others to devise various chromatographic procedures for sample preparation prior to spectrophotometric determination. The current method is an advancement over those procedures since the sulfonamide is determined directly on the separation medium. Presumably, the TLC method could be adapted for measurement of specific sulfonamide drug levels when sulfonamide mixtures are used. TLC conditions for separation of up to 15 different sulfonamides on one plate have been reported (19).

Finally, the lower limit of sensitivity of the TLC procedure is at least 20 times lower than is required for normal sulfonamide plasma concentration levels. This factor enabled the development of the very simple extraction procedure. In most laboratories, TLC has been utilized mainly as a qualitative tool. These new assay procedures for trimethoprim and sulfamethoxazole in plasma demonstrate that TLC can also provide a rapid, sensitive, accurate, and specific means for quantitative determinations.

REFERENCES

(1) G. H. Hitchings, *Postgrad. Med. J., Suppl.*, **45**, 7(1969).

(2) S. R. M. Bushby and G. H. Hitchings, *Brit. J. Pharmacol.*, **33**, 72(1968).

(3) S. R. M. Bushby, *Postgrad. Med. J., Suppl.*, **45**, 10(1969).

(4) G. H. Hitchings and J. J. Burchall, *Advan. Enzymol.*, **27**, 417(1965).

(5) S. R. M. Bushby and G. H. Hitchings, *Brit. J. Pharmacol. Chemother.*, **33**, 72(1968).

(6) J. C. Pechere, *Pathol. Biol.*, **18**, 343(1970).

(7) D. E. Schwartz, B. Koechlin, and R. E. Weinfeld, *Chemotherapy, Suppl.*, **14**, 22(1969).

(8) D. E. Schwartz, W. Vetter, and G. Englert, *Arzneim.-Forsch.*, **20**, 1867(1970).

(9) M. A. Brooks, J. A. F. de Silva, and L. M. D'Arconte, *Anal. Chem.*, **45**, 263(1973).

(10) F. Moncalvo, V. Bernareggi, G. Bugada, A. Cassola, and G. Levi, *Gazz. Med. Ital.*, **131**, 176(1972).

(11) C. W. Sigel and M. E. Grace, *J. Chromatogr.*, **80**, 11(1973).

(12) A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, **128**, 537(1939).

(13) H. B. Falk and R. G. Kelley, *Clin. Chem.*, **11**, 1045(1965).

(14) M. Kraml and A. Boudreau, *Clin. Biochem.*, **4**, 123(1971).

(15) J. Rieder, *Chemotherapy*, **17**, 1(1972).

(16) T. Bican-Fister and V. Kajganovic, *J. Chromatogr.*, **16**, 503(1964).

(17) M. Sarsunova, V. Schwarz, E. Feketeva, and J. Protiva, *Pharmazie*, **21**, 219(1966).

(18) G. Wagner and J. Wandel, *ibid.*, **21**, 105(1966).

(19) R. A. DeZeeun, *J. Chromatogr.*, **48**, 27(1970).

(20) U. R. Cieri, *ibid.*, **49**, 493(1970).

(21) C. A. Brunner, *J. Ass. Offic. Anal. Chem.*, **56**, 689(1973).

(22) S. Crisp, *Analyst*, **96**, 671(1971).

(23) H. M. Miller, *J. Ass. Offic. Anal. Chem.*, **53**, 1100(1970).

(24) B. R. Rader, *J. Pharm. Sci.*, **62**, 1148(1973).

(25) C. W. Sigel, M. E. Grace, and C. A. Nichol, *J. Infect. Dis., Suppl.*, **128**, 580(1973).

ACKNOWLEDGMENTS AND ADDRESSES

Received December 6, 1973, from *The Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, NC 27709*

Accepted for publication January 31, 1974.

The authors thank Mr. Wendell Wilkerson and Dr. Robert Szot for the sulfonamide determinations using the Bratton-Marshall procedure and Mr. James Murphy for helpful discussions during this work.

[x] To whom inquiries should be directed.

Interaction of 8-Hydroxyquinoline Sulfate with Components of Tuberculin Purified Protein Derivative Solutions III: Binding of 8-Hydroxyquinoline by Tuberculin Purified Protein Derivative

H. R. HELD[x] and S. LANDI

Abstract □ The interaction between the preservative 8-hydroxyquinoline sulfate and macromolecules present in tuberculin purified protein derivative solutions such as tuberculoprotein, nucleic acid, and polysaccharide was studied. In buffered solution (pH 7.38), 8-hydroxyquinoline sulfate is dissociated to 8-hydroxyquinoline and sulfuric acid, and it is the base 8-hydroxyquinoline that forms a reversible association with these macromolecules. The degree of binding of 8-hydroxyquinoline to tuberculoprotein, nucleic acid, and polysaccharide was shown to be a function of the concentration of these macromolecules. In commercial solutions of tuberculin purified protein derivative, the low concentrations of purified protein derivative used have little effect on the concentration of 8-hydroxyquinoline sulfate. Thus, for all practical purposes the antimicrobial activity of 8-hydroxyquinoline sulfate was not affected.

Keyphrases □ 8-Hydroxyquinoline sulfate—interaction with components of tuberculin purified protein derivative □ Tuberculin purified protein derivative—interaction of 8-hydroxyquinoline sulfate with solution components, binding of 8-hydroxyquinoline □ Binding—8-hydroxyquinoline to tuberculoprotein, nucleic acid, and polysaccharide (components of tuberculin purified protein derivative) □ Antimicrobial agents—interaction of 8-hydroxyquinoline sulfate with components of tuberculin purified protein derivative

8-Hydroxyquinoline sulfate[1] is added to tuberculin purified protein derivative solutions as an antimicrobial agent (2). In previous reports, the authors described how 8-hydroxyquinoline sulfate disappeared from these solutions when dispensed in glass vials stoppered with rubber closures. They showed that, in

[1] Chinosol (1), Eastman Organic Chemicals, catalog number 1776.

a buffered solution (pH 7.38), 8-hydroxyquinoline sulfate is dissociated into 8-hydroxyquinoline and sulfuric acid and that most of the loss of 8-hydroxyquinoline sulfate from the solution was caused by sorption of 8-hydroxyquinoline by the rubber closures (3). Furthermore, it was shown that 8-hydroxyquinoline interacted with polysorbate 80, used as an antiadsorption agent in tuberculin purified protein derivative solutions (4), and that 8-hydroxyquinoline reacted with traces of metals present in tuberculin purified protein derivative solutions to form dark precipitates. These metals also affected the antimicrobial properties of 8-hydroxyquinoline (5, 6).

