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A Photometric Method for the Determination of Serum Titres by Latex Particle Agglutination

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A simple photometric method for the determination of latex particle agglutination in immunochemical systems is described. This photometric latex test (PLT) allows the determination of serum titres by the numerical evaluation of measured turbidities. A mathematical algorithm for data processing by a desk-top computer is designed. From the experiments with a model immunochemical system (human serum albumin — rabbit anti-serum) it was found that with the PLT the reliability and reproducibility in the serum titre determination was significantly increased in comparison to standard latex-fixation tests with the visual detection of agglutination. It was found that the PLT is at least as sensitive as passive hemagglutination tests. The PLT can easily be applied in routine immunochemical and serologic work and represents an improvement of existing quantitative latex agglutination tests.

INTRODUCTION

Since 1956 when Singer and Plotz¹ reported for the first time on the use of synthetic polymer latices for serologic purposes, the number of papers dealing with latex agglutination tests have vastly increased. This technique, applied initially in the serology of rheumatoid arthritis, was soon applied in serologic tests for the determination of the antibody content in the sera of patients suffering from various diseases. In the extensive review of Singer², covering the literature until the end of 1960, about 80 references are quoted, and this number has doubled until now.

Singer and Plotz have designed the first latex test, the so-called »latex-fixation test« (LFT), which is used in many laboratories with or without minor modifications. The LFT is a quantitative test for the determination of serum titres, allowing the estimation of the antibody content of sera. In the LFT the determination of latex agglutination is made by visual examination. This is one of the major drawbacks of latex agglutination tests since it involves the danger of subjective errors and does not allow the introduction of automated procedures.

In the design of an objective serologic test one of the main problems is the choice of a proper detecting method. Among these methods, transmittance measurement is widely used in various laboratory tests and autoanalysing

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equipment. Since polymer latices are opalescent systems which exhibit light scattering easily measurable by transmission methods³, and latex particle agglutination as an aggregation process can be followed by changes in light scattering quantities, it appears that measurement of transmittance could serve as a powerful and reliable method for an objective evaluation of agglutination, with much prospect of an application in automated tests.

Attempts were made⁴⁻⁶ to introduce transmittance measurement as a method for the detection of latex agglutination. The methods applied were a modification of the LFT and included incubation of the agglutinating systems at elevated temperature and subsequent twofold centrifugation. Optical transmittance of the remaining supernatant was taken as an indicator of latex agglutination. No attempts were made, however, to use the methods for quantitative work, *i. e.* for the determination of serum titres.

In this paper a simple photometric method for the determination of latex particle agglutination is described. This objective photometric method, named here the »photometric latex test« (PLT), allows the determination of the serum titre by the numerical evaluation of measured turbidities.

The principle of the method consists in preparing mixtures of suspensions of the antigen-coated latex particles with the immune serum dilutions and allowing them to stand at rest at room temperature until the process of particle agglutination and sedimentation of agglutinates in the gravitation field is finished. Systems without agglutination remain stable and have a constant turbidity, whereas in agglutinated systems the turbidity decreases. The method was developed by experimenting with a model immunochemical system (human serum albumin — rabbit antiserum), but it can easily be applied in routine serologic work.

EXPERIMENTAL

Materials

Polystyrene latices were monodisperse samples prepared in this laboratory⁷ ranging in size from 200 to 940 m μ . All latex samples were extensively dialysed against distilled water. Because of their high negative electric charge the dialysed latex dispersions were very stable at concentrations higher than 1%. The concentration of latices was determined gravimetrically by evaporation and drying in vacuum over P₂O₅ at 100° C. For comparative purposes some experiments were performed with a commercial monodisperse polystyrene latex sample »Bacto 0.81«, a product of Difco Laboratories, Detroit, Mich., U. S. A.

