IMMUNOCHEMICAL AND ELECTROPHORETIC PROPERTIES OF ESTRUAL RABBIT UTERINE FLUIDS

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ABSTRACT

IMMUNOCHEMICAL AND ELECTROPHORETIC PROPERTIES OF ESTRUAL RABBIT UTERINE FLUIDS

By Kent R. Stevens

The uteri of parous or mature virgin Dutch Belted rabbits were ligated at the cervical or both ends of the uteri. After ligation intervals of 1, 2, 4, 5, 7, 16, or 20 weeks, the accumulated fluid was aspirated from the uteri and the pH and volume were recorded.

The fluids were centrifuged and the protein concentration of each sample was determined by biuret protein analysis according to procedures outlined by Gornall (39). The pellet of particulate matter, which resulted from centrifugation, was smeared on a microscope slide, stained with the pyronin Y - methyl green stain of Paape et al. (84) and the relative cellular concentration was determined by direct microscopic count.

The uterine fluids from each ligation interval were pooled and concentrated with a dialysis membrane ultrafilter. Antisera to these concentrated fluids and to rabbit blood sera were produced in mature guinea pigs.

By Kent R. Stevens

The protein components of rabbit uterine fluids were studied and compared with rabbit blood sera by (1) agar gel double diffusion tests of Ouchterlony (83), (2) moving boundary electrophoresis, (3) agar gel electrophoresis, (4) paper electrophoresis and (5) immunoelectrophoresis by the procedures of Wieme (110).

Serous, clear to turbid fluids, which appeared colorless to slightly yellow, were aspirated from uteri after each ligation interval. The pH of these fluids, taken immediately after aspiration, was 7.62 ± 0.008. The pH increased markedly with time and handling after collection.

The volume increased with ligation time from 2.48 ml after 1 week of ligation to 71.00 ml after 20 weeks of ligation. The protein concentration decreased from 5.13 mg/ml after 1 week of ligation to 1.82 mg/ml after 20 weeks of ligation. The total protein per uterus increased with ligation time in spite of the decreasing protein concentration.

Thirteen protein antigens were identified in uterine fluids by agar gel double diffusion tests. Ten of these were also found in blood serum. Immunoelectrophoresis revealed 20 antigens in uterine fluids, 15 of which were

also found in blood serum. The five proteins which appeared to be peculiar to uterine fluids were described as two prealbumins and three beta globulins. The prealbumin fraction was also identified by moving boundary electrophoresis in fluids from uteri ligated for more than five weeks. Analysis by moving boundary electrophoresis also revealed an alpha globulin in uterine fluids that was not found in blood serum.

The mobilities of uterine fluid proteins appeared greater than those of blood in the paper electrophoretic analyses, but not in agar gel electrophoresis. Neither of these procedures revealed a protein component peculiar to uterine fluids.

The results of the different electrophoretic analyses are compared, and the possible significance of the protein components peculiar to uterine fluids is discussed.

IMMUNOCHEMICAL AND ELECTROPHORETIC PROPERTIES OF ESTRUAL RABBIT UTERINE FLUIDS

By

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BIOGRAPHICAL SKETCH

Kent R. Stevens was born in Brooklyn, New York on January 4, 1939. He received his elementary education at Plaza School and his high school education at Baldwin High School, from which he was graduated in 1956.

After one year at Rutgers University, he transferred to the Long Island Agricultural and Technical Institute where he majored in Animal Science. He was graduated in 1959 with an A.A.S. degree.

In September of 1959, he enrolled at Michigan State University. In 1961 he received his Bachelor of Science degree in Dairy Science. He began his graduate work in the dairy department at Michigan State University with a Ralston Purina Research Fellowship. He received his Master of Science Degree in August 1963 in the Dairy Department with a major in Animal Reproductive Physiology.

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INTRODUCTION

Mammals are unique because they are able to control the fertilization environment of the gametes. For example, the female controls the temperature, gas tension, pH, etc. of the uterine and tubal environment in which sperm must reside before fertilization. The secretions of these organs add moisture and other constituents to the fertilization medium. To what extent each of these factors contributes to fertility in an individual is not known.

It has recently been shown that sperm are altered in some way in the uterine environment. Sperm, incubated in the uterus of a rabbit, have the capacity to fertilize a freshly ovulated egg, whereas sperm which have not been incubated in the uterus do not. Thus, capacitation of the sperm occurred after sufficient incubation time in the uterus. However, if sperm are incubated in the uterus in a dialysis bag, the sperm are not capacitated. Hence, it appeared that capacitation occured through the interaction of the sperm with the uterine endometrium, or of the sperm with the macromolecules from the uterine secretions. This macromolecule could be an enzyme, hormone or other proteinaceous substance which acts upon sperm to capacitate them.

The secretions of the estrual uterus have not been studied extensively. Little is known of their rate of secretion, composition or more importantly, of their role in reproduction. It was the purpose of this thesis to describe some of the physical characteristics of uterine fluids of the rabbit, and especially to study the immunochemical and electrophoretic properties of the proteins of these fluids. This work was intended to be a foundation from which further studies to elucidate the physiological role of these fluids may be initiated.

REVIEW OF LITERATURE

The evidence available concerning the occurrence of estrual uterine secretions is contradictory. Bishop (15) doubly ligated the uteri of rabbits and failed to observe accumulation of fluids in the ligated uteri, although fluid did accumulate in oviducts which were doubly ligated (13). He concluded that the uterus did not normally contribute any fluid to the reproductive tract (13), but that the oviduct secretions bathed the entire tract and kept it moist (15). These reports do not support previous work in this area, because the presence of luminal fluids has been reported in the rabbit as well as in many other species.

In 1891, Woskressensky (113), while studying rabbit oviduct secretions, noticed that hydrometra developed within 6 weeks after ligating the uteri at the cervical and tubal extremities. In 1898, C. J. Bond (21) used the same procedure to occlude the uteri of the guinea pig and rabbit and observed the accumulation of " a colorless, watery fluid, neutral or faintly alkaline in reaction, (and) of low specific gravity."

Bond also attempted to induce hydrometra by ligating either but not both ends of the uterus. He observed that

fluid did not accumulate if the uterus was ligated only at the tubo-uterine end, but that it did accumulate if only the cervical extremity was ligated. He concluded that "the communication between the cavities of the fallopian tube and uterine cornu, in animals is so arranged anatomically, that no regurgitation of fluid secretion is allowed from the uterine to the tubal cavity even under pressure." This impassable nature of the tubo-uterine junction was later confirmed for the rabbit (10, 24), and guinea pig (10), and also demonstrated in the cow (17), pig (10), dog (58), monkey (47), cat (10), rat (64) and mouse (6). In the rabbit, this constriction of the tubo-uterine junction is maximal during estrus (112).

In 1906, Bond (22) reported further observations on factors affecting the accumulations of uterine fluids. Firstly, if one rabbit uterus was ligated and the other was allowed to become pregnant, no fluid accumulated in the ligated uterus. Secondly, the ligated uteri of ovariectomized rabbits accumulated fluids at a markedly decreased rate compared to intact animals. Lastly, ligated uteri which were transplanted to " distant parts of the abdominal cavity," also accumulated uterine fluids. The first and second of these observations have been substantiated (51, 69).

To date, uterine fluids have been found not only in the rabbit (18, 22, 113) and the guinea pig (18), but also in the whale (78), cow (43, 80, 94, 97), sheep (48), pig (54), opossum (46), cat (25), dog (31, 37), monkey (103), hamster (51), rat (64), mouse (6) and bankvole (25). They have also been suggested to occur in the human (23, 104).

Of the species in which uterine fluids have been shown to occur, the rat, mouse and bankvole are unique, in that the fluids normally accumulated in the uteri during estrus (6, 25, 64). Fluid accumulation became evident with the constriction of the cervix at the onset of estrus (36). The uterine size increased with the collection of fluid until about ten hours after estrus, when the cervix relaxed and the fluids were released into the vagina (12, 20). After copulation occurred, the fluids of the mouse seemed to clot and did not leave the uterus through the cervix as swiftly as in the rat (12).

In the other animals, fluid was also secreted during estrus (31, 37) - the greatest rate of secretion occuring around the time of ovulation (68). Only small quantities of uterine fluids could be found since the fluids did not normally accumulate as in the rat, mouse and bankvole. Greater quantities of fluid could be collected by occluding the

uteri during estrus by ligation (3, 21, 51, 113).

Characteristics of Uterine Fluids

Uterine fluids most generally have been described as a watery (21), colorless (21, 78, 90) to opalescent (78, 90, 98) fluid which is alkaline (21, 78) in reaction and contains some epithelial cells (97, 98) and leukocytes (12, 56, 78, 90) in estrus animals.

In the cow, the fluids were opaque and yellowish in color during proestrus, watery and slightly gray during estrus and mucus-like and yellowish during post-estrus with some tendancy to clot (80). In the pig, the fluids have been described as yellow, with a characteristic odor (67).

The cellular content of the uterine fluid seemed to differ with the species, investigator and method of removing the uterine secretions from the uterus. Olds and VanDemark (80, 81), "stripped" the cow uterus of its uterine fluid and found many epithelial cells and cellular fragments. Other workers, collected fluid from ligated uteri or uteri distended at estrus and reported low numbers of leukocytes (56), epithelial cells (98) or red blood cells (56). The presence of cellular elements may be hormonally influenced, since fluid from uteri of ovariectomized mice treated

with estrogen and progesterone, was found to be less cloudy and more free of cellular elements than fluid from ovariectomized mice treated only with estrogen (52).

Shih et al. (98) and Ringler (90) aspirated fluids from uteri of estrus rats, yet Ringler reported the consistent presence of black granules in the uterine fluids, whereas Shih et al. did not mention the black granules. Perhaps their presence is a peculiarity of the strain of rat studied.

Sergin (97) has reported that bovine uterine fluids were isosmotic with bull semen, which itself was slightly hypertonic as indicated by freezing point depression values. The freezing point of bull semen was -0.61° C (102), while that of cattle blood serum was only -0.585° C (102). Olds (79) and Olds and VanDemark (81) also demonstrated the slight hypertonicity of bovine uterine fluid by freezing point depression studies. They determined that 80% of the osmotic pressure was due to sodium and potassium compounds in the uterine fluids.

On the other hand, uterine secretions have been reported to be hypotonic in the pig (54), mouse (54) and rabbit (66). Shih et al. (98) implied that uterine fluids of the dog, rabbit and rat were hypotonic because of their low protein concentration.

Hydrogen ion concentration (pH) measurements have been determined for the uterine secretions of several species. In the cow, these values range from 5.85 as measured by Skaarup-Thygessen (99, 100) to 7.91 as measured by Gupta (43). The former worker reported that the pH of the uterine secretions were more alkaline in cows with uterine infections (99, 100). Other pH values are listed in Table 1.

In rats, the average pH was more alkaline, with several values reported between 7.55 and 7.67 during estrus (Table 1). Blandau observed that the pH of rat fluids increased with time after removal from the uterus (15). This has also been observed in cow uterine fluids (43).

Table 1. Reported pH values of uterine fluids of the cow, rat and rabbit.

Cow		Rat		Rabbit	
pH	References	pH	References	pH	References
5.85	99	7.55	98	7.6	117
6.8	61	7.57	90	7.78	98
7.1	80	7.67	45	7.86	108
7.91	43			8.1-8.5	1

The pH of rat and mouse uterine fluids before estrus was 7.24 and 7.11 respectively (45). These values increased to 7.67 and 7.46 in the rat and mouse respectively, shortly

after estrus (45). In the cow, no correlation was observed between the pH and the stages of the estrous cycle (87). In the human, the pH of the uterine luminal environment increased from 6.5 during the "secretory" phase to 6.8 during the "proliferative" phase (33).

Bishop (16) suggests that the loss of carbon dioxide from uterine fluids may have been responsible for the high pH values as reported by Blandau et al. (19). Since carbon dioxide occurs in the fluids as (1) dissolved carbon dioxide which has no effect on pH, as (2) carbonate which is acidic, or as (3) bicarbonate which is alkaline; Bishop's suggestion implies that there was a loss of carbon dioxide in the form of carbonate. This could not be applicable in vivo because the concentration of carbon dioxide in fluids, in the form of carbonic acid, is determined by the carbon dioxide tension in the uterus - which Campbell (27, 28) has shown to be identical with blood plasma in the rabbit. Bishop's suggestion is entirely possible for fluids removed from the uteri. The high bicarbonate content of rabbit uterine secretions contributed to their high pH. It has been shown that there was twice as much bicarbonate in rabbit uterine fluid than rabbit blood plasma (108).

