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Biophysical Study of Bioactive-Substance Conformation and Interaction with Drugs in Solution

Molecular interaction between salicylate and nucleic acid base derivatives in non-polar solvents

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Abstract □ The interaction of salicylic acid (S.A), salicylamide (S.M) with nucleic acid base derivatives such as 9-ethyl adenine (A), 1-cyclohexyl uracil (U), 2′, 3′-benzylidine-5′ trityl-cytidine (C), guanosine-2′, 3′, 5′-isobutylate (G) has been spectroscopically investigated to determine the binding mechanism. NMR and IR spectra were measured in nonpolar solvents. The association constant K of the formation of complex was calculated from the IR spectra. Compounds S.A and A form a 1 : 1 or 1 : 2 cyclic hydrogen-bonded complex depending on the sample concentration. Compounds S.A and U form a 1 : 1 or 1 : 2 hydrogen-bonded complex on the sample concentration. Compounds S.A and C form a 2 : 1 hydrogen-bonded complex at low concentration (0.0016M). Compound S.A binds compound G, but its binding does not completely break the self-association of compound G. Compound S.M binds compounds A, U, C, G very weakly.

Keywords □ Salicylic acid, Salicylamide, Adenine, Uracil, Cytidine, Guanosine, Association constant, Molecular interaction, Nonpolar solvents.

Salicylate, one of the oldest synthetic drugs, remains the most widely used analgesic and antipyretic agent. It is frequently used in pregnancy period without any caution. Since it was reported that salicylate was teratogenic in rat (By Warkany and Takacs, 1959)[1], salicylate has been shown to be teratogenic or embryotoxic in a variety of species including the mouse[2], hamster[3], guinea pig[4], cat[5], dog[6] and even in human primate (monkey)[7]. The teratogenicity of salicylate in human also was reported[8]. Extensive studies about the mechanism of salicylate teratogenicity were carried out by many investigators.

Salicylate interferes with the biosynthesis of mucopolysaccharide[8], inhibits the activity of dehydrogenase, decarboxylase, and aminotransferase enzymes[9], and impaires protein synthesis in animal tissues[10]. Salicylate also inhibits the activity of nucleic acid polymerase prepared from the rat liver[11] and interferes with the biosynthesis of nucleic acids in adult mice[12]. A further and perhaps more relevant action is that salicylate (2mM) inhibits the RNA polymerase activity in rat foetuses and the incorporation of labelled orotate into the nucleic acids of rat foetuses[13]. Salicylate also inhibits the growth of some human embryonic cells in culture[14]. The inhibitory action of salicylate on RNA biosynthesis may be related to its teratogenicity and embryotoxicity.

Hydrogen bonds between bases play an important role in DNA replication, RNA transcription and in maintaining the structual integrity of the base pairs of DNA. Therefore, it seems worthwhile to examine the molecular interaction

between salicylate and nucleic acid bases. Aspirin and sodium salicylate are more widely used for analgesic and antipyretic drug than is any other salicylate. salicylic acid was identified as the causative agent in the teratogenesis of aspirin and sodium salicylate[15,16]. Salicylamide hardly acted as teratogen[16]. Thus, salicylic acid and salicylamide are investigated and their abilities of hydrogen bonding with nucleic acid bases are compared.

EXPERIMENTAL METHODS

Materials: 9-ethyladenine (A), 1-cyclohexyluracil (U), and 2′,3′-benzylidine-5′-trityl-cytidine (C) were purchased from Cyclo chemical Co., Los Angeles. Guanosine-2′,3′,5′-isobutyrate (G) is a gift from Dr. Y. Kyogoku. Commercial salicylic acid (S.A) and salicylamide (S.M) were recrystallized from chloroform. Chloroform-d was purchased from E. Merck, Darmstadt. It was purified by filtration through an alumina gel column, 5 cm in length. Chloroform was treated with one-half its volume of water several times, dried over calcium chloride overnight, and fractionally distilled from phosphorus pentoxide through a 120-cm column packed with glass helices. The distillate was refluxed with phosphorus pentoxide and redistilled fractionally. The distillate from phosphorus pentoxide was tested for water by measuring the infrared spectrum in quartz cells, any band of water was not shown.