Human serum albumin (HSA) was a crystalline sample of high purity, free of electrolytes, and kept in a lyophilized form. Hyperimmune anti-HSA (A-HSA or A) and anti-total human serum (A-HS) rabbit sera were also used as lyophilized undiluted samples. All details of these materials will be given in a forthcoming paper. Several experiments were performed with commercial hyperimmune anti-HS (Lot No. 1393 G) and anti-HSA (Lot No. 1481 L) sera produced by Behringwerke AG, Marburg-Lahn, Germany. Normal rabbit sera were from animals previously involved in no immunologic experiments. All sera were inactivated before agglutination experiments in a thermostated water bath at 56° C for 1 hour.

Buffers of a defined ionic strength μ (0.01—0.5 moles/l) and pH (8.0—8.1) were prepared by using tris(hydroxymethyl)aminomethane as the buffer substance. All buffer solutions contained 0.02% of sodium azide as preservative.

Latex-Antigen Reagent (LSA-Reagent)

A concentrated suspension (1—2%) of a polystyrene latex was mixed with a certain quantity of HSA (antigen) dissolved in a buffer of desired pH and a desired ionic strength. The latex concentration was chosen empirically in order to give, in final latex dilutions, optical densities near unity. The concentration of

HSA should be such as to ensure the presence of an excess amount of the antigen and, consequently, to realize a maximal saturation of the latex particle surface. Table I gives the final concentrations of latices (c_{LS}) and HSA (c_{HSA}) together with the mean particle diameters \bar{D} .

TABLE I
Composition of LSA-reagents

Latex Sample	\bar{D} (m μ)	c_{LS} (mg/ml)	c_{HSA} (mg/ml)
LS 145-1	939.3	1.00	0.20
LS 142-1	726.1	0.75	0.20
LS 137-4	534.3	0.75	0.30
LS 97-6	399.2	1.00	0.50
LS 54-2	197.3	3.00	3.00
<i>Bacto 0.81</i>	812.0	1.00	0.20

After mixing latex particles with the antigen the suspension was left to incubate overnight at +4°C. In order to remove the excess antigen, the suspension was centrifuged at 24,000 \times G for 30 minutes (only in the case of particles of 200 m μ in diameter the time had to be prolonged to 1 hour). The clear supernatant was discarded and the remaining compact sediment was resuspended in the same volume of the buffer. This procedure was repeated. The final suspension served as the LSA-reagent in further work and was kept at +4°C.

Latex Agglutination Method

Rabbit sera were diluted by twofold increments with the appropriate buffer starting with a dilution 1:10. The general term of this geometric series was consequently $d' = 10 \times 2^n$, where d' is the serum dilution and n represents the order of dilution. All dilutions were performed in commercial test tubes (12 \times 75 mm). To 1 ml. of a serum dilution a constant volume (in most cases 0.1 ml.) of the LSA-reagent was added. The increase of the serum dilution produced by reagent addition was corrected by multiplying d' by a volume correction factor C defined as the ratio of the final volume of the system to the volume of serum dilution. In the pertinent case (1 ml. of serum dilution + 0.1 ml. of LSA-reagent) $C = 1.1$, and the actual serum dilution d in the final mixture amounts to 11×2^n . The LSA-serum systems were thoroughly mixed by shaking the tubes and left to stand without disturbance at room temperature for 24 hours. After that time the major part of the agglutinated latex particles sedimented, whereas the stable suspension of nonagglutinated particles remained turbid. The last tube in the dilution series contained a pure buffer and served as a control.

Measurement of Latex Agglutination and Evaluation of Results

The turbidities of latex suspensions were measured by a Beckman Model B spectrophotometer with a test tube attachment. Measurements were made with the light of the 436 m μ wavelength. The exit slit of the monochromator was shortened in the vertical direction to 8 mm. in order to allow work with 12 \times 75 mm. test tubes and the volumes as small as 1 ml. The light beam has to pass through the part of the tube having exclusively cylindrical geometry which is a necessary precaution against focusing errors from the curved bottom of the tube. Since no absolute measurements of turbidity are necessary, only optical density being proportional to turbidity is to be recorded.

