (Initial)	24	48	72	96	120
1	82.4	80.0	76.2	73.4	72.0	72.2
2	68.0	65.0	64.0	62.0	60.0	60.5
3	86.6	84.0	82.6	82.6	80.2	81.0
4	93.6	91.0	88.0	86.0	85.0	85.0
5	82.0	79.0	76.0	73.0	73.0	74.5
6	54.0	52.0	51.0	49.5	48.0	48.5
7	66.2	64.5	64.0	61.4	60.0	59.5
8	45.5	42.5	42.0	39.0	38.0	39.0

from Fig. 1 that just after the fracture, the total phosphorus content of the exudate rose much above the normal level. The value dropped sharply 24 hours after fracture and remained near the blood level upto 72 hours, after which a further decrease was observed. The calcium concentration in the exudate, soon after the fracture, had a tendency to fall below the corresponding value in the blood and kept at a level lower than the normal blood level throughout the period of the experiment. However, it may be mentioned that calcium content of the exudate 72 hours after the fracture was slightly more than the initial value.

The alkaline phosphatase level of the exudate was much higher than that in the circulating blood during the half hour interval after the fracture. It fell gradually to a steady state in 96 hours.

Statistical analysis of the data (cf. Table VII) would indicate that the fall in the values of total phosphorus and alkaline phosphatase, three days after the fracture, as compared with the figures just after the fracture was significant at 5 per cent level. The slight rise in the calcium content of the exudate three days after the fracture, when compared with the initial value, was also significant at 5 per cent level.

Ratio of calcium to total phosphorus :

The data have further been statistically analysed to see if there occurred any change in the Calcium/Total P ratio as a result of anaesthesia as well as fracture in blood and exudate.

TABLE VII

Statistical analysis of the data on the effect of fracture on the total phosphorus, calcium and the alkaline phosphatase contents of the exudate

	Difference in (3 days after fracture-just after fracture)		
	Total P	Calcium	Alk. phosphatase
Mean difference of 8 dogs	− 71.6125	+ 0.6375	− 6.9167
S. E. of mean difference	14.363	0.0822	0.6651
t	− 4.986	+ 7.755	− 10.399
P	< 0.01	< 0.001	< 0.001
The differences are significant.			

It would appear from the results presented in Table VIII that although the calcium / total phosphorus ratio of blood decreased slightly after anaesthesia, the fall was not statistically significant. However, three days after the fracture the Ca/P ratio decreased by 7. per cent in blood and increased by 7.8 per cetn in the exudate.

TABLE VIII

Ratio of calcium to total phosphorus in blood and exudate

	BLOOD			EXUDATE	
	Normal	After anaesthesia	3 days after fracture	½ hr. after fracture (initial)	3 days after fracture
Ratio Ca/P	0.346	0.335	0.274	0.281	0.359
Per cent change from initial to 3 days after facture	−	−	−7.2	−	+7.8
	+ Increase			− Decrease	

DISCUSSION

The results presented above would show that the concentration of calcium, total phosphorus and alkaline phosphatase are altered in serum and exudate as a result of the fracture of the middle third of the femur bone. The observed increase in the total phosphorus and the alkaline phosphatase levels of the serum after the fracture would indicate that there is a flow of phosphorus compounds from the tissues into the circulation to meet the increased

demand for these substances at the site of injury. Progressive increases in the level of inorganic phosphate and alkaline phosphatase in the sera of fractured patients and experimental animals were also observed by several investigators (Moorhead *et al.*, 1923; Satanowski, 1925; Peden, 1937 and Wilkins and Regen, 1935). Most of these workers could not observe any significant change in the serum calcium values during the first week following fracture. In the present experiments, serum calcium has been shown to decrease by 10 to 19 per cent five days after the fracture had been produced. The calcium to phosphorus ratio in the serum also declines three days after the fracture.

In the exudate, however, the total phosphorus and alkaline phosphatase rise above the corresponding values in the venous blood, registering about 10 to 31 and 10 to 19 per cent increase respectively during the first half-hour after the fracture. The initial rise is followed by a gradual fall. In most of the dogs, the total phosphorus values decrease much below the blood level 120 hours after the fracture. The calcium level shows a tendeney to fall below the serum level soon after the fracture, but the calcium to phosphorus ratio of the exudate increases by 7.8 per cent.

Thus the experimental evidence indicates that soon after fracture, the phosphate-ion concentration is increased locally at the site of injury by a mechanism in which alkaline phosphatase may be involved. This is followed by a marked decrease in the amount of total phosphorus and a moderate decrease in the concentration of calcium in the haematoma fluid after 72 hours, the time at which callus formation usually takes place. Swenson (1946) had shown earlier that after the fracture, the haematoma fluid was more acidic than the venous blood and that the pH shifted gradually to the alkaline side in the course of next few days. Hence it is quite possible that the subsequent change to alkalinity in the fracture haematoma, results in the precipitation of bone salts, from the locally supersaturated solution, on the bone matrix.

The above observations support the view that total phosphorus and calcium play important roles along with alkaline phosphatase in the process of ossification after fracture.

CONCLUSIONS

1. Ether anaesthesia has no effect on the total phosphorus, calcium and alkaline phosphatase contents of the serum.

2. The total phosphorus content of the serum rises above the normal level soon after the fracture of the femur bone. This is followed by a gradual increase which reaches its maximum after 120 hours.

3. The alkaline phosphatase content of the serum falls below the normal level 24 hours after the fracture and then it rises progressively reaching a maximum after 120 hours.

4. The calcium level of the serum decreases gradually reaching a constant level 72 to 96 hours after the fracture.

5. The total phosphorus and alkaline phosphatase contents of the exudate during the half hour interval after fracture are much higher than those in the circulating blood. The values then drop, reaching a minimum after 96 to 120 hours.

6. The calcium level in the exudate, soon after the fracture, has a tendency to fall below the corresponding value in blood.

7. The calcium / phosphorus ratio decreases in serum and increases in the exudate three days after the fracture.

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