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Studies on the Permeabilities of Serum Protein into Uterine Lumen and Specific Protein in Uterine Fluid in Rat

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Summary

The present experiments were designed to examine (a) time of passage of serum protein into uterine lumen by mean of FITC protein tracing, (b) changes of passage rate of serum protein in estrus cycle, (c) common and specific protein to serum protein in uterine fluid using immunochemical analysis.

FITC serum protein passed readily into uterine lumen of metestrus rats, reaching a peak at 6 hrs after injection of FITC serum protein. The rate of passage varied significantly at different stages of estrus cycle, the least occuring at proestrus and the most at diestrus. It has been demonstrated immunochemically that at least four protein components in uterine fluid were common with serum protein and that eight specific proteins in uterine fluid which are undetectable in serum were observed in Oucterlony test and Disc immunodiffusion reactions.

In previous reports, it has been observed that protein content in uterine fluid increased in proestrus stage by accumulation of uterine fluid and reached to maximum from 22:00 hr of proestrus to 6:00 hr of estrus (1). Then, it has been shown that the appearance of specific proteins was detected by Disc electrophoresis of uterine fluid from proestrus to estrus and the alterations of each protein fraction in uterine fluid were affected by ovarian hormones (2). It seemed probable that the origin of uterine fluid protein is dependent on a combination of the release of secretory cells in endometrium and the transudation from the vasculer system. However, the permeant rate of protein in serum and the protein components secreted into uterine lumen in estrus cycle was not clear. This investigation was undertaken to obtain information on the rate of the passage of serum protein to uterine fluid and the specific protein components secreted from the epithelium of uterus.

Materials and Methods

Mature virgin female rats of Wistar strain, weighing from 180 to 250g were used.

Uterine fluid was collected by aspiration with tuberculin sylinge or flushing with a 0.15 M sodium chloride. Then the endometrium was obtained by shaving. The determination of protein content was performed by the method of Daughaday, using bovine serum albumin as a standard protein (3). The content of fluorescein isothiocyanate (FITC) in uterine fluid and serum was measured by fluorescence spectrophotometer (Hitachi 203).

Labelling and Purification of Serum Protein. The protein passage from serum into uterine lumen was determined by the method of direct protein tracing previously described (4). Serum protein solution and carbonate bicarbonate buffer (pH 9.1, 0.5 M) were equally added and this solution was stirred throughly at 0°C–2°C. One mg FITC dissolved in a little buffer solution was added to one hundred mg of serum protein, and then stirring was continued for six hours at 0°C–2°C. Removal of unreacted FITC was accomplished by dialysis for phosphate buffer saline (0.01 M, pH 7.1 containing 0.15 M sodium chloride) and by gel filtration with a Sephadex G 25 colum chromatography. For intravenous tracing, the rat was injected with two hundred mg conjugates (FITC serum protein) per kilogram of body weight.

Polyacrylamide Disc Electrophoresis. Electrophoresis of uterine fluid and serum was performed in same manner previously described (5). After electrophoresis the gel was removed from the tube and stained with Amido Black dye. Background stain was taken off by mean of electric current (6).

Preparation of Antisera. Antibodies to rat uterine fluid and serum were prepared in Japanese White rabbits weighing 2.5 to 4.0 kilograms. Serum diluted five-fold with 0.15 M sodium chloride or ten-times concentrated uterine fluid (approximately 12 mg of protein/ml) was emulsified with an equal of Freund's complete adjuvant. Each animal was intracutaneously injected with two ml of emulsion every other week during six weeks.

To remove the crossreactive antibodies to serum, antisera developed against uterine fluid was absorbed with serum. The mixture was allowed to stand two hours at 37°C and replaced overnight at 5°C. The formed precipitation was removed by centrifuging at 5°C.

Double and Disc Immunodiffusion. Ouchterlony test was carried out by using 1.0% agar (Difco special agar norble) dissolved in a barbital buffer (pH 8.6, 0.025 M) containing 0.1% sodium azide. In the immunophoretic studies, electrophoresed acrylamide gels were embedded in the same agar medium (8). Antisera were introduced into parallel trough. In each case, diffusion was allowed to proceed for seven days at room temperature. The plate was washed with 0.15 M sodium chloride stained with a 1% Amido Black dissolved in 7% acetic acid solution, and then washed by several times with 7% acetic acid for removal of excess stain.