A constant (maximal) value of optical density indicates a stable suspension, whereas a decrease in optical density indicates latex agglutination resulting from the sedimentation of aggregated particles.

In order to diminish deviations in the optical thickness of the test tubes, the tubes were classified into groups, having the smallest deviation in optical density when filled with a standard latex suspension. A classification by the internal tube diameter proved to give satisfactory results.

The numerical values of optical densities were processed by a desk-top computer Olivetti *Programma 101* for the evaluation of serum titres.

Passive Hemagglutination Method

Boyden's method⁸ with tanned erythrocytes was applied by following the procedure given in Nowotny's manual⁹. Fresh sheep red blood cells were taken and after the treatment with tannic acid the cells were coated with HSA. All suspensions and solutions were prepared with physiological saline. The hemagglutination was read by visual examination. Five degrees of hemagglutination were differentiated, with the last two degrees (\pm and $-$) being regarded as a negative result. Double dilutions of antiserum (0.2 ml.), starting with a dilution 1:10, were mixed with an equal volume of HSA-coated red blood cells. In this case $C = 2$ and $d = 20 \times 2^n$.

RESULTS

In a latex agglutination test at lower serum dilutions the systems are pronouncedly agglutinated, whereas at higher dilutions the systems remain stable. There is always an intermediate transition region where the degree of agglutination gradually changes. A typical agglutination curve represented as a dependence of turbidity τ vs. binary logarithm of serum dilution $\log_2 d$ is shown in Fig. 1. The serum titre t is defined as the limiting value of d

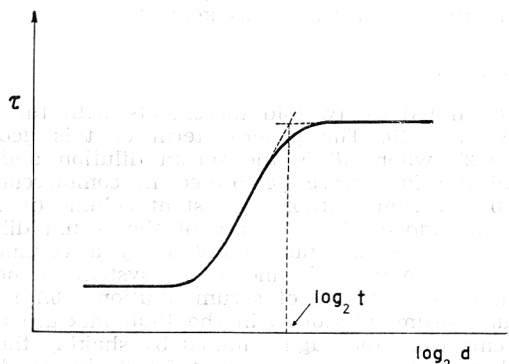


Fig. 1. The scheme of a typical agglutination curve; τ — relative turbidity (optical density), d — serum dilution, t — serum titre.

at which agglutination is still detectable. In the case of the agglutination curves of the type shown in Fig. 1 t can be determined from the intersection of the straight line going through the points with constant τ with the straight line going through the points at the steep part of the curve. This intersection gives the value of $\log_2 t$ and can be evaluated both graphically and by automatic computation, the latter method being more objective and precise.

The mathematical algorithm for the titre evaluation is designed as follows: Beginning with the turbidity (optical density) of the very last (stable) suspension in the series, the arithmetic mean turbidities $\bar{\tau} = k$ are computed automatically. With the first value of $\bar{\tau}$ being significantly different from $\bar{\tau}$ (*i. e.* when $\tau < p \bar{\tau}$, where p is a discrimination factor discussed below) the computer starts to calculate, by the least squares method, the slopes of the

best straight lines passing through subsequent points. The slopes are compared successively and the computing proceeds as long as the slope rises. When the slope starts to decrease (*i. e.* when the curve approaches its left low part), the computation automatically stops and the straight line parameters a and b are computed for all the points on the steep part of the curve. From the straight line equation

$$\tau = a + b \log_2 d$$

the value of $\log_2 t$ is computed by inserting k for τ^* .

The most crucial question in this procedure is the determination of p . This factor was estimated from the coefficients of variation s_r of turbidity measurements. Working with classified test tubes \bar{s}_r was found to range from 1.4% to 1.8%. It was supposed that all values $\tau \geq \tau(1 - 3s_r/100)$ still belonged to the group of constant τ -values. Therefore $p = 1 - 3s_r/100$ would discriminate, with 99% of confidence, all τ values significantly lower than $\bar{\tau}$. In order to increase the computing reliability, it was found appropriate to round off s_r to 2%, *i. e.* to work with $p = 0.94$.

