PAPER-ELECTROPHORETIC STUDIES ON THE PROTEIN FRACTIONS OF BOVINE SERUM COLLECTED AT REGULAR INTERVALS

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Since the paper-electrophoretic method was devised by WIELAND and FISCHER (1948), many workers have undertaken a number of supplemental studies. Improvements in this method have been proposed and discussed for appraisal.[9] Though the paper-electrophoretic method is inferior in accuracy to Tiselius' electrophoretic method, it has been widely applied in biology, chemistry, medicine and many other fields, because of its several excellent points: simpler construction, easier technique, smaller amount of samples, etc.[15] Especially, it has been used by clinicians as one of the important helpful methods for diagnosing many kinds of diseases. Moreover, it has been recently applied for purification or extraction of samples and also for study of the antigen-antibody reaction.

In veterinary medicine, it is used for studying serum protein, milk or other body fluids of healthy and diseased animals, and it is also applicable for clinical diagnosis of various diseases.[6,21] On the other hand, little information is available on the physiological range in percentages of the protein fractions of bovine serum,[2,4,7,10,18,20] especially on their daily variations along with the sexual cycle.

CHOPARD and other workers investigated the variation of the bovine serum protein during pregnancy by means of the paper-electrophoretic method; they reported an increase in the total protein contents. However, no information is available for a continuous observation of the protein fractions during the non-pregnant period. On the other hand, MOBERG in the Swedish Red and White Breed (SRB) and the Swedish Highland Breed (SKB) and TASHIRO in the Japanese Black Breed noted independently that the blood cell components varied with the sexual cycle. It is interesting to make a study on whether the serum protein fractions show similar cyclic changes as do the blood cells. For this purpose the present author has made a paper-electrophoretic study on the variation of serum protein obtained from 3 non-pregnant cows at regular intervals during 2 months.

Materials and Methods

1 Materials

Sera used in this experiment were collected every 3 days from the 3 cows, as shown in table 1, and from 1 cow on the 45th day post partum as control. The outlines of the clinical histories and the findings at autopsy of these 3 cows are as follows:

No. 1 During 8 months from the last parturition to the beginning of the experiment, she showed estrus only 3 times, failing conception with 3 times of copulation. No abortion could be observed in this period. From the histological points of view, the uterus was normal as examined by uterine biopsy, but the right fallopian tube was impassable on both the premortem and the post-mortem tuboinsufflation test.

No. 2 During 2 years after the last calving, this cow failed in conception with 13 times of coitus or artificial insemination. According to her anamnesis she had 13 times of estrus and no observable abortion. At the beginning of the experiment, she was diagnosed by biopsy as having chronic catarrhal endometritis. But she got well without any treatment by the end of the experiment. On the premortem tuboinsufflation test, both oviducts were impassable, while on the post-mortem test, only the right tube was found to be impassable. At autopsy, a yellowish-brown colored, string-like, dry solid was found in the uterus. It appeared to be a degenerated fetal membrane.

No. 3 As in the case of No. 2, 13 times of insemination were carried out during 20 months without conception or abortion. In the early stage of the experiment she showed only a slight sign of endometrial catarrh, but she was clearly suffering from chronic endometritis at several days before slaughter. In the tuboinsufflation test carried out at the early stage of the experiment, the left oviduct was found to be impassable, but both oviducts became again passable at the later stage, which was being accordant with the results of the post-mortem tuboinsufflation test.

During the whole course of the experiment, from June 28 to August 15, 1957, 20 ml of blood was taken aseptically from the jugular vein of each cow every 3 days before feeding (between 15.00~16.00).

Sera taken from the same cow on the same day were divided into 2 parts: one part was frozen and the other was lyophilised. The ampoules containing each 0.5 ml of material used were kept in the refrigerator at −30°C for 193 to 219 days, except control sera (No. 4) which was kept for 8 to 28 days.

2. Apparatus

Kobayashi's paper-electrophoretic apparatus (type A*) was used in this experiment.[11] As shown in figs. 1 and 2, filter papers (D_1 & D_2) of uniform size and quality were placed serially at the horizontal level.

