CHAPTER III

RESULTS

1. Total protein and inorganic phosphate concentration in the uterine fluid

Determination of total protein and inorganic phosphate (P_i) concentration in pooled uterine fluid collected from many groups of rats used in the following experiments confirmed the previous results (3) that the concentration of total protein and P_i in the IUD fluid were significantly increased 4 and 15-fold respectively of those in control fluid (Table 1). The average concentration of P_i in the IUD fluid was 40.33 ± 22.14 µg/ml.

2. Number of normal fetuses observed in control pregnant rats

The pregnancy of recipient rats were induced as described under "Methods" and the number of normal fetuses in both uteri were observed on day 15 of pregnancy.

It was shown in Table 2. that the number of normal fetuses in each uteri varied from 3-7 with more or less similar average number of 4.63 ± 1.22 in the left uterus and 5.00 ± 1.12 in the right uterus.

3. Bioasaay for the contraceptive activity of inorganic phosphate

Since Yaovapolkul (3) found that the fluid-volume of the IUD-bearing horn increased from 0.3 ml to 0.5 ml with the net increase of about 0.2 ml during oestrus stage, in this experiment therefore, 0.2 ml

Table 1. Concentration of total protein and inorgnic phosphate in the uterine fluid

	Group No.	# rats/group	Total protein (µg/µl)	Inorgnic phosphate (µg/ml)
control fluid	I	9	3.20	3.00
	II	11	2.50	2.00
	III	15	1.80	2.00
	IV	10	2.36	3.50
	V	15	3.48	2.50
	VI	36	2.13	4.00
	VII	14	2.85	2.50
	VIII	17	1.45	2.00
Mean ± SD.			2.47 ± 0.64	2.69 ± 0.70
IUD fluid	I	9	11.20	80.00
	II	15	9.40	16.50
	III	10	10.00	30.00
	IV	10	10.00	58.50
	V	10	12.60	22.00
	VI	14	9.00	35.00
Mean ± SD.			10.37 ± 1.21	40.33 ± 22.14

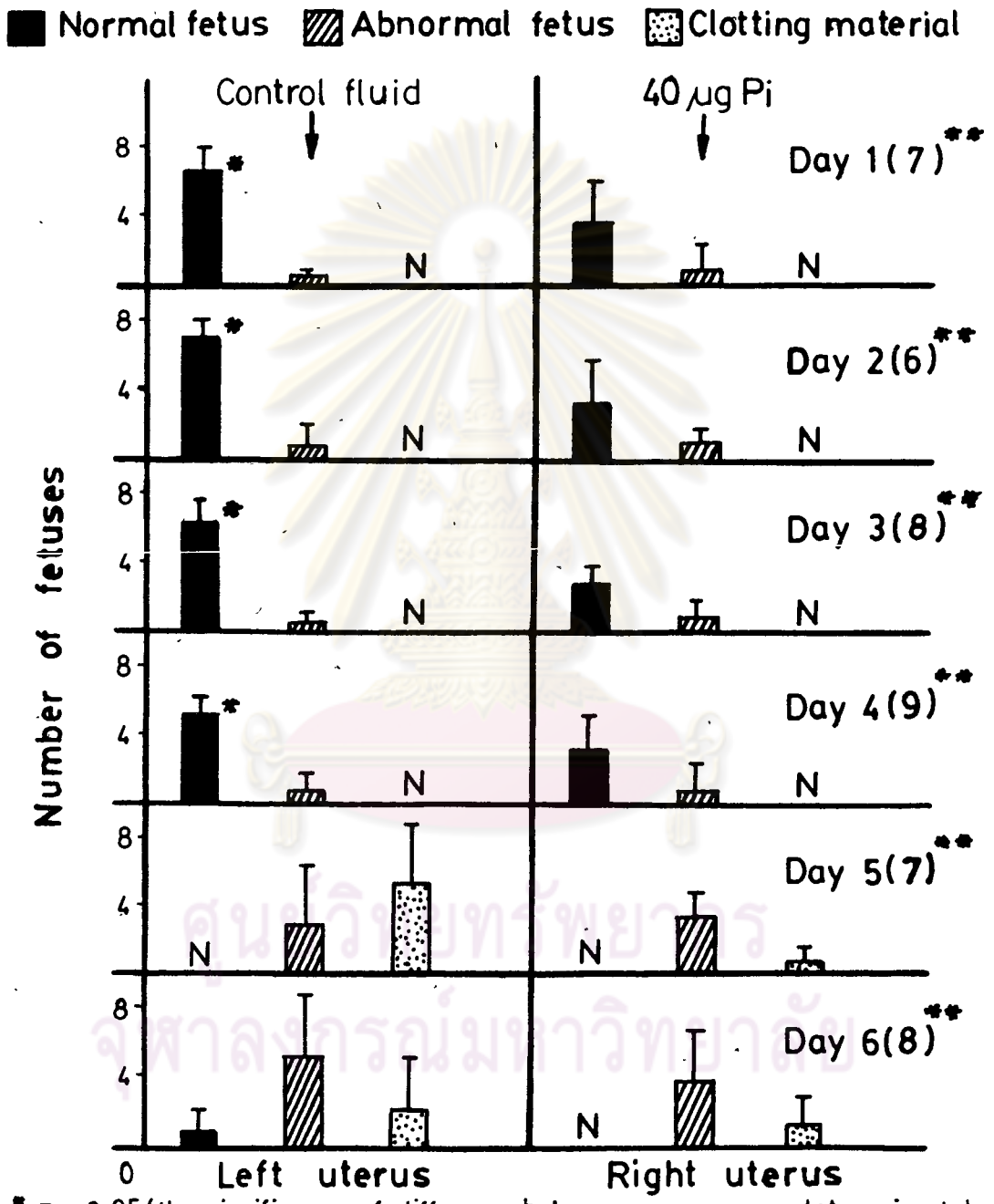
Table 2. Number of fetuses in control pregnant rats(day 15 of pregnancy).

Rat No.	No. of normal fetuses	
	L	R
1	6	7
2	3	5
3	5	3
4	6	5
5	4	5
6	4	5
7	6	6
8	3	4
Mean± SD.	4.63 ± 1.22	5.00 ± 1.12

of testing fluid was injected into the right uterus and the same volume of proper control fluid into the left uterus of the same recipient rat. The concentration of P_i in phosphate buffer was 200 µg P/ml so that the amount of P_i administered per horn was 40 µg which was in the range observed in the IUD-bearing horn as previously shown in Table 1.

