A METHOD FOR THE MEASUREMENT OF FREE SALICYLATE

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY
FUSAKO MAEHIRA

1971

ABSTRACT

A METHOD FOR THE MEASUREMENT OF FREE SALICYLATE

By

Fusako Maehira

A method is proposed for the simplified estimation of unbound salicylates in serum using Sephadex dextran gels by batch procedures.

After equilibration of sample solution with dry Sephadex, the measurement of total salicylates in the original sample and salicylates in the extrafluid of Sephadex gels will give unbound salicylates in the sample.

The precision of the method was 13.5% of coefficient of variation about the percentage of unbound salicylates at pH 7.4.

The study of the interaction between protein and salicylate molecules indicated that the method developed here is comparable to the equilibrium tube dialysis method.

A METHOD FOR THE MEASUREMENT OF FREE SALICYLATE

By

Fusako Maehira

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Pathology

1971

To my parents,

Mr. and Mrs. Wulff,

and Semih

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. A. E. Lewis, my major professor, for counsel and guidance in this task, and for limitless patience and for the unselfish gift of many hours of his time.

I am thankful to Dr. T. M. Brady, Dr. D. F. Cowan, and Dr. C. C. Morrill for serving on my committee that reviewed critically this manuscript and gave useful comments and suggestions.

I am also indebted to my scholarship sponsor, United States Civil Administration of the Ryukyus Islands, for providing the grant to complete my graduate work including this project.

To Mrs. Margaret Schick and Mrs. Louise K. Mueller, and other laboratory staff at Olin Health Center, I wish to express my sincere appreciation for providing the convenience to use some of their facilities.

My thanks are extended to the laboratory staff at Edward W. Sparrow Hospital for their cooperation in collecting the specimens, and to the staff of the graduate laboratory of the Department of Pathology, and to fellow graduate students for advice and assistance throughout this task.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

INTRODUCTION

In general, cases of salicylate intoxication arise out of three situations: (1) accidental ingestion, (2) suicidal intent, and (3) prolonged high dosage treatment in rheumatic conditions. In the first two situations, the total salicylate level will give enough information for physicians. In the third situation, the measurement of serum free salicylate may provide useful information about the dynamic state of the drug action from the standpoint of both the optimal and toxic effects since free drug in the blood circulation is considered as the biologically active form and the protein bound drug as the reservoir form (Tompsett, Schultz, and McDermott, 1947; Davis, 1948; Reynolds and Cluff, 1960; Brodie and Hoegben, 1957).

Presently available methods for measuring the unbound fraction of serum salicylates are cumbersome and time-consuming. Generally these are the equilibrium tube dialysis technique (Davidson and Smith, 1961) or gel filtration column chromatographic methods with Sephadex resins (Potter and Guy, 1964; Lomax, 1970).

The purpose of the investigation reported here was to develop and evaluate a more rapid and efficient method for determining free salicylates in serum using equilibration of serum directly with dry Sephadex resins. A cross-linked dextran Sephadex gel will not admit large molecules, while there is no restriction of the diffusion of smaller molecules. Dry Sephadex resins added to serum will take up water and solutes except for proteins, polypeptides, and certain protein-bound substances depending

upon molecular size and pore size of each type of Sephadex. After equilibration the relationship between salicylate concentrations in the solvent medium and within the gel is summarized in the equation below. Derivation of this equation is presented in Part A of Appendix II.

$$Si = \frac{St \cdot Vt - Ss \cdot (Vt - Vi)}{Vi}$$

where St = total salicylate, mg.%

Si = free salicylate inside of Sephadex gels, mg.%

Ss = salicylate in extra-fluid of Sephadex gels, mg.%

Vt = total volume of sample, ml.

Vi = volume of Sephadex gels = WR x a, ml.

WR = water regain of Sephadex, ml. per gm. of gels

a = amount of Sephadex used, gm.

The intra-gel solution may be regarded as an ultrafiltrate or dialysate of serum. The concentration of unbound salicylate in the gel, multiplied by an appropriate Donnan correction factor, will give the concentration of unbound salicylate in the original serum sample. If the Donnan effect is negligible in the system, intra-gel salicylate is directly equal to the unbound salicylate in the sample serum.

REVIEW OF LITERATURE

Pharmacology of Salicylates

Pharmacologic effects of salicylates are usually regarded as inhibitory actions described as antipyretics, antinociception, and anti-inflammation, antagonism of smooth muscle responses, and antihemostasis. Among those effects, salicylates are used principally for their anti-pyretic, analgesic, and anti-inflammatory effects.

Antipyretic Effects. Salicylates lower body temperature arising from various natural pathologic states in man (Barbour, 1919), but they do not appreciably lower normal body temperature. The signal given to the temperature-regulating center in the hypothalamus by endogenous pyrogen (Atkins, 1960) may be translated into action by a change there in the balance of mediating amines which have been suggested to be 5-hydroxy tryptamine (5-HT) mediating a rise and noradrenaline a fall of body temperature (Feldberg and Myers, 1963, 1964, 1965; Feldberg *et al.*, 1966, 1967).

The earlier workers thought that salicylate acted in the hypothalamus (Guerra and Brobeck, 1944), but so little aspirin penetrated into the brain of dogs after parenteral administration that Lim *et al.* (1967) concluded that it acts outside of the brain. The latter belief is supported by the recent findings of Cooper *et al.* (1968) that intravenous sodium salicylate lessened fever in the rabbit when endogenous pyrogen was injected intravenously, but not when pyrogen was injected into a

cerebral ventricle. Therefore, salicylates could exert their antipyretic action outside the hypothalamus possibly either by inhibiting the release of endogenous pyrogen from leukocytes or by obstructing passage of pyrogen into the brain (Gander *et al.*, 1967; Cooper *et al.*, 1968).

Antinociception. Although salicylates have long been widely used clinically for the relief of pain, there are conflicting views as to whether the site of its action is central or peripheral. Much of the controversy may lie in the experimental models of pain employed for the evaluation of analgesics. The evidence obtained from intensive studies by Lim *et al.* (1964) and discussed in greater detail by Lim (1966), and by Collier (1969), suggest strongly that salicylates block pain at a peripheral chemo-sensitive receptor, whereas morphine and other narcotics act in the central nervous system. The possible mechanism of blocking pain in a humoral system may include the interaction with the mediating substances liberated by injuries (Collier, 1969). It is suggested that the reason why the peripheral analgesics fail to block traumatic cutaneous pain may be due to their inability to block axons which are not chemo-sensitive. On the other hand, the central analgesics will block or inhibit all impulses regardless as to whether they arise from axons or receptors. Salicylates are effective in conditions in which the pain-evoking stimulus is chemical (viz., the rheumatic group, early phase of malignant disease, headaches of vascular and muscle-contraction origin, and acute inflammation following trauma).

Anti-inflammatory Action. It is generally agreed that salicylates are useful remedies in the treatment of the rheumatic diseases and on this basis alone they can be described as antirheumatic drugs. If administered in adequate dosage, they are capable of reducing the acute pain, swelling,

immobility, and stiffness of the affected joints in acute rheumatic fever and in rheumatoid arthritis. No single site of action can be designated which adequately explains the anti-inflammatory and antirheumatic activities of the salicylates. The mechanisms which have received most attention are concerned with an inhibition of the release, or of the action of chemical mediators of inflammation such as kinin and histamine, and with the uncoupling effects of salicylates on oxidative phosphorylation processes (Brody, 1955, 1956), since the movement of many substances across such membranes depends on the supply of ATP (Smith, 1966) and uncoupling of oxidation will interfere with selective permeability. Salicylates seem more likely therefore to interfere with humoral mediation of the defensive reaction, either by directly antagonizing the mediator, by inhibiting its release, or by hastening its destruction (Collier, 1969).

Antihemostasis. An antihemostatic effect of salicylates was first reported in animals by Link (1943), who observed that salicylic acid delayed blood clotting time in the rat. Meyer and Howard (1943) found that salicylate also diminished the prothrombin content of human blood. In normal people, an oral dose of about 6 gm. of aspirin was needed to produce significant prolongation of prothrombin time (Quick and Clescari, 1960). Aspirin also affects other aspects of hemostasis. There is prolonged bleeding time (Weiss and Aledort, 1967), and decreased aggregation of platelets in blood (Zucker and Peterson, 1968). Antihemostatic action of salicylates may be regarded as an expression of antidefensive activity of salicylates. Unlike some of its other antidefensive effects, the delay of hemostasis has not become the basis of a clinical use of salicylates.

Salicylate Toxicity

General. The salicylates are one of the safest groups of chemotherapeutic agents, but may cause certain toxic reactions. Those may vary from mild and inconsequential symptoms to the more serious and alarming manifestations of acute salicylate poisoning, which may terminate fatally. Idiosyncrasy to salicylates is comparatively rare and usually shows itself in the form of skin rash or asthma. A potentially more serious situation appeared to be revealed by the reports of the high incidence of gastrointestinal bleeding during medication. Overdosage with salicylates can produce a large number of toxic symptoms, including tinnitus, deafness, and transient vomiting, which are common but not clinically serious. The appearance of other toxic manifestations, including hyperventilation, severe vomiting, hemorrhage, dizziness, drowsiness, mental upsets, and pulmonary edema, during salicylate therapy are indications that the drug must be stopped or its dosage modified. Infants and young children appear to be more sensitive to certain toxic effects of salicylates than are adults. One or more of the central and automatic nervous systems, neuroendocrine functions, and metabolic processes, may be involved in the toxic actions of the drugs, in contrast to the therapeutic effects of salicylates on the humoral mechanism actions.