3. Methods

1) Electrophoretic methods: For the paper-electrophoresis the frozen sera were melted at room temperature To the lyophilised sera was added some quantity of distilled water to cause them to regain their volumes before lyophilisation. Each 0.005 ml of the samples

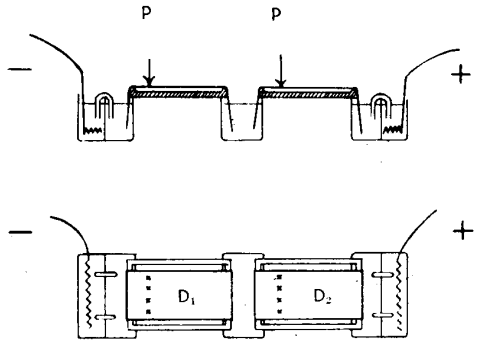
* This apparatus was manifactured by the Natume Co., Tokyo.

TABLE 1. *Experimental Cows*

COW NO.	BREED	BIRTH OF DATE	NUMBER OF PARTURITIONS	LAST PARTURITION	TIME OF TAKING SAMPLE	POST-MORTEM TUBOINSUFFLATION TEST	
						Pressure (mm Hg)	Patency
1	Holstein	1 Feb. 1946	8*	1 Oct. 1956	2-days' interval	right 120	−
						left 120～40	+
2	Holstein	2 Mar. 1942	10	16 Jun. 1955	〃	right 160	−
						left 60～30	+
3	Guernsey	3 Apr. 1945	6	11 Oct. 1955	〃	right 120～20	+
						left 140～20	+
4**	Holstein	18 Mar. 1947	6	14 Nov. 1957	45th day post partum, before reappearance of estrus		

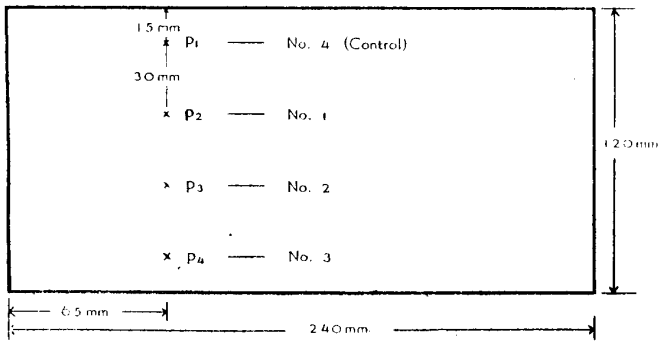
Notes: * including 4 abortions.
** control cow.

FIGURE 1. *Apparatus for Electrophoresis*



Notes: P: Position of dropping of material.
D_1 & D_2: Filter papers.

FIGURE 2. *Position of Spotting Point*



was dropped on the filter papers by a micro-pipette, in the manner shown in fig. 2. The samples taken from the three cows (Nos. 1, 2, and 3) on the same day were electrophoresed at the same time with the control material (No. 4). The frozen sera were electrophoresed in the morning (7.00～12.00) and the lyophilised ones in the afternoon (12.30～18.15).

2) Conditions: The experiments were carried out under the following conditions: amperage: 10 mA, filter paper: Toyo filter paper No. 51,* time of electrophoresis: 5 hours, buffer: veronal/acetate buffer (pH 8.5) with the composition shown in table 2.

3) Treatments after electrophoresis: The filter papers were dried with a gas flame, immediately after electrophoresis, stained with 0.05 per cent blomphenol-blue solution for 20 minutes, and then decolorized with 1 per cent acetic acid solution for 20 minutes. After being dried in air for about 24 hours, they were exposed to ammonia gas for a moment to develop color of blomphenol-blue, and then fixed with paraffin. By using Kobayashi's filter paper electron densitometer (type III), the density of the papers was measured.

TABLE 2. *Composition of Buffer Solution used*

Sodium 5, 5-diethylbarbiturate	8.82 g
Sodium acetate	4.68 g
N/10 acidum aceticum	80.8 ml
With aqua destillata up to 2 l	(pH 8.5 $\mu=0.045$)

4) Statistical analysis and correction of the data observed: Results of measurement were graphed on a section paper. Three perpendicular lines were drawn from the 3 main lowest valleys to the abscissa of this pattern, which was divided into 4 sections, corresponding to the main fractions of serum protein: albumin, α-, β- and γ-globulin. The areas of each section were measured by using a planimeter and the results were represented in percentage.

In order to determine whether any difference in the patterns exists in repeated trials with the same material, the electrophoresis was repeated 5 times with the serum of the

* The quality of "Toyo filter paper No. 51" is similar to that of Whatman paper No. 1.

control animal (No. 4). The results obtained from this preliminary experiment were statistically analysed.

