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EVALUATION OF THE PREGNANCY TEST BY THE OCCURRENCE OF POLYAMINE IN THE BOVINE AND HORSE BLOOD PLASMA*

By

Hirokazu SHIMIZU and Masami SATO

Laboratory of Animal Reproduction, Faculty of Agriculture, Tohoku University, Sendai, Japan

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It is desirable to confirm early the pregnancy in the dairy cows by some effective means, since whether the cows become pregnant within a certain period decides greatly their productivity in either milk or calves. Most pregnancy diagnosis tests for the farm animal had hitherto been based on the detection of the hormonal changes in the blood and urine. Under practical conditions, the examination of the cervical secretion, (the qualitative and quantitative changes of the cervical mucin) and the rectal palpation of the uterus and ovary have been highly evaluated for this purpose. But these two methods tend to be somewhat subjective, and further the latter needs special and mature skills.

It is of interest to consider other methods from the biochemical view by which the blood changes accompanied with pregnancy can be demonstrated, and to find whether any are likely to provide information of value in pregnancy diagnosis. Some attempts have been made to perfect methods for diagnosing pregnancy based on the detection of the changes in the blood compositions (1).

A few years ago, Morgantini proposed (2) a pregnancy diagnosis test for cows, a colour reaction of the blood serum by heating the serum with cupric carbonate. The test resulted in positive at various stages of pregnancy from the 30th day onwards. He described that this might be caused by the presence of spermine. This method was originally proposed for the cancer diagnosis by Tokuoka (3).

In general, the biochemical significance of spermine, spermidine and others still remains obscure (4). If the detection of spermine with a simple procedure is so superior for pregnancy test, as Morgantini mentioned, it should be convenient to apply the test in the field with the practical object. The authors

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therefore followed the Morgantini's test at first, but and failed in finding any difference in the results of between pregnant and non-pregnant plasma of the cows and horses. Accordingly they tested further by a more sensitive method, the for analysis of amines to know if the occurrence of these amines could be connected to any sexual conditions in cows and horses.

Materials and Methods

Dairy cows with various conditions in pregnancy and lactation, and a bull, of the University Farm, and a few horses were used for the blood analysis. It was necessary to keep some of the whole blood, to which anticoagulant had been added, in the refrigirator overnight because of transportation.

i) For Morgantini's test, one ml of the blood plasma diluted with one ml of water was boiled for one minute adding 0.1 g of cupric carbonate ($CuCO_3 \cdot Cu(OH)_2 \cdot H_2O$, malachite). The colour developed was examined after the solution was cooled down. The same reaction was carried out with trichloro-acetic acid (TCA) soluble fraction of the plasma.

ii) For the more sensitive and semi-quantitative method by paper-chromatography, the following procedure was adopted: that is, 25 ml of the plasma was deproteinized at 5 per cent of TCA in final concentration. After centrifuging off the precipitate, TCA was extracted from the supernatent by shaking with an equal volume of ether changing several times, until the supernatent became around pH 5. Then the solution was neutralized with N-NaOH and was filterated. This was concentrated under reduced pressure by suction while heating in the water bath at 45~50°C. The residue was dissolved with a small volume of distilled water and then was placed in micro-Kjeldahl apparatus to obtain the volatile amine fraction by vapour distillation for 15 minutes under the strong alkaline condition. The fraction was catched with N-HCl and the distillate was dried up again to eliminate the remaining HCl. This process was repeated two more times. The yellowish-white residue was finally yielded, and was dissolved with 0.1 ml of distilled water for application of the paper-chromatography. The paper-chromatography was run one dimensionally with two solvent systems as follows: water-saturated phenol, and butanol, ethanol, water: 4, 2, 3, by volume. Development of the colour was performed by spraying 1 per cent ninhydrin solution of water saturated butanol, and by subsequent heating the paper for one minute in the oven at 110°C.

Results and Discussion

i) *Morgantini's reaction*: The results of the Morgantini's colour reaction of the blood plasma with cupric carbonate was lilac-blue (positive reaction) in almost all cases and light green (negative reaction) appeared in one out of 17. Therefore, the positive reaction did not correspond to the pregnancy,

conversely it happened in the non-pregnant cases, too. The same results were demonstrated in the TCA-soluble fraction of the blood plasma and in the horse whole plasma (Table 1).

Table 1. Results of Morgantini's reaction on the bovine and horse plasma.

