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Shinsaku Natori : Antibacterial Effect of Lichen Substances and Related Compounds. VIII.* Some Observations on Inactivation of Antitubercular Activity by Surface Active Agents and Serum.

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In the previous paper* the author reported on the decrease of bacteriostatic activity of dibenzofuran derivatives by the addition of serum, serum albumin, or some non-ionic surface active agents.

Since Dubos and Davis[1] recommended the use of Tween-albumin medium for the cultivation of tubercle bacilli, there have been many reports which deal with the effect of non-ionic surface active agents on antibacterial activity.[2] Although the increase of antibacterial activity has been observed in many instances, the decrease, as was seen in this series, has also been pointed out in some cases.[3~9] Physicochemical explanations for this phenomenon have also been made.[9~11]

The inactivation of antibacterial agents by the addition of serum is a well-known fact, and the albumin fraction (fraction V) is assumed to play a chief rôle in the action of serum.[3~5,7,12~15]

In the present work, the dibenzofuran derivatives and some other antitubercular agents were submitted to comparative tests for their antibacterial activities in the presence of some interfering agents, which included a surface active agent, Tween 80, and serum albumin, and which were expected to interact with the antibacterial substances, while the interaction between Tween 80 or serum albumin and the drugs was pursued by physicochemical methods.

Materials and Methods

Antibacterial Substances—Following 8 compounds were employed : Streptomycin sulfate (J. P. VI), isoniazid (J. N. F. II, isonicotinic acid hydrazide), 4,4′-diaminodiphenyl sulfone (m. p. 174~175°), 2-methyl-1,4-naphthoquinone (m.p. 104~106°), usnic acid (m.p. 202~203°), oleic acid (J. P. VI), 3-aminodibenzofuran,[16] and sodium 3-dibenzofurylaminomethanesulfonate.[17]

Agents Added to Examine Interaction—Polysorbate 80 (J. N. F. II, "Tween 80") was employed as the non-ionic surface active agent and, in this connection, polyethylene glycol 1500 (Carbowax

* Part VII. S. Natori : This Bulletin, **5**, 553(1957).
** Hongo, Tokyo (名取信策).

1) R. J. Dubos, B. D. Davis : J. Exptl. Med., **83**, 409(1946).
2) H. Mima : Kagaku no Ryoiki, **7**, 689(1953).
3) H. S. Forrest, *et al.* : Nature, **160**, 94(1947).
4) A. S. Youmans, G. P. Youmans : J. Bacteriol., **56**, 245(1948).
5) B. D. Davis, R. J. Dubos : J. Exptl. Med., **86**, 215(1947).
6) A. Stoll, *et al.* : Schweiz. Z. allgem. Pathol. Bakteriol., **13**, 752(1950).
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12) J. C. Hoogerheide : J. Bacteriol., **49**, 277(1945).
13) R. J. Dubos : J. Exptl. Med., **85**, 9(1547).
14) T. Ukita, *et al.* : Yakugaku Zasshi, **71**, 234(1951).
15) R. Tompsett *et al.* : J. Bacteriol., **53**, 581(1947).
16) S. Shibata, S. Natori, Y. Sumi : Yakugaku Zasshi, **72**, 1333(1952).
17) S. Natori : This Bulletin, **5**, 539(1957).

1500 ") was also examined. Horse serum (inactivated), bovine serum albumin (Armour Laboratories), egg albumin, and lecithin were examined as the interacting proteins. A blood extender, polyvinylpyrrolidone (" Kollidon 25 "), and two model protein compounds, urea and guanidine, were also employed.

Antibacterial Activity—Antibacterial activity was examined by the serial dilution method, using *Mycobacterium tuberculosis* A.T.C.C. No. 607 as the test organism. A medium, prepared by the composition of Kirchner's medium but devoid of serum, was used as a basal medium, to which the interfering agent was added to make the final concentration shown in Table I.

Three-day culture of the organism in Tween 80-asparagine-glycerol medium was diluted with 10 volumes of physiological saline and 0.1 cc. of the suspension was inoculated to each tube containing 3 cc. of the test solution. Each test was duplicated more than twice and the results were read after incubation for 3 days at 37°.

Interaction with Tween 80—The increase of solubility by the addition of Tween 80 was examined as follows: A definite excess amount of the antibacterial agent was placed in a Monod's shake culture tubes and a definite amount of Krebs-Ringer-phosphate buffer or 0.05% solution of Tween 80 in the buffer was added. The suspension was shaken for 10 hrs. at 37° and centrifuged immediately after that time. The supernartant was diluted to an appropriate concentration to be measured by the method shown below.

