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Influence of Progesterone on Orthopox Viruses *in vitro* and *in vivo*

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With 3 figures

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Summary

The influence of progesterone on virus-host-interactions was investigated for orthopox viruses *in vitro* and *in vivo*. Virus induced cytopathogenic effects in Vero cells were markedly reduced in presence of the hormone. Dose dependent restriction of viral replication was evaluated by infectivity titration and immunological antigen determination. Progesterone concentrations of 5 µg/ml tissue culture medium depressed the production of new viral particles nearly totally. The results obtained by the two different methods indicate that probably later stages of viral synthesis are affected when lower dosages of progesterone are used. The protective activity of progesterone against pathogenic actions of orthopox viruses *in vivo* was confirmed by using rabbits for intradermal infections.

Key words: Orthopox, progesterone, antiviral activity

Introduction

Numerous hormones influence or regulate the metabolic situation of probably all kinds of organisms. Concerning infectious diseases there is a special interest in the action of hormones on pathogenic microorganisms.

A frequently named example for the effect of hormones on infection is the spontaneous disappearance of ringworm at puberty (17). Disease due to paracoccidoides brasiliensis occurs post-puberty predominantly in males. It was hypothesized that the action of estrogen on fungus may be responsible for the distinct resistance of females (14). Several authors report about inhibitory effects of progesterone on the growth and virulence of various bacteria, fungus and parasites (4, 5, 8, 9, 15, 19).

However, the action of progesterone on virus replication seems to be rarely investigated. Sexual differences have been observed in the susceptibility of mice for infections with ectromelia virus (10). Morbidity and mortality were significantly higher in males than in females.

Albano (2) demonstrated that progesterone reduces the cytopathogenic effect of poliomyelitis virus in monkey kidney cultures. Treatment of mice with progesterone gave a 100 % protection against an adapted strain of poliomyelitis virus. Other steroids were less effective or even ineffective (3).

In the view of these findings an investigation into the effect of progesterone on the virulence and replication of orthopox viruses was carried out *in vitro* and *in vivo*.

Material and Methods

Cells

Vero cells were grown to confluence in Hepes buffered medium 199 in Earle's salts (Biochrom, W-Berlin) supplemented with 10 % fetal bovine serum. Confluent monolayer cultures were maintained in MEM Eagle (modified) with Hanks' salts (Flow Laboratories, Meckenheim, FRG). Both media contained antibiotics.

Animals

Five white, male rabbits with a weight of 2.4—2.9 kg were used for *in vivo* experiments. The animals were healthy and had no previous contact with orthopox viruses.

Viruses

Studies were performed with laboratory passaged strains of orthopox virus cameli type CP-1, vaccinia virus strain Bern and cowpox virus strain OPV-85[1]. Conditions of virus propagation and preparation of stocks have been described already (7).

Hormone preparations

4-pregnene-3,20-dione (Sigma Chemical, St. Louis) dissolved in absolute ethanol (10 mg/ml) and diluted in MEM was applicated to the cell cultures. For each experiment the hormone solution was freshly prepared under steril precautions.

Three rabbits were treated with hydroxyprogesteronecaproate dissolved in oil (Proluton Depot®, Schering, W-Berlin).

Determination of progesterone related cytotoxicity

Confluent Vero monolayer cultures were treated with different concentrations of progesterone and progesterone free diluent, respectively. For a period of up to 7 days the cells were controlled for morphological alterations. Viability was tested by serial subculturing.

Investigation of viral cytopathogenicity

Confluent Vero cells cultured in 25 cm^2 flasks (Nunc, Roskilde) were simultaneously inoculated with CP-1 (2.88—7.18 $\log_{10}$ $TCID_{50}$) and progesterone (9—75 µg, i. e. 0.6—5 µg/ml medium). The cultures were microscopically investigated as for type and extent of cytopathogenic effect. Pictures were taken at day 6 p. i.

Evaluation of virus replication

10^2 $TCID_{50}$ CP-1 per well and various dosages of progesterone were applicated to confluent Vero cells in 24-well microplates (Becton Dickinson Labware, Oxnard). Each combination was tested in four replicate wells. After an incubation for 72 hours the cultures of corresponding wells were harvested and stored at − 80 °C.

The yield of virus was measured by infectivity titrations and immunological antigen determinations.

Procedures of infectivity titrations were previously described by KROPP (7).

