Acta Medica Okayama

Volume 24, *Issue* 2 1970 *Article* 6

APRIL 1970

Binding of photosensitizing dyes with some biopolymers

Shigeo Yasui*

*Okayama University,

Binding of photosensitizing dyes with some biopolymers*

Shigeo Yasui

Abstract

To obtain some information of the biological action of Kankohso 101dinicotinate and Kankohso 301-nicotinate, observations were made on the binding mode of these substances with protein, chondroitin sulfate and nucleic acids and the following results were obtained; 1. Kankohso 10 I-dinicotinate binds reversively with bovine serum albumin or serum r-globulin, resulting in metachromasia. By binding with proteins the absorption maximum of the dye shifts toward the long wave length side and the absorbance decreased distinctly. The data show that there are more than one kind of binding sites and the binding with bovine serum albumin is weak in acidic solution and strong in alkaline solution. 2. Kankohso 10 I-dinicotinate produces strong metachromasia with sodium chondroitin sulfate and the color of the solution changes from violet blue to reddish violet. The absorption maximum at 592 mp. decreases without shifting its wave length ,and the shoulder appears at 555 mp. be. comes distinct peak. The strongest metachromatical changes occurs at the concentration of the chondroitinsulfate whose sulfonate radicals is equal to the molecules of Kankohso 10 I-dinicotinate. 3. Kankohso IOI-dinicotinate produces metachromasia with nucleic acid, where absorption spectrum is shifted toward long wave length and absorbance is decreased at a certain concentration. 4. Kankohso 301.nicotinate binds weakly with bovine serum albumin, the binding of which is reversible and the maximum binding number is 1.1 per molecule of albumin. Metachromasia cannot be produced by binding. Kankohso 30I.nicotinate does not bind with bovine serum γ-globulin. This compund does not produce metachromasia with sodium chondroitin sulfate but produces weak metachromasia with nucleic acid, indicating some affinity to nucleic acid.

---

*PMID: 4195456 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **24**, 185—204 (1970)

BINDING OF PHOTOSENSITIZING DYES WITH SOME BIOPOLYMERS

Shigeo YASUI

Department of Public Health, Okayama University Medical School, Okayama, Japan (Director : Prof. M. Ogata)

Received for publication, December 24, 1969

Investigation on the binding of drugs with serum protein is important from the aspects of the concentration of drug in the blood, its biological activity, metabolism and excretion. Further, the investigation on the binding with biopolymers such as nucleic acid and chondroitin sulfate and the resulting metachromasia is of great interest both in affinity of drug toward living body and staining of tissue sections.

In the present investigation binding of biopolymers with the nicotinates of Kankohso 101 (Platonin or NK 19) (1—7) and Kankohso 301 (T7 or NK 343) (8—14) were observed. Kankohso 101 is a photosensitizing dye of cyanine series and used as a drug for stimulation of the function of the reticuloendothelial system, regulation of the imbalance of nervous system, bactericidal, prevention of the side-effects of some anti-cancer agent and so on. Kankohso 301 is an aminovinyl type compound, the intermediate of synthesis of cyanine, and has the biological activity of the promotion of wound healing and hair growth.

In this experiment the binding capacity of the Kankohso 101-dinicotinate and Kankohso 301-nicotinate (15) (abbreviated K-101-NA and K-301-NA) with protein, polysaccharide and nucleic acid were observed for the purpose to get some information for the mechanism of biological action of K-101 and K-301, because these nicotinates show similar biological activities as those of K-101 and 301 (15) except the increased solubility in water. These nicotinates were synthesized by substitution of the iodo-anions at the quaternary nitrogen with nicotinic anions.

In this paper the binding modes of K-101-NA and K-301-NA with bovine serum albumin (BSA) and γ-globulin (BS γ-G) as investigated by the dialysis equlibrium method, metachromasia method and gel-filtration method are reported. Further, metachromasia of the dyes induced by binding with chondroitin sulfate, DNA and RNA are also reported.

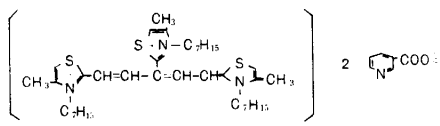
MATERIALS AND METHODS

The dyes used for the present experiment, K-101-NA and K-301-NA, were prepared in this laboratory by the method previously reported (15). Their structural formulae, scientific names and physico-chemical properties may be referred to Table 1. These dyes were combined with bovine serum albumin (Sigma),

Table 1 STRUCTURAL FORMURAE, SCIENTIFIC NAMES AND PROPERTIES OF KANKOHSO

Kankohso 101-dinicotinate

(other names : Platonin-or NK19-dinicotinate)

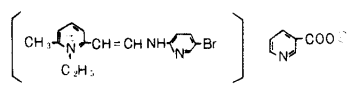


2, 2'-{3'-(2-(3-heptyl-4-methyl-2-thiazolin-2-ylidene) ethylidene)propenylene} bis {3-heptyl-4-methyl} thiazolium dinicotinate

Absorption maximum 595mμ (in MeOH)
Melting point 85°C

Kankohso 301-nicotinato

(Other names : T₂- or NK343-nicotinate)



6-{2-(5-bromo-2-pyridyl) amino} vinyl} -1 -ethyl 2-picolinium nicotinate

Absorption maximum 404mμ (in MeOH)
Melting point 179-80°C (d)

γ-globulin (Sigma), DNA of herring sperm (Sigma), RNA of torula yeast (Sigma) and sodium chondroitin sulfate (Ishizu).

Binding of the dyes with bovine serum protein was made by dialysis equilibrium method. A cellophane, 2 cm in diameter, 12 cm long and one end closed, was inserted in a large test tube 2.5 cm in diameter. Two and a half ml of 1/20 M phosphate buffer solution containing 0.3 % NaCl (pH 7.0) was placed outside the cellophane tube and 2.5 ml of 1 % bovine serum protein in the same buffer solution was placed inside the tube, and each 2.5 ml of K-101-NA (about 10^{-4} to 5×10^{-6}) and K-301-NA (about 8×10^{-4} M to 2×10^{-5} M) in the same buffer solution was added to the solution inside and outside the cellophane tube. Then the test tubes were kept in a dark place at low temperature (0°—5°C) for 48 hours shaking slowly to allow to reach an equilibrium. After 48 hours the difference in concentration of K-101-NA or K-301-NA inside and outside the cellophane tube was measured and the quantity of the dyes bound with the protein was

calculated. The concentration of the dyes in the solution was measured from the absorbance at the absorption maximum.

For the investigation of the influence of pH on the binding of the dyes (K-101-NA, 5×10^{-5} M and K-301-NA, 5.4×10^{-5} M) with serum albumin, phosphate buffer solution containing 0.3 % NaCl (the ionic strength ajusted to 0.05) was used under the same conditions as just described.