Fig. 2 shows typical experimental agglutination curves obtained with the LSA-reagents prepared from latices of different particle size. All measurements

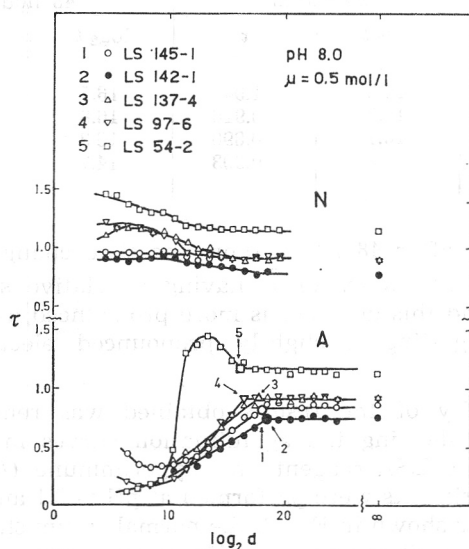


Fig. 2. Experimental agglutination curves obtained with LSA-reagents of different latex particle size, and with hyperimmune anti-HSA (A) and normal (N) rabbit sera; pH 8.0, $\mu = 0.5$ moles/l. Big points denote serum titres.

were made at pH = 8.0 and the ionic strength $\mu = 0.5$ moles/l. The dilutions were made of the anti-HSA hyperimmune rabbit serum (A) and the normal rabbit serum (N). With the reagents containing latices with the particle size equal or larger than 400 $m\mu$ the agglutination curves (A) were of the type shown in Fig. 1. With those containing 200 $m\mu$ particles the curve initially shows a turbidity increase which is changed to a sudden drop. This can be

* The computer program written for the Programma 101 is available on request.

explained by the fact that the specific turbidity curve¹⁰ goes through a maximum which, for the wavelength 436 m μ and polystyrene particles, corresponds to a 750 m μ particle diameter. With particles of 400 m μ or larger the agglutination causes a decrease in turbidity, whereas with particles of 200 m μ the turbidity of aggregates increases. Only the sedimentation of greater aggregates observable at $\log_2 d < 13$ diminishes the turbidity. The agglutination curves of this type cannot be processed by the algorithm mentioned and another program should be designed. Normal sera did not show any specific immunochemical interaction at the pertinent ionic strength, and the curves are smooth with a weak tendency towards a turbidity decrease with an increasing d . This slight decrease is probably connected with a very slow electrolyte coagulation effect which does not disturb specific agglutination effects. All measurements were made after 24 hours. After this time the agglutination process was finished as can be concluded from data in Table II.

TABLE II

Comparison of titres determined after 24 and 48 hours, respectively, after mixing; Polystyrene latex LS 137-4, pH 8.0, hyperimmune anti-HSA rabbit serum, $c_{r\text{SA}} = 68.0 \mu\text{g./ml.}$

μ (moles/l)	24 hours		48 hours	
	$\log_2 t$	$\bar{\tau}$	$\log_2 t$	$\bar{\tau}$
0.01	16.3	1.044	16.7	1.031
0.05	16.8	0.916	16.8	0.845
0.10	16.1	0.890	16.3	0.807
0.50	14.3	0.903	14.7	0.822

The values measured after 48 hours show the titre changes well within the experimental error. The values of $\bar{\tau}$ (having a relative standard error less than 1%) decrease, and this decrease is more pronounced as the ionic strength increases, again suggesting a slightly pronounced electrolyte coagulation effect.

The reproducibility of the results obtained was remarkable. This can be seen from Fig. 3 showing the agglutination curves measured during 2.5 months with the same LSA-reagent and hyperimmune (A) and normal (N) rabbit sera. The experiments were performed at pH = 8.1 and $\mu = 0.05$ moles/l. Contrary to the results shown in Fig. 2, the normal serum showed agglutination effects which can be ascribed to nonspecific interactions, as will be explained in detail in a forthcoming paper. Except for a small decrease in $\bar{\tau}$ caused by a slow decrease of the latex particle concentration (because of the spontaneous aggregation and sticking on the glass walls of the reagent container), the titres varied 6% (for A) and 7% (for N), respectively. This is within the experimental error, as shown in Discussion.