Before the statistical analysis, correcting rates due to the spotting positions of P_2, P_3, P_4 to P_1 on the filter papers of D_1 and D_2 were respectively calculated. At the same time, a correcting rate due to arrangement of the papers of D_1 and D_2 were also calculated. On the other hand, the other correcting rates relating to the day of experiment were calculated from the averages of the each fraction of the control animal No. 4. By applying these 3 series of correcting rates, each datum of Nos. 1, 2 and 3 was corrected.

5) Analysis for variation of the serum protein fractions as *cyclus extragenitalis*: The period of one estrous cycle was divided equally into the following 5 stages: the estrus, 1st, 2nd, 3rd and 4th stage, respectively. Total protein value of each frozen sera collected at the 5 various stages was measured by the micro-Kjeldahl method and analysed statistically by the two-way lay-out method. Similarly, in order to clarify whether the serum protein fraction value varies in the estrous cycle, in other words whether it shows *cyclus extragenitalis*, the serum protein fraction values obtained by the paper-electrophoresis corresponding to the 5 stages were analysed by the repeated two-way lay-out, three-way lay-out and four-way lay-out methods.

RESULTS

1) The results of the preliminary experiment on the control animal No. 4 are shown in tables 3 and 4. These data were analysed statistically by the three-way lay-out method.

TABLE 3. *Protein Fractions: Control · Frozen*

		P_1				P_2				P_3				P_4			
		Alb.	Glob.			Alb.	Glob.			Alb.	Glob.			Alb.	Glob.		
			α	β	γ		α	β	γ		α	β	γ		α	β	γ
		%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
1	D_1	44.1	21.1	6.5	28.2	36.5	18.4	10.2	34.9	38.0	16.9	9.1	36.2	40.8	19.9	6.9	32.4
	D_2	47.3	20.5	7.1	25.1	40.2	18.1	8.7	33.0	38.6	18.6	8.9	33.9	44.6	20.1	6.6	28.7
2	D_1	38.3	17.7	8.8	35.1	41.4	16.9	8.2	33.5	40.6	18.2	7.9	33.2	46.0	16.6	7.5	29.9
	D_2	41.4	21.4	8.1	29.2	40.4	19.7	7.6	32.4	37.5	25.0	8.3	29.2	43.9	20.0	7.9	28.2
3	D_1	41.8	18.9	8.0	31.4	40.0	20.7	7.6	31.7	40.8	21.2	9.3	28.7	37.2	20.8	7.0	35.1
	D_2	39.0	18.5	9.0	33.6	38.9	20.3	8.3	32.5	42.3	19.9	8.6	29.3	38.3	15.1	9.4	37.1
4	D_1	35.2	21.8	10.1	32.8	32.6	18.1	9.8	39.5	36.3	21.2	8.7	33.8	36.3	20.2	8.6	34.9
	D_2	34.7	19.4	9.0	36.8	36.4	19.9	7.9	35.8	30.7	20.7	10.4	38.2	38.8	20.1	8.4	32.7
5	D_1	39.2	19.7	10.9	30.3	37.6	21.7	9.1	31.7	36.7	18.5	10.7	34.2	38.0	19.6	8.2	34.3
	D_2	37.5	19.4	10.0	33.1	37.9	21.1	9.6	31.4	37.3	20.6	9.5	32.7	36.0	20.7	12.2	31.2

As shown in table 5, differences due to the repeated trials (R) on albumin, β- and γ-globulin fractions on the frozen materials were significant. As for the γ-globulin, differences due to spotting point (P) of material were also significant. On the other hand, in the lyophilised materials, significant differences due to the arrangement of the filter papers (D) and due