Olds (82) has determined the buffering capacity of bovine

uterine fluid. The buffering capacity is defined as the number of gram equivalents of strong acid or base required to produce a change of one pH unit of one liter of fluid (107). The buffering capacity of bovine uterine fluid collected from cow reproductive tracts, 1 to 8 hours after slaughter, was 0.0139 against acid and 0.0308 against base for an average capacity of 0.0129. The buffering capacity of bovine blood serum, with an average pH of 7.9 was 0.0129. Olds stated that the high pH of the serum was due to the loss of carbon dioxide from the serum during handling, and this could affect the buffering capacity of the sera. In fact, in experiments with fresh follicular fluid, Olds found the buffering capacity to be 0.0243 against acid, but only 0.0067 in fluid from which the carbon dioxide was removed. Thus, it appeared that 70% of the buffering capacity of follicular fluid was due to the carbonate content of the fluid.

Composition of Uterine Fluids

In 1898, Bond (21) stated that rabbit and guinea pig uterine fluids contained sodium chloride, serum albumin, calcium phosphate and mucin. Since then, more information has been gathered concerning the constituents of these uterine secretions. Heap et al. (48) have identified sodium, potassium, calcium, phosphorous, iron, copper,

aluminum, magnesium and carbon ions in uterine washings of rabbits, sheep, rats and cows. Carbohydrate, protein and lipids were also identified. Since Heap et al. analyzed uterine washings, their data are expressed in total content of substance analyzed per uterus washed. It is therefore not possible to compare the concentrations of uterine contents of the species reported by them.

Sodium and Potassium

In quantitative studies on rat uterine fluid, the mean concentration of sodium in uterine fluid of estrogen treated ovariectomized rats, ranged from 108 to 123 mM/l (90). The sodium content of blood plasma from these rats ranged from 110 to 140 mM/l (90). The potassium concentration of this uterine fluid ranged from 37 to 56 mM/l, while the concentration in blood sera averaged 4 mM/l (90). These values are similar to those of Howard and DeFeo (55), who observed that the sum of sodium and potassium compounds in the uterine fluids was approximately the same as the comparable sum for blood serum. However, the potassium concentration in uterine fluid was about ten times the concentration in blood serum. The potassium concentration increased at the expense of the sodium concentration. The

Table 2. The concentrations of some components of uterine fluids from cows, rats and rabbits.

	Cow		Rat		Rabbit	
Components	Conc.	Ref.	Conc.	Ref.	Conc.	Ref.
Water gm/l	916	81	982	98	979	98
Sodium mM/l	95.6	81	169	98	158	98
	161.6	43	123	90	110	68
			115.3	55		
Potassium	4.7	81	4.3	98	6.1	98
mM/l	19.9	43	42.0	90	10.7	68
			37.4	55		
Calcium	3.8	81	1.5	98	4.7	98
mM/l	4.3	43				
Inorganic P	2.4	81	0.0	98	0.2	98
mM/l	2.1	43	0.6	90		
Chloride	102	81	98	98	98	98
mM/l					55	68
Total N	740	81	100	98	80	98
mg/100 ml	497	43	41	90		
Carbon di-			61.8	98	53.6	98
oxide mM/l					31.4	66
Protein	3,106	81	510	98	270	98
mg/100 ml	4,600	43	240	90		

sodium and potassium values reported by Shih et al. (98) for the rat, resemble the values reported above for rat blood serum (Table 2.).

In the uterine fluid of estrus rabbits, Shih et al. (98) found 158 mM of sodium/l and 6.1 mM of potassium/l.

Lutwak-Mann obtained 110 mM of sodium/l and 10.7 mM of potassium/l from rabbit uterine fluid obtained 12 hours after mating (68). By comparison, rabbit blood sera contained 158 mM/l and 4.1 mM/l of sodium and potassium, respectively (102).

The sodium and potassium concentration of dog uterine fluid was 162 mM/l and 5.2 mM/l, respectively (98), while the concentration for dog blood serum was 150 mM/l and 4.4 mM/l respectively (102).

Gupta obtained 161.6 mM of sodium/l and 19.9 mM of potassium/l from bovine uterine fluid removed from uteri in vivo (43). Olds and VanDemark (81) reported 95.6 mM of sodium/l and 4.7 mM of potassium/l from uterine fluid removed from excised cow uteri (81). The sodium concentration of bovine blood serum was 142 mM/l and the potassium concentration was 4.8 mM/l (102).

Generally then, the potassium concentration in uterine fluids of animals studied was always higher than the potassium concentration in blood sera of these animals. The sodium concentration, by comparison, was usually but not always lower.

Calcium

Calcium concentrations for cow uterine fluids have been reported to be 3.8 mM/l (81) and 4.3 mM/l (43) while in the rat, rabbit and dog, values of 1.5 mM/l, 4.7 mM/l and 3.5 mM/l have been found, respectively (98). The calcium concentrations in blood sera have been reported to be 5.4, 5.3 and 7.0 mM/l for cattle, dog and rabbit, respectively (102). The calcium content of uterine fluids, therefore, appeared to be lower than that of blood serum.

The high potassium to calcium ratio found in uterine fluids, in the light of previous work (57, 115), may be an indication of growth - probably of the uterus.

Chloride

The chloride content of the cow, rat and dog uterine fluid was 102 (81), 98 (98) and 167 (98) mM/l, respectively. In the rabbit, chloride concentrations of 98 mM/l (98) and 55 mM/l (68) have been reported. These values appear to be similar to the chloride values obtained for blood serum of these species except in the dog, where the chloride concentration has been determined to be 106 mM/l (102).

Inorganic Phosphorous

The phosphorous content of uterine fluids is very low for the rat, rabbit and dog, as reported by Shih et al. (98). The uterine secretions of the cow, on the other hand, contained 2.1 (43) or 2.4 mM/l (81) of inorganic phosphorous. The inorganic phosphorous content of blood serum appeared to be 1.8 to 2.1 mM/l in the bovine, 1.8 mM/l in the dog and 1.9 mM/l in the rabbit (102). The inorganic phosphorous content, therefore, was lower in uterine fluids than in blood serum in all animals studied, except the cow.

Total Nitrogen

Dog and rabbit uterine fluids contained 80 mg of total nitrogen per 100 ml (98). Reports of 100 mg (98) and 41 mg (90) per 100 ml have been found for the rat, while 740 mg (81) and 497 mg (43) per 100 ml were found for cow uterine fluids.

Carbon Dioxide

The carbon dioxide concentration in uterine fluids has been determined in the dog, rabbit and rat to be 3.0, 53.6 and 61.3 mM/l respectively (98). In rabbits, the bicarbonate content of uterine fluids has been reported to be 50.1 mM/l (108) and 49.3 mM/l (68). The bicarbonate content of

uterine fluids removed 24 hours after mating was 31.4 mM/l (65) and 40.4 mM/l (68). In comparison, the bicarbonate content of rabbit blood serum was only 24.00 mM/l (108).

Protein

Protein concentrations for the uterine fluids of the cow were calculated from protein nitrogen determinations and were found to be 3,106 mg (43) and 4.600 mg (81) per 100 ml of secretions. This was considerably greater than the 510, 270 and 380 mg per 100 ml of uterine fluid from the rat, rabbit and dog, respectively (98). Ringler (90) only found about 240 mg of protein per 100 ml of rat uterine fluids. Protein concentrations of blood sera from cattle, dog, rat and rabbit have been reported to be 6.9, 6.9, 6.3 and 7.2 gm per 100 ml, respectively (102). This is consistent with Junge and Blandau's (56) report that rat uterine fluids contain about 5% of the protein concentration found in rat blood serum.

The protein components of rat uterine fluids have been studied by electrophoretic (56, 90) and immunological methods (3). The free electrophoretic patterns of uterine fluids of castrate rats treated with estrogens demonstrated the presence of four or five protein peaks depending on the

treatment (90). Fluids from estrone-treated rats, or rats with ligated uteri contained five electrophoretic components, while fluid from estriol-treated rats contained only four components. Ringler (90) demonstrated that the treatments did not significantly alter the mobilities of the four components common to all fluids. The fifth protein component of estrone-treated rats, had a mobility that was greater than that of plasma albumin, while the mobilities of the other four components did not differ significantly from the mobilities of the protein components of rat blood plasma, namely beta, alpha$_2$, alpha$_1$ globulins and albumin. In earlier studies, Ringler (88) reported six protein components in rat uterine fluid by both free electrophoresis and ultracentrifugation studies. However, he could find only three ultracentrifugal components in rat blood plasma.

The relative amounts of each protein component in uterine fluids of the rat differed between and within estrogen treatments, and were not similar to the protein distribution of rat blood plasma. However, when rat uteri were ligated for over 90 days, the relative protein composition was altered so that the proportions of the protein components approached those seen in rat blood plasma.

The prealbumin component which Ringler (90) found in

uterine secretions, but not in rat blood serum, has been found by other workers (50, 96) to be present in rat blood serum. Since this is so, Ringler concluded that the uterine proteins were similar or the same as proteins of the blood serum of rats. Ringler noted that the percentage composition of the albumin-like and prealbumin components of uterine fluid was about 59%. Since this was near the 56% albumin concentration found in rat plasma, Ringler suggested the possibility that some of the plasma albumins were altered as they passed through the uterine wall. Exopeptidases, which can enzymatically alter the proteins, have been identified in the uterine wall (101).

Junge and Blandau (56) identified only four components in the uterine fluid of estrus rats by moving boundary electrophoresis. The major protein component had a mobility greater than that of the albumin fraction of blood sera. The concentration of this component was similar to the fast moving protein component described by Ringler (90).

Neither the protein content determined by total nitrogen determination, nor the electrophoretic mobilities, differed in samples collected at the beginning or the end of heat (56).

By paper electrophoretic methods, Junge and Blandau (56) found no components with mobilities in the serum albumin

range. However, a protein component with a mobility between the beta and gamma globulins was found by this technique. This procedure did not demonstrate the similarity to plasma components found by moving boundary electrophoresis.

Albers and Neves e Castro (3) collected uterine fluids from estrus and ligated rat uteri. Antibodies against the uterine fluids and blood serum were produced in two groups of three rabbits. The blood was then collected from these rabbits by heart puncture 2 weeks after the initial injection.

On the basis of agar gel diffusion techniques, Albers and Neves e Castro (3) found four antigenic components in rat uterine fluid. Three of these were also found in rat serum and one was peculiar to the uterine fluid. The protein components found by this method represented only the minimal number of demonstrable antigen-antibody complexes present in uterine fluid.

When agar gel electrophoresis was carried out (3), five protein components were found. They were described as a prealbumin, an albumin, two alpha globulins and a beta globulin. No gamma globulins were identified. Both of the previous investigators also recognized the prealbumin component which represented about 3.6% of the total protein.

Albers and Neves e Castro (3) did not perceive any difference in the protein patterns of uterine fluids which were collected by ligation or at the time of estrus. They found however, that the curvature of the precipitation lines differed from blood serum to uterine fluid. This, they concluded was not to be taken as proof or disproof of identity. It was still possible therefore, that the other proteins were also found in blood as postulated by Ringler (90).

Other Components of Uterine Fluids

In the cow, 1.2 gm of fat/ 100 ml and 80 mg of reducing sugars/ 100 ml were present in uterine fluids removed from excised uteri (81). Only 41.8 mg of reducing sugar/ 100 ml of uterine fluids was obtained from fluids found in vivo (43). In addition, cow uterine fluids contained 2.4 mg of magnesium per 100 ml and an undetermined amount of complex polysaccharides which were identified chromatographically (43).

