22 Rose, M. L., Parrott, D. M. V., and Bruce, R. G., Immunology *35* (1978) 415.
23 Smith, C. W., and Goldman, A. S., Pediat. Res. *2* (1968) 103.
24 Barlow, B., Santulli, T. V., Heird, W. C., Pitt, J., Blanc, W. A., and Schullinger, J. W., J. Pediat. Surf. *9* (1974) 587.
25 Diaz-Juoanen, E. P., and Williams, R. C., Clin. Immun. Immunopath. *3* (1974) 248.
26 Parmely, M. J., Beer, R. A., and Billingham, R. E., J. exp. Med. *144* (1976) 358.
27 Smith, J. W., and Schultz, R. D., Cell. Immunol. *29* (1977) 165.
28 Weiler, I. J., Hickler, W., and Sprenger, R., Am. J. Reprod. Immun. *4* (1983) 95.
29 Asano, K., Muramatsu, K., and Okamoto, K., Jpn. J. Parasit. *38* (1989) 106.

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Shape change of human blood platelets: Reliable and fast detection by quasi-elastic light scattering

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Abstract. Quasi-elastic light scattering has been used for the first time to obtain reliable information about the morphology of platelets under physiological conditions within a short time. By measuring two independent parameters (electrophoretic mobility and diffusion coefficient) it is possible to distinguish between different stages of shape change on the one hand, and between shape change and binding of particles to the platelet surface without shape change on the other hand.
Key words. Light scattering; electrophoresis; platelets; shape change; hyperlipaemia.

The initial events in the genesis of disorders like atherosclerosis[1,2], hypertension[3] or Raynaud's[4] disease are thought to be closely related to platelet shape change and release reaction. Although turbidimetric measurements of platelet aggregability proved to be very suitable in determining haemostatic disorders in routine clinical examinations[5], the investigation of platelet shape change requires more complicated techniques. The measurement of light transmission under the undefined optical conditions of the aggregometer cannot give quantitative results[6]. Even the simultaneous measurement of 90° light scattering in a lumi-aggregometer does not improve shape change measurement, since the intensity of the scattered ligth I decreases rapidly with the increase of the angle: for $\Theta = 10°$:$I = 2.8 \times 10^5$ pulses/s, $\Theta = 20°$:$I = 2.9 \times 10^4$ pulses/s, $\Theta = 90°$:I = 360 pulses/s, measured with a 30 mW HeNe-laser. Another disadvantage of aggregometry is the fact that hyperlipaemic plasma, i.e. plasma containing chylomicra, cannot be investigated. Furthermore, contamination of the sample with larger cells such as leukocytes or erythrocytes may cause big errors from the optical point of view regardless of their physiological interaction.

Quasi-elastic light scattering (QELS) does not suffer from these disadvantages. As it is a method of measuring the Doppler shift frequency of the light scattered by moving particles the basic theory differs very much from that underlying turbidimetric experiments[7].

It is possible to identify discoid, resting platelets in buffer solutions after gel-filtration or washing of the platelets, or directly in platelet-rich plasma (PRP), even in the presence of chylomicra or contaminations with other cells. The quasi-simultaneous measurement of electrophoretic and diffusion light scattering (EDLS) allows shape changes to be followed, i.e. to distinguish between different stages of bleb and pseudopod formation, and concomitant conversion from discoid to spherical bodies. The resultant parameters are the electrophoretic mobility μ, which is proportional to the surface charge density σ_e and to the zeta potential ζ, and the diffusion coefficient D, which is reversely proportional to the hydrodynamic radius R_H or the effective size of the cell. Both parameters change significantly during shape change. The electrophoretic mobility decreases by 40% and the diffusion coefficient decreases by about 50%. The change in electrophoretic mobility is caused by the formation of pseudopods, i.e. other parts of the membrane (open canalicular system) are exposed[8], which also increase the effective size. Therefore the thermal motion of activated cells is slowed down compared to that of discoid cells, which leads to a decreased diffusion coefficient. A similar effect on the electrophoretic mobility could be caused by simple binding of nonactivating substances to the platelet membrane, but in contrast to shape change this will only slightly affect the diffusion coefficient. Distinguishing between these two effects makes the simultaneous measurement of both parameters necessary.

Materials and methods

EDLS was applied to compare gel-filtered platelets (GFP) with PRP, discoid platelets at 37 °C with activated platelets at 20 °C, and to investigate the influence of chylomicra on platelets.

Blood was taken from young male volunteers (age 20–30 years), who were fully informed about the nature and the consequences of the study and who had not taken any medication for at least two weeks before the venipuncture. 50 ml of fresh whole blood was treated with citrate as an anticoagulant (15.75 g sodium citrate, 0.74 g citric acid per 500 ml distilled water) 1:10 (v/v). After centrifugation at 200 × g for 10 min the PRP was taken off. Platelets were separated from plasma proteins on a Sepharose 2B column (Pharmacia Fine Chemicals, Uppsala, Sweden). The platelets were eluted from the column with phosphate buffered saline (PBS). During all stepts of preparation the temperature was close to 37 °C and at the end of the preparation the PRP or GFP were kept undisturbed at 37 °C in a waterbath for about 30 min to recover from the manipulation. The pH of the solutions was maintained at 7.4. All chemicals for buffer solutions were obtained from Merck and were of p.a. quality. For experiments on the influence of chylomicra on platelets, blood was taken at 09.00 h from fasting donors as described above. After venipuncture the donor took a standardized breakfast with milk, butter and cream and gave blood again 2 hours later. PRP was prepared and compared to the PRP from the fasting state.

For scanning electron microscopy 400 µl of platelet suspension were fixed with 1 ml of 3% glutaraldehyde. For resting platelets it is highly recommended to prewarm the fixative to 37 °C and also to maintain this temperature for the first 30 min of fixation. For other temperatures an analogous procedure was necessary, when the temperature-dependent activation of platelets was studied.