TABLE 4. *Protein Fractions: Control · Lyophilised*

		P_1				P_2				P_3				P_4			
		Alb.	Glob.			Alb.	Glob.			Alb.	Glob.			Alb.	Glob.		
			α	β	γ		α	β	γ		α	β	γ		α	β	γ
		%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
1	D_1	35.2	18.1	9.6	37.1	40.7	16.0	9.2	34.1	40.6	16.1	6.9	36.5	41.6	17.3	9.1	32.1
	D_2	40.8	19.2	9.1	30.9	41.2	19.8	7.9	31.0	41.4	18.5	9.2	30.9	39.7	17.0	8.2	35.1
2	D_1	41.5	16.9	9.6	32.0	38.8	19.5	7.7	33.9	41.5	15.6	8.7	34.2	45.6	20.0	8.4	26.0
	D_2	44.2	18.6	6.6	30.6	42.4	17.8	9.7	30.1	36.9	17.6	9.3	36.2	38.2	20.0	8.9	32.9
3	D_1	37.7	19.6	8.8	33.9	38.3	19.8	8.2	33.7	34.9	20.8	9.0	35.3	38.9	20.3	9.3	31.6
	D_2	40.8	19.0	6.9	33.3	38.7	18.4	9.4	33.4	36.5	21.5	7.7	34.2	39.6	18.7	7.9	33.9
4	D_1	44.0	18.7	6.7	30.7	40.1	15.8	8.2	35.9	36.9	21.2	9.3	32.6	38.6	19.1	10.0	32.4
	D_2	42.5	17.0	7.4	33.1	40.3	18.6	8.5	32.6	39.5	20.1	8.2	32.2	42.3	16.4	9.9	31.4
5	D_1	40.2	18.0	5.6	36.3	43.1	17.2	6.4	33.3	40.8	16.5	8.5	34.2	34.3	19.7	9.8	36.2
	D_2	37.9	22.5	8.7	30.9	36.6	23.7	8.0	31.7	33.0	24.3	9.2	33.4	35.2	23.2	6.8	34.8

TABLE 5. *Results of Statistical Analysis: Control*

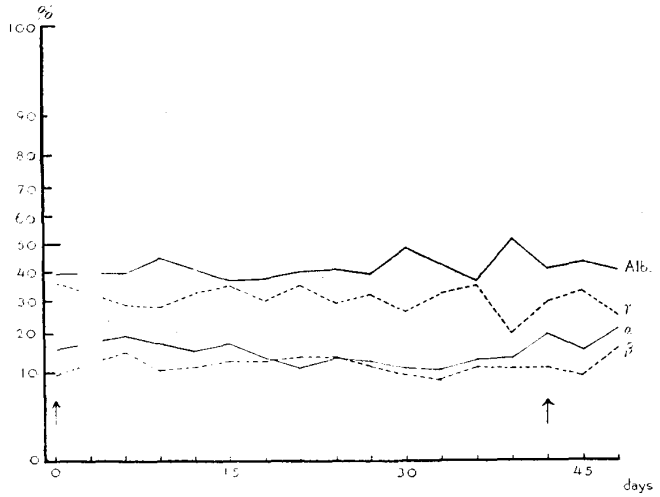
			MEAN SQUARES							
			D.F.	D	D.F.	P	D.F.	R	D.F.	Error
Frozen	Alb.			14898.40		34032.13		192716.98**		11558.38
	Glob.	α	1	16321.60	3	5863.83	4	9470.46	12	12948.91
		β		15633.10		14464.37		59340.28**		10350.36
		γ		28037.03*		29829.43**		62913.84**		4771.74
Lyophilised	Alb.			28394.28		33594.09		64376.35		24906.27
	Glob.	α	1	86304.10**	3	4042.27	4	59476.69**	12	6915.70
		β		409.60		18452.60		9414.84		19261.08
		γ		33062.50		13378.17		18571.60		14422.65

Notes: D: Difference due to the arrangement of the filter papers.
P: Difference due to the position of spotting points.
R: Difference due to the repeated trials.
D.F.: Degree of freedom.
*: $p<0.05$. **: $p<0.01$.

to the repeated trials (R) were found in α-globulin fraction ($p<0.01$). However, there was observed no correlation among these 3 factors (D, P & R).

2) The results of the electrophoresis in both frozen and lyophilised materials of the animals Nos. 1, 2 and 3 are graphed in figs. 3~14, in which no regularity was observed. In some instances there are even inconsistent results obtained from the 2 filter papers (D_1 & D_2) electrophoresed with the same materials. In addition, a statistical analysis made

FIGURE 3. *Changes in Each Protein Fraction No. 1 Frozen D_1*



Note: ↑ shows estrus.