Viral antigen was quantified by a modification of the biotin-avidin enzyme immunoassay described by PFAHLER et al. (13).

Briefly, the wells of flat bottomed microelisa plates (Immunolon M 129, Dynatech, Plochingen, FRG) were coated with antigen probes at a 1 : 400 dilution level in blocks of 4 replicate wells.

Sequentially the plates were filled (100 µl/well) and incubated (1 h / 37 °C) with standard positive camel serum (1 : 200), biotinylated rabbit anti-camel globulin (30 µg/ml) and avidin peroxidase conjugate (1 : 2,000). Finally H_2O_2 activated O-phenylene-diamine was added (100 µl/well). After each incubation step the plates were washed three times.

Results of both assays were expressed as percentage of the titer and the antigen amount, respectively, found in progesterone free positive control wells. Referring to these calculations a calibration curve was prepared for quantifying the antigen detected by the ELISA.

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[1] We thank Prof. Dr. MAHNEL for supplying us with this virus strain.

Investigation of viral virulence in vivo

In a preliminary experiment each of two rabbits was inoculated with 3.7 $\log_{10}$ $TCID_{50}$ vaccinia virus in 50 µl on scarified skin areas (4 × 4 cm) of the depilated flanks. 250 mg Proluton® were intramuscularly injected to one of the two rabbits every other day for 8 consecutive days, starting at the day of inoculation.

Analogous a second experiment was performed with cowpox virus infecting each of three rabbits with 4.48 $\log_{10}$ $TCID_{50}$. While one animal served as untreated control, the other two rabbits were daily and every other day, respectively, treated with 250 mg Proluton® i. m. Every day skin lesions were photographed and assessed for size, number, appearance and degree of evolution.

Results

Effects of progesterone on non-infected cells

Tests of cytotoxicity of progesterone for Vero cells demonstrated that concentrations of more than 10 µg/ml were without significant influence on cell morphology and viability during the first 24 hours. 5 µg progesterone per ml slightly increased the percentage of rounded cells during a 7 days incubation period. Nevertheless, subculturing was still possible at this hormone concentration.

Finally, sensibility of Vero cells decreased with increasing age of the monolayer.

Effects of progesterone on virus infected cells

A qualitative impression of the influence of progesterone on viral cytopathogenicity *in vitro* is given by Fig. 1. Independent of the initial dosage of viral infection (up to $10^{7.18}$ $TCID_{50}$) 5 µg progesterone per ml medium seemed to suppress totally the development of virus induced cytopathogenic effects.

In further tests we investigated the influence of progesterone on virus replication. The results of infectivity titration were compared with those of the enzyme immunoassay (Fig. 2). It is evident that progesterone reduced the yield of virus, although the values obtained by infectivity titration are lower than those of the ELISA. Higher concentrations of the hormone resulted in higher viral inhibition.

Effect of progesterone on virus-host interaction in vivo

A first experiment demonstrated the effect of progesterone on vaccinia virus induced skin lesions of rabbits. After inoculation the scarification area of the untreated control animal developed confluent ulcerated papules with a purple center and small areolas. The maximum reaction was noticed at day 6 and 7 p. i. In comparison the progesterone treated animal only developed 4 flat lesions with central scabs and marked areolas.

A second investigation reconfirmed these results using a more virulent virus (cowpox virus strain OPV-85) in a higher concentration and applying progesterone at two different dosages and intervals. Within six days the scarification area of the untreated control animal changed into a completely necrotic and hemorrhagic infiltrated tissue surrounded by swollen oedematous skin (Fig. 3 a). After 5 days the rectal body temperature exceeded 40 °C and reached its peak at day seven with 40.8 °C. Inappetence and apathy indicated a serious clinical course. The second animal, which was treated with 250 mg Proluton® every other day, developed about 25 solitary hemorrhagic ulcers of 1—3 mm in diameter at day six (Fig. 3 b). Numerous small red nodules (1 mm in diameter) in the skin of the ears and the depilated flanks point at a generalized infection. Contrarily, after six days the scarification area of the third animal, which was daily treated with 250 mg Proluton®, only developed individual flat lesions with shallow central excavations (about 3 mm in diameter) but without hemorrhagic infiltration, subcutaneous oedema or generalization. The number of pox lesions was similar to that of the second animal (Fig. 3 c). The rectal body temperature never reached 40 °C. Behaviour, activity and appetite was normal during the whole observation period.

