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HEPARIN AND THE RELEASE OF HISTAMINE

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DISSERTATION COMMITTEE

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HEPARIN AND THE RELEASE OF HISTAMINE

CHAPTER I

INTRODUCTION

Although histamine and heparin have been known for many years to be normal constituents of the human organism, their physiological function is not fully established. In spite of many efforts to show them as essential to the normal economy of the body, they still remain in the category of drugs. This is especially true of histamine.

The primary effects of administered histamine were elucidated by Dale (1910). At this early date, the similarity of the reactions from injected histamine to the symptoms of anaphylaxis was noted. But not until the work of Lewis (1927) was it suggested that a "histamine-like" substance could be concerned in body reactions. Also, Best and Dale (1927) demonstrated that histamine was a normal body component. Dale (1929) stated a theory of anaphylaxis that remains little changed even today; viz, by the union of antigen and antibody, during anaphylaxis, histamine is released and is responsible for much of the symptomology of anaphylactic shock.

Important work attempting to substantiate a relationship between histamine and anaphylaxis appeared in the 1930's.

Dragstedt and Feulnegg (1932) presented the first good evidence that histamine activity appeared in considerable amounts in the blood of dogs during anaphylactic shock. Code (1939) measured the blood histamine of anaphylactically shocked dogs and guinea pigs and found it to be increased 80 times over normal in the dog and 18 times over normal in the guinea pig. Riley and West (1952) added a significant chapter to the histamine story when they showed a striking correlation between the amount of releasable histamine in many tissues and the number of mast cells present.

Substances capable of freeing histamine from its binding sites in animal tissue had been known for many years. However, they were materials which, given in doses sufficient to release any substantial quantity of histamine, were likely to do gross tissue damage. Proteolytic enzymes (Rocha e Silva, 1940) or snake venoms (Feldberg and Kellaway, 1937) are good examples of such agents. With the demonstration that substantial amounts of histamine can be released by the action of many organic bases, it became possible to make a new approach to the study of histamine binding and mechanisms of release. The monumental work concerning the basic organic histamine liberators was done by MacIntosh and Paton (1949). However, Alam and co-workers, working with curare (1939), and Schild (1947), working with strychnine, had shown these

substances to be potent liberators of histamine in the perfused dog gastrocnemius muscle. The advantage of such drugs in histamine studies is that very little damage seems to occur to the tissue from which histamine is released.

Heparin was discovered in Howell's laboratory (1916) because of its anticoagulant ability. Its metachromatic staining properties were described by Jorpes (1937). With the demonstration of its presence in mast cells by Holmgren and Wilander (1937), the implication was strong that heparin was the indispensable, naturally occurring anticoagulant. This question has not been finally settled; but, since normal blood seems to contain such a minimal amount, it would be hard to view heparin as playing so vital a role in maintaining blood fluidity.

With Hahn's chance observation (1943) of the effect of injected heparin on the turbidity of lipemic dog plasma, a new aspect of investigation was opened regarding the possible normal physiology of heparin. As Hahn first showed, the turbidity of fatty plasma is dispelled by parenteral administration of heparin. Weld (1944) confirmed Hahn's observations and extended them to include the cat, the rabbit, and man. Indeed, all animal species thus far tested show the plasma-clearing effect of heparin. Both Hahn and Weld demonstrated that this clearing of turbidity was produced only when heparin was given parenterally. It could not be produced by the simple addition of heparin to drawn blood. From this fact, it seemed reasonable that other body tissues were making

a contribution to the chearing factor. It was shown by Brown and Anfinsen (1952) that this clearing factor had the properties of an enzyme to which heparin was an activator and cofactor. Brown and Anfinsen were also able to show that, once formed by the living animal, the clearing factor could operate in vitro. An enzyme with properties duplicating those of the plasma-clearing factor was isolated from heart muscle by Korn (1955) and named lipoprotein lipase.

The turbidity of lipemic plasma is due to the presence of globules of neutral fat, approximately 1 micron in diameter, coated with a thin layer of protein. Clearing factor was shown to cause a dissolution of these chylomicra and actual conversion of neutral fat into fatty acids and glycerol by Swank (1951). The amount of heparin needed to cause clearing factor production, also reported by Swank, is very small -- only 1/5 to 1/25 of the amounts needed to cause any significant increase in coagulation time. In speculating upon a physiological role for heparin, it is interesting to note that the small amount necessary to produce clearing factor is within the range of the concentration of heparin actually encountered in normal blood. Robinson and French (1953) have shown that, while the rate of clearing was proportional to the amount of heparin injected, the completeness of clearing was dependent upon the presence in the plasma of albumin which seemed to be acting as a temporary receiver of the fatty acids coming from the neutral fat of the chylomicra.

Histamine and heparin are related to each other at least in the

fact that they coexist in mast cells. It is believed by most authors that the metachromatic granules of the mast cells (and blood basophils) represent the bulk of intracellular heparin. The location of histamine seems also to be in small intracellular granules, some of which may be the same granules wherein heparin is found. The manner of binding of histamine in the mast cells is not known with certainty. One idea is that histamine is linked to heparin in a loose attraction that depends upon the basicity of histamine and the acid property of heparin. Support for this concept comes from the ability of organic compounds to act as histamine liberators because, being stronger bases than histamine, they presumably could displace histamine from a combination with heparin. This has been called the "ion exchange" theory and has recently been stated fully by Paton (1957).

The increase in coagulation time seen in the dog after anaphylactic shock is another observation that suggests a relationship between heparin and histamine. The liver of the dog is the shock organ of anaphylaxis in this species and the release of histamine from the liver is considered a cardinal event of the shocking process. The liver of the dog has a high content of heparin and contains many mast cells. It has been assumed, therefore, that the appearance of heparin in the blood was only incidental, being liberated when mast cells are broken. But, with the occurrence of mast cells in the connective tissue of many species, a possibility exists that the liberation of heparin occurs concomitantly with

the liberation of histamine under many circumstances other than anaphylactic shock in the dog.

If it is true that at least one of the methods of binding of histamine in tissues depends upon an attraction to heparin, then the fate of heparin during the release of histamine is important. This is especially true since so many of the drugs in human therapeutic use are histamine liberators. In addition to curare and strychnine, already mentioned, morphine (Nasmyth and Stewart, 1949), quinine (Burstein and Parrot, 1949), and atropine (Schachter, 1952) have been shown to be histamine liberators.

The testing of the possibility that release of heparin occurs with histamine release was the starting point for the work described in this dissertation. It was assumed that, since such a small amount of heparin could activate the clearing-factor enzyme, the presence of clearing factor after injection of known histamine liberators would be a sensitive indicator of heparin release.

It will be seen that this method of testing for heparin release quickly led into the larger question of an affinity between heparin and drugs in therapeutic use. Most of the drugs tested for heparin affinity in this present study were taken from a list compiled by Paton (1955). In addition to demonstrating and quantitating the affinity between these drugs and heparin, it was hoped that some further knowledge might be gained regarding the larger question of the mechanism of histamine release.

