ginine-sensitive enzymes may not hydrolyze this bond. Considering this evidence, it is suggested that the phosphoproteins might have a hydrophilic interaction with liver and pulpal alkaline phosphatases, similar to that of homoarginine. An alkaline phosphatase hydrolyzes a variety of compounds having the phosphomonoester linkage at alkaline pH. The optimum pH for the bovine intestinal enzyme acting on p-nitrophenyl-phosphate and phosphoserine was observed at 9.8 and 8.7, respectively. However, when dentine phosphoprotein and phosvitin served as substrates, the optimum pH-value for these reactions was between 5.5 and 6.0. This result also coincided with observations on human placental alkaline phosphatase with phosvitin and casein[2]. In the alkaline pH range (pH 8–9), the phosphatase activity was 50% for dentine phosphoprotein and 30% for phosvitin compared with that observed at the optimum pH. In studies on the phosphatases of rat calvaria[15] acting on different substrates, 2 activity peaks, one in the acid and the other in the alkaline pH range, were observed with p-nitrophenyl-phosphate and phosphoserine, but casein as a substrate was attacked only at acid pH. This result shows that the calvaria alkaline phosphatase could not hydrolyze the phosphoester linkage of phosphoprotein. Our present study of protein phosphatase activity by alkaline phosphatases from different organs provides new information on the substrate specificity of the enzymes.

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Purification of a nuclease from human serum[1]

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Summary. The purification procedure for a nuclease from human serum is described. It includes ammonium sulfate precipitation, chromatography on DEAE-Sephadex and on Sephacryl-S 200, and preparative electrophoresis. The enzyme, purified about 2000-fold, is homogeneous in a sodium dodecyl sulfate electrophoretic system, where it has a mol.wt of 78,000. The pH optimum lies around pH 6.5; it is a sugar-nonspecific endonuclease.

Ribonuclease and deoxyribonuclease activities[3-12] have been described in mammalian and human serum. Inhibitors of these enzymes in the serum have also been shown[13,14]. The variation of such enzymatic activities in some diseases has also been studied. The results are sometimes contradictory; both constant[6] and decreased[15] deoxyribonuclease activities have been observed during malignant disease. We therefore decided to purify the different nucleases of human serum in order to define the characteristic conditions for the activity determination of each enzyme and to yield meaningful results. In the following paper the purification of a sugar-nonspecific nuclease from human serum is described.

Material and methods. Materials were obtained as follows: Deoxyribonuclease I (2000 U/mg) (EC 3.1.4.5), RNA yeast, PM2 DNA, standard proteins for SDS electrophoresis from Boehringer Mannheim GmbH (FRG); herring sperm DNA, prepared according to Zahn et al.[16], was a gift from H. Mack Illertissen (Germany); (^{3}H) *E.coli* DNA was prepared as described[9]. Sephacryl S 200 Superfine and DEAE Sephadex A 25 from Pharmacia Fine chemicals AB, Uppsala (Sweden); all other reagents were from the highest analytical grade as supplied by Serva Heidelberg (FRG) and Merck Darmstadt (FRG).

Heat denatured DNA was prepared by heating native DNA for 10 min to 100 °C in a boiling water bath and chilling in ice. Human serum was obtained from healthy donors.

Nuclease activity was routinely assayed, unless otherwise stated, by adding a 20-µl sample to an incubation mixture consisting of 200 µl 0.05 M Tris-HCl pH 7.5, 10 mM MgCl; 50 µl herring sperm DNA (500 µg/ml in 0.05 M NaCl); 40 µl (^{3}) *E.coli* DNA (10,000 cpm). This mixture was incubated for 30 min at 37 °C, then it was put into an ice bath and 200 µl ice cold 1.2 N trichloroacetic acid added. The mixture was allowed to stand in ice for 15 min, and after centrifugation the radioactivity of the supernatant was determined by liquid scintillation counting in 2 ml Aquasol. The test was standardized with deoxyribonuclease I. The in situ detection of deoxyribonuclease in DNA-containing polyacrylamide gels after micro-disc-electrophoretic separation was performed as described elsewhere[9,10] Protein concentration was determined by the method of Lowry et al.[17]. Sodium dodecylsulfate electrophoresis was performed as described by Laemmli[18]. Gels contained 10%