Serious Toxic Symptoms

Acid-base disturbance. Toxic amounts of salicylates may exert three independent actions which affect acid-base balance directly. One is direct stimulation of the respiratory center. The magnitude of this action is related to the degree of poisoning, but not to the age of patients. Its primary effects are to increase alveolar ventilation,

reduce the plasma pCO_2, and cause a respiratory alkalosis with a tendency towards an alkaline blood pH. The second is increased metabolic production of carbon dioxide. The effect results from the uncoupling of salicylate on oxidative phosphorylation (Brody, 1956; Packer, 1959; Charnock, 1962). The reduction of ATP synthesis enhances the rate of oxidation reaction of a variety of metabolic intermediates as a compensatory process. Therefore the increased CO_2 production is proportional to the degree of intoxication. Its primary effects are to increase the plasma pCO_2 and to cause a respiratory acidosis with a tendency towards a lower blood pH. The third action is to increase accumulation of organic acid anions in the plasma. The effect may be caused by multiple inhibitory actions of salicylate on dehydrogenase and amino transferase enzymes (Smith, 1966). Its magnitude is inversely related to age, the very young child being particularly sensitive, while the adult is almost completely resistant to this action of the drug. Primary effects are a reduction in the plasma bicarbonate leading to a metabolic acidosis with a tendency towards an acid blood pH.

The final result of the interaction of these three effects determines the blood pH, and the direction and deviation of blood pH represents the net effect of the intensities of the three separate actions of the drug (Figure 1).

Hyperthermia. In therapeutic amounts, salicylates act as antipyretics, but at toxic levels they manifest the opposite effect. The mechanism of this hyperthermia in salicylate poisoning is basically by the uncoupling action of salicylate on oxidative phosphorylation reactions. Because of this uncoupling of ATP synthesis from lower energy inorganic compounds, normal trapping and the storage of the energy produced during

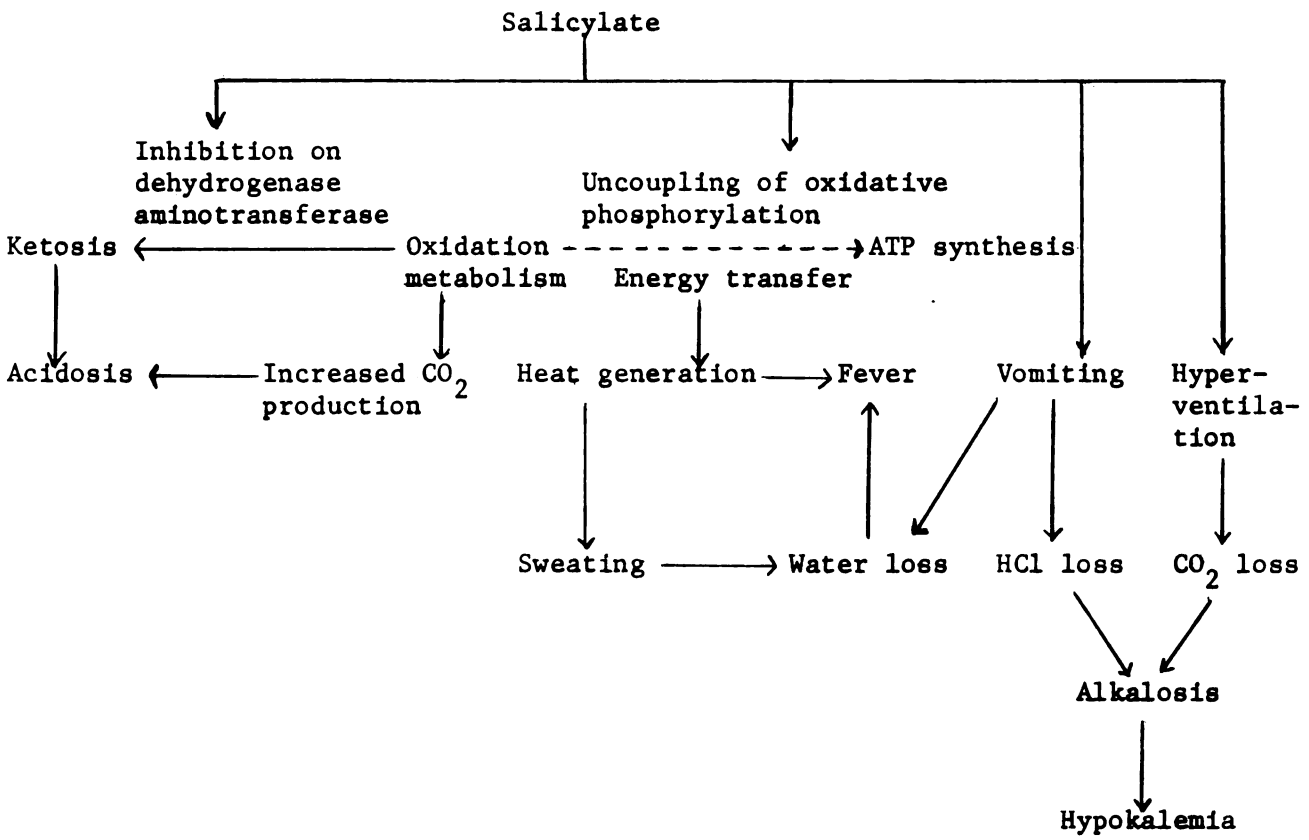


Figure 1. Schematic diagram of toxic effects of salicylate.
------- negative effect, ——— positive effect.

oxidative reactions is partially blocked. The energy is therefore dissipated in other forms, principally as heat. This may induce a state of water deficiency by hypotonic sweating. Dehydration of this nature may be worsened because the water intake of salicylate poisoned children is frequently inadequate as a result of coma or vomiting. Thus, hyperpyrexia accelerates dehydration and dehydration potentiates hyperpyrexia.

Electrolyte imbalance. The concentrations of cations and anions in the extracellular fluid may be altered by a variety of causes. Vomiting, if sever and prolonbed, will produce a primary salt and acid deficiency, and the compensatory changes in response to hyperventilation cause an increased renal excretion of sodium bicarbonate. Hypokalemia is an especially important manifestation of salicylate intoxication due to respiratory alkalosis (Robin, Davis, and Rees, 1959). An alkalemia will cause an increased renal loss of the ion.

Gastrointestinal hemorrhage. Salicylate therapy may be associated with a relatively high incidence of gastrointestinal bleeding. Stubbé (1958) found fecal bleeding some of 70% of normal and rheumatic fever patients after aspirin administration in quantities ranging from 0.75 to 3 gm. per day. Occult bleeding of a greater magnitude must be considered as one of the possible causes of anemia.

Numerous mechanisms have been suggested to explain the occurrence of gastrointestinal bleeding after salicylate administration. Those fall into three main groups: (1) salicylates have a systemic mode in action, (2) salicylates act solely on the gastric mucosal cell, and (3) salicylates produce a local irritant effect on the stomach but additional local or central mechanisms contribute to the initiation of the gastrointestinal bleeding (Smith, 1966).

Free and Protein Bound Drugs in Drug Action

General. To be effective, a drug must not only possess the intrinsic capacity of impinging on a particular receptor site, but it must also have characteristics which allow it to reach its objective in an adequate concentration. It is usually not possible to measure the drug at its site of action, but it is possible to measure the level of the drug in plasma. Since plasma is the physiological medium of exchange between tissues, the level of a drug in plasma may be considered to be in physico-chemical eqilibrium with its concentration at the locale of action.

The drug exists in the plasma partly in the free state and partly bound by protein. It is generally thought that it is the concentration of the free form and not the total concentration of the drug that determines its therapeutic activity (Davis, 1943; Tompsett, Schultz, and McDermott, 1947; Reynolds and Cluff, 1960). Free drug as the biologically active form will gain access to its locus of action by crossing various hurdles such as the blood-brain barrier, the boundaries of various tissue cells and even intracellular barriers. The protein-bound drug provides a reservoir form that keeps the free drug continuously available by release over a long time (Brodie and Hogben, 1957; Stafford, 1963).

Drug-protein interaction may be represented in a general way as: Drug + Protein $\rightleftarrows$ Drug-Protein complex. As this is a reversible reaction we may apply the Law of Mass Action. The association constant is then defined as: $K = \frac{[\text{Drug-Protein}]}{[\text{Drug}][\text{Protein}]}$. The higher the value of the constant, the stronger is the association because more of the drug exists in the bound form.

Protein-binding of Salicylates. A large part of salicylate in blood is bound to plasma protein. As reported by Smith *et al.* (1946), up to 80%

of the salicylate ion is bound in normal plasma. Of this fraction, at least 85% is bound to albumin, with the remaining 15% adhering to alpha and beta globulins (Davidson and Mandel, 1965; Reynolds and Cluff, 1966). Binding involves primarily the free carboxyl group of salicylate, but the o-phenolic group markedly enhances the attraction for proteins. The binding on protein appears to be predominantly to the epsilon-amino and possibly the guanidino groups of the protein molecule. Aspirin itself, however, undergoes little or no binding, unless the acetyl group is hydrolized to provide a free carboxyl group (Davidson and Smith, 1961).

Measurement of Salicylates

Existing colorimetric methods of measuring salicylate level are based on the following color reactions: (1) the purple color given with ferric nitrate after extraction with ethylene dichloride (Brodie, Udenfriend, and Coburn, 1944); (2) the red color given with sodium nitrate and copper sulphate in acetic acid after a preliminary extraction with iso-amyl alcohol at pH 2 (Reid, 1948); (3) the yellow color given with sodium hydroxide after previous nitration (Volterra and Jacobs, 1947); and (4) blue color produced after incubation with an alcoholic solution of the Folin-Ciocalteu phenol reagent (Weichselbaum and Shapiro, 1945; Smith and Talbot, 1950). Among those, the method of Brodie *et al.* has been most thoroughly established due to its low blank value. A semi-micro technique of a modified Brodie method has also been reported by Routh and his co-workers (1956). Their method, though accurate, involves the use of the toxic ethylene dichloride and requires several mechanical shakings, centrifugations, and aspirations which are tedious and time consuming. In an attempt to bypass the extraction procedure and shorten the time required for the determination, other investigators have

employed so-called single solution methods. Their reagent may consist of protein precipitants and ferric salts (Trinder, 1954), or a nitric acid solution of ferric nitrate (Keller, 1947).