Methods: Nuclear magnetic resonance spectra were recorded on a 80-MHz, FT-NMR spectrometer equipped with a temperature-control unit. Chemical shifts were read relative to the resonance of internal standard, tetramethylsilane. Infrared spectra were recorded on a Beckman I.R. 20 A infrared spectrophotometer. Quartz cells (10 mm) were used in 3 μ region and potassium bromide cells (1.0 mm) were used in 6 μ region.

RESULTS

1. *Adenine.*

The molecular interactions between adenine and salicylates were reported by B.S.Yu *et al*[17]. He showed that adenine and salicylic acid formed a 1 : 1 or 1 : 2 cyclic hydrogen bonded complex depending on the sample concentration. Also, he showed that the association between adenine and salicylamide was weaker than the self-association of adenine. Because his experiment was carried out in high sample concentration i.e. NMR (0.08 M), IR (0.01 M), it is questionable that salicylates can strongly bind adenine in physiological condition (i.e. at low concentration). Thus, we showed that salicylic acid could strongly bind adenine at low concentration (0.0016 M). In the IR spectrum of salicylic acid solution in $CHCl_3$, a slightly broad band is observed at 3,510 cm^{-1} and a quitely broad band is observed at 3,350 cm^{-1} (Fig. 1). In the 6μ region, a strong band is observed at 1,690 cm^{-1}, a medium band at 1,670 cm^{-1}, and two weak bands are observed at 1,622 cm^{-1} and 1,595 cm^{-1}. The apparent extinction coefficients of the 3,510 cm^{-1} and the 1,690 cm^{-1} band increase, and the 3,200 cm^{-1} and the 1,670 cm^{-1} band decrease with the dilution of the solution. Thus the band at 3,510 cm^{-1} is assignable to nonbonded OH stretching vibration and the band at 3,200cm^{-1} is assignable to bonded OH stretching vibration. The band at 1,690 cm^{-1} is assignable to the conjugated carbonyl stretching vibration and the band at 1,670 cm^{-1} is assignable to the bonded carbonyl stretching vibration.

The 1,622 cm^{-1} and 1,595 cm^{-1} band are considered to be related to the aromatic C=C stret-

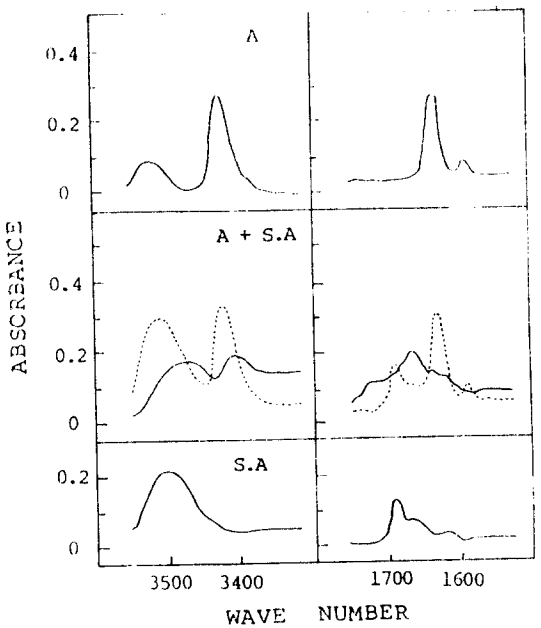

Fig. 1: Infrared spectra of adenine, salicylic acid and 1:1 mixed solution. These spectia show the observed spectra (solid line) and the calculated sum of the individual spectrum of components (dashed line). The concentrations were 0.0016 M in $CHCl_3$ for the measurements in 3 μ region (10 mm quartz cell) and in 6 μ region (1 mm KBr cell).