Purification of the sugar-nonspecific nuclease from human serum

Purification step	Specific activity (U/mg)
Serum	0.042
Ammonium sulfate (20%–50%)	0.057
DEAE Sephadex A 25	39.8
Sephacryl S 200	74.6
Electrophoresis	(Protein concentration too low)

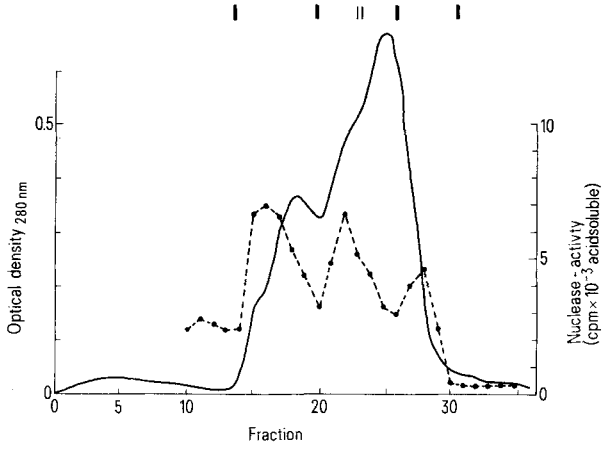

Fig. 1. Chromatography of DEAE Sephadex A 25 fractions from human serum on Sephacryl S 200. The experimental conditions are described in the text. The nuclease activity (● - - - ●) and the absorbance at 280 nm (——) were recorded.

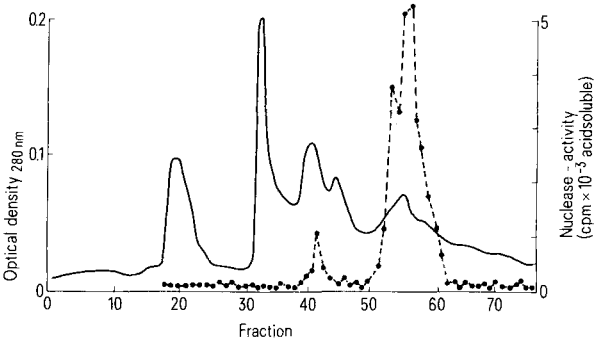

Fig. 2. Preparative polyacrylamide gel electrophoresis of Sephacryl S 200 fractions from human serum. The experimental conditions are described in the text. The nuclease activity (● - - ●) and the absorbance at 280 nm (——) were recorded.

acrylamide. Bovine serum albumin and RNA polymerases were used as standards for the molecular weight estimation. The analysis of breakage of PM 2 DNA was performed according to Paoletti et al.[19].

Results and discussion. Purification of the serum nuclease. Solid ammonium sulfate was added at 4 °C to human serum with stirring to achieve 20% saturation. After 2 h the solution was centrifuged and the supernatant was recovered. This supernatant was brought to 50% saturation. After 2 h at 4 °C the resulting precipitate was collected by centrifugation and dissolved in 50 mM Tris-HCl pH 7.5 and dialyzed against the same buffer.

This sample was then applied to a DEAE Sephadex A 25 column (20×2 cm) previously equilibrated with 50 mM Tris-HCl pH 7.5. After extensive washing with the same buffer until no protein was eluted from the column the nuclease was eluted with 0.35 M NaCl in the same buffer. Samples of 5 ml were collected, tested for nuclease activity as described, and the fractions containing activity were pooled. The eluate from the DEAE Sephadex column was then concentrated with 15% polyethyleneglycol to a final volume of about 10 ml. This concentrated protein solution was put on to a Sephacryl S 200 column (100×2.5 cm) previously equilibrated with 0.08 M Tris-Phosphate pH 6.9 and elution was carried out with the same buffer. 4-ml aliquots were collected and tested for enzyme activity