Fluorometric methods of measuring salicylates were first described by Saltzman (1948). Although the method is highly specific for salicylic acid, the need for a fluorometer militated against its general employment in hospital laboratories. Recently, in studies requiring high sensitivity with micro samples, fluorometric analysis has been employed. When salicylic acid is excited at around 310 n.m. it emits fluorescence at around 400 n.m. (Udenfriend *et al.*, 1957; Chirigos and Underfriend, 1959; Potter and Guy, 1964). Acetylsalicylic acid does not fluoresce, but is detected only after hydrolysis of the acetyl group (Potter and Guy, 1964). Of the metabolic products of salicylic acid, gentistic acid also exhibits weak fluorescence. However, this occurs at 440 n.m. when activated at 315 n.m.

Measurement of Free Salicylates

The measurement of free salicylates in the research field has been mostly done by the tube dialysis technique using a semipermeable membrane combined with the colorimetric analysis of dialysate (Reynolds and Cluff, 1960; Davidson and Smith, 1961). Two column chromatographic methods have recently been reported using the gel filtration principle with Sephadex* resins. Free and protein-bound salicylates were separated according to their molecular sizes by column chromatography and the concentrations of salicylates in the eluents were measured either by Trinder's colorimetric method (Lomax, 1970) or by a fluorometric method (Potter and Guy, 1964).

---

*Pharmacia Fine Chemicals, Inc., 800 Centennial Avenue, Piscataway, N.J. 08854.

MATERIALS AND METHODS

Experiment 1. Free Diffusion Test

In fractionation of plasma salicylate using Sephadex dextran gels, the primary requirement is that unbound salicylate molecules must freely diffuse into the gels, while the excluded protein-bound salicylate and proteins remain in the external fluid phase of the Sephadex water mixture.

Method. Aqueous solutions of salicylate with concentrations of 25 mg.% and 50 mg.% were prepared. The free diffusion test was performed using Sephadex G-25 Coarse and G-50 Coarse with the amounts of 0.1 gm., 0.3 gm., 0.5 gm., and 1.0 gm., respectively.

The different amounts of Sephadex gel were measured into 5 ml. of 2 different concentrations of salicylate aqueous solution in 15 x 100 mm. test tubes. The tubes were stoppered with cork covered with parafilm and were shaken at moderate speed (40 to 60 times per minute) on a mechanical shaker* for 75 minutes and 3 hours, respectively. The tubes were centrifuged for 3 to 5 minutes at 1000 to 1500 rpm. Then, the concentrations of salicylate in the supernatant and in the original salicylate aqueous solution were measured by the modified method of Trinder (1954). Salicylate inside of Sephadex (Si) was calculated by the formula presented in the introduction and discussed in Appendix II, Part A.

Modified Trinder Method for Salicylate Determination. The serum, 0.2 ml. to the unknown tube, and 0.2 ml. of working standard solution to the

*Eberbach Corporation, Ann Arbor, Mich., 115 volts, 60 cycle AC.

standard tube, was added to 1.8 ml. of deionized water in the 12 x 75 mm. tubes. Into the blank tube, 2 ml. of deionized water was measured. To each tube, 2 ml. of Trinder reagent was pipetted and they were mixed well. After standing for 5 minutes at room temperature, they were centrifuged at high speed (2000 rpm) for 5 minutes, then all of the clear supernate was transferred with disposable pipettes to the 9 x 75 mm. cuvette and transmittance, %T, was read at 540 n.m. against the blank in a Coleman Junior spectrophotometer.* After converting %T to absorbance, the concentration of salicylate was calculated as follows:

$$\text{Salicylate (mg.\%)} = \frac{25 \cdot Ax}{As}$$

where Ax = absorbance of unknowns

As = absorbance of standard (25 mg.%)

Statistical analysis of the precision of the method will be presented under the experiment of salicylate-protein interaction in the results. All reagents are listed in Appendix I.

Experiment 2. Correction for the Affinity of Salicylate to Sephadex Gels

In free diffusion tests, certain interactions of salicylate with Sephadex matrix was found. When the amount of Sephadex gel is fixed, then the degree of deviation from free diffusion caused by the interaction between salicylate molecules and Sephadex will be also fixed. In order to find the factor which corrects such deviation from free diffusion of unbound salicylate molecules into gels, the following experiment was carried out under the same conditions as that with serum.

---

*Coleman Instruments, Inc., Maywood, Ill., U.S.A.

Method. Since the sample size was reduced from 5.0 to 3.0 ml., 0.2 gm. of Sephadex G-50 was used, which is the maximum amount of gel leaving enough supernate for further analysis. The subsequent procedures of the experiment were identical to those in the free diffusion test of Experiment 1.

Experiment 3. Time to Reach Equilibrium

The rate of uptake of water and solutes by the dry Sephadex gels is time-dependent. Before the analysis of salicylate in the supernate, equilibrium must be established between the inside and outside of Sephadex. The determination of time to reach equilibrium was performed by measuring the length of time required for protein and salicylate in the external solution of gels to reach constant concentration.

Method. Pooled serum with 25 mg.% of salicylate concentration was prepared. Using 3 ml. of this serum sample, the fractionation was carried out with 0.2 gm. of Sephadex G-50. Duplicate tests were run for every 30-minute increment of equilibrium time and both protein and salicylate concentrations in the supernate were measured. The procedures of fractionationg and salicylate determination are the same as in the free diffusion test. For determination of protein the Biuret method was used. The statistical analysis of the reproducibility of the Biuret method was also carried out using commercial control serum, Moni-trol II.*

Biuret Method for Protein Determination. Into 19 x 105 mm. cuvettes, 8.0 ml. of Biuret reagent was measured. Using T.C. pipettes, 0.1 ml. of serum to unknown tube, Lab-trol* to standard tube, and Moni-trol II to

---

*DADE Division, American Hospital Supply Corp., Miami, Fla. 3315.

control tube was added. The content of each tube was mixed well. After letting the tubes stand for 30 minutes at room temperature, transmittance (%T) of each tube was read at 540 n.m. setting 100 %T with Biuret reagent. After the transmittance was converted to absorbance the concentration of protein was calculated as follows:

$$\text{Total protein (gm.\%)} = \frac{Ax \cdot (\text{total protein value of standard})}{As}$$

where Ax = absorbance of unknown

As = absorbance of standard

The accuracy of the method was tested using 10 sets of commercial control serum, Moni-Trol II. All reagents are listed in Appendix I.

Experiment 4. Effect of Serum Protein Concentration on the Distribution of Free and Bound Salicylates

This test was performed in order to determine if the Donnan effect will be considerable or negligible in the proposed system.

Method. The protein concentration of pooled serum was adjusted to the maximum possible range handled in the clinical laboratory, that was 4, 5, 6, 7, 8, 9, and 10 gm.% by adding 25 gm.% Normal Serum Albumin (Human)* or by diluting with physiological saline. Three different concentrations of salicylate, 10 mg.%, 25 mg.%, 50 mg.%, were prepared for each of those samples with different protein concentrations. The fractionation test was performed with 3.0 ml. of each sample and with 0.2 gm. of G-50 gels, and for 75 minutes equilibrium time. Salicylate determination of the original samples and supernates are described above.

---

*Salt poor, no preservative, provided by the American National Red Cross.

Experiment 5. pH Control Studies

I. The effect of small changes in pH on the binding nature of protein and salicylate was examined within the pH range readily obtained in the clinical laboratory.

Method. The pH of the pooled serum containing approximately 25 mg.% of salicylate was adjusted to 7.1, 7.4, and 7.8 with the range of $\pm$ 0.05 pH units by bubbling a gas mixture of CO_2 (10.03%) balanced by N_2 which was washed through physiological saline. The shift of pH by CO_2 gas depends on the flow rate and the time length of bubbling the gas. Since the flow rate of CO_2 could not be reproduced accurately, the length of time of flushing CO_2 gas had to be set up on the extra prepared aliquots of the specimens during each experimental run. The pH was measured with a blood gas pH meter.* After the fractionation experiments were carried out on the pH adjusted pooled sera, the statistical analysis was performed on the percentage of unbound salicylate of 3 different pH groups.

II. In order to test validity of pH control, pH at zero time and pH after equilibrium time were compared.

Method. The pH of pooled serum containing 25 mg.% of salicylate was adjusted to approximately 7.4 at 37°C. by bubbling CO_2 gas (10.03%) through it for 5 minutes. From this pH-adjusted serum, 3.0 ml. of the sample was transferred to a 12 x 100 mm. test tube containing 0.2 gm. Sephadex G-50 as quickly as possible. Carbon dioxide gas was again flushed into serum and Sephadex gel mixtures for 30 seconds, and pH at zero time was measured. Tubes were stoppered with parafilm-covered cork and were

---

*Instrumentation Laboratory, Inc., 9 Galen Street, Watertown, Mass. 02172.

shaken as described previously. Duplicate tubes were removed every 30 minutes from the mechanical shaker and the pH was measured.

Experiment 6. Estimation of Average Association Constant of Protein-Salicylate by Batch Procedures With Sephadex Gels

Method. The serum salicylate fractionation test was run on pooled serum with varying concentrations of salicylate and constant amounts of protein. The sample, 3.0 ml., was measured into a 12 x 100 mm. test tube and its pH was adjusted to 7.4 $\pm$ 0.05 at 37°C. by bubbling a gas mixture of CO_2 (10.03%) balanced by N_2 for a suitable time which had been set up by measuring the pH of extra prepared aliquots of the sample after bubbling CO_2. Immediately 0.2 gm. of Sephadex G-50 Coarse within $\pm$ 0.5 mg. range was added to the tube which was then stoppered with parafilm-covered cork. The cork-stoppered tube was sealed with additional parafilm tape. All tubes were shaken at the speed of 40 to 60 times per minute with a mechanical shaker for 75 minutes and centrifuged for 3 to 5 minutes at 1000 to 1500 rpm.