For the examination of antibiotic activity of K-101-NA, 0.1 ml of *Staph. aureus* FDA suspension was added to 20 ml of semi-synthetic medium containing K-101-NA in varied concentrations and cultured in the Monod's tube shaking gently at 37°C for 8 hours. Final concentration of K-101-NA was 0.125, 0.25, 0.5, 0.75, 1.0 and 1.5 mcg/ml. The growth inhibition was determined by nepherometry at 650 mμ after 8 hours incubation.

For the observation of metachromasia of the dyes induced by binding with protein, 0.01—2 % bovine serum protein in 0.1 M phosphate buffer solution (pH 7.2 and 5.9) was added to the dyes, 9×10^{-6} M in final concentration, and the absorption spectra were recorded by a recording spectrophotometer at 13—14°C.

For the gel-filtration by Sephadex, Sephadex G-25 was used. It was packed in a column of 1 cm in diameter to a height of 20 cm and 1/20 M phosphate buffer solution containing 0.3 % NaCl (pH 7.3) was used as the filtering solution. Two percent bovine serum protein in buffer sulution was mixed with an equal volume of 4×10^{-4} M K-101-NA and 0.9 ml of the mixture was filtered at a flow rate of 0.24 ml per minute in the dark at 5°C. The estimation of bovine serum protein was carried by the biurett reaction.

For the observation of metachromasia of the dye produced by binding with sodium chondroitin sulfate, 2.1×10^{-5} M solution of K-101-NA was mixed with an equal volume of 10^{-2}—10^{-6} M solution of sodium chondroitin sulfate (M. W. 575) and after 15 minutes incubation at 23°C, absorption spectra were drawn.

For the detection of metachromasia of the dyes bound to nucleic acid, DNA and RNA of 1 % to 0.0001 % solution in 0.01 M Tris-buffer solution containing 0.06 % NaCl (pH 7.4) was mixed with an equal volume of aqueous solution of K-101-NA (1.3×10^{-5} M) or K-301-NA (1.6×10^{-5} M) and after 30 minutes incubation at 15—16°C, absorption spectra were obtained on the mixture. The experiment was scheduled to be completed within 4 hours after preparation of the nucleic acid solution.

RESULTS

The experiments on binding of K-101-NA with bovine serum protein as studied by dialysis equilibrium method revealed that the binding cannot simply be expressed by the adsorption equation of KLOTZ (16). As is well known in the reversible binding of some low molecular compounds or ions with protein, the relation between the concentration of the free low molecular compound (C) and the number of the molecules bound with one protein molecule (r) can be given by the following equation (adsorp-

tion equation of KLOTZ);

$$1/r = 1/Kn \cdot 1/C + 1/n \quad \text{.....(A)}$$

Where n is the maximum number of the low molecular compound to be bound with one protein molecule. K is a constant. And if the compound binds reversibly with protein only at one site, then the relation between 1/C and 1/r should be given as a straight line. But the data obtained on K-101-NA (Table 2) did not give a straight line but a curve (Fig. 1). This shows a possibility of several different binding modes or irreversible binding between the dye and BSA. But in the range of concentration of 4×10^{-5} to 1.3×10^{-5} M of the dye it seemed to give a straight line (Fig. 1), and it was suggested that the maximum binding capacity might be obtained by the equation of SCATCHARD (17). The equation is equivalent to the

Table 2 TEST OF BINDING OF KANKOHSO 101-NA WITH BOVINE SERUM ALBUMIN BY DIALYSIS EQUILIBRIUM METHOD, VALUES AFTER 48 HOURS INCUBATION AT 0°~5°C

Final Concentration of Kankohso 101-NA ($\times 10^{-5}$M)		Amount of Bound Kankohso	Binding Rate
Inside of cellophane tube	Outside of cellophane tube	101-NA with Protein ($\times 10^{-5}$M)	(%)
8.450	4.900	3.550	42.0
3.910	2.175	1.735	44.4
2.525	1.350	1.175	46.3
1.768	0.910	0.858	48.5
1.320	0.670	0.650	49.2
0.840	0.344	0.496	59.2
0.545	0.182	0.363	66.6

equation (A) and gives a more precise maximum binding number: $r/C = Kn - Kr$ .....(B).

By applying equation (B) the data obtained on the K-101-NA gives a hyperboric curve as demonstrated in Fig. 2. Thus, it is indicated clearly that either the dye binds irreversibly with BSA or the dye has two or more kinds of binding sites for the albumin molecule, and by this equation the maximum binding capacity cannot be obtained, which will be taken as a crossing point of the line with the abscissa.

The binding of K-101-NA with BS_{γ}-G (Table 3) gave the similar results as in the binding of the dye with BSA. The curve showing the relation between 1/r and 1/C suggests that either the dye binds irreversibly with protein or the dye binds with protein molecule with bonds of more than one kind (Fig. 3). This relation is clearly indicated by drawing the curve with the values of r/C and r (Fig. 4).

The binding rate of K-101-NA with BSA at a certain concentration gave higher values in alkaline media than acidic media (Fig. 5). Thus in

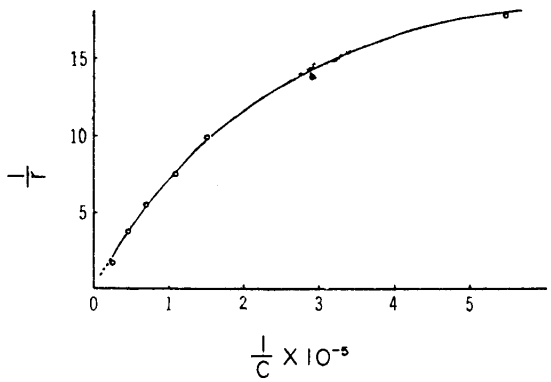


Fig. 1. Binding of Kankohso 101-NA with bovine serum albumin plotted by equation (A)
r: Number of bound Kankohso 101-NA per molecule of bovine serum albumin
C: Concentration of free Kankohso 101-NA

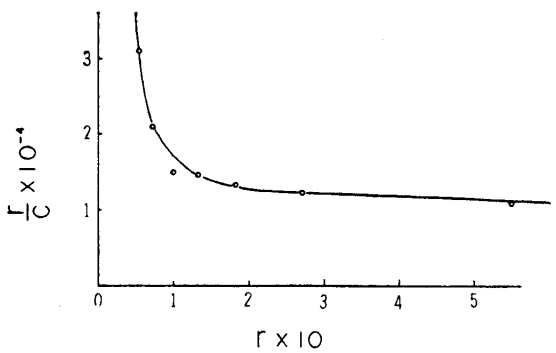


Fig. 2. Binding of Kankohso 101-NA with bovine serum albumin plotted by equation (B)
r and C are the same as those in Fig. 1.