CHAPTER II

MATERIALS AND METHODS

In the work, many determinations were made of the decrease in optical density of lipemic rat blood plasma. The plasma was obtained from adult white rats of the Holtzman strain which, after being starved for 24-30 hours, were placed on a diet consisting of powdered Purina chow and cottonseed oil in a proportion of 3 to 1. In one type of experiment, injections of drugs were made into these rats, blood was drawn and changes in optical density of the plasma were measured. In all other experiments, the rats on the high fat diet served only as donors of lipemic plasma. In these latter experiments, other groups of rats were given intraperitoneal injections of various drugs, the blood was drawn and the plasma was separated. The two plasmas were then mixed, incubated at 37° C, and, at intervals, the optical density was determined.

All optical density measurements were made by means of a Klett-Summerson Photoelectric Colorimeter, Model 900.3, using a number 42 blue filter or number 66 red filter. Optical density readings are reported as "Klett optical density units."

Blood was obtained from all rats under light ether anesthesia, by

cardiac puncture, with a dry syringe and number 20 gauge hypodermic needle. The blood was placed in 15-ml centrifuge tubes containing either 2.0% or 6.0% sodium citrate as an anticoagulant. The tubes were kept in a crushed ice bath until centrifugation. In most cases, centrifugation was done in an ordinary International centrifuge. In a few experiments, a refrigerated International centrifuge was used, with the temperature maintained at 5° C. In all cases, centrifugation was done at a relative centrifugal force of 859 times gravity for 20 minutes.

All drugs used in this work were dissolved in 0.9% sodium chloride solution. They were as follows:

Medicinal Powdered Peptone (Frederick Stearns Co.)

Para-methoxyphenylethylmethylamine, hereafter referred to as compound 48/80 (Wellcome Research Laboratories)

Dextran, 6% in saline, (Abbott and Co.)

Atropine Sulfate (Merck and Co.)

Strychnine Sulfate (Eimer and Amend)

Stilbamidine Di-isethionate (Merck and Co.)

Trimethaphan Camphorsulfonate (Hoffman-LaRoche)

Tubocurarine Chloride (Squibb and Co.)

Quinine Hydrochloride (Merck and Co.)

Amphetamine Sulfate racemic (Merck and Co.)

Protamine Sulfate (Wyeth)

Heparin Sodium (Abbott and Co.)

Polyvinyl Pyrrolidone (Schenley)

Morphine and codeine were also used, but the manufacturer's name was not available.

The histological stain, Toluidine Blue-O (National Aniline); reagent grade acetone (Mallinckrodt); benzene (Baker); and commercial grade mineral oil were used in one experiment.

Whenever a buffer was used, it was always .05 M. phosphate (the appropriate combination of dibasic sodium phosphate and monobasic potassium phosphate) prepared to give the desired pH.

All glassware was washed in hot water with detergent, rinsed repeatedly in tap water, given a final rinse in distilled water, and dried in an oven at 100° C.

In one type of experiment, acute toxicity studies were done in adult white male and female mice of the C57BL strain. The drugs tested were given intraperitoneally using a tuberculin syringe and a 24-gauge hypodermic needle.

Details of the procedures followed when tests were made of the affinity of drugs for heparin in the presence of toluidine blue will be given when that experiment is presented.

CHAPTER III

EXPERIMENTS AND RESULTS

Five groups of experiments were performed in this work. They were as follows:

1. Attempts to show a release of heparin occurring concomitantly with the release of histamine, by the production of a lipemia-clearing factor.

2. The demonstration and quantitation of the degree of affinity between heparin and certain organic substances which possess the ability to liberate histamine.

3. The testing of the ability of histamine-liberating substances to inhibit a heparin-activated lipemia clearing factor.

4. Attempts to show a reduction in the toxicity of certain histamine-liberating drugs in the presence of heparin.

5. The testing of a suspected affinity for heparin in compounds having chemical characteristics similar to those of compounds known to have an affinity for heparin.

Experiment I

To test for the release of endogenous heparin, histamine-liberating drugs were injected into rats with an alimentary lipemia and blood was then drawn to measure the rate of clearing.

Four groups of rats with a lipemia were injected with one of the following substances: dextran, compound 48/80, peptone, or heparin. The dosage of each drug was 1 mg/kg, given intraperitoneally. One group of rats, with a similar lipemia, was kept untreated to give a measure of spontaneous clearing. The plasma from the heparin-treated animals gave an indication of the maximum degree of clearing to be expected.

From each rat, 2.0 ml of blood were drawn and mixed with 1.0 ml of 2.0% sodium citrate. After centrifugation, the plasma was removed, incubated, and the optical density was measured periodically.

Table I shows no difference in the rate of clearing of plasma from dextran or peptone-treated animals when compared with that from the control group. This was interpreted as meaning that no endogenous heparin was released into the blood by the action of the histamine liberators. The total lack of clearing in the rats which were given compound 48/80 seemed unimportant at the time. A possible explanation of this will be suggested later. The rate of clearing in the heparin-treated animals indicated that a high level of clearing factor could be formed at this dosage.

Table I

Decrease in Optical Density of Plasma from Rats with an Alimentary Lipemia Treated with Various Histamine Liberators

	Average Decrease in Klett Units From Time Zero		
	15-Min.	30-Min.	60-Min.
Dextran-treated (6 rats)	6.0	10.0	17.6
Compound 48/80-treated (6 rats)	0.0	0.0	0.0
Peptone-treated (6 rats)	9.0	9.4	15.2
Control Group (6 rats)	8.4	10.4	18.0
Heparin-treated (6 rats)	30.4	68.6	84.4

Experiment II

Considerable difference in the amount of turbidity was seen in the plasma from individual rats in Experiment I. This complicated the interpretation of the results. Therefore, it was decided to utilize one group of rats, on a high fat diet, as donors of lipemic plasma. This plasma would serve as the substrate for any clearing factor possibly produced by the injection of drugs into the rats of other groups. To give an equal turbidity for all reaction tubes, the plasma from this donor group was pooled. From Table II, it can be seen that the results of this procedure show no appreciable difference from those of Experiment I. Treatment of rats with heparin produced plasmas with a high amount of clearing factor, while treatment with 48/80 produced plasmas with very little or no clearing ability. Peptone or dextran-treated rats showed little difference in clearing rate from the untreated control group.

Because of the observation of spontaneous clearing in the untreated group of both Experiments I and II, it seemed profitable to determine whether clearing could be stimulated by the presence of high albumin concentration. Albumin is known to be a temporary carrier of the fatty acids coming from dissolution of the chylomicra. Stimulation of clearing was observed in the presence of an increased albumin concentration. This study was not pursued further, however, and the details will not be reported here.