Interaction with Serum Albumin—The equilibrium dialysis[18] was employed. The solution (5 cc.) of the antibacterial agents in Krebs-Ringer-phosphate buffer, with or without the addition of serum albumin (0.1%), was placed in a cellophane bag and dialysed against the buffer (15 cc.) in a test tube at 25°±1°. The concentration of the agent, dialysed outside of the bag, was determined by the method shown below.

Determination of the Agents—The concentration of the agents was determined by ultraviolet absorption at the following wave lengths: Isoniazid (261 mμ), 4,4'-diaminodiphenyl sulfone (290 mμ), 2-methyl-1,4-naphthoquinone (250 mμ), usnic acid (289 mμ), 3-aminodibenzofuran (310 mμ), and sodium 3-dibenzofurylaminomethanesulfonate (313 mμ). Oleic acid was determined by titration.

Results and Discussions

Antibacterial Activity in the Presence of the Supposed Interfering Agents—The results shown in Table I indicate that the significant decrease of antibacterial activity against *M. tuberc.* A.T.C.C. No. 607 was observed in some cases. The addition of

Table I. Antibacterial Activity of Some Antitubercular Agents against *M. tuberc.* A.T.C.C. No. 607 in the Presence of Interfering Agents

Compd. \ Interfering agent	Min. inhibitory concn. (in log of reciprocal mol. concn.)									
	None	Tween 80 (0.05%)	Carbowax 1500(0.015%)	Horse serum (10%)	Bovine serum albumin(0.2%)	Egg albumin (0.2%)	Lecithin (0.2%)	Polyvinylpyrrolidone(0.2%)	Urea ($10^{-4}M$.)	Guanidine ($10^{-4}M$.)
Streptomycin sulfate	6	5.5	6	5.5	6	6	6	5.5	5.5	6
Isoniazid	4	<3.5 (+)	4	3.5	3.5	4	3.5	3.5	3.5	4
4,4'-Diaminodiphenyl sulfone	4.5	3.5 (+)	4.5	4	4	4	3.5 (+)	4.5	4.5	4
2-Methyl-1,4-naphthoquinone	4	3.5	4	<3.5 (+)	<3.5 (+)	3.5	4	<3.5 (+)	3.5	3.5
Usnic acid	5	4.5	5	4 (+)	4 (+)	5	5	5	5	5
Oleic acid	4	<3.5 (+)	4	<3.5 (+)	<3.5 (+)	3.5	4	4	4	4
3-Aminodibenzofuran	5.5	<4 (+)	5.5	4 (+)	4.5 (+)	5.5	5.5	5.5	5.5	5.5
Sodium 3-dibenzofuryl-aminomethanesulfonate	5.5	<4 (+)	4.5 (+)	<4 (+)	4 (+)	5	5.5	5.5	5.5	5

(+) Significant decrease of antibacterial activity.

18) I.M. Klotz, *et al.*: J. Am. Chem. Soc., **68**, 1486(1946); F. Karush, M. Sonnenberg: *Ibid.*, **71**, 1369(1949).

Tween 80 decreased the activity of isoniazid, 4,4′-diaminodiphenyl sulfone, oleic acid, 3-aminodibenzofuran, and sodium 3-dibenzofurylaminomethanesulfonate, but no obvious change was observed in other three substances. The activity of isoniazid[19] and 4,4′-diaminodiphenyl sulfone[4] was reported to be incresed by the addition of surface active agents under some different conditions.

Results with whole serum and serum albumin are similar and the activity of 2-methyl-1,4-naphthoquinone, usnic acid, oleic acid, 3-aminodibenzofuran, and sodium 3-dibenzofurylaminomethanesulfanate was reversed remarkably, while three clinically useful agents, streptomycin, isoniazid, and 4,4′-diaminodiphenyl sulfone, were indifferent. On the contrary egg albumin and lecithin did not exhibit any antagonistic effect in almost all cases. The artificial blood extender, polyvinylpyrrolidone, exhibited no effect except in the case of 2-methyl-1,4-naphthoquinone.

Interaction with Tween 80—The solubility of six compounds in the buffer and in 0.05% solution of Tween 80 in the buffer, measured by the above-mentioned method, are shown in Table II.