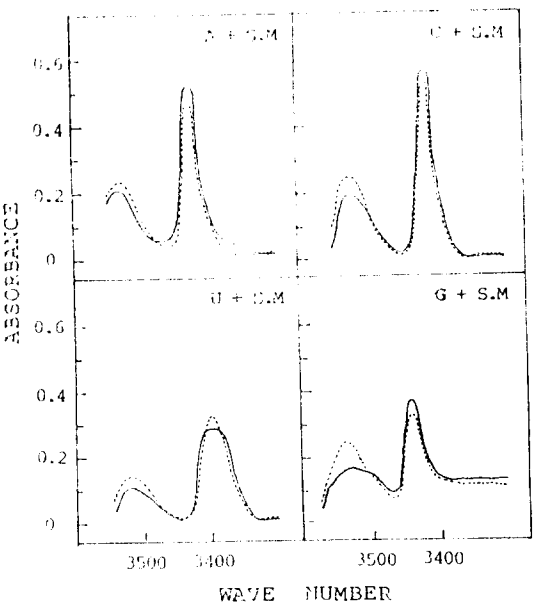

Fig. 2: Infrared spectra of the equimolar mixture solution of salicylamide and one of the A, U, C, G compounds. These spectra show the observed spectra (solid line) and the calculated sum of the individual spectrum of components (dashed line). the concentrations were 0.0016 M in $CHCl_3$ for the measurements in 3 μ region (10 mm quartz cell) and in 6 μ region (1 mm KBr).

ching vibration. Benzoic acid also showed similar pattern to salicylic acid despite that it couldn't form intra-molecular hydrogen bond. Salicyclic acid has been known to form both intra-molecular and inter-molecular hydrogen bond simultaneously[18]. Intra-molecular hydrogen bonding (H-bonded spectral behavior) is retained at the lowest concentration. In contrast, the spectral changes resulting from inter-molecular hydrogen bonding are lost at low concentration as the association is disrupted[19]. Thus, salicylic acid is considered to form mainly cyclic dimer in $CHCl_3$. The spectrum of adenine shows two strong bands at 3,527 and 3,416 cm^{-1} which are respectively due to the anti-symmetric and symmetric NH stretching vibration of the non-bonded amino group[20]. In the 6 μ region, adenine shows a strong sharp band at 1,629 cm^{-1} and a weak band at 1,586 cm^{-1}, which were assigned to the coupled vibration of the NH_2 scissors vibration and ring stretching motions[20]. When adenine and salicylic acid solutions are mixed, the nonbonded bands at 3527, 3510, 3416, 1690 cm^{-1} decrease drastically in intensity and the bonded band at 1,670 cm^{-1} increases in intensity and new association bands appear. These spectral changes are apparently caused by hydrogen bonding. When salicylamide and adenine solutions are mixed, the spectral change

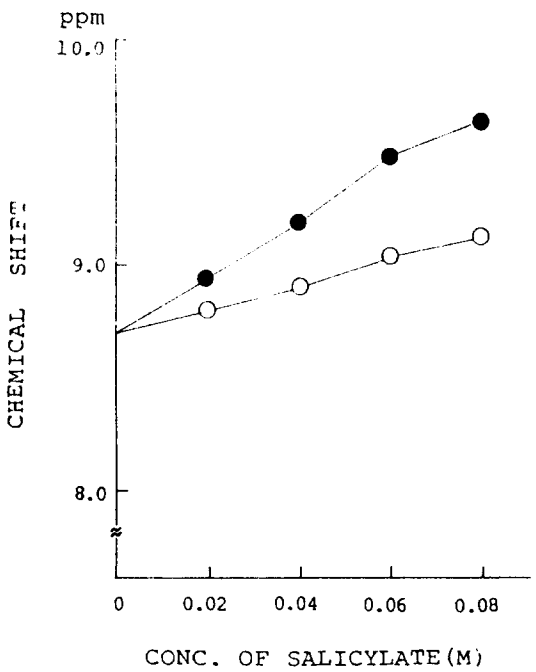

Fig. 3: Effects of the concentration of salicylic acid (•) and salicylamide (○) on the chemical shift of the imino proton of uracil, keeping the concentration of uracil constant (0.05 M).

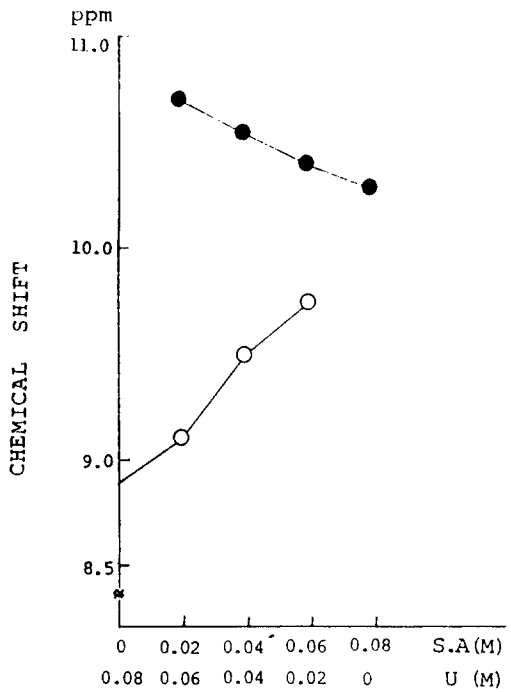

Fig. 4: Stoichiometric pairing of salicylic acid and uracil: dependence of the chemical shifts of the imino proton of uracil (○) and the carboxyl proton of salicylic acid (•) in a mixing experiment of uracil and salicylic acid. Total concentration was constant at 0.08 M.

is very slight (Fig. 2).