In a previous report (4), it was mentioned that the interaction of 8-hydroxyquinoline sulfate with some macromolecules other than polysorbate 80 such as tuberculoprotein, nucleic acid, and polysaccharide, which are present in tuberculin purified protein derivative solutions, would be reported subsequently. Since it is known that preservatives and other organic substances of small molecular weight interact with proteins (7–19), polysaccharides (20–23), and nucleic acids (19, 24–26) and that the bound preservative has no antimicrobial activity (15, 27), it was important to measure the degree of interaction that may take place between 8-hydroxyquinoline sulfate and these macromolecules.

The solubility method (28), the equilibrium dialysis method (28), and the spectrophotometric method were used to measure the degree of interaction between the components of tuberculin purified protein derivative and 8-hydroxyquinoline sulfate.

EXPERIMENTAL

Reagents—8-Hydroxyquinoline sulfate[1], 8-hydroxyquinoline[2], and polysorbate 80 (polyoxyethylene 20 sorbitan monooleate)[3] were used.

Buffer Solution (pH 7.38)—Isotonic phosphate-buffered solution (2), pH 7.38 (0.145% KH_2PO_4, 0.76% Na_2HPO_4, and 0.48% NaCl) was used. This buffer is also used to prepare the diluent (4, 5) for the preparation of tuberculin purified protein derivative solutions[4].

Tuberculin Purified Protein Derivative—Tuberculin purified protein derivative consists of tuberculoprotein, with its main impurities being mycobacterial nucleic acid and polysaccharide. It was of interest to determine the interaction of 8-hydroxyquinoline with each of these substances separately. The tuberculoprotein as well as the nucleic acid and the polysaccharide used was prepared from the culture filtrate of *Mycobacterium tuberculosis* grown on Long's synthetic medium (29). The first step of the preparation consisted of precipitation of tuberculin purified protein derivative with trichloroacetic acid (4% final concentration). Besides tuberculoprotein, the dry tuberculin purified protein derivative also contained 0.4% polysaccharide, 5% nucleic acid, and traces of metals (5). The tuberculin purified protein derivative was redissolved in a phosphate-buffered saline to a volume of approximately 4% of the original volume of the crude filtrate. This provided a stock solution of tuberculin purified protein derivative designated "tuberculin purified protein derivative stock solution" (29).

Tuberculoprotein—To decrease the nucleic acid content of the purified protein derivative, the tuberculin purified protein derivative stock solution was precipitated with an equal volume of saturated ammonium sulfate solution (pH 7.1). The precipitate was washed free of ammonium sulfate by repeatedly treating it with 1% trichloroacetic acid solution. Finally, to remove most of the metals, the precipitate was redissolved in buffer solution (pH 7.38) to form a 2% solution of purified protein derivative, and an excess of 8-hydroxyquinoline sulfate was added. This darkened the color of the purified protein derivative solution immediately, and a black precipitate formed within a few days and was removed by filtration[5] (pore size 0.45 μm). To the clear filtrate, trichloroacetic acid (1% final concentration) was added and the precipitate was dried with acetone and ether. The tuberculoprotein powder so obtained contained less than 0.1% nucleic acid and, when dissolved in buffer (pH 7.38), it did not form precipitates with 8-hydroxyquinoline sulfate.

Polysaccharide—The supernate from the trichloroacetic acid precipitation of the culture filtrate was neutralized with sodium hydroxide, concentrated under vacuum, and dialyzed against running tap water. After dialysis, the contents of the dialysis bags were concentrated under vacuum until they became viscous. To this viscous concentrate an equal volume of acetone was added, and the precipitate obtained was dried in a vacuum desiccator until it became brittle enough to be ground in a mortar. The yellowish powder so obtained was a crude tuberculopolysaccharide of relatively high molecular weight and was designated Crude Polysaccharide A. This polysaccharide was purified by passing it through a gel filtration column[6] using water as an eluant (30). The fractions collected from the column were scanned by UV spectrophotometry and were tested by the anthrone reaction (31). The fractions showing the presence of polysaccharide were pooled and precipitated with ethanol. After centrifuging and vacuum drying, the polysaccharide, a snow-white powder, was designated Purified Polysaccharide A.

The supernate of the acetone precipitation was evaporated under vacuum to dryness. The evaporation residue consisted of crude polysaccharides of medium and low molecular weights and was designated as Crude Polysaccharide B.

Nucleic Acid—The supernate from the ammonium sulfate precipitation of tuberculin purified protein derivative was dialyzed against running tap water. Then the contents of the dialysis bags were concentrated under vacuum and precipitated with acidified ethanol, washed with ethanol and ether, and vacuum dried. The white powder so obtained consisted of nucleic acid (29). A sufficient amount of this nucleic acid was further purified by paper electrophoresis (29).

Molecular Weight and Light Absorption Spectrum of Tuberculin Purified Protein Derivative—The molecular weight ranges of the tuberculoprotein, of the polysaccharides, and of the nucleic acid used were estimated from the elution patterns obtained when these substances were subjected to gel filtration on a column[6] (Fig. 1). The elution patterns of Blue Dextran, human serum albumin, egg albumin, ribonuclease, and phenol with approximate molecular weights of 2,000,000, 67,000, 45,000, 14,700, and 96, respectively, are shown for comparison (Fig. 1). The absorbance, measured at various wavelengths and concentrations, is recorded only to show the relative locations of the peaks. Therefore, no values are given for the absorbance (Fig. 1).

The UV light absorption spectra of the preparations used are shown in Fig. 2.

Preparation of Solutions—A 5% solution of the test substance in buffer (pH 7.38) was prepared first. Solutions of lower concentration were obtained by diluting the 5% solution with buffer (pH 7.38) to the required concentration. When the 5% solution of tuberculoprotein was prepared, the required amount of 1 *N* NaOH was added to maintain the pH of the buffer at 7.38. In the case of nucleic acid, the following procedure was used. Nucleic acid and an equivalent amount of sodium bicarbonate were dissolved in as little buffer as possible while stirring until the development of carbon dioxide stopped and the pH of the solution was pH 7.38. Then more buffer was added to obtain concentrations of nucleic acid of 5% or lower.