In quasi-elastic light scattering experiments the Doppler shift of the scattered light caused by the movement of the particles is measured. The angle Θ between incident and scattered light is called scattering angle and is bisected by the scattering vector k. Particles moving in the direction of k produce a maximal frequency shift, whereas particles moving in a perpendicular direction do not shift the light at all. This happens because the shift frequency $\Delta\nu$ is proportional to the scalar product of the scattering vector k and the velocity vector v of the particles.

$$\Delta\nu = \mathrm{k} \cdot \mathrm{v}$$

The absolute value of k is given by $\mathrm{k} = (4\pi\mathrm{n}/\lambda_0)\sin(\theta/2)$. λ_0 is the wavelength of the laser in vacuum and n is the refractive index of the medium. The Doppler shift produced by a moving particle is so small (about 30 Hz) compared to the frequency of the laser light (about 10^{14} Hz), that no detector of sufficient resolution is available. Therefore the technique of light beating resulting from interference between shifted and unshifted light is the only practical way to detect the shift frequency.

The diffusion measurement (thermal, Brownian motion) is a homodyne experiment, i.e. shifted and unshifted light are components of the scattered light, because in a certain time interval there are particles which have a velocity component in direction of k, and particles which have not. In electrophoretic measurements all particles move according to the electric field (the electrophoretic cell is set up parallel to k so that the frequency shift is a maximum) and in order to have unshifted light a reference beam is guided around the sample (heterodyne experiment).

Figure 1 shows the experimental setup for EDLS measurements. The light of a HeNe-laser (NEC model GLG5360, 10 mW, wavelength $\lambda_0 = 632.8$ nm) is fo-

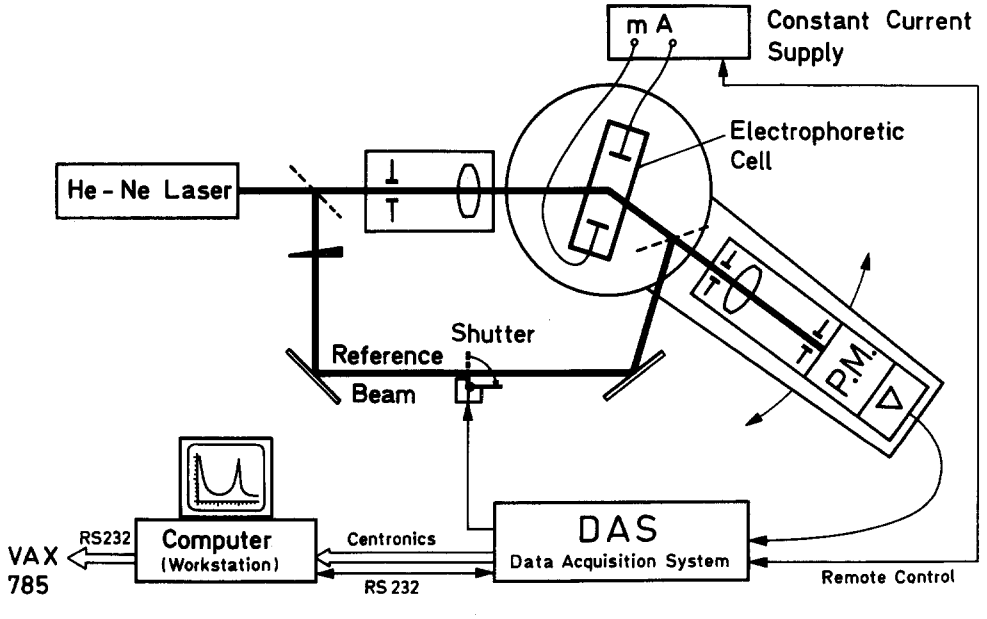

Figure 1. Schematic diagram of the instrument for the quasi-simultaneous measurement of electrophoretic- and diffusion light scattering (EDLS). The light from a He-Ne laser is focused onto the sample and the scattered light is detected by a photomultiplier. The PM output pulses are counted by the DAS, which sends the data to the computer for evaluation.

cussed into the sample cell, which is temperature-controlled. The scattered light is gathered into the photomultiplier by the detector optics and the resultant photocurrent is amplified and converted to voltage pulses, which are counted by an interactive data acquisition system[9]. This microcomputer also controls the experiment by switching the constant current supply of the electrophoretic cell and the shutter of the reference beam. Data are sent to a computer for further evaluation. Various input parameters can be easily changed and adapted to a given sample. This setup provides quasi-simultaneous measurements of electrophoretic mobilities and diffusion coefficients within a few minutes. Figure 2 shows the timetable for this kind of experiment.

Diffusion measurements can be carried out in the electrophoretic cell as well. This was proven by comparative measurements in a standard cuvette. The electrophoretic cell is a slightly modified version of the one designed by Smith and Ware[10]. The metal blocks are electrochemically coated with gold to make the surface inert. The illuminated volume is located within a narrow gap ($0.75 \times 2.5 \times 3.7$ mm) to obtain a high electric field strength. The platinized platinum electrodes prevent formation of big gas bubbles. Since the illuminated volume is far from the electrodes, electrolytic reaction products can hardly influence the sample under investigation. The sample volume is about 0.5 ml. To minimize electroosmosis, the surfaces of the spacers, as well as that of the windows surrounding the gap, are covered with a methylcellulose coating to obtain a neutral surface[11,12]. This tedious procedure is not necessary for relative measurements, but for experiments on platelets the glass windows were siliconized to prevent adhesion.

For evaluation of data, power spectra can be calculated with a gliding window Fast Fourier Transformation (FFT) algorithm or correlation functions by a software-correlator. Electrophoretic measurements are evaluated as power spectra with peaks centered at the shift frequency due to the surface charge density of the particles. Diffusion experiments with monodisperse samples give single exponential correlation functions (fig. 3). The characteristic value of the correlation function is the decay time, which is longer for big particles than for small ones, because they move more slowly. The decay time is inversely proportional to the diffusion coefficient and directly proportional to the hydrodynamic radius. If the sample is heterogeneous, i.e. there are particles of different sizes, the time analysis of the data results in a sum of exponential functions and so the correlation function will contain the decay rates of all differently sized particles. To cover a wide range of decay times in heterogeneous samples a logarithmic correlator is used. Furthermore, an indirect Laplace transformation of a correlation function allows the calculation of a size distribution in terms of intensity or volume with respect to the contribution of differently-sized particles to the signal[13].

Scanning electron microscopy was used to verify the interpretation of the light-scattering experiments, i.e. to visualize the shape change on the platelets determined by the changes of diffusion coefficient and electrophoretic mobility.

