It is believed therefore that this technique is not only useful in screening unknown antitumor compounds but also in analyzing condition of manifestation of action or mode of action of certain compounds. Determination of resistant grade of resistant strains of Yoshida sarcoma was also defined by comparing IC_{50} of the resistant and the normal strains by this technique.

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Summary

A new method of screening of antitumor compounds employing Yoshida sarcoma cell suspension cultured *in vitro* was presented. Evaluation of the antitumor effect was carried out by determination of cytomorphological change and inhibition of cell proliferation by cell counting.

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74. **Toyozo Uno and Koichiro Miyajima** : Determination of Surface-active Agent. III.[*2] Volumetric Determination of Anionic Surface-active Agent using Neutral Red as an Indicator.[*3].

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Titrimetric determination of surface-active agents using dyes has been studied and widely used, because of simplicity of procedure, accuracy of results, and convenience in practical use. Two series of volumetric methods of surfactant determination has been known, namely, as Hartley's and Epton's methods.1,2)

Hartley1) reported the determination of cationic surface-active agent (CSAA) with anionic surface-active agent (ASAA) using Bromophenol blue as an indicator in ammonia alkaline medium. This method depends upon the difference of the color of Bromophenol blue-CSAA complex (blue) and alkaline Bromophenol blue (blue purple), however the recognition of the end-point is not easy, because of the similarity of these two colors.

Epton2) also determined the ASAA with CSAA using Methyleneblue and organic solvents, such as chloroform or ethylene dichloride. Methyleneblue forms a complex with ASAA, which is insoluble in water and soluble in organic solvents. This method has been studied by many workers.3~6) Recently Aoki and Iwayama7) reported the determination of ionic surface-active agents using Bromocresol green-Neutral red (NR) com-

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[*2] Part II : This Bulletin, **9**, 326 (1961).

[*3] This work was presented at the Kinki branch meeting of Pharmaceutical Society of Japan, February, 1961.

1) G.S. Hartley, D.F. Runnicles : Proc. Roy. Soc. (London), **168**, 424 (1938).
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3) A.S. Weatherburn : J. Am. Oil Chemist's. Soc., **28**, 233 (1951).
4) H. Glazer, T.D. Smith : Nature, **169**, 497 (1952).
5) W. Kimura, H. Ihara : Yukagaku, **9**, 124, 296, 480, 482, 484 (1960),
6) M. Aoki, Y. Iwayama : Yakugaku Zasshi, **79**, 522, 526 (1959).
7) *Idem* : *Ibid.*, **80**, 1745 (1960).

plex and chloroform. This method also depends on the difference of the solubility of NR-ASAA, Bromocresol green-CSAA in chloroform and in water. Therefore, this color change is not due to the pH change.

In the recent paper,*[2] we reported the titrimetric determination of CSAA with precipitating reagents using Methyl Orange as an indicator, taking the advantage of their micelle formation ability. This paper deals with the determination of several kinds of ASAA using NR as an indicator. When NR is added to the solution of ASAA, complexes of NR with ASAA are produced, but ASAA include and solubilize these complexes, and alkaline color of NR is not observed even in alkaline medium (pH : 8∼9). However, when this solution is titrated with the standard solution of CSAA, the alkaline color of NR appears at the end-point.

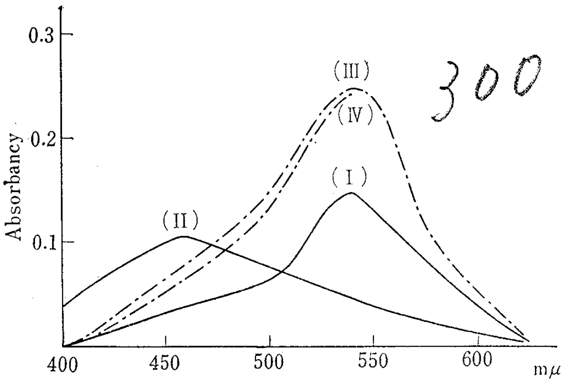

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Fig. 1. Absorption Spectra of Neutral Red in Water and in $M/100$ Sodium Lauryl Sulfate Solution

Ⅰ : 0.15 cc. of $M/1000$ Neutral red solution in 10 cc. water (pH : 6.0)
Ⅱ : 0.15 cc. of $M/1000$ Neutral red solution in 10 cc. water (pH : 8.5)
Ⅲ : 0.15 cc. of $M/1000$ Neutral red solution in 10 cc. of $M/100$ sodium lauryl sulfate solution (pH : 6.0)
Ⅳ : 0.15 cc. of $M/1000$ Neutral red solution in 10 cc. of $M/100$ sodium lauryl sulfate solution (pH : 8.5)

As shown in Fig. 1, absorption spectrum of NR in acid (pH : 6.0) and alkaline (pH: 8.5) medium has the absorption maximum at 535 and 460 mμ. (I, Ⅱ), respectively. When two or three drops of $M/1000$ NR solution were added to 10 cc. of $M/100$ sodium lauryl sulfate (SLS) solution, precipitate was formed at first and then dispersed into SLS solution while stirring. Absorption spectra of this solution are Ⅲ (pH : 6.0), and Ⅳ (pH: 8.5). Both have the same absorption maximum at 545 mμ. However, the alkaline color gradually appears at pH 9, and at pH 10 the absorption maximum transfers to 460 mμ.