After fractionation procedures, salicylate in the sample serum and salicylate in the supernate were determined by the modified Trinder method, and albumin concentration by the dye-binding Albustrate method, respectively. The calculations for bound and unbound salicylate are as follows:

$$Si = \frac{St \cdot Vt - Ss' \cdot (Vt - Vi)}{Vi} = 3\ St - 2\ Ss' \ ; \ Ss' = Ss \times 1.07$$

$$Sb = St - Si \ ; \ Salb = Sb \times 0.85$$

where St = total salicylate, mg.%

Si = free salicylate inside of Sephadex gels which is also equal to unbound salicylate in the sample, mg.%

Ss = experimental value of salicylate in supernatant, mg.%

Ss' = corrected Ss about the deviation from free diffusion, mg.%

Sb = protein-bound salicylate, mg.%

Salb = albumin-bound salicylate, mg.%

Vt = sample volume, 3 ml.

Vi = Volume of Sephadex = WR x a, 1 ml.

WR = water regain of gels, 5.0 ml. per gm. of gels

a = amount of gels used, 0.2 gm.

Serum Albumin Determination. Albstrate reagent,* 1.0 ml., was measured into 10 x 105 mm. cuvettes and 4.0 ml. of distilled water was added to each tube. Using a T.C. pipette, 0.01 ml. of sample was added into unknown tubes but not into the reagent blank and the contents of the tubes were mixed well. Transmittance (%T) of each test against the reagent blank was read at 630 n.m. Moni-trol I as standard and Moni-trol II as control were run with the unknowns. After converting transmittance to absorbance, the concentration of serum albumin was calculated by the following formula:

$$\text{Albumin (gm.\%)} = \frac{Ax \cdot (\text{albumin value of standard})}{As}$$

where Ax = absorbance of unknown

As = absorbance of standard

The precision of the method was tested about 10 sets of commercial serum control, Moni-trol II. All reagents are listed in Appendix I.

---

*General Diagnostics, Division Warner-Lambert, Morris Plains, N.J. 07950.

RESULTS

A certain affinity of salicylate molecules with the Sephadex gels was revealed as shown in Table 1. At equilibrium state if there is free diffusion of salicylate molecules, the total salicylate, St, and salicylate outside of gels, Ss, and salicylate inside of gels, Si, are all expected to be equal. According to the data, Ss was always lower than St, which made Si high by the calculation. Sephadex type G-50 gave less deviation from free diffusion than G-25 type. The two different equilibriums time did not show any significant difference.

Finding the factor (St/Ss) which corrects for deviation from free diffusion, there was no significant difference in St/Ss ratios between 25 mg.% and 50 mg.% of total salicylate groups as indicated in Table 2. The comparison of two groups showed that p was greater than 50% at d.f. = 7. Thus, taking the average of St/Ss ratios of two groups, the correction factor was found to be 1.07.

The uptake of water and solutes by dry dextran gels was completed within 30 minutes and no further change occurred beyond this time (Table 3).

The results of the serum salicylate fractionation in which the effect of the increased concentration of serum protein on the percentage of unbound salicylate was examined are shown in Table 4 and Figure 2. There was no significant effect observed on the percentage of diffusible unbound salicylate inside of Sephadex by changing the concentration of protein. Therefore, the Donnan effect is not appreciable in the system developed

Table 1. Free diffusion test of salicylate molecule into Sephadex gels

Equilib. time	Sephadex Type	Sephadex gm.	St (mg.%)	Ss (mg.%)	Si (mg.%)	St/Ss
75 min.	G-25	0.1	25.0	25.0	25.0	1.00
		0.3	25.0	24.7	26.7	1.01
		0.5	25.0	21.5	35.5	1.11
		1.0	25.0	16.7	33.3	1.50
		0.1	49.3	48.3	58.8	1.02
		0.3	49.3	46.2	66.9	1.07
		0.5	49.3	43.6	66.8	1.13
		1.0	49.3	37.1	61.5	1.33
	G-50	0.3	26.4	24.3	31.1	1.09
		0.5	26.4	---	---	---
		0.3	50.9	48.2	57.0	1.06
		0.5	50.9	---	---	---
3 hrs.	G-25	0.3	28.6	24.3	52.9	1.18
		0.5	28.6	25.0	39.4	1.14
		0.3	53.3	49.7	73.7	1.07
		0.5	53.3	47.0	76.4	1.13
	G-50	0.3	26.4	24.5	30.8	1.08
		0.5	26.4	---	---	---
		0.3	50.9	48.3	57.0	1.05
		0.5	50.9	---	---	---

--- no supernatant

St = total salicylate

Ss = salicylate in supernatant

Si = salicylate inside Sephadex

Table 2. Correction factor (St/Ss) for the deviation of salicylate from free diffusion

n	St (mg.%)	Ss (mg.%)	Si (mg.%)	St/Ss	Comparison of St/Ss of two groups
1	25.0	24.0	27.0	1.042	
2	25.0	22.6	29.8	1.106	
3	25.0	22.9	29.2	1.092	
4	25.0	23.3	28.4	1.073	
5	24.3	21.6	29.7	1.125	
6	24.3	23.0	26.9	1.057	$n = 8$
7	26.4	24.3	31.1	1.086	$\bar{D} = -0.025$
8	26.4	24.5	30.8	1.078	$s = 0.47$
					$t = 0.148$
1	48.2	45.3	54.0	1.064	$p > 50\%$ at d.f. = 7
2	48.5	45.1	55.3	1.075	
3	48.5	46.4	52.7	1.045	
4	48.5	46.8	51.9	1.036	
5	48.5	47.6	50.3	1.019	
6	50.4	45.3	60.6	1.113	
7	50.9	48.2	57.0	1.056	
8	50.9	48.3	57.0	1.054	
			Average	1.070	

Table 3. Time to reach equilibrium--protein and salicylate determinations in the external fluid of Sephadex

Time (hours)	0	0.5	1	1.25	1.5	2	3
Protein (gm.%)	6.52	9.96	9.96		9.96	9.89	9.76
	6.52	9.96	9.82		9.89	9.96	9.67
	6.60	10.14	10.07		10.14	10.14	
	6.62	10.22	10.07		10.07	10.22	
Salicylate (mg.%)	26.4	34.7	34.7		34.7	35.9	
	26.1	36.5	35.1		35.9	35.9	
	28.6	38.3		40.7			38.3
	27.9	39.1		39.9			39.4

Table 4. Effect of serum protein concentration on the distribution of unbound salicylate inside of Sephadex

Protein (gm.%)	St (mg.%)	Ss (mg.%)	Si	
			(mg.%)	(%)
4.19	12.7	16.4	5.3	41.7
5.59	12.7	16.0	6.1	48.0
6.09	12.7	16.1	5.9	46.5
6.82	12.7	16.5	5.1	40.2
7.80	13.3	17.1	5.7	42.9
8.77	12.7	16.0	6.1	48.0
9.59	12.7	16.0	6.1	48.0
4.17	26.0	30.8	16.4	63.1
5.39	25.7	31.4	14.3	55.6
6.09	26.3	32.1	16.5	62.7
8.36	27.4	32.3	17.6	63.8
9.71	26.3	32.1	14.7	55.9
8.90	26.3	32.1	14.7	55.9
9.71	26.7	31.6	16.9	63.3
4.22	50.2	58.7	33.2	66.1
5.52	51.1	60.3	32.7	64.0
6.08	49.4	60.3	27.6	55.9
6.87	52.4	62.7	31.8	60.7
9.45	52.5	64.7	28.1	53.5
9.92	51.5	61.2	32.1	62.3
11.51	50.7	60.5	31.1	61.3

Ss are the average of duplicate fractionation tests.

pH of pooled serum was 8.5.