FIGURE 4. *Changes in Each Protein Fraction No. 1 Frozen D_2*

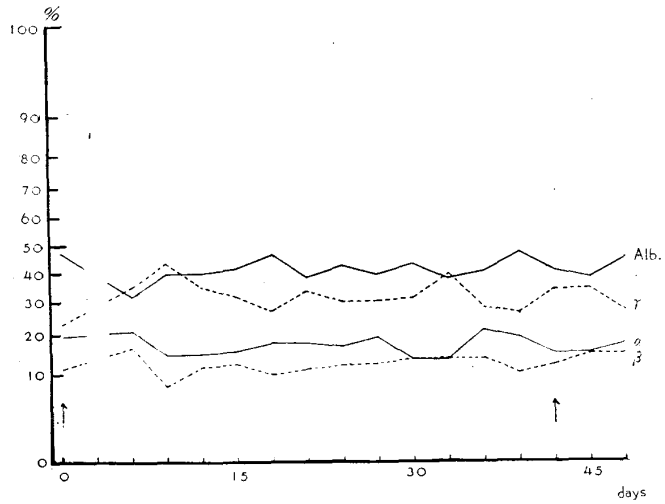


FIGURE 5. *Changes in Each Protein Fraction No. 2 Frozen D_1*

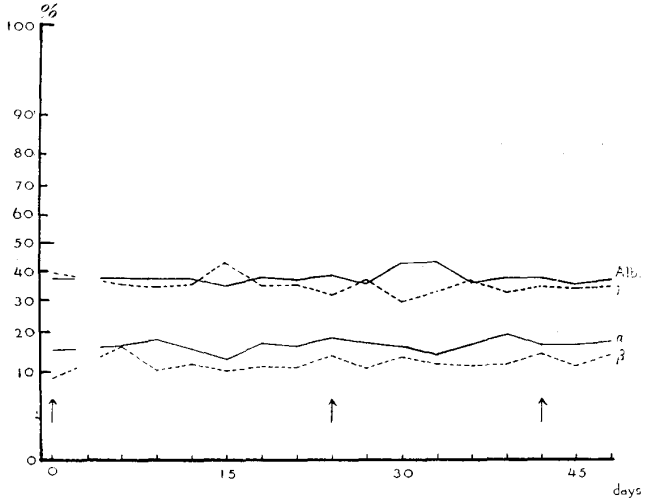


FIGURE 6. *Changes in Each Protein Fraction No. 2 Frozen D_2*

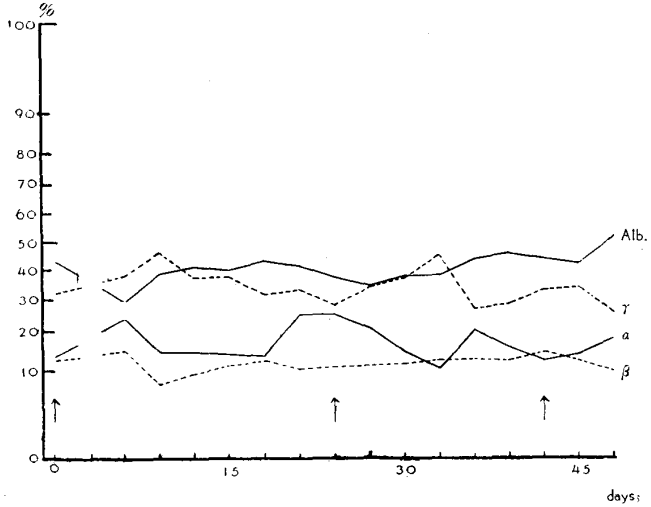


FIGURE 7. *Changes in Each Protein Fraction No. 3 Frozen D_1*