Table II

Decrease in Optical Density of Lipemic Rat Plasma after Addition of Plasma from Rats Treated with Various Histamine Liberators

	Average Decrease in Klett Units From Time Zero		
	15-Min.	30-Min.	60-Min.
Dextran-treated (4 rats)	4.3	8.8	15.0
Compound 48/80-treated (5 rats)	0.0	1.2	3.1
Peptone-treated (6 rats)	7.2	8.4	14.4
Control Group (6 rats)	7.6	8.9	15.8
Heparin-treated (5 rats)	42.3	51.0	68.3

Experiment III

In view of the absence of an increase in the rate of lipemia clearing as a result of the injection of histamine liberators in Experiments I and II, it was considered possible that heparin might have been released but failed to enter the circulation in any substantial amount. Heparin is a fairly large polymer composed of units of sulfated glucosamine and glucuronic acid (Wolfrom, 1950). Its molecular weight is from 12,000 to 15,000 and it is a rather hydrophilic substance. With these properties, it seems highly probable that it might be retained in the insterstitial spaces after its release. Therefore, it was proposed to make tissue homogenates, add the histamine liberator, and test the supernatant for the presence of heparin. To test for heparin in the supernatant, it was decided to use the dye, toluidine blue, and measure the amount of metachromasia produced when it combined with heparin. In a preliminary experiment, it was found that one histamine liberator, compound 48/80, had a strong affinity for heparin. It was also found that this affinity would interfere with the metachromatic staining of toluidine blue. A search of the literature disclosed that this was reported by MacIntosh (1955). It seemed obvious then that such a method of testing for heparin release would be unfeasible if the histamine liberator itself had an affinity for heparin. But this also posed the question: which, if any, of other known histamine liberators might have heparin affinity?

This information would be extremely important in determining which of these drugs could be operating as histamine liberators by virtue of an affinity for endogenous heparin.

To test this possible affinity for heparin, the method of MacIntosh (1941) for the colorimetric assay of heparin was modified slightly. The maintenance of a pH near that of blood plasma was the most important modification.

The procedure consisted of placing 50 micrograms of toluidine blue in a 15.0-ml tube with 5 units of heparin. Various amounts of the substance under test, dissolved in 1.0 ml 0.9% sodium chloride solution, were added and the volume brought to 5.0 ml with phosphate buffer (Na_2HPO_4-KH_2PO_4) at a pH of 7.3 to 7.35. Control tubes containing only toluidine blue and heparin were brought to a similar volume with buffer. On top of this aqueous phase was placed 5 ml of 70-30 mixture of mineral oil and benzene. The tubes were shaken vigorously for 60-70 seconds. By this shaking, the heparin-toluidine blue combination was extracted into the oil-benzene layer and the uncombined toluidine blue was left in the aqueous layer. After a mild centrifugation for two minutes to re-establish the two layers, the oily layer was carefully drawn off. A 2.0 ml sample of the aqueous phase was then pipetted out and mixed with 2.0 ml of reagent grade acetone. The color of this mixture was then measured in the Klett-Summerson colorimeter, using a number 66 red filter. If the substance under test had an affinity for heparin, it

displaced some toluidine blue from its combination with heparin. This resulted in a reduction of the metachromasia of toluidine blue and was discernible before the extraction with the oil-benzene mixture. (See Plates I and II.) To quantitate this affinity, the readings of toluidine blue in the control tube and in the tube containing the substance under test were compared on a standard curve. The numerical difference of these two readings was a measure, in micrograms of toluidine blue displaced, of the affinity between heparin and the histamine liberator under test. To prepare the standard curve, a procedure was followed similar to that of actual tests. Graded amounts of toluidine blue were diluted to 5.0 ml with buffer. A 2.0 ml aliquot was withdrawn, diluted with 2.0 ml acetone and the color measured in the Klett colorimeter. From the data of several sets of such determinations, a straight line curve was constructed. On the final curve, Klett readings were plotted against micrograms of toluidine blue in the original 5.0 ml dilution. Toluidine blue and heparin do not combine stoichiometrically. Some toluidine blue remains uncombined except in the presence of a large excess of heparin. The amount of toluidine blue in the control tube must, therefore, always be subtracted from the amount in the experimental tube. Each value recorded in Tables III, IV, and V represents the average of several determinations for the drug involved.

It can be seen from Table III that, in respect to ability to dispell the metachromasia of toluidine blue-heparin complex, compound 48/80

Plate I

Displacement of Toluidine Blue from Heparin by a Histamine Liberator



In this example, compound 48/80 was added to Tubes 1 through 4. Tubes 5 and 6 were kept as controls.

Tube 1 contained 100 micrograms of compound 48/80; Tube 4 contained 10 micrograms. Note the gradual loss of color, indicating less displacement of toluidine blue with decreasing amounts of the histamine liberator.

Plate II

Extraction of Heparin-Toluidine Blue Complex Into Oil-Benzene Layer



After extraction, the metachromatic heparin-toluidine blue complex appears in the oil-benzene layer. Obvious differences of color are now apparent in the oily layer as well as in the aqueous phase. No heparin-toluidine blue complex is seen in Tubes 1 or 2. It becomes barely visible in Tube 3, while Tube 4 is only a little different from the control tubes.

is much the strongest of any substance tested. Tubocurarine, stilbamidine, and trimethaphan camphorsulfonate appear to have about equal affinities for heparin, by this test.

Table IV shows the results with several histamine-liberating substances which have little or no affinity for heparin under the circumstances of this test.

Tables III and IV show the affinity for heparin of several histamine-liberating compounds taken from a list by Paton (1955).

Table V shows the degree of heparin affinity of some other known histamine liberators; viz, dextran, polyvinyl pyrrolidone, and peptone. Dextran and polyvinyl pyrrolidone have a use in human therapeutics as plasma expanders, but neither is known to be a histamine liberator in man upon a first injection. These substances do, however, produce an anaphylactoid reaction in other species. Dextran is effective in and specific to the rat (Vorhees and co-workers, 1951), while polyvinyl pyrrolidone produces its histamine-liberating effects only in the dog (Halpern and Briot, 1953). Peptone is known to cause damage to mast cells (Wilander, 1939), and is generally accepted as a releaser of histamine. From Table V, it can be seen that dextran shows no affinity for heparin, while polyvinyl pyrrolidone has an affinity about twice that of stilbamidine. Peptone's affinity for heparin is about equal to that of stilbamidine.

The reason for the testing of mescaline will be explained in the discussion.

Table III

Micrograms of Toluidine Blue Displaced by Various Histamine Liberators

(Group I)

Drug Used	Micrograms of Drug Used	Micrograms of Toluidine Blue Displaced
Tubocurarine Chloride	1,000	17.5
	500	22.0
	250	11.0
	100	9.0
Trimethaphan Camphorsulfonate	1,000	18.0
	500	14.0
	250	12.0
	100	9.0
Stilbamidine Di-isethionate	1,000	16.0
	500	11.0
	250	8.0
	100	6.0
Quinine Hydrochloride	1,000	13.0
	500	9.0
	250	5.0
	100	3.0
Compound 48/80	100	34.0
	50	26.0
	25	9.0
	10	4.0

Table IV

Micrograms of Toluidine Blue Displaced by Various Histamine Liberators

(Group II)

Drugs Tested	Micrograms of Drug Used	Micrograms of Toluidine Blue Displaced
Strychnine Sulfate	1,000	0.5
	500	0.0
	250	1.0
	100	0.0
Atropine Sulfate	1,000	2.0
	500	1.0
	250	1.0
	100	2.0
Amphetamine Sulfate	1,000	0.0
	500	0.5
	250	0.5
	100	0.5
Morphine Sulfate	1,000	0.0
	500	0.5
	250	0.5
	100	0.5
Codeine Sulfate	1,000	1.0
	500	0.0
	250	0.5
	100	0.0

Table V

Micrograms of Toluidine Blue Displaced by Various Histamine Liberators

(Group III)

Drug Tested	Micrograms of Drug Used	Micrograms of Toluidine Blue Displaced
Dextran	1,000	0.0
	500	0.5
	250	0.0
	100	0.0
Polyvinyl Pyrrolidone	1,000	31.0
	500	24.0
	250	15.5
	100	11.6
Peptone	1,000	16.5
	500	7.0
	250	4.0
	100	2.5
Mescaline	1,000	6.6
	500	3.6
	250	0.5
	100	0.0

Experiment IV

The ability of histamine liberators to affect the clearing action of heparin-activated lipoprotein lipase was tested by a somewhat similar procedure to that followed in Experiments I and II.