Results

As already pointed out, we were interested in morphological changes of human blood platelets during activation. In order to start with a well-defined reference sample, i.e. discoid, resting platelets, we took care to keep the platelets at about 37 °C during the taking of blood and the whole preparation process. We subsequently incubated the sample at 37 °C for 30 min so that the platelets could recover from manipulation. For the QELS measurements the following conditions turned out to give the best results: scattering angle $\Theta = 20°$; temperature T = 37 °C except for measurements of temperature-de-

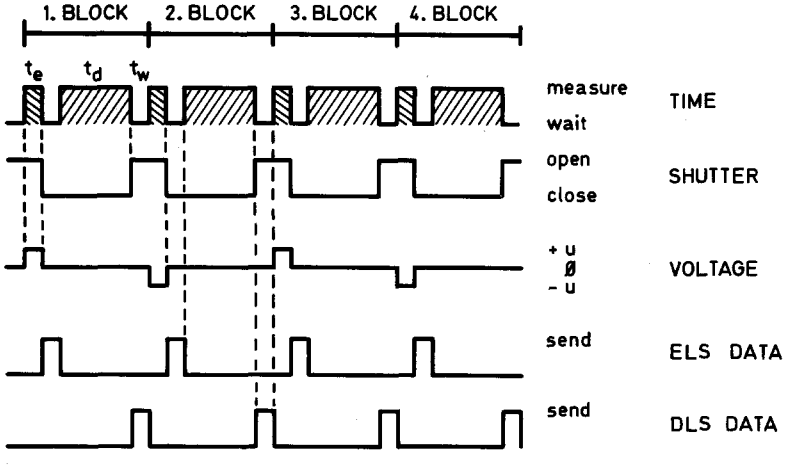

Figure 2. Timetable of a quasi-simultaneous EDLS measurement. During electrophoresis (ELS) ($t_e = 2$–5 s) the shutter of the reference beam is open and the voltage for the electrophoretic cell is switched on in contrast to the diffusion measurement (DLS) ($t_d >= 60$ s). Within a waiting time ($t_w = 5$ s) between ELS and DLS, data are sent from the DAS to the computer. In successive blocks the direction of the electric field is reversed. An average signal of 4 to 10 blocks gives results of good quality.

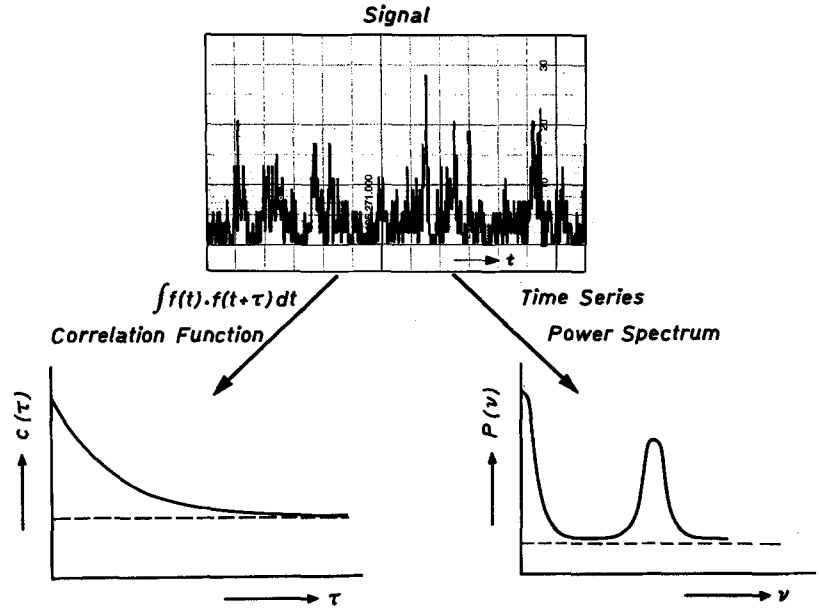

Figure 3. The signal – a stochastic number of voltage pulses proportional to the scattered light intensity – is evaluated 1) by a correlation function in homodyne experiments (diffusion), which is a time analysis of the data, or 2) by a power spectrum in heterodyne experiments (electrophoresis), which is a frequency analysis of the data. The decay time of the correlation function is indirectly proportional to the hydrodynamic radius and the shift frequency in the power spectrum is proportional to the electrophoretic mobility of the particles.

pendent changes; field strength E = 10–30 V/cm; conductivity of the solution L = 0.6–1 mS. Under these conditions erythrocytes and leukocytes do not interfere with the platelet signal, firstly, because they settle very quickly and secondly, because they scatter significantly only up to a scattering angle of $\Theta = 10°$, as elaborated in special experiments with media of appropriate density (data not shown). At $\Theta = 20°$ the scattering intensity of erythrocytes or leukocytes drops to the background level.

Lowering the temperature from 37 °C to 20 °C causes significant differences in the electrophoretic mobility (fig. 4a) and the diffusion coefficient (fig. 4b), which indicates a change in platelet shape. In this case the mean electrophoretic mobility decreases by 40% and the mean diffusion coefficient by 50%. According to the findings of Hourdille et al.[14] the decrease in the net negative charge, i.e. electrophoretic mobility of platelets, with activation, might be caused by the movement of GPI_b to the inside and $GPII_b/III_a$ to the outside of the plasma membrane. As the sialic acid content of GPI_b is much higher than that of $GPII_b/III_a$ the number of accessible negative surface charges would be considerably reduced. In order to find out whether these processes are responsible for the decreased electrophoretic mobility at 20 °C it would be necessary to use additional methods like immunofluorescence, because electrophoretic light scattering is not able to give information on the molecular scale.

