suggests that antibodies against antigen Dd may be universally present in man and what the latter detects might only be their raised amounts. Should it indeed be so then it would be interesting to seek an explanation for it.

1 This work was supported through U.G.C. Grant No. F.23-230/75/SR II. H.K. participated in this study, first as J.R.F. of U.G.C. and then as S.R.F. of I.C.M.R. We are grateful to Dr Baruch S. Blumberg for his invaluable suggestions and to Professor H. Walter for the gift of IgG, IgM and IgA immune sera.

2 Author for reprint requests: P.K.S., Department of Anthropology, Saugar University, Sagar 470003, India.

3 Shrivastava, P.K., Dermatologica *144* (1972) 310.
4 Kaur, N., and Shrivastava, P.K., Dermatologica *149* (1974) 559.
5 Kaur, H., Sehajpal, P.K., and Shrivastava, P.K., Acta anthropogenet. *1* (1977) 28.
6 Rose, N.R., and Bigazzi, P.E., Methods in Immunodiagnosis. John Wiley, New York 1973.
7 Brzecka, K., and Gajos, E., Arch. Immun. Terap. doświad. *17* (1979) 314.
8 Mancini, G., Carbonara, A.O., and Heremans, J.F., Immunochemistry *2* (1965) 235.
9 Berrens, L., Dermatologica *134* (1967) 432.
10 Kaur, H., Sehajpal, P.K., Khanna, A.K., and Shrivastava, P.K., Hum. Hered. *30* (1980) 204.
11 Gajdusek, D.C., Nature, Lond. *179* (1969) 666.
12 Kaur, H., Kaur, K., and Shrivastava, P.K., Acta anthropogenet. *2* (1978) 61.

Endotoxin-like activity in *Pseudomonas paucimobilis* (group IIK biotype 1) and *Flavobacterium multivorum* (group IIK biotype 2)

D.L. Smalley

Department of Pathology, The University of Tennessee Center for the Health Sciences, Memphis (Tennessee 38163, USA), 19 May 1982

Summary. 7 strains of *Pseudomonas paucimobilis* and 7 strains of *Flavobacterium multivorum* (formerly groups IIK biotype 1 and biotype 2, respectively) showed endotoxin-like activity in vitro by the limulus amoebocyte lysate test.

Pseudomonas paucimobilis and *Flavobacterium multivorum* are typically yellow-pigmented, non-fermentative, gram-negative bacilli which were previously designated as groups IIK biotype 1 and biotype 2[1]. These organisms have often been isolated from human clinical specimens, hospital environments, and other sources[2,3]. However, these microbes have recently been reported to be causative agents of leg ulcer infection[4], of bacteremia[5,6] and of peritonitis[7]. Because of their roles as opportunistic pathogens, several investigations have been undertaken to better understand these bacteria. In one study, the cellular fatty acid composition of both groups was reported[8], and another study revealed that *P. paucimobilis* (biotype 1) was not immunologically related to *P. aeruginosa* or *P. cepacia* although complement-mediated bactericidal immunity was demonstrated[9]. Because of the need to further understand the pathogenesis of these organisms, especially since they are known to cause bacteremia[5,6], the present study was undertaken to test for the production of endotoxin in both groups of organisms.

Methods. 7 strains of *P. paucimobilis* and 7 strains of *F. multivorum* (kindly provided by Dr Robert Weaver, Centers for Disease Control, Atlanta, GA) were grown overnight at 35 °C on trypticase soy agar (BBL Microbiology Systems, Cockeysville, MD). The bacteria were harvested by washing the surface of each plate with 3 ml of sterile, pyrogen-free water. The cell suspensions were washed twice in the pyrogen-free water and then diluted to 50% transmission at 535 nm on a spectrophotometer. Bacterial suspensions were further diluted to yield $1:10^5$, $1:10^6$, and $1:10^7$ bacteria and were tested at each of these concentrations. Drying of aliquots of cells was done at 60 °C in an oven until the weights were constant.