Results

Experiment I: the Rate of Passage of FITC Serum Protein into Uterine Lumen

To observe a time-interval of passage of FITC serum protein into the uterine lumen, uterine fluid was obtained by flushing at intervals of 1/6, 1/2, 1, 2, 4, 6, 12 and 24 hours after injection of FITC serum protein. The rats were used from metestus to diestrrus stage, because the protein content in uterine fluid during these periods is not alterative as in the previous report (1). The results were shown as the ratio of FITC content to one hundred mg of serum protein. The mean values of this parameter are plotted against time for each specimen, as shown in Fig. 1. The disappearance rate of FITC serum protein from the circulation was very rapid

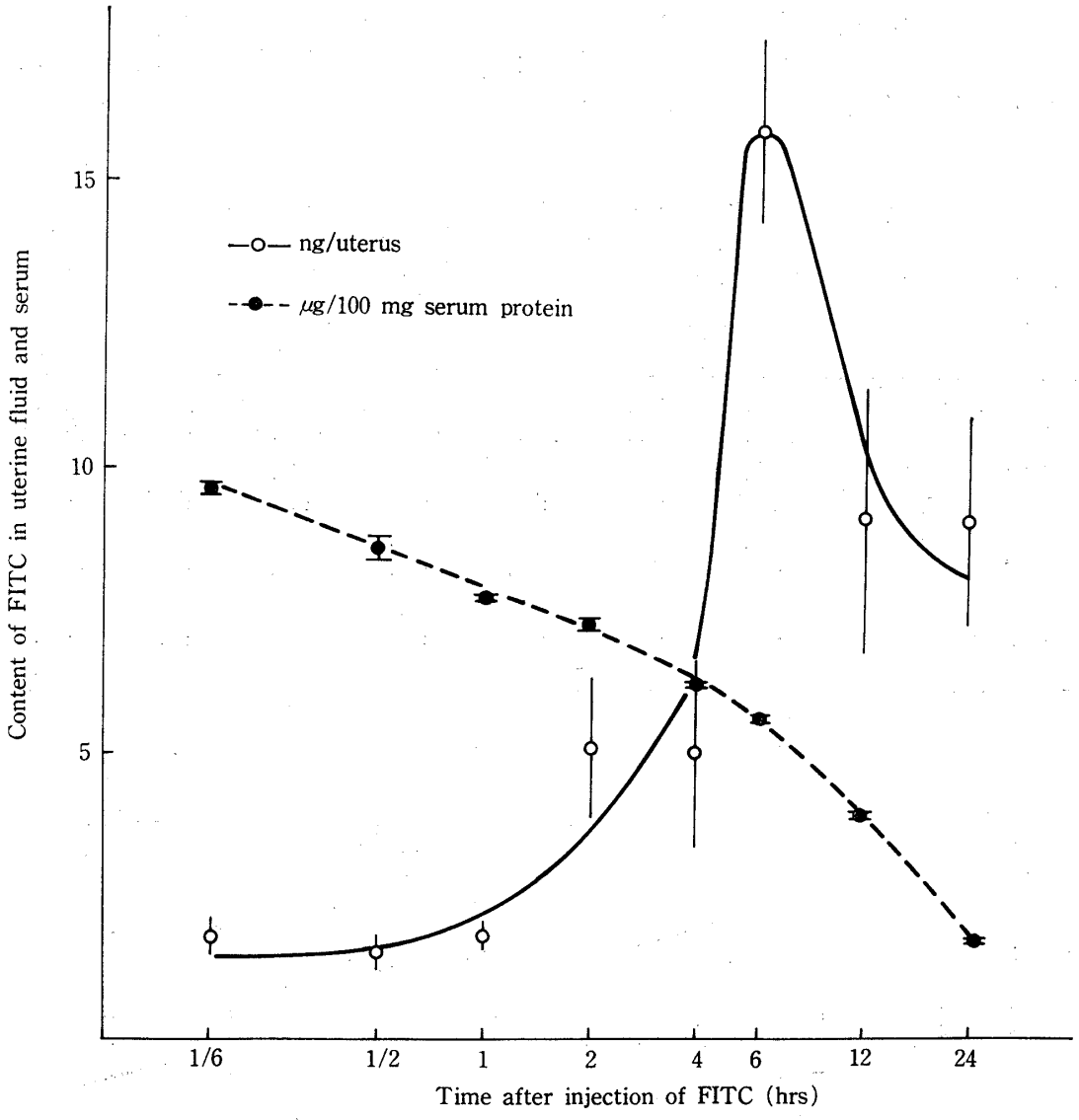


FIG. 1. Content of FITC in uterine fluid and serum from metestrus to diestrus after injection of 200 mg FITC labelled serum protein per kg body weight. Time was represented as logarithmic rate.

until two hours after injection of FITC serum protein, and then slow thereafter. In the uterine fluid, the transport of FITC serum protein to uterine fluid began from two hours and reached a peak by six hours after injection of FITC serum protein.

Experiment II: the Permeability of Serum Protein to Uterine Lumen in Estrus Cycle.

Uterine fluid was collected at 18:00 hr of diestrus, 6:00, 14:00 and 22:00 hr of proestrus, 6:00 and 18:00 hr of estrus, and 18:00 hr of metestrus, and these times were equivalent to six hours after injection of FITC serum protein, respectively.