3.1 Effect of inorganic phosphate injected on various days of pregnancy

The contraceptive effect of 40 µg P_i injected into the right uterus of the recipient rats on day 1-6 of pregnancy comparing to control fluid was evidenced by the decrease in number of normal fetuses in the right uterus to zero when injected on day 5 and 6 of pregnancy (Fig. 3). On this critical day 5, the number of normal fetuses in the left uterus also decreased to zero. On day 6 of pregnancy the number of normal fetuses decreased to zero in the right uterus and approached zero in the left control horn. During day 1-4 of pregnancy, injection of P_i resulted in a significant decrease ($P<0.05$) in the number of normal fetuses in the right uterus as compared to the left uterus receiving 0.2 ml of control uterine fluid. Besides, abnormally small fetuses were found in both uteri and remarkably increased on day 5 and 6 of pregnancy. In addition, the presence of clotting materiaes in both uteri of the recipient rats recieving P_i on day 5 and 6 of pregnancy also supported the harmful effect of P_i on fetal development. These results, firstly showed that P_i exerted the most drastic contraceptive effect when injected on day 5 of pregnancy and secondly, P_i injected in the right uterus of day 5 pregnant rats also diminished the number of normal fetuses to nil in the left uterus.



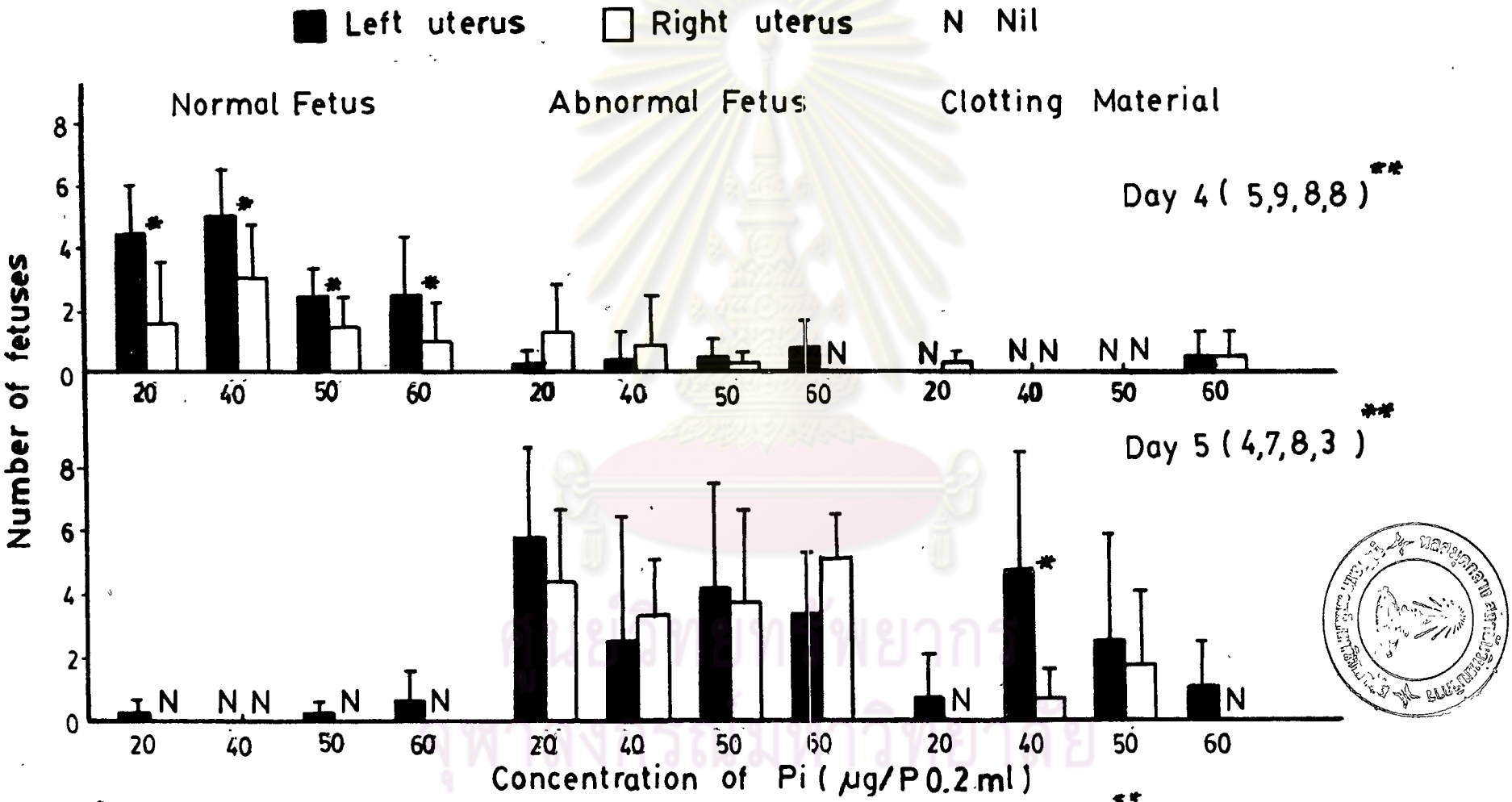
* $P<0.05$ (the significance of difference between means was determined by t-test)

** Number of rats used.

Figure 3.

3.2 Effect of inorganic phosphate at different concentrations in combination with control fluid

Since, the previous results of P_i concentration in IUD fluid showed wide variation between 20-80 µg/ml (Table 1) and injection of P_i on day 5 of pregnancy resulted in the most drastic effect on fetal development, we decided to vary the P_i concentration from 20 µg/0.2 ml to 60 µg/0.2 ml (100-300 µg/ml) to test the contraceptive effect in the right uterus of the recipient rats. Day 4 and 5 of pregnancy were chosen to compare between the critical day and the others. Control uterine fluid of the same volume was injected simultaneously in the left uterus. Fig. 4 apparently showed that at every concentration of P_i used the number of normal fetuses in the right uterus receiving P_i on day 5 of pregnancy decreased to zero. Transfer effect of P_i into the left uterus was also observed by the significantly decrease in number of normal fetuses at every concentration of P_i on day 5 comparing to day 4 of pregnancy. Variation of P_i concentration from 100-300 µg/ml had no correlation with the number of normal fetuses in both uteri whether injected on day 4 or 5 of pregnancy. Abnormally small fetuses and clotting materials were found in both uteri on day 4 and noticably increased when P_i was injected on day 5 of pregnancy. However, on day 4 of pregnancy, clotting materials in both uteri were found only when P_i 60 µg/0.2 ml (300 µg/ml) was injected into the right uterus. These results showed that injection of P_i on day 5 of pregnancy exerted the stronger contraceptive effect on fetal development and this effect can be transferred into the left control horn.



*P<0.05 (the significance of difference between means was determined by t-test), ** Number of rats used.

