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DNASES IN MILK AND BLOOD SERA FROM DIFFERENT SPECIES

By

Roar Gudding

GUDDING, R.: *DNases in milk and blood sera from different species.* Acta vet. scand. 1979, *20,* 404—416. — DNases were demonstrated in samples of colostrum and blood serum from man and various domestic animals. The measurable DNase activity recorded was highest in samples from cat and dog and lowest in samples from goat, horse, pig and sheep. In contrast to DNases produced by certain bacteria, these enzymes were thermo-labile and the activity was maximal in the area pH 5.0—5.5.

A modification of an agar medium originally described for the demonstration of bacterial DNases was found to be suitable for assays of DNases from colostrum, milk and serum.

DNases; blood serum; milk; colostrum.

Enzymes which depolymerize DNA are found in various animal tissues. The comprehensive literature on the properties and the demonstration of these enzymes has been reviewed by *Bernandi* (1971) and *Laskowski* (1971).

The nomenclature of these enzymes is confusing, as 2 or more terms have been used for the same enzymes. DNase I or pancreatic DNase is the designation of DNases produced in the pancreas and which are present in the intestine. The DNases present in tissues are called acid DNase, DNase II, acid spleen DNase or hog spleen DNase, the latter indicating the organ and the species from which it usually has been extracted.

In examinations of bacterial DNases in quarter milk samples with the Toluidine Blue DNA Agar (TDA) (*Lachica et al.* 1971) DNases which seem to be of non-bacterial origin may represent a diagnostic problem. The present study was carried out in order to get information about the amount of naturally occurring DNases in milk and serum from different species, and to examine some properties of these enzymes, particularly those of

importance for distinguishing these DNases from the DNases produced by bacteria. It was also of interest to compare the agar diffusion method with a modified TDA and the spectrophotometric method for the assay of these DNases.

MATERIALS AND METHODS

Materials

Samples of colostrum, milk and serum from the following species were examined: cattle, sheep, pig and man. From cattle, sheep and pig, samples were taken on the day of partus and the 3 or 4 subsequent days. The first sample of human colostrum originated from the 2nd day after partus. In addition, milk and serum samples from dog and serum samples from horse, goat, cat and rabbit were included. With the exception of the colostrum and milk from man, the samples originated from 2 to more than 10 different individuals. All samples were taken from adult individuals, with the exception of 5 serum samples from 2—8 months old calves. All the donors were healthy individuals with no infection or inflammation of the mammary gland. In the agar diffusion analyses the samples were generally examined without any further preparations. For examinations by the spectrophotometric method, bovine colostrum, milk and serum and human milk were concentrated by the following procedure. The sample was added $(NH_4)_2SO_4$ to 40 % saturation and centrifuged. The supernatant fluid was saturated to 80 % with $(NH_4)_2SO_4$, and after centrifugation the precipitate was dissolved in distilled water and dialyzed against tap water. The samples were stored at — 20°C for up to 1 year.

For control purposes DNase I* (bovine pancreas), DNase II* (bovine spleen) and DNase II* (hog spleen) were included in the experiments.

Agar diffusion method

The TDA described by *Lachica et al.* (1971) was modified as NaCl and $CaCl_2$ were omitted and the pH was adjusted to 5.5 with 0.1 M acetate buffer. EDTA was added to a final concentration of 10^{-4} M. The agar was melted at 100°C. The samples were applied in wells with a diameter of 10 mm punched out in the 2 mm agar layer. The concentration of DNase is given in

* Sigma, St. Louis, Mo., USA.

diffusion units as described for proteolytic enzymes by *Sandvik* (1962).