Two groups of white rats of the Sprague-Dawley Holtzman strain were used. Group One, on a high fat diet, served as the source of lipemic plasma. Group Two was injected intraperitoneally with 1 mg/kg of heparin, and the plasma from this group served as the source of clearing factor. With the rats under ether anesthesia, 6.0 to 7.0 ml of blood were drawn by cardiac puncture, mixed with 2.0 ml of 6% sodium citrate, and kept in an ice bath. The blood was centrifuged; the plasma was drawn off, pooled, and used immediately.

Into each of a series of Klett colorimeter tubes was placed 1.0 ml of the lipemic plasma, 1.0 ml of the plasma containing heparin-activated clearing factor, and 1.0 ml of 0.9% sodium citrate solution containing the histamine-liberating drug under test. The volume was brought to 4.0 ml in all cases by the addition of phosphate buffer at a pH of 7.3 to 7.35. Control tubes contained lipemic plasma, plasma with clearing factor, buffer, but no histamine-liberating drug. After the addition of plasma containing clearing factor, all tubes were read immediately for optical density in the Klett colorimeter using a number 66 red filter. The tubes were then placed in a 37° C water bath for

incubation. The optical density was read at timed intervals and a decrease in optical density was taken as evidence of clearing by the heparin-activated enzyme.

Protamine sulfate, which is known to inhibit clearing factor (Brown. W. D., 1952), was tested to give a base value for estimating the degree of inhibition.

From Table VI it can be seen that protamine sulfate and compound 48/80 gave practically complete inhibition of clearing in concentrations one-tenth that of most of the other drugs tested. Clearing in the presence of stilbamidine was approximately 50% that of the control tubes.

Table VII shows the results obtained by using the histamine-liberating drugs which had little or no affinity for heparin in the toluidine blue test of Experiment III. No inhibition of clearing was shown. Most of the drugs tested showed no inhibition of clearing at this concentration (0.25 mg/ml). It seemed possible that inhibition might occur with increased concentration of the drugs tested.

Table VIII shows the effect of raising the concentration of the various histamine liberators that gave no inhibition of clearing factor at a concentration of 0.25 mg/ml. In this test system, no inhibition was obtained from any of the substances tested, even at concentrations as high as 1.0 mg/ml.

Table VI

Decrease in Optical Density of Lipemic Plasma in Presence of Histamine-Liberating Drugs

	Average Decrease in Klett Units from Time Zero		
	15-Min.	30-Min.	60-Min.
Tubocurarine 0.250 mg/ml	8.3	13.2	17.6
Quinine 0.250 mg/ml	8.0	13.8	17.4
Trimethaphan Camphorsulfonate 0.250 mg/ml	9.2	14.1	18.6
Protamine 0.025 mg/ml	1.2	1.8	2.4
Stilbamidine 0.250 mg/ml	3.9	6.2	8.7
Compound 48/80 0.025 mg/ml	1.0	1.2	2.1
Control Group	9.1	14.0	18.2

Table VII

Decrease in Optical Density of Lipemic Plasma in Presence of Histamine-Liberating Drugs

	Average Decrease in Klett Units from Time Zero		
	15-Min.	30-Min.	60-Min.
Strychnine 0.25 mg/ml	7.5	15.0	23.5
Morphine 0.25 mg/ml	9.0	16.5	25.5
Atropine 0.25 mg/ml	9.5	14.5	24.0
Amphetamine 0.25 mg/ml	9.0	14.5	24.0
Control Group	9.0	15.2	24.0

Table VIII

Decrease in Optical Density of Lipemic Plasma in Presence of Histamine-Liberating Drugs

	Average Decrease in Klett Units from Time Zero		
	15-Min.	30-Min.	60-Min.
Quinine			
0.5 mg/ml	8.6	13.1	16.1
1.0 mg/ml	8.4	14.1	16.2
Strychnine			
0.5 mg/ml	8.1	12.8	15.6
1.0 mg/ml	7.9	13.0	15.8
Tubocurarine			
0.5 mg/ml	7.8	12.7	15.0
0.75 mg/ml	8.3	13.2	15.4
Atropine			
0.5 mg/ml	8.9	14.3	16.5
1.0 mg/ml	8.6	15.1	16.1
Trimethaphan Camphorsulfonate			
0.5 mg/ml	8.8	15.4	16.6
1.0 mg/ml	8.6	15.2	16.4
Control Group	8.3	14.2	16.2

Experiment V

The ability of heparin to reduce the toxicity of tubocurarine chloride and strychnine sulfate was tested in white mice of the C57bl strain. White male and female adult mice of 20-30 grams in weight were fasted for 24 hours. They were then given an intraperitoneal injection of the drug at a dose level previously shown to be lethal to 50% of the animals. Different amounts of heparin were given, either in the same injection or by previous injections, to the experimental groups. The control group received only the drug under test. Protection from the drug effect was considered positive if the animal survived 24 hours.

Table VIII shows that, when the LD 50 of tubocurarine (0.55 $\pm$ .05 mg/kg) is mixed with heparin in a proportion of 1 to 5 by weight, the mortality is reduced to 20%. This decrease in toxicity of tubocurarine was seen even if the heparin was administered from 45-75 minutes prior to giving the tubocurarine. An amount of heparin equal to the tubocurarine given was without effect on toxicity.

The intraperitoneal LD 50 of strychnine has proved difficult to determine. It seems to be more of a range than a precise value, as is the case with tubocurarine. Preliminary studies have established this range to be between 2.0 and 2.7 mg/kg. In an experiment similar to that done with tubocurarine, mice were injected with strychnine at a dosage of 2.3 mg/kg. In the same injection, these mice also received

heparin in an amount approximately five times that of strychnine. The mortality of this group was 71% (49 dead out of 69). A control group which received only strychnine had a mortality of 67% (47 dead out of 70).