Endotoxin detection was done by the limulus amoebocyte lysate test (Mallinckrodt, Inc., St. Louis, MO) using a microdilution procedure previously described[10]. Briefly, lysate was prepared with pyrogen-free water as recommended by the manufacturer, and equal volumes of lysate and bacterial suspensions were added in triplicate to microtiter plate wells, mixed, and incubated at 37 °C for 1 h. In order to determine the presence or absence of gelation, 0.01 ml of aqueous crystal violet was added to each well and then the lysate was viewed as previously described[10].

In addition, sensitivity of the lysate was confirmed using a standard reference endotoxin EC-2 (Mallinckrodt, Inc., St. Louis, MO) which yielded a minimum lysate sensitivity of 0.125 ng. EC-2 endotoxin was also run with each bacterial strain as a positive control.

Results and discussion. All 7 strains of both *P. paucimobilis* and *F. multivorum* yielded positive limulus tests at a concentration of $1:10^5$ bacteria per ml. 4 out of 7 *P. paucimobilis* strains exhibited positive endotoxin-like activity at $1:10^6$, and 5 of 7 *F. multivorum* strains yielded positive reactions at $1:10^6$ bacteria per ml. Thus, members of groups IIK biotype 1 and biotype 2 were highly reactive in in vitro gelation of lysate with the reference endotoxin. The sensitivity of the lysate test was 0.125 ng, and the present bacteria containing endotoxin-like substance is shown in the table.

Although only whole cells were tested, all strains of both genera were consistently positive in the lysate assay at a $1:10^5$ dilution. This suggests that these bacteria do possess some in vitro biological properties associated with endotoxin-like activity. These data are consistent with cellular fractionations by gas-liquid chromatography which detected hydroxy fatty acids in both genera. The difference of

Endotoxin-like activity of *Pseudomonas paucimobilis* and *Flavobacterium multivorum*

	P. paucimobilis	*F. multivorum*
Endpoint dilution*	10^5	10^5
Dry weight of bacteria, ng/ml	0.50	0.45
'Endotoxin' in bacteria, %	25	28

*Results expressed at the highest dilution of bacterial suspension at 50% transmission in which 100% of strains induced gelation.

endotoxin-like substance in the bacterial genera appeared minimal. The chromatography analysis[8] did, however, reveal a large percentage of branched-chain fatty acids in *F. multivorum* that were not present in *P. paucimobilis* and may account for the difference in activity. The presence of endotoxin-like activity also may partially explain the virulence of these organisms during infection in man. This evidence suggests that additional studies, including the pyrogenic assay and Shwartzman reaction, may be helpful in further defining the endotoxicity of these organisms.

1 Tatum, H.W., Ewing, W.H., and Weaver, R.E., in: Manual of clinical microbiology, 2nd edn, p.270. American Society for Microbiology, Washington, D.C. 1974.
2 Holmes, B., Owen, R.J., Evans, A., Malnick, H., and Willcox, W.R., Int. J. Syst. Bact. *27* (1977) 133.
3 Reinhardt, D.J., Adams, D., Dickson, P., and Traina, V., Devs ind. Microbiol. *20* (1979) 705.
4 Peel, M.M., Davis, J.M., Armstrong, W.L.H., Wilson, J.R., and Holmes, B., J. clin. Microbiol. *9* (1979) 561.
5 Slotnick, I.J., Hall, J., and Sacks, H., Am. J. clin. Path. *72* (1979) 882.
6 Southern, P.M., and Kutscher, A.E., J. clin. Microbiol. *13* (1981) 1070.
7 Dhawan, V.K., Rajashekaraiah, K.R., Metzger, W.I., Rice, T.W., and Kallick, C.A., J. clin. Microbiol. *11* (1980) 492.
8 Dees, S.B., Moss, C.W., Weaver, R.E., and Hollis, D., J. clin. Microbiol. *10* (1979) 206.
9 Smalley, D.L., and Ourth, D.D., J. clin. Microbiol. *12* (1980) 439.
10 Prior, R.B., and Spagna, V.A., J. clin. Microbiol. *13* (1981) 167.