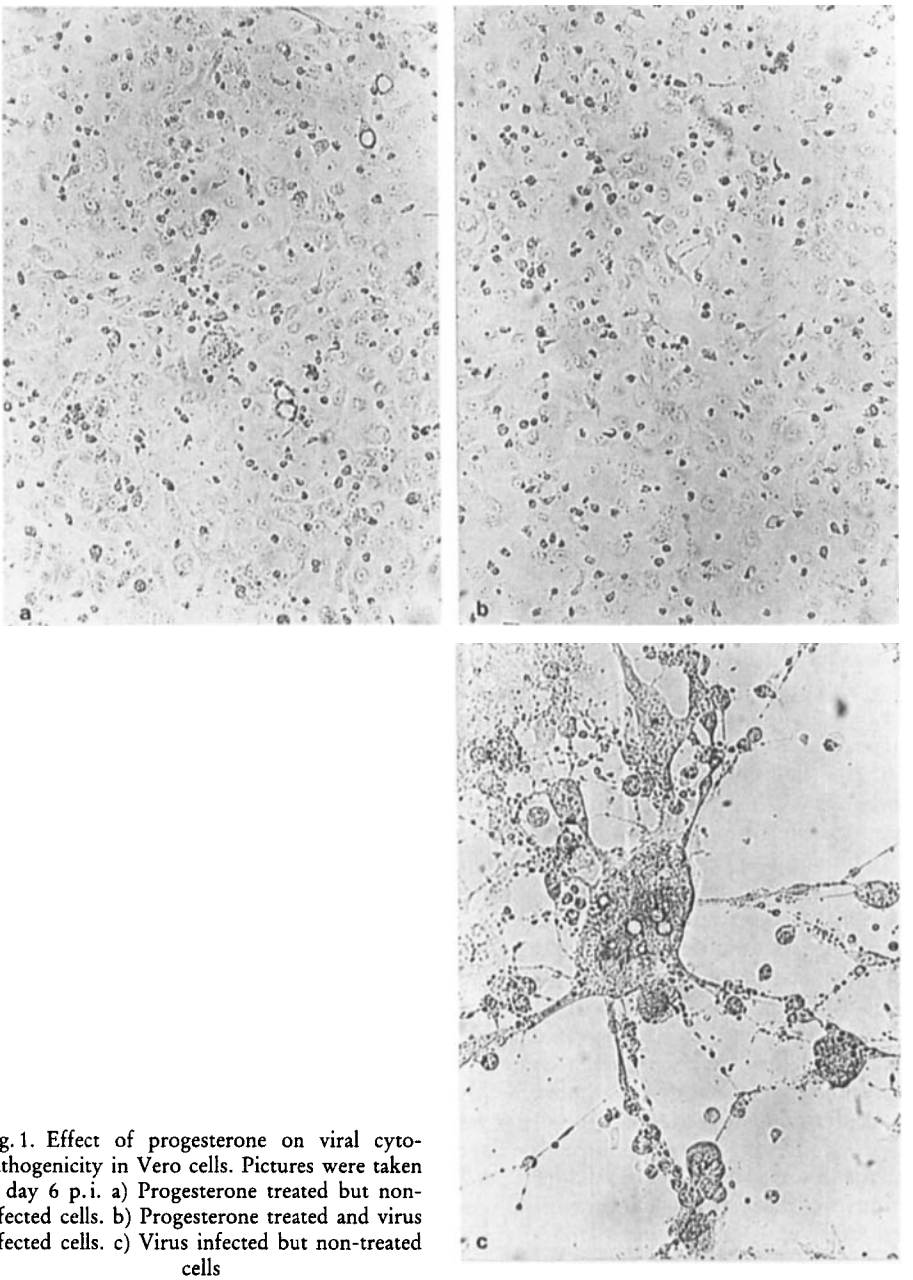

Fig. 1. Effect of progesterone on viral cytopathogenicity in Vero cells. Pictures were taken at day 6 p.i. a) Progesterone treated but non-infected cells. b) Progesterone treated and virus infected cells. c) Virus infected but non-treated cells

Discussion

The results of this study indicate, that progesterone seems to be active against orthopox viruses *in vitro* and *in vivo*. Virus infected cell cultures containing 5 µg progesterone per ml medium showed no morphological differences to non-infected control cultures. Even high viral dosages of about 1.5 infectious particles per cell (i.e. 7.18 $\log_{10}$ $TCID_{50}$ in 25 cm^2 flasks) did not induce cytopathogenic alterations (Fig. 1).