Table IX

Effect of Heparin on the Toxicity of Tubocurarine in White Mice

Experiment I		
Group 1	.55 mg/kg tubocurarine	9/20 dead/total
Group 2	.55 mg/kg tubocurarine plus .55 mg/kg (approx.) Na Heparin	8/20 dead/total
Experiment II		
Group 1	.55 mg/kg tubocurarine	18/32 dead/total
Group 2	.55 mg/kg tubocurarine plus 2.75 mg/kg (approx.) Na Heparin	8/31 dead/total
Experiment III		
Group 1	.55 mg/kg tubocurarine	8/21 dead/total
Group 2	2.75 mg/kg (approx.) Na Heparin*	3/21 dead/total

* Heparin given 45-75 minutes before tubocurarine

Controls given saline i. p. before tubocurarine

CHAPTER IV

DISCUSSION

The question of the fate of heparin when histamine is released from cells which contain them both bears upon the fundamental point of the physiological "use" of heparin. Release of histamine, without release of heparin, was achieved by the use of compound 48/80 in rats (Mota, 1953) and in dogs (Quivy, 1955). These studies utilized the anticoagulant properties of heparin in judging its release. In this present work, the more sensitive method of measuring clearing factor production was used, but with the same result. That the release of endogenous heparin can cause clearing factor production was shown by Havel and Boyle (1954). These workers demonstrated that clearing factor was regularly present during anaphylaxis in the dog. Heparin was shown to be the anticoagulant released during this type of shock in the dog by Jacques and Waters (1940). It should be emphasized, however, that the dog is quite unique in this regard. No other species shows a measurable increase in the heparin of the blood during anaphylaxis. If heparin is released by situations that release histamine, and does not reach the blood stream, then its disposition is another question. Riley

Shepherd and West (1955), using compound 48/80 to deplete the rat skin of histamine, noted a great reduction of metachromatic staining material in the mast cells. However, the total tissue content of heparin was not greatly reduced. They suggest, among other possible explanations, that heparin complexed with compound 48/80 and was taken up by local fibroblasts. Such complexing of compound 48/80 with heparin and its subsequent uptake by fibroblasts has been demonstrated by Higginbotham and Dougherty (1956). These workers have postulated that one function of heparin is to serve as a detoxifying agent of many noxious substances. They further suggest that some mast cells may be the result of fibroblast injestion of complexes between noxious substances and heparin.

In Experiments I and II, it was noted that rats with an alimentary lipemia showed virtually no clearing when given compound 48/80. With this knowledge of the complexing of heparin and compound 48/80 in vitro, it becomes possible to explain this inhibition of clearing by the postulation of an in vivo neutralization of the heparin which is acting as cofactor to the clearing enzyme.

If heparin can act as a detoxicant, then conceivably, endogenous histamine is held by heparin, at least temporarily, until it can be excreted. This binding of histamine to heparin is a necessary assumption for the operation of Paton's "ion exchange" theory of histamine release by organic bases. Paton (1955), in discussing the possible binding of histamine by tissue acids and their release by organic bases, implied

that most basic histamine liberators have at least enough affinity for heparin to displace histamine. He presents evidence to the effect that ribonucleic acid possesses an affinity for many of the substances tested for heparin affinity in this present work. The criterion Paton uses to judge the affinity of RNA for the histamine liberators is the production of a visible precipitate. The concentration needed to produce this precipitate are so great (at least in the case of morphine and amphetamine) that a concept of these substances displacing histamine through a competitive affinity seems highly questionable. This may be the mechanism in the case of compound 48/80 or stilbamidine. Indeed, the uptake of stilbamidine into mast cells has been beautifully demonstrated by Riley (1953). One impetus to this present work was the desire to gather evidence as to which of the basic histamine liberators could possibly be operating as such by complexing with heparin. Based on the work presented here, it appears probable that (of the substances tested) only compound 48/80, tubocurarine, quinine, stilbamidine, trimethaphan camphorsulfonate, polyvinyl pyrrolidone, and peptone have sufficient attraction for heparin to allow the postulate that they release histamine by a matter of simple displacement.

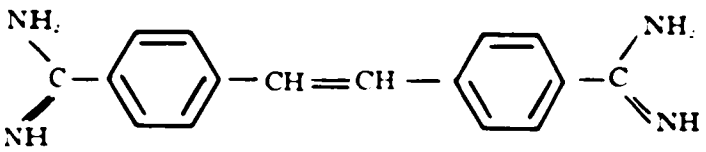
During these experiments, the question arose as to what chemical configurations of the drugs under test could account for their affinity for heparin. In inspecting the structural formula, it was noted that, with the exception of trimethaphan camphorsulfonate, all substances

with a definite affinity for heparin had nitrogen atoms in such states as to favor a high degree of reactivity. (See Plates III and IV.) To evaluate this idea of nitrogen reactivity being responsible for combination with heparin, substances with quaternary nitrogens were tested. By the toluidine blue test, neither neostigmine, acetylcholine, nor succinylcholine showed any affinity for heparin. Methoxy groups were noticed occurring with regularity in the compounds with an affinity for heparin. Because of this and also because of a marked similarity to the units of compound 48/80, mescaline was tested for a possible heparin affinity. (See Plate V.) A small but constant displacement of toluidine blue was seen as is recorded in Table V. It seems worth noting here that, of the substances tested for heparin affinity, those with greatest affinity are polymers. Compound 48/80 is said to be a mixture of dimers, trimers and tetramers complexed with formaldehyde (Baltzly, et al., 1949). Polyvinyl pyrrolidone is a rather large polymer resulting from the combination of acetylene with N-vinylpyrrolidone. Protamine, a substance with a strong attraction for heparin, is a long, chain-type molecule approaching protein size. Peptone, of course, is a mixture of polypeptides. Dextran, however, is also a polymer, but shows no affinity for heparin by the toluidine blue test. It might be important in explaining this non-attraction for heparin that dextran is a polymer of glucose and contains no nitrogen.

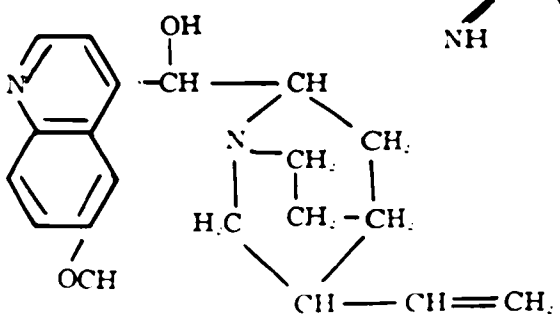
To test the affinity of these histamine-liberating substances for

Plate III

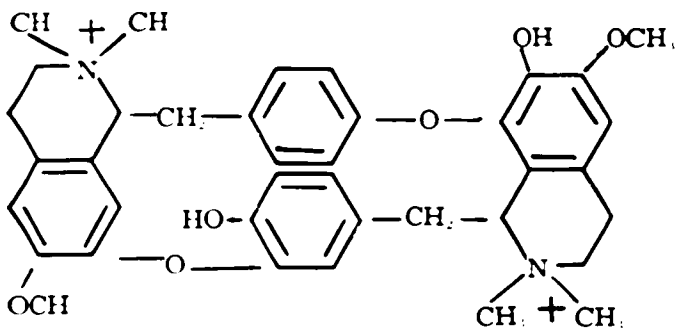
Structural Formula Of Histamine Liberators With An Affinity For Heparin