These findings were confirmed by electron microscopy (fig. 5). Blood platelets are not homogeneous, and there are many possible reasons for their heterogeneity. Therefore the analysis of the correlation functions gives a dis-

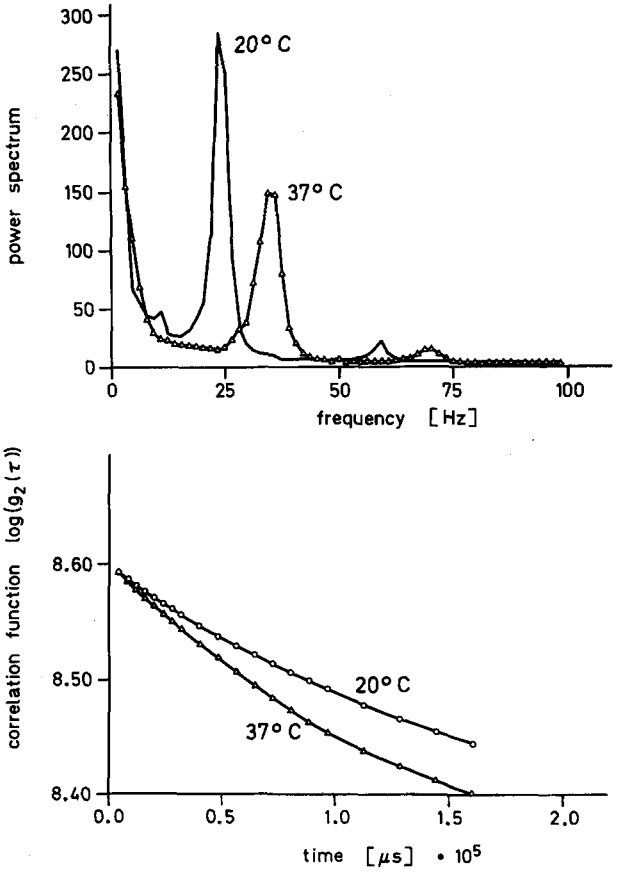

Figure 4. Cooling of PRP from 37 °C to 20 °C results in platelet shape change, that can be seen in a decrease in electrophoretic mobility (*a*) and in diffusion coefficient (*b*) indicating that the negative surface charge density of the platelets is reduced and the effective size increases because of the formation of pseudopods.

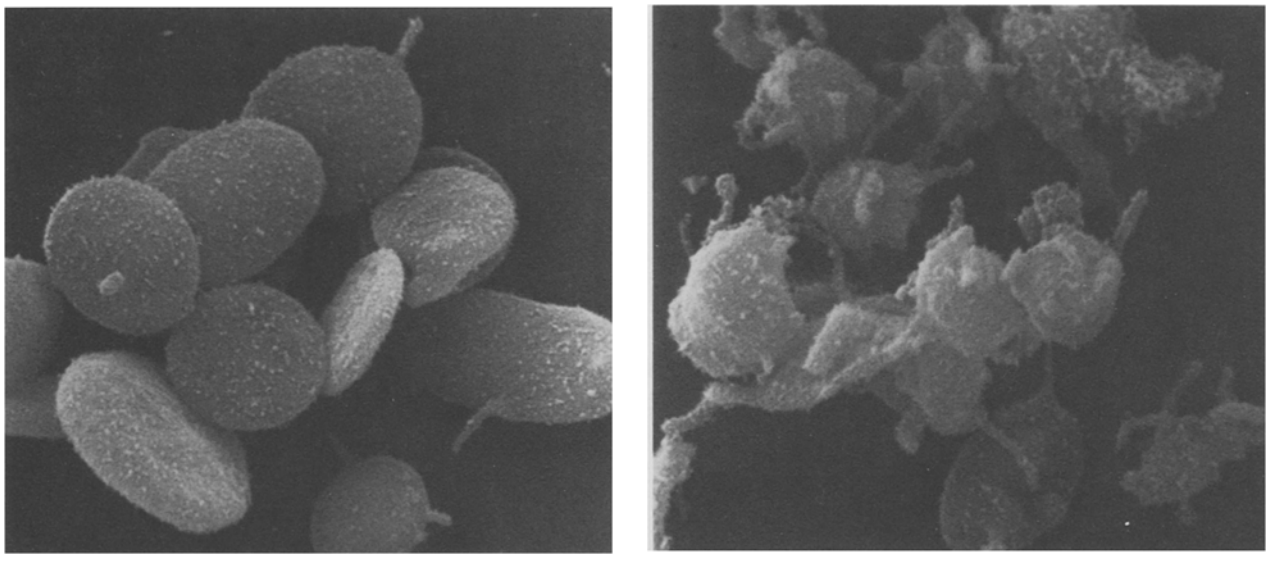

Figure 5. Scanning electron micrographs showing discoid, resting platelets at 37 °C (*a*) and activated platelets with a spherical body and pseudopods at 20 °C (*b*). The platelets (PRP) are fixed with 3% glutaraldehyde at the appropriate temperature.

tribution of platelet sizes with a maximum at the mean diffusion coefficient or hydrodynamic radius. The volume size distribution functions of discoid platelets at 37 °C (full line), and of activated 'spiny sphere' platelets at 20 °C (dashed line), showed big differences (fig. 6).

The electrophoretic mobility μ is proportional to the zeta potential ζ (Smoluchovski's equation).

$$\mu = \frac{\zeta \cdot \varepsilon_r}{\eta}$$

ε_r is the relative dielectric constant and η is the dynamic viscosity of the solution. Taking mean values for μ, η, and ε_r at 37 °C and 20 °C for PRP, the zeta potential ζ results in -23 mV and -26 mV, respectively (table). Although some assumptions have to be made, a rough estimation of the surface charge density σ_e can be carried out. A correction of the electrical double layer is not necessary, because it is only about 0.6 nm and is therefore very small compared to the long axis of the platelets (about 3 μm). With a simplified Gouy-Chapman equation[15]

$$\sigma_e = 2 \cdot [N \cdot \varepsilon \cdot k \cdot T/2000]^{1/2} \cdot I^{1/2} \cdot \sinh [e \cdot \zeta/2 \cdot k \cdot T]$$

the surface charge S of an unactivated platelet is 94000 and of an activated one is 85400. I is the ionic strength of the medium (0.3 mol/l) and the surface charge S is given by σ_e times the surface area divided by the electron charge. These results indicate that discoid, resting platelets in plasma have a higher surface charge density than platelets with a changed shape. This is contradictory to results of earlier studies, but the unphysiological preparation and measuring conditions published[16] (room temperature or below, physiological salt solution, observation of single platelets) may be responsible. Our results are not influenced by any aggregating agent[17], but give the change in surface charge density due to shape change only. We suspect that others started with platelets that were already activated before the addition of aggregating agents.