Table 3 TEST OF BINDING OF KANKOHSO 101-NA WITH BOVINE SERUM γ-GLOBULIN BY DIALYSIS EQUILIBRIUM METHOD, VALUES AFTER-48 HOUR INCUBATION AT 0°~5°C

Final Concentration of Kankohso 101-NA ($\times 10^{-5}$M)		Amount of Bound Kankohso 101-NA with Protein($\times 10^{-5}$M)	Binding Rate (%)
Inside of cellophane tube	Outside of cellophane tube		
7.050	5.030	2.020	28.7
3.600	2.300	1.300	36.1
2.570	1.600	0.970	37.7
1.755	1.100	0.655	37.3
1.361	0.824	0.537	39.5
0.740	0.386	0.354	47.8
0.538	0.271	0.267	49.6

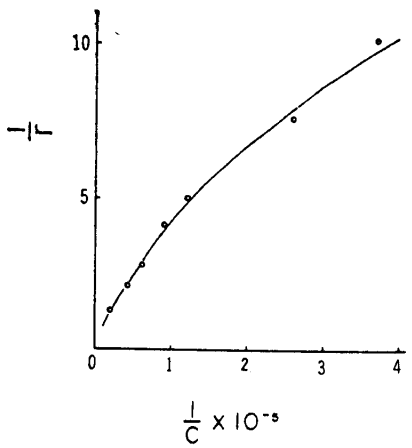


Fig. 3. Binding of Kankohso 101-NA with bovine serum γ-globulin plotted by equation (A)
r : Number of bound Kankohso 101-NA per molecule of bovine serum γ-globulin
C : Concentration of free Kankohso 101-NA

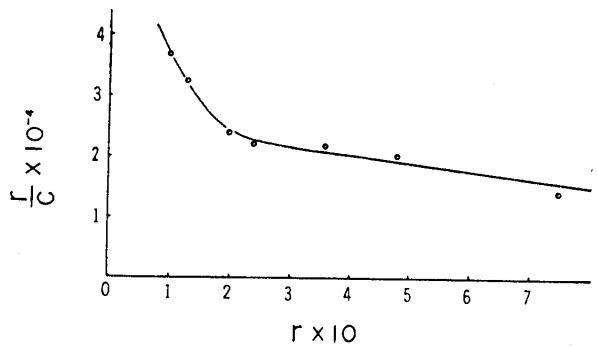


Fig. 4. Binding of Kankohso 101-NA with bovine serum γ-globulin plotted by epuation (B)
r and C are the same as those in Fig. 3.

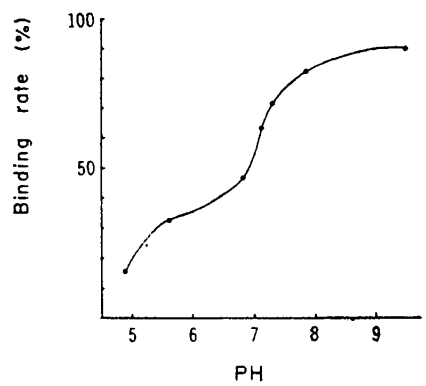


Fig. 5. Influence of pH on binding of Kankohso 101-NA with bovine serum albumin

0.5 % BSA solution only 16 % of the dye added (5×10^{-5} M) was bound to the protein at pH 4.8, while about 90 % of the dye was bound to the protein at pH 9.5 under the same conditions. The curve showing the binding rate of the dye with protein (Fig. 5) resembles the titration curve of the carboxylic acid residual group of BSA with alkali, showing that the dye binds with BSA by electrostatic binding between its quater-

nary nitrogen cation and carboxylic anion of the protein, though some binding forces other than ionic binding will participate in their binding in higher concentration as just pointed.

The binding between K-301-NA and BSA (Table 4) was also presented

Table 4 TEST OF BINDING OF KANKOHSO 301-NA WITH BOVINE SERUM ALBUMIN BY DIALYSIS EQUILIBRIUM METHOD, VALUES AFTER 48-HOUR INCUBATION AT 0°~5°C

Final Concentration of Kankohso 301-NA ($\times 10^{-5}$M)		Amount of Bound Kankohso 301-NA with Protein($\times 10^{-5}$M)	Binding Rate (%)
Inside of cellophane tube	Outside of cellophane tube		
43.40	39.0	4.40	10.2
25.41	22.0	3.41	13.4
14.55	12.15	2.40	15.8
8.22	6.73	1.49	18.1
2.224	1.75	0.474	21.3
1.275	1.00	0.275	21.7

in Figs. 6 and 7 showing the relation between 1/C and 1/r or r and r/C. The experimental data gave linear plots showing that the dye has only one kind of binding site with BSA and the maximum binding number of the dye is 1.1 per molecule of BSA. However, K-301-NA did not bind with BSγ-G. The binding between the dye and BSA was also shown to be changed largely depending upon the pH of the media (Fig. 8). The binding rate of the dye to the protein increased at higher pH region (pH 8—10) and minimized at lower pH reaching the minimum value at pH 6.5.

K-101-NA shows metachromasia binding with BSA and BSγ-G (Figs. 9—11). The absorption maximum of the dye in the medium of pH 7.2 shifted from 591 to 596 mμ at 1.8% BSA and at the same time, its absorbance decreased by about 17% (Fig. 9). The color of the solution changed from bluish purple to deep blue. The maximum absorption of the dye shifted toward the side of longer wave length with the increase of the protein

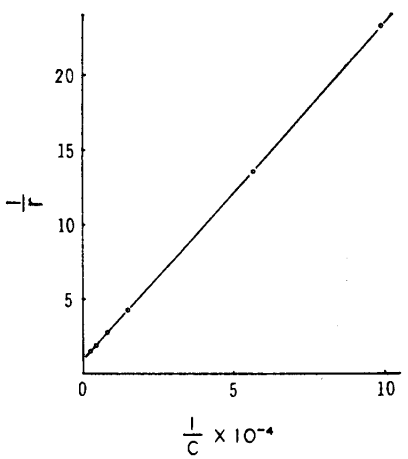


Fig. 6. Binding of Kankohso 301-NA with bovine serum albumin plotted by equation (A)

r: Number of bound Kankohso 301-NA per molecule of bovine serum albumin

C: Concentration of free Kankohso 301-NA

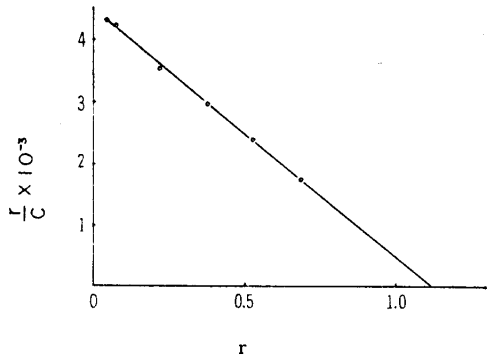


Fig. 7. Binding of Kankohso 301-NA with bovine serum albumin plotted by equation (B) r and C arc the same as thosee in Fig. 6.