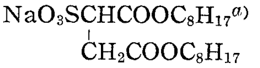
When the solution of same molar concentration of NR and SLS were mixed, dark precipitate with green fluorescence formed. Recrystallizing from benzene, plate crystals were obtained, which correspond to monohydrate of 1:1 complex. These crystals are insoluble in water, and soluble in ASAA solution and organic solvents. The absorption spectra of these crystals in SLS solution and the solution of NR in SLS solution were found to be similar. When CSAA solution is added to the solution of spectrum Ⅳ, pink color disappears and turns to yellow at the end-point. It seems that these phenomena suggest that at first, CSAA, the titrant, combines with free SLS, and then combines SLS which include the 1:1 complexes. At the end-point, the complexes are destroyed, and NR is set free from the complexes, and the alkaline color of NR appears. In low concentration such as $M/400 \sim M/800$ SLS solution, alkaline color of NR appears, when pH is adjusted to 8.5 with ammonia-ammonium chloride buffer solution. However, in the course of titration with CSAA solution, a yellow color of SLS solution turns to pink, and the pink color disappears again at the end-point. Similar phenomenon was also observed more obviously in the determination of CSAA with sodium tetraphenylborate using Methyl Orange as an indicator in low concentration. This phenomenon seems to be caused by the following effects.

1) Adsorption effect of slightly soluble CSAA-ASAA precipitate.
2) Ionic effect of inorganic salts formed.
3) Micelle effect of dissolved CSAA-ASAA precipitate.

These three effects make the critical micelle concentration (c. m. c.) of the solution lower. Among these three effects, it seems that adsorption effect of the precipitate is the most important, considering the facts that the color of the solution returns to pink when CSAA-ASAA precipitate or benzene is added to the alkaline (yellow color) solution. This phenomenon is observed in certain range of the concentration near the c. m. c. of the solution. When the concentration is lower than the c. m. c., such a phenomenon cannot be observed.

Considering these phenomena, SLS and other commercially available ASAA were determined and the results were found to agree with those of Epton's method. This method is simpler than Epton's method, and the end-point is observed clearly, because ion exchange reaction takes place more rapidly than in the case of Epton's method. Other dyes applicable for this titration could not be found, but dyes which are the pH indicator and combines with the ASAA would be useful for this method. No interference was observed even by addition of non-ionic surface-active agent, such as Tween 40, and Tween 85. Addition of sodium sulfate scarcely influences the titration. Results of determination are shown in Table I.

Table I. Determination of Anionic Surface-active Compounds
(Titrant : *M*/100 cetyltrimethyl ammonium chloride)

ASAA.	mg./cc. (Added)	by this method		by Epton's method	
		mg./cc. (Found)	Recovery (%)	mg./cc. (Found)	Recovery (%)
$C_{12}H_{25}SO_4Na$	3.049	3.009	98.70	3.009	98.70
$C_{12}H_{25}SO_4Na$[a]	2.895	0.989	34.12	0.994	34.30
$C_{18}H_{37}SO_4Na$[a]	3.910	1.474	37.60	1.492	38.10
$C_{12}H_{25}C_6H_4SO_3Na$[a]	4.510	1.780	39.46	1.780	39.46
$C_{12}H_{25}C_6H_4SO_3NH\text{-}C_3H_7$[a]	4.327	2.234	51.62	2.242	51.81
 $NaO_3SCHCOOC_8H_{17}$[a] $CH_2COOC_8H_{17}$	4.724	1.353	30.43	1.353	30.43

a) These are commercially available compounds.

Experimental

Method——(1) Procedure : To 10～20 cc. of *M*/50～*M*/200 sample solution, 2 or 3 drops of *M*/1000 NR solution is added, the solution is adjusted to pH 8.5 with NaOH, and titrated with *M*/100 cetyltrimethyl ammonium chloride solution until the red color disappears.

(2) Preparation of Reagents : *M*/100 standard cetyltrimethyl ammonium chloride solution; cetyltrimethyl ammonium chloride (Reagent grade) (3.2 g.) is dissolved in H_2O to make 1 L.,

pH value of this solution is 6.5. *M*/1000 NR solution; NR (Reagent grade) (0.3 g.) is dissolved in H_2O to make 1 L.

pH value of this solution is 4.5. NH_4OH-NH_4Cl solution; NH_4Cl (0.07 g.) and NH_4OH (0.57 cc.) (s. g. : 0.90) are dissolved in H_2O to make 1 L.

pH value of this solution is 9.70.