2. *Uracil.*

NMR Spectra: When protons take part in hydrogen bonding, they become less shielded and their resonances shift downfield. As the concentration of salicylic acid increases keeping the concentration of uracil constant at 0.05 M, the imino proton signal of uracil shifted downfield. But in the case of salicylamide, the imino proton signal of uracil shifted downfield only slightly (Fig. 3). To obtain information on the characteristics of complex in $CDCl_3$, experiments were performed at constant total concentration (Fig. 4). As the relative concentration of salicylic acid increased, the imino proton signal of uracil shifted downfield. As the relative concentration of uracil increased, the carboxylic hydroxyl proton signal of salicylic acid shifted downfield. From these results, it can be inferred that the association between uracil and salicylic acid is stronger than the self-association of either compound. It may be assumed by the shape of curves that 1 : 1 complex is formed[21].

IR Spectra: The spectrum of uracil shows one strong band at 3,395cm^{-1} which is due to the NH stretching vibration of the nonbonded imino group[20] (Fig. 5). In the 6μ region, uracil shows a strong band at 1,687 cm^{-1} and a medium band at 1,710 cm^{-1}. The 1,710 cm^{-1} band is due to the nonborded 2-C carbonyl and the 1,687 cm^{-1} band to the nonbonded 4-C carbonyl stretching

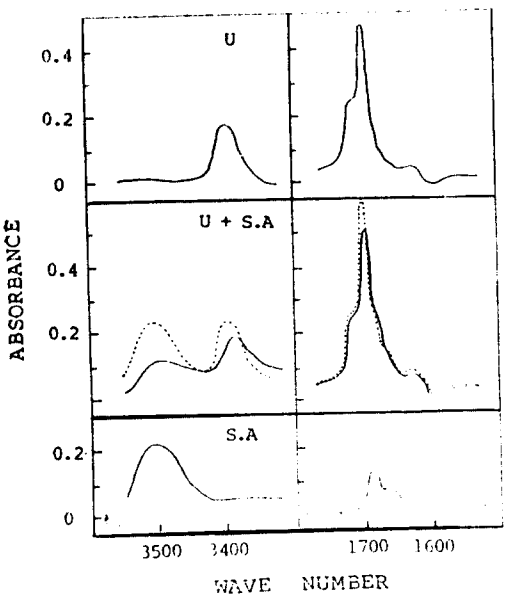

Fig. 5: Infrared spectra of uracil, salicylic acid and 1:1 mixed solution. These spectra show the observed spectra (solid line) and the calculated sum of the individual spectrum of components (dashed line). The concentrations were 0.0016 M in $CHCl_3$ for the measurements in 3 μ region (10 mm quartz cell) and in 6 μ region (1 mm KBr cell).

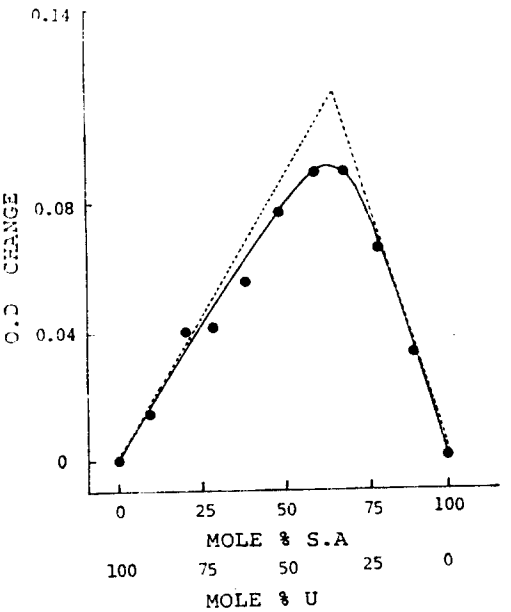

Fig. 6: Change in the absorbance of the non-bonded band at 3395 cm^{-1} as a function of the molar ratio of uracil and salicylic acid. Absorbance of the pure solution is adjusted to zero.

vibrations coupled with the NH bending mode[20]. When salicylic acid and uracil solutions are mixed, the nonbonded bands at 3,510, 3,395, 1,690, 1,687 cm^{-1} decrease drastically in intensity and the bonded band at 1,670 cm^{-1} increases and new association bands appear.