Animal No.		Animal condition	Results (Positive cases)
Cattle: *Pregnant*	1	P-4	+
	2	P-4	+ (2/2)
Pregnant-lactating	3	P-3, L	+
	4	P-4, L-7	−
	5	P-5, L	+
	6	P-7, L	+ (3/4)
Lactating	7	L	+
	8	L	+
	9	L	+
	10	L-9	+
	11	L-9	+
	12	L-13	+ (6/6)
Nonpregnant-nonlactating	13	(−)	+
	14	(−)	+
	15	(−)	+
	16	(−)	+ (4/4)
Male	17	Young bull	+ (1/1)
Horses: *Pregnant*	18	P-2	+
	19	P-2	+
	20	P-4	+
	21	P-4	+ (4/4)
Nonpregnant	22	(−)	+ (1/1)
Gelding 1	23		+
Gelding 2	24		+ (2/2)

Figures followed with P or L indicate the months of pregnancy or lactation. The horses Nos. 18, 20 and 19, 21 are the same individuals.

ii) *Amine analysis by the paper-chromatography*: A more sensitive method for determing di- and polyamine was introduced for the blood plasma of the cattle and horses. The results are presented in Table 2 and Figures 1 and 2.

Spots obtained in the paper-chromatography of the pregnant cows indicated also no particularity, compared with those of non-pregnant ones: *i.e.*, two spots appearing on the paper were entirely common to those of pregnant, pregnant-lactating, lactating and nonpregnant-nonlactating cows and a young bull, in addition, of pregnant, and non-pregnant mares, and geldings. Arranging spots in order from the higher rate of flow (Rf) value to the lower, 0.25 and 0.08 in the phenol system were obtained. The colour of the each spot was violet and included yellow in the centre and purplish orange, respectively. The Rf of these spots was less variable in the phenol system than in butanol. Quite similar pattern was demonstrated in the horse plasma. Judging from

Table 2. Results of paper-chromatographical analysis of amines in the bovine and horse plasma.

Animal				Amines		
No.		Name	Conditions	Sp.-Sd.	Pt.	Cd.
Cattle :	1	H-6	P(1)	+	+++	−
	2	J-4	P(2)	+	+++	−
	3	H-K	P(7)	+	+++	−
	4	NH-408	P(7)	+	+++	−
	5	H-R_4	P(2)L(5)	+	+++	−
	6	H-R_4	L(3)	+	+++	−
	7	NH-413	L(4)	+	+++	−
	8	H-H_5	L(6)	+	+++	−
	9	J-2	L(11)	+	+++	−
	10	H-7	—	+	+++	−
	11	H-7	—	+	+++	−
Horses :	1	Mare 1	P(2)	+	+++	−
	2	Mare 2	P(2)	+	+++	−
	3	Mare 1	P(4)	+	+++	−
	4	Mare 2	P(4)	+	+++	−
	5	Mare 3	—	+	+++	−
	6	Gelding 1		+	+++	−
	7	Gelding 2		+	+++	−

Abbveviations used in the table:
H-Holstein, J-Jersey, NH-Japanes Native Breed × Holstein.
Sp.-spermine, Sd-spermidine, Pt-putrescine, Cd-cadaverine

the guiding and over-mounting trials of the amines, the spots obtained seemed likely to correspond to spermine or spermidine and putrescine. The spots with lower Rf, could not be well identified, since spermine and spermidine run in conjunction, under the present method employed. The amount of putrescine on the paper was considerably exceeding in comparison with the other amine. A similar relation was also obtained in the pregnant, non-pregnant mares and geldings.

From the results in the present investigation, the tests based on either colour reaction by cupric carbonate or the detection of amine with paper-chromatography prove the existence of spermine, which reported to have appeared particularly in the pregnant plasma, seemed to show no applicability for the pregnancy diagnosis test.

It happens frequent that some cancer diagnoses methods can serve as well for the pregnancy tests. Tokuoka reported (3) that his colour reaction for cancer diagnosis also showed positive with pregnant serum in human (42.8%), before Morgantini applied the method for pregnancy tests in the bovine blood serum. Both of them claimed spermine *per se* was the reactive substance in the serum.