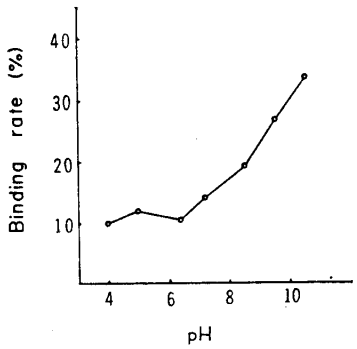


Fig. 8. Influence of pH on binding of Kankohso 301-NA with bovine serum albumin

concentration and the absorbance also decreased gradually (Fig. 10). Observation on the binding of the dye with BSγ-G gave also the similar result (Fig. 11).

K-301-NA did not produce any metachromasia by binding with BSA. The above experiments suggest a relatively strong binding between K-101-NA and BSA, possively an irreversible binding in a higher concentration. With this dye-protein complex the binding force between the molecules was

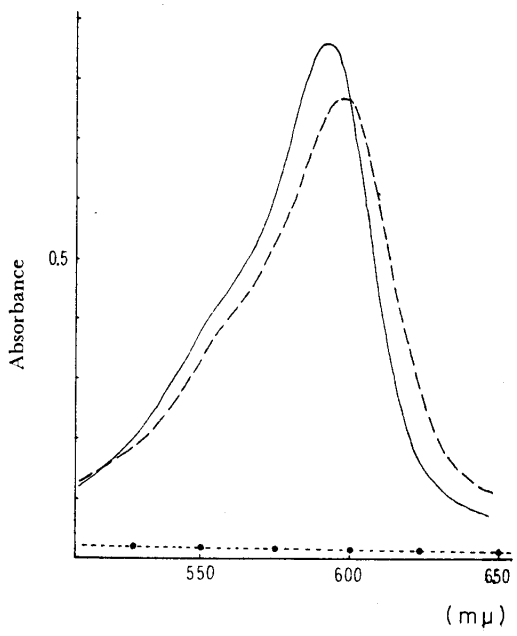


Fig. 9. The changes in absorption spectra of Kankohso 101-NA produced by binding with BSA at pH 7.2
Solid line : Absorption spectra of Kankohso 101-NA in the buffer solution, Broken line: Absorption spectra of the same dye in 1.8 % BSA solution, Broken line with filled circles: Absorption spectra of 2 % BSA

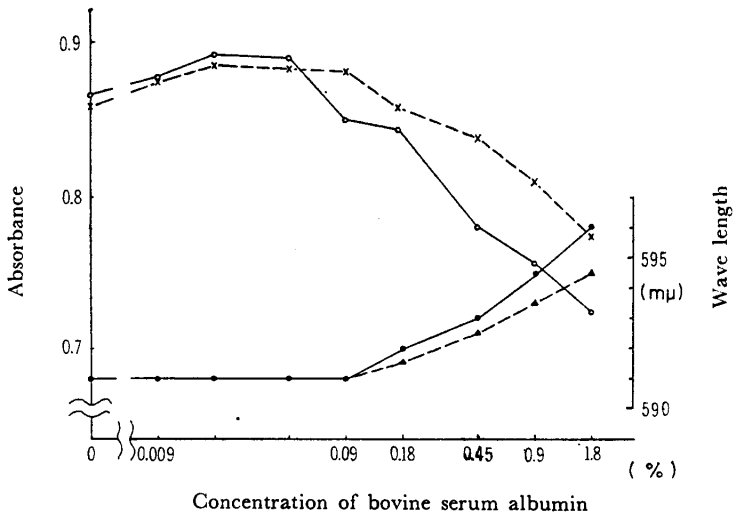


Fig. 10. Metachromasia of Kankohso 101-NA produced by BSA
Changes in absorbance and shift of wave length at the absorption maximum plotted against increasing concentration of BSA
Solid line with open circles: Absorbance at pH 7.2, Broken line with cross: Absorbance at pH 5.9, Solid line with filled circles: Shift of wave length at pH 7.2, Broken line with triangles: Shift of wave lengt at pH 5.9

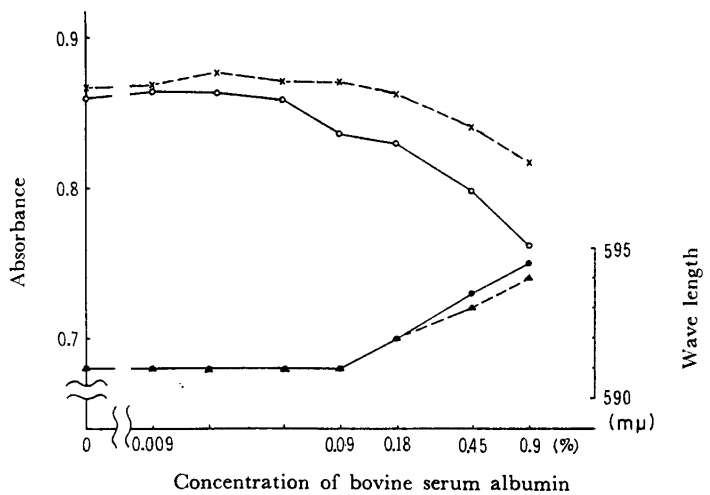


Fig. 11. Metachromasia of Kankohso 101-NA produced by BSγ-G
Changes in absorbance and shift of wave length at the absorption maximum are plotted against increasing concentration of BSγ-G
Solid line with open circles: Absorbance at pH 7.2, Broken line with cross: Absorbance at pH 5.9, Solid line with filled circles: Shift of wave length at pH 7.2, Broken line with triangles: Shift of wave length at pH 5.9

studied by Gel-filtration technique using Sephadex G-25. The experiment revealed that the dye could be released from the dye protein complex by Sephadex G-25 (Fig. 12). The peak of the dye concentration found by eluting the protein dye complex appeared at the same location as the peak found by eluting the pure dye solution. The similar experiment was carried out on K-101-NA and BS_{γ}-G and nearly the same results on the dye with BSA were obtained. The data indicate that K-101-NA bound considerably with BSA and BS_{γ}-G gives distinct metachromasia but the binding with the protein is not so strong and separated by eluting through Sephadex.