Thus, 4-C carbonyl group appears to be involved in hydrogen bonding, but 2-C carbonyl group doesn't. To obtain informations on the characteristics of complex in $CHCl_3$, an additive property such as spectrophotometric absorbance was applied[22] (Fig 6). The result indicates a complex 1:2 type. As the total sample concentration increases, the result gradually shows a complex 1:1 type concurred with that obtained from NMR method (not presented). When salicylamide and uracil solutions are mixed, the spectral change is very slight (Fig 2).

3. Cytidine

NMR Spectra: When salicylic acid (0.08 M) is added to the 0.006M cytidine solution, the amino proton signal of cytidine shifted downfield slightly. As the concentration of cytidine increases keeping the concentration of salicylic acid solution constant at 0.08 M, the carboxylic hydroxyl proton signal of salicylic acid shifted upfield (Fig 7). This result may be due to the breaking of salicylic acid self-association by cytidine. As the concentration of cytidine increases keeping the concentration of salicylamide constant at 0.08 M, the amino proton signal of salicylamide didn't shift (Fig 7).

When salicylamide (0.08 M) is added to the 0.006 M cytidine solution, the amino proton signal of cytidine didn't shift. Thus salicylamide

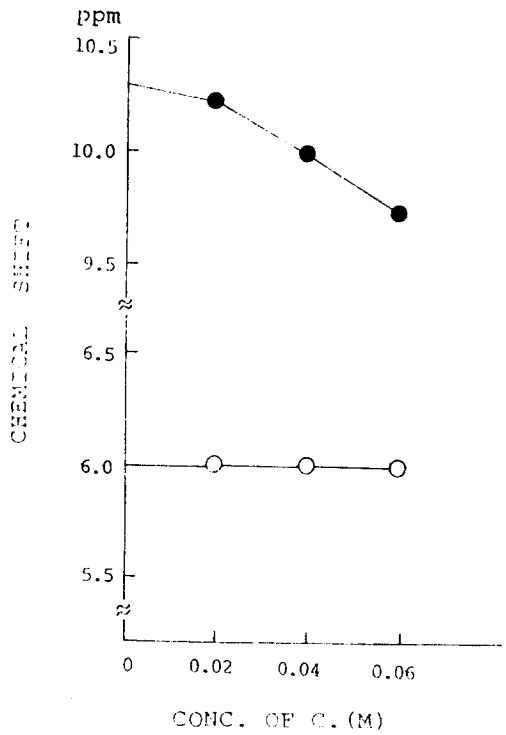

Fig. 7: Effects of the concentration of cytidine on the chemical shifts of carboxyl proton of salicylic acid (•) and amino proton of salicylamide, (○) keeping the concentrations of salicylates constant (0.08 M).

doesn't bind cytidine.

IR Spectra: The spectrum of cytidine shows two strong bands at 3,533 and 3,415 cm^{-1} which are respectively due to the anti-symmetric NH stretching vibration of the nonbonded amino group[23] (Fig 8). In the 6 μ region, cytidine shows two strong bands at 1660, 1645 cm^{-1} and a weak band at 1,595 cm^{-1}. When salicylic acid and cytidine solutions are mixed, the nonbonded bands at 3,533, 3,510, 3,415, 1,690 cm^{-1} decrease drastically in intensity. To obtain informations on the characteristics of complex in $CHCl_3$, an additive property such as spectrophotometric absorbance was applied[22] (Fig 9). The result indicates a complex 2:1 type. When salicylamide