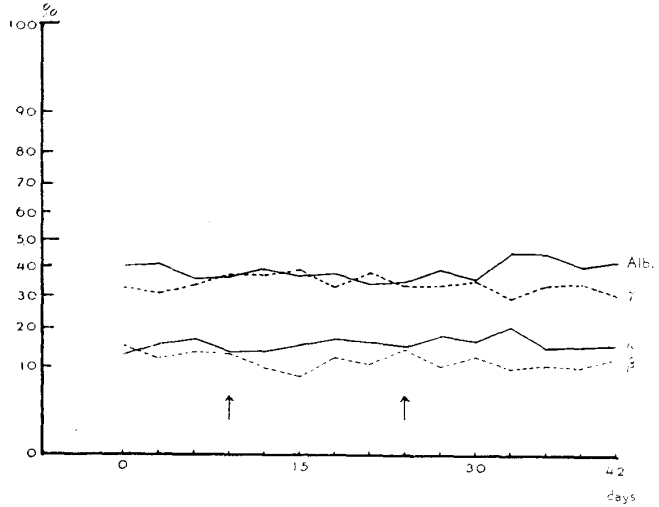


FIGURE 8. *Changes in Each Protein Fraction No. 3 Frozen D_2*

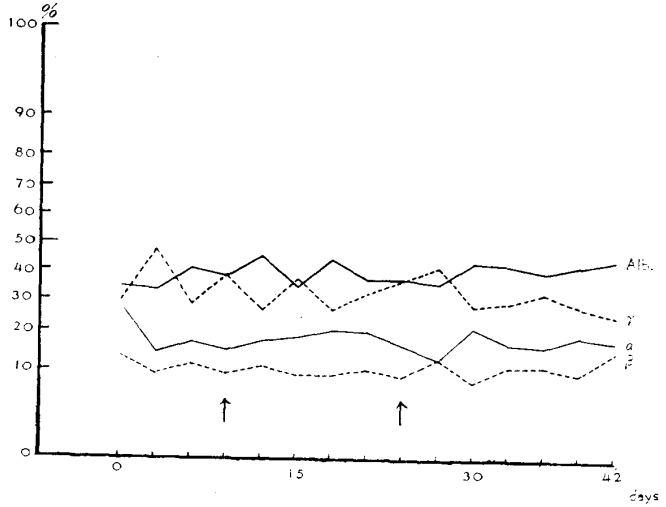


FIGURE 9. *Changes in Each Protein Fraction No. 1 Lyophilised D_1*

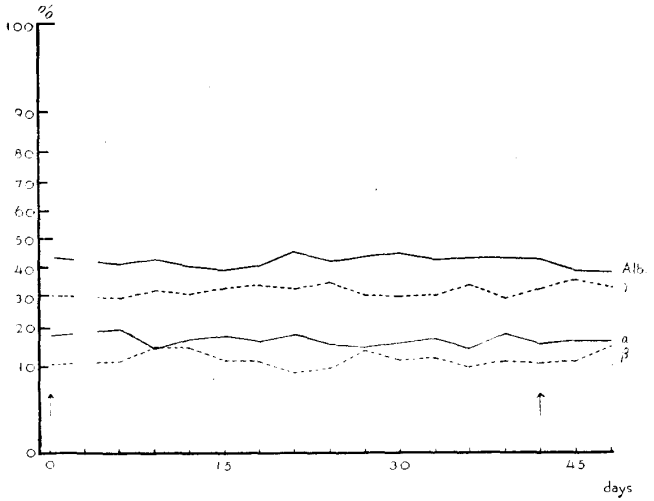


FIGURE 10. *Changes in Each Protein Fraction No. 1 Lyophilised D_2*

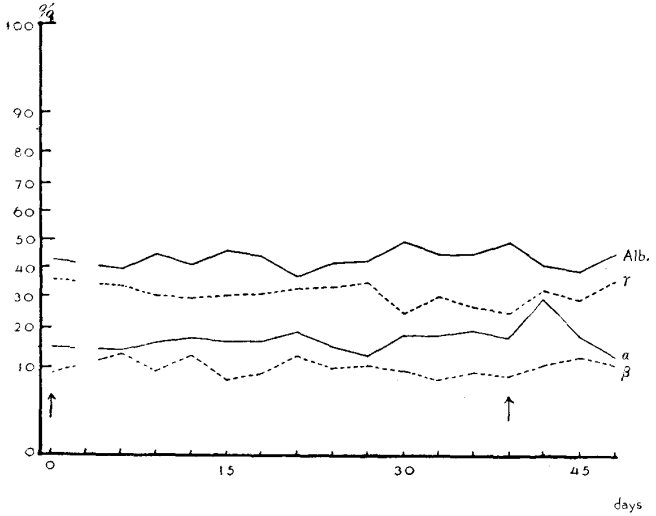