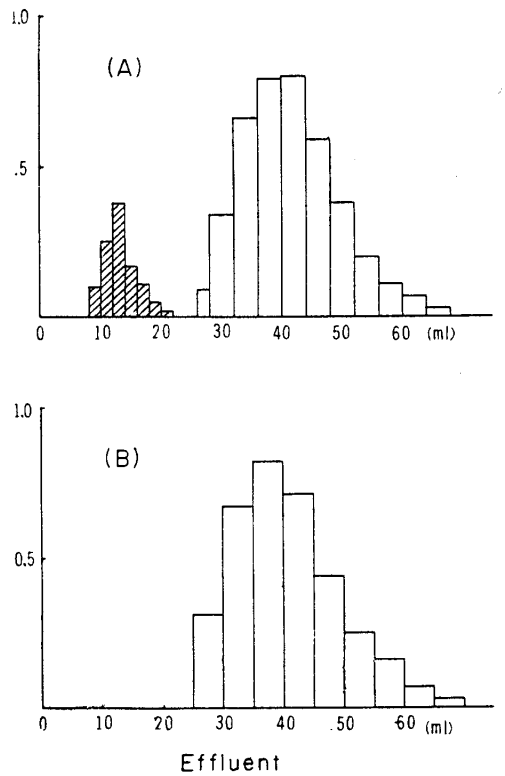


Fig. 12. Gel-filtration of the Kankohso 101-NA-BSA complex (A) and the pure dye solution (B)
Hatched column : Absorbance of BSA at 540 mμ by biurett reaction, Open column : Absorbance of Kankohso 101-NA at 590 mμ

The minimal inhibitory concentration of K-101-NA against *Staph. aureus* FDA declined from 0.25 mcg to 1 mcg with increase in the peptone concentration in the medium from 0.5 to 1 %, but further increase in

peptone concentration (1.5 %) gave no stronger effect (Table 5). The dye exhibited a stronger growth inhibitory action on the bacteria in the range of pH 7.4 to 8.5 than the acidic medium, pH 6.0 (Table 6).

Table 5 MININAL INHIBITORY CONCENTRATION OF KANKOHSO 101-NA AGAINST *STAPH. AUREUS* FDA IN THE MEDIUM CONTAINING EXCESS PEPTONE (mcg/ml)

Amount of Peptone in the Medium	0.5 % (Control)	1 %	1.5 %
Minimal Inhibitory Concentration	0.25	1.0	1.0

Table 6 MINIMAL INHIBITORY CONCENTRATION OF KANKOHSO 101-NA AGAINST *STAPH. AUREUS* FDA AT VARIOUS pH's OF THE MEDIUM (mcg/ml)

pH of the Medium	6.0	7.4	8.5
Minimal Inhibitory Concentration	1.0	0.25	0.25

Absorption spectra of K-101-NA are changed by binding with chondroitin sulfate (Fig. 13). The maximum absorption at 592 mμ of the dye decreases in the presence of chondroitin sulfate without shifting its wave length and leads to the minimum value at 10^{-5} M of chondroitin sulfate, and a new peak of the absorption at 555 mμ appeared, whose absorbance was not influenced so much by the change in the chondroitin sulfate concentration. At this point the color of solution changed from a violet blue to a reddish purple. As the concentration of chondroitin sulfate increased further, reversion of metachromasia took place and the absorption spectrum recovered to the original one. At 10^{-2} M, the effect of chondroitin sulfate could no longer be observed. Fig. 14 shows the change in absorbance at 592 and 555 mμ with the changed concentration of chondroitin sulfate. The absorption at 555 mμ appeared clearly forming a distinct peak at the concentration of 10^{-5} M chondroitin sulfate where the dye showed the strongest metachromasia but its height hardly changed keeping nearly the same value as given by the original one and the absorbance at 592 mμ decreased considerably taking the value of about 1/2 of the original level. There was no shift in wave length, and the change in color was deduced to be solely due to the decrease in absorbance at 592 mμ. Detailed investigation by changing the concentration of chondroitin sulfate made it clear that at the concentration showing the strongest metachromasia the number of sulfonic acid radicals in condroitin sulfate is equal to the number of molecules of K-101-NA.

The color of K-101-NA changed from violet blue to bright blue by combinig with DNA. In the metachromasia produced by combining with DNA the absorption maximum at 592 mμ of the dye shifted to the long wave length side, the magnitude increased with the increase in DNA con-

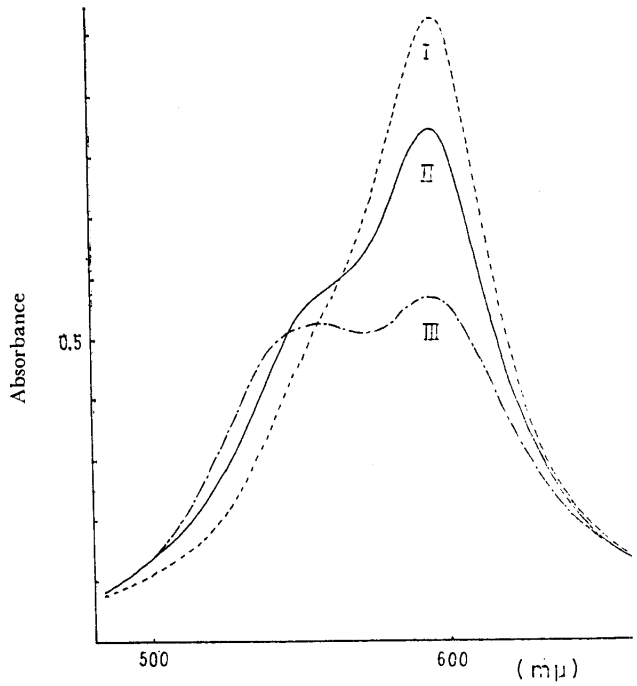


Fig. 13. The changes in absorption spectra of Kankohso 101-NA after binding with sodium chondroitin sulfate

Broken line I : Absorption spectra of Kankohso 101-NA in water, Solid line II : Absorption spectra of the same dye in 10^{-3} M solution of sodium chondroitin sulfate, Broken line III : Absorption spectra of the same dye in 10^{-5} M solution of sodium chondroitin sulfate

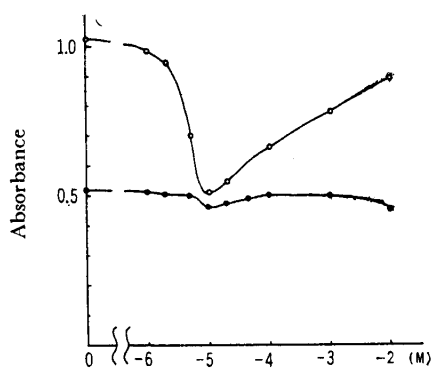


Fig. 14. The changes in absorbance of Kankohso 101-NA at the absorption maximum and the shoulder plotted against increasing concentration of sodium chondroitin sulfate

Solid line with open circles : Absorption maximum (592 mμ). Solid line with filled circles : Shoulder (555 mμ)

centration, by 15 mμ in 0.5 % DNA solution (Figs. 15, 16). The absorption intensity at 592 mμ decreases with increase in the DNA concentration first reaching the minimum at 5×10^{-3} % of DNA concentration being decreased by about 25 % but later the absorption intensity increases again with further increase in the DNA concentration. The shoulder appearing on the short wave length side also shifted toward the long wave length side with the increase in DNA concentration by 10 mμ in 0.5 % DNA solution. The pattern of the curve showed no distinct change independent of the shift of the maximum absorption.