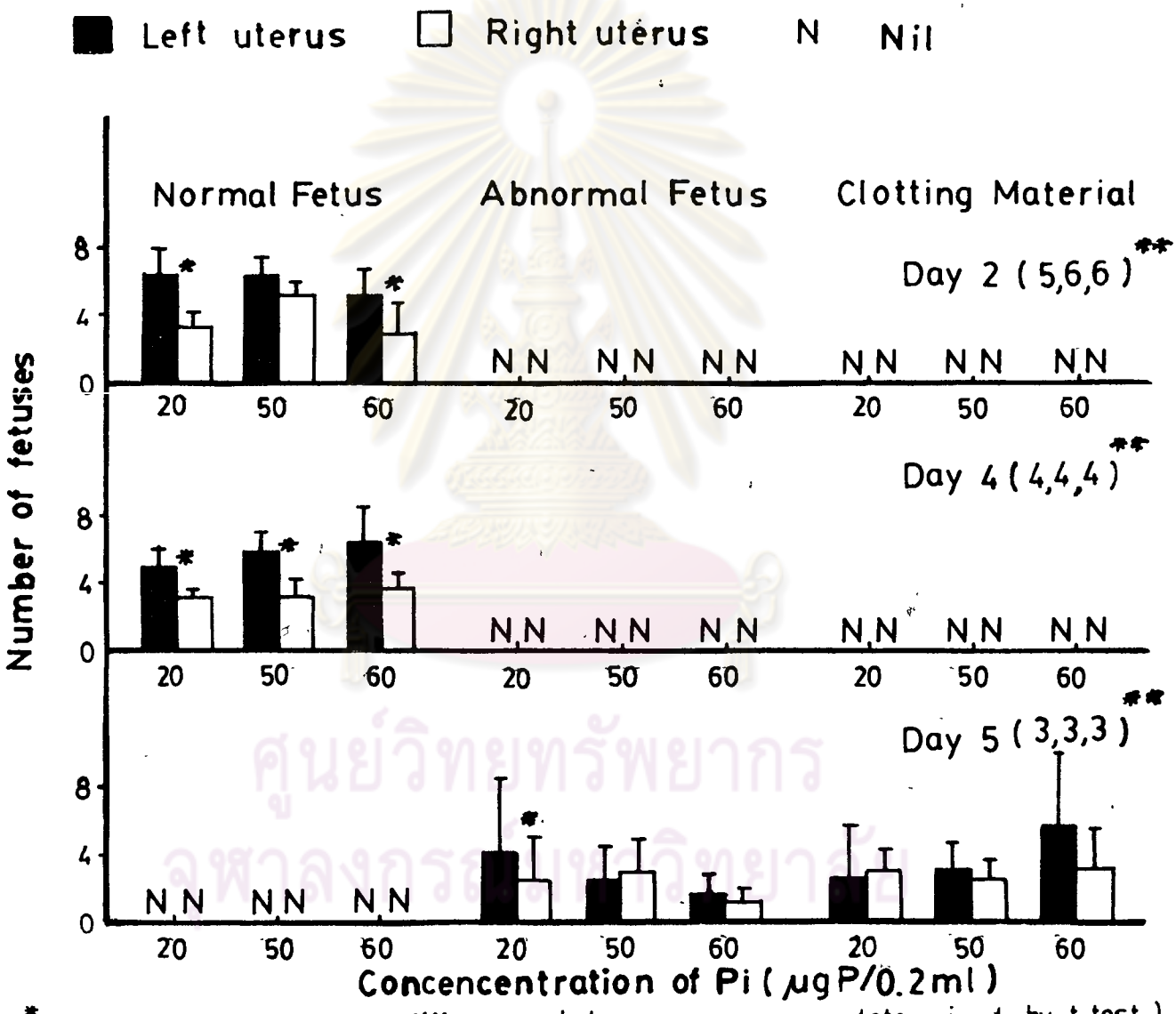
Figure 4.

3.3 Effect of inorganic phosphate at different concentrations in combination with normal saline

In thonext experiment, control uterine fluid injected into the left uterus was replaced by physiological saline solution, in order to prove if P_i alone or P_i combined with some biomolecules in the uterine fluid mediate the contraceptive activity. Fig. 5 showed that various concentrations of P_i injected into the right uterus comparing to normal saline in the left uterus resulted in diminishing the number of normal fetuses to nil in both uteri at every concentrations of P_i when the injection was carried out on day 5 of pregnancy. In addition, abnormally small fetuses and clotting materials were also found in both uteri on this critical day 5. On the contrary, injection on day 2 and 4 of pregnancy allowed normal fetal development in both uteri, although the number of normal fetuses in the right uterus decreased significantly from the left uterus at every concentration of P_i. No traces of abnormal fetuses or clotting materials were observed when the administration was performed on day 2 and 4 of pregnancy. All the results strongly suggested that injection of P_i on day 5 of pregnancy in combination with control uterine fluid or normal saline caused the most drastic contraceptive effect in both uteri.

3.4 Effect of injection on day critical

It was of interest therefore, to test the effect of normal saline or control uterine fluid injected into both uteri on this critical day 5. The results in Fig. 6 showed that normal saline and control fluid



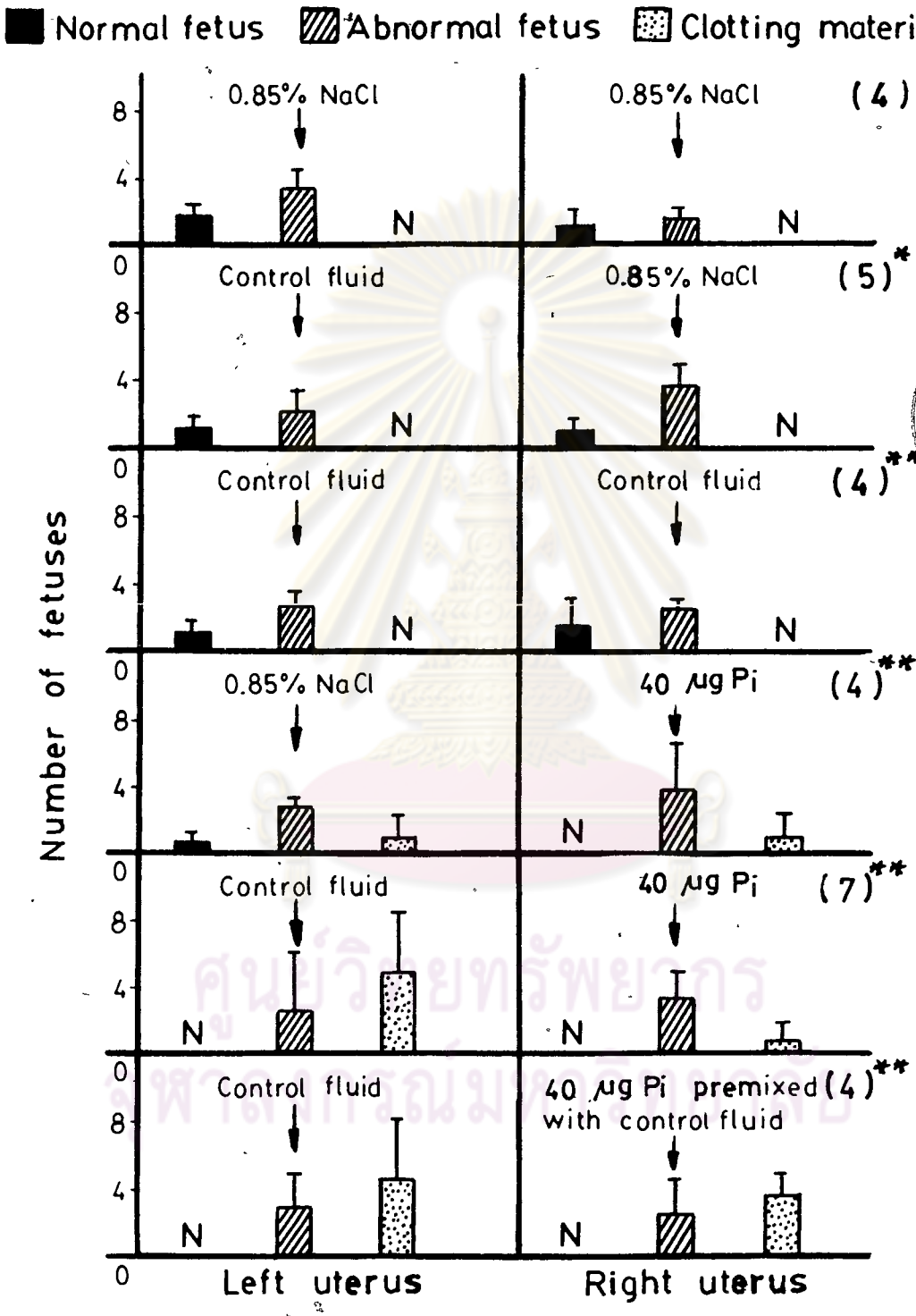
*P<0.05 (the significance of difference between means was determined by t-test)