Other components have also been identified and quantitatively analyzed in rabbit uterine fluid. Gregoire (41) has chromatographically identified five main amino acid components of rabbit uterine fluid. Glycine, glutamic acid, serine, alanine and threonine were present in 26.8, 11.9

10.4, 6.4 and 4.0 mg per 100 ml of uterine fluid. Traces of tryptophan, methionine, valine, taurine, and glutamine were identified when the amount of fluid chromatographed was increased from 50 to 100λ. Beta alanine was identified when 200λ of deproteinized uterine fluids were chromatographed. It is interesting to note that the relative order of concentrations of amino acids in bull semen is similar to that found in rabbit uterine fluids (35).

Inositol has been found in rabbit uterine fluids in an average concentration of 7.2 mg/100 ml (42). It may act as a reserve carbohydrate for use by spermatozoa or a developing egg (42). Another sugar, glucose, has been identified in rabbit uterine secretions. Uterine fluids of estrus rabbits contained between 0 and 160 mg of glucose/l (98). At 24 hours after mating, the secretions contained 100 to 180 mg/l (65). Intravenous administration of glucose slightly "enhanced" the concentration of glucose in uterine fluids (68). Only small amounts of lactic acid, fructose, citric acid and sialic acid were found in rabbit uterine fluids (68).

Enzyme and Hormone Content

Uterine fluids of the rat contain beta glucuronidase and alkaline phosphatase, but not acid phosphatase (90).

The enzyme concentrations were much greater in the uterine fluids than were found in rat blood plasma. The concentration of beta glucuronidase in fluid from ligated uteri was six times greater than fluid from estrone-stimulated uteri. The alkaline phosphatase concentration was about twice as great.

Leone et al. (63) identified 5-nucleotidase, ATPase and purine nucleotidase in rabbit uterine fluid.

Dipeptidases have been localized in rat and rabbit uteri and their respective fluids (4). The highest levels of dipeptidase found during the estrous cycle occurred at pro-estrus and estrus. At these times, the uterus and uterine fluids had similar activity. Secretions of rabbit uteri obtained 12 hours after injection of human chorionic gonadotrophin had higher dipeptidase activity than the corresponding uterine tissue homogenates. It appeared that estrogen stimulated the enzymatic activity. However, the greatest dipeptidase activity was found in uteri during pseudopregnancy.

Estrogens have not been identified in uterine fluids of the rat. When C^{14} labelled acetate was administered to rats, no labelled steroids could be recovered from uterine fluid (53). Ringler (89) subcutaneously injected estrone-16-C^{14}

into rats. He could find only a minute amount of radioactivity in the uterine fluids which were removed from these rats. A bioassay of the phenolic fraction of the extract was negative for estrogens. Ringler (89) concluded that once estrogens exerted their effect upon the uterus, they either left the uterus or were broken down.

Uterone

Homburger et al. (53) proposed the name "uterone" for mouse endometrial secretions because they found that it had some biological activity. They injected 0.1 to 0.2 ml of mouse uterine fluids into certain strains of castrate female mice and noted a statistically significant decrease in the weight of the adrenals and an increase in uterine size. The increase in uterine size was stated to be due to simple myometrial hyperplasia, but no explanation was given for the decrease in adrenal size. Similar experiments in immature mice showed similar changes, but these were not statistically significant. There were no anatomical or micro-morphological changes in the ovaries of immature animals indicating that uterine fluids contained no gonadotrophins.

When more than 32 mg of freeze-dried uterine secretions

were injected into castrate mice, some of the animals developed acute disorders of the eye, characterized by intrabulbar hemorrhage with marked exophthalmus (black eye syndrome). The condition was occasionally induced by causing the animals to resorb their own accumulated uterine fluids by administration of testosterone. This disorder usually progressed quickly and was terminated by death of the animal in a few days. However, since this syndrome was not produced in all strains of mice, it may be of limited significance.

To the contrary, Warren (107) found no micromorphological alteration of the reproductive tract, mammary glands or pituitary of castrated animals given injections of uterine secretions amounting to ten times that administered by Homburger et al. (53).

Factors Affecting the Accumulation and Composition of Uterine Fluids

Hormones

The occurence of uterine secretions has been associated with estrus. Fluid accumulations have not been demonstrated in the ligated horn of a pregnant animal (22, 69) and fluid secretion was slight in ovariectomized animals (22, 41, 51).

Long and Evans (64) noticed that uterine fluids accumulated in the uteri of normal rats with the onset of estrus. Estrus rat uteri contained eighteen times as much fluid by weight than did diestrus uteri (107). De Marco (31) noted that fluid secretions could be obtained only from the uteri of dogs in heat.

Castrate animals secrete uterine fluids at greatly diminished rates (22, 41, 51). Gregoire (41) obtained 3.84 ml of uterine fluid from intact rabbits, but only 1.73 ml from castrated rabbits after ligation of the uteri for 72 hours. In mice (51), less than 20% of castrated mice accumulated measurable amounts in their uteri 1 month after ligation. The incidence of hydro-uteri, however, increased with ligation time.

In 1924, Allen and Doisy (8) demonstrated that injection of follicular fluid into castrated mice caused a typical estrus vaginal smear and accumulation of fluid in the uterus. In the same year, they induced uterine fluid formation in immature rats with hog follicular fluid (7). Follicular fluids contained the hormone estradiol (71, 72). Marrian (70) used the criterion of uterine fluid accumulation in mice as an index of response to estrogens prepared from

urine of pregnant women. Ringler (90) and Homburger et al. (52) castrated rats and mice, respectively, and administered estrogens in order to increase the volumes of uterine secretions. Different estrogens had a different effect on the production of uterine secretions (90). When ovariectomized rats were injected with estradiol, estrone or estriol for 3 days and the uterine fluids collected on the fourth day, the resulting volumes were 0.24, 0.38 and 0.80 ml, respectively. Uteri of proestrus, control rats accumulated 0.37 ml of fluid. Increased dosages of estradiol or estrone did not result in greater accumulation of fluid. It has been shown that estriol induced greater uptake of water by the uterus than the other two estrogens (90), thus explaining the higher volumes obtained from uteri stimulated with estriol. In the light of these findings, it seems strange that Gregoire (41) obtained greater volumes from uteri of ovariectomized rabbits treated with progesterone, than from uteri of ovariectomized rabbits treated with estrogens.

Estrogen stimulation was associated not only with increases in the volume of fluid obtained, but also with an increase in the relative concentrations of components in these secretions. Heap (49) found that the sodium, potassium, chloride, nitrogen and carbohydrate content of rat

uterine washings were greater at estrus than at diestrus. In the rabbit, he found that potassium, phosphorous, nitrogen and carbohydrate occurred in greater amounts in the luteal phase uterine washings than estrus phase. Lutwak-Mann (65), on the other hand, demonstrated that radioactive phosphate, sulphate, sodium and iodide ions were present in greater amounts in non-pregnant animals than in pregnant animals. The potassium ion concentration, however, was higher in pregnant animals. The ionic concentrations also appeared to increase during luteal phase in uterine washings of the sheep and cow (48).

In castrated mice treated with estrogen and/or progesterone, the incidence of hydro-uteri was greater when estrogen and progesterone were administered together than when either was administered alone (52). In the opinion of the present author, the hormone interaction suggested the necessity of a hormone balance for maximal uterine secretion.

Testosterone administration to ovariectomized rats inhibited uterine fluid secretion (53). Furthermore, after estrogens have stimulated production of uterine fluid in castrated rats, administration of testosterone caused marked regression of the distended uterus within 7 days and complete fluid disappearance in 21 days (53). In intact animals, the

testosterone effect was abolished by the administration of stilbesterol. The testosterone causes "atrophy of the endometrium and... a change in the structure of the uterine wall which permits effusion of the hydro-uterine fluid into, and probably through, the uterine stroma ...." The regressive effects of testosterone on established hydro-uterus was greatly diminished by cortisone administration as well as by stilbesterol administration.

Ligation

As previously indicated, fluids do not accumulate in the uteri of most species unless the uteri are occluded. It has been shown that the accumulation of uterine secretions distends the uterus, and induces the uterus to grow (92) by "work hypertrophy" (69) much as physical distention will induce growth.

Heap et al. (40, 44) contended that ligation altered the ratio of components of uterine fluids and therefore concluded that this fluid was not normal. In the rabbit, uterine washings from ligated and nonligated uteri were analyzed for specific substances. The ratio of components in uteri which were ligated was different from nonligated uteri (48). The sodium, potassium, phosphorous, total

nitrogen and carbohydrate concentrations were increased two to ten fold by ligation. However, Ringler (90) did not find a difference in sodium, potassium or phosphorous levels between unligated uteri and uteri which were ligated for 45, 90 or 360 days.

In mice, the protein carbohydrate ratio remained the same during ligation time. The concentration of each of these components, however, decreased with ligation time (51). Ringler (90) observed that the concentration of protein components in rat uterine fluids, increased sharply with ligation time. Also, the ratio of the protein components was altered so that as ligation time increased, the ratio of protein components of uterine fluid approached that of rat blood (90). Immunoelectrophoretic studies did not reveal any differences in the protein components of uterine fluid from ligated or nonligated uteri of rats (3).

Drugs

De Marco (31), using uterine fistulae in dogs, found that subcutaneous injection of pilocarpine stimulated the flow of uterine fluid during estrus. Flow was increased by about four times with pilocarpine. This flow could be diminished with subsequent administration of atropine and

pituitrin. Using the same procedures, Shih et al. (98) found that uterine fluid was secreted at a rate of 0.2 to 0.4 ml per hour with pilocarpine, whereas De. Marco reported volumes of 0.5 to 1.75 ml per hour. Shih et al. (98) found no difference in chemical composition between normal uterine secretions and secretions stimulated by pilocarpine in dog uteri. The stimulation of secretion by pilocarpine was noted in the oviduct of estrus rabbits (13). Shih et al. (98) and Bishop (13) emphasized that this effect was noted only in animals whose reproductive tracts were under the influence of estrogen. Bishop (13) stated that pilocarpine probably acted on the process of tubal secretion by encouraging the release of intracellular material already formed.

Pilocarpine stimulation of estrus uteri is reminiscent of the observations of Sauer et al. (95) who demonstrated that pilocarpine elicited contractions only in the estrin-stimulated uterus and not in the castrate or pseudopregnant uterus. These contractions may be implicated with the increased secretion rate of the uterine fluids after pilocarpine administration.

To the contrary, Sturgis (103) reported that pilocarpine inhibited uterine secretions in the monkey. Atropine abolished this inhibition. Atropine had no effect on the

rate of uterine secretion when administered alone. Intravenous administration of saline and injections of adrenalin increased uterine secretion. Secretion paralleled the increase in systolic pressure. From these observations, Sturgis concluded that "it appears possible that changes in the volume of circulating blood in the pelvis (of the monkey) may be of primary importance in the rate of secretion of uterine fluid." It has been reported, on the basis of this conclusion, that the accumulation of uterine fluid in the monkey was suggestive of a transudate and not a secretion (13).

Functions of Uterine Fluid

Bond (23) observed that "it appears that the uterine secretion is associated with the destructive processes which ordinarily go on in the generative canal in the female, and not with the constructive processes which take the form of increased growth of tissue and which are associated with pregnancy." The presence of epithelial cells in uterine fluids (80, 97, 98) supports this hypothesis. The secretion of uterine fluids may provide a method of removing the degenerated cells of the estrus uterine endometrium.

Sperm Transport and Motility

In rats, it has been suggested that the uterine secretions aid in the transport of sperm in the female reproductive tract (62, 64, 93, 109). However, Leonard (62) expressed the opinion that uterine fluids would influence fertilization only in cases of reduced numbers of viable sperm in the uterus. In mice (60) and cows (80), the uterine secretions of proestrus and estrus uteri permitted greater survival than at other times during the cycle. It has also been found that mouse sperm remain motile for 9 to 12 hours (73) in the mouse uterus, but, they remain motile for 24 to 30 hours when they were removed from the uteri and placed in glucose-Ringer's (73). The loss of motility may be due to the increase in concentration of leucocytes in the uterine lumen after insemination (12, 73).

If uterine secretions are a factor in sperm transport and motility, they do so with species specificity, since introduction of sperm of one animal into the uterine fluids of other species resulted in coaggulation and death of the foreign sperm (94, 114).