Metachromasia of K-101-NA was also induced by combining with RNA but it showed nearly the same tendency as in the case of DNA (Figs. 17, 18). In 0.5 % RNA solution the absorption maximum shifted by 12 mμ toward the long wave length side and at 5×10^{-3} % RNA concentration the absorption intensity decreased by about 30 % of the original level. The color of K-101-NA changed from violet blue to bright blue by changing the RNA concentration just as in the case of DNA.

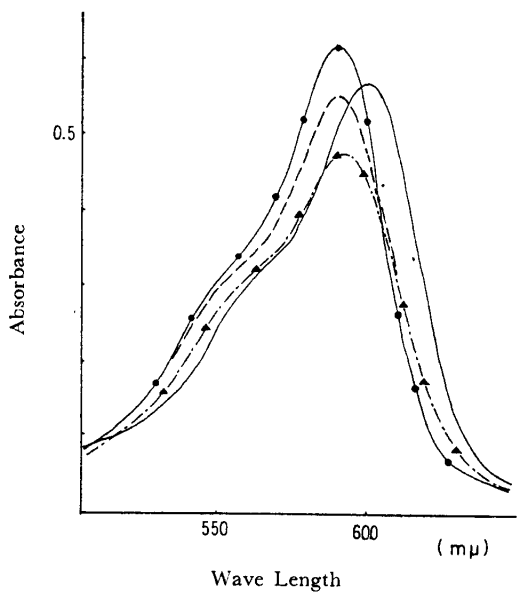


Fig. 15. The changes in absorption spectra of Kankohso 101-NA produced by binding with DNA

Solid line with filled circles : Absorption spectra of Kankohso 101-NA in the buffer solution, Broken line : Absorption spectra of the same dye in 0.0005 % DNA solution, Broken line with triangles : Absorption spectra of the same dye in 0.005 % DNA solution, Solid line : Absorption spectra of the same dye in 0.05 % DNA solution

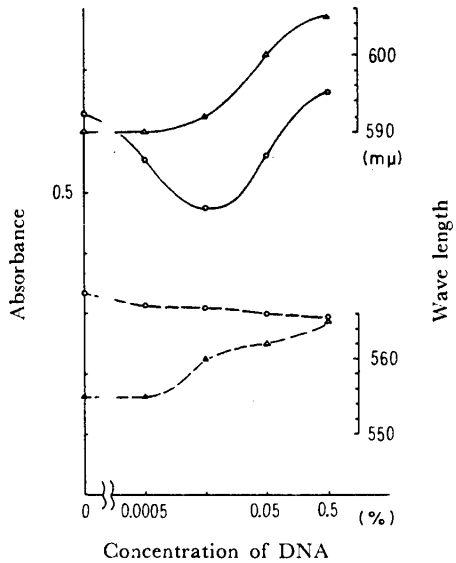


Fig. 16. The changes in absorbance and wave length of Kankohso 101-NA at the absorption maximum and the shoulder plotted against increasing concentration of DNA

Solid line with open circles : Absorbance of the absorption maximum, Solid line with triangles: Wave length of the absorption maximum, Broken line with open circles : absorbance of the shoulder, Broken line with triangles: Wave length of the shoulder

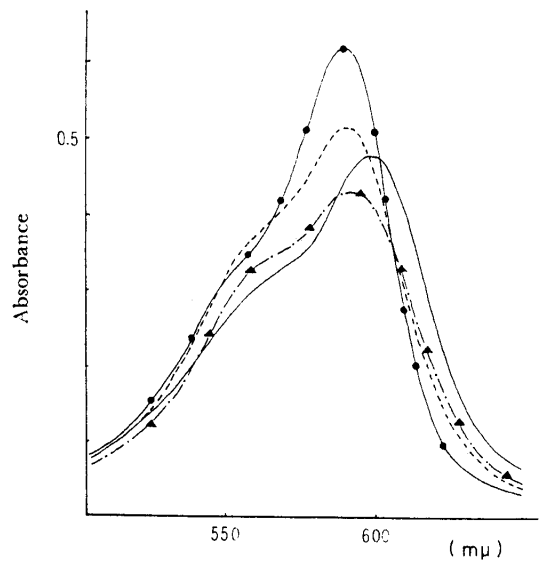
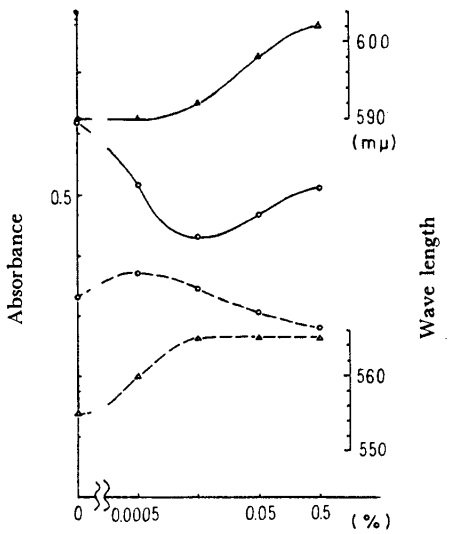


Fig. 17. The changes in absorption spectra of Kankohso 101-NA produced by binding with RNA

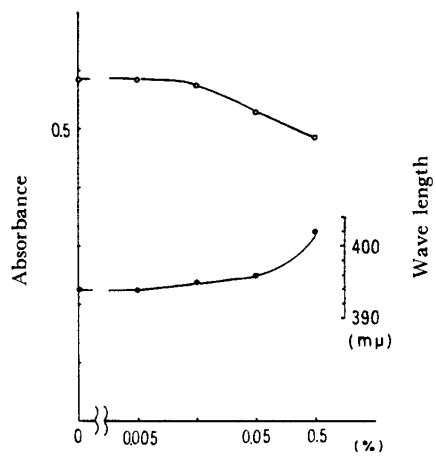
Solid line with filled circles : Absorption spectra of Kankohso 101-NA in the buffer solution, Broken line : Absorption spectra of the same dye in 0.0005 % RNA solution, Broken line with triangles : Absorption spectra of the same dye in 0.005 % RNA solution, Solid line : Absorption spectra of the same dye in 0.05 % RNA solution



Concentration of RNA

Fig. 18. The changes in absorbance and wave length of Kankohso 101-NA at the absorption maximum and the shoulder plotted against increasing concentration of RNA
Solid line with open circles : Absorbance of the absorption maximum, Solid line with triangles : Wave length of the absorption maximum, Solid line with triangles : Wave length of the absorption maximum, Broken line with open circles : Absorbance of the shoulder, Broken line with triangles : Wave length of the shoulder

The absorption curve drawn with K-301-NA combined with nucleic acid hardly presented a clear picture of the shift of the absorption maximum at 394 mμ being concealed in the absorption of nucleic acid, but the maximum absorption decreased in intensity by 15 % and shifted toward long wave length side by 9 mμ with the increase in nucleic acid concentration (Fig.19). The changes were the same in both DNA and RNA.