FIGURE 11. *Changes in Each Protein Fraction No. 2 Lyophilised D_1*

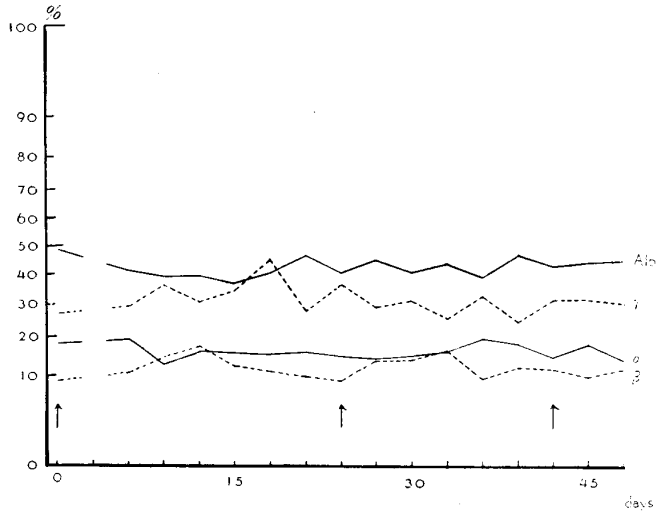


FIGURE 12. *Changes in Each Protein Fraction No. 2 Lyophilised D_2*

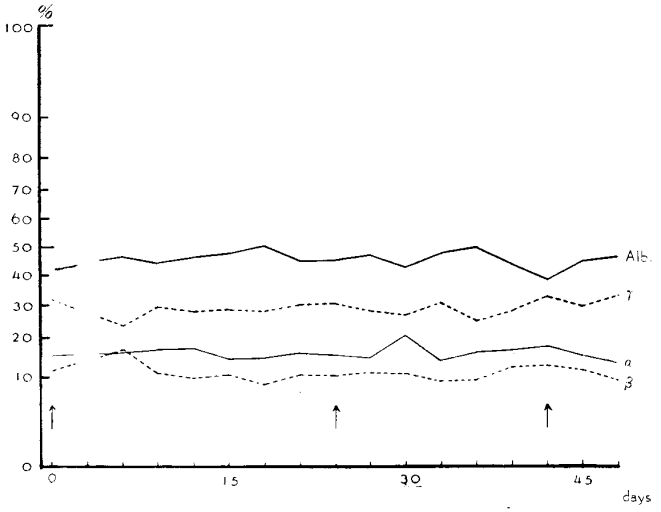


FIGURE 13. *Changes in Each Protein Fraction No. 3 Lyophilised D_1*

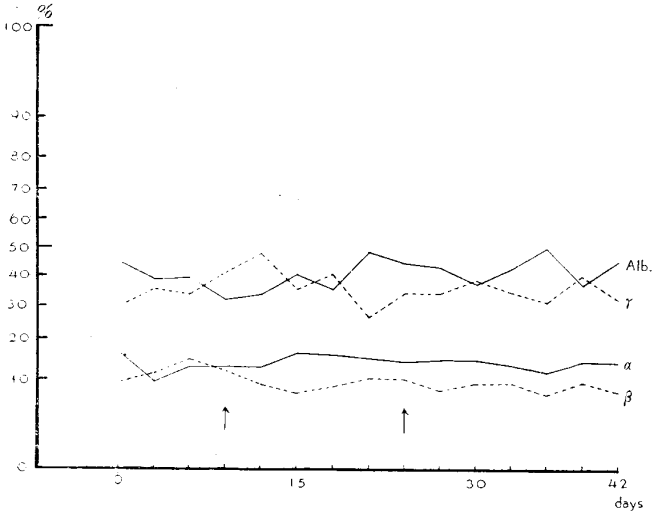
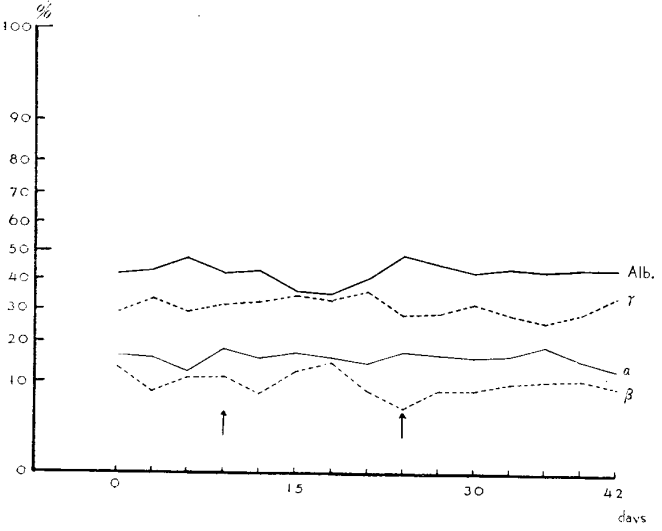