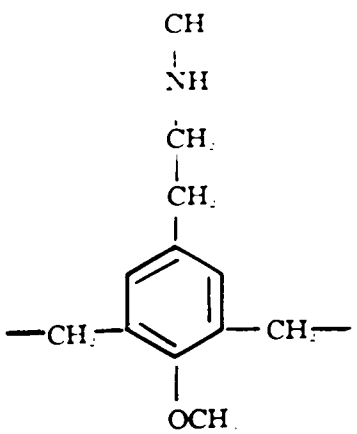
Stilbamidine



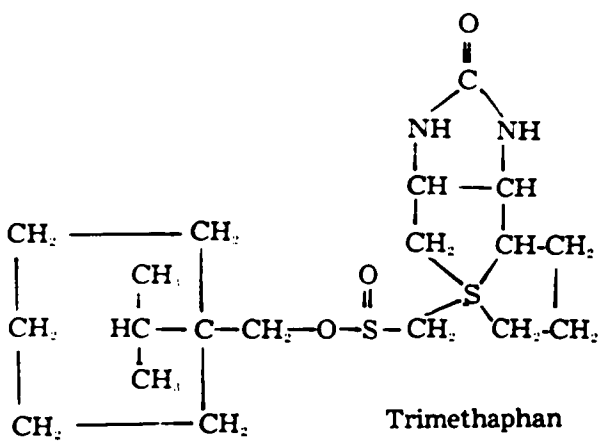
Quinine



Tubocurarine



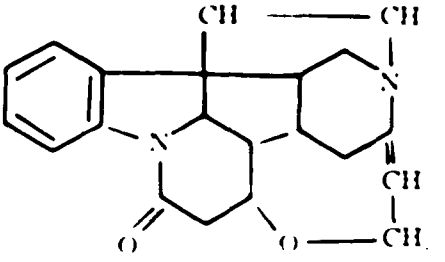
Compound 48/80



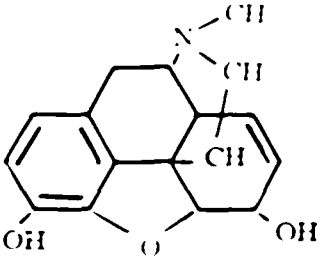
Trimethaphan Camphorsulfonate

Plate IV

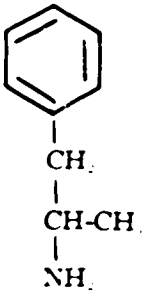
Structural Formula Of Histamine Liberators With No Affinity For Heparin



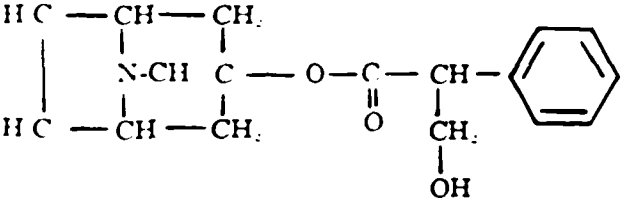
Strychnine



Morphine



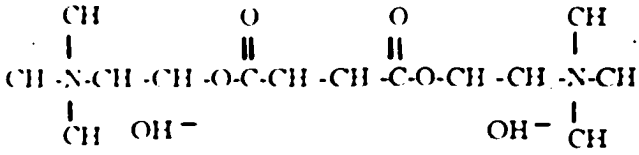
Amphetamine



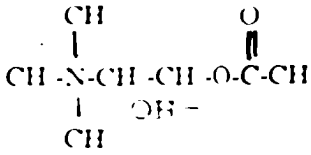
Atropine

Plate V

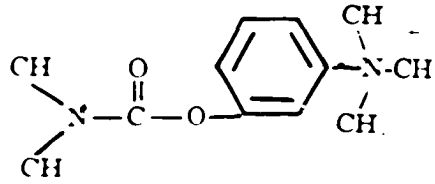
Structural Formula Of Compounds Tested For A Possible Affinity For Heparin



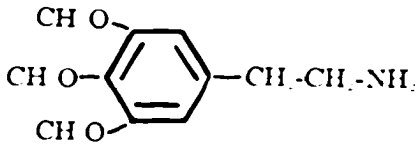
Succinylcholine



Acetylcholine



Neostigmine



Mescaline

heparin in a more physiological situation, the study of the possible inhibition of heparin-activated clearing factor was undertaken. The results show clearly that the substance with the highest affinity for heparin by the toluidine blue test, compound 48/80, also gives the greatest amount of clearing factor inhibition. Also of note is that, in consideration of their nearly equal affinity for heparin by the toluidine blue test, the presence of 1 mg of stilbamidine gives considerable inhibition, while 1 mg of tubocurarine shows little inhibitory power. All other substances tested, even in very high concentrations, gave no inhibition. Not one of the substances with negligible affinity for heparin by the toluidine blue test showed the slightest tendency to inhibit clearing factor.

In the course of these experiments, it was noticed that the same dosage of heparin did not always produce clearing at the same rate. This became quite perplexing. It was later discovered that the rate of clearing was high when the amount of lipemia was high. This could indicate that the action of the clearing enzyme was being affected by the availability of substrate. This would characterize the enzyme as having a first order reaction rate.

Higginbotham and Dougherty (1956) have reported the reduction of toxicity of 48/80 in white mice, with heparin. In this present work, the toxicity of tubocurarine was strikingly reduced by the injection of a large dose of heparin either before or with the injection of tubocurarine.

Since tubocurarine has been shown to have a definite affinity for heparin in the presence of toluidine blue, these results were not surprising. It does seem remarkable, however, that an amount of heparin just equal to the curarine administered had no apparent effect on its toxicity. If larger numbers of animals were tested, an effect might be seen. Heparin gives no reduction in the toxicity of strychnine in experiments similar to those done with tubocurarine. The question behind these experiments, however, was not how much the toxicity of a drug could be reduced, but whether or not heparin complexed with it. This is certainly true of tubocurarine.

CHAPTER V

SUMMARY AND CONCLUSIONS

1. As judged by the rate of clearing of lipemic plasma, dextran, peptone, and compound 48/80 do not cause the liberation of heparin into the blood stream of the rat. Compound 48/80 inhibits naturally occurring clearing factor.

2. Of the histamine liberators tested, compound 48/80, stilbamidine di-isethionate, quinine hydrochloride, trimethaphan camphorsulfonate, tubocurarine chloride, polyvinyl pyrrolidone, and peptone show an affinity for heparin by the in vitro displacement of toluidine blue. A quantitation of this affinity is given.

3. Strychnine sulfate, atropine sulfate, morphine sulfate, amphetamine sulfate, and dextran show little or no ability to displace toluidine blue from its combination with heparin up to the ratio of 20 to 1 of the drug to toluidine blue.

4. Because of the presence of highly reactive nitrogens or methoxy groups, succinylcholine, acetylcholine, neostigmine, and mescaline were tested for a suspected affinity for heparin by the toluidine blue test. Of this group, only mescaline showed any

attraction for heparin.

5. Compound 48/80 shows ability to inhibit a heparin-activated clearing factor approximately equal to that of protamine sulfate.

6. Stilbamidine di-isethionate had a decided ability to inhibit heparin-activated clearing factor. At the same concentrations, tubocurarine, quinine hydrochloride and trimethaphan camphorsulfonate show little or no ability to inhibit clearing factor. Even at high concentrations, no other drug tested gave an inhibition of clearing factor.