On the other hand, the biochemical significance of spermine was barely known in the higher animal, in spite of its early discovery and evidences as to its wide distribution in the various organs (4, 16–18). While Ugami and his coworkers (5–11) discovered mammamine, a new specific peptide characte-

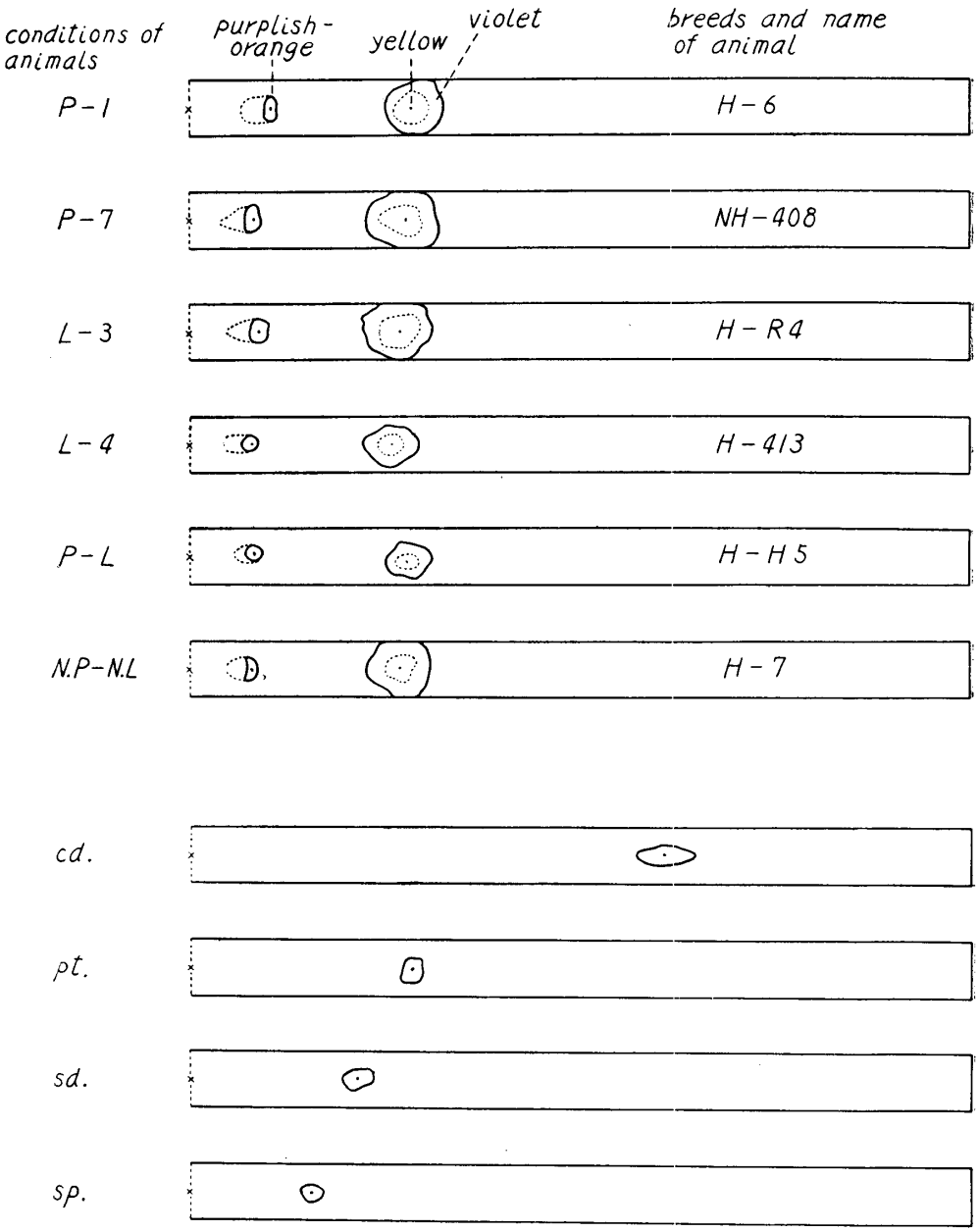

Figure 1. The paper-chromatography of amines in the bovine blood plasma (phenol system).

ristically existing in the mammary glands of the rats since the end part of gestation, maintaining much higher concentration throughout lactation period, they suggested there were some connections between the occurrence of the amine and the mammary gland functions. Later, they identified mammamine as spermidine (9, 12).

As regards the pregnancy tests resemble ingd the cupric carbonate method, biuret and ninhydrin reaction had been reported (13, 14). However these two were not proved as to their usefulness for such purpose at least (1).