The number of ADP binding sites calculated by Hampton and Mitchell[16] is the same as the number of charges

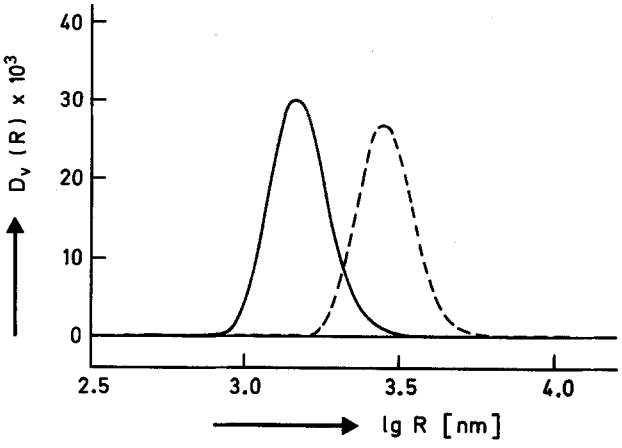

Figure 6. The comparison of the volume size distributions of discoid, resting platelets at 37 °C (full line) and of activated 'spiny sphere' platelets at 20 °C (dashed line) in PRP show the increase in effective size of the platelets because of shape change.

The table gives the mean values (n = 54) of the electrophoretic mobility μ, the dynamic viscosity η, the relative dielectric constant ε_r, the zeta potential ζ, the hydrodynamic radius R_H and the number of negative surface charges of the platelet plasma membrane in PRP at 37 °C (unactivated) and at 20 °C (activated).

T [°C]	μ [cm²/Vs] $\times 10^{-4}$	η [Pa · s] $\times 10^{-3}$	ε_r	ζ [mV]	R_H [nm]	S
37	−1.67	1.68	90	−26	1470	94000
20	−1.08	1.18	84	−23	2880	85400

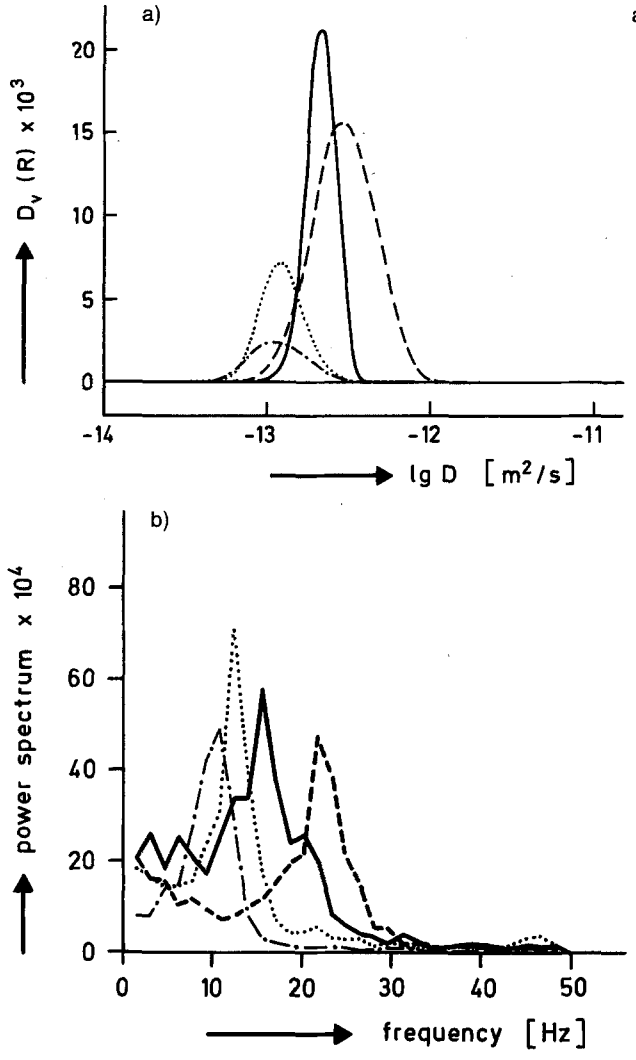

Figure 7. Comparison of the thermal motion, i.e. diffusion (*a*), and electrophoretic mobility (*b*) of GFP and PRP and their temperature-dependent activation (37 °C → 20 °C). Discoid GFP at 37 °C: dashed line; activated GFP at 20 °C: dotted line; discoid PRP at 37 °C: full line; activated PRP at 20 °C: dashed dotted line. The difference in discoid, resting PRP and GFP is due to adhesive plasma proteins in PRP, but the changes caused by activation are similar, indicating that the method can be used to detect platelet shape change in PRP and GFP.

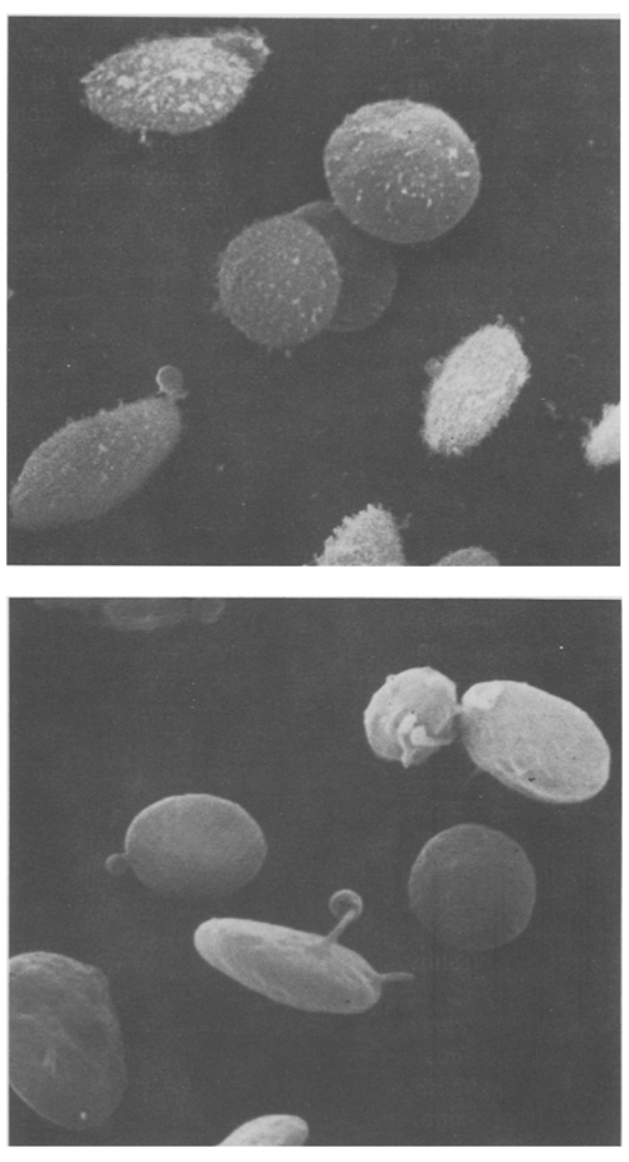

Figure 8. The scanning electron micrographs show that preparation of PRP (*a*) and GFP (*b*) at 37 °C results in discoid, resting platelets. In PRP the platelets are covered with plasma proteins.

we found on the surface of activated platelets. The negative charge of activated platelets is mostly caused by the sialic acids of $GPII_b/III_a$, which is the receptor of ADP. The agreement of the two independently obtained results could be mere coincidence, but with our method it is impossible to test this. The combination with other techniques would be necessary to reveal whether there is a real relationship or not. The surface charge density is an important overall parameter for classifying the binding activity of receptors to substrates like ADP, thrombin, serotonin etc.