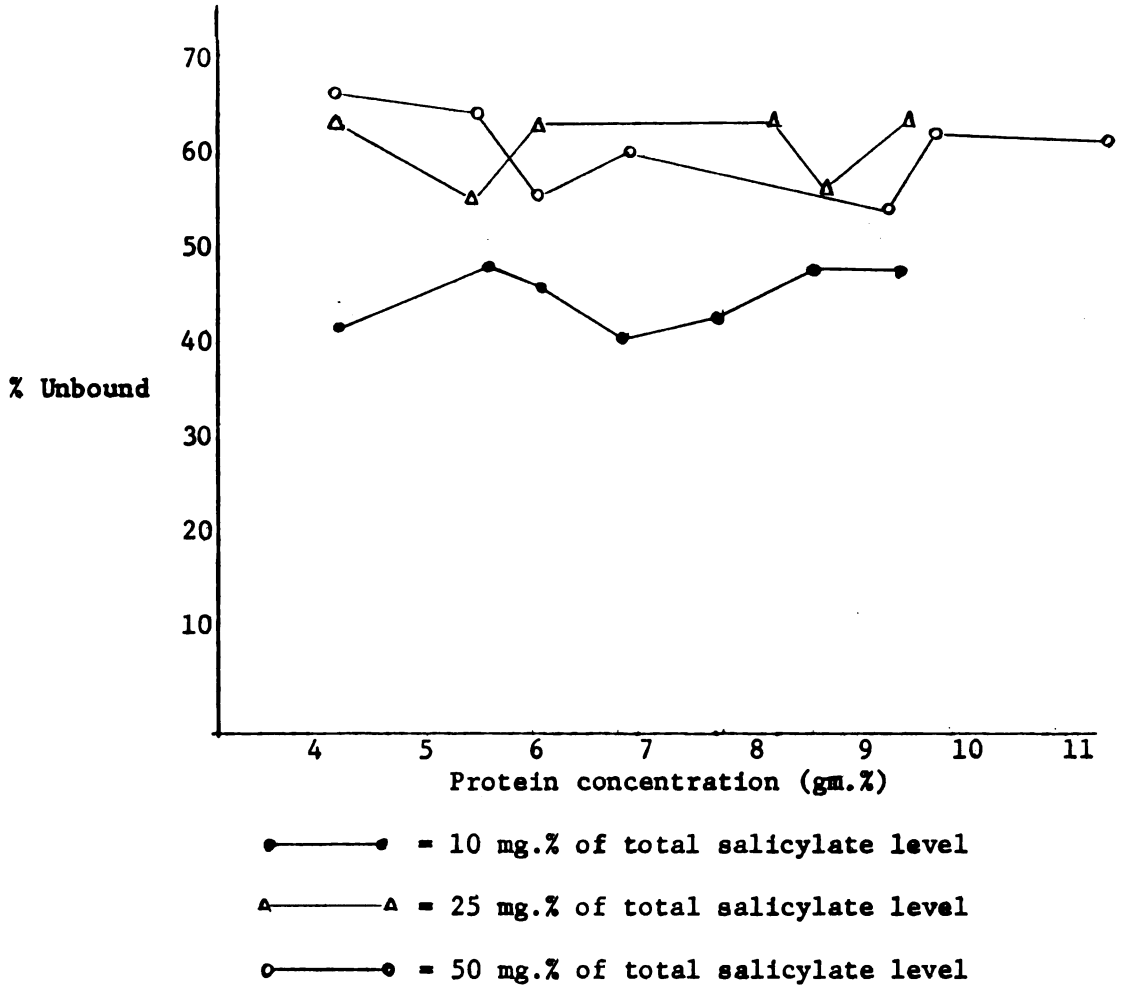


Figure 2. Relationship between protein concentration and unbound salicylate.

here. The coefficient of variation of protein determination by the Biuret method was 4.4%.

The effect of a small change of pH at 7.1, 7.4, and 7.8 on the percentage of unbound salicylate was found to be insignificant as indicated in Table 5-I and Table 5-II. The statistical comparison of unbound salicylate between each of two pH groups shows that p is greater than 50% in all cases. The reproducibility of the proposed method can be evaluated from the statistical analysis of data on 19 replicate samples of pooled human serum. At pH 7.4, when the mean of the percentage of unbound salicylate was 32.9, the standard deviation was 4.5 (coefficient of variation = 13.5%). Observations on pH change during the equilibration period reveal that the pH decreases an average of 0.13 pH units irrespective of the time allowed for equilibration.

Using the method developed here, the estimation of the average association constant of albumin-salicylate was investigated about different salicylate levels at pH 7.4 and at constant protein concentrations. The results are shown in Table 6 and Figure 3. The average association constant of protein-salicylate was found to be 5.8×10^3 and the average number of binding sites per protein molecule was estimated as 5.2. The comparison of the experimental values with the reference method, tube dialysis technique by Davidson and Smith (1961) is listed in Table 7. The calculation formulas are presented in Appendix II, Part C. The precision of both albumin and salicylate determinations was tested using containing 25 mg.% of salicylate for the modified Trinder method. The coefficient of variation for the Albustrate method was 4.1% and for the Trinder method was 2.8%.

Table 5-I. Effect of pH on the percentage of unbound salicylate in three different pH groups

	pH 7.1 (%)	pH 7.4 (%)	pH 7.8 (%)
	35.4	29.2	31.5
	35.4	29.2	26.1
	26.5	35.4	35.4
	26.5	41.7	29.5
	26.5	38.6	41.7
	38.6	38.6	38.6
	28.3	26.5	38.6
	32.3	35.6	32.3
	32.3	32.6	35.4
		38.6	
		28.3	
		37.8	
		28.3	
		32.3	
		28.3	
		30.4	
		32.8	
		30.4	
		30.4	
Mean ± S.D.	33.0 ± 5.3%	32.9 ± 4.5%	34.3 ± 5.0%
Variance	28.3	20.2	24.5
Coeff. of var.	16.1%	13.5%	14.5%
n	9	19	9

Table 5-II. Effect of pH on the unbound salicylate; comparison of three pH groups

Comparison between	Comparison of means					Comparison of variance	
	V'	s'	t	d.f.	p	Variance ratio	p
pH 7.1 and pH 7.4	22.2	4.76	0.005	26	>50%	$F_{8,18} = 1.40$	>10%
pH 7.4 and pH 7.8	21.5	4.64	0.773	26	>50%	$F_{8,18} = 1.21$	>10%
pH 7.1 and pH 7.8	26.4	5.14	0.537	16	>50%	$F_{8,18} = 1.16$	>10%

Calculation formula (Lewis, 1966).

Table 5-III. Validity of pH during equilibration time

0 hr	0.5 hr	1 hr	1.5 hr		pH difference
7.370	7.193				-0.177
7.272	7.170				-0.102
7.354		7.248			-0.106
7.365		7.200			-0.165
7.345			7.247		-0.098
7.358			7.233		-0.125
				average	-0.129

Table 6. Interaction between human serum albumin and salicylate

St moles/l.	Sunb moles/l.	Sb moles/l.	Salb moles/l.	Alb moles/l.	r	r/f
$x10^{-4}$	$x10^{-4}$	$x10^{-4}$	$x10^{-4}$	$x10^{-4}$		$x10^{4}$
4.92	1.72	3.19	2.71	2.33	1.22	0.70
8.33	3.84	4.49	3.82	2.00	1.91	0.50
4.71	0.36	4.34	3.69	5.86	0.63	1.75
8.76	1.09	7.67	6.52	6.34	1.03	0.95
8.83	1.30	7.53	6.40	5.85	1.09	0.84
12.60	2.17	10.43	8.87	6.28	1.41	0.65
15.20	2.75	12.45	10.58	6.29	1.68	0.61
18.25	3.19	14.92	12.68	6.34	2.00	0.63
26.57	6.30	20.27	17.23	6.38	2.70	0.43
29.32	6.88	22.44	19.07	6.29	3.03	0.44
32.65	11.37	21.29	18.10	6.34	2.86	0.25
35.33	10.72	24.62	20.93	6.20	3.38	0.32
40.47	17.16	23.17	19.69	6.20	3.18	0.19

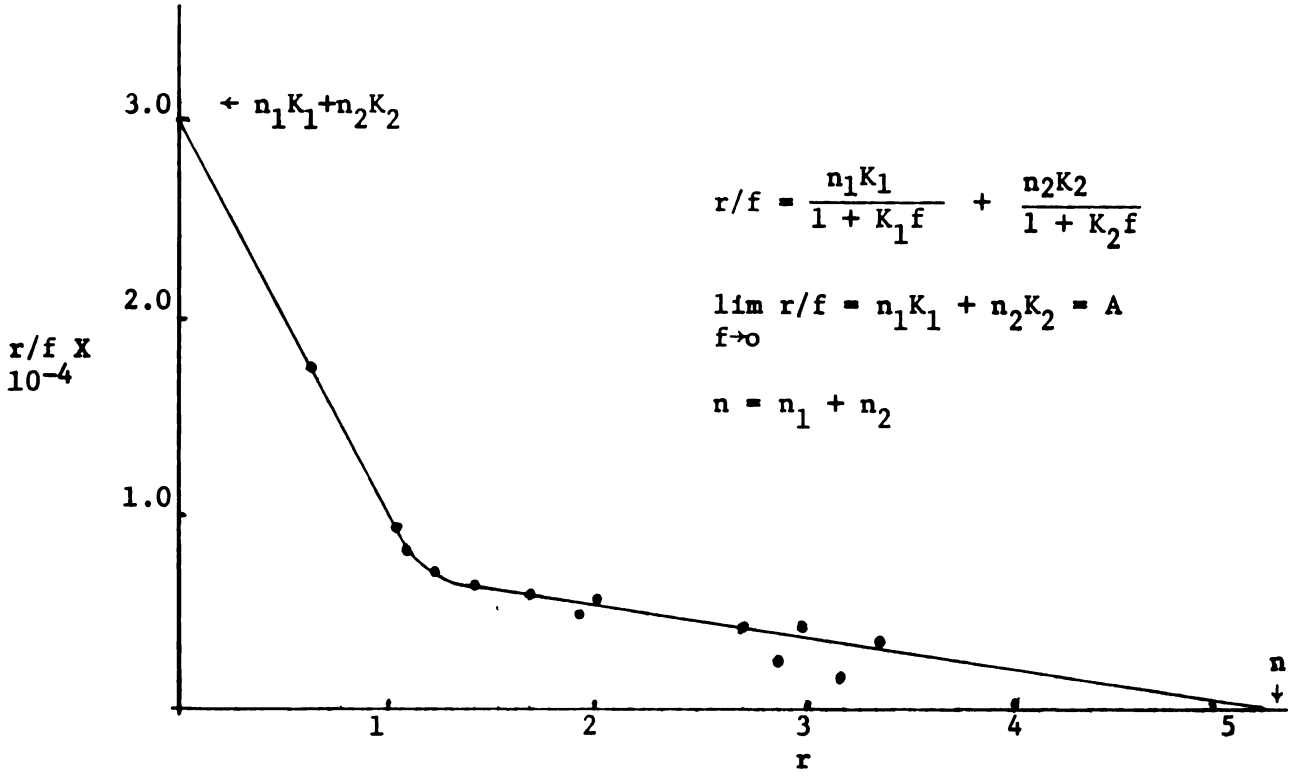
r = Salb/Alb, f = Sunb

Salb = Albumin-bound salicylate = Sb x 0.85

Sb = Protein-bound salicylate

St = Total salicylate

Sunb = Unbound salicylate = f



r = Albumin-bound salicylate/Albumin

f = Free salicylate

n = Average number of binding sites per molecule of protein

K_1, K_2 = Association constants in two types of binding

Figure 3. Binding of human albumin with salicylate at pH 7.4, pooled human serum.