**Number of rats used.

Figure 5.

Figure 6. Effect of injection on day critical (day 5 of pregnancy)

Two hundred microliters of test fluid was injected into the left and right uteri on day 5 of pregnancy. Number and development of fetuses in each rat were observed on day 15 of pregnancy.



** Number of rats used.

Figure 6.

injected into both uteri exerted a milder contraceptive effect as evidenced by a decrease in number of the normal fetuses in both uteri and the presence of many abnormally small fetuses, but none of the clotting material was observed. Injection of 40 μg P_i (200 μg P/ml) into the right uterus in combination with normal saline in the left uterus showed more harmful effect on fetal development as indicated by the decrease of normal fetuses in the right uterus to nil and the presence of clotting materials in both uteri. When 40 μg P_i was administered into the right uterus in combination with control uterine fluid in the left uterus, none of the normal fetuses was observed in both uteri. This extensive contraceptive effect was also observed when 40 μg P_i premixed with control fluid was injected into the right uterus in combination with merely uterine fluid in the left. All these results implied that free P_i (40 μg/0.2 ml) injected into the right uterus could migrate into the left uterus and exerted its contraceptive effect. In addition, binding of P_i to some biomolecules in the uterine fluid might intensify the effect of P_i.

4. Translocation of ^{32}P-P_i in vivo

In order to prove that free P_i injected into the right uterus of the recipient rats on day 5 of pregnancy can be translocated into the left uterus and consequently exerted similar antifertility action, ^{32}P-labelled P_i (0.32 MBq) in 0.2 ml 0.006 M cold phosphate buffer pH. 7.0 was injected into the right uterus. The same amount of control fluid was injected into the left uterus. At various times after injection the rat was sacrificed, and the radioactive ^{32}P-P_i was determined in both right and left uteri, which were divided into 3 fraction : fraction 1, flushing

(F_1); fraction 2, endometrial homogenate (F_2); fraction 3, muscular cells homogenate (F_3).

The results from "long term" study (1 hr-4 days) were shown in Fig. 7. The radioactivity of ^{32}P-P_i was detected in both uteri, but the per cent recovery was very low (0.005-0.18%). The time after injection was therefore reduced to 5-20 min as shown in Fig. 8. The results from both "long term" and "short term" study in vivo indicated the ^{32}P-P_i can migrate from the right uterus into the left uterus when the injection was performed on day 5 of pregnancy. The high recovery of radioactivity in the right uterus firstly appeared in F_1, flushing, at 5 min and consequently shifted to F_3, the muscular tissue of the right uterus in 10 min. The ^{32}P-P_i was detected in F_1, F_2 and F_3 of the left uterus 5 min after injection. However, the radioactivity was accumulated mostly in F_3 which was the muscular fraction of both uteri, which implied that the muscular tissue might be the target tissue for the contraceptive activity of P_i. The radioactivity of ^{32}P-P_i was also found significantly (>0.1%) in F_2, the endometrial lining, which also stayed quite permanently as in F_3. Fig. 9 showed the kinetics of ^{32}P-P_i translocation in vivo. ^{32}P-P_i injected intraluminally into the right uterus was translocated from the flushing into the muscular tissue of the right uterus within 10 min after injection. In the left uterus, maximal recovery (0.2%) was also found in F_3, the smooth muscle at, 10 min after injection. The radioactivity of ^{32}P-P_i incoperated into this smooth muscle fraction seemed to be retained at constant level (approximately 0.05%) until 4 days after injection. The incorporation of ^{32}P-P_i was also checked in several other tissues such as adipose tissue, ovary, liver, and in blood circulation but

Figure 7. Long term study on the distribution of ^{32}P-P_i injected into the right uterus

^{32}P-P_i (0.32 MBq) in 0.2 ml 0.006 M cold phosphate buffer, pH. 7.0 was injected into the right uterus the same time as 0.2 ml of control fluid into the left uterus of the recipeint rats on day 5 of pregnancy. Rats were sacrificed at various times after injection, and the incorporation of ^{32}P-P_i into the right uterus ☐ and the left uterus ▨ was determined in 3 fractions as described in "Methods"

F_1 - intraluminal flushing

F_2 - endometrial lining

F_3 - smooth muscle

One rat was used for each time interval.

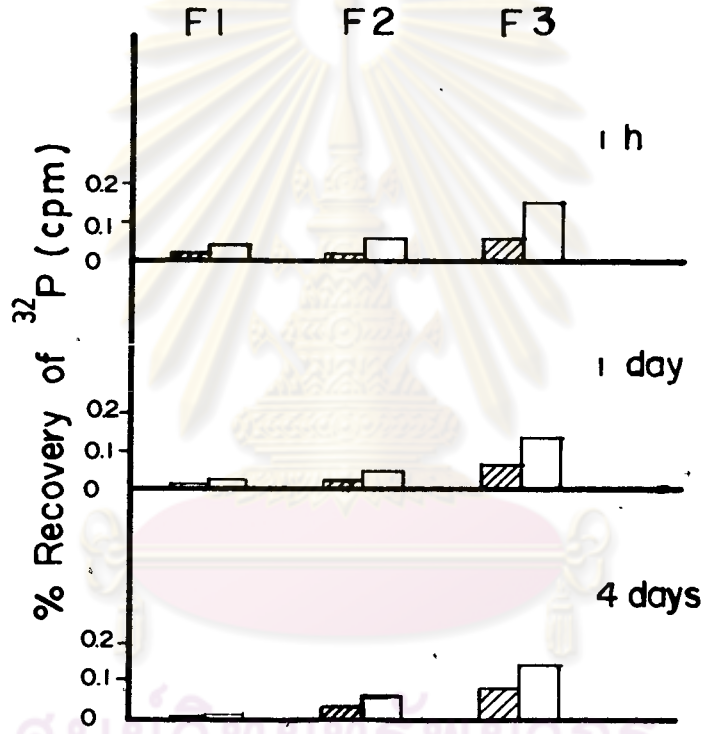


Figure 7.