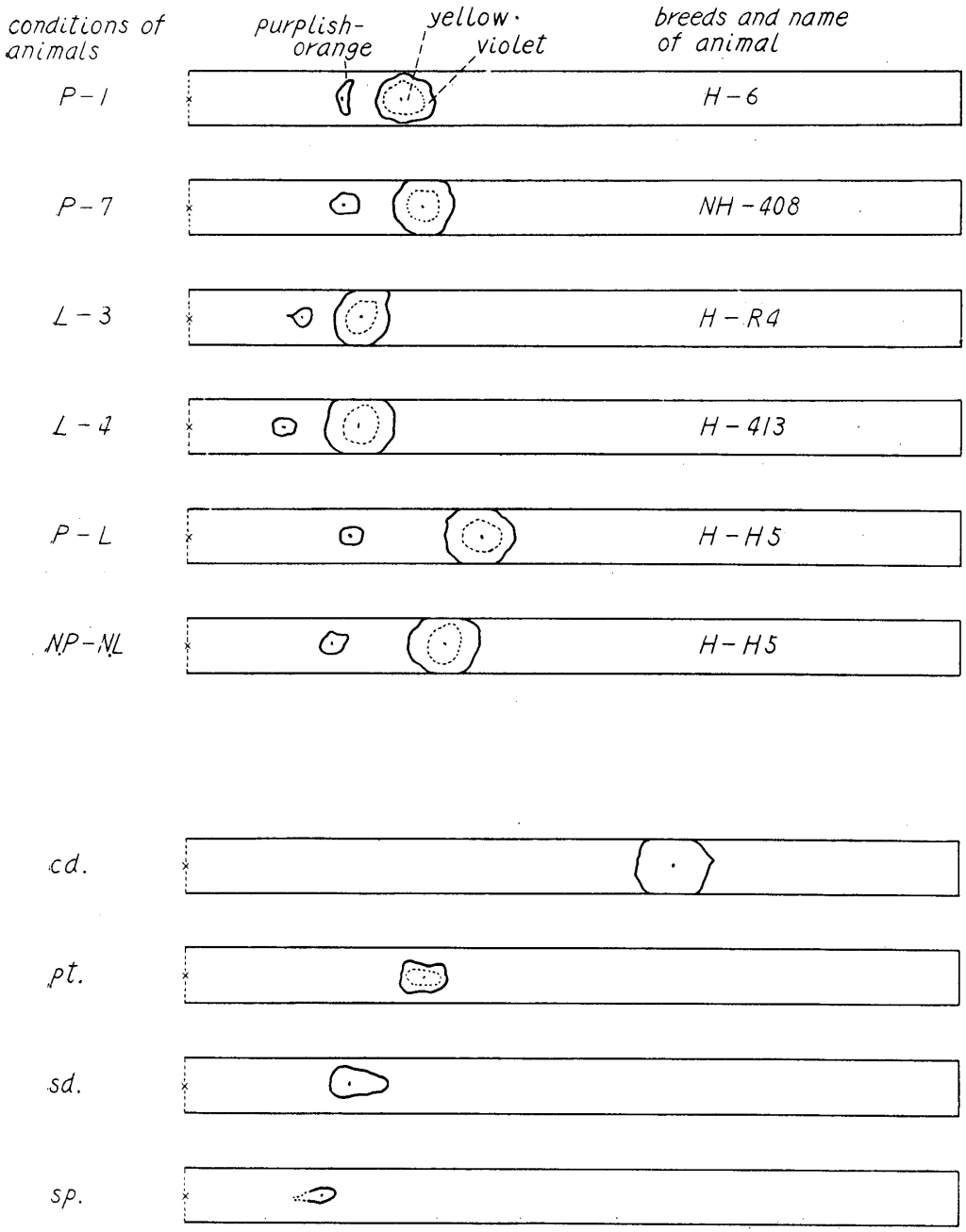

Figure 2. The paper-chromatography of amines in the bovine blood plasma (butanol system).

While the determination of amine oxidase which increases in the human blood has been demonstrated, it is not applicable as the test, it had not been applied to farm animals (1, 15).