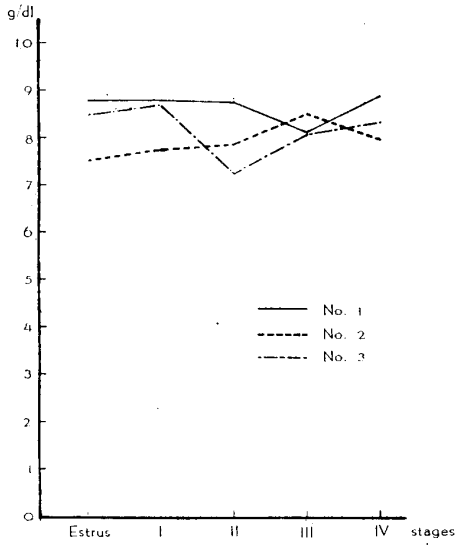
FIGURE 14. *Changes in Each Protein Fraction No. 3 Lyophilised D_2*



by means of the repeated two-way lay-out, three-way lay-out and four-way lay-out methods did not show any significant difference.

3) The total protein concentration of each sample was measured by the micro-Kjeldahl method, and the results obtained are shown in fig. 15. In the cases of Nos. 1 and 3, there existed a slight decrease of the total protien value during diestrus, at the time of the 2nd or 3rd stage of the cycle, while in the case of No. 2, in which a degenerated fetal membrane was found in the uterus, such a tendency was not noted. However, the present author could not consider this temporary decrease of the total protein as a specific change along with the sexual cycle, because of the inadequate number of cases.

FIGURE 15. *Concentration of Total Protein in Serum (micro-Kjeldahl method)*



Discussion

According to Pieper and Molinski and others, the paper-electrophoretic method is highly reproducible. In the present author's experiment, however, reproducibility was not satisfactory, though the conditions were kept as constant as possible. As for the cause of the poor reproducibility, the following several factors would play an important role; quality of the filter paper, the spotting points of material, voltage, amperage, electrophoretic time, buffer and stain solution, etc. Other factors which might affect the results obtained are temperature, humidity, illumination, atmosphere, etc., during the course of the experiments.[12] Unfortunately, the present author could not entirely control such meteorological factors. In addition, the animals used in this experiment did not always represent regularity in the sexual cycle or normality in the conditions of the genital organs through the whole course of the experiment. So, these animals were not in the most desirable state for examination of the serum protein fractions along with the sexual cycle. However, there was an accordance with Chopard's report in which he stated that albumin tends to decrease and γ-globulin tends to increase in some cases of infertile cows. It is doubtful whether these finding would always reflect the variation of the serum protein fractions of infertile cattle, because the age factor of the experimental animals would probably be concerned in this respect. Hartung also stated an opinion similar to that of the present author that serum protein fractions in the cow vary with age. On the other hand, in older cows, 10~12 years old, the fraction rate of α-globulin to β-globulin in these measurements was the reverse to Hartung's data. Such an inconsistent

result may probably be due to the difference in the technique of dividing the fractions.

As for the relation between the total serum protein contents estimated by the micro-Kjeldahl method and the sexual cycle, again, the author could not find out any close correlation. Further study will be required to clarify these problems.

The paper-electrophoretic method used in the present experiment is a modified one in respect of buffer solution and drying method, from the standard method decided by the Electrophoresis Committee, Tokyo, 1957.[15] One must keep the conditions of an experiment as constant as possible, and pay careful attention to separate each of the protein fractions on a filter paper. In order to obtain a consistent result, dialysis of material before electrophoresis was recommended by GRAF and LIST. For the same purpose, when protein concentration of the materials is very high, dilution of the materials by adding some quantity of buffer solution was tried by NEELY and NEILL.

As for the influence of storage on the serum protein fractions, MATHEW and BUTHALA reported that pig serum stored at −20°C for 26 weeks was found to be completely intact. An experiment on the influence of storage on frozen and lyophilised bovine serum, now being carried out by the present author, will be reported in the future.

CONCLUSION

The protein fractions were analysed by paper-electrophoresis for bovine sera collected from 3 cows at 3-day intervals. The data obtained were treated statistically, but they failed to show any significant variation, except a slight tendency of decrease of albumin contents and increase of γ-globulin. This is probably due to experimental errors which were considerably large. On the other hand, variation of total protein value along with the sexual cycle measured by means of the micro-Kjeldahl method were moderately significant; a slight decrease of total protein at the middle stage of the cycle occurred in 2 cases.

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