In order to know whether PRP and GFP will give comparable results, we investigated resting, discoid platelets in plasma and after gel filtration at 37 °C and compared the temperature-dependent activation of both samples by cooling to 20 °C.

The volume-distribution for nonactivated GFP at 37 °C (fig. 7a, dashed line) is broader and the mean diffusion coefficient is shifted to a higher value than for PRP (full line). This means that the thermal mobility of gel-filtered platelets is slightly higher than for protein-coated platelets in PRP, i.e. the effective size of platelets in buffer without protein coating is smaller. Comparison of the electrophoretic mobility of unactivated platelets in GFP (dashed line) and PRP (full line) (fig. 7) shows that the electrophoretic mobility in plasma is significantly lower, owing to the membrane-bound proteins.

Nevertheless, the relative changes in electrophoretic mobility and diffusion coefficient, as a consequence of the shape change with drop of temperature, are of comparable extent (fig. 7, GFP: dotted line, PRP: dashed dotted line). Formation of pseudopods diminishes the difference

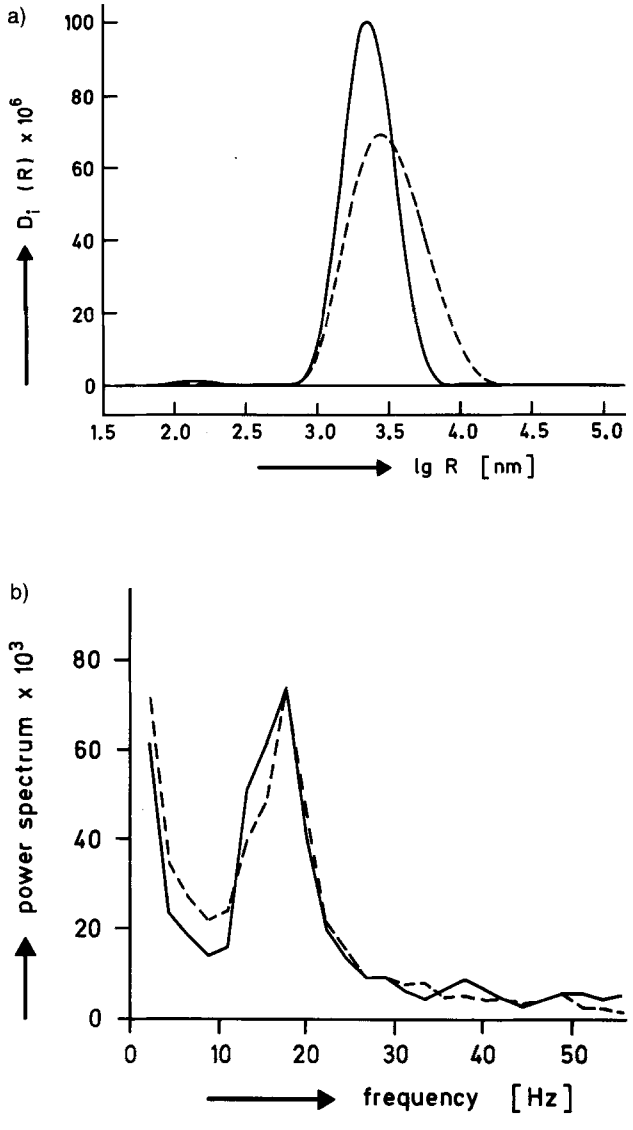

Figure 9. Comparison of PRP of fasting donors (dashed line) and 2 hours after intake of a fatty meal (full line). In the intensity size distribution (*a*) chylomicra can be detected in the postprandial hyperlipaemic plasma, but chylomicra did not change the size or the electrophoretic mobility (*b*) of platelets at 37 °C.