Capacitation

Tubal insemination with freshly ejaculated sperm about

the time of ovulation resulted in no fertilizations. In contrast, tubal insemination about the time of ovulation with sperm that have been incubated in the uterus for 6 to 10 hours resulted in near normal fertilization (11, 29). Thus the sperm developed the capacity to fertilize ova as a result of their exposure to the uterine environment. Hence, the change has been termed capacitation (11).

Sperm, incubated within a dialysis membrane in the uterus did not become capacitated (75). Consequently, this author is of the opinion that capacitation may be due to a macromolecule (such as a protein) in uterine fluids. All species in which capacitation has been demonstrated, also have been shown to produce uterine fluids.

Uterine Defense

Broome and Lamming (26) have demonstrated that E. Coli and Staph. Aureus live very well in washings of rabbit and cow uteri. These workers therefore, have concluded that the uterine defense factor is not free in the uterine lumen.

MATERIAL AND METHODS

Collection of Uterine Fluid

To collect useable volumes of uterine fluids from rabbits, it was necessary to occlude the cervical or both extremities as described below.

Parous or mature virgin Dutch Belted rabbits were anesthetized with anesthetic grade ether or sodium pentabarbitol (30 mg/kg body weight). Each rabbit was secured to an inclined operating board. The lower abdominal region was shaved and swabbed with a 0.79% solution of 17% benzalkonium chloride (Zephiran) in 50% ethanol. A 2 inch incision was made ending 2 inches anterior to the pelvis. The uteri were exteriorized and a Supramid synthetic suture (Jen-Sal Inc.) or silk suture was tied around each cervix with care to avoid rupture or occluding blood vessels. In certain rabbits, one or both tubo-uterine junctions were also tied in the same manner. The ovaries were examined for the presence of normal estrual follicles, and the uteri were then returned to their normal position. The abdominal musculature was closed by blanket stitching with size 0 braided silk suture. A urea-sulphonamide antiseptic powder was applied to the closed incision. The skin was closed

with 11 mm Michel wound clips and a urea-sulfonamide antiseptic powder was applied to the wound. The rabbit was then returned to its cage. No special post-operative care was necessary.

One to 20 weeks later, rabbits with ligated uteri were anesthetized, and their uteri exteriorized as described above. The uteri were inspected for fluid accumulations. A 26 ga disposable needle attached to a syringe of appropriate size was passed into the lumen of the uterus with care to avoid piercing uterine blood vessels. The fluids were then aspirated. After removal of the fluid, the volume acquired was recorded, and the pH of the fluid was measured with a Beckman Zeromatic pH meter equipped with one drop electrodes. The meter was adjusted with two buffers before use to insure accurate measurements.

Protein Determination

Uterine fluids were centrifuged at 2,000 X g for 10 min at 5° C and then frozen at -20° C until subsequently thawed for biuret protein analysis, by the method of Kingsely (59) as modified by Gornall et al. (39). The reagent for the biuret test reacted with the peptide bonds of proteins to form the compound biuret. Biuret, in turn, formed a charac-

teristic purple complex with the copper sulfate of the reagent. The amount of this purple colored complex, as indicated by the optical density of the protein-biuret solution at 540 mμ was an indirect measure of protein concentration.

One ml of biuret reagent was added to 0.5 ml of uterine fluid. The mixture was agitated and then incubated for 30 min at room temperature in 5 ml test tubes. The mixture was decanted into a 1.5 ml cuvette. The optical density was determined at 540 mμ in a Beckman DB Spectrophotometer and compared with a standard curve obtained previously by reacting known amounts of bovine serum albumin with the biuret reagent.

Cell Counts

Preliminary studies showed that the number of cells present in uterine fluids was too small to be counted accurately with a hemocytometer. Therefore, the pellets formed by centrifugation of uterine fluid samples were smeared on glass slides, dried and stained with the pyronin Y-methyl green stain procedure of Paape et al. (84). This procedure was chosen because it allowed greater differentiation of the cell types than did other stains.

Concentration of Fluids

The uterine fluids were concentrated with a dialysis membrane ultra-filter (LKB)[a]. The filter consisted of a nylon frame over which a length of 11/16 inch cellulose tubing was drawn. The cellulose tubing was closed by tying at one end of the frame. The other end of the filter was connected to a rubber tubing which was attached to a vacuum pump to evacuate the filter to a pressure of about 200 mm Hg. The rubber tube was then occluded with a pinch clamp.

The evacuated filter was immersed into the uterine fluids in a 250 ml graduated cylinder. Water and other low molecular weight compounds passed through the dialysis membrane into the region of lower pressure.

When the inside of the filter became filled with dialysate fluid, the inside of the filter was evacuated as before. The fluids were collected in a clean, dry trap placed in the vacuum line. These dialysates were tested for biuret protein content.

Collection of Blood Serum

Normal rabbit blood was removed from healthy Dutch Belted rabbits by cardiac puncture. The donor rabbit was

[a]Available from Fisher Scientific Co.

anesthetized with an intraveinous administration of sodium pentabarbitol (Nembutal) and placed on its back on an operating board. The thoracic region was sterilized with tincture of Zephiran. The chest region was palpated for maximum sensation of heart beat. An 18 ga needle attached to a 20 ml syringe was introduced through the sternum at the point of maximum palpable heart beat, and into the heart. Twenty ml of blood was then withdrawn slowly from the heart.

The blood was introduced into a 50 ml beaker, covered with aluminum foil and allowed to clot at room temperature for 4 to 6 hours. The clot was loosened from the side of the beaker and allowed to retract overnight at 5^{o} C to express the serum. The resulting serum was centrifuged at 2,000 X g for 10 min to remove residual cells. The serum was stored at -20^{o} C until thawed at room temperature for use.

Preparation of Antisera to Uterine Fluids

Guinea pigs were used to produce antibodies to rabbit antigens. Uterine fluids collected after 1 or 2 weeks of ligation were concentrated and standardized to 1% biuret protein. The uterine fluid was mixed with equal parts of Freund's complete adjuvant containing paraffin oil, mannide mono-oleate and killed mycobacterium butyricum. According

to Freund (34, 35), the use of this adjuvant enhances antibody production to antigens in low concentration in the test sample. The uterine fluid and adjuvant were emulsified by mixing in a Serval Omnimixer and then 1.0 ml of emulsified antigen and adjuvant were injected subcutaneously into each of six mature guinea pigs. One week later, these guinea pigs were injected with 1.0 ml of a similar mixture, however, the adjuvant used did not contain the killed bacteria. Two weeks after the second injection, 10.0 ml of blood was removed from each guinea pig by cardiac puncture using a 20 ga needle. The blood was allowed to clot. The serum was decanted into 15 ml glass centrifuge tubes, and centrifuged at approximately 2,000 X g for 10 min to remove residual red blood cells. The serum was stored in 1.0 ml quantities at -20° C in 5 ml glass test tubes.

Preparation of Antisera to Rabbit Blood Serum

Twenty ml of blood was removed from each of five mature Dutch Belted rabbits and was allowed to clot as described above. The resulting serum was decanted into 15 ml glass centrifuge tubes and centrifuged at approximately 2,000 X g for 10 min. The sera were pooled and stored in 2 ml quantities in glass test tubes at -20° C.

Antisera against blood serum was prepared as described above for rabbit uterine fluid, except that the serum was not diluted or standardized to 1% biuret protein concentration before being emulsified with adjuvant.

Agar Gel Diffusion Test

The double diffusion precipitation test of Ouchterlony (83) assumes that protein antigens and antibodies, when placed in separate wells in an agar medium, will diffuse out from the wells at their characteristic diffusion rates and form antigen-antibody complexes which precipitate and appear as lines. This test was carried out in petri dishes which were prepared in the following manner. (Illustrated in Appendix Fig. 1)

Four to six strips of Whatman no. 1 filter paper (1/2 in X 1 in) were folded approximately in half and placed over the lip of the base of a petri dish. These strips were held in place by a 1/2 in X 11 in strip of filter paper which was flattened around the inside wall of the base of the petri dish. This filter paper ring and filter paper strips were moistened with distilled water so that they adhered to the glass. Thirty ml of an 0.85% solution of agar agar no. 3 [a] in 0.85% saline, buffered with 0.005 M phosphate buffer at

[a] available from Consolidated Laboratories Inc.

pH 7.4 and containing 0.001% mertiolate, was then poured into the petri dish and allowed to solidify at room temperature.

The wells, from which the antigens and antibodies diffused into the agar, were cut into the agar plate with a Feinberg cutter which produces a pattern having a center well and six peripheral wells which are equidistant from the center well (Appendix Fig. 1). The bottom of each well was sealed with a drop of molten agar.

The wells were filled with antigen solution or antisera and the plates were held at 5° C. The wells were refilled at irregular intervals as necessary. The precipitation line formation was recorded at intervals on Kodak high contrast copy film.

Moving Boundary Electrophoresis

Proteins are amphoteric, that is, they contain both acidic and basic groups. Since proteins are electrolytes, they migrate in an electric field, and the direction of migration depends on the net charge of the protein molecule. The distance that the protein travels in an electric field per unit of time depends largely on the magnitude of the net charge. The hydrogen ion concentration of the medium containing the protein greatly influences not only the charge

of the protein, but also the magnitude of the charge.

Free electrophoresis of rabbit uterine fluids and rabbit blood sera was carried out in barbitol buffer with a pH of 8.6 and an ionic strength of 0.10 at 1.5 to 2.0° C in an Aminco Portable Electrophoresis unit equipped with a cylindrical lens and rotating slit [a].

Ten ml of sample, adjusted to 1.5 to 2.0% biuret protein was placed in Visking cellulose tubing and dialyzed for 12 hours against each of two changes of barbitol buffer, each about 100 times the volume of the sample. The ionic strength of the dialyzed sample was compared to that of the buffer by measuring the conductivity of each prior to the electrophoretic analysis. The progress of the electrophoretic separation of proteins was recorded on Polaroid film (type 46 L) at 30 minute intervals after the first hour of electrophoresis.

The electrophoretic mobilities were calculated according to the procedure of Alberty (5). The relative protein concentrations were calculated by tracing the electrophoretic pattern on graph paper, cutting out the protein peaks and weighing these paper peaks (105).

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[a] available from American Instruments Co., Catalog # 5-8510

Paper Electrophoresis

Paper electrophoresis was carried out in a model R Spinco paper electrophoresis cell.

Eight Schleicher and Schuel 2043 mgl paper strips were placed in the cell. One liter of barbitol buffer with a pH of 8.6 and an ionic strength of 0.075, was added to the cell and the cell was then covered. After 30 min, 0.006, 0.008 or 0.010 ml of sample was applied to each paper strip with a Spinco sample applicator. The cell was then sealed. The electrophoresis was conducted at room temperature for approximately 16 hours with a current of 3 ma.

Upon completion of electrophoresis, the paper strips were removed, blotted and dried in a preheated oven at 110° C for 30 min.

When dry, the stips were placed in a staining rack which was immersed in methanol for 6 min and then into a 0.1% solution of bromphenol blue in methanol for 30 min. After staining, the strips were immersed in each of three rinse solutions of 5% aqueous glacial acetic acid for 6 min each. The strips were then dried at 110° C in a preheated oven for 15 min.

The dried paper strips were immediately placed in a tray which contained a small blotter moistened with 30%

aqueous ammonia hydroxide. The tray was then covered. After 15 to 30 min in the tray, the strips were removed individually and scanned on a model RB Analytrol equipped with two 500 mμ interference filters and B-5 cam. The results were recorded on graph paper.

Agar Gel Electrophoresis

Electrophoresis in agar gels utilizes the same principles of electrophoresis as free electrophoresis. The agar gel matrix is used to contain the buffer and sample instead of glass vessels as in free electrophoresis.

Eight and one-half grams of Agar agar no. 3 was dissolved in 1.0 liter of barbitol buffer (pH = 8.4, μ = 0.05) in an autoclave at 15 psi for 10 min. The hot liquid was then filtered through Whatman no. 1 filter paper in a Buchner funnel under negative pressure applied by a vacuum pump. The filtered agar was then poured into enamel trays on a level surface until the entire bottom of the tray was covered with agar. This agar was allowed to solidify in place at room temperature, to form a level surface. Glass slides (5 cm X 7.5 cm) were then placed on the solidified agar base and hot liquid agar was poured on top of them to a depth of 1.5 mm. This agar was then allowed to solidify

in place at room temperature. After solidification, the trays were covered with a glass lid which was lined with moistened cheese cloth to provide a water-saturated atmosphere, and stored at 5° C until used.