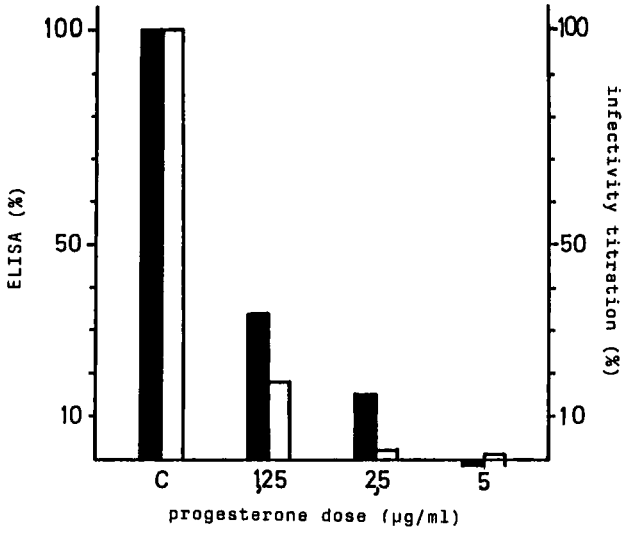

Fig. 2. Effect of progesterone on viral growth in Vero cells. Two different methods were applied to quantify viral reproduction. Results are expressed as percentage of the progesterone-free control sample (C)

■ ELISA
□ Infectivity titration

Quantitative examinations of the viral reproduction demonstrate that 5 µg progesterone/ml reduce the yield of infectious particles for 99 % in comparison with controls (Fig. 2). However, 10^2 $TCID_{50}$/ml (i. e. 1 infectious particle per 5,000 cells) have been used for inoculation and the same titer was detected by infectivity titration after 3 days incubation in progesterone containing medium. That is to say, the initial inoculatory viral particles either did not replicate at all and have been redetected at the end of the incubation period or — after penetration and uncoating — have been replicated with a multiplication factor of approximately 1 within three days.

Lower dosages of progesterone resulted in a higher yield of virus. However, using two different methods, considerable differences in the percentage of inhibition were measured for corresponding progesterone concentrations. For 2.5 µg progesterone per ml the infectivity titration gave a 7.5 times lower value than the ELISA. Similarly, for 1.25 µg/ml the percentage of inhibition was two times higher by infectivity titration than the results obtained by the ELISA. With respect to this observation it should be mentioned, that only infective complete viral particles are registered by infectivity titration whereas soluble antigens of low molecular weight react in the ELISA independently of the presence of morphological intact virions. Consequently it my be concluded, that at least in certain concentrations progesterone affects later stages of viral reproduction.

In vivo progesterone reduced the development of pox virus induced skin lesions in rabbits as for quantity and quality (Fig. 3). The temporary course of evolution of the skin lesions was more slowly and the general state of health was undisturbed. The results of the first study with vaccinia virus strain Bern were reproduced applying the more aggressive cowpox virus strain OPV-85 even in a higher dosage for inoculation. Comparing the two rabbits, which received progesterone in different intervals and therefore different amounts, obviously the number of primary pox lesions was approximately the same for both animals, but the quality of skin eruptions clearly depended on the hormone dosage and/or the frequence of application. Additionally, the animal with fewer injections developed secondary generalized pox lesions which may indicate a situation of immunosuppression. Refering to these findings it has been reported, that immunosuppression by progesterone does not depend on its circulating level but rather on changes in its concentration. SHERBLOM et al. (16) demonstrated, that even high levels of progesterone did not have immunosuppressive activity after the concentration reached a plateau.