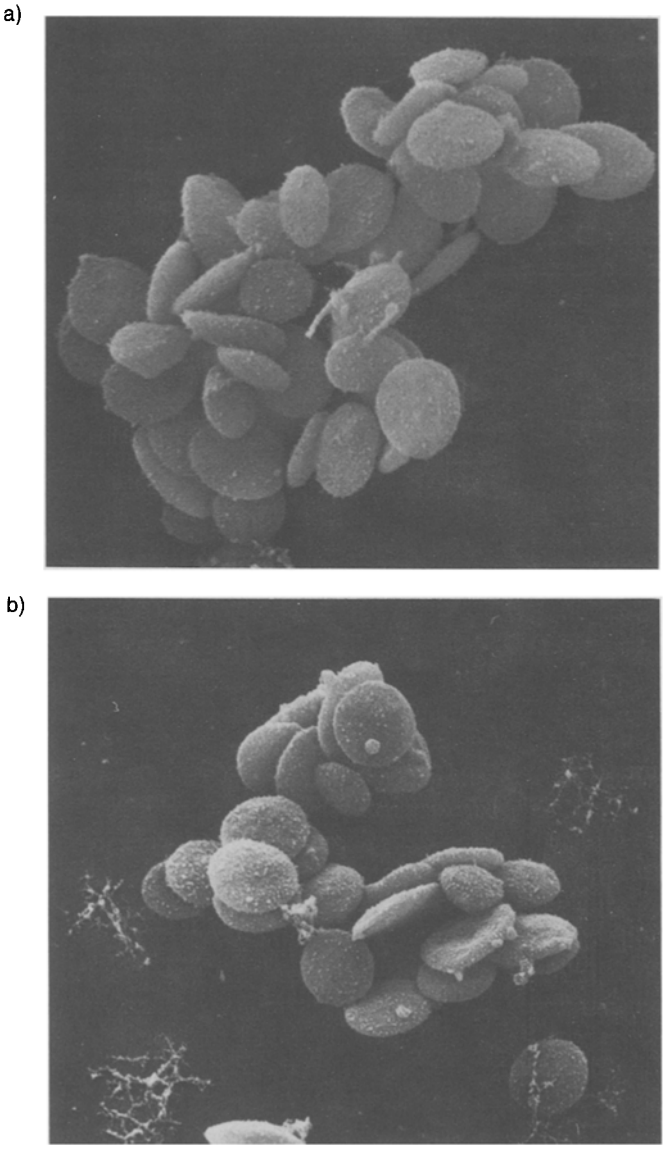

Figure 10. In agreement with QELS, scanning electron microscopy shows no difference in platelets at 37 °C taken from a fasting donor (*a*) and 2 h after a fatty meal (*b*).

between GFP and PRP in diffusion, whereas in electrophoresis a small difference remains. Electron micrographs show that both preparations are of good quality, resulting in discoid, nearly unactivated platelets at 37 °C (fig. 8). In conclusion, we found that gel-filtration is not necessary for EDLS measurements and that the technique can distinguish between adhesion of plasma proteins and activation.

Hyperlipaemic plasma was investigated for two reasons. Firstly, contradictory results on the influence of chylomicra have been published[18,19]. It was suspected that chylomicra, being the precursors of atherogenic lipoproteins like LDL, might activate platelets[20]. Secondly, the influence of chylomicra is important from a practical point of view, i.e. whether or not it is necessary to take blood from fasting donors. Figure 9 shows the comparison of the intensity distributions and the electrophoretic mobilities before (dashed line) and after (full line) a fatty meal. Chylomicra are small compared to platelets (the radius of chylomicra is about 100–200 nm) and therefore they do not show up very easily in the intensity size distribution, whereas in a volume size distribution the peak of the platelets gets very small and the peak for chylomicra is prominent (data not shown). The difference in the two platelet populations is not significant, but indicates that the platelets prepared before the meal had not had enough time to recover fully from the preparation process. These experiments do at least show that chylomicra do not activate platelets or bind to them, and that hyperlipaemic blood can be investigated by QELS. The results are in very good agreement with the scanning electron micrographs (fig. 10).

As described before, QELS data of sufficient quality can be obtained only by calculating the average of several blocks within one measurement. Thus the essential measuring time of some minutes exceeds the time scale of the shape change by a large amount. With QELS, processes in the range of minutes like relaxation of platelet shape after mechanical stress or cooling can be followed, but on-line kinetic studies of platelet shape change are impossible. We were interested in the effects of different influences like temperature, activating agents, chylomicra etc, and not in the velocity of these effects. Therefore the impossibility of following the time-course was not important for us. We investigated the stable products after the changes, in order to get information about how sensitive platelets are to external physical or chemical influences. Although our electrophoretic chamber is not constructed to mix platelet suspensions directly with activating agents, preliminary experiments showed that dose-dependent activating effects on platelets after addition of thrombin, ADP or epinephrine can easily be followed by QELS. Detailed results will be presented in a subsequent publication.

Discussion

We used the temperature-dependent activation for the classification of normal platelets. Of 57 donors, 54 had platelets which underwent a severe shape change when the temperature was decreased from 37 °C to 20 °C. Since this was a characteristic behaviour for most of the platelet samples, we categorized them as normal. For some blood donors we observed quite significant differences in the temperature-dependent activation, which matches well with other observations [21]. From a practical point of view, EDLS measurement of the temperature dependence offers a fast and clear classification of blood donors during serial studies, in order to sort out platelet samples that can be expected to give non-representative results. Because of the complexity of physiological processes and the numerous factors that can influence platelets, we are not yet able to assign deviations from normal temperature-dependent platelet activation to clinical parameters. We suspect that temperature-dependent platelet shape change could be important in thermoregulation during hypothermia [22].

Considering that serotonin constricts arteries and veins and regulates skin flow in certain pathological conditions such as Raynaud's disease, where there is an excess sensitivity to cold [4], we checked the release of serotonin (5-HT) with decreasing temperature. Up to now we could not find significant release at different temperatures measured by high-pressure liquid chromatography and electrochemical detection (HPLC-EC) (unpublished results). Although the total serotonin content of platelets was in the range of 300–1700 ng/10^9 platelets [23] and 75–85% were liberated by addition of 0.4 U/ml thrombin for 1 min, neither decrease of temperature to 20 °C nor addition of 10 μM ADP or 10 μM epinephrine caused serotonin release from platelets. This contradicts the evidence that the passive outflow of serotonin from platelets shows marked temperature sensitivity [24], and the suggestion that the conformation of the serotonin transporter may change as a function of temperature [25].

The physiological relevance of temperature-dependent activation and the existence of a critical temperature of 30 °C for some donors is still unclear. It needs further investigation, because several correlations between disorders and temperature have been found, like cold-related thrombosis, which could be observed as the higher winter mortality from ischaemic heart disease and from stroke [26]. The results concerning temperature are important from a practical point of view, because working with blood platelets at room temperature clearly does not result in resting platelets. This is of great importance, for example when one is looking for the submaximal dose of an aggregating agent. If one starts with preactivated platelets, the dose measured will be much lower and therefore will not accurately reflect platelet reactivity [27]. Comparative experiments on temperature activation in an aggregometer were impossible, because commercially available instruments are restricted to a small temperature range around 37 °C or are fixed at 37 °C.

The suitability of QELS for the quantitative study of the shape change of human blood platelets is shown in this paper. Although QELS and electron microscopy lead to the same results, the effort for sample preparation and data evaluation, as well as the time involved, is much less for QELS.

The results obtained with this new method may lead to a better understanding of shape change. If we are successful in applying commercially available instruments such as zeta-sizers for these experiments, a new clinical tool will be accessible to supply very important data on the activation of human blood platelets.