The total content of FITC in uterine fluid in each group is shown in Table 1. The passage rate of labelled protein decreased during estrus period, although the content of total protein of uterine fluid increased in this stage. Further, the ratio of FITC to one milligram of protein content in uterine fluid became remarkably less in estrus period (Table 1 and Fig. 2). A clearance of FITC serum protein in circulation did not differ in the estrus cycle.

TABLE 1. *Alteration of Protein and FITC in Uterine Fluid at Various Stages of Estrus Cycle*

	Stage of estrus cycle						
	D 18[a]	P 6	P 14	P 22	E 6	E 18	M 18
A, Protein content in uterine fluid (μg)	231.8 ±13.7	407.6 ±28.7	512.4 ±21.2	807.1 ±61.6	500.5 ±18.2	239.3 ±9.5	274.3 ±11.2
B, FITC content in uterine fluid (ng)	15.5 ±2.8	16.8 ±3.9	8.1 ±2.3	3.0 ±0.6	18.2 ±2.5	8.0 ±1.5	17.8 ±4.3
C, B/A (ng/1 mg protein)	72.1 ±7.4	42.9 ±6.4	16.3 ±4.0	3.7 ±0.5	36.4 ±6.7	31.8 ±4.1	59.1 ±11.3
D, FITC concentration in serum (μg/100 mg protein)	4.11 ±0.36	5.03 ±0.26	4.95 ±0.23	4.99 ±0.24	4.61 ±0.29	5.64 ±0.55	4.64 ±0.14

Difference of protein content (A) and ratio of FITC to protein (C) at estrus cycle

(A), Protein content in uterine fluid

(C), B/A	D 18	P 6	P 14	P 22	E 6	E 18	M 18
D 18		*	**	**	**	—	—
P 6	**		—	**	—	*	—
P 14	**	**		**	—	**	**
P 22	**	**	—		**	**	**
E 6	**	—	*	**		**	**
E 18	**	—	—	**	—		—
M18	—	—	**	**	*	**	

(a) D18 means time of 18:00 hr at diestrus, and P, E and M were represented proestrus, estrus and metestrus respectively. –, $P>0.05$; *, $P<0.05$; **, $P<0.01$