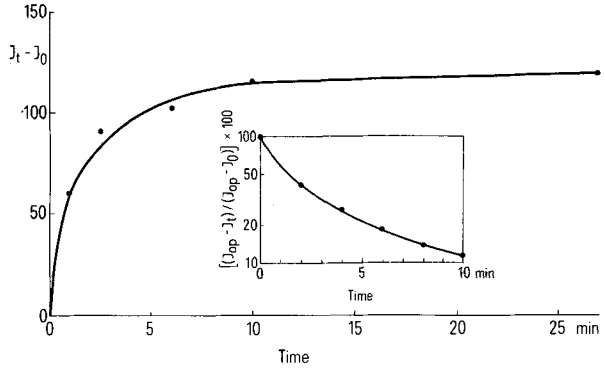

Fig. 3. Kinetics of opening of closed PM 2 DNA circles by human serum nucleases. 100 µl of purified neutral human serum DNase were added to 0.9 ml of substrate (4 µl/ml of PM 2 DNA in 0.1 M Tris-HCl pH 7.4, mM $MgCl_2$, 1 mM $CaCl_2$). At the times indicated, 0.2-ml samples of the reaction mixture were withdrawn and added to 1.8-ml ethidium bromide solution (1 µg/ml in 10 mM Tris-HCl pH 7.4; 20 mM NaCl, 5 mM EDTA). Fluorimetric measurements were performed as described in 'Materials and methods'. $J_o = 100$; $J_{op} = 230$. The percentage of closed circles remaining is given by $[(J_{op} - J_t)/(J_{op} - J_o)] \times 100$.

(fig. 1). The active fractions were pooled in 3 parts and the middle one (fig. 1, II) used for further purification. These pooled fractions were then applied to a preparative electrophoresis apparatus (Ultraphor Colora Messtechnik, Lorch, FRG) with continuous elution. Separation gel 60 ml, 0.38 M Tris-HCl pH 8.9, 7.5% acrylamide; spacer gel 25 ml, 0.059 M Tris-H_3PO_4 pH 6.9, 2.5% acrylamide. Separation was done at constant power (40 W) for 36 h with the anode at the bottom. Elution was done with 0.124 M Tris-HCl pH 8.1 at a pump rate of about 0.5 ml/min and 5 ml fractions were collected and nuclease activity was determined (fig. 2). The whole purification procedure is summarized in the table. We estimate that the enzyme was purified more than about 2000-fold. An exact calculation is not possible because there are additional nuclease activities present in human serum, as shown by the in situ detection of deoxyribonucleases in DNA-containing polyacrylamide gels. In addition, the protein concentration of the final preparation is too low to give exact values for the calculation of the specific activity.

Purity of the preparation. When the final preparation is analyzed on polyacrylamide gels containing sodium dodecyl sulfate only 1 band is detectable. Also using the in situ detection of deoxyribonucleases in DNA-containing acrylamide gels after electrophoretic separation only 1 nuclease activity could be detected, corresponding to only 1 protein band if a parallel gel were stained with Coomassie blue. From these results one can deduce a high purity of the final preparation.

Estimating the molecular weight of the enzyme from the linear relationship between electrophoretic mobility in sodium dodecyl sulfate acrylamide gels and the logarithm of the molecular weights of standard proteins, 1 sharp protein band, could be detected, corresponding to a mol.wt of about 78,000. Estimation of the mol.wt from Sephacryl S 200 chromatography gives a mol.wt of the same order of magnitude. So it is probable that the protein characterized by a mol.wt of 78,000 represents the active enzyme and not a subunit.