In order to compare the properties of our latices with the properties of a commercial latex (Difco Bacto 0.81) and to measure latex agglutination with different hyperimmune sera, both anti-HS and anti-HSA, several titrations were made. The results are shown in Fig. 4. Almost identical titres were obtained for the LSA-reagents prepared by using both LS 145-1 or the Difco

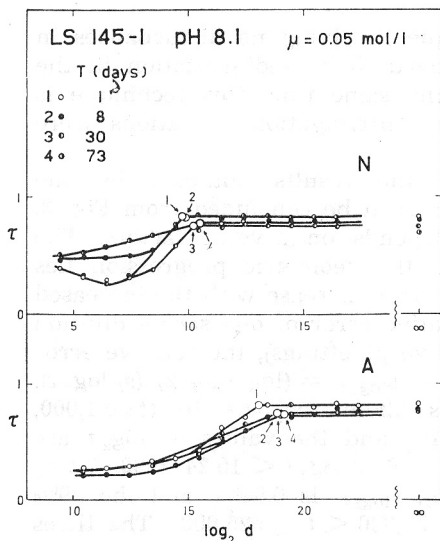


Fig. 3. Experimental agglutination curves of LSA-reagent (LS 145-1) with hyperimmune anti-HSA (A) and normal (N) rabbit sera in dependence of the age of LSA-reagent; T — age (in days), pH 8.1, $\mu = 0.05$ moles/l. Big points denote serum titres.

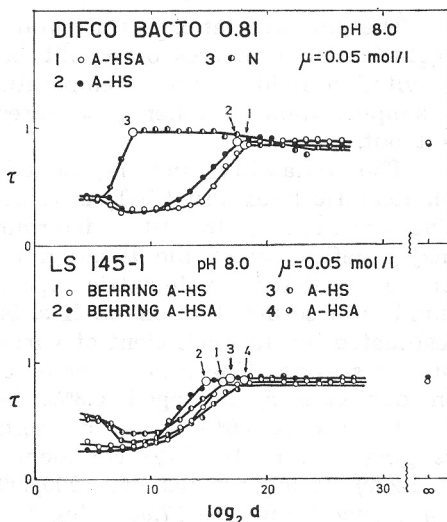


Fig. 4. Experimental agglutination curves of LSA-reagents prepared from different latex samples (LS 145-1, *Bacto 0.81*) and obtained with rabbit sera of our and Behringwerke production; A-HSA — hyperimmune anti-HSA serum, A-HS — hyperimmune anti HS serum, N — normal serum; pH 8.0, $\mu = 0.05$ moles/l. Big points denote serum titres.

latex (the upper part: curves 1 and 2; the lower part: curves 4 and 3). On the other hand, a well pronounced agglutination could be detected with all hyperimmune sera investigated. The titres of these sera varied significantly, as was expected, indicating a variation in the antibody content of sera.

Finally, the binary logarithm of the titre of our anti-HSA serum determined by passive hemagglutination amounted to $\log_2 t = 14.3$ ($t \approx 41,000$).

DISCUSSION

Standard techniques for the detection of latex agglutinates are based on a visual estimation of the degree of particle agglutination. In order to accelerate the separation of agglutinated particles from single ones, it is a common practice to centrifuge the suspensions. It is obvious that such a procedure should entail a great danger of subjective and systematic errors: on the one hand, it is easy to overlook agglutinates in the transition region, on the other, the operation of centrifuging is not easily performed in a reproducible manner, especially when coarse agglutinates have to be separated by short centrifugations.