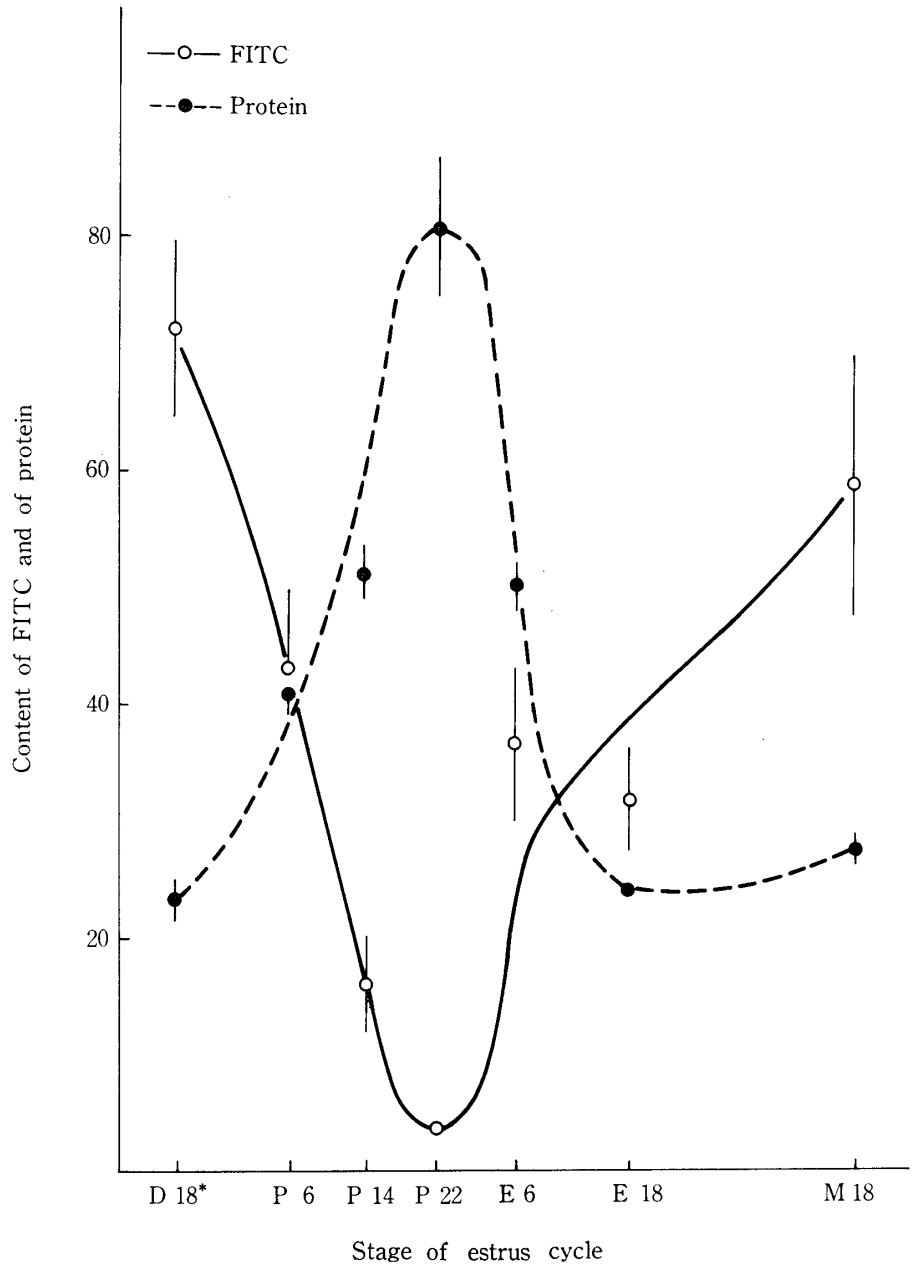
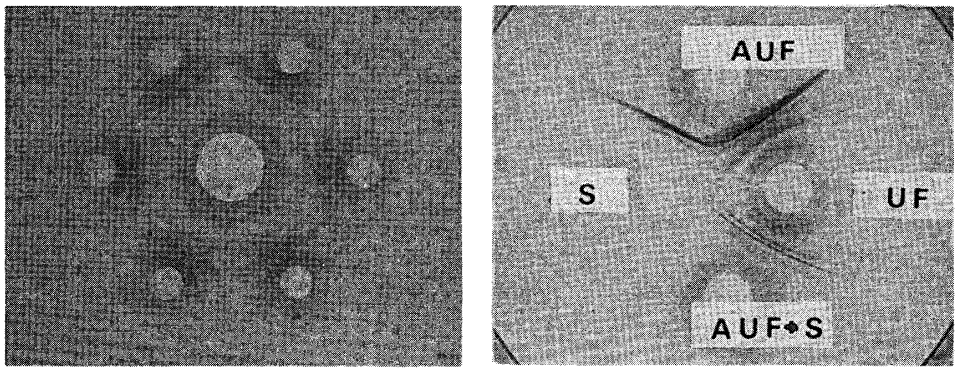