Properties of the enzyme. The purified final preparation was stable at −20 °C for at least 1 month. Using 0.05 M phosphate or 0.1 M Tris-HCl buffer with native DNA as substrate the enzyme shows a broad pH optimum around

pH 6.5. The optimal temperature was about 40 °C. Native DNA, denatured DNA, and RNA are digested by the pure enzyme. The highest activity, twice as high as with native DNA, was detected with denatured DNA. Using closed circular PM 2 DNA, a time dependent increase of the fluorescence in the presence of ethidium bromide during the action of the serum nuclease can be monitored (fig. 3). According to Paoletti et al.[19] this is due to a single endonucleolytic scission in the DNA molecule opening the closed circular DNA. From these data an endonucleolytic action of the enzyme seems probable, but the kinetics observed are nonlinear, i.e. the rate of the logarithmic change of fluorescence decreases with time (inset fig. 3). This deviation from linearity could be the result of a change in the initial velocity of the reaction or a parallel decrease in fluorescence due, for example, to concomitant exonuclease action. From the data available this cannot be decided yet.

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Galactose-l-phosphate uridyl transferase activity in red cells of various animal species

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Summary. Monkey red cells were chosen as controls in tests of human red cell galactose-l-phosphate uridyl transferase, after comparing activities and isozyme patterns of the enzyme from several domestic or laboratory-bred species.

In order to estimate the day-to-day variability in an assay for galactose-l-phosphate uridyl transferase in human red cells[1], we include a 'standard' sample-either fresh cells from the same daily donor or aliquots from a sample of frozen packed cells. Monitoring the between-test reproducibility is important when the assay is used to define the transferase phenotype, e.g., to distinguish carriers of galactosemia from noncarriers. Daily donation is inconvenient, however, and the stability of the enzyme in packed cells is questionable. We therefore considered using animal red cells for the standard and assessed them as a replacement as described below.

We required a transferase with activity similar to that in human red cells, for which the assay was designed. Transferase activities have already been measured in several species, including rats[2-4], hamsters[3], rabbits[3], kangaroos and other marsupials[5-7], cats[8], sea lions[9], monkeys[3,10] and mice[11,12]. Although activities were measured by different methods, and comparisons were difficult, they appeared species-specific, with higher values when the young were tested[3-4].

In our search for a compatible source, we selected mature animals of species which were readily available. We also tested the stability of transferase in stored samples from species with the desired levels of activity.

Materials and methods. Blood was drawn from mature animals of the following 8 species (table 1) bred or maintained at the Department's laboratory farm: Hartley guinea-pigs, Wistar rats, Nys: (FG) rabbits, retired riding horses, Suffolk sheep, Holstein cows, green monkeys and mixed breed goats. A few comparisons were made with young of the species, including a newborn lamb, a newborn calf, and a 2-month-old kid.

Samples were drawn by cardiac puncture from the monkeys, rats and guinea-pigs, with the rats and guinea-pigs having been immobilized by exposure to carbon dioxide, and the monkeys immobilized by intramuscular injection of phencyclidine hydrochloride (0.7 ml of a 20 mg/ml solution). Rabbits were bled from the marginal ear vein, and the sheep, cows, horses, and goats were bled by jugular puncture.

Cells were hemolyzed by freezing and thawing 0.5 ml aliquots of the blood samples 3 times in a Dry Ice-ethanol bath. 'Ghosts' were separated by centrifugation at $1400 \times g$ for 5 min.

The reaction mixture was combined in the following order just before use: 0.6 ml of 10 mM uridine-5'-diphosphoglucose (UDPG); 0.6 ml of 27 mM galactose-l-PO_4; 0.8 ml of freshly prepared 6 mM nicotinamide adenine dinucleotide phosphate (NADP); 2.0 ml of freshly prepared 0.75 M Tris-acetate buffer, pH 8.0; 0.8 ml of saturated digitonin; 0.09 ml of 27 mM disodium ethylene diaminetetraacetic acid; 0.13 ml of 0.1 M magnesium chloride; and 1.0 ml of demineralized water. The total volume was 6.0 ml (enough for 9 samples). The stock solutions were made earlier, except as noted, and freezing at −20 °C for as long as 6 weeks did not affect their stability.

The reaction mixture was divided into 5 0.2 ml portions which were warmed at 37 °C for 3-5 min. At 30-sec intervals, 20 µl of saline (0.85%) or hemolysate was added