Determination of 8-Hydroxyquinoline Sulfate—The 8-hydroxyquinoline sulfate concentration of a solution was determined by measuring its absorbance[7] at 239 nm after dilution with buffer (pH 7.38) and comparing it with the absorbance at the same wavelength of a standard solution.

Since tuberculoprotein, polysaccharide, and nucleic acid also absorb UV light (Fig. 2), their own absorption had to be taken

[2] Fisher Scientific Co., catalog number 0-261.
[3] Tween 80, Atlas Chemical Industries, Brantford, Canada.
[4] Mantoux.
[5] Millipore.
[6] Sephadex G200, Pharmacia Fine Chemicals, Uppsala, Sweden.
[7] Beckman model DU spectrophotometer.

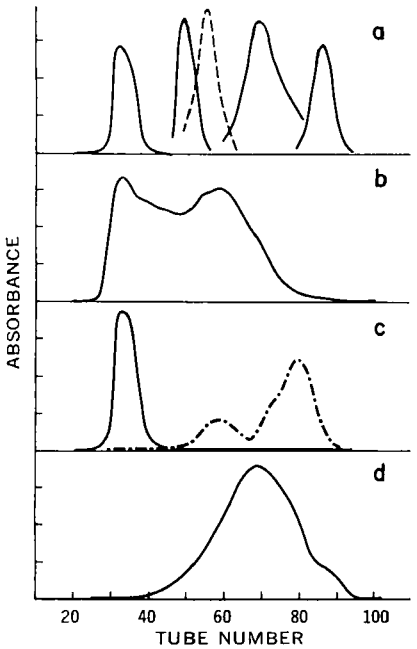

Figure 1—*Elution pattern of various substances after passing through one column. Key: a (from left to right), Blue Dextran, human serum albumin, egg albumin, ribonuclease, and phenol of molecular weights 2,000,000, 67,000, 45,000, 14,700, and 96, respectively; b, tuberculoprotein; c, Purified Polysaccharide A of high molecular weight (the solid line) and Crude Polysaccharide B (the broken line representing a mixture of crude polysaccharides of medium and lower molecular weight); and d, mycobacterial nucleic acid.*

into consideration when 8-hydroxyquinoline was measured in their presence. The absorption at 239 nm due to the known amount of tuberculoprotein or polysaccharide present was subtracted from the absorption of the mixture to obtain the absorption due to 8-hydroxyquinoline sulfate.

In the case of nucleic acid, this correction was considered too large to obtain precise results. Therefore, the following procedure was used to remove most of the nucleic acid from the samples. To 1 ml of the solution containing nucleic acid and 8-hydroxyquinoline sulfate was added 3 ml of acidified ethanol. The mixture was centrifuged[8] in closed Pyrex glass tubes at 5000 rpm for 10 min. A 0.1-ml aliquot of the clear supernate was added to 9.9 ml buffer solution (pH 7.38), and the UV absorption at 239 nm was measured. An identical solution containing the same amount of nucleic acid but no 8-hydroxyquinoline sulfate was submitted to the same procedure; to obtain the absorption due to 8-hydroxyquinoline sulfate, the absorption of this solution was subtracted from the absorption of the solution containing nucleic acid and 8-hydroxyquinoline sulfate.

Solubility Method—The solubility of 8-hydroxyquinoline sulfate in buffered solution (pH 7.38), containing various amounts of the tested macromolecular substance, was evaluated by placing into glass ampuls 1.5 ml of this solution and an excess of 8-hydroxyquinoline sulfate (40 mg of a mixture consisting of equivalent amounts of 8-hydroxyquinoline sulfate and sodium bicarbonate). The ampuls were flame sealed and agitated for 48 hr at 23°. The contents of the ampuls were then clarified either by centrifugation in glass-stoppered tubes or by filtration[9] (pore size 0.45 μm), and the 8-hydroxyquinoline sulfate in the clear solution was determined spectrophotometrically. To reduce evaporation losses, pressure rather than vacuum was applied to the filter.

Dialysis Method—The dialysis membranes consisted of small, thin nylon bags[10] which had been preequilibrated against 8-hydroxyquinoline sulfate by keeping them for 10 days in 1 liter of buffered solution (pH 7.38) containing 0.08% 8-hydroxyquinoline sulfate. Into each bag was placed 2 ml of a buffered solution (pH 7.38) containing 0.08% 8-hydroxyquinoline sulfate and various amounts of the tested macromolecular substance. Each nylon bag was then placed in a glass-stoppered weighing bottle containing 2 ml of a 0.08% solution of 8-hydroxyquinoline sulfate in buffered solution (pH 7.38). Each bottle was then stoppered tightly with the end of the nylon bag protruding to the outside of the bottle, thus providing also a tight closure for the nylon bag. To minimize evaporation losses, the weighing bottles were then placed in a larger closed glass container which also contained some buffer (0.08% 8-hydroxyquinoline sulfate), and the whole system was agitated for 4 days at 23°. After this time, an equilibrium between the inner and outer solutions of the dialysis membranes was reached, and samples were taken from the inside and outside of the nylon bags to determine the concentration of 8-hydroxyquinoline.

Spectrophotometric Method—If a solution contains two substances that do not interact, the light absorption of the solution at each wavelength will be the sum of the light absorption of each substance. If an interaction occurs, then the light absorption of the mixture will not be the same at all wavelengths and a shift in the spectrum will occur. Obviously, the shift will increase with an increasing percentage of the bound substance present. Therefore, these spectrophotometric observations had to be done at fairly high concentrations of 8-hydroxyquinoline sulfate (0.05%) and of the interacting substances, namely, 1 and 5% polysorbate 80, 0.3% tuberculoprotein, 1% polysaccharide, and 0.2% nucleic acid. At these concentrations, some absorbance measurements[7] had to be done in quartz cells, allowing light paths of 1, 0.1, and 0.01 cm.

Different light paths (0.1–10 cm) were also employed when measuring the spectrum of 8-hydroxyquinoline sulfate at various concentrations. The values for the 2- and 10-cm light paths were obtained by calculation from the absorbance of the 1-cm light path.

On the other hand, the quantitative determination of 8-hydroxyquinoline sulfate (1-cm cells) was not affected by the shift in the spectrum due to the presence of tuberculin purified protein derivative, since these measurements were done after a high dilution of the sample.