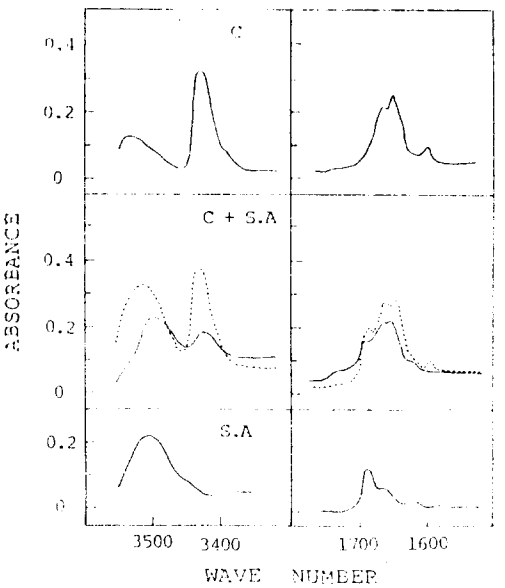

Fig. 8: Infrared spectra of cytidine, salicylic acid and 1:1 mixed solution. These spectra show the observed spectra (solid line) and the calculated sum of the individual spectrum of components (dashed line). The concentrations were 0.0016 M in $CHCl_3$ for the measurements in 3 μ region (10 mm quartz cell) and in 6 μ region (1 mm KBr cell).

and cytidine solutions are mixed, the spectral change is very slight (Fig 2).

4. Guanosine

NMR Spectra: As the concentration of salicylic acid increases keeping the concentration of guanosine constant at 0.02 M, the chemical shift of the amino proton and C_8-proton of guanosine fluctuated. As the concentration of guanosine increases keeping the concentration of salicylic acid at 0.08M, the chemical shift of the carboxylic hydroxyl proton of salicylic acid fluctuated. In the case of salicylamide, similar pattern is observed, but the degree of fluctuation is small (not presented).

IR Spectra: In the spectrum of guanosine in $CHCl_3$, bands due to nonbonded anti-symmetric

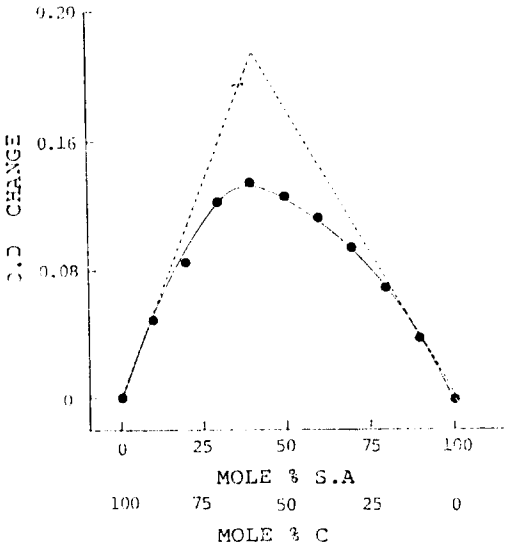

Fig. 9: Changes in the absorbance of the non-bonded band at 3415 cm^{-1} as a function of the molar ratio of cytidine and salicylic acid. Absorbance of the pure solution is adjusted to zero.

and symmetric NH stretching vibrations appear at 3,515 and 3,407 cm^{-1}, while the band at 3305 cm^{-1} is association band due to the hydrogen bonding of the guanosine residues with themselves (Fig. 10)[23]. In the 6 μ region, guanosine shows a strong band at 1690 cm^{-1}, and two midium band at 1,740, 1,630 cm^{-1}. When salicylic acid and guanosine solutions are mixed, spectral change is occured. When salicylamide and guanosine solutions are mixed, spectral change is occurred (Fig 2). Quantitative measurements were made of the interac-

Table I: Association constants for hydrogen bonding.

	K_{25}(liter/mol)		K_{25}(liter/mol)
A—A	3.1±0.3	S.A—S.A	186
A—U	100±20	A—S.A	10^3~10^4
U—U	6.1±0.6	U—S.A	10^6~10^7
C—C	28±3	C—S.A	10^6~10^7
G—C	10^4~10^5	G—G	10^3~10^4