Aging of the erythrocyte. XV. Isoosmotic lysis times

G. Bartosz[1]

Department of Biophysics, Institute of Biochemistry and Biophysics, University of Łódź, 90-237 Łódź (Poland), 1 June 1981

Summary. Isoosmotic lysis times of various density (age) fractions of human, pig and ox erythrocytes in glycerol and ammonium chloride solutions decreased with increasing cell density.

The question of age-releated changes in erythrocyte osmotic properties seems to be of importance since simple tests based on osmotic fragility are employed in creening for red cell membrane disorders[2-5] and in some cases the observed alterations in values of appropriate parameters might be due to changes in the mean age of erythrocyte populations studied. Data concerning this point are contradictory: some authors found increased osmotic fragility of in vivo aged erythrocytes of serveral mammalian species[6-9] but others did not observe any significant correlation between osmotic fragility and cell age[10-12]. In those studies various techniques of red cell fractionation according to age were employed and the cells were of different species origin. This report describes isoosmotic lysis of erythrocytes from 3 mammalian species, separated according to age, by the same method.

Material and methods. Human, pig and ox blood was taken into citrate. Erythrocytes were separated according to density (and age)[13] by the method of Murphy[14].

Isoosmotic lysis times (ILT_{50}) were determined by introducing 50 µl of appropriately diluted erythrocyte suspension in phosphate-buffered saline to 1.5 ml of 300 mM glycerol or 163 mM NH_4Cl in a spectrophotometric cuvette and monitoring changes in optical density at 640 nm (initial value: 1). Control samples incubated in saline did not show changes in optical density greater than 0.01 during the incubation period. The time needed for a decrease in optical density to 0.5 was defined as ILT_{50} in the appropriate solution. The lysis was carried out at ambient temperature (20 ± 1 °C).

Results and discussion. Successful separation of erythrocytes from all species according to density (age) was demonstrated, among other tests, by an increase in the mean cell hemoglobin concentration in successive red cell fractions. The average ratio of this parameter in 17% bottom cells and in 17% top cells was 1.07 for ox erythrocytes, 1.11 for pig red cells and 1.18 for human cells. These values may reflect interspecies differences in separation efficiency of in the rate of age-related changes in the red cell hemoglobin concentration.

Absolute ITL_{50}-values averaged from all density fractions are given in table 1. They coincided with values obtained for non-fractionated erythrocyte samples, therefore there was no discernible effect of the separating centrifugation on the parameters studied. Glycerol lysis time was not determined for human erythrocytes since rapid lysis in this version of the test[2,3], precluded accurate measurements. Changes in ILT_{50} accompanying increasing cell density shown in table 2, demonstrate a significant acceleration of lysis in both sulutions for red cells of all species examined.

Table 1. ILT_{50} (sec) of erythrocytes from various species in glycerol and NH_4Cl solutions

Species	Glycerol Mean	SD	n	NH_4Cl Mean	SD	n
Human				155	31	5
Pig	450	96	5	111	35	6
Ox	830	264	5	156	61	6

Table 2. Relative ILT_{50}-values of different density fractions of erythrocytes (fraction 1, lightest cells; fraction 6, densest cells; mean ± SD, n = 5-6 (different individuals)

Fraction No.	Glycerol Pig	Ox	NH_4Cl Human	Pig	Ox
1	100	100	100	100	100
2	86 ± 8*	93 ± 9	78 ± 12*	95 ± 4	87 ± 10
3	79 ± 14*	82 ± 10*	77 ± 13*	91 ± 9	74 ± 12*
4	73 ± 17*	81 ± 12*	70 ± 19*	79 ± 8**	67 ± 10**
5	51 ± 9**	74 ± 10**	65 ± 16**	78 ± 10*	64 ± 12**
6	47 ± 10**	74 ± 6**	65 ± 19*	78 ± 13*	67 ± 14**

Statistical significance of differences with respect to fraction 1: * $p < 0.05$, ** $p < 0.01$ (one-tailed Student's t-test).