FIG. 2. Content of FITC (ng/1 mg protein) and of protein ($\mu g \times 10^{-1}$) in uterine fluid obtained 6 hrs after FITC protein injection.
*D18, Value at 18:00 hr of diestrus; P, Proestrus; E, Estrus; M, Metestrus

Experiment III: Immunochemical Analysis of Protein Components in Uterine Fluid

The uterine fluid collected from rats at 22:00 hr of proestrus stage were used for the preparation of antibodies against the protein in uterine fluid. At this time, the protein content in uterine fluid came to its maximum amount, and the passage of serum protein into uterine lumen decreased excessively as shown in Experiment II. When unabsorbed anti-rat uterine fluid antisera (AUF) were



A B

FIG. 3. Precipitin bands formed in Ouchterlony plates when rabbit anti-rat uterine fluid antibodies were absorbed with rat serum. Uterine fluid ten-fold concentrated was used.

A. Central well, anti-rat uterine fluid antibodies absorbed with serum; Peripheral wells, uterine fluid

B. AUF, anti-rat uterine fluid antibodies; S, rat serum; UF, rat uterine fluid; AUF+S, absorbed anti-uterine fluid antibodies with serum

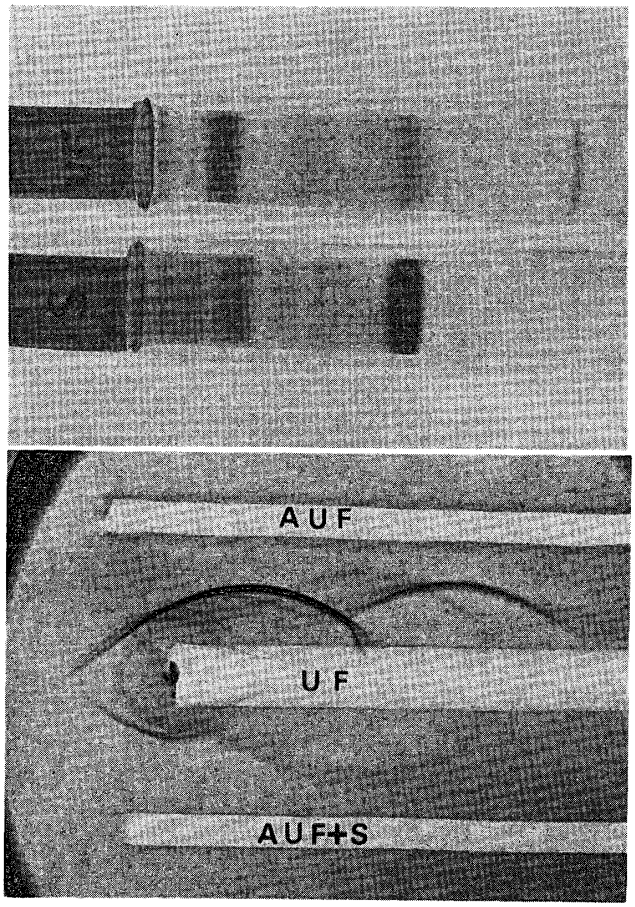


FIG. 4. Acrylamide gel electrophoresis (A) and immunophoretic analysis (B) of rat serum and uterine fluid. Acrylamide gel was stained with Amido Black (A) and unstained gel was embedded in agar (B). UF, uterine fluid; S, serum; AUF, anti-rat uterine fluid antibodies; AUF+S, absorbed anti-rat uterine fluid antibodies with serum

allowed to react against their homologus antigens in Ouchterlony plates, many precipitin bands were observed.