Our first attempts to introduce a more objective detection of agglutinates were made by observing suspensions in an ultramicroscope. This method, however, appeared to be very unreliable since a number of particles remained single even after the agglutination process was over, making the determination of the degree of agglutination extremely difficult. The centrifugation of agglutinated systems provoked difficulties in many cases, as the sedimented agglutinates showed a pronounced tendency to redisperse after resuspending the sediments.

The simplest latex agglutination technique involving no disturbances in agglutination processes or equilibria has proved to be sedimentation in the gravitation field at room temperature. At the same time this technique is a simplification of other procedures as the centrifugation operations were left out.

The reliability and reproducibility of the results obtained by the photometric latex test (PLT) is remarkable, as can be concluded from Fig. 3. The precision of the titre determination depends on several factors. The major deficiency of the titration method in the geometric progression lies in the fact that the volumetric experimental errors increase with the increased number of serum dilutions n . If s_r is the relative error of one serum dilution (estimated by the coefficient of variation of two pipettings), the relative error of t is $s_t = ns_r$. The relative error of $\log_2 t$ is $s_{\log_2 t} = (\log e / \log 2) (s_t / \log_2 t)$. In our case s_r averaged 0.8%. For titres about $\log_2 t = 10$ ($t \approx 1,000$, $n = 6-7$) s_t is 5.6%, $s_{\log_2 t}$ amounts to 0.81% and the values of $\log_2 t$ are ranging within the 99% confidence limits $9.76 < \log_2 t < 10.24$ ($890 < t < 1,230$). If $\log_2 t = 18.5$ ($t \approx 200,000$, $n = 15$), $s_{\log_2 t}$ is 0.94%, and the 99% confidence limits are $17.98 < \log_2 t < 19.02$ ($255,000 < t < 525,000$). The titres in Fig. 3 range from 9.72—10.45 for the normal serum, and from 18.08—19.18 for the hyperimmune anti-HSA serum. One can see that the experimental values are slightly exceeding the 99% confidence limits. This can be ascribed to an additional error arising from turbidity measurement and varying from 0.5% to 1% (estimated by standard errors). In the ideal case the experimental error in $\log_2 t$ determination should amount to 1—2%. Actually, it was found that the scattering of data is somewhat greater suggesting an error about 5% and being probably a result of the sensitivity of the antigen-antibody interaction to various factors (conformational changes of antigen at the latex particle surface, impurities present in the system etc.). It is obvious that the presentation of titres in the logarithmic form should have an advantage over the usual way in the presenting the titres in the reciprocals of serum dilutions, since the latter are suggesting an experimental precision which cannot be achieved in reality¹¹.

It is clear that the automated computation of titres cannot increase the overall precision of the titre values if the titration is performed by the geometric series dilutions. This method served merely as a very useful tool for an objective titre evaluation instead of using the more subjective graphical method. Nevertheless, by automated computation the titre values are determined faster than by graphical evaluation. If the precision in preparing serum dilutions is increased — and this easily be done by the techniques common in analytical chemistry — automated computation will add significantly to the precision of the titre determination.

The sensitivity of the detection of HSA-antibodies by the described photometric latex agglutination test is considerable. If we suppose that the immunoglobulin concentration in a rabbit serum amounts roughly to 10 mg/ml (taking the protein concentration in adult vertebrate sera to average 7.5% with 14.0% of the Ig-fraction¹²) and all the Ig molecules are immunochemically active, the sensitivity of the PLT with the LS 145-1 at pH = 8 ranges from 0.01—0.06 $\mu\text{g/ml}$ of the antibody content. This is, however, an underestimation, since immunochemically active Ig molecules are supposedly only a fraction of the whole Ig population. From the values given for passive hemagglutination

methods¹³ it can be concluded that the PLT is at least as sensitive as passive hemagglutination. This sensitivity could hardly be achieved by the standard latex fixation tests with the visual detection of agglutination.