The glass slides were cut from the agar tray with a clean, sharp, thin blade. Eight-mm silts were cut in the agar 3.75 cm from either end of the long axis of the glass slide (Appendix Fig. 1.). The slits were placed 4 mm from each edge and 5 mm from each other so that 3 slits could be cut on one slide in the manner described. Small strips of Whatman no. 1 filter paper (7 mm X 25 mm) were introduced into the slits in order to remove excess buffer from the slit by capillary action (Appendix Fig. 1.). When the filter paper strip was nearly saturated, disposable 10λ capillary tubes were filled with 1% protein solution in barbitol buffer. The filter paper strips were removed and the 10λ of protein solution was immediately pipetted into the slit. After about 5 min, the slide was turned agar-side down and placed in the electrophoresis cell described by Wieme (110) (Appendix Fig. 2). The region surrounding the slide was filled with petroleum ether (bp 30° - 60° C) and the electrode troughs were filled with babitol buffer

(pH = 8.4, μ = 0.1). A potential of 150 volts was applied. Amperage was maintained at approximately 30 ma by cooling the cell by forcing a stream across the top of the cell and allowing the evaporation of petroleum ether to cool the slide. The petroleum ether always covered the slide during the electrophoretic trial which lasted 30 min. The slide was removed from the cell and placed into a fixing bath containing 5% acetic acid in 70% ethanol which fixed the proteins in the agar matrix.

The slides were dried by placing the agar-side down upon an 11 cm circle of Whatman no. 1 filter paper and allowing the water to evaporate from the filter paper overnight.

The dried slide was stained by immersing in a 0.1% solution of amido black in acetate buffer as described by Crowle (30). The slide was stained for 10 min and then cleared in 2% acetic acid until the agar background became clear. The slide was then rinsed in tap water for about 1 min to remove the acetic acid and then was air dried.

Immunoelectrophoresis

Immunoelectrophoresis is an electrophoresis system in which the separated protein components are located by immunological methods. The procedure is based on the prin-

ciples of electrophoretic separation of proteins and antigen-antibody precipitation.

Agar gel electrophoresis of the sample was carried out as previously described, and a trough was cut in the agar at a right angle to the sample slit. This trough extended to within 1/4 in of each end of the slide and was located approximately 1/4 in from the sample slit. The bottom of the trough was sealed by placing a drop or two of molten agar in the trough and allowing it to disperse and solidify evenly over the base of the trough.

The trough was filled with antisera which was allowed to diffuse from the trough at 5^{o} C for periods up to 3 weeks The trough was refilled when the level of antisera became low. The progress of the precipitin line formation was followed by photographing the electrophoresis plates with Kodak high contrast copy film.

RESULTS AND DISCUSSION

Collection of Uterine Fluid

After ligation of the uterus for periods of 1 to 20 weeks, varying amounts of fluids were aspirated from some uteri (Fig. 1 and 2). These fluids were colorless to slightly yellow or amber. A few deep red or brown secretions were found, but these samples were not representative and therefore were thought to be abnormal. It is felt that the deep color was due to the inclusion of blood in the fluids as a result of either ligation or previous collection procedures. The "normal" fluids were serous in consistency and ranged from clear to slightly turbid.

Cellular Content

Hemocytometer counts of uterine fluids were extremely variable because of the usually low number of cells in the fluids. When the pellets, formed after centrifugation, were smeared on slides and stained with the pyronin Y-methyl green stain, four prominent cell types could be found - Epithelial cells, lymphocytes, monocytes and neutrophils. These cells averaged 76%, 14%, 8% and 2% of the total cellular content, respectively. Red blood cells seldom

Fig. 1

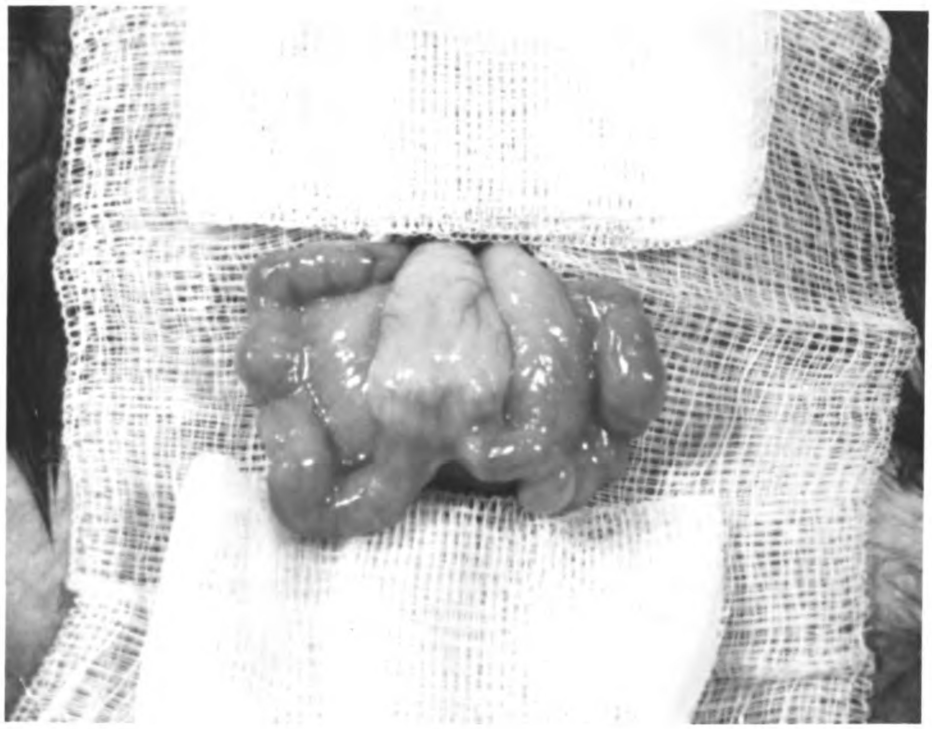
Normal Rabbit uteri *in vivo*

The vagina in the center of the figure gives rise to two separate cervices and uteri which may be seen on both sides of the vagina. Note the size of the uteri

Fig. 2

Ligated rabbit uteri *in vivo*

The anatomical configuration is the same as in Fig. 1. The uteri are filled with large amounts of clear fluid. These uteri were ligated for 8 weeks. Note the uterine vasculation.



accounted for more than 1% of the total cell content.

Volumes Collected

The volumes of fluid that accumulated during the ligation period are shown in Table 3. Many uteri did not accumulate visible quantities of fluid. Some of these, when re-ligated, did accumulate fluids. This evidence indicated that the first ligatures in these cases, were too loose. For this reason, only uteri which contained obvious amounts of fluid were included in these data. The increase in accumulated volumes appeared approximately proportional to the duration of ligation.

Table 3. Effect of ligation time on the volume of rabbit uterine fluid collected.

Ligation time	No. uteri	Volume of uterine fluid per uterus	
		Avg.	Std. error
(wk)		(ml)	
1	35	2.48	0.24
2	116	4.31	0.23
4	6	9.93	1.97
5	9	10.48	1.44
7	7	31.36	1.87
16	6	35.17	10.74
20	6	71.00	10.51

pH of Uterine Fluids

At the outset of this study, the pH of uterine fluids was measured after the samples had been stored at 5° C for 8 hours or more. The pH of these samples averaged 8.44 ± 0.03. When samples were withdrawn and immediately dispensed into a Beckman one drop electrode, the pH averaged 7.86 ± 0.03. When the intra-uterine pH was measured, an average pH of 7.65 was recorded, indicating that the uterine fluid was altered between the time it was aspirated and the time its pH was measured. When pH was measured in the syringe after a minimum of handling, the average pH was 7.62 ± 0.008. This increase in pH with time after collection was in agreement with results for rat's (19) and cow's (43) uterine fluids. This value corresponds closely to the intra-uterine pH. Both of these values differed consistently from the average pH of 7.3 measured in the fluid in the peritoneal cavity. Rabbit uterine fluids contained twice the bicarbonate of rabbit blood (108), which may explain its high pH. A loss of dissolved carbon dioxide with time and handling may cause the pH to rise to the higher values found in this study. The low standard errors of the higher pH values may be due to the standardization of experimental procedures.

Protein Concentration of Uterine Fluids

The protein concentration of rabbit uterine fluids declined significantly with ligation time. However, the increased volumes collected after longer ligation times caused the total protein content to increase markedly with ligation time. The relationships of protein concentration and total protein per uterus with ligation time may be seen in Table 4.

Table 4. Effect of ligation time on protein concentration and total protein of rabbit uterine fluids.

Ligation time	No. uteri	Protein Concentration		Total protein/uterus	
		Avg.	Std. error	Avg.	Std. error
(wk)		(mg/ml)		(mg)	
1	27	5.13	0.12	13.83	0.15
2	92	3.18	0.16	12.63	0.27
4	25	3.05	0.11	30.16	0.14
5	9	2.16	0.08	23.03	1.13
7	7	2.24	0.12	70.53	15.32
16	6	2.05	0.02	69.69	22.28
20	6	1.82	0.03	126.76	.6.85

More amber colored samples were noticed after 1 or 2 weeks ligation than after subsequent periods of ligation. Whether this amber color was normal or caused by the inclusion of blood was not known. Blood cells were not found in all of these amber colored samples, suggesting that the

amber color might have been a normal characteristic of these uterine fluids. On the other hand, the red cells could have been hemolyzed, causing the amber color. Introduction of some blood into the lumen of the uterus at the time of ligation may have been responsible for the higher protein concentrations for uterine fluid collected after 1 or 2 week ligation times. The data in Table 4 suggests that such an initial blood contamination may have been progressively diluted with accumulating uterine fluid.

Protein Components of Uterine Fluids

Agar Gel Double Diffusion

When the double diffusion technique described by Ouchterlony (83) was employed, protein antigens were found to be present in uterine fluids. The number of precipitin lines identified represented only the minimum number of antigens present, because the presence of identifiable antigen-antibody complexes depends on (1) the concentration of antigens and antibodies in the system, (2) separation of the precipitin lines for each of the protein components, (3) the solubility of the antigens, antibodies and their respective complexes in the buffer, and (4) the ability of the immunized animals to produce antibodies to

the test antigen (30).

In order to determine the concentration of antigen required to produce the maximum number of identifiable precipitin lines when diffused against its respective antibody, titer plates were made with various concentrations of antigen on a single plate. In both blood serum and uterine fluid titer plates, a 1.0% biuret protein concentration of antigen resulted in the maximum number of identifiable precipitin lines.

Antibodies to pooled rabbit uterine fluids collected after 1 or 2 weeks of ligation time reacted similarly with uterine fluids from all ligation times, as can be seen in Fig. 3. Thirteen continuous precipitin lines could be identified in uterine fluids for each ligation time. This indicated that fluids from all ligation times contained the same components as fluid from the uteri ligated for 1 week. It did not, however, mean that all proteins of fluids from uteri of the longer ligation periods were also present in fluids collected after only 1 week of ligation. To test this hypothesis, antibodies must be made to fluids collected after long periods of ligation and diffused against uterine fluids from all ligation times. This, however, was not

Included in the present research.

To test whether the uterine fluids contained antigens also found in blood, guinea pig anti rabbit blood serum was diffused against rabbit uterine fluid with the result that six precipitin lines formed (Fig. 4). Presumably these represent antigens that passed from blood into uterine fluid. In order to test if rabbit uterine fluids contained any protein components which were not also found in blood, antisera to uterine fluid was absorbed with rabbit blood serum by mixing one part of antisera to two parts of blood serum. In this way, antibodies to uterine fluid proteins alone would be available for diffusion and reaction, since all the antibodies to blood serum proteins would be precipitated before diffusion took place.