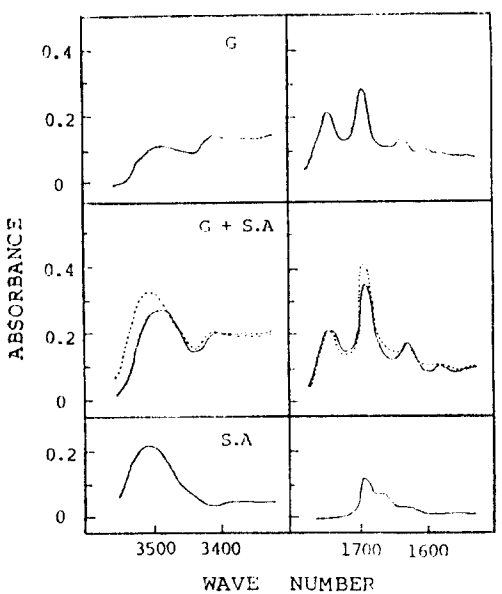

Fig. 10: Infrared spectra of guanosine, salicylic acid and 1:1 mixed solution. These spectra show the observed spectra (solid line) and the calculated sum of the individual spectrum of components (dashed line). The concentrations were 0.0016 M in $CHCl_3$ for the measurements in 3 μ region (10 mm quartz cell) and in 6 μ region (1 mm KBr cell).

tions between salicylic acid and nucleic acid bases. Association constants are listed in Table I. Association constants are determined by previously reported IR method[20,24]. The constants of nucleic acid base-nucleic acid base association were previously reported by Y. Kyogoku[26]. The constant of salicylic acid-salicylic acid association was obtained by using the following formula:

$$A = \left(\frac{1}{2K}\right) a_m^2 l^2 \left(\frac{C_o}{A}\right) - \left(\frac{1}{2K}\right) a_m l, \quad \text{where A}$$

is due to the absorbance of the band due to the nonbonded OH group and C_o is the concentration of salicylic acid. If A is plotted against C_o/A, a straight line should be obtained. The unknown quantities a_m and K can be computed from the slope s and the intercept i of this

line as follows: $a_m = -\frac{s}{il}$, $K = \frac{s}{2i^2}$ The constants of salicylic acid-nucleic acid bases were obtained by using the following formula:

$$mA + nB \rightleftarrows A_mB_n,$$

$$K = \frac{(m+n)^{m+n-1}\frac{l}{L}}{m^m n^n \left(1-\frac{l}{L}\right)^{m+n} C^{m+n-1}}$$

where l is the height of observed curve, L is the length from cross point to base line, and C_o is total concentration (Fig 6, 8). The association constants determined by this method are only reliable within the order of magnitude. We couldn't determine the association constant of salicylic acid-guanosine association and the association constants of salicylamide-nucleic acid bases because spectral change is slight. The constants of salicylic acid-adenine association and salicylic acid-uracil association are larger than the constant of adenine-uracil association. The constant of salicylic acid-cytidine association is larger than that of cytidine-guanosine association.

DISCUSSION

The interaction of salicylic acid with nucleic acid bases are expected to be very strong because the spectra show remarkable changes and very strong apparent new association bands appear. While in the case of salicylamide, spectral change is slight. Thus, the interactions of salicylamide with nucleic acid bases are very weak. These facts are consistent with the result of clinical experiments that salicylic acid was teratogenic and salicylamide hardly acted as teratogen[15]. When IR spectra are analyzed, the contribution to the absorbanec of the monomer band from other dimers, trimers and tetramers can be neglected at low concentration employed (0.0016 M). But the infrared spectra of guanosine is somewhat more complicated, since the molecular structure shows evidence of considerable self-association even at the lowest concentration[23]. Guanosine derivatives take strong tetrameric forms through H-bonding involving imino protons, amino protons and carbonyl groups and nitrogen atoms in the crystalline state[27]. These facts may be able to explain the reason that the chemical shifts of the NH_2 proton and C_8-proton of guanosine are fluctuated as the salicylate concentration increased. By comparing the values of the association constants of the complexes, we can imagine that salicylic acid may affect the RNA transcription.

The use of $CHCl_3$ and $CDCl_3$ as solvents in these experiments enabled observation of these interactions free of strong solute-solvent association effects. This environment may mimic in someways the inside of the enzyme (RNA polymerase)-DNA complex from which water may be excluded. It has been suggested that the inside of the double-stranded DNA molecule is largely hydrophobic. If one considers that the RNA transcription process occurs in a lipophilic rather than a aqueous environment, it may be worthwhile to research the direct interaction between salicylate and nucleic acid base moiety in a nonpolar systems. Further NMR experiments in low concentration should lead to a more precise fundamental understanding of the salicylate-nucleic acid base complex formation.

ACKNOWLEDGEMENT

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