Spectrophotometric method

The DNase activity was also assayed by the determination of acid-soluble oligonucleotides formed by DNA degradation, based on principles of a method described by *Bernandi et al.* (1966). The reaction mixture contained 1 ml native DNA (150 mg per 100 ml), 1 ml 0.1 M acetate buffer (pH 5.5) and 1 ml crude enzyme solution. The DNA solution generally contained 10^{-3} M EDTA. In analyses of DNase I 0.1 M acetate buffer (pH 6.0) and 0.05 M Tris-HCl (pH 6.5 and 7.0), respectively, and a DNA solution with 10^{-3} M-$MgSO_4$ were also used. The reaction mixture was incubated for 30 min at 37°C. The enzyme reaction was terminated by the addition of 1 ml 5 % trichloroacetic acid. After centrifugation of the solution at 40000 × g for 20 min the absorbance at 260 nm of the solution and its 2-fold dilutions was recorded. The controls were identical with the samples with the exception that the trichloroacetic acid was added previously to the enzyme.

Influence of $MgSO_4$/EDTA and of different pH levels

The DNase activity was tested in the presence of $MgSO_4$ or EDTA added as 1 ml of 10^{-3} M, 10^{-2} M and 10^{-1} M to 100 ml TDA, respectively. Using the spectrophotometric method 1 ml of a solution of $MgSO_4$ or EDTA with a concentration of 10^{-4} M and 10^{-3} M, respectively, was added to the reaction mixture. Controls without $MgSO_4$ and EDTA were always included. The pH was 5.5 during this experiment.

The influence of pH on the DNase activity was tested at the following pH values: 5.0, 5.5 and 6.0 (0.1 M acetate buffer), 6.0, 6.5 and 7.0 (0.05 M Tris-maleate buffer), 6.5, 7.0, 8.0 and 9.0 (0.05 M Tris-HCl buffer). EDTA was included in the agar or the reaction mixture in these experiments, except that $MgSO_4$ substituted EDTA when DNase I was tested.

Effect of heat denaturation

The preparation of the TDA with unheated and heated DNA was performed according to the principles described for streptococcal DNases by *Gudding* (1979). In the spectrophotometric

analyses native DNA resolved at maximum 60°C was compared with heat denatured DNA.

Thermostability

Aliquots of 0.2 ml of concentrated bovine colostrum and serum, DNase I, DNase II (bovine spleen) and DNase II (hog spleen) were transferred into thin-walled 1 ml glass ampoules which were sealed. The ampoules were submerged in water at temperatures of 50, 55, 60, 62, 64, 66, 68, 70, 72, 74, 76, 80, 90 and 100°C for 2 min and subsequently cooled in ice-water. Untreated samples of colostrum, milk and serum from all the other species were heated at 72°C for 2 min. The DNase activity after the heat treatment was tested by the agar diffusion method.

Isoelectric focusing

The samples of bovine colostrum, DNase I, DNase II (bovine spleen) and DNase II (hog spleen) were analysed by isoelectric focusing as described for streptococcal DNases (*Gudding*). The TDA modified as described in the present paper was used in the agar overlayer.

Enzymoserological examinations

The cross-wise inhibition test described by *Sandvik* (1974) was used for the serological analyses of bovine colostrum, bovine serum and canine serum. The DNases were tested with antisera against DNases of Staphylococcus aureus ATCC* 10832, Streptococcus pyogenes NVH** 3144, S. agalactiae NVH 3148, S. dysgalactiae NVH 3152, S. equi NVH 3150, S. equisimilis NVH 3159 and Streptococcus group G NVH 3155. All antisera were produced by the immunization of rabbits.

RESULTS

DNase activity was demonstrated in samples of colostrum and blood serum from all species tested when examined by a modified TDA (Table 1). The zones in the modified TDA as a result of DNA depolymerization had the pink colour as pro-

* American Type Culture Collection, Rockville, Maryland, USA.

** The culture collection at the Department of Microbiology and Immunology, Veterinary College of Norway, Oslo.

Table 1. Optimal pH values for DNase activity and corresponding enzyme activity in untreated samples of colostrum, milk and serum determined by the agar diffusion method*.