Specific uterine fluid protein could be best observed when uterine fluid was diffused against antibodies to uterine fluid which had been pretreated with serum to absorb the common antibodies (AUF+S) (Fig. 3AB). The reaction indicates that AUF which may be crossreactive with serum, has been removed by the treat-

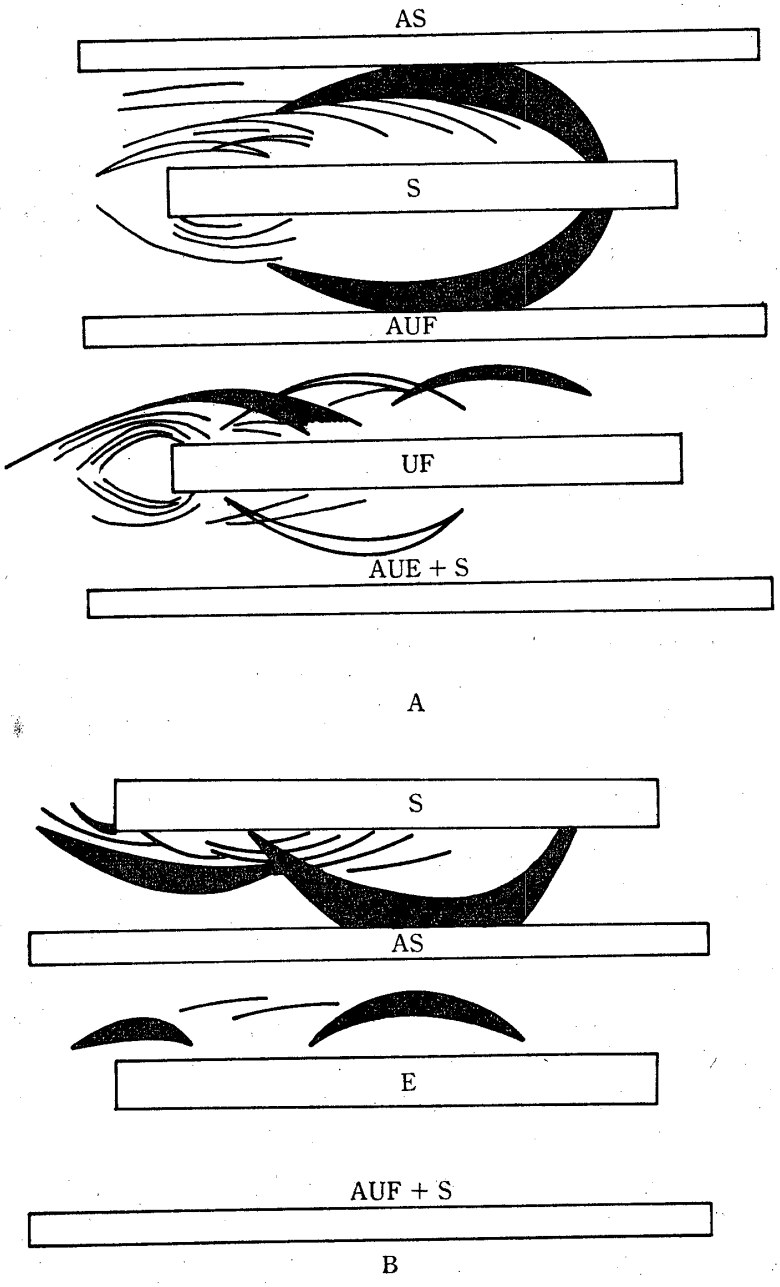


FIG. 5. Disc immunophoretic diffusion analysis of protein components in uterine fluid (A) and saline extracts of endometrium (B). S, serum; UF, rat uterine fluid; E, saline extracts of endometrium; AUF, anti-rat uterine fluid antibodies, AUF+S, absorbed anti-rat uterine fluid antibodies with serum; AS, anti-rat serum antibodies