7. The toxicity of tubocurarine in white mice is reduced by previous or concomitant injection of heparin in an amount five times the dose of tubocurarine.

8. The toxicity of strychnine sulfate in white mice is unaffected by injections of heparin five times the dosage of strychnine.

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APPENDIX A

Data Relative to Table I

Groups of Rats	Klett Readings			
	Zero	15-Min.	30-Min.	60-Min.
Dextran-treated				
Rat 1	395	391	384	378
Rat 2	240	232	230	226
Rat 3	330	325	320	218
Rat 4	285	278	276	266
Rat 5	400	396	392	384
Rat 6	175	169	163	158
Compound 48/80-treated				
Rat 1	258	258	258	260
Rat 2	279	279	277	278
Rat 3	240	239	240	241
Rat 4	298	298	296	297
Rat 5	170	170	170	170
Rat 6	335	335	333	334
Peptone-treated				
Rat 1	385	387	387	372
Rat 2	260	252	250	243
Rat 3	295	288	288	280
Rat 4	340	326	325	322
Rat 5	210	202	202	196
Rat 6	190	182	182	174
Heparin-treated				
Rat 1	132	108	66	52
Rat 2	325	302	255	238
Rat 3	153	115	88	78
Rat 4	380	338	306	298
Rat 5	265	236	198	181
Rat 6	208	174	144	129

APPENDIX A (Continued)

Groups of Rats	Klett Readings			
	Zero	15-Min.	30-Min.	60-Min.
Control				
Rat 1	262	251	249	246
Rat 2	187	180	178	169
Rat 3	192	182	181	174
Rat 4	98	91	89	82
Rat 5	310	300	294	290
Rat 6	238	234	226	211

APPENDIX B

Data Relative to Table II

Groups of Rats	Klett Readings			
	Zero	15-Min.	30-Min.	60-Min.
Dextran-treated				
Rat 1	178	173	169	164
Rat 2	182	178	172	166
Rat 3	170	165	162	154
Rat 4	176	171	175	168
Compound 48/80-treated				
Rat 1	180	180	179	177
Rat 2	182	182	180	180
Rat 3	179	178	179	179
Rat 4	184	185	184	180
Rat 5	181	181	179	178
Peptone-treated				
Rat 1	176	168	168	162
Rat 2	174	168	175	160
Rat 3	180	176	170	165
Rat 4	181	174	170	165
Rat 5	178	170	168	165
Rat 6	178	171	170	164
Control Group				
Rat 1	128	120	118	114
Rat 2	130	120	119	114
Rat 3	129	121	120	113
Rat 4	130	124	121	115
Rat 5	130	124	122	112
Rat 6	128	120	119	113
Heparin-treated				
Rat 1	178	136	129	109
Rat 2	176	132	124	105
Rat 3	170	126	121	101
Rat 4	176	134	120	105
Rat 5	176	133	121	107

APPENDIX C

Data Relative to Tables III, IV and V

Drug Tested for Heparin Affinity	Micrograms of Drug	Klett Reading	Micrograms of Toluidine Blue Displaced
Tubocurarine	1,000	46	37.0
"	"	48	33.0
"	"	42	29.0
"	"	52	36.0
"	"	44	30.0
"	500	55	38.0
"	"	52	35.5
"	"	58	40.0
"	"	49	34.0
"	"	59	40.5
"	250	38	26.0
"	"	40	27.5
"	"	39	27.0
"	"	36	24.5
"	100	27	18.5
"	"	29	20.0
"	"	26	18.0
"	"	27	18.5
Trimethaphan Camphorsulfonate	1,000	47	32.0
"	"	54	37.0
"	"	48	33.0
"	"	46	31.0
"	"	49	34.0

APPENDIX C (Continued)

Drug Tested Heparin Affinity	Micrograms of Drug	Klett Reading	Micrograms of Toluidine Blue Displaced
Trimethaphan Camphorsulfonate (Continued)	500	41	28.0
"	"	40	27.5
"	"	39	27.0
"	"	42	29.0
"	"	38	26.0
"	250	39	27.0
"	"	38	26.0
"	"	40	27.5
"	100	27	18.5
"	"	26	18.0
"	"	26	18.0
"	"	29	20.0
Stilbamidine Di-isethionate	1,000	51	35.0
"	"	46	31.0
"	"	41	28.0
"	"	40	27.5
"	"	43	29.5
"	"	42	29.0
"	500	38	26.0
"	"	36	24.5
"	"	35	24.0
"	"	36	24.5
"	250	34	23.0
"	"	32	22.0
"	"	30	20.5
"	"	30	20.5
"	"	31	21.0
"	100	27	19.0
"	"	29	20.0
"	"	29	20.0

APPENDIX C (Continued)

Drug Tested for Heparin Affinity	Micrograms of Drugs	Klett Reading	Micrograms of Toluidine Blue Displaced
Quinine Hydrochloride	1,000	40	27.5
"	"	42	29.0
"	"	40	27.5
"	"	38	26.0
"	"	39	27.0
"	500	36	24.5
"	"	34	23.0
"	"	32	22.0
"	"	34	23.0
"	"	35	24.0
"	250	26	18.0
"	"	28	19.0
"	"	27	18.5
"	100	25	17.0
"	"	23	16.0
"	"	25	17.0
"	"	22	15.0
Compound 48/80	100	70	48.0
"	"	74	50.0
"	"	68	47.0
"	"	72	49.0
"	"	74	50.0
"	"	71	48.5
"	"	70	48.0
"	50	61	42.0
"	"	56	38.5
"	"	54	37.0
"	"	59	40.5
"	"	51	35.0
"	"	50	34.0
"	"	55	36.0

APPENDIX C (Continued)

Drug Tested for Heparin Affinity	Micrograms of Drug	Klett Reading	Micrograms of Toluidine Blue Displaced
Compound 48/80 (Continued)	25	33	23.0
"	"	37	25.0
"	"	34	23.0
"	"	35	24.0
"	"	30	20.5
"	"	31	21.0
"	10	25	17.0
"	"	20	14.0
"	"	19	13.0
"	"	22	15.0
"	"	20	14.0
Strychnine Sulfate	1,000	22	15.0
"	"	19	13.0
"	"	18	12.5
"	"	20	14.0
"	500	19	13.0
"	"	19	13.0
"	"	17	12.0
"	"	18	12.5
"	250	20	14.0
"	"	21	14.5
"	"	23	16.0
"	"	21	14.5
"	100	19	13.0
"	"	20	14.0
"	"	18	12.5
"	"	20	14.0

APPENDIX C (Continued)

Drug Tested for Heparin Affinity	Micrograms of Drug	Klett Reading	Micrograms Toluidine Blue Displaced
Atropine Sulfate	1,000	19	13.0
"	"	23	16.0
"	"	22	15.0
"	"	24	16.5
"	"	21	14.5
"	500	21	14.5
"	"	20	14.0
"	"	21	14.5
"	"	21	14.5
"	250	22	15.0
"	"	19	13.0
"	"	21	14.5
"	"	22	15.0
"	100	22	15.0
"	"	22	15.0
"	"	20	14.0
"	"	22	15.0
Amphetamine Sulfate	1,000	18	12.5
"	"	21	14.5
"	"	19	13.0
"	"	19	13.0
"	500	21	14.5
"	"	20	14.0
"	"	20	14.0
"	250	19	13.0
"	"	21	14.5
"	"	21	14.5
"	100	18	12.5
"	"	22	15.0