Sample	Species	Number of samples	Optimal pH	DNase activity (diffusion units per 0.1 ml)	
				mean	range
Colostrum	cow	>10	5.5	170	95—250
Milk	cow	>10	5.5	<1	
Serum	cow	>10	5.5	65	40—160
Serum	calf	5	5.5	82	70— 95
Colostrum	sheep	6	5.0	35	20— 65
Milk	sheep	6	5.0	<1	
Serum	sheep	>10	5.5	10	6— 30
Colostrum	pig	5	5.0	48	30— 85
Milk	pig	5	5.0	<1	
Serum	pig	>10	5.0	7	4— 13
Milk	dog	2	6.5	2	1— 3
Serum	dog	>10	6.5	280	185—410
Serum	cat	2	6.5	260	230—290
Serum	rabbit	5	5.0	104	50—180
Serum	horse	>10	5.5	3	1— 7
Serum	goat	>10	5.5	8	3— 12
Colostrum	man	1	5.5	32	32— 32
Milk	man	1	5.0	<1	
Serum	man	2	5.0	16	12— 20
DNase I (pancreas)	cow		6.5		
DNase II (spleen)	cow		5.5		
DNase II (spleen)	pig		5.5		

* Toluidine Blue DNA Agar.

duced by staphylococcal DNases in TDA at pH 9.0. Dilutions of commercial DNase I, DNase II (bovine spleen) and DNase II (hog spleen) also produced pink zones in the modified TDA with diameters corresponding to the amount of enzyme applied in the wells.

The concentration of the DNases in samples from the mammary glands varied considerably, both within a species and especially between samples from different species. The highest concentrations were recorded in bovine colostrum. Also in quarter samples of cows collected in the dry period the DNase activity was high. The DNase activity of bovine colostrum or samples collected during the dry period could even be detected in TDA with a pH of 7.5, 8.0 or 9.0. During the first 3 to 4 days

after partus the DNase activity in mammary secretions declined gradually, and DNase activity could not be detected by the agar diffusion method in most unconcentrated samples of milk collected after the 5th day of lactation (Table 1). In canine milk the enzyme was found regularly even in unconcentrated samples.

In blood serum the concentrations of the DNases varied from 280 (dog) to 3 diffusion units per 0.1 ml (horse). The distinctness of the pink zones seemed partly to be correlated to the concentration of the DNase as serum from dogs, cats, rabbits and to some extent cows produced zones with the most distinct pink colour.

The addition of $MgSO_4$ or EDTA did not influence, or influenced the activity of the crude DNases only very slightly when assayed by the agar diffusion method and the spectrophotometric method, respectively (Table 2).

Table 2. Relative activity of DNases with and without $MgSO_4$ or EDTA assayed by the agar diffusion method and the spectrophotometric method, respectively.

Enzyme	Agar diffusion method			Spectrophotometric method		
	no addition	$MgSO_4$*	EDTA*	no addition	$MgSO_4$**	EDTA**
Crude DNase (bovine colostrum)	100	100	120	100	100	120
DNase I (bovine pancreas)	100	150	20	100	200	1
DNase II (bovine spleen)	100	100	150	100	100	200
DNase II (hog spleen)	100	100	200	100	100	600

* 1 ml of 10^{-2} M to 100 ml TDA.
** DNA solution containing 10^{-3} M-$MgSO_4$ or EDTA, respectively.

The comparative studies of DNases from bovine pancreas and spleen and from hog spleen showed that $MgSO_4$ caused a slight increase, and EDTA a marked decrease, in the activity of the DNase I. The activity of hog DNase II was significantly increased in the presence of EDTA. This effect was, however, much less evident when bovine DNase II was tested (Table 2).