Table 7. Binding of human serum albumin with salicylic acid; comparison of reference method values with the proposed method

Experimental batch procedure with Sephadex gels	Davidson and Smith (1961) tube dialysis method
$n = 5.2$, $n_1K_1+n_2K_2 = 3.0 \times 10^4$	$n = 5.4$, $n_1K_1+n_2K_2 = 2.2 \times 10^4$
Ave. $K = 5.8 \times 10^3$	Ave. $K = 4.1 \times 10^3$
$n_1 = 0.8$ $K_1 = 1.66 \times 10^4$	$n_1 = 1.4$ $K_1 = 1.50 \times 10^4$
$n_2 = 4.4$ $K_2 = 3.98 \times 10^3$	$n_2 = 4.0$ $K_2 = 0.25 \times 10^3$
pH = 7.4	pH = 5.4
Pooled human serum	Human crystalline albumin

DISCUSSION

The methods developed to date for the determination of serum-free salicylate are either cumbersome or require an expensive instrument, a fluorometer, which is still not available in many laboratories. In the tube-dialysis technique using a semipermeable membrane (Davidson and Smith, 1961), it takes at least 16 hours to reach equilibrium and requires a relatively large volume of sample, a minimum of 5 ml. of serum. Two column chromatographic methods have been reported using the gel filtration principle with Sephadex resins. The column chromatographic procedures used by Potter and Guy (1964) may be the best method for the micro techniques, but it requires a fluorometer for the salicylate determination and the column must be standardized for each batch of fresh buffer. The method reported by Lomax (1969) combines column chromatography with photometric determinations but requires measurements of both salicylate and protein of all eluent fractions.

In all of those methods, it is difficult to prevent or control pH changes of the specimens. Although the statistical comparison of the percentage of unbound salicylate at pH 7.1, 7.4, and 7.8 shows no significant change in the present study, the mean unbound salicylate of the three pH groups increases with the increase of pH (Table 5-I). The greater tendency of protein to bind salicylate at the lower pH has been observed by Potter and Guy (1964).

In the batch procedure proposed here the serum sample immediately after the separation from whole blood is pipetted into a test tube, and

free salicylate is taken up by dry dextran gels. This sampling step should be done as quickly as possible to avoid any loss of CO_2 from the sample, which may cause a shift in pH. During equilibration, pH was lowered an average up to 0.13 which may be caused by Sephadex itself since the dextran gels have many hydroxy groups. However, that much shift of pH does not significantly affect the results according to the study of pH effects. If the time factor is important for the test, the equilibration time may be reduced to 45 minutes since equilibration is complete within 30 minutes.

In the free diffusion test, a certain interaction between salicylate molecules and Sephadex gels was observed. As one of the properties of Sephadex dextran gels, it was reported by Marsden (1965) that aromatic substances (homocyclic or heterocyclic) and solutes containing hydroxy and carboxy groups have greater affinity for Sephadex gels than non-aromatic substances of similar molecular size. Salicylic acid belongs to those substances with an aromatic ring and hydroxy and carboxyl groups. The actual interaction between salicylate solute and Sephadex matrix is not known. However, such deviation of salicylate from free diffusion through Sephadex gels was corrected by an experimentally determined factor as shown in the results.

According to the Donnan equilibrium theory (Tanford, 1961; Greene and Power, 1931), because of the effect of nondiffusible protein ions present in only fluid external to Sephadex, the diffusible free salicylate ions on both sides of a semipermeable membrane are not equal even at equilibrium condition. Thus, the concentration of any of the filterable or unbound ions in the gels has to be multipled by an appropriate Donnan correction factor in order to give the concentration of any of these diffusible ions in the original sample. In the proposed system, if the

Donnan effect is significant, then the increase of the protein concentration on one side of the semipermeable membrane is expected to decrease the concentration of diffusible free salicylate molecules on the other side. However, the results of the experiment showed that in this particular system of protein-binding with salicylate anions at the physiological range of protein concentration, the Donnan effect was negligible. This may be because a net charge of protein is negative at the physiological pH, 7.4; thus, it does not affect the distribution of salicylate anions across the semipermeable membrane. Also this may be because the dissociation constant of salicylate is so small that ionization of both salicylic acid and salicylate-bound proteinate molecules is not enough to follow the Donnan theory. The Donnan theory is founded on the assumption that the base proteinate and diffusible molecules on both sides of the membrane are completely ionized, and only the ionized constituents of both sides of the membrane enter into the Donnan equilibrium (Greene and Power, 1931). Another explanation can be offered. If water regain of Sephadex gels in aqueous solution is used in the calculation formula for Si presented in the introduction instead of using water regain in the serum phase, consideration of the Donnan correction factor will be unnecessary as shown in Appendix II, Part B. Water regain, H_2O per gm., of dry Sephadex can be obtained from the instruction manual of Sephadex by Pharmacia Fine Chemicals, Inc.* (1968). From the evidence of experimental results, the diffusible free salicylate inside of Sephadex directly represents the unbound salicylate in the original serum sample.

In order to test the validity of the method developed here for estimating free salicylate, the interaction between protein and salicylate

---

*Pharmacia Fine Chemicals, Inc., 800 Centennial Avenue, Piscataway, N.J. 08854.

molecules was studied by the proposed batch procedures with Sephadex G-50 Coarse grains. Measuring unbound salicylate (Sunb) by the method developed, protein bound salicylate (Sb) was calculated from total salicylate (St) and Sunb. Albumin concentration was used for the protein concentration in calculation of the association constant (K) and albumin-bound salicylate was obtained by multiplying protein-bound salicylate by 0.85 since approximately 85% of protein-bound salicylate is bound to albumin while the remaining 15% adheres to alpha and beta globulins (Davidson and Mandel, 1965; Reynolds and Cluff, 1960). The technique of plotting the data for Figure 3 is that of Scatchard (1949), also utilized by Karush (1950), Teresi and Luck (1952), and Davidson and Smith (1961). If more than one type of binding is involved, a curvilinear plot is obtained. The nature of the curve indicates that the binding of protein by salicylate ions cannot be represented by a single intrinsic association constant, as there are at least two groups of binding sites on the protein molecule. As described by the above investigators, according to the assumption in which the binding can be expressed by only two different values of association constants, the average association constant (K) and K_1 and K_2 for the two groups of binding sites, n_1 and n_2, and the average number of binding sites (n) per protein molecule were calculated from the formula in Appendix II, Part C.

The results, the average association constant ($K = 5.8 \times 10^3$), the average number of binding sites per protein molecule ($n = 5.2$), were obtained with pooled human serum at pH 7.4. The value of n need not be interger (Karush, 1950), since the proteins may not be homogeneous with respect to binding properties. The study using the tube dialysis method of Davidson and Smith (1961) gave average $K = 4.1 \times 10^3$, $n = 5.4$, using

crystalline human serum albumin at pH 5.4. The results obtained by two methods are presented in Table 7. Comparing those results by two techniques, the average number of binding sites per protein molecule and the average association constant are reasonably well agreed, taking account of the different experimental conditions such as pH and protein used, the possible electrostatic interaction of buffer ions for the binding sites (Scatchard, 1949; McMenamy and Oncley, 1958) in the tube-dialysis method, and some uncertainty of finding total value of association constants, $n_1K_1 + n_2K_2$, at the intercept of y axis by extrapolation of such a curve as shown in Figure 3. The binding of salicylates exhibits two types of binding sites, one of relatively high affinity but small in number, a second of low affinity but in greater number. The former, n_1 and K_1, are regarded as more structure-specific (Goodman, 1958). The K_1 value of 16,600 with a binding site number of 0.8 obtained in the experiment are in the same range as the values of 15,000 and 1.4 reported by Davidson and Smith. The type 2 binding seems to be much less affected by structure; the different values for K_2 and n_2 are probably within the error of the method.

The correlation between free serum salicylate level and either its pharmacological or toxicological effects has not been studied yet in the clinical field. Carrying out such a study, such information as patient's blood pH, urine pH, salicylate excretion of 24-hour urine, and clinical manifestations as well as the total and free salicylate levels will be necessary. A simple, reliable method of determination of free serum salicylate as developed here will be useful in such investigations in the future.

SUMMARY AND CONCLUSIONS

In the treatment of such diseases as rheumatic conditions using salicylates, from both pharmacological and toxicological aspects, the measurement of unbound salicylate level in the patient's blood will provide useful information. In this project, the development of simplified, reliable procedures for the determination of serum unbound salicylate was sought using the common laboratory instruments.

The principle of this method is based on the molecular sieving gel filtration with Sephadex dextran gels in batch procedures. Originally this technique was applied to unbound serum calcium determination by Schatz (1962). The serum sample was shaken with dry Sephadex gels until equilibrium was reached. From the total salicylate and salicylate in extrafluid of Sephadex, free salicylate intrafluid of Sephadex was calculated according to the formula given.

The distribution of salicylate molecules between Sephadex and solvent deviates from the theoretical ideal, and therefore a correction factor must be applied. This was empirically determined to be 1.07.

The significance of the Donnan equilibrium theory in the proposed system was examined. Increased concentration of protein in the extrafluid of Sephadex gels did not affect the percentage of unbound salicylate in the intrafluid of Sephadex. Therefore, free salicylate inside the gels was considered to be equal to unbound salicylate in the sample.

Theoretically some effect on protein binding of changes in pH might be expected. Comparisons of the binding occurring at pH 7.1, 7.4, and

7.8 showed no significant difference, and since this encompasses the range that might be encountered in clinical material, corrections for pH effect need not be made on individual specimens.

The coefficient of variation of the method at pH 7.4, testing 19 samples, was 13.5%.

Using the procedures developed, the interaction between protein and salicylate molecules was studied using pooled human serum at pH 7.4; the average association constant of albumin-salicylate was found to be 5.8×10^3, and the average number of binding sites per protein molecule was 5.2.

REFERENCES

REFERENCES

Atkins, E.: Pathogenesis of fever. Physiol. Rev., 40, (1960): 580-646.

Barbour, H. G.: The antipyretic action of dextrose. Proc. Soc. Exptl. Biol. Med., 16, (1919): 136-138.

Brodie, B. B., and Hogben, C. A. M.: Some physico-chemical factors in drug action. J. Pharm. & Pharmacol., 9, (1957): 354-380.