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1 Packham, M. A., and Mustard, J. F., Sem. Hemat. *23* (1986) 8.
2 Sevitt, S., Atherosclerosis *61* (1986) 107.
3 Erne, P., Resink, T. J., Bürgisser, E., and Bühler, F. R., J. Card. Pharm. *7* (1985) S103.
4 Siebold, J. R., and Jagenau, A. H. N., Arthritis and Rheumatism *27* (1984) 139.
5 Holmsen, H., in: Platelet Responses and Metabolism, vol. 1, p. 4. CRC Press, Boca Raton, Florida 1987.
6 Latimer, P., Appl. Optics *22* (8) (1983) 1136.
7 Deranleau, D. A., Dubler, D., Rothen, C., and Lüscher, E. F., Proc. natl Acad. Sci. USA *79* (1982) 7297.
8 Steen, V. M., and Holmsen, H., Eur. J. Haemat. *38* (1987) 383.
9 Fuchs, H. F., Jorde, C., and Glatter, O., Rev. Sci. Instrum. *60* (5) (1989) 854.
10 Smith, B. A., and Ware, B. R., Contemp. Top. analyt. clin. Chem. *2* (1978) 29.
11 Herren, B. J., Shafer, S. G., van Alstine, J., Harris, J. M., and Snyder, R. S., J. Colloid Interface Sci. *115* (1987) 46.
12 Spurej, E., in: Trägerfreie Elektrophorese mit Laser-Doppler Detektion, Diplomarbeit (1988).
13 Schnablegger, H., and Glatter, O., submitted to Applied Optics (1990).
14 Hourdille, P., Heilmann, E., Combrie, R., Winckler, J., Clemetson, K. J., and Nurden, A. T., Blood *76* (1990) 1503.

15 Hunter, R. J., in: Zeta Potential in Colloid Science, p. 27. Academic Press, London 1981.
16 Hampton, J. R., and Mitchell, J. R. A., Nature *211* (7) (1966) 245.
17 Boisseau, M. R., Lorient, M. F., Born, G. V. R., and Michal, F., Proc. R. Soc. Lond. B. *196* (1977) 471.
18 Aznar, J., Santos, M. T., and Valles, J., Thromb. Res. *48* (1987) 567.
19 Jakubowski, J. A., Ardlie, N. G., Chesterman, C. N., Mc Gready, J. F., and Morgan, F. J., Thromb. Res. *39* (1985) 725.
20 Nordoy, A., and Lagarde, M., Eur. J. clin. Invest. *14* (1984) 339.
21 Trenchard, P. M., Br. J. Haemat. *67* (2) (1987) 248.
22 Bühler, F. R., Amstein, R., and Fetkovska, M., J. Cardiovasc. Pharmac. *10* (3) (1987) S32.
23 Ortiz, J., Artigas, F., and Gelpi, E., Life Sci. *43* (1988) 983.
24 DaPrada, M., Pletscher, A., and Bartholini, G., Life Sci. *4* (1965) 1773.
25 De Oliveira, A. M., Shoemaker, H., Segonzac, A., and Langer, S. C., Neuropharmacology *28* (8) (1989) 823.
26 Taccola, A., Gutti, G. B., Zelaschi, G. P., Pisati, P., and Terzi, R., Minerva Medica *75* (1984) 975.
27 Kawahara, J., Sano, H., Fukuzaki, H., Saito, K., and Hirouchi, H., Am J. Hypertens. *2* (1989) 724.

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Common antigenic properties of a g-type (goose) and a c-type (duck) egg white lysozyme: Antibody responses in rabbits and mice

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Abstract. Embden goose (GEWL) and Barbary duck (DEWL) egg white lysozymes possess different amino acid sequences corresponding to the g-type and c-type, respectively. GEWL was shown to be a better immunogen than DEWL in both rabbits and mice. The antigenicity of the two lysozymes was tested using different techniques (i.e. indirect ELISA, inhibition tests and immunoabsorption experiments). Injection of either GEWL or DEWL into rabbits and mice induced both specific antibodies and cross-reacting antibodies. Moreover, anti-GEWL antibodies, in contrast to anti-DEWL antibodies, did not cross-react with hen egg white lysozyme (HEWL), a c-type lysozyme. While the structure of GEWL was not modified after binding to plastic, DEWL was denaturated, but it did keep some native epitopes. It was concluded that g-type and c-type lysozymes, which have different amino acid sequences, exhibit strong common antigenic properties.
Key words. Bird lysozymes; immunogenicity; antigenicity; ELISA.

Lysozyme (E.C. 3.2.1.17) is an enzyme found in various tissues and secretions, which hydrolyses 1,4-β linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine in the bacterial cell wall. Lysozyme has been extensively used as a model system in a large number of studies[18] of protein sequence determinations[17,18], X-ray crystallography[6,22], mechanisms of enzyme action[6], molecular evolution[18,19], and immunology[4,5]. Lysozymes have been found not only in mammals, birds, fishes and invertebrates but also in bacteriophages, bacteria, fungi and plants[18]. These lysozymes belong to different enzyme families and in many instances are quite different structurally.

The type of lysozymes found in hen egg white (c-type, molecular mass: 14.5 kD), whose sequence and properties are well-known, has been found to be present in the egg white of only two orders of birds, the Galliforms and the Anseriforms[23]. In contrast, the lysozyme which was originally found in goose egg white (g-type, molecular mass: 21 kD)[8,9] has been detected in a large number of avian orders[23]. The taxonomic distribution of g-type lysozyme is thus broader than that of c-type lysozyme.

Schoentgen et al.[24] found only slight amino acid sequence homology between g-type and c-type lysozymes, which was localized around the active site of the enzymes. However, a partial common domain structure for both types of lysozymes has been determined by crystallography[12].

The immunological properties of hen egg white lysozyme (HEWL) have been studied by many workers[3,4,16,20]. The c-type lysozyme contains several major immunologically-active regions around the disulphide bridges, and in such regions particular amino acid residues (such as Arg 68) have a stronger impact than others on the antigenic reactivity[4,15]. Anti-HEWL polyclonal serum[15,16,20] has been shown to cross-react with different c-type lysozymes (i.e. duck, quail, turkey...) which had been differentiated by monoclonal antibodies[13,25,26]. However, it has been reported that antibodies directed against the c-type HEWL do not cross-react with the g-type lysozyme, and vice versa, either in a microcomplement fixation procedure or in immunodiffusion tests[2,3].

It has been shown that when enzyme-linked immunosorbent assay (ELISA) tests are used to investigate cross-reactions between proteins, the results may vary because some proteins are denatured by binding to the plastic. In this study we were able to demonstrate a strong anti-