When such a test was performed, five lines developed against uterine fluids obtained after all ligation times. Two of these lines did become continuous with blood after prolonged diffusion time as seen in Fig 5. These results are similar to results in Fig 3 where 3 precipitin lines of uterine fluids were not continuous with blood serum. In a similar plate, which also contained rabbit peritoneal fluid, three antigens peculiar to uterine fluids were also

Fig. 3

Double diffusion plate no. 1

Center well is filled with antisera to uterine fluid. Starting clockwise from the right-hand well, the peripheral wells contain 1. uterine fluid after 1 week of ligation 2. uterine fluid after 4 weeks of ligation 3. uterine fluid after five weeks of ligation 4. uterine fluid after sixteen weeks of ligation 5. uterine fluid after twenty weeks of ligation 6. blood serum.

Fig. 4

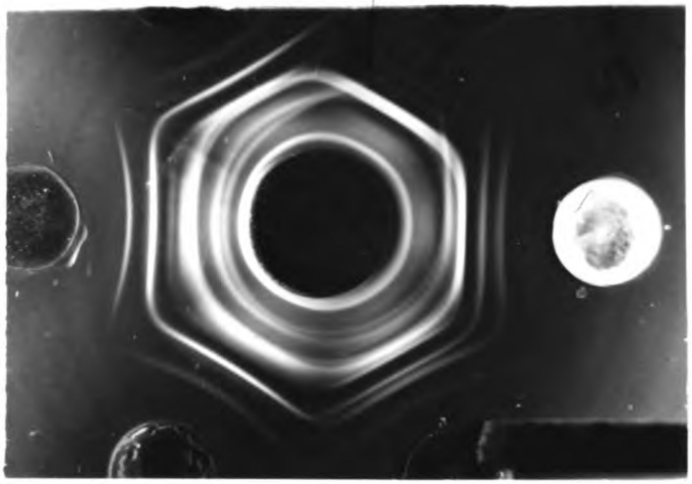
Double diffusion plate no. 2

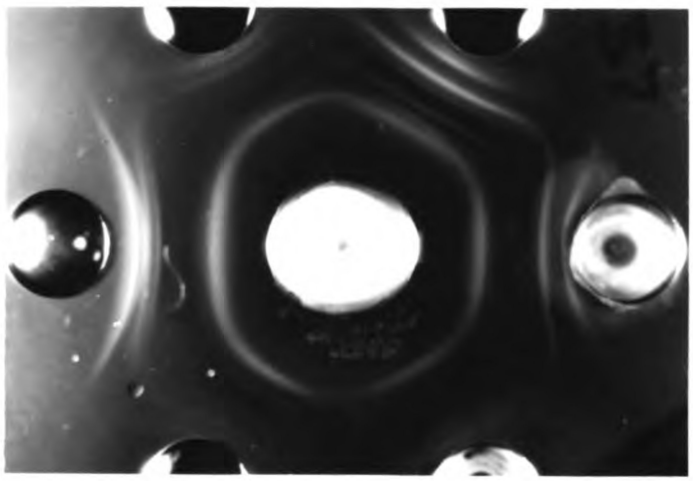
Center well is filled with antisera to blood serum. The peripheral wells are filled with antigen as described for figure no. 3.

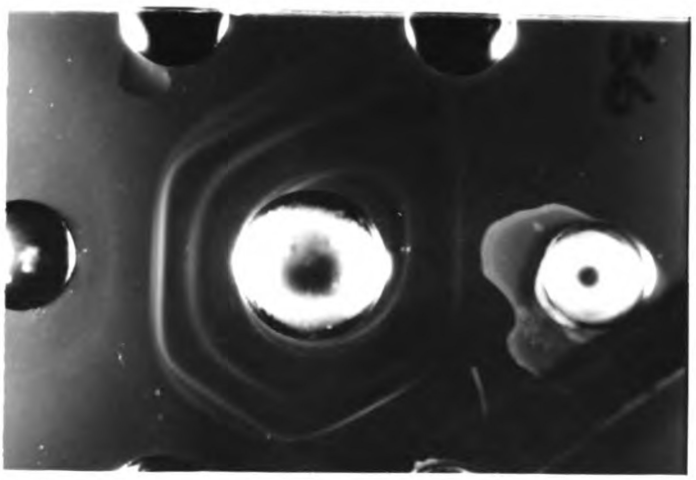
Fig. 5

Double diffusion plate no. 3

Center well is filled with antisera to uterine fluid which has been absorbed with blood sera The peripheral wells are filled with antigen as described for figure no. 3.







demonstrated. This seems to indicate that there are at least three protein components in rabbit uterine fluids that are not found in rabbit blood serum or peritoneal fluid. In rats, Albers and Neves e Castro (3) have reported that only one protein could be found in rat uterine fluid that was not also contained in rat blood serum.

The precipitation bands were not as sharp when uterine fluid proteins were reacted with antibodies to rabbit blood serum as were the precipitation bands formed when rabbit blood serum proteins reacted with antibodies to uterine fluids. It is possible that the uterine fluids collected after 1 or 2 weeks of ligation time may have contained some rabbit blood because of contamination of uterine fluid with blood due to ligation or collection procedures (see p 54). Antibodies produced against these contaminated uterine fluids would therefore also contain antibodies against blood. On the other hand, uterine fluids should not have contaminated the blood serum samples. This may explain the greater sharpness of precipitin lines formed between blood serum and antibodies to uterine fluid. Concentration differences in the protein components of course may have also contributed to this effect.

An attempt was made to test the similarity of uterine secretions and blood sera of rabbits, rats and guinea pigs by diffusing these unconcentrated fluids against antisera to rabbit uterine fluids and rabbit blood serum. Precipitin lines formed only against the rabbit blood serum and uterine fluids. This indicated that there was no immunological similarity between the proteins of the biological fluids tested from the different species. If these tests were carried out at optimum precipitation conditions, these results are surprising, since one would expect similar proteins in these fluids in view of the comparative similarity of the species. Perhaps greater protein concentration of the rat and guinea pig uterine fluids would reveal the antigenic similarity to rabbit uterine fluid that was expected.

Moving Boundary Electrophoresis

Typical moving boundary electrophoretic patterns of uterine fluid and blood serum are presented in Fig. 6, 7 and 8. One analysis was made for pooled uterine fluids collected after different ligation times except for the 1-2 week fluids of which 3 replicate analyses were made. The respective concentrations and mobilities of the components of

Fig. 6

Moving boundary electrophoresis
Pattern no. 1

Uterine fluid collected after one week
of ligation

Fig. 7

Moving boundary electrophoresis
Pattern no. 2

Uterine fluid collected after twenty weeks
of ligation

Fig. 8

Moving boundary electrophoresis
Pattern no. 3

Normal rabbit blood serum.

these fluids were very similar as can bee seen from Tables 5 and 6.

A maximum of eight protein components were found in uterine fluids while only seven could be found in blood serum. The electrophoretic patterns appeared to be similar for each of the uterine fluids collected after different ligation times except for the addition of a prealbumin peak, a protein peculiar to each uterine fluid pool obtained after 5 weeks of ligation

The mobilities for blood serum components listed in Table 5 are in good agreement with similar data reported by Moore (74) and Deutsch and Goodloe (32) for rabbit blood serum.

Two protein components of uterine fluids had mobilities which were unlike the mobilities of protein components in blood sera. These components had mobilities similar to a prealbumin and an alpha globulin. Their absence from blood serum electrophoretic patterns represented either an actual absence from the serum or a concentration too low to be detected by free electrophoresis. Thus, these results indicated that there were two protein components peculiar to uterine fluids.

Table 5. Electrophoretic mobilities of ascending protein components of uterine fluid and blood serum.

Sample	Ligation time	Electrophoretic component							
		1	2	3	4	5	6	7	8
Ut. flu.	1-2	1.6	2.9	3.5	4.5	5.1	5.7	6.9	——
		1.8	3.0	3.6	4.5	5.1	5.6	6.8	——
		1.8	3.0	3.8	4.8	——	5.8	7.4	——
Ut. flu.	5	2.3	3.3	4.0	4.8	——	5.8	7.3	——
Ut. flu.	7	2.1	3.0	3.9	——	5.2	5.8	6.8	7.8
Ut. flu.	16	1.5	2.7	3.5	4.2	4.8	5.8	6.6	7.8
Ut. flu.	20	——	3.2	3.8	4.9	——	6.1	7.0	7.9
Blood serum		1.5	2.3	3.5	4.3	5.2	——	6.9	——
		1.4	2.4	3.7	4.5	5.4	——	7.1	——
		1.6	2.9	3.6	4.9	5.2	——	6.8	——
					4.3				

Table 6. Average relative protein concentration of uterine fluids and blood serum (percent)

Sample	Ligation time	Electrophoretic component							
		1	2	3	4	5	6	7	8
Ut. flu.	1-2	3	1	4	6	6	11	68	—
Ut. flu.	5	3	5	11	5	—	14	67	—
Ut. flu.	7	3	4	6	—	11	13	59	9
Ut. flu.	16	5	6	2	2	3	17	56	9
Ut. flu.	20	—	3	6	13	—	13	58	7
Blood serum		8	3	8	4	—	9	68	—

Uterine fluids contained a high percentage of albumin as did rabbit blood sera. The relative concentrations seemed to be directly related to the electrophoretic mobility; that is, as the relative concentration decreased, so also did the mobility. In blood serum this was not the case, alpha, beta and gamma globulin components seemed to be present in about the same concentration. While the concentrations of the uterine fluid proteins appeared different from those of blood, the mobilities of these components appeared very similar.

Paper Electrophoresis

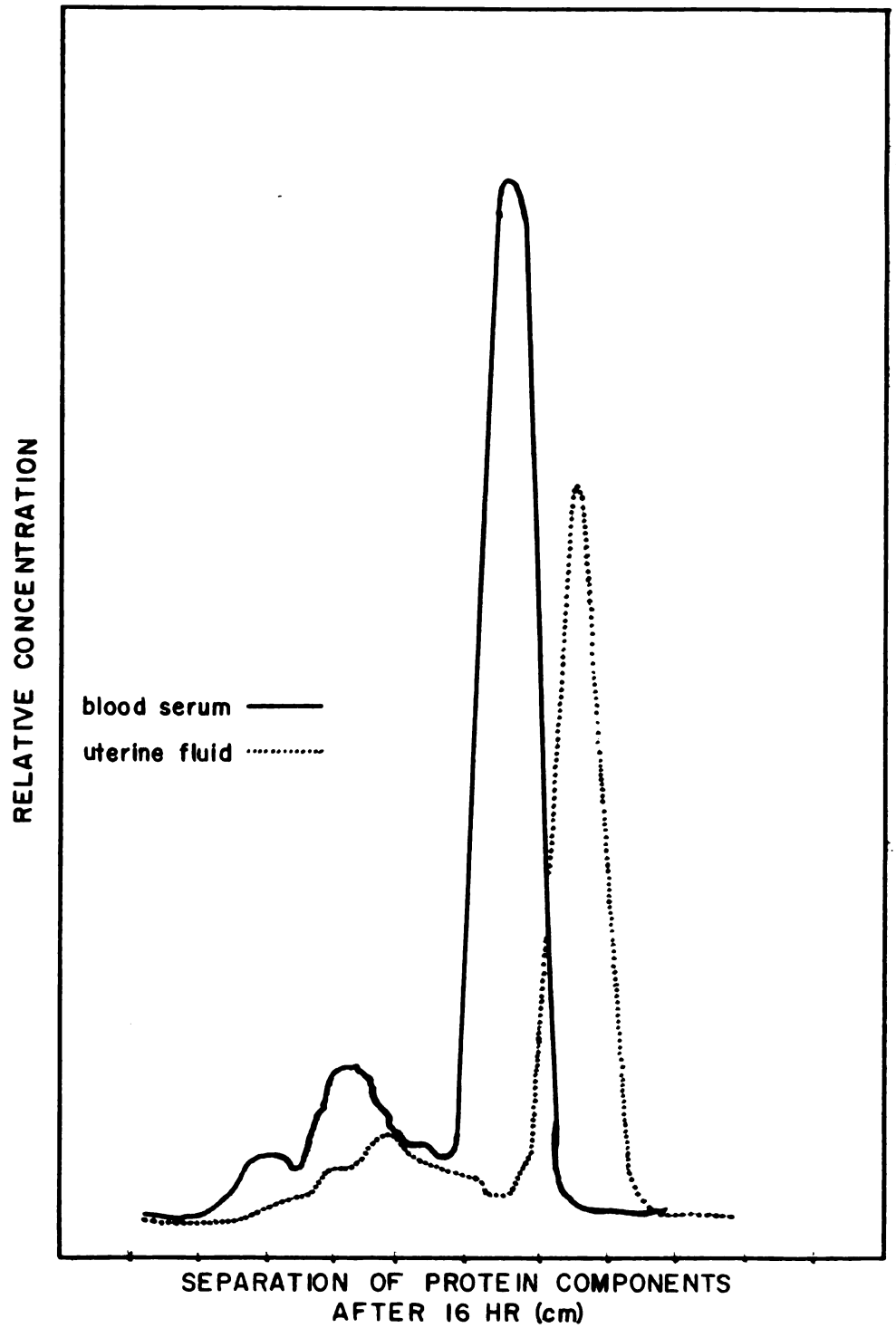
The paper electrophoretic separation of blood serum and uterine fluids revealed four protein components in each fluid. The relative concentration of the protein components were similar for each fluid. In ascending order of their mobility, blood serum components represented 4, 13, 4 and 79 percent of the total protein concentration, while uterine fluid components represented 7, 11, 5 and 77 percent of the total protein concentration.