The DNase activity of the samples from all species, except those from dogs and cats, was highest at a pH of 5.0 or 5.5 when tested by the agar diffusion method (Table 1). The activity of the DNase in bovine colostrum and the DNase II (bovine spleen) at different pH values is presented in Fig. 1. The activity at different pH values of the samples from the other species was similar to the results of the bovine colostrum as presented in Fig. 1. However, the enzyme activity at higher pH

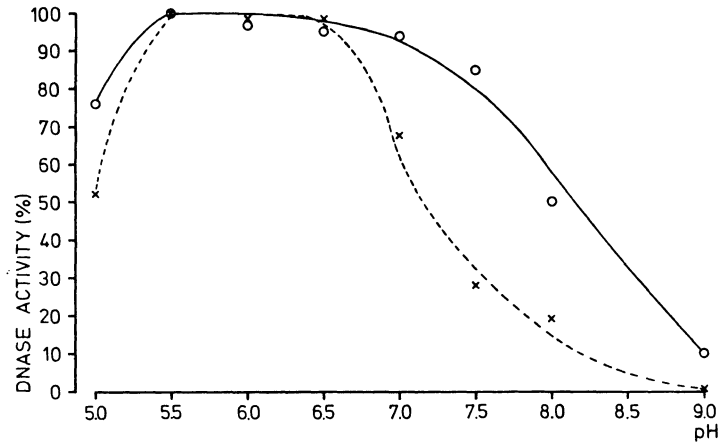

Figure 1. Activity of the DNase in bovine colostrum o——o and DNase II (bovine spleen) x--x at various pH values measured by the spectrophotometric method.

values (pH $\geqslant$ 7.0) declined more rapidly for DNases with low pH optima (man, pig, rabbit, sheep) than for samples with pH optima of 5.5. The canine and feline DNases were less active at lower pH values than the DNases of bovine colostrum.

The pH optima for DNase I, bovine DNase II and hog DNase II were found to be 6.5, 5.5 and 5.0, respectively, when examined by the spectrophotometric method. Corresponding results obtained by the agar diffusion method are shown in Table 1. In addition to larger zones, the colour of the zones was more distinct at the optimal pH of the agar. The shape of the activity curve at different pH values for DNase I was similar to that of bovine DNase II (Fig. 1). The activity of hog DNase II was relatively lower at pH values higher than 6.5 than that of bovine DNase II.

The zones in the TDA were slightly wider (20—30 %) and the enzyme activity recorded in the spectrophotometer showed a marked increase (approx. 250 %) when the DNases acted on native DNA compared with heat denatured DNA.

When Tris-maleate buffer was used, the increase in u. v.-light absorbance as a result of DNA depolymerization was lower than when acetate buffer and Tris-HCl buffer with the same pH values were used. In the agar diffusion method the zones in the TDA were smaller when Tris-maleate was included in the agar. However, for examinations of DNases from canine and feline serum by this method, the choice among the 3 buffers was indifferent.

The sensitivity of the agar diffusion method and the spectrophotometric method is compared in Table 3. Except for the DNase I there was generally a good agreement between the 2 methods when the enzymes were tested at their optimal pH.

Table 3. Highest double dilutions of different DNases measurable by the agar diffusion method and the spectrophotometric method at optimal pH.

Enzyme	Agar diffusion method	Spectro-photometric method
Crude DNase (bovine colostrum)	160	160
Crude DNase (human colostrum)	32	32
DNase II (bovine spleen)	64	64
DNase II (hog spleen)	16	32
DNase I (bovine pancreas)	2560	80

The thermostability of the DNases of bovine colostrum and bovine serum is presented in Fig. 2. The stability of DNases from bovine and hog spleen was similar to that of bovine serum. DNase I was slightly more thermo-stable than the DNase in bovine colostrum. All DNases were completely or almost completely inactivated at 72°C for 2 min.