Figure 8. Short term study on the distribution of $^{32}P\text{-}P_i$ injected into the right uterus

$^{32}P\text{-}P_i$ (0.32 MBq) in 0.2 ml cold phosphate buffer was injected into the right uterus the same time as 0.2 ml of control fluid into the left uterus of the recipient rats on day 5 of pregnancy. Rats were sacrificed various times after injection, and the incorporation of $^{32}P\text{-}P_i$ into the right uterus ☐ and the left uterus ▨ was determined in 3 fractions as described in "Methods"

F_1 - intraluminal flushing

F_2 - endometrial lining

F_3 - smooth muscle

One rat was used for each time interval.

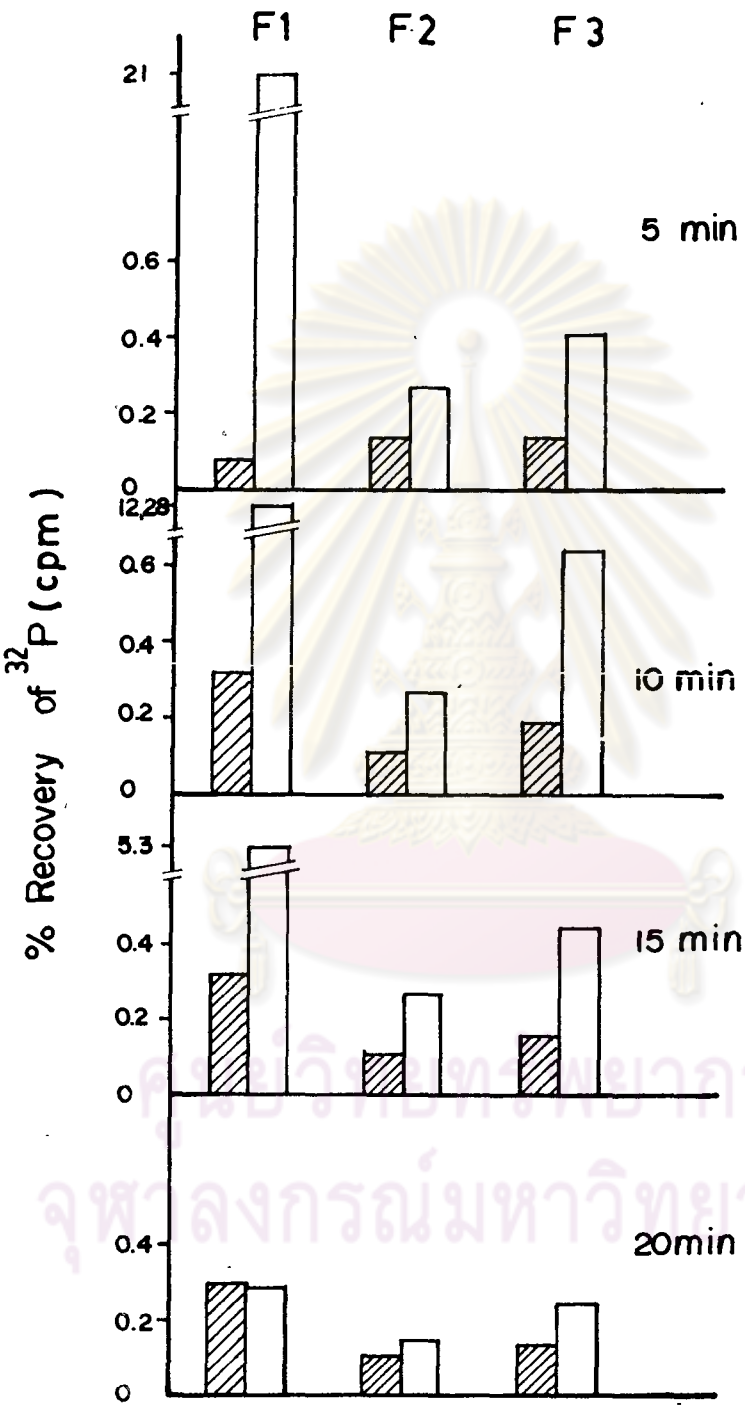


Figure 8.