Brodie, B. B., Udenfriend, S., and Coburn, A. F.: The determination of salicylic acid in plasma. J. Pharmacol. Exptl. Therap., 80, (1944): 114-117.

Brody, T. M.: The uncoupling of oxidative phosphorylation as a mechanism of drug action. Pharmacol. Rev., 7, (1955): 335-363.

Brody, T. M.: Action of sodium salicylate and related compounds on tissue metabolism *in vitro*. J. Pharmacol. Exptl. Therap., 117, (1956): 39-51.

Charnock, J. S., Opit, L. J., and Hetzel, B. S.: An evaluation of the effect of salicylate on oxidative phosphorylation in rat-liver mitochondria. Biochem. J., 83, (1962): 602-606.

Chirigos, M. A., and Udenfriend, S.: A simple fluorometric procedure for determining salicylic acid in biological tissues. J. Lab. Clin. Med., 54, (1959): 769-772.

Collier, H. O. J.: A pharmacological analysis of aspirin. Adv. in Pharmacol. & Chemotherap., 7, (1969): 333-405.

Cooper, K. E., Grundman, M. J., and Honour, A. J.: Observation on sodium salicylate as an antypyretic. J. Physiol., 196, (1968): 56P-57P.

Davidson, C., and Smith, P. K.: The binding of salicylic acid and related substances to purified proteins. J. Pharmacol. Exptl. Therap., 133, (1961): 161-170.

Davidson, P., and Mandel, H. G.: Non-narcotic analgesics and antipyretics. I. Salicylates, in *Drills Pharmacology in Medicine*, ed. by Diapalma, J. R., 3rd ed., McGraw-Hill Book Co., New York (1965): 297.

Davis, B.: The binding of sulfonamide rings by plasma proteins. A factor in determining the distribution of drugs in the body. J. Clin. Invest., 22, (1943): 753-762.

Davis, B. D.: The binding of chemotherapeutic agents to proteins and its effect on their distribution and activity, in *Evaluation of Chemotherapeutic Agents*, Columbia University Press, New York, 1948.

Feldberg, W., and Myers, R. D.: A new concept of temperature regulation by amines in the hypothalamus. Nature, 200, (1963): 1325.

Feldberg, W., and Myers, R. D.: Effects of temperature of amines injected into the cerebral ventricles. A new concept of temperature regulation. J. Physiol., 173, (1964): 226-237.

Feldberg, W., and Myers, R. D.: Changes in temperature produced by micro injections of amines into the anterior hypothalamus of cats. J. Physiol., 177, (1965): 239-245.

Feldberg, W., and El Harway, M. B. E.: Effect of 5-hydroxytryptophan acting from the cerebral ventricles on 5-hydroxytryptamine output and body temperature. J. Physiol., 186, (1966): 401-415.

Feldberg, W., Hellon, R. F., and Lotti, V. J.: Temperature effects produced in dogs and monkeys by injections of monoamines and related substances into the third ventricle. J. Physiol., 191, (1967): 501-515.

Gander, G. W., Chaffee, J., and Goodale, F.: Studies on the antipyretic action of salicylates. Proc. Soc. Exptl. Biol. & Med., 126, (1967): 205-209.

Goodman, D. S.: The interaction of human serum albumin with long-chain fatty acid anions. J. Am. Chem. Soc., 80, (1958): 3892-3898.

Greene, C. H., and Power, M. H.: The distribution of electrolytes between serum and the *in vivo* dialysate. J. Biol. Chem., 91, (1931): 183-202.

Guerra, F., and Brobeck, J. R.: The hypothalamic control of aspirin antipyresis in the monkey. J. Pharmacol. & Exptl. Therap., 80, (1944): 209-216.

Karush, F.: Heterogeneity of the binding sites of bovine serum albumin. J. Am. Chem. Soc., 72, (1950): 2705-2713.

Keller, W. J., Jr.: A rapid method for the determination of salicylates in serum or plasma. Am. J. Clin. Path., 17, (1947): 415-417.

Lewis, A. E.: *Biostatistics*. Reinhold Publishing Corp., New York., 1966.

Lim, R. K. S., Guzman, F., Rogers, D. W., Goto, K., Braun, C., Dickerson, C. D., and Engle, R. J.: Site of action of narcotic and non-narcotic analgesics determined by blocking Bradykinin-evoked visceral pain. Arch. Intern. Pharmacodyn., 152, (1964): 25-58.

Lim, R. K. S.: Salicylate analgesia, in *Salicylates: A Critical Bibliographic Review*, ed. by Smith, M. J. H., and Smith, P. K., Wiley, New York, (1966): 155-202.

Lim, R. K. S., Leung, P. M., and Guy, J. L.: Salicylate level in CNS in aspirin analgesia and antipyresis. Fed. Proc., 26, (1967): 619.

Link, K. P., Overman, R. S., Sullivan, W. R., Huebner, C. F., and Sheel, L. D.: Studies on the hemorrhagic sweet clover disease. XI. Hypoprothrombinemia in the rat induced by salicylic acid. J. Biol. Chem., 147, (1943): 463-473.

Lomax, G. D.: The determination of protein-bound salicylates. J. Med. Lab. Technol., 27, (1970): 51-71.

Marsden, N. V. B.: Solute behavior in tightly cross-linked dextran gels. Ann. N. Y. Acad. Sci., 125, (1965): 428-457.

McMenamy, R. H., and Oncley, J. L.: The specific binding of L-tryptophan to serum albumin. J. Biol. Chem., 233, (1958): 1436-1447.

Meyer, O. O., and Howard, B.: Production of hypoprothrombinemia and hypocoagulability of the blood with salicylates. Proc. Soc. Exptl. Biol. & Med., 53, (1943): 234-237.

Packer, L., Austen, F. K., and Knoblock, E. C.: Effects of salicylates on respiration and phosphorylation in heart mitochondria. Proc. Soc. Exptl. Biol. & Med., 100, (1959): 239-244.

Potter, G. D., and Guy, L. G.: A micro method for analysis of plasma salicylate. Proc. Soc. Exptl. Biol. & Med., 116, (1964): 658-660.

Quick, A. J., and Clescari, L.: Influence of acetylsalicylic acid and salicylamide on the coagulation of blood. J. Pharmacol. & Exptl. Therap., 128, (1960): 95-98.

Rodkey, F. L.: Direct spectrophotometric determination of albumin in human serum. Clin. Chem., 11, (1965): 478-487.

Reid, J.: Does sodium salicylate cure rheumatic fever? Quart. J. Med., 17, (1948): 139-151.

Reynolds, R. C., and Cluff, L. E.: Interaction of serum and sodium salicylate changes during acute infection and its influence on pharmacological activity. Bull. Johns Hopkins Hosp., 107, (1960): 278-290.

Robin, E. D., Davis, M. D., and Rees, S. B.: Salicylate intoxication with special reference to the development of hypokalemia. Am. J. Med., 26, (1959): 869-882.

Routh, J. I., Paul, W. D., Arredondo, E., and Dryer, R. L.: Semimicro method for the determination of salicylate levels in blood. Clin. Chem., 2, (1956): 432-438.

Saltzman, A.: Fluorometric method for the estimation of salicylate in blood. J. Biol. Chem., 174, (1948): 399-404.

Scatchard, G.: The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci., 51, (1949): 660-672.

Scatchard, G., Scheinberg, I. H., and Armstrong, S. H.: Physical chemistry of protein solutions. V. The combination of human serum albumin with thiocyanate ion. J. Am. Chem. Soc., 72, (1950): 540-546.

Schatz, B. C.: Determination of protein bound calcium. Thesis, Graduate School, Univ. S. Calif., 1962.

Sephadex-Gel Filtration in Theory and Practice, Pharmacia Fine Chemicals, Inc., 800 Centennial Avenue, Piscataway, N.J. 08854.

Smith, M. J. H.: Anti-inflammatory activity of salicylates, in *Salicylates: A Critical Bibliographic Review*, ed. by Smith, M. J. H., and Smith, P. K., Wiley, New York, (1966): 203-232.

Smith, M. J. H., and Talbot, J. M.: The estimation of plasma salicylate levels. Brit. J. Exptl. Path., 31, (1950): 65-69.

Smith, P. K., Gleason, H. L., Stoll, C. B., and Orgorzalek, S.: Studies on the pharmacology of salicylates. J. Pharmacol. & Exptl. Therap., 87, (1946): 237-255.

Stafford, W. L.: Protein binding of salicylates, in *Salicylates: An International Symposium*, ed. by Dixon, A. S. J., Smith, M. J. H., Martin, B. K., and Wood, P. H. N., Churchill, London, (1963): 75-78.

Stubbé, L. Th. F. L.: Occult blood in faeces after administration of aspirin. Brit. Med. J., II, (1958): 920-925.

Tanford, C.: *Physical Chemistry of Macro Molecules*. John Wiley and Sons, Inc., New York, (1961): 225-227.

Teresi, J. D., and Luck, J. M.: The combination of organic anions with serum albumin. J. Biol. Chem., 194, (1952): 823-828.

Tompsett, R., Schultz, S., and McDermott, W.: Relation of protein binding to the pharmacology and antibacterial activity of penicillin X, G, dihydro F, and K. J. Bact., 53, (1947): 581-595.

Trinder, P.: Rapid determination of salicylate in biological fluids. Biochem. J., 57, (1954): 301-303.

Underfriend, S., Duggan, E. D., Vasta, M. B., and Brodie, B. B.: A spectrophotofluorometric study of organic compounds of pharmacological interest. J. Pharmacol. & Exptl. Therap., 120, (1957): 26-32.

Volterra, M., and Jacobs, M. D.: A simple method for the estimation of salicylates in blood. J. Lab. & Clin. Med., 32, (1947): 1282-1283.

Weichselbaum, J. E., and Shapiro, F. M.: A rapid and simple method for the determination of salicylic acid in small amounts of blood plasma. Am. J. Clin. Path., 15, (1945): 42-45.