The separation pattern of crude DNase, DNase I and DNase II by isoelectric focusing is shown in Fig. 3. The pI levels were estimated to 5.7 (DNase I), 5.7 and 7.2 (bovine DNase II) and 7.2 (hog DNase II) The DNase from bovine colostrum was se-

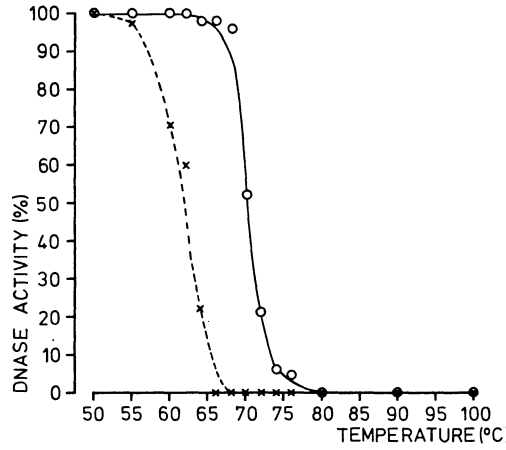

Figure 2. DNase activity after heating for 2 min at temperatures from 50°C to 100°C. Crude DNase (bovine colostrum) o——o and crude DNase (bovine serum) x - - x.

parated into 2 fractions which seemed to be localized identically with the fractions of bovine DNase II. However, the relative activity of the fractions was different, as the fractions of bovine DNase II exhibited equal activity in contrast to the DNase from colostrum of which the fractions with the lowest pI predominated.

None of the antisera against bacterial DNases inhibited the animal DNases in the cross-wise inhibition test.

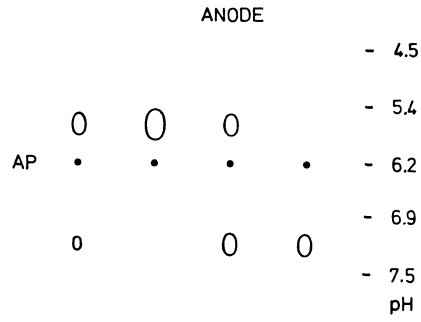

Figure 3. Isoelectric focusing zymogram developed by Toluidine Blue DNA Agar of some naturally occurring DNases. From left to right: Crude DNase (bovine colostrum), DNase I (bovine pancreas), DNase II (bovine spleen), DNase II (hog spleen). AP: Application points.

DISCUSSION

The principle of agar diffusion has been used successfully in assays of proteinases, lipases, lecithinases, amylases, phosphatases, DNases and other enzymes of bacterial and non-bacterial origin (*Sandvik* 1962, *Fossum et al.* 1965, *Schill & Schumacher* 1972, *Fossum & Whitaker* 1974, *Liven* 1976 and *Gudding* 1979). These methods are sensitive and simple, and they allow the analyses of small amounts of crude enzymes. *Liven* found agar diffusion tests suitable for measuring DNase activity in pig intestinal content. In the present study the DNase activity of samples of colostrum, milk and blood serum from different species has been measured with a modified TDA. It should be emphasized that the DNases were demonstrated in colostrum from all species examined, and the concentrations were generally higher than those of DNases of blood sera from the same species.

In most of the previous studies of DNases the enzyme activity has been recorded as the increase in absorbance at 260 nm due to DNA hydrolysis. Due to the phenomenon of hypochromism, results given by spectrophotometry may be difficult to interpret, and dilutions of both the enzyme and of the reaction mixture are necessary in order to get reliable results (*Liven*).

The sensitivity of the agar diffusion method is of the same order as that of the spectrophotometric method for assays of DNases from bovine and human colostrum and DNase II from hog and bovine spleen, respectively (Table 3). The application of a modified TDA seems especially favourable in assays of DNase I. Consequently, in examinations of normally occurring DNases and especially when series of samples with crude DNases are to be examined, the TDA is advantageous if the composition of the agar is adjusted according to the properties of these enzymes.