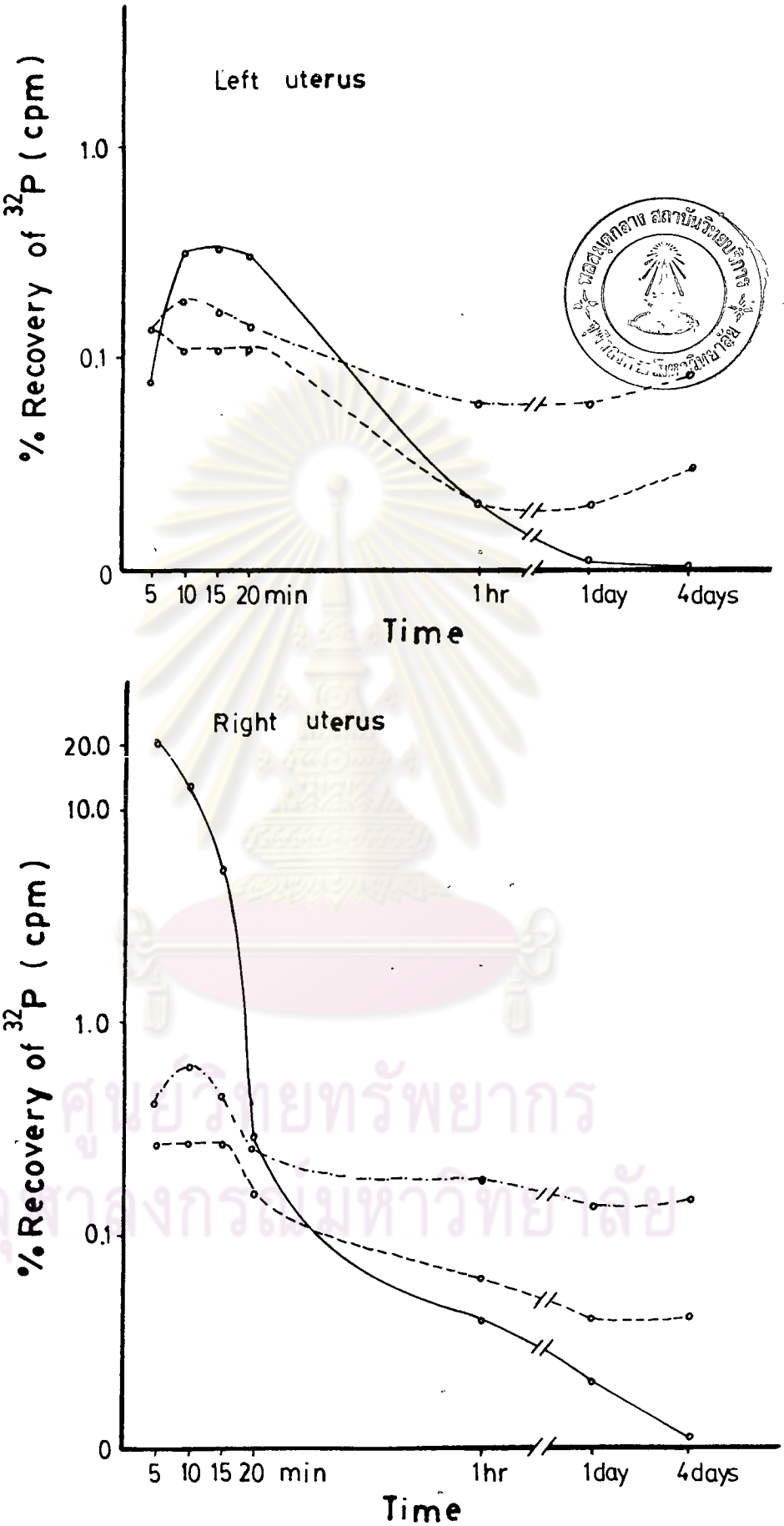

Left uterus
% Recovery of ^{32}P (cpm)
1.0
0.1
0
5 10 15 20 min
1hr
1day
4days
Time
Right uterus
% Recovery of ^{32}P (cpm)
20.0
10.0
1.0
0.1
0
5 10 15 20 min
1hr
1day
4days
Time

Figure 9.

the radioactivity observed was all lower than 0.1%. From these results it can be concluded that ^{32}P-P_i injected on day 5 of pregnancy could be translocated from the right uterus into the left uterus and its incorporation was directly into the uterine muscular tissue (F_3). Constantly accumulation of radioactivity in F_3 in both "short term" and "long term" experiments suggested that the target tissue for the antifertility effect of P_i might be the uterine smooth muscle.

5. Binding of ^{32}P-P_i with uterine fluid in vitro

5.1 Gel filtration profile of sole ^{32}P-P_i and uterine fluid

When sole ^{32}P-P_i or uterine fluid (control or IUD fluid) was eluted on a Sephadex G-25 column as described in "Methods", Fig. 10 a and b showed that both the control fluid and IUD fluid were eluted at the void volumn of the column, fraction 6-8 and ^{32}P-P_i was eluted in fractions 10-16.

5.2 Binding of ^{32}P-P_i at various times of incubation

The binding of ^{32}P-P_i with some biomolecules in the uterus fluid at 37°C was shown in Fig. 10 c and d. In the presence of uterine fluid, either control or IUD fluid, ^{32}P-P_i obviously bound to some biomolecules and formed another peak (fraction 8, F_8) which was eluted before P_i alone. This binding could be observed even at 0 hr of incubation and it increased with incubation time. Heating of the mixture of ^{32}P-P_i and uterine fluid at 100°C for 10 min (Fig. 10 e and f) did not prohibit this binding so that the chromatogram were more or less similar to those of 0 hr incubation (Fig. 10 c and d). These results showed that both control

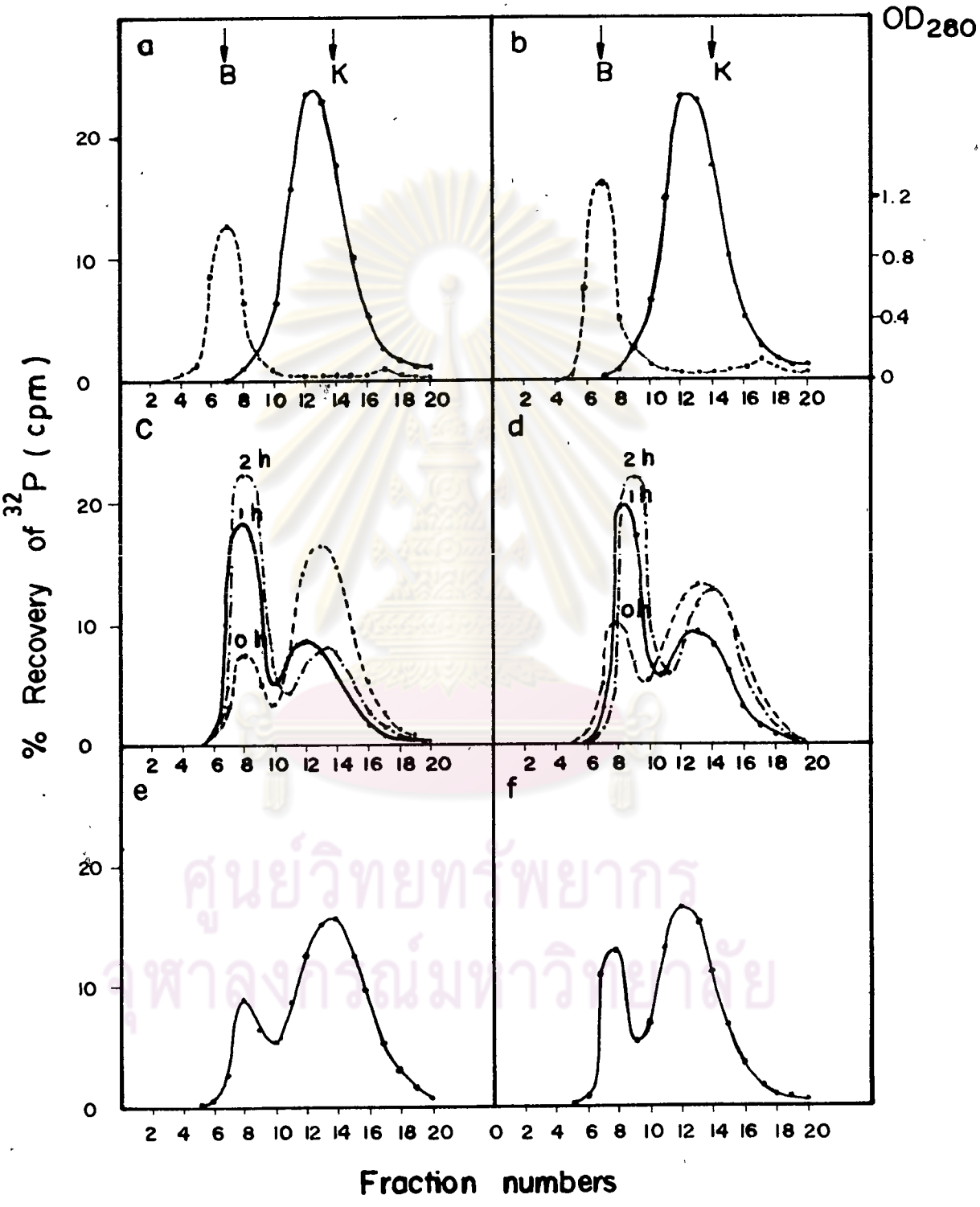

a
b
c
d
e
f
B
K
2 h
1 h
0 h
OD280
1.2
0.8
0.4
0
% Recovery of 32P (cpm)
Fraction numbers

Figure 10.

and IUD fluid contained some biomolecules that bind to ^{32}P-P_i and this component has the M.W. about 5,000 dalton.