(3) Determination of factor of *M*/100 cetyltrimethyl ammonium chloride solution. To 10 cc. of cetyltrimethyl ammonium chloride solution measured accurately, a drop of *M*/200 Methyl Orange solution is added and adjusted to pH 3 with HCl. This solution is titrated with *M*/100 sodium tetraphenylborate solution standarized with KCl, until the pink color appears.

Discussion of conditions——(1) Effect of the hydrogen ion concentration : As the range of the color change of NR is from 6.8 to 8.0, it is impossible to titrate at less than pH 6.8, because color change does not occur in such acid solution. When pH value is within 6.8～8.0, color change is not sharp. As shown in Table Ⅱ, good results are obtained within pH 8.0～9.0. Above pH 9, dissociation of 1:1 complex is occurred, and titration cannot be carried out.

(2) Effect of the concentration of the solution : As shown in Table Ⅲ, when the concentration of the solution is higher than *M*/200, the color of the solution changes pink to yellow at the end-point. *M*/400～*M*/800, color of the solution changes to yellow before titration, and in the course of titration the solution changes to pink, and finally at the end-point, changes to yellow again. When the concentration is lower than *M*/1000, this titration cannot be carried out, because the concentration of the solution is lower than the c. m. c. of the solution.

(3) Effect of the volume of *M*/1000 NR solution : When the volume of the indicator is less than 0.05 cc., the color of the solution is too weak to recognize the color change of the solution at the

TABLE II. Effect of the Hydrogen Ion Concentration
(Sample solution : $M/100$ sodium lauryl sulfate)

pH Value		mg./cc.	mg./cc.	Recovery	Note
Before titration	After titration	(Added)	(Found)	(%)	End-point
7.85	7.20	3.049	3.074	100.81	not clear
8.10	8.00	3.049	3.009	98.70	clear
8.50	8.40	3.049	3.009	98.70	clear
9.00	8.90	3.049	3.009	98.70	clear
9.50	9.40	3.049	3.009	98.70	comparatively clear
9.70	9.65	3.049	—	—	not clear

TABLE III. Effect of the Concentration of the Solution
(Sample solution : Sodium lauryl sulfate)

Molar concentration of sample solution	mg,/20 cc. (Added)	mg./20 cc. (Found)	Recovery (%)	Color change of the solution
1/50	60.98	60.17	98.68	P—YW
1/100	30.49	30.09	98.70	P—Y
1/200	15.25	15.04	98.63	P—Y
1/400	7.63	7.49	98.10	Y—P—Y
1/800	3.82	3.74	98.00	Y—P—Y
1/1000	1.91	—	—	Y——

P : Pink Y : Yellow W : White

TABLE IV. Effect of the Volume of the Indicator
($M/1000$ N.R. solution)

Volume of the indicator (cc.)	mg./cc. (Added)	mg,/cc. (Found)	Recovery (%)	Volume of the indicator (cc.)	mg./cc. (Added)	mg,/cc. (Found)	Recovery (%)
0.05	30.49	30.09	98.70	0.25	30.49	30.09	98.70
0.10	30.49	30.09	98.70	0.35	30.49	30.09	98.70
0.15	30.49	30.09	98.70	0.50	30.49	29.85	97.80

end-point. When the volume of the indicator is more than 0.5 cc., the color begins to change even before the end-point and low results are obtained.

(4) Effect of the non-ionic surface-active agents : As shown in Table V, when the volume of 1% Tween 40 or Tween 85 solution is more than 5 to 10 cc. of sample solution, titration could not carried out, because an interaction between non-ionic surface-active agent and NR takes place, and also non-ionic surface-active agent solubilize the CSAA-ASAA precipitate.

TABLE V. Results obtained when Non-ionic Surfactants were added
(Sample solution : $M/100$ sodium lauryl sulfate)

Non-ionic surfactant (1 w/w %)	Volume of non-ionlc surfactant (cc.)	mg./cc. (Added)	mg./cc. (Found)	Recovery (%)
Tween 40	0	30.49	30.09	98.70
〃	1	30.49	30.09	98.70
〃	5	30.49	30.09	98.70
〃	10	30.49	—	—
Tween 85	0	30.49	30.09	98.70
〃	1	30.49	30.09	98.70
〃	5	30.49	30.09	98.70
〃	10	30.49	—	—

Neutral red-sodium lauryl sulfate complex, m.p. 162~163°. *Anal.* Calcd. for $C_{27}H_{44}O_5N_4S$: C, 60.4; H, 8.26; N, 10.4. Found : C, 60.1 ; H, 8.49; N, 10.4.

Summary

A new volumetric method of determination of anionic surface-active agent with cationic surface-active agent using Neutral red as an indicator at pH 8~9 was studied. This method can be applied for the anionic surface-active agents or sulfate or sulfonate type, and not be applied for soaps. Addition of non-ionic surfactant and sodium sulfate scarcely influences the titration. The mechanism of this titration is also discussed.

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