The most essential factor to consider when preparing the TDA for examinations of DNases in milk and serum is the pH of the agar. The DNases from all species, except dog and cat, had a pH optimum of 5.0 and 5.5, respectively, which is similar to the commercially available preparations of DNase II. These results are in fairly good accordance with those of *Bernandi & Griffé* (1964) and *Cordonnier & Bernandi* (1968). By spectrophotometry they found a pH optimum of hog DNase II of 4.8 and 4.9, respectively. However, as there is no sharp pH optimum

for any of these DNases, it may be more correct to use the term "pH range for optimal activity" instead of "pH optimum".

As Tris-maleate buffer is found to be disadvantageous in analyses of these DNases and, also, as phosphate buffer has an inhibitory effect on spleen DNases (*Bernandi & Griffé*), there seems to be a lack of a suitable buffer system in the pH area 5.5 to 7.0. At pH 5.5 the activity of all the DNases tested in the present study may be characterized as satisfactory. Consequently, the adjustment of the pH of the TDA to 5.5 by use of an acetate buffer is recommended in examinations of acid DNases in serum and mammary secretions, and as concluded by *Liven*, this pH is also suitable for agar diffusion analyses of DNases present in the pig's intestine.

The enhancement of the activity of DNase II by EDTA as recorded by *Bernandi & Griffé* was confirmed when the commercial hog DNase II was analyzed by the spectrophotometric method. However, this effect was less evident for bovine DNase II and the DNase of bovine and human colostrum, and in analyses of DNases from bovine colostrum, milk and serum, the exclusion of EDTA influenced the sensitivity of the agar diffusion method very slightly.

All DNases tested in the present study prefer native DNA as substrate, and this is in agreement with the data presented by *Bernandi & Griffé* who examined hog DNase II. However, in the agar diffusion method this property cannot be taken sufficiently into account as the preparation of TDA with unheated DNA has practical disadvantages (*Gudding*).

The effect of heat upon the DNases concentrated from bovine colostrum and serum (Fig. 2) was comparable with the results of *Bernandi & Griffé* who found that 62°C for 20 min gave a 50 % thermal inactivation of hog DNase II. The normally occurring DNases are thermo-labile, and this property is one of the most important ones in distinguishing these DNases from DNases produced by most staphylococci and streptococci (*Erickson & Deibel* 1973, *Gudding*).

The isoelectric focusing experiments indicate a similarity between bovine DNase II and the DNase from pancreas of the same species (bovine DNase I) and the DNase from the same tissue of the pig (hog DNase II), respectively. The observation of a difference in activity in the 2 DNase fractions from bovine colostrum may be an indication that the DNases present in

biological fluids comprise DNases from different tissues and with different properties.

However, the pH range for optimal activity, the enhancement of activity in the presence of EDTA, the preference of native DNA, the thermostability and the similar isoelectric pattern, indicate that the DNA-hydrolyzing enzymes which have been detected in serum, colostrum and milk are mainly acid spleen DNases like the ones described by *Bernandi & Griffé* and *Cordonnier & Bernandi.*

The great variation in the concentrations of DNases recorded in samples from different species may reflect real differences. However, another reasonable explanation may be the presence of inhibitors or even activators which determine the measurable activity of the enzyme. Such substances have been described, but the significance of the endogenous inhibition or activation factors is unknown (*Lesca & Paoletti* 1969, *Colombara et al.* 1971). As shown in the present study antibodies against some bacterial DNases did not inhibit the normally occurring DNases.

The biological role of DNases is unclear. However, there is increasing evidence for the assumption that some DNases are involved in the cell division and multiplication (*Slor & Lev* 1971). Although the average concentration of DNases in serum of calves were higher than that of cows, the difference is so small that no conclusion can be drawn from this observation.

The pH range for optimal activity and in particular the thermostability of the enzymes are the most important properties for distinguishing the normally occurring DNases from DNases produced by certain bacteria.