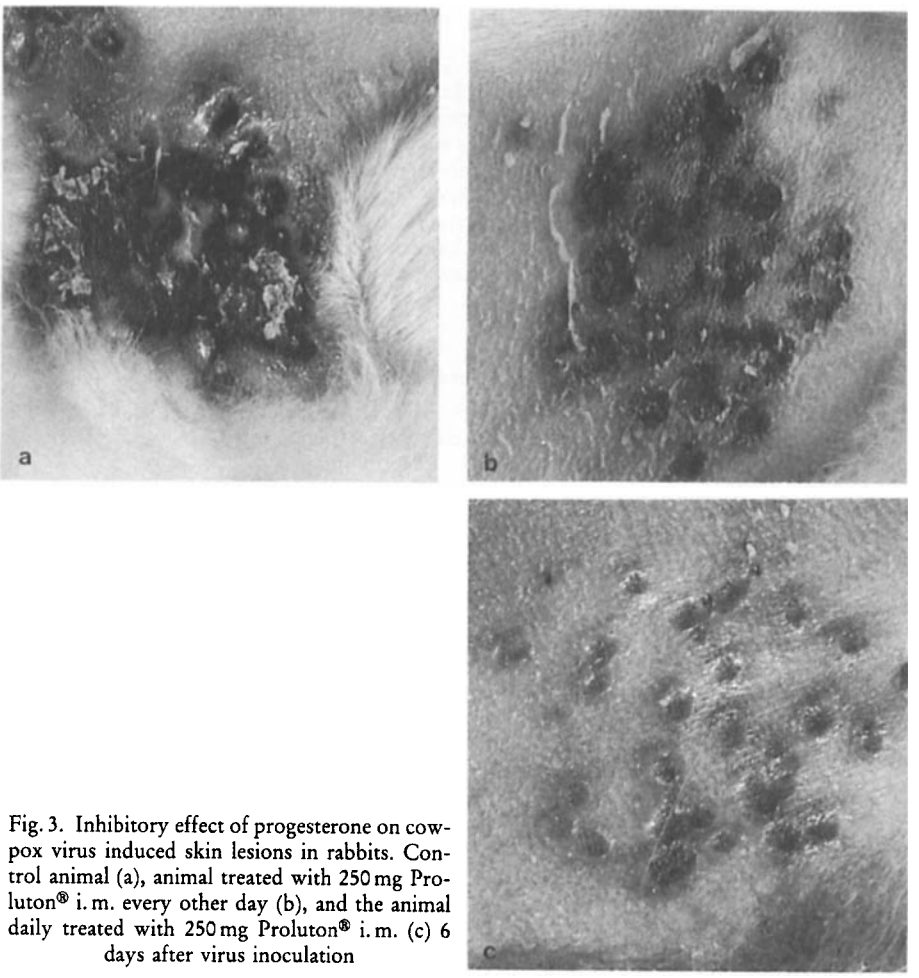

Fig. 3. Inhibitory effect of progesterone on cowpox virus induced skin lesions in rabbits. Control animal (a), animal treated with 250 mg Proluton® i. m. every other day (b), and the animal daily treated with 250 mg Proluton® i. m. (c) 6 days after virus inoculation

The high dosages of progesterone applied in our experiments are clearly beyond the range of physiological values. However, it should be considered, that other steroid hormones like highly potent synthetic glucocorticoid preparations are therapeutically used in amounts of several gramms per day.

Additionally, progesterone is rapidly inactivated or transformed (11, 12, 19). For the same reason the degree of inhibition might be underestimated.

Although numerous biochemical actions of progesterone are known, its antiviral activity is not yet understood. Nevertheless, it should be mentioned, that progesterone has been shown to regulate the expression of some genes (1) and to stabilize cellular membranes (6). But there are no investigations done about the influence of progesterone on viral genes, membranes and other viral components. This report just scratches the surface of progesterone dependent virus-host interactions, which might point out new channels of alleviating the clinical course of acute and serious viral diseases.

Zusammenfassung

Es wurde der Einfluß von Progesteron auf die Virus-Wirt-Wechselbeziehung am Beispiel der Orthopocken-Viren *in vitro* und *in vivo* untersucht. Die virusinduzierten zytopathogenen Effekte in Vero-Zellkulturen wurden in Gegenwart des Hormons deutlich reduziert. Die Hemmung der Virus-Replikation in Abhängigkeit von der Dosis wurde mittels Infektionstitration sowie immunologischer Antigenbestimmung gemessen. Eine Progesteron-Konzentration von 5 µg/ml Zellkulturmedium unterdrückte die Bildung von Viruspartikeln nahezu vollständig. Die Ergebnisse der beiden unterschiedlichen Methoden lassen vermuten, daß durch niedrigere Progesteron-Konzentrationen spätere Stadien der Virussynthese gehemmt werden. Durch intradermale Infektion von Kaninchen konnte die protektive Wirkung von Progesteron gegen zytopathogene Mechanismen der Orthopox-Viren *in vivo* bestätigt werden.