In sum, no information was ever given that any amine of the blood plasma in farm animals occurred specificially or increased promptly at the time of conception. As to the existence of the amines, especially spermine in the bovine blood, previous workers claimed (16, 17) that no, or only a trace of

amine could be detected. Recently Rosenthal and Tabor indicated (18) existence of 5–20 μg of spermine per ml of the blood of mice, rats and men, but they ascribed its origin principally to the leucocytes. But our results seemed to demonstrate the existence of spermine or spermidine and putrescine in the blood plasma of cattle and horses, judging from the sensitivity of ninhydrin to the colour development of spermine mounted on paper, though their concentrations appear to be of much less extent than that of the whole blood as Rosenthal *et al.* reported. This disparty may be mainly due to the difference of the analytical method employed.

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Summary

A pregnancy test based on the colour reaction of the blood plasma boiled with cupric carbonate was followed after Morgantini to ascertain the possibility of practical usage in cattle and horses, but the results came out entirely negative in usefulness. Accordingly, poly- and diamine in the blood plasma were examined by paper-chromatographical means, since spermine had been regarded as the reactive substance in the colour reaction. The results demonstrated that the pattern consisted of putrescine and spermine or spermidine and was not different from the various sexual conditions of the cows and even from that of a bull calf, as well as in horses. So that the pregnancy test based on the colour reaction and on detection of spermine by more sensitive method was also proved to be not available for practical usage. But the occurrence of amines in the bovine and horse plasma was shown as likely.

Addendum

Scince this report was submitted for publication, the papers of Dr. H. Blaschko, Oxford University, England has come to our attention; he demonstrated the differences of amine oxidase activity in the blood plasma of various mammals, and, in addition, the changes of sperimine oxidase activity accompanied with the rumen growth in the ruminant (J. Physiol., **145**: 124, and 384, 1959). Dr. Blaschko has kindly informed us by a personal communucation that the oxidase prevented the accumulation of spermine in the bovine plasma.

References

1) Cowie, A.T. (1948). "Pregnancy diagnosis tests: A review" Commonwealth Agricultural Bureaux Joint Publication No. 13, Commonwealth Bureaux.
2) Morgatini, F. (1954). Ann. Fac. Med. Vet. Pisa, **7**, 153 cited from Vet. Bull,, **26**, p. 101 No. 627, 1955, or Anim. Breed. Abst., **23**, 1150, 1955.
3) Tokuoka, S. (1950). Acta Schol. Med. Univ. Kioto, **27**, 241.
Tokuoka, S. (1955). Saishin-igaku, **9**, 889 (in Japanese).
4) Guggenheim, M. (1951). "Die biogenen Amine" pp. 308-29, Karger, Basel.
5) Ugami, S., K. Kuretani, M. Ohashi and M. Sato (1955). Jap. J. Anim. Reprod., **1**, 27 (in Japanese).
6) Ugami, S.K. Kuretani, M. Ohashi and M. Sato (1955). Jap. J. Anim. Reprod., **1**, 53 (in Japanese).
7) Ugami, S. K. Kuretani, M. Ohashi and M. Sato (1955). Jap. J. Anim. Reprod., **1**, 103 (in Japanese).
8) Ugami, S.K. Kuretani, M. Ohashi and M. Sato (1955). Jap. J. Anim. Reprod., **1**, 131 (In Japanese).
9) Ugami, S., K. Kuretani, M. Ohashi and M. Sato (1956). Jap. J. Anim. Reprod., **2**, 7 (in Japanese).
10) Ugami, S., K. Kuretani, M. Ohashi and M. Sato (1955). J. Jap. Biochem. Soc., **27**, 133 (in Japanese).
11) Ugami, S., K. Kuretani, M. Ohashi and M. Sato (1955). J. Jap. Biochem. Soc., **27**, 303 (in Japanese).
12) Kuretani, K. (1957). Rep. Sci. Res. Inst., **33**, 80 (in Japanese).
13) Dienst, A. (1922). Arch. Gynäk., **117**, 56, 117.
14) Dienst, A. (1926). Mschr. Geburtsh. Gynäk., **72**, 249.
15) Zeller, E.A. (1942). "Diamin-oxydase" in Adv. Enzymol. **11**, pp. 93-112, Intersci. Publ., N.Y.
16) Dudley, H.W. and O. Rosenheim (1925). Biochem. J., **19**, 1034.
17) Wrede, F. (1926). Zft. Physiol. Chem., **153**, 296.
18) Rosenthal, S.M. and C.W. Tabor (1956). J. Pharmacol. Exp. Therap, **116**, 131.