ment described above. Many bands were discerned between uterine fluid and AUF, and four precipitins were formed between AUF and rat serum (Fig. 3B). Eight protein components with the persistent bands in the reaction between AUF+S and uterine fluid may be considered immunologically specific protein in uterine fluid. These results indicate that four antigenic proteins in uterine fluid were common to serum protein and that at least eight specific uterine fluid proteins were immunochemically distinct from serum antigens. Protein patterns of rat uterine fluid at 22:00 hr of proestrus stage were compared with those of serum from same stage by mean of polyacrylamide gel Disc electrophoresis (Fig. 4A). Many bands were observed in uterine fluid and a certain band was distinguishably mobile compared with those of serum protein. After electrophoresis, the separated protein were allowed to diffuse against AUF and AUF+S. There were four common proteins between uterine fluid and serum including albumin (Fig. 4B and 5A). Twelve spurs in reaction between uterine fluid and AUF were observed; eight spurs were revealed in reaction between uterine fluid and AUF+S. In the specific proteins of uterine fluid, two proteins and other proteins manifested the mobilities of α-β-globulin and γ-globulin, respectively. Prealbumin spur observed by immunoelectrophoresis was not demonstrated in Experiment III. Saline extracts of endometrium did react to four precipitins against anti-rat serum antibodies, but not at all to AUF+S as shown in Fig. 5B.

Discussion

Schiller et al. (9) and Chadwick et al. (10) have shown that rapid disappearance of fluorescein labelled protein is due to its distribution throughout the animal tissues. The rate of disappearance of FITC labelled protein in this investigation was in agreement with the other writers (9–11). From our results, studies on passage of sodium and iodoantipyrine (12) or transport of bovine serum albumin into rabbit uterine lumen (13), it may be suggested that the time of transport of serum components was four to twelve hours and that the common protein between serum and uterine fluid was transported not by secretion, but by diffusion in metestrus to diestrus stage rats.

The contents of protein and FITC in flushing from the uterine lumen have altered significantly at different stages of the estrus cycle. The value of protein content was least at metestrus and diestrus, and most at 22:00 hr of proestrus (1). On the other hand, the content of FITC protein in flushing from uterine lumen was significantly lower at 22:00 hr of proestrus than at other estrus stages.

The results in this experiment may suggest that the rate of passage of macromolecules (serum protein) depended also on the barrier controlled by ovarian hormones which was suggested by Marley and Robson (12).

Eight arcs between AUF+S and uterine fluid were obtained in Ouchterlony tests. Then it has been indicated that eight protein components, which have not

been observed in serum, were present in the rat uterine fluid. On the other hand, when AUF or anti-rat serum antibodies was allowed to react against serum and uterine fluid, four and five precipitative bands were formed respectively. These results have shown that there were at least four common proteins with serum in uterine fluid.

Albers and Castro, using Ouchterlony gel diffusion and immunoelectrophoresis procedures, have shown the presence of at least five protein components, and one of them was specific in rat uterine fluid (7).

Kunitake et al. have detected nine protein components by Disc electrophoretic technique and shown four common protein components with serum; however, they have not investigated specific protein in uterine fluid immunochemically (8). Although the number of common proteins with serum in uterine fluid was approximately agreement with the reports described above (7, 8), the number of the specific protein components in uterine fluid, which were not detected in serum, was much more. From the results of Experiment II and immunochemical analysis, uterine fluid at estrus contained much more specific protein components secreted from the uterine epithelium than the common protein components of serum.

It seems that this difference between our results and others (7, 8) depended on procedure and the stage of uterine fluid collection. They collected uterine fluid from rats ligated of uterine tube or with injections of estrogen. On the other hand, we have used the uterine fluid at 22:00 hr of proestrus in which the proportion of albumin was minimal compared with other protein components in uterine fluid (1). By a combination of Disc electrophoresis and immunoprecipitation with AUF and AUF+S, further investigation was achieved into the properties of uterine fluid proteins. In four common proteins with serum in uterine fluid, one precipitin was albumin and three arcs were in the gamma globulin region. The specific protein in uterine fluid revealed two arcs in the alpha globulin region and other precipitins were observed most in slow migration region — probably gamma globulin. The presence of some serum protein in rat uterine fluid that has been demonstrated by the tracing method of FITC labelled serum protein physiologically and by immunochemical analysis, would likewise imply the transudation of a certain amount of blood protein into the uterine lumen. Furthermore, from the difference of proportion of common protein components between uterine fluid and serum, it was infered that there was a mechanism involving specific transport processes controlled by ovarian hormones. Although there were a great variety of protein components in uterine fluid due to estrus cycle or injection of ovarian hormones (1, 2). It was of very interest that specific protein components contained in uterine fluid were greatest at the estrus period (22:00 hr of proestrus). It was possible that these specific proteins were secreted from uterine mucosa.

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