References

1. AHE, D. V. D., J.-M. RENOIR, T. BUCHOU, E.-E. BAULIEU, and M. BEATO, 1986: Receptors for glucocorticosteroid and progesterone recognize distinct features of a DNA regulatory element. Proc. Natl. Acad. Sci. USA **83**, 2817—2821.
2. ALBANO, A., 1956: Ormoni steroidei e virus poliomielitico *in vitro*. Boll. Ist. sieroter. milan. **35**, 580—585.
3. ANDERSON, A. J., and V. BOLIN, 1946: The influence of various hormones on the resistance of swiss mice to adapted poliomyelitis virus. Endocrinology **39**, 67—71.
4. BASHER, H. A., D. R. FOWLER, A. SEAMAN, and M. WOODBINE, 1985: The effect of progesterone on growth and virulence of Listeria monocytogenes. Zbl. Bakt. Hyg. A **259**, 351—358.
5. JEFFERSON, W. E. jr., and G. SISCO, 1961: The influence of exogenous steroids on the growth of *aspergillus niger* and *torula utilis*. J. gen. Physiol. **44**, 1029—1045.
6. KAYA, H., and T. SAITO, 1985: Effect of progesterone and its 17-hydroxyl derivative on human erythrocyte membrane. Japan. J. Pharmacol. **39**, 299—306.
7. KROPP, E.-M., 1985: Kamelpocken — eine synoptische Darstellung sowie der Nachweis von Antikörpern in ostafrikanischen Dromedarseren mit einem ELISA. Vet. Med. Diss., München.
8. LAGRANGE, E., 1962: L'action des ultrasons sur la bilharziose experimentale de la souris. C. R. Soc. Biol. (Paris) **156**, 1725—1727.
9. LESSER, E., 1953: The effect of some hormones and related compounds on the bacteria-free culture of Entamoebe invadens and free-living amoeba. Canad. J. Zool. **31**, 511—518.
10. MUNZ, E., M. REIMANN, R. HOFFMANN und E. GÖBEL, 1974: Perorale Immunisierungsversuche mit Vaccinia-Virus gegen Mäusepocken. Zbl. Bakt. Hyg., I. Abt. Orig. B **159**, 10—30.
11. NAIM, N., and M. A. E. NAIM, 1985: Mechanism of the enzymatic conversion of progesterone with *Trichuris spiralis*. Rev. Lat.-Amer. Microbiol. **27**, 213—217.
12. OJANOTKO-HARRI, A., 1985: Metabolism of progesterone by healthy and inflamed human gingiva *in vitro*. J. steroid. Biochem. **23**, 1031—1035.
13. PFAHLER, W. H. E., M. REIMANN, and E. MUNZ, 1986: A biotin-avidin amplified enzyme immunoassay for detection and quantification of orthopox virus cameli antibodies in dromedaries. J. Vet. Med. B **33**, 477—484.
14. RESTREPO, A., M. E. SALAZAR, L. E. CANO, E. P. STOVER, D. FELDMAN, and D. A. STEVENS, 1984: Estrogens inhibit mycelium-to-yeast transformation in the fungus Paracoccidioides brasiliensis: implications for resistance of females to paracoccidioidomycosis. Infec. Immunity **46**, 346—353.
15. SHÄR, G., E. P. STOVER, K. V. CLEMONS, D. FELDMAN, and D. A. STEVENS, 1986: Progesterone binding and inhibition of growth in *Trichophyton mentagrophytes*. Infect. Immunity **52**, 763—767.
16. SHERBLOM, A. P., R. M. SMAGULA, C. E. MOODY, and G. W. ANDERSON, 1985: Immunsuppression, sialic acid, and sialyltransferase of bovine serum as a function of progesterone concentration. J. Reprod. Fert. **74**, 509—517.
17. WEIR, D. M., 1973: Natural and acquired immunity. In: Medical Microbiology, 12th Edn., Vol. 1, pp. 134—163, Eds. R. CRUICKSHANK, J. P. DUGUID, B. P. MARMION, and R. H. A. SWAIN. Churchill Livingstone, Edinburgh.
18. YEILDING, K. L., G. M. TOMPKINS, J. S. MUNOY, and D. J. COWLEY, 1960: The effects of steroids on electron transport. J. Biol. Chem. **235**, 3413—3417.
19. YOTIS, W. W., and R. STANKE, 1966: Bacteriostatic action of progesterone on staphylococci and other micro-organism. J. Bact. **92**, 1285—1289.