RESULTS

Interaction between Components of Tuberculin Purified Protein Derivative and 8-Hydroxyquinoline at pH 7.38—When 8-hydroxyquinoline sulfate is dissolved in a buffered solution (pH 7.38), it is dissociated into 8-hydroxyquinoline and sulfuric acid; it is the base 8-hydroxyquinoline that interacts with polysorbate 80 (4), with metals (5), or with other macromolecules such as tu-

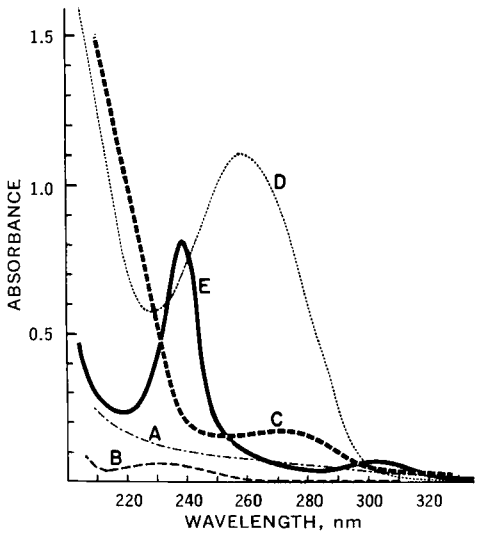

Figure 2—*UV absorption spectra in buffered solution (pH 7.38). Key: A, Purified Polysaccharide A (1000 μg/ml); B, polysorbate 80 (100 μg/ml); C, tuberculoprotein (100 μg/ml); D, nucleic acid (50 μg/ml); and E, 8-hydroxyquinoline sulfate (5 μg/ml).*

[8] Servall SS-3 centrifuge.
[9] Millipore HAWP1300.
[10] Supplied by Holland Rantos Co., New York, N.Y.

Table I—Degree of Binding of 8-Hydroxyquinoline by Tuberculoprotein Calculated[a] from the Solubility Increase of 8-Hydroxyquinoline Sulfate due to the Presence of Tuberculoprotein

Tuberculoprotein		Binding of 8-Hydroxyquinoline, Expressed as	
% w/v	Tu[b]/ml	Ratio of Total/Free	% of Total
5		1.94	48.45
3.75		1.709	41.49
2.50		1.470	31.97
1.25		1.235	19.03
1.0		1.188	15.82
0.625		1.1175	10.51
0.2	100,000	1.0376	3.62
0.1	50,000	1.0188	1.85
0.01	5,000	1.00188	0.188
0.001	500	1.00019	0.019
0.0001	50	1.00002	0.002
0	0	1.00000	0.000

[a] Calculated from the slope (0.188) of curve a (Fig. 3), using the following equation: % bound = 100% − (100/R)%. R = ratio of total/free. [b] The biological potency of tuberculin is expressed in tuberculin units (Tu). One Tu is biologically equivalent to 0.00002 mg of PPD-S (36), which is the international standard for tuberculin purified protein derivative.

berculoprotein, polysaccharide, and nucleic acid. Nevertheless, since in the literature dealing with tuberculin the concentration of this preservative is given as percentage of 8-hydroxyquinoline sulfate, the authors have expressed all measurements in 8-hydroxyquinoline sulfate equivalents.

Interaction Evidenced by Solubility and Dialysis Methods—*Tuberculoprotein*—Figure 3 (curve a) shows the degree of interaction of tuberculoprotein with 8-hydroxyquinoline sulfate, as determined by the solubility method. The degree of interaction, expressed as the ratio R = total/free preservative, was small when compared to the interaction of 8-hydroxyquinoline sulfate with nonionic surfactants, such as polysorbate 80 (Fig. 3, curve e) (4).

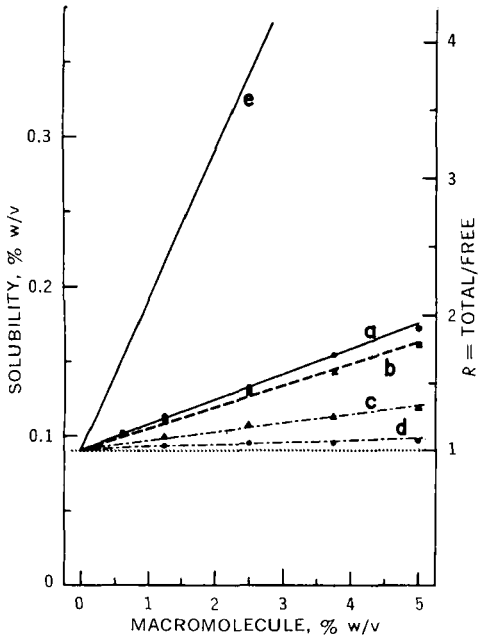

Figure 3—*Solubility of 8-hydroxyquinoline sulfate in a buffered solution (pH 7.38, 23°) containing various amounts of macromolecular substances present in tuberculin purified protein derivative. The increase in solubility represents the bound 8-hydroxyquinoline. The binding, calculated as the ratio R = total/free, is shown at the right scale. Key: a, tuberculoprotein; b, nucleic acid; c, Crude Polysaccharide A; d, Purified Polysaccharide A; and e, polysorbate 80. The slopes for the interaction curves (solubility method) were: a, 0.188; b, 0.160; c, 0.068; d, 0.018; and e, 1.06.*

Table II—Degree of Binding of 8-Hydroxyquinoline by Tuberculoprotein Calculated[a] from the Ratio C_i/C_o (Dialysis Method at pH 7.38 using a Nylon Dialysis Bag)

Tuberculoprotein, % w/v	Concentration of 8-Hydroxyquinoline Inside (C_i) and Outside (C_o) of Nylon Bag			Binding of 8-Hydroxyquinoline, Expressed as	
	C_i, %	C_o, %	C_i/C_o	Ratio of Total/Free	% of Total
5	0.105	0.057	1.84	1.84	45.6
3.75	0.102	0.062	1.65	1.65	39.4
2.5	0.093	0.065	1.43	1.43	30.0
1.25	0.086	0.071	1.21	1.21	17.4
0.625	0.082	0.074	1.11	1.11	9.9
0	0.08	0.08	1.0	1.0	0

[a] The ratio C_i/C_o is numerically equal to the ratio R = total/free preservative. For calculation of the degree of binding, the equation given as a footnote in Table I was used. The slope of the interaction curve (dialysis method) was 0.171.