The mobilities of each of the uterine fluid components was greater than the comparable mobilities for blood sera, as illustrated in Fig. 9.

Fig. 9

Paper electrophoretic pattern of blood serum and uterine fluid.

Typical electrophoretic patterns of blood sera and uterine fluid after 16 hours of electrophoresis at room temperature in a Spinco model R paper electrophoresis cell.


RELATIVE CONCENTRATION
blood serum
uterine fluid
SEPARATION OF PROTEIN COMPONENTS
AFTER 16 HR (cm)

There were no consistent analytrol peaks which revealed any protein components that were peculiar to uterine fluids.

Agar Gel Electrophoresis

Agar gel electrophoresis of uterine fluids obtained after the different ligation periods revealed the same number of uterine fluid components for each ligation period. Seven protein components were located in each of these fluids, while eight components were located in blood serum. The additional component of blood serum had a mobility similar to an alpha globulin. The mobilities of the blood and uterine fluid protein components were similar (Fig. 10).

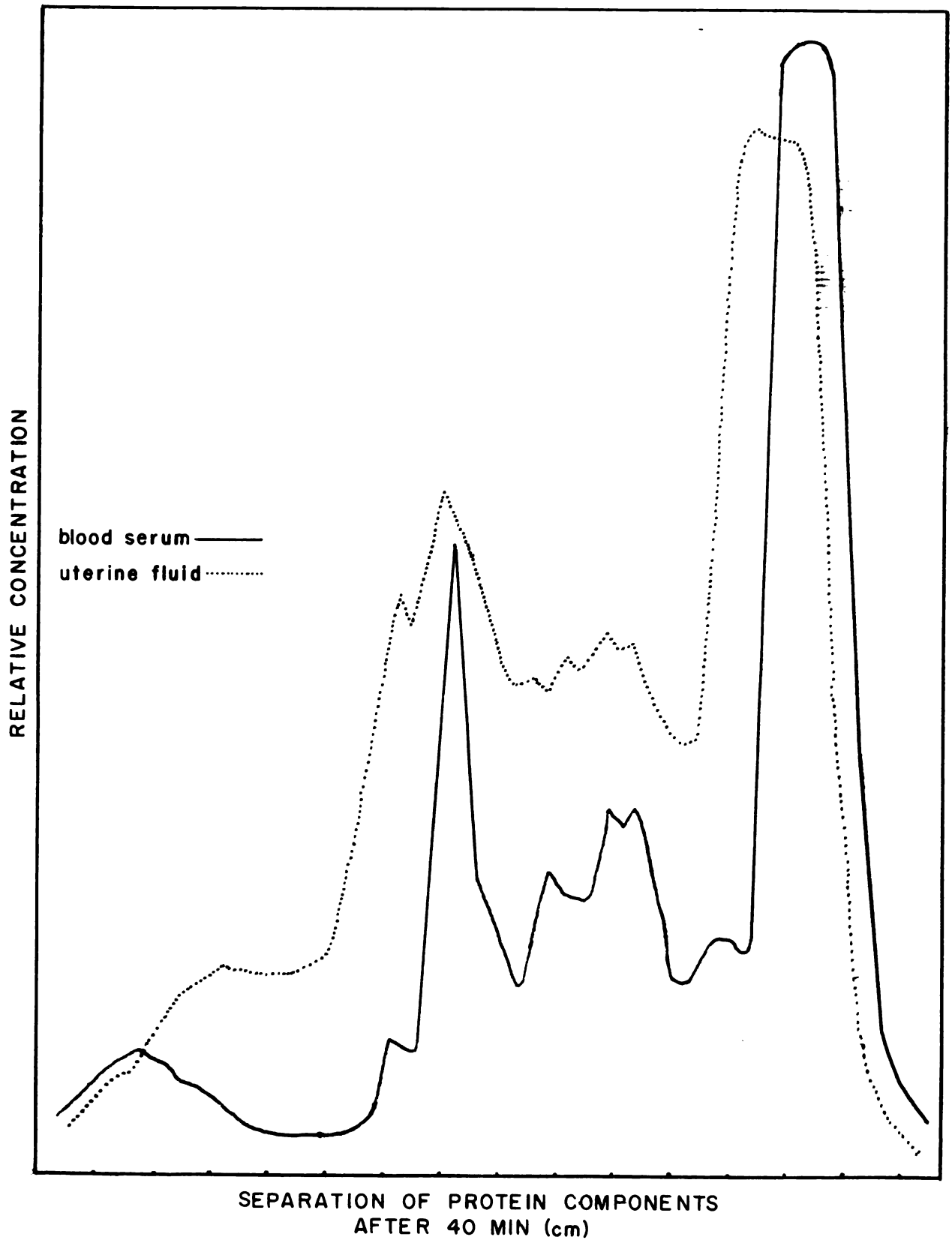
Uterine fluid protein components did not separate as sharply as did the blood serum proteins, and there appeared to be more "trailing" of the gamma globulin fraction of uterine fluid. These results seem to indicate a wider molecular weight or protein charge range for uterine fluid protein components.

Relative concentrations of protein components could not be determined by this procedure because of the different abilities of the protein components to take up the stain.

Fig. 10

Agar gel electrophoretic pattern of blood serum and uterine fluid

typical electrophoretic patterns of blood serum and uterine fluid after 40 min of separation at 150 v in the Wieme (110) agar gel electrophoresis unit. (Appendix Fig. 2)


RELATIVE CONCENTRATION
blood serum
uterine fluid
SEPARATION OF PROTEIN COMPONENTS
AFTER 40 MIN (cm)

Immunoelectrophoresis

Immunoelectrophoresis was carried out with uterine fluids collected after 4 or 5 weeks of ligation, since the immuno-diffusion data indicated the similarity of uterine fluids from all ligation times. Immunoelectrophoretic separations of proteins revealed 20 precipitin lines for uterine fluids and 15 for blood when diffused against their respective antisera as shown in Fig. 11. This figure also shows the presence of at least five precipitin lines in uterine fluids that are not also detected in blood serum, while the remaining lines appear to be similar to those of blood. Two of these components appear as prealbumin fractions and at least three remain near the origin thereby resembling beta globulins. To test this observation, antisera to uterine fluid was absorbed with blood serum before diffusing it against electrophoresed uterine fluid. Although five precipitin lines were expected, only two arcs were detected in uterine fluids that were not found in blood serum. One arc was detected in the prealbumin region and the other was found near the beta globulin region (Fig. 12). This supports the evidence presented in Fig. 11, although the components did not separate as completely as in the

former figure.

To further demonstrate the similarity of blood serum and uterine fluid, separated uterine fluid proteins were diffused against antisera to uterine fluid in one trough and antisera to blood serum in another trough as shown in Fig. 13. The same concentrations that were optimal for precipitation in double diffusion systems were used in these tests. Fig. 13 shows that antisera to blood reacts visibly with only two uterine fluid components. The difference in results between tests where uterine fluid was diffused against antisera to blood and where blood sera were diffused against antisera to uterine fluids is discussed for double diffusion tests (p 59), and should apply for these tests as well.

These data indicate that there was at least two protein components in uterine fluids that were not also found in blood serum (Fig. 12). More probably, there were as many as five components peculiar to uterine fluids (Fig. 11). Although figure 13 indicates that as many as 18 components may be peculiar to uterine fluids. However, the Ouchterlony data presented above, suggested that the anti-blood serum concentration in figure 13 was too low to effect maximal precipitation.

Fig. 11

Immunoelectrophoretic pattern no. 1

The pattern above the trough represents the reaction of uterine fluid with antisera to uterine fluid and antisera to blood sera. The lower pattern represents the reaction of rabbit blood serum with these same antisera. Anode is at left.

Fig. 12

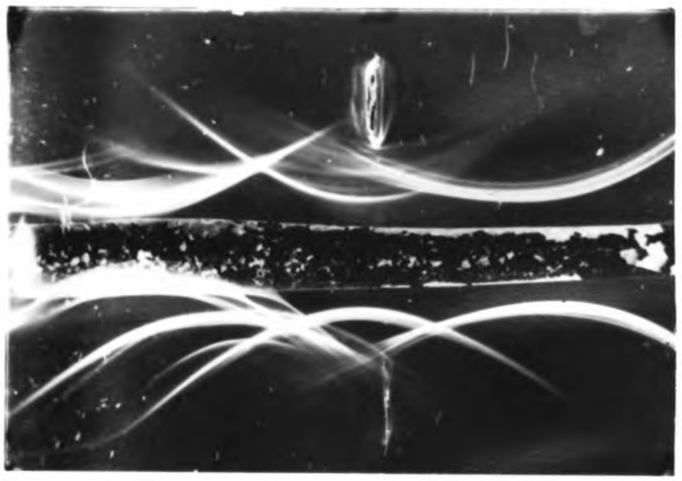
Immunoelectrophoretic pattern no. 2

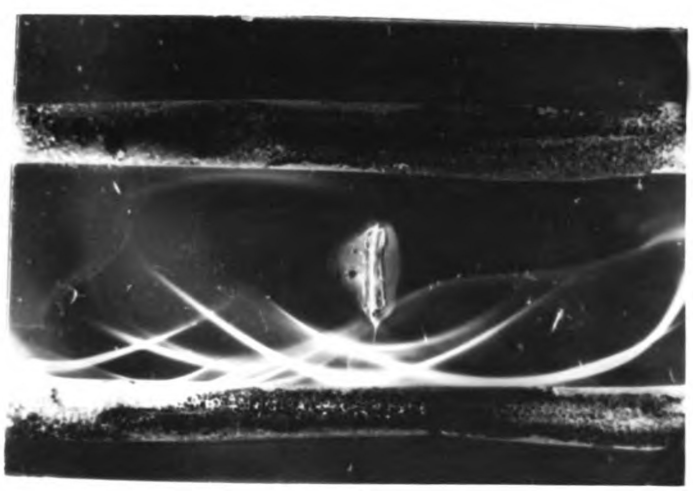
The pattern above the trough represents the reaction of uterine fluid with antisera to uterine fluid which has been absorbed with rabbit blood serum. Blood serum was electrophoresed in the region below the trough. Anode is at left.

Fig. 13

Immunoelectrophoretic pattern no. 3

Uterine fluid proteins were electrophoresed between the two troughs. The upper trough contains antisera to rabbit blood serum. The lower trough contains antisera to rabbit uterine fluids. Anode is at left.





The reported presence of prealbumin (56, 90) and beta globulin (3, 56) fractions specific for uterine fluids, is similar to results reported here. However, the data reported here indicated that rabbit uterine fluids contained two prealbumin fractions and at least three beta globulin fractions instead of a single component in each of the two protein classes as reported for rats.

SUMMARY AND CONCLUSIONS

A serous, colorless to slightly yellow fluid accumulated in uteri ligated for all ligation intervalls. The pH of this fluid averaged 7.62 at the time of collection. The average pH increased to 7.86 with time and handling after collection. The average pH of fluids stored at 5^{o} C for 8 hours or more was 8.44.