Concentration of nucleic acid

Fig. 19. The changes in absorbance and wave length of Kankohso 301-NA at the absorption maximum plotted against increasing concentration of nucleic acid
Solid line with open circles : Absorbance, Solid line with filled circles : Wave length

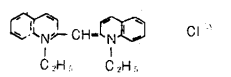
DISCUSSION

As shown in the above experiment, among the substances tested, protein, chondroitin sulfate and nucleic acid, K-101-NA or K-301-NA showed an interesting combining mode with BSA and BS$_7$-G. Generally, anionic dyes having sulfonic acid group like methyl orange bind strongly with BSA showing

metachromasy and a number of study have been reported concerning their binding (18, 19). However, there are only a few research reports on the binding of cationic dyes or organic cationic compounds with protein, as many of them do not bind with serum protein. According to KLOTZ (20), organic cationic compounds generally do not bind with bovine serum protein. In Table 5 some of these cationic compounds are presented. (*A*) is one of the photosensitizing dyes of cyanine type and (*B*) is of styryl type. Both of them have quaternary nitrogen of positive charge as K-101-NA (cyanine type) or K-301-NA (aminovinyl type) but they do not bind with bovine serum albumin or globulin. (*C*) is a basic dye and also it does not bind with serum albunin. The data are similar to those given by KUSUNOKI (21) who reported that the basic dyes such as methylene blue, toluidine blue and rose anilin hydrochloride did not interact with BSA and BS_{γ}-G even at a higher cencentration of the protein. The azo dye having quaternary ammonium ion (*D*) also did not interact with BSA and BS_{γ}-G as observed by dialysis equilibrium test and spectrophotometric study on metachromasy.

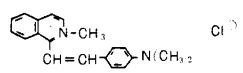
Table 5 ORGANIC CATIONIC COMPOUNDS NOT BINDING WITH SERUM PROTEIN

(*A*)



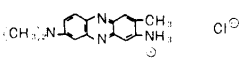
1, 1'-Diethyl-2, 2'-cyanine chloride

(*B*)



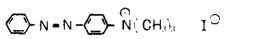
1-(p-Dimethyl amino styryl)-isoquinoline metho chloride

(*C*)



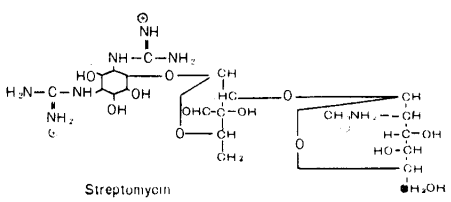
Neutral red

(*D*)



p-Azobenzen trimethyl ammonium iodide

(*E*)



Streptomycin

On the other hand, it is reported that some cationic compounds having quaternary ammonium group combine with protein provided with a long chain alkyl group in their molecular structures (22, 23). For example, benzylcetyldimethylammonium chloride binds with serum albumin stoichiometrically. In this case the number of molecules of the detergent bound with one protein molecule was approximately equal to the number of the carboxyl groups of the protein. Thus organic cationic compound binds with protein if it has some long hydrophobic group or groups. It is to be stressed, however, that the binding force of the cationic compound with protein is much weaker compared to the anionic compound of similar molecular size. For example, tetraethylammonium ion and trimethylphenylammonium ion do not shift the isoionic point of a protein when they are added to the protein solution (24). Roseaniline hydrochloride does not bind with BSA (21), while bromphenol blue, an anionic dye of the similar size having sulfonic acid group, binds irreversibly with BSA, as has been confirmed by the gel-filtration experiment, in which the BSA-bromphenolblue complex was eluted through Sephadex G-25 (25). Another example is that methyl orange, an anionic dye, binds with BSA strongly (18), whereas *p*-azobenzene-trimethyl ammonium (*D*), a cationic dye whose molecular size is comparable to that of methylorange, does not bind with the protein.

The observations cited above suggest that K-301-NA might not combine with BSA absolutely, while K-101-NA might show some affinity to the protein, because K-301-NA is a cationic compound poor in hydrophobic group, while K-101-NA has three n-heptyl radicals in its molecule.

As expected the present experiment proved that K-101-NA had a fairly good affinity with BSA though the binding was reversible being dissociated by eluting through Sephadex G-25. The dialysis equilibrium test proved that the dye has two or more kinds of binding sites with BSA. But as far as K-301-NA is concerned, the result is far from the initial expectation and it shows some affinity to BSA.

The test by using BS_{γ}-G revealed that K-101-NA binds with BS_{γ}-G which shows absolutely no affinity to both cationic and anionic compounds (20, 26). Binding mode between K-101-NA and BS_{γ}-G was similar to that of BSA, as was deduced from the dialysis equilibrium test and metachromasia.

As reported previously, K-101-NA has a marked antibiotic activity (27) and its minimal growth inhibitory concentration against *Staph. aureus* FDA is comparable to that of K-101-NA. As was supposed from the characteristics of K-101-NA showing a fairly good affinity to BSA, the

antibiotic activity of K-101-NA was reduced along with the increase in peptone concentration of the medium. In addition, it is predictable from the higher binding rate of K-101-NA to BSA in alkaline medium that the antibiotic activity increases in acidic medium and decreases in alkaline medium. Unexpectedly, however, antibiotic activity of K-101-NA decreased in the acidic medium and increased in the alkaline and neutral media indicating that K-101-NA has a stronger affinity to the bacterial cells than peptone.

As described above K-101-NA shows a distinct metachromasia by combining with chondroitin sulfate and nucleic acid and yet the metachromatical color changes induced by these two substance are in opposition to each other. This characteristic of the dye is helpful for microscopic observasion of tissues and is used for tissue staining by some authors (28, 29, 30), though the color change of K-101-NA produced by chondroitin sulfate and nucleic acid is not so marked as other photosensitizing dyes. The present quantitative study on metachromasia given by K-101-NA binding with chondroitin sulfate reveales that the strongest metachromatical change takes place when the concentration of the dye is equal to that of sulfate group in the chondroitin sulfate. The result is comparable to the data presented by LAVINE and SCHUBERT (26) using methylene blue-chondroitin sulfate system but referring to K-101-NA only one of the two quaternary ammonium groups of K-101-NA participates in the combination with the sulfonate group of chondroitin sulfate. On the other hand, K-301-NA is also cationic compound but metachromasia is not produced by chondroitin sulfate.