Based on the slope of the interaction curve (Fig. 3, curve a), the amount of 8-hydroxyquinoline sulfate bound to tuberculoprotein could be calculated. Table I shows that in a concentrated stock solution (1% tuberculoprotein), approximately 15.8% of the total 8-hydroxyquinoline sulfate present was bound to the tuberculoprotein, while in a dilute solution [0.0001% tuberculoprotein, 50 tuberculin units (Tu)/ml)], a negligible amount of approximately 0.002% of the total 8-hydroxyquinoline sulfate present was bound to tuberculoprotein.

When the dialysis method was used for the determination of the interaction of 8-hydroxyquinoline sulfate with tuberculoprotein (Table II), the findings obtained by the solubility method were confirmed. In fact, Tables I and II show that the degree of binding of 8-hydroxyquinoline sulfate to tuberculoprotein as determined by these two methods was of the same order.

The interaction between tuberculoprotein and 8-hydroxyquinoline sulfate was reversible. This was evidenced by the fact that the nylon bags, from the dialysis experiment containing tuberculoprotein and 8-hydroxyquinoline sulfate, lost all of their 8-hydroxyquinoline sulfate when dialyzed against a large volume of buffer at pH 7.38.

Nucleic Acid—If mycobacterial nucleic acid and 8-hydroxyquinoline sulfate were simultaneously present in buffered solution (pH 7.38), the nucleic acid interacted reversibly with 8-hydroxyquinoline. Figure 3 shows that the degrees of interaction between 8-hydroxyquinoline sulfate and nucleic acid (curve b) and tuberculoprotein (curve a) as determined by the solubility method are of the same order.

Polysaccharide—Figure 3 (curves c and d) represents the interaction between 8-hydroxyquinoline sulfate and Crude Polysaccharide A and Purified Polysaccharide A, as indicated by the solubility method. The degree of interaction of 8-hydroxyquinoline sulfate with Crude Polysaccharide A and B was practically the same (curve c) but much less than the interaction between 8-hydroxyquinoline sulfate and tuberculoprotein (curve a) or nucleic acid (curve b). However, the degree of interaction with Purified Polysaccharide A was extremely small (curve d). Presumably, part of the interaction shown by the crude polysaccharides was due to some impurities.

Interaction Evidenced by Spectrophotometric Methods—By using the solubility and dialysis methods, it was found that the degree of interaction between 8-hydroxyquinoline and substances of relatively high molecular weight, which are present in tuberculin purified protein derivative, is small. Therefore, additional evidence of this interaction was sought by measuring the spectrum of 8-hydroxyquinoline in buffered solution alone and in buffered solution containing a fairly high concentration of these substances. The evidence that interaction had occurred was based on the change in the spectrum of 8-hydroxyquinoline in the presence of these substances. Polysorbate 80, which is known to interact strongly with 8-hydroxyquinoline (4), was included for comparison. To see the effect of the interaction, Figs. 4–7 each shows three curves obtained by measuring the absorption of UV light, namely of 8-hydroxyquinoline, of the tested macromolecular substance, and of the mixture of 8-hydroxyquinoline with the macromolecular sub-

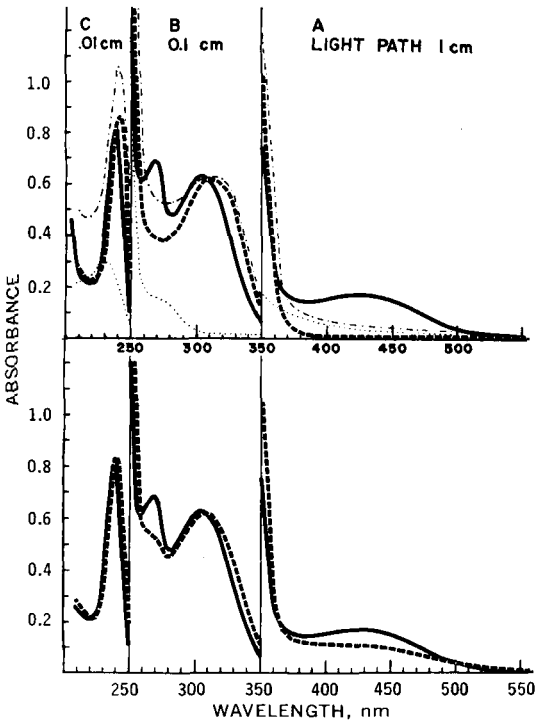

Figure 4—*Effect of polysorbate 80 on the absorption spectrum of 8-hydroxyquinoline sulfate in buffered solution (pH 7.38). Key: . . ., 5% polysorbate 80; –·–·, 5% polysorbate 80 and 0.05% 8-hydroxyquinoline sulfate; ——, 0.05% 8-hydroxyquinoline sulfate; and - - -, 0.05% 8-hydroxyquinoline sulfate in the presence of, and corrected for, absorption due to 5% polysorbate 80 (upper section) and 1% polysorbate 80 (lower section).*

stance. In addition, to see more easily the effect of the macromolecule on the spectrum of 8-hydroxyquinoline, Figs. 4–7 show a fourth curve which was obtained by subtracting the absorption of the macromolecule from that of the mixture of 8-hydroxyquinoline with the macromolecule. This calculated curve represents the light absorbed by 8-hydroxyquinoline when in the presence of the macromolecule.

Polysorbate 80—Figure 4 shows the spectrum of 8-hydroxyquinoline (0.05%) alone and in presence of 5% polysorbate 80 (Fig. 4, upper section) and 1% polysorbate 80 (Fig. 4, lower section). Polysorbate 80 caused the disappearance of the absorption peak at 270 nm, and there was a spectral shift over the whole spectrum from 220 to 500 nm which was especially pronounced between 370 and 500 nm.

In the case of polysorbate, the spectral change was also visually recognizable by the disappearance of the yellow color of the 8-hydroxyquinoline solution when polysorbate 80 (5% final concentration) was added to it.