Weiss, H. J., and Aledort, L. M.: Impaired platelet connective tissue reaction in man after aspirin injestion. Lancet ii, (1967): 495-497.

Zucker, M. B., and Peterson, J.: Inhibition of adenosine diphosphate-induced secondary aggregation and other platelet functions by acetylsalicylic acid ingestion. Proc. Soc. Exptl. Biol. & Med., 127, (1968): 547-551.

APPENDICES

APPENDIX I

Reagents

I. Modified Trinder Method for Salicylate Determination:

Stock Standard Solution (250 mg./100 ml.):

Dissolve 290 mg. of sodium salicylate in distilled water, dilute to 100 ml. with distilled water. Keep in the refrigerator.

Working Standard Solution (25 mg./100 ml.):

Just before use dilute 10 ml. of stock solution to 100 ml. with distilled water.

Trinder Color Reagent:

With the aid of heat, 4.0 gm. of mercuric chloride are dissolved in 85 ml. of distilled water. The solution is cooled and 12 ml. of 1N-HCl and 4.0 gm. of ferric nitrate, $Fe(NO_3)_3 \cdot 9H_2O$, are added. When all the ferric nitrate has dissolved, the volume of the solution is made to 100 ml. with distilled water. Store in dark bottle at room temperature. This solution is stable indefinitely.

II. Biuret Method for Protein Determination:

Biuret Reagent:

Dissolve one tablet of Biuret reagent* in 70 ml. of hot distilled water using stirring rod if necessary. Add 30 ml. of 10% sodium hydroxide. Mix thoroughly and filter for use. Active ingredients of the tablet are copper sulfate, sodium potassium tartrate, and potassium iodide.

*Scientific Products, Division of American Hospital Supply Corp., Evanston, Ill.

III. Serum Albumin Determination by Dye Binding Method (Rodkey, 1960):

Albstrate Reagent:*

Contains dye (bromcresol green), buffer, surface active agent, and preservative. The reagent is stable indefinitely when stored in the refrigerator.

*General Diagnostics, Division Warner-Lambert, Morris Plains, N.J. 07950.

APPENDIX II

Calculation Formula

PART A.

Calculation Formula for Unbound Salicylates (Schatz, 1962)

$$Vt \cdot St = Vs \cdot Ss + Vo \cdot Ss + Vi \cdot Si$$

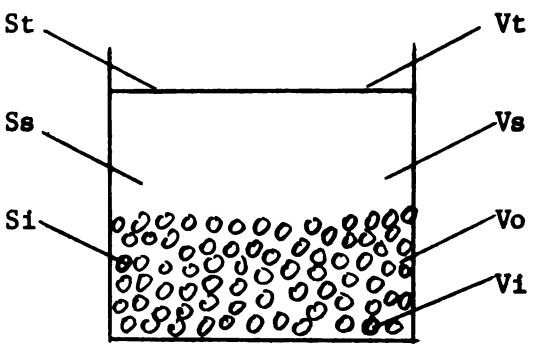
$$Vi \cdot Si = Vt \cdot St - (Vo + Vs) \cdot Ss$$

$$Si = \frac{Vt \cdot St - (Vo + Vs) \cdot Ss}{Vi}$$

since $Vt = Vs + Vo + Vi$

$$Vt - Vi = Vs + Vo,$$

$$Si = \frac{Vt \cdot St - (Vt - Vi) \cdot Ss}{Vi}$$



where St = total salicylate, mg.%

Si = free salicylate inside of Sephadex gel, mg.%

Ss = salicylate in extrafluid of Sephadex gels, mg.%

Vt = total volume of sample, ml.

Vo = void volume, ml.

Vi = volume of Sephadex gels = WR x a, ml.

WR = water regain of Sephadex, ml. per gm. of gels

a = amount of Sephadex used, gm.

PART B.

Relationship Between Water Regain of Séphadex and Donnan Correction Factor

$$D.F. = \frac{\text{WR in serum}}{\text{WR in aqueous solution}} \text{ ------ (1)}$$

According to Donnan Theory,

$$Sunb = Si \times D.F. \text{ ------ (2)}, \quad Si = \frac{St \cdot Vt - Ss \cdot (Vt - Vi)}{Vi} = \frac{St \cdot Vt - Ss \cdot (Vt - Vi)}{WR \times a} \text{ ------(3)}$$

From (1), (2), and (3)

$$Sunb = \frac{St \cdot Vt - Ss \cdot (Vt - Vi)}{\text{WR in serum} \times a} \times \frac{\text{WR in serum}}{\text{WR in aqueous solution}}$$

$$= \frac{St \cdot Vt - Ss \cdot (Vt - Vi)}{\text{WR in aqueous solution} \times a}$$

where Sunb = unbound salicylate in sample, mg.%

D.F. = Donnan correction factor.

PART C.

Interaction Between Protein and Small Molecules (Scatchard, 1949; Scatchard, Scheinberg, and Armstrong, 1950; Karush, 1950; Davidson and Smith, 1961)

The relationship between free and protein-bound drugs will be expressed as follows: $f + p^+ \underset{\leftarrow}{\overset{K}{\rightarrow}} b$ where f = free drug, moles/l.

From Law of Mass Action,

$$K = \frac{(b)}{(p^+)(f)} \text{ ------(1)}$$

Now, $(tp) = (p^+) + (b)$

$$(p^+) = (tp) - (b) \text{ ------(2)}$$

b = protein bound drug, moles/l.

tp = total protein, moles/l.

p^+ = ionized protein, moles/l.

K = association constant

r = b/tp = average association, expressed by average number of moles of bound drug per mole of protein.

From (1) and (2),

$$\frac{(b)}{[(tp) - (b)](f)} = K$$

$$(b) = K(tp)(f) - K(b)(f)$$

$$(b) + K(b)(f) = K(tp)(f)$$

$$(b) = \frac{K(tp)(f)}{1 + K(f)}$$

$$\frac{(b)}{(tp)} = r = \frac{K(f)}{1 + K(f)}$$

$$r = \frac{K(f)}{1 + K(f)}$$

If there are n binding sites per protein molecule which have intrinsic association constants Ki and if there is no interaction among the bound ions, then the dependence of r on f is given by:

$$r = \frac{\sum_i Ki \cdot f}{1 + Ki \cdot f} \quad (i = 1, 2, \text{------ } n) \quad \text{---------- (3)}$$

Simple Theory:

The system has only one intrinsic association constant and Ki's are equal.

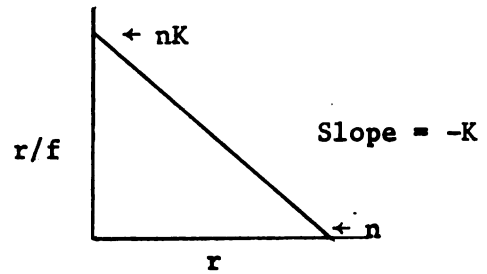
$K_1 = K_2 = \text{-------} = K_n = K$, then, Equation (3) will be:

$$r = n \cdot \frac{K \cdot f}{1 + K \cdot f} = \frac{n \cdot K \cdot f}{1 + K \cdot f}$$

$$r(1 + K \cdot f) = n \cdot K \cdot f$$

$$r = n \cdot K \cdot f - r \cdot K \cdot f$$

$$r/f = n \cdot K - r \cdot K \text{ ------ (4)}$$



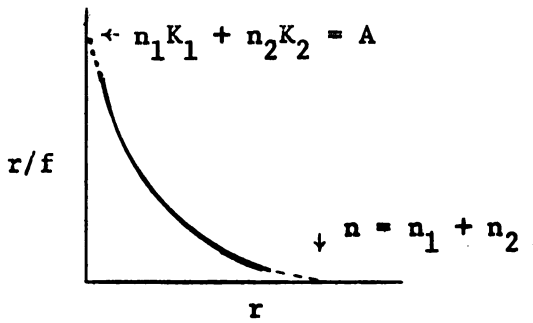
Plotting r/f vs. r, the straight line will be obtained.

Two Constant Equation:

Plotting r/f vs. r, the deviation from linearity, such as in Figure 2, in the experiment, indicates that the binding involves more than one binding constant. On the basis of the assumption employed by Scatchard, Scheinberg, Armstrong (1950) and Karush (1950), in which the binding can be described by only two different values of K at two types of binding sites, then Equation (3) will be expressed as follows:

$$r = \frac{n_1 \cdot K_1 \cdot f}{1 + K_1 \cdot f} + \frac{n_2 \cdot K_2 \cdot f}{1 + K_2 \cdot f}$$

$$r/f = \frac{n_1 \cdot K_1}{1 + K_1 \cdot f} + \frac{n_2 \cdot K_2}{1 + K_2 \cdot f} \text{ ------ (5)}$$



where $n = n_1 + n_2$ ---------- (6)

and $\lim_{f \to o} r/f = n_1K_1 + n_2K_2 = A$ ----- (7)

where A is the intercept on the r/f axis of a plot of r/f vs. r. The value of n and K were determined by employing Equations (6) and (7) together with two sets of values of r/f and f in Equation (5).

VITA

The author was born in Okinawa, Japan, on April 26, 1941. She graduated from the University of Nagasaki with her first B.S. in Pharmacy in March 1963. After three years' practice as a pharmacist, she transferred to the University of Hawaii to learn Medical Technology in September 1966 and completed one year of professional training at Tripler Army General Hospital in Honolulu, and received her second B.S. degree in Medical Technology from the University of Hawaii in June 1968. In August 1968, she was registered as a medical technologist with the American Society of Clinical Pathologists, and in September of the same year with the California State license. After working at St. Francis Hospital in Honolulu and St. Johns Hospital in Santa Monica, Los Angeles, she enrolled in a program of Graduate School in Clinical Laboratory Science in the Department of Pathology, Michigan State University, in September 1969, with the aid of the scholarship offered by the United States Civil Administration of the Ryukyus Islands.