Actually, the titre obtained by passive hemagglutination is significantly lower than latex agglutination titres obtained with latices having $D > 500$ m μ . The values of $\log_2 t$ for smaller latices are nearer to the hemagglutination value, but in most cases they had $\log_2 t > 15$ (the $\log_2 t$ values 14.3 and 14.7 for LS 137-4 at $\mu = 0.5$ moles/l in Table II appear to be too low since measurements with other LSA-reagents prepared with LS 137-4 particles gave the values greater than 16, as is the case with the curve 3, Fig. 2, where $\log_2 t = 17.6$ was obtained). It should be noted that one has to be careful when comparing hemagglutination with latex agglutination, since both reactions were performed at different pH and ionic strength. The present results suggest, however, that the PLT appears to be more sensitive than passive hemagglutination.

In our laboratory the PLT was successfully applied in an extensive study of latex agglutination in the immunochemical HSA-hyperimmune rabbit antiserum system and has also been used for the detection of rheumatoid factors in human sera. The results of these investigations will be presented elsewhere.

It can be concluded that the PLT represents a substantial improvement in the existing quantitative latex tests since it increases the reliability and reproducibility in the serum titre determination, simplifies the experimental procedures, and opens good prospects of the automation of serologic work with polymer latices based on turbidity measurement.

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REFERENCES

1. J. M. Singer and C. M. Plotz, *Am. J. Med.* **21** (1956) 888.
2. J. M. Singer, *Am. J. Med.* **31** (1961) 776.
3. Gj. Deželić and J. P. Kratochvil, *J. Colloid Sci* **16** (1961) 561.
4. G. C. Bernhard, W. Cheng, and D. W. Talmage, *J. Immunol.* **88** (1962) 750.
5. H. Mathies and V. Helming, *Klin. Wschr.* **43** (1965) 1173.
6. H. Mathies, *Z. Rheumaforsch.* **26** (1967) 363.
7. N. Deželić, J. J. Petres, and Gj. Deželić, *Preparation of Monodisperse Polystyrene Latices*, *Kolloid-Z. u. Z. Polymere* (in press).
8. S. S. Boyden, *J. Exp. Med.* **93** (1951) 107.
9. A. Nowotny, *Basic Exercises in Immunochemistry*, Springer-Verlag, Berlin, 1969, p. 145.
10. W. Heller and J. McCarty, *J. Chem. Phys.* **29** (1958) 78.
11. F. Haurowitz, *Immunochemistry and the Biosynthesis of Antibodies*, Interscience Publishers, New York, N. Y., 1968, p. 69.
12. D. H. Moore, *Clinical and Physiological Applications of Electrophoresis*, in: *Electrophoresis* (Edited by M. Bier), Vol. 1, Academic Press, New York, N. Y., 1959, p. 370.
13. E. A. Kabat and M. M. Mayer, *Experimental Immunochemistry*, 2nd Ed., Thomas, Springfield, Ill., 1961, p. 121.

IZVOD**Fotometrijska metoda za određivanje titra seruma pomoću aglutinacije čestica lateksa***N. Deželić i Gj. Deželić*

Opisana je jednostavna fotometrijska metoda za određivanje aglutinacije čestica lateksa u imunokemijskim sistemima. Titar seruma određuje se pomoću fotometrijskog lateks-testa (PLT) iz mjerenih mutnoća numeričkim putem. Izrađen je matematički algoritam za obradu mjerenih podataka pomoću stolnog elektronskog računala. Na osnovu eksperimenata s modelnim imunokemijskim sistemom (humani serum albumin — antiserum kunića) nađeno je da PLT značajno povećava pouzdanost i reproducibilnost u određivanju titra seruma u usporedbi sa standardnim testovima fiksacije na lateks s vizuelnom detekcijom aglutinacije. Nađeno je da je PLT, u najmanju ruku, jednako osjetljiv kao i testovi pasivne hemaglutinacije. PLT se može lako primijeniti u rutinskom imunokemijskom i serološkom radu i predstavlja poboljšanje postojećih kvantitativnih testova aglutinacije lateksa.

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