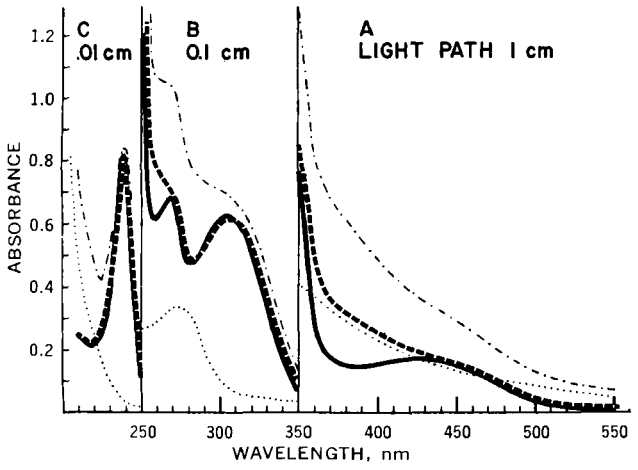

Figure 5—*Effect of tuberculoprotein on the absorption spectrum of 8-hydroxyquinoline sulfate in buffered solution (pH 7.38). Key: . . ., 0.3% tuberculoprotein; –·–·, 0.3% tuberculoprotein and 0.05% 8-hydroxyquinoline sulfate; ——, 0.05% 8-hydroxyquinoline sulfate; and - - -, 0.05% 8-hydroxyquinoline sulfate in the presence of 0.3% tuberculoprotein and corrected for absorption due to tuberculoprotein.*

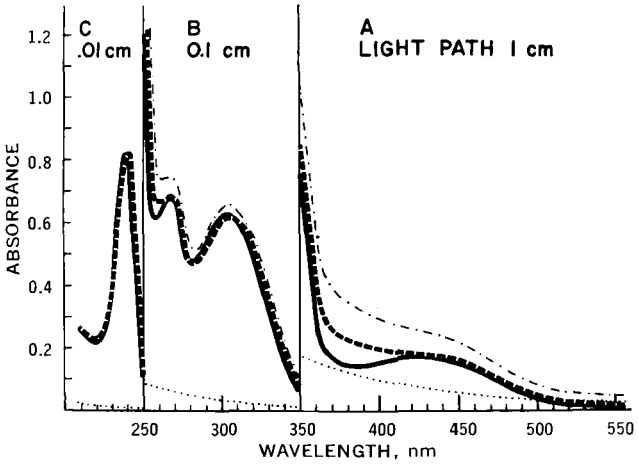

Figure 6—*Effect of polysaccharide on the absorption spectrum of 8-hydroxyquinoline sulfate in buffered solution (pH 7.38). Key: . . ., 1% polysaccharide; –·–·, 1% polysaccharide and 0.05% 8-hydroxyquinoline sulfate; ——, 0.05% 8-hydroxyquinoline sulfate; and - - -, 0.05% 8-hydroxyquinoline sulfate in the presence of 1% polysaccharide and corrected for absorption due to polysaccharide.*

Tuberculin Purified Protein Derivative—Tuberculoprotein as well as Purified Polysaccharide A or nucleic acid caused an increase in absorption from approximately 350 to 420 nm (Figs. 5–7). In addition, tuberculoprotein and Purified Polysaccharide A caused the disappearance of the light absorption peak at 270 nm (Figs. 5 and 6). No attempt was made to measure the effect of nucleic acid on the spectral peak of 8-hydroxyquinoline at 270 nm, since the absorption of nucleic acid was too strong in this region (Fig. 7).

DISCUSSION

The fact that tuberculoprotein, mycobacterial nucleic acid, and polysaccharide present in tuberculin purified protein derivative interacted with the preservative 8-hydroxyquinoline sulfate was not surprising, since it was well known that other representatives of these groups of macromolecules interact with numerous substances. For instance, proteins such as plasma proteins (7–13), lactoglobulins (14), gelatin (15), bacterial proteins (16), and others (17–19) interact with numerous drugs, preservatives, detergents, dyes, and other substances. Polysaccharides such as starches (20), dextrins (21, 22), sucrose esters (23), and others (19) interact to a low degree with drugs, dyes, and other substances. Nucleic acids (19, 24–26) interact with peptides, dyes, antibiotics, and other substances. However, Table I shows that the loss of preservative due to its binding to tuberculoprotein was negligible because of the relatively low concentration in which tuberculin purified protein derivative is used[11]. Furthermore, the degree of interaction with some impurities contained in tuberculin purified protein derivative solutions, such as nucleic acid and polysaccharide, was still smaller and, therefore, the loss of preservative due to their presence was also negligible.

The small degree of interaction of 8-hydroxyquinoline with tuberculin purified protein derivative as measured by the solubility method and the dialysis method was confirmed by observing changes in the light absorption spectrum of 8-hydroxyquinoline

[11] Mantoux testing (0.0001%) and for Heaf testing (0.2%).

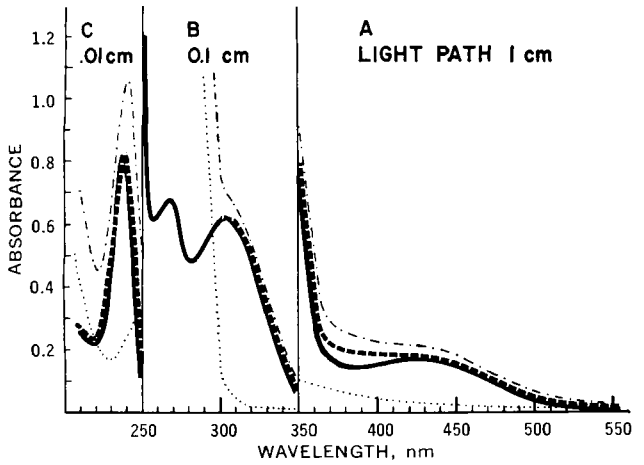

Figure 7—*Effect of nucleic acid on the absorption spectrum of 8-hydroxyquinoline sulfate in a buffered solution (pH 7.38). Key: . . ., 0.2% nucleic acid; —·—·, 0.2% nucleic acid and 0.05% 8-hydroxyquinoline sulfate; ——, 0.05% 8-hydroxyquinoline sulfate; and - - -, 0.05% 8-hydroxyquinoline sulfate in the presence of 0.2% nucleic acid and corrected for absorption due to nucleic acid.*