Table II. Solubility of Antibacterial Agents

Compound	Solubility (mol./L.) in Krebs-Ringer-phosphate buffer	Solubility (mol./L.) in 0.05% soln. of Tween 80	Ratio
4,4′-Diaminodiphenyl sulfone	1.1×10^{-3}	1.2×10^{-3}	1.1
2-Methyl-1,4-naphthoquinone	1.5×10^{-3}	1.5×10^{-3}	1.0
Usnic acid	1.4×10^{-4}	2.0×10^{-4}	1.4
Oleic acid	1.2×10^{-5}	2.9×10^{-5}	2.4
3-Aminodibenzofuran	0.7×10^{-3}	1.0×10^{-3}	1.4
Sodium 3-dibenzofurylaminomethanesulfonate	0.9×10^{-2}	0.7×10^{-2}	0.8

The decrease of antibacterial activity by the addition of surface active agents has been elucidated chiefly by the occlusion of compounds into the micelle of surface active agents.[9~11] Apparent increase of solubility, which corresponded to the decrease of antibacterial activity, also seemed to support this view. However, the increase of solubility was not exactly parallel to the decrease of biological activity in their extent. This might suggest that the effect of surface active agents should not be observed merely from the interaction between antibacterial substances and surface active agents, but the effect of surface active agents on the organisms, especially the influence on permeability of bacterial cell, must also be taken into consideration.

Interaction with Serum Albumin—Results of equilibrium dialysis of the six antibacterial agents with or without the addition of serum albumin are shown in Table III.

Table III. Equilibrium Dialysis of the Antibacterial Agents in the Presence of Bovine Serum Albumin

Compd.	Initial concn. in cellophane bag ($\times10^{-5}M.$)	Calcd. concn. in equilibrated soln. ($\times10^{-5}M.$)	Observed concn. in equilibrated soln. ($\times10^{-5}M.$) with albumin (0.1%)	Observed concn. in equilibrated soln. ($\times10^{-5}M.$) without albumin	Time required for the dialysis of ½ amount of the compd. (hrs.) with albumin (0.1%)	Time required for the dialysis of ½ amount of the compd. (hrs.) without albumin	interaction
Isoniazid	40	10	10.2	9.8	5.0	4.5	(−)
4,4′-Diaminodiphenyl sulfone	10	2.5	2.4	2.4	5.5	5.0	(−)
2-Methyl-1,4-naphthoquinone	20	5	4.4	5.0	11	9	(+)
Usnic acid	6	1.5	0.5	1.5	—	3.0	(++)
3-Aminodibenzofuran	10	2.5	2.1	2.5	5.0	3.5	(+)
Sodium 3-dibenzofuryl-aminomethanesulfonate	10	2.5	2.0	2.2	8	6	(±)

19) K. Yanagisawa, *et al.*: Saishin Igaku, **10**, 225(1955); T. Aoyagi, D. Mizuno: Unpublished data.

Isoniazid and 4,4′-diaminodiphenyl sulfone did not show any sign of interaction but, in the case of other four drugs, the concentration of the drugs in the external solution after equilibration showed some difference between the presence and the absence of serum albumin. The velocity of the equilibration also showed some differences in all compounds. It took more time to reach the equilibrium in the case of 2-methyl-1,4-naphthoquinone, irrespective of the presence or absence of albumin.

The compounds, whose antibacterial activity was reversed by serum or serum albumin, agreed with those in which interaction with serum albumin was observed. Although the experimental results shown here were rather small in number, they were assumed to support the theory[4,5,15] that the decrease of antibacterial activity is due to the absorption or bonding of drugs with some serum components, probably with albumin. However, the magnitude of interaction was not parallel to that of the decrease of antibacterial activity, and participation of other factors, including the effect on the organism, might not be neglected.

Specific activity of albumin fraction in serum proteins on the interaction with drugs has been assumed chiefly due to its chemical components.[20] Although the explanation may be suitable for interaction with anions, the specificity of albumin fraction against neutral or cationic substances, such as 3-aminodibenzofuran, does not seem to be explainable in the same way. The fact that the activities of the compounds susceptible to serum or serum albumin, such as 3-aminodibenzofuran and three out of 20 compounds in Youmans' experiment,[4] were also reduced by surface active agents might suggest that the serum albumin fraction provides a similar influence both upon the drug and the organism through its surface activity or other properties.

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Summary

Influence of some agents, such as Tween 80 and serum albumin, on the antibacterial activity of eight compounds including dibenzofuran derivatives was examined. Interaction between antibacterial agents and Tween 80 or serum albumin was also examined by solubility and equilibrium dialysis, and some discussions were made on the cause of decrease of antibacterial action by the addition of surface active agents or serum albumin.

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20) I.M. Klotz: "The Proteins," Vol. I, 726.