REFERENCES

Bernandi, G.: Spleen acid deoxyribonuclease. *In* Boyer, P. D., ed.: The Enzymes 1971, *4*, 271—287.

Bernandi, G. & M. Griffé: Studies on acid deoxyribonuclease. II. Isolation and characterization of spleen-acid deoxyribonuclease. Biochemistry 1964, *3*, 1419—1426.

Bernandi, G., A. Bernandi & A. Chersi: Studies on acid hydrolases. I. A procedure for the preparation of acid deoxyribonuclease and other acid hydrolases. Biochim. biophys. Acta (Amst.) 1966, *129*, 1—11.

Colombara, E., M. Ihl & M. A. Tagle: Serum deoxyribonuclease I and its inhibitor in growing and pregnant rats. J. Nutr. 1971, *101*, 1525—1530.

Cordonnier, C. & G. Bernandi: A comparative study of acid deoxyribonucleases extracted from different tissues and species. Canad. J. Biochem. 1968, *46,* 989—995.

Erickson, A. & R. H. Deibel: Production and heat stability of staphylococcal nuclease. Appl. Microbiol. 1973, *25,* 332—336.

Fossum, K., O. Lyngset & J. Aamdal: Quantitative determination of proteolytic, lipolytic and haemolytic activities in semen of some animal species. Acta vet. scand. 1965, *6,* 318—328.

Fossum, K. & J. R. Whitaker: Simple method for detecting amylase inhibitors in biological materials. J. Nutr. 1974, *104,* 930—936.

Gudding, R.: The demonstration and characterization of deoxyribonucleases of streptococci group A, B, C, G and L. Acta vet. scand. 1979, *20,* 102—121.

Lachica, R. V. F., C. Genigeorgis & P. D. Hoeprich: Metachromatic agar-diffusion methods for detecting staphylococcal nuclease activity. Appl. Microbiol. 1971, *21,* 585—587.

Laskowski, M.: Deoxyribonuclease I. *In* Boyer, P. D., ed.: The Enzymes 1971, *4,* 289—311.

Lesca, P. & C. Paoletti: A protein inhibitor of acid deoxyribonucleases. Proc. nat. Acad. Sci. (Wash.) 1969, *64,* 913—919.

Liven, E.: The applicability of agar-diffusion test in measuring deoxyribonucleases in pig intestinal content. Acta vet. scand. 1976, *17,* 244—254.

Sandvik, O.: Studies on casein precipitating enzymes of aerobic and facultatively anaerobic bacteria. Thesis, Veterinary College of Norway, Oslo 1962, 116 pp.

Sandvik, O.: The occurrence of antibodies against staphylococcal deoxyribonucleases in blood sera from different species. Acta vet. scand. 1974, *15,* 631—635.

Schill, W. B. & G. F. B. Schumacher: Radial diffusion in gel for micro determination of enzymes. I. Muramidase, alpha-amylase, DNase I, RNase A, acid phosphatase and alkaline phosphatase. Analyt. Biochem. 1972, *46,* 502—533.

Slor, H. & T. Lev: Acid deoxyribonuclease activity in purified calf thymus nuclei. Biochem. J. 1971, *123,* 993—995.

SAMMENDRAG

DNaser i melk og blodserum fra forskjellige arter.

DNaser ble påvist i prøver fra kolostrum og blodserum fra menneske og forskjellige husdyr. DNaseaktiviteten var høyest i prøver fra hund og katt og lavest i prøver fra geit, hest, gris og sau. I motsetning til DNaser produsert av visse bakterier, var disse enzymene termolabile og aktiviteten var størst i pH-området 5,0 til 5,5.

En modifikasjon av et agarmedium beskrevet for påvisning av bakterielle DNaser ble funnet å være velegnet ved undersøkelse av DNaser fra kolostrum, melk og serum.

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Reprints may be requested from: Roar Gudding, the National Veterinary Institute, Postbox 8156 Dep., Oslo 1, Norway.