when tuberculoprotein, polysaccharide, or nucleic acid was present. No attempt will be made here to draw any conclusions from the spectral changes as to which groups of 8-hydroxyquinoline and of the macromolecules do interact except to say that the absorption peak of 8-hydroxyquinoline at 270 nm may be caused by association between several molecules of 8-hydroxyquinoline, since this peak disappeared at higher dilution but became more pronounced with increasing concentration of 8-hydroxyquinoline (Fig. 8). Thus, if the presence of polysorbate 80, tuberculoprotein, or polysaccharide reduced the size of the peak at 270 nm, it would indicate that the association of 8-hydroxyquinoline was retarded by interacting with these macromolecules, and this would explain the increase in solubility of 8-hydroxyquinoline in the presence of these macromolecules. Furthermore, the changes in the light absorption spectrum due to interaction are of interest, since they can be determined in a few minutes, while the solubility and dialysis studies require days to be completed. The spectral shifts shown in Figs. 4–7 were determined 5–10 min after the mixing of 8-hydroxyquinoline sulfate with the test substance, and there was no further change afterward. This would indicate that the interaction between 8-hydroxyquinoline and purified protein derivative may take place very fast after the mixing of these two substances.

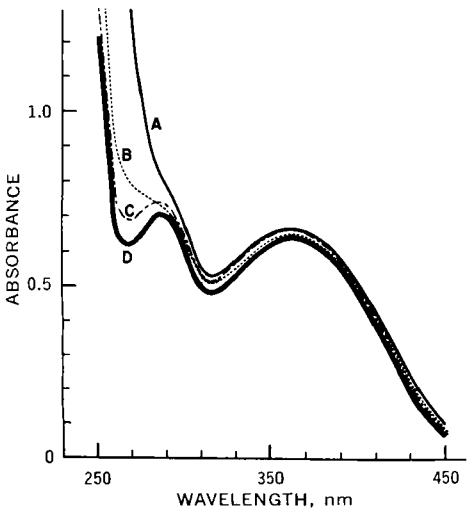

Figure 8—*Effect of various concentrations of 8-hydroxyquinoline sulfate on its absorption spectrum at pH 7.38. Concentration of 8-hydroxyquinoline sulfate was: A, 5 μg/ml; B, 25 μg/ml; C, 100 μg/ml; and D, 500 μg/ml. The light path was 10, 2, 0.5, and 0.1 cm for A, B, C, and D, respectively.*

One difficulty in obtaining valid measurements of the interaction between the components present in tuberculin purified protein derivative solutions and 8-hydroxyquinoline sulfate was due to the fact that tuberculoprotein is a very complex mixture of proteins which differ in their physical properties and which cover a wide range of molecular weights (32, 33); hence, the percentage distribution pattern of these proteins will vary from lot to lot of purified protein derivative. Therefore, the values determined for the interaction can only be expected to be strictly valid for the particular lot of tuberculoprotein, polysaccharide, or nucleic acid used. However, the degree of interaction of 8-hydroxyquinoline and, for instance, tuberculoprotein prepared from different lots of culture filtrate can be expected to be of the same order.

Some difficulties encountered when 8-hydroxyquinoline sulfate is used as a preservative (34) can be overcome if phenol, which is the preservative used in our tuberculin purified protein derivative solutions, is employed. In fact, work done in these laboratories (35) showed that the degree of interaction between phenol and the components present in tuberculin purified protein derivative solutions is much smaller for phenol than for 8-hydroxyquinoline sulfate. This is particularly true in the case of tuberculin solution for Heaf testing where the presence of glycerol binds 61.5% of 8-hydroxyquinoline sulfate as compared to 28.5% of phenol (35).

REFERENCES

(1) "The Merck Index," 8th ed., Merck & Co., Inc., Rahway, N.J., 1968, pp. 555, 1462.

(2) M. Magnusson, J. Guld, K. Magnus, and H. Waaler, *Bull. WHO*, **19**, 799(1958).

(3) S. Landi and H. R. Held, *ibid.*, **33**, 395(1965).

(4) S. Landi and H. R. Held, *J. Pharm. Sci.*, **55**, 18(1966).

(5) *Ibid.*, **57**, 605(1968).

(6) S. Landi, H. R. Held, and H. Pivnick, *Bull. WHO*, **39**, 809(1968).

(7) I. M. Klotz, F. M. Walker, and R. B. Pivan, *J. Amer. Chem. Soc.*, **68**, 1486(1946).

(8) I. M. Klotz and J. M. Urquhart, *J. Phys. Chem.*, **53**, 100(1949).

(9) N. K. Patel, P. C. Sheen, and K. E. Taylor, *J. Pharm. Sci.*, **57**, 1370(1968).

(10) H. J. Weder and M. H. Bickel, *ibid.*, **59**, 1505(1970).

(11) M. J. Cho, A. G. Mitchell, and M. Pernarowski, *ibid.*, **60**, 196(1971).

(12) M. C. Meyer and D. E. Guttman, *ibid.*, **57**, 895(1968).

(13) W. G. Unger, *J. Pharm. Pharmacol.*, **24**, 470(1972).

(14) A. Wishnia and T. W. Pinder, *Biochemistry*, **5**, 1534(1966).

(15) H. B. Kostenbauder, *Develop. Ind. Microbiol.*, **3**, 286(1962).

(16) J. E. Starr and J. Judis, *J. Pharm. Sci.*, **57**, 768(1968).

(17) F. Haurowitz, "Chemistry and Biology of Proteins," Academic, New York, N.Y., 1950, pp. 186–193.

(18) G. C. K. Roberts and O. Jardetzky, *Advan. Protein Chem.*, **24**, 499(1970).

(19) J. H. Perrin and P. A. Hart, *J. Pharm. Sci.*, **59**, 431(1970).

(20) Z. Mansour and E. P. Guth, *ibid.*, **57**, 404(1968).

(21) J. Cohen and J. L. Lach, *ibid.*, **52**, 132(1963).

(22) J. L. Lach and W. A. Pauli, *ibid.*, **55**, 32(1966).

(23) S. M. Blaug and D. S. Ebersman, *ibid.*, **53**, 35(1964).

(24) P. D. Lawley, *Biochim. Biophys. Acta*, **69**, 160(1956).

(25) U. Bachrach and S. Persky, *ibid.*, **179**, 484(1969).

(26) E. J. Gabbay, K. Sanford, and C. S. Baxter, *Biochemistry*, **11**, 3429(1972).