5.3 Replacement of uterine fluid with BSA

When the same amount of BSA (1.25 mg protein) was used instead of uterine fluid, Fig. 11 b showed that there was no other peak at fraction 8, indicating that the binding between ^{32}P-P_i and the component in uterine fluid was rather specific, hence such binding was not observed with BSA.

5.4 Chasing with cold P_i

In order to study thenature of binding between ^{32}P-P_i and the component in the uterine fluid (F_8), the mixture of ^{32}P-P_i already incubated with control or IUD fluid at 37°C, 1 hr was chased with cold P_i at 1,000 fold concentration higher than that of ^{32}P-P_i and further incubated at 37°C for another hour. Fig. 12 a and b (·----·) showed that the incorporation of ^{32}P in F_8 was decreased to 43 % and 37 % respectively. Preincubation of uterine fluid with excess amount of cold P_i for 1 hr, before adding ^{32}P-P_i also resulted in decreasing incorporation of ^{32}P in F_8 to 24 % and 28 % as shown in Fig. 12 a and b (·-·-·). These results demonstrated that ^{32}P-P_i bound to F_8 could be exchanged with cold P_i and vice versa. It also implied that covalent binding should not exist between P_i and the biomolecule(s) in F_8.

5.5 SDS-gel electrophoresis

Pooled fractions 1-9 and 10-15 from Sephadex G-25 column with the radioactivity range of 3,000-30,000 cpm were lyophilized and

Figure 11. The gel filtration profiles of ^{32}P-P_i and BSA

The binding of ^{32}P-P_i (1.62 MBq/0.5 ml) and BSA (1.25 mg protein/0.5 ml) was tested on Sephadex G-25 column in the way as previously done with the uterine fluid

a. individual application of ^{32}P-P_i (—·—) and BSA (- - -)

b. mixture of ^{32}P-P_i and BSA (—·—) incubated at 37°C for 1 h before application onto the column.

(B and K stand for Blue dextran and potassium chromate marker)

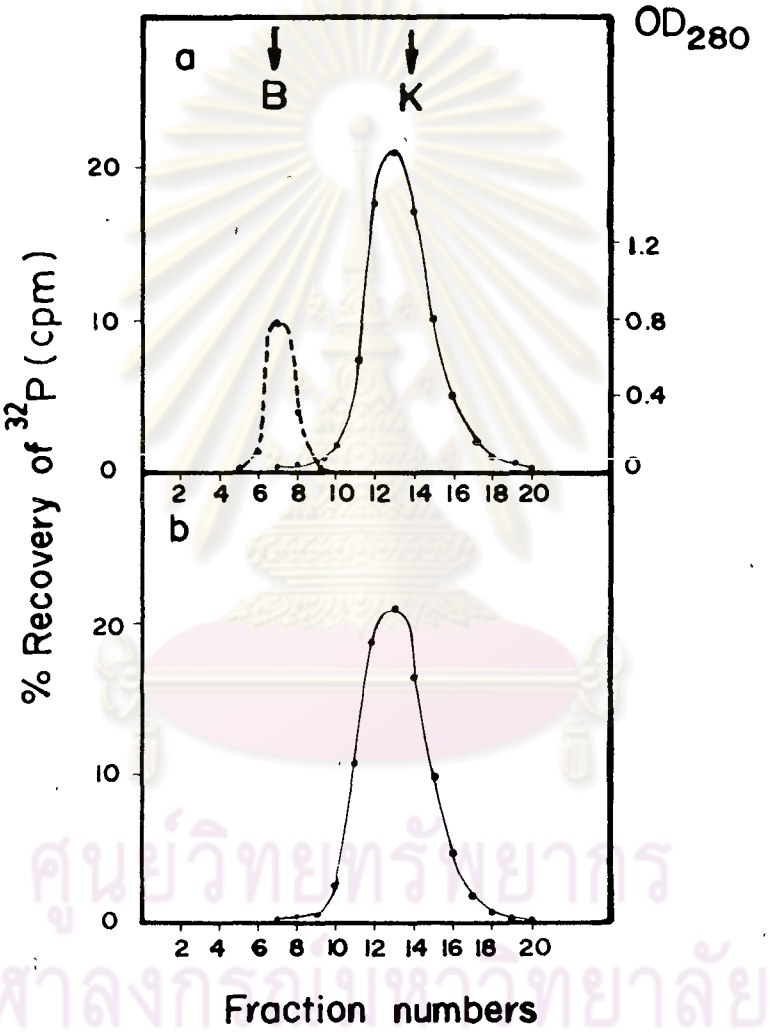


Figure 11.

Figure 12. Exchange of P_i bound to the component in uterine fluid.

The radioactive profile of,

a. (——) ^{32}P-P_i (1.62 MBq/0.5 ml) mixed with control fluid (1.25 mg protein/0.5 ml) and incubated at 37°C for 1 h without chasing

(- - - -) ^{32}P-P_i and control fluid preincubated at 37°C, 1 h chasing with cold P_i (1,000-fold) by further incubation at 37°C for one more hour

(— · —) cold P_i and control fluid preincubated at 37°C, 1 h chasing with ^{32}P-P_i (10^{-3} fold) by further incubation at 37°C for one more hour

b. (——) ^{32}P-P_i (1.62 MBq/0.5 ml) mixed with IUD fluid (1.25 mg protein/0.5 ml) and incubated at 37°C for 1 h without chasing

(- - - -) ^{32}P-P_i and IUD fluid preincubated at 37°C, 1 h chasing with cold P_i (1,000-fold) by further incubation at 37°C for one more hour

(— · —) cold P_i and IUD fluid preincubated at 37°C, 1 h chasing with ^{32}P-P_i (10^{-3} fold) by further incubation at 37°C for one more hour.

↓ ↓
(M and ^{32}P-P_i stand for macromolecule in the uterine fluid and free ^{32}P-P_i)