The volume of rabbit uterine fluids collected, averaged 2.48 ml after 1 week of ligation. The average volume increased with ligation time to 71.00 ml after 20 weeks of ligation.

The average protein concentration decreased from 5.13 mg/ml after 1 week to 1.82 mg/ml after 20 weeks of ligation. There is some doubt that the decrease in protein concentration was due to ligation per se. It is more probable that the decreasing protein concentration represents a dilution of blood serum contaminants in the uterine fluids, which were introduced at the time of ligation or previous collection. In spite of the decreasing protein concentrations, the total protein content increased with ligation time because of the high volumes which accumulated during the longer ligation times.

Although some of the results of the analyses made on protein components of uterine fluids appear to be contradictory, certain inferences and conclusions may be drawn.

Double diffusion tests demonstrated the presence of 13 components in uterine fluid, 10 of which were also found in blood serum. Immunoelectrophoresis expanded the protein spectrum by electrophoretic as well as diffusion means to reveal 20 uterine fluid proteins, 15 of which were also found in blood serum, leaving at least 5 protein components which were found only in uterine secretions. Two of these components appeared as prealbumins and three appeared as beta globulins.

Moving boundary electrophoresis also detected a prealbumin fraction in fluids from uteri ligated more than 5 weeks, while the immunoelectrophoresis revealed the prealbumin fraction in fluids collected after all ligation times. Thus, it appeared that the prealbumin fraction was found in all uterine fluids, but that its concentration increased with ligation time.

Rat uterine fluids have revealed prealbumin fractions that have not been found in rat blood sera. Ringer (90) suggested that blood albumins are altered in some way as

they pass through the uterine membranes into the uterine lumen. Exopeptidases have been found in human and rabbit uteri which are capable of altering these proteins (101).

Peptidases have also been found in uterine fluids of the rabbit and rat (4). These enzymes may have altered the uterine fluid proteins during the long ligation periods of the present study, since the prealbumin fraction only appeared in free electrophoretic patterns of fluids collected after 5 weeks of ligation. The paper electrophoretic mobilities of uterine fluids were consistently greater than those of blood thereby supporting this contention. On the other hand, agar gel electrophoresis does not indicate this trend. The molecular seiving effect may have prevented this trend from manifesting itself.

The alpha globulin fraction from the Tiselius electrophoretic analysis of uterine fluids, which appeared to be peculiar to uterine fluids, could not be located by immunoelectrophoretic procedures. This suggested that this uterine fluid protein had an antigenic analogue in blood which differed electrophoretically. The immunochemical similarity of this protein in uterine fluid and blood suggests that this uterine fluid protein was of blood

serum origin. If this was, in fact, the case, then we may assume that its electrophoretic mobility was altered as it passed into the lumen of the uterus. This hypothesis parallels Ringler's (90) suggestion concerning the alteration of blood serum albumins to form uterine fluid prealbumins, as outlined above.

Junge and Blandau (56) and Albers and Neves e Castro (3) have demonstrated the presence of a beta globulin in rat uterine fluids which was not found in rat blood sera. The immunochemical tests performed in this study suggested that in rabbit uterine fluids, there were three beta globulins which were peculiar to the uterine fluids.

These data suggest, as has already been stated (90), that "...luminal fluid is an ultrafiltrate of plasma supplemented by secretions from the uterus."

The consistent presence of the proteins peculiar to uterine fluids and the persistent absence of these proteins from peritoneal fluid and blood serum may indicate some biological value. These proteins may have an important, even necessary role in reproduction. Sperm must reside in the uterine milieu, and hence with these proteins, before fertilizing an egg. It is tempting to speculate that these

proteins may affect sperm motility, viability, capacitation or some related phenomenon of which we may be unaware at present. They may conceivably act as enzymes or hormones which affect sperm so as to help insure their proper reproductive potential.

The present studies, however, merely serve as a base from which hypotheses, such as these, may be investigated.

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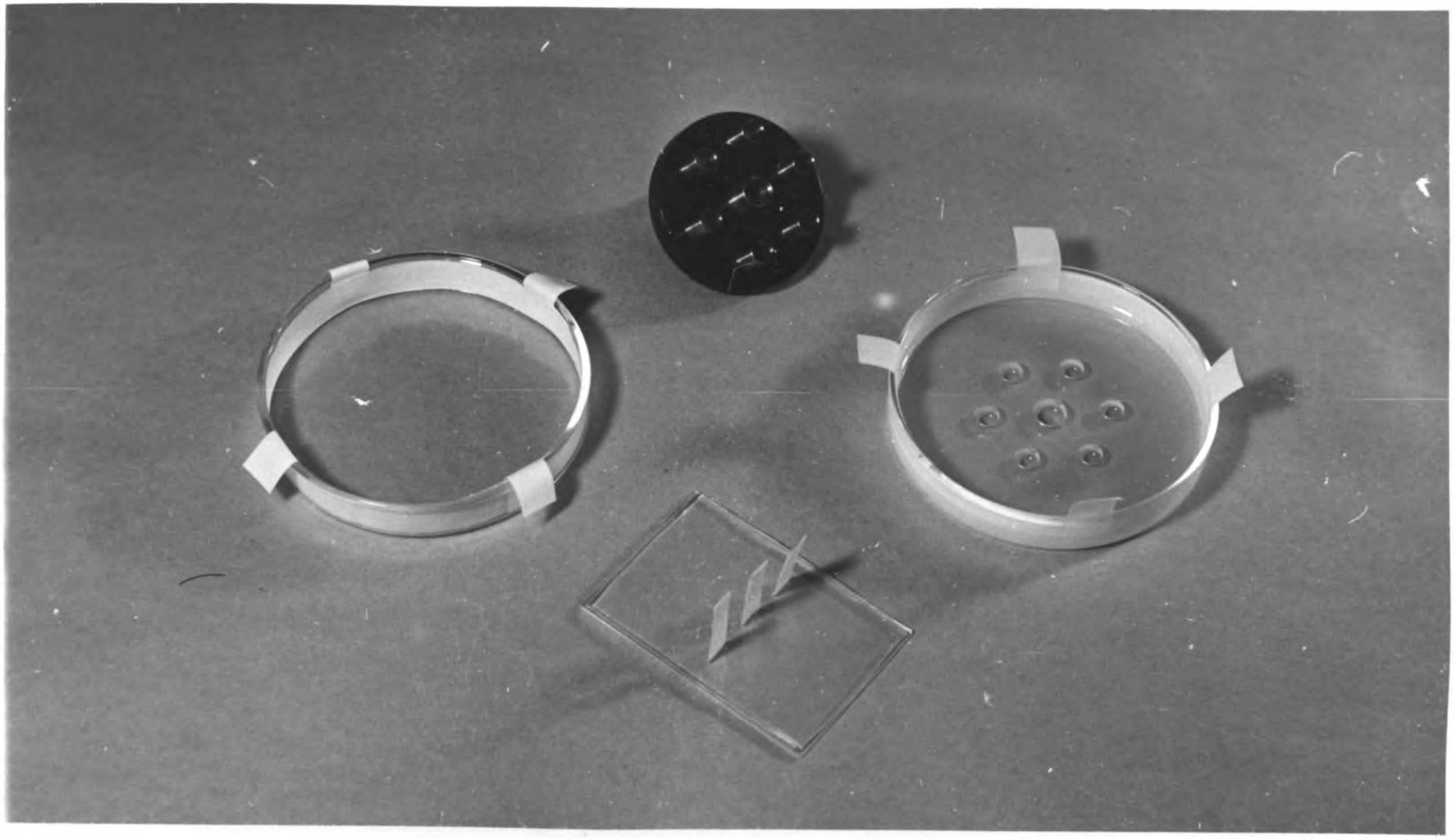
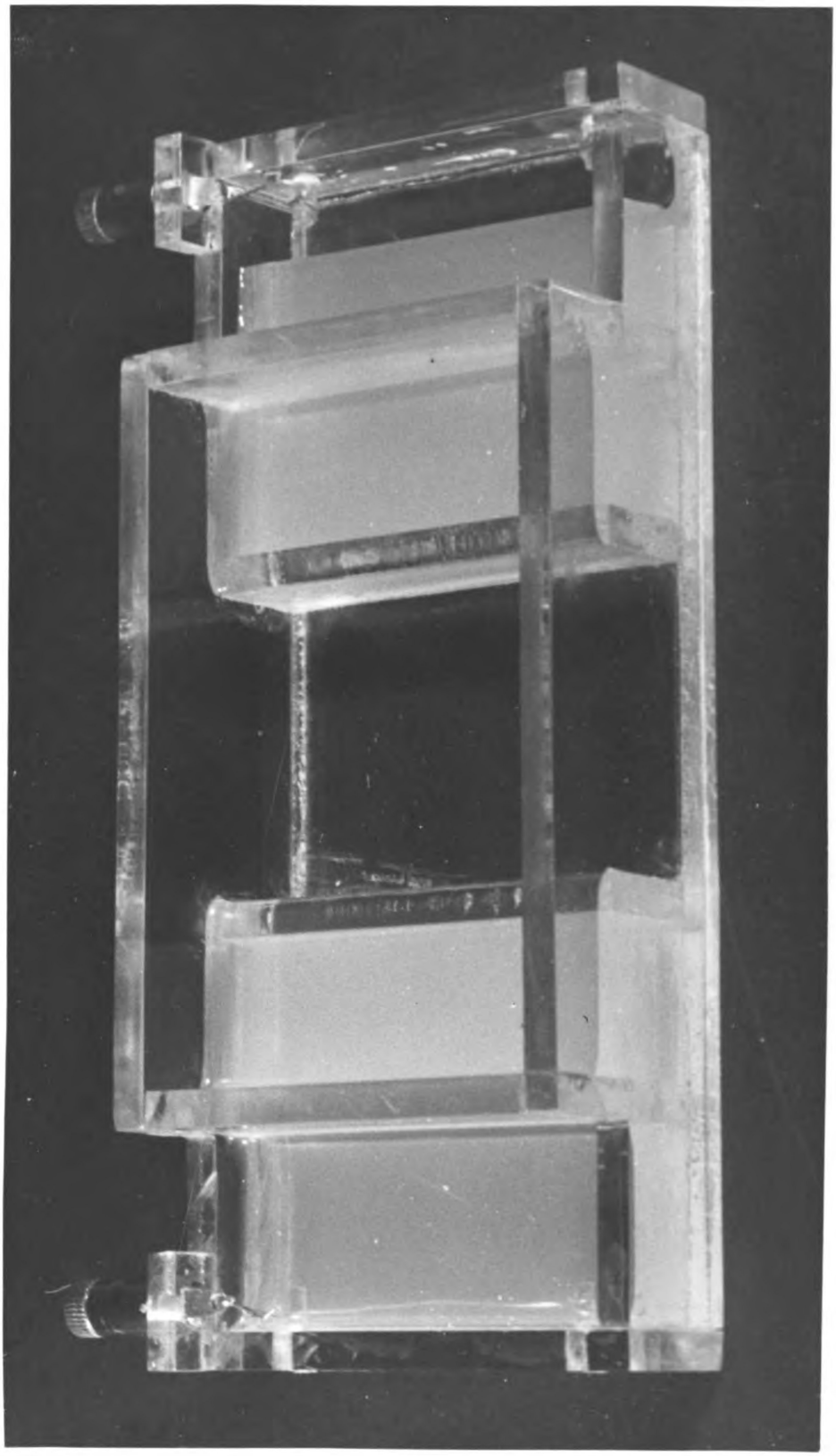
Appendix Fig. 1

This figure illustrates the arrangement of the filter paper strips in the base of the petri dish (left) prior to pouring the agar and cutting the wells (right) with the Feinberg cutter (top). An agar gel electrophoresis slide is pictured at the bottom of the figure. The filter paper strips, which remove water from the slits, are in place. See text for explanation.



Appendix Fig. 2

The agar gel electrophoresis unit of Wieme (110) is illustrated in this Fig. Both sides of the cell are filled with agar and allowed to solidify. The electrodes are then freed of agar (see right electrode). Electrophoresis slide is placed agar side down between the agar shelves in the center of the cell. Area surrounding slide is filled with petroleum ether to cool the slide.



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