(27) F. D. Pisano and H. B. Kostenbauder, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 310(1959).

(28) N. K. Patel and H. B. Kostenbauder, *ibid.*, **47**, 289(1958).

(29) S. Landi and H. R. Held, *Appl. Microbiol.*, **13**, 132(1965).

(30) E. Weiss and J. L. Long, *ibid.*, **18**, 843(1969).

(31) L. H. Koehler, *Anal. Chem.*, **24**, 1576(1952).

(32) S. Landi, H. R. Held, and M. C. Tseng, *Progr. Immunobiol. Stand.*, **4**, 512(1970).

(33) S. Landi and H. R. Held, *Ann. Sclavo*, **13**, 862(1971).
(34) S. Landi, H. R. Held, and H. Pivnick, *Bull. WHO*, **39**, 809(1968).
(35) S. Landi and H. R. Held, Proceedings of the 38th Symposium on Preservatives, San Francisco, Calif., 1973, in press.
(36) American Thoracic Society, *Amer. Rev. Resp. Dis.*, **99**, 460(1969).

ACKNOWLEDGMENTS AND ADDRESSES

Received January 16, 1974, from the *Tuberculosis Department, Connaught Laboratories Limited, Toronto-Willowdale, Ontario, Canada.*

Accepted for publication March 21, 1974.

[x] To whom inquiries should be directed.

Influence of Sodium Salicylate on Metabolism of Lathyrogen-Treated 3T6 Fibroblasts in Culture

JOSEPH J. ALEO

Abstract □ The effect of sodium salicylate on the metabolism of 3T6 fibroblasts treated with the lathyrogen β-aminopropionitrile was studied. A 5 m*M* concentration of the lathyrogen and/or 0.5 m*M* sodium salicylate was used to study proliferation, collagen synthesis, and mucopolysaccharide synthesis as measured by $^{35}SO_4$ incorporation. Sodium salicylate was able to reverse all lathyrogen-mediated changes to control or near control values. The diversity of these biosynthetic activities suggests that the influence of sodium salicylate on lathyrogen-treated cells is a result of a general metabolic effect rather than a specific one; the exact mechanism of action remains unknown.

Keyphrases □ Sodium salicylate—effect on metabolism of fibroblasts treated with β-aminopropionitrile, proliferation, collagen synthesis, and mucopolysaccharide synthesis □ Lathyrogens, β-aminopropionitrile—used to treat fibroblasts, effect of sodium salicylate on fibroblast metabolism □ β-Aminopropionitrile—used to study effects of sodium salicylate on metabolism of lathyrogen-treated fibroblasts □ Fibroblasts (3T6), β-aminopropionitrile treated—effects of sodium salicylate on metabolism, proliferation, collagen synthesis, and mucopolysaccharide synthesis □ Metabolism—β-aminopropionitrile-treated fibroblasts, effects of sodium salicylate

The lathyrogen β-aminopropionitrile fumarate in a concentration of 5 m*M* has been shown to depress cell proliferation while enhancing the synthesis of collagen, noncollagenous protein, glycogen, and mucopolysaccharides (1–4). Lesser concentrations of the lathyrogen do not exhibit these changes. Both the higher and lower concentrations, however, do affect collagen maturation, which is thought to be due to an inactivation of lysyl oxidase resulting in a deficiency of lysine-derived aldehydes (5). The discrepancies in data among studies are no doubt due to differences in cell lines used, experimental conditions, and, most important, concentrations of the lathyrogen employed.

Among the antirheumatic drugs, salicylates have been shown to have a beneficial influence on changes in experimental lathyrism by decreasing the elevated proportion of extractable collagen in tissues and the disappearance of the typical histological changes seen in the disease (6–8). Salicylates have also been shown to modify collagen and mucopolysaccharide synthesis to a greater extent than noncollagenous protein (9). Other researchers reported that they stimulate the turnover of collagen in general and its maturation in particular (10) while inhibiting the growth of human embryonic cells (11).

In the present study, an attempt was made to ascertain whether the influence of sodium salicylate was limited primarily to correcting the collagen maturation defect brought about by low concentrations of the lathyrogen (1 or 2 m*M*) or whether sodium salicylate affects other metabolic alterations such as those resulting from a 5 m*M* lathyrogen concentration.

EXPERIMENTAL

Materials and Methods—The 3T6 fibroblasts (12) were grown in the presence of 10% CO_2 in 60 × 15-mm plastic petri dishes in the Dulbecco–Vogt modification of Eagle's medium containing 10% calf serum. Five-day-old cultures were trypsinized and resuspended into a common culture having approximately 100,000 cells/ml. The common culture was then divided into four equal portions: β-aminopropionitrile was added to one at a concentration of 5 m*M*; sodium salicylate was added to the second at a concentration of 0.5 m*M*; β-aminopropionitrile (5 m*M*) and sodium salicylate (0.5 m*M*) were added to the third; and the fourth served as a control. The dosages of the lathyrogen and sodium salicylate used were established by assessing the toxicity of various concentrations. It was found that 5 m*M* β-aminopropionitrile and 0.5 m*M* sodium salicylate produced the maximum response without affecting cell viability.

Replicate cultures were prepared by dispensing 5 ml of cell suspension into each culture plate and incubating them at 37°. The medium was changed three times weekly for the duration of the experiment. Cells were harvested at 2, 4, 7, 9, 11, and 14 days. All media were pooled and saved for analysis, so the data represent total synthesis of material to the day of harvest. For analysis the medium was decanted and centrifuged at 200×*g* for 10 min to remove any dislodged cells or debris, and the cell layer was detached by using a razor blade or by incubation with 0.02% ethylenediaminetetraacetic acid in a phosphate buffer. Quantitation was based on both the cell count and DNA content (13), taking the average of three identically treated plates separately analyzed.

Chemical Analysis—*Collagen Synthesis*—Hydroxyproline in the medium and cell layer was determined by the method of Prockop and Udenfriend (14). Recoveries by this method averaged 86.8 ± 4.2%. All readings[1] were made at 560 nm. The medium was dialyzed exhaustively and then hydrolyzed in 6 *N* HCl for 16 hr at 104° before analysis. Unused medium served as a blank.

The solubility of the cell layer was determined as follows. Salt-

[1] Hitachi-Perkin-Elmer spectrophotometer.