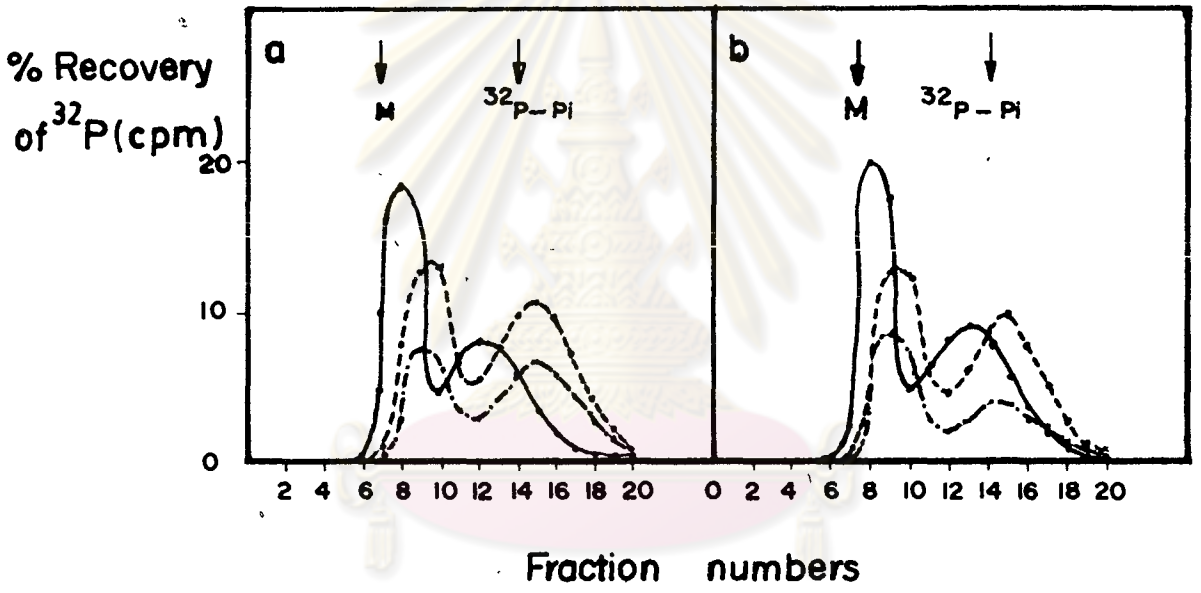


Figure 12.

subjected to SDS gel electrophoresis. Each gel was longitudinally cut, one half was stained for protein and the other half was fractionated and counted for radioactivity as shown in Fig. 13. The gel electrophoretic profiles in Fig. 13 a, b, c and d showed that all the proteins in both control and IUD fluid was eluted from Sephadex G-25 column in the fractions 1-9 and there was no more in the fractions 10-15. There were four dominant peaks of protein numbering I, II, III and IV in the order of decreasing M.W., Protein IV (P_4) which had the lowest M.W. among the four was increased significantly in the IUD fluid. However, there was no incorporation of ^{32}P in P_4 or any other peaks as evidenced by the low radioactivity, less than 100 cpm in every gel fraction. This results also confirmed that ^{32}P-P_i found associated with the macromolecule(s) distributed in fractions 1-9 of the Sephadex G-25 column should not be phosphorelated covalently to any proteins.

Figure 13. The SDS-gel electrophoretic profiles of the ^{32}P-associated fractions eluted from Sephadex G-25 column.

Pooled fractions 1-9 and 10-15 showing ^{32}P incorporation 3,000-30,000 cpm obtained from the application of ^{32}P-P_i and uterine fluid mixture as shown in Fig. 10 c and d were lyophilized and subjected to the SDS gel electrophoresis as described in "Methods". The protein profiles (·----) were scanned at 650 nm, and the radioactivity in each fraction of the same gel was presented in a continuous line (•———•)

a. pooled fractions 1-9 obtained from the mixture of ^{32}P-P_i and control fluid (total cpm = 2.4×10^4)

b. pooled fractions 10-15 obtained from the mixture of ^{32}P-P_i and control fluid (total cpm = 3.4×10^3)

c. pooled fractions 1-9 obtained from the mixture of ^{32}P-P_i and IUD fluid (total cpm = 3.4×10^4)

d. pooled fractions 10-15 obtained from the mixture of ^{32}P-P_i and IUD fluid (Total cpm = 8.3×10^3).

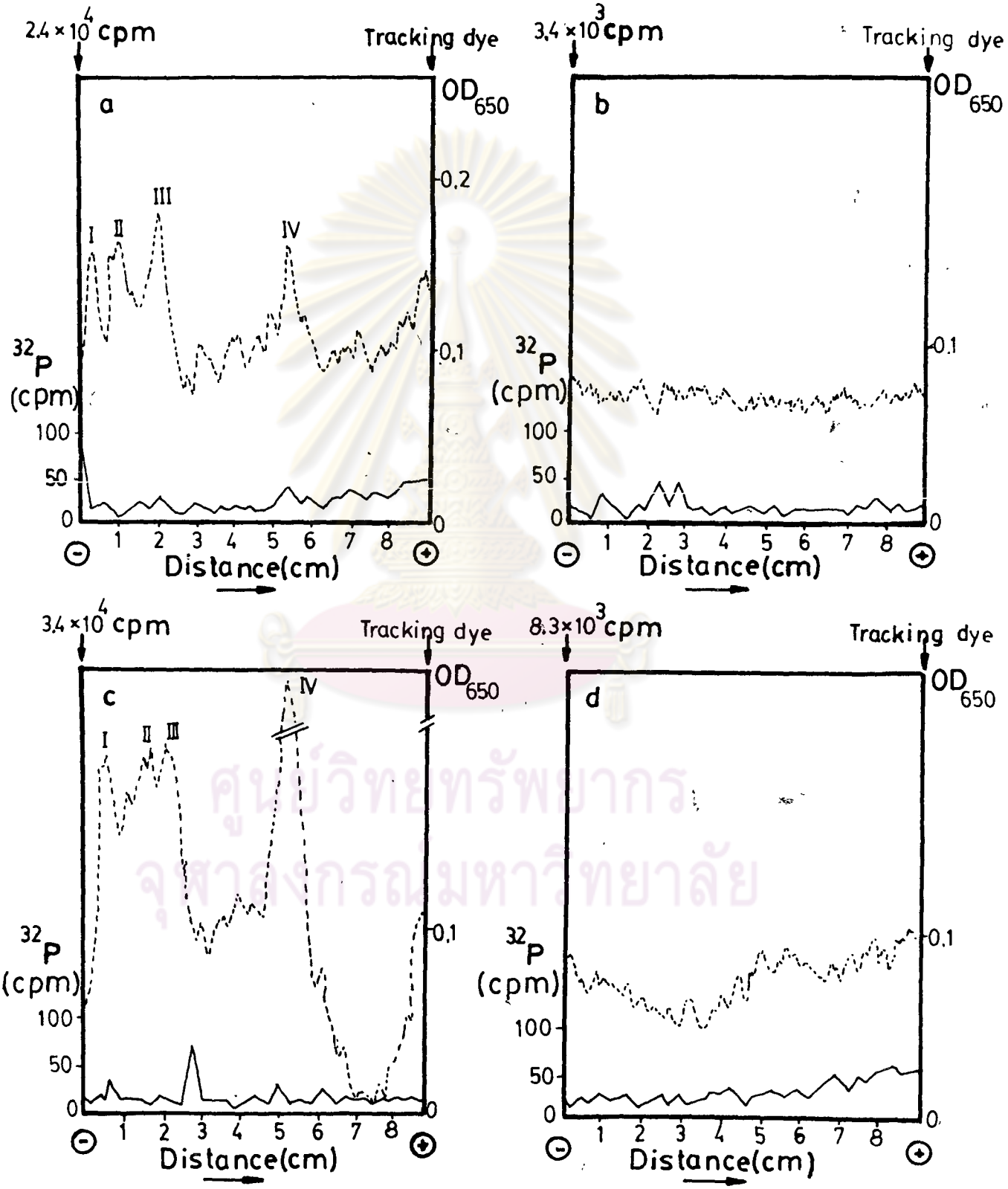


Figure 13.