The affinity of K-101-NA to nucleic acid may be corelated to the bactericidal activity of the dye. The binding will occur between phosphoric acid group of nucleic acid an quaternary nitrogen group and metachromatic color change of K-101-NA induced by nucleic acid resembles very closely the metachromasia elicited by amino-acridines combined with nucleic acid (32). The maximum point of decrease in absorbance of the absorption maximum of K-101-NA bound with DNA is given by 5×10^{-3} % DNA concentration (approx. 1.5×10^{-4} M : concentration of phosphate radical in DNA) indicating no special relation between the concentrations of the two differing from that observable between acridine orange and nucleic acid, where the maximum point of change in absorption appears in the polymers in which number of molecules of acridine orange is equal to that of phosphate group of DNA (33).

K-301-NA does not show metachromasia by BSA and chondroitin sulfate but metachromasia is induced by nucleic acid indicating affinity

of the drug to DNA or RNA. The detailed observation on the combining mode between them needs to be carried out in the futhure.

SUMMARY

To obtain some information of the biological action of Kankohso 101-dinicotinate and Kankohso 301-nicotinate, observations were made on the binding mode of these substances with protein, chondroitin sulfate and nucleic acids and the following results were obtained;

1. Kankohso 101-dinicotinate binds reversively with bovine serum albumin or serum γ-globulin, resulting in metachromasia. By binding with proteins the absorption maximum of the dye shifts toward the long wave length side and the absorbance decreased distinctly. The data show that there are more than one kind of binding sites and the binding with bovine serum albumin is weak in acidic solution and strong in alkaline solution.

2. Kankohso 101-dinicotinate produces strong metachromasia with sodium chondroitin sulfate and the color of the solution changes from violet blue to reddish violet. The absorption maximum at 592 mμ decreases without shifting its wave length and the shoulder appears at 555 mμ becomes distinct peak. The strongest metachromatical changes occurs at the concentration of the chondroitinsulfate whose sulfonate radicals is equal to the molecules of Kankohso 101-dinicotinate.

3. Kankohso 101-dinicotinate produces metachromasia with nucleic acid, where absorption spectrum is shifted toward long wave length and absorbance is decreased at a certain concentration.

4. Kankohso 301-nicotinate binds weakly with bovine serum albumin, the binding of which is reversible and the maximum binding number is 1.1 per molecule of albumin. Metachromasia cannot be produced by binding. Kankohso 301-nicotinate does not bind with bovine serum γ-globulin. This compund does not produce metachromasia with sodium chondroitin sulfate but produces weak metachromasia with nucleic acid, indicating some affinity to nucleic acid.

ACKNOWLEDGEMENT

The author wishes to express appreciation to Prof. MASANA OGATA, Department of Public Health, Okayama University Medical School for enthusiastic guidance throughout the study. Thanks are given to Mr. HIROSHI HARA for assistance in the experimental work.

REFERENCES

1. Commentary of Japanese Standard of Cosmetic Ingredients, p. 62, 1st ed. Yakujinippohsha, Tokyo, 1968
2. ARAKAWA, N.: *Kankohshikiso* **19**, 1, 1953
3. HONJO, H.: *Kankohshikiso* **43**, 27, 1957
4. HONJO, H.: *Kankohshikiso* **48**, 1, 1958
5. NAKAI, T.: *Kankohshikiso* **65**, 1, 1962
6. ICHIHASHI, H. and KONDO, I.: *Gann* **58**, 529, 1967
7. KOGATA, S.: Okayama-Igakukai-Zasshi **70**, 1878, 4451, 1958
8. Commentary of Japanese Standard of Cosmetic Ingredients, p. 68, 1st ed. *Yakujinippohsha*, Tokyo, 1968
9. KANAMORI, H., IHARA, J., TAKAMORI, T. and SUZUKI, S.: *Kankohshikiso* **20**, 17, 1953
10. KANAMORI, H.. LING, C. and HAKOKI, K.: *Kankohshikiso* **25**, 14, 1954
11. NISHIJIMA, S.: *Kankohshikiso* **26**, 20, 1954
12. NISHIJIMA, S.: *Kankohshikiso* **27**, 1, 1954
13. ITO, K. and KURODA, K.: *Kankohshikiso* **49**, 30, 1958
14. MAESAWA, T.: *Kumamo.o Med. J.* **8**, 93, 101, 125, 145, 187, 210, 1955
15. SUZUE, K. and YASUI, S.: *J. Japanese Cosmeetic Chemist Association* **5**, 54, 1969
16. KLOTZ, I. M.: *Arch. Biochem. Biophys.* **9**, 109, 1946
17. SCATCHARD, G.: *Ann. N. Y. Acad. Sci.* **51**, 660, 1949
18. KKOTZ, I. M.: *J. Am. Chem. Soc.* **68**, 2299, 1946
19. GOLDSTEIN, A.: *Pharm. Rev.* **2**, 102, 1949
20. KLOTZ, I. M., GELEWITZ, E. W. and URQUHART, J. M.: *J. Am. Chem. Soc.* **74**, 209, 1952
21. KUSUNOKI, T.: *J. Biochem.* **40**, 277, 1953
22. GLASSMAN, H. N.: *Ann. N. Y. Acad. Sci.* **53**, 91, 1950
23. CHINARD, F. P.: *J. Biol. Chem.* **176**, 1439, 1948
24. SCATCHARD, G. and BLACK, E. S.: *J. phys. and colloid Chem.* **53**, 88, 1949
25. YASUI, S.: Unpublished data
26. KLOTZ, I. M. and URQUHART, T. M.: *J. Am. Chem. Soc.* **71**, 1597, 1949
27. MURAKAMI, S., NAGAO, N. and BANNO, M.: *Kankohshikiso* **8**, 33, 1951
28. MASAKI, K.: *Oral Surgery* **2**, (2), 98, 1956
29. OKADA, A.: *Rep. Tubec. Res. Inst.*, Kyoto Univ. 12, No. 1, 11, 1963, No. 2, 157, 1964
30. MASAKI, K. and others: *Kankohshikiso* **39**, 17, 1956
31. LEVINE, A. and SCHUBERT, M.: *J. Am. Chem. Soc.* **74**, 91, 1952
32. MORTHLAND, F. W., DE BURYN, P. P. H. and SMITH, N. H.: *Expel. Cell. Res.* **7**, 201, 1954
33. BRADLEY, D. F. and WALF, M. K.: *Proc. Nat. Acad. Sci. U. S.* **45**, 944, 1959