APPENDIX C (Continued)

Drug Tested for Heparin Affinity	Micrograms of Drug	Klett Reading	Micrograms of Toluidine Blue Displaced
Morphine Sulfate	1,000	20	14.0
"	"	19	13.0
"	"	19	13.0
"	"	20	14.0
"	500	20	14.0
"	"	21	14.5
"	"	21	14.5
"	"	21	14.5
"	250	22	15.0
"	"	20	14.0
"	"	20	14.0
"	"	22	15.0
"	100	19	13.0
"	"	22	15.0
"	"	21	14.5
Codeine Sulfate	1,000	21	14.5
"	"	22	15.0
"	"	23	16.0
"	"	20	14.0
"	500	19	13.0
"	"	19	13.0
"	"	21	14.5
"	"	20	14.0
"	250	22	15.0
"	"	20	14.0
"	100	19	13.0
"	"	20	14.0

APPENDIX C (Continued)

Drug Tested for Heparin Affinity	Micrograms of Drug	Klett Reading	Micrograms of Toluidine Blue Displaced
Dextran	1,000	21	14.5
"	"	19	13.0
"	"	18	12.5
"	"	20	14.0
"	500	22	15.0
"	"	20	14.0
"	"	21	14.5
"	250	20	14.0
"	"	19	13.0
"	100	19	13.0
"	"	19	13.0
Polyvinyl Pyrrolidone	1,000	68	47.0
"	"	62	42.5
"	"	65	44.5
"	"	65	44.5
"	"	66	45.0
"	500	56	38.5
"	"	59	40.5
"	"	52	35.5
"	"	50	34.0
"	"	58	40.0
"	250	39	27.0
"	"	42	29.0
"	"	46	31.0
"	"	38	26.0
"	"	43	29.5
"	100	34	23.0
"	"	36	24.5
"	"	31	21.0
"	"	34	23.0

APPENDIX C (Continued)

Drug Tested for Heparin Affinity	Micrograms of Drug	Klett Reading	Micrograms of Toluidine Blue Displaced
Peptone	1,000	37	25.0
"	"	42	29.0
"	"	40	27.5
"	"	41	28.0
"	"	39	27.0
"	"	40	27.5
"	500	35	24.0
"	"	33	22.5
"	"	36	24.5
"	"	30	20.5
"	"	34	23.0
"	250	24	16.5
"	"	25	17.0
"	"	23	16.0
"	"	25	17.0
"	100	25	17.0
"	"	21	14.5
"	"	23	16.0
Mescaline	1,000	26	18.0
"	"	27	18.5
"	"	29	20.0
"	"	27	18.5
"	500	24	16.5
"	"	23	16.0
"	"	23	16.0
"	250	21	14.5
"	"	19	13.0
"	"	19	13.0
"	100	19	13.0
"	"	19	13.0
"	"	20	14.0

APPENDIX C (Continued)

Drug Tested for Heparin Affinity	Micrograms of Drug	Klett Reading	Micrograms of Toluidine Blue Displaced
Control Group	(No Drug)	18	12.5
"	"	19	13.0
"	"	19	13.0
"	"	21	14.5
"	"	20	14.0
"	"	17	12.0
"	"	17	12.0
"	"	18	12.5
"	"	20	14.0
"	"	20	14.0
"	"	19	13.0
"	"	19	13.0
"	"	18	12.5
"	"	21	14.5
"	"	20	14.0
"	"	18	12.5
"	"	17	12.0
"	"	17	12.0
"	"	17	12.0
"	"	18	12.5
"	"	19	13.0
"	"	22	15.0
"	"	21	14.5
"	"	22	15.0
"	"	18	12.5
"	"	17	12.0
"	"	16	11.0
"	"	18	12.5
"	"	18	12.5
"	"	19	13.0
"	"	20	14.0
"	"	20	14.0
"	"	20	14.0
"	"	18	12.5

APPENDIX D

Data Relative to Table VI

Drug Used	Concentration of Drug in Milligrams per Milliliter	Klett Readings			
		Zero	15-Min.	30-Min.	60-Min.
Tubocurarine	0.250	64	55	50	37
"	"	61	54	49	34
"	"	48	40	35	30
"	"	46	39	32	24
"	"	40	44	28	23
"	"	41	34	27	24
Quinine	0.25	62	53	49	45
"	"	60	52	46	42
"	"	49	42	35	32
"	"	48	41	34	31
"	"	41	34	28	23
"	"	41	35	29	23
Trimethaphan	0.25	63	53	48	44
"	"	65	56	51	47
"	"	50	42	37	32
"	"	48	39	34	29
"	"	40	31	26	22
"	"	42	32	27	24
Protamine	0.025	62	60	59	59
"	"	60	60	60	59
"	"	51	50	50	49
"	"	51	50	49	48

APPENDIX D (Continued)

Drug Used	Concentration of Drug in Milligrams per Milliliter	Klett Readings			
		Zero	15-Min.	30-Min.	60-Min.
Stilbamidine	0.25	62	58	56	53
"	"	61	57	54	53
"	"	48	45	43	39
"	"	50	46	43	41
"	"	41	37	35	33
"	"	40	37	34	32
Compound 48/80	0.025	60	60	60	59
"	"	60	60	59	58
"	"	49	48	48	47
"	"	50	49	49	47
"	"	42	40	40	40
"	"	41	39	39	39
Control	(No Drug)	61	52	45	42
"	"	59	50	44	43
"	"	59	49	44	41
"	"	60	50	46	40
"	"	60	51	47	42
"	"	49	40	34	31
"	"	51	41	37	32
"	"	49	41	36	33
"	"	49	42	37	31
"	"	50	40	36	32
"	"	50	42	37	32
"	"	42	40	28	24
"	"	44	35	31	26
"	"	40	31	26	23
"	"	40	31	26	24
"	"	41	33	26	23
"	"	40	32	25	23

APPENDIX E

Data Relative to Table VII

Drug Used	Concentration of Drug in Milligrams per Milliliter	Klett Readings			
		Zero	15-Min.	30-Min.	60-Min.
Strychnine	0.25	69	61	53	45
"	"	67	60	51	44
"	"	64	57	50	41
"	"	64	56	50	40
"	"	63	56	49	40
"	"	65	57	51	41
Morphine	0.25	68	60	51	42
"	"	68	59	51	43
"	"	65	55	49	40
"	"	64	54	47	38
"	"	63	54	47	38
"	"	62	54	46	36
Atropine	0.25	67	57	52	44
"	"	68	59	53	44
"	"	64	55	50	39
"	"	64	55	50	39
"	"	62	52	47	38
Amphetamine	0.25	68	60	53	44
"	"	68	58	52	43
"	"	63	52	49	40
"	"	64	53	49	41
"	"	63	55	48	41
"	"	63	54	49	40

APPENDIX E (Continued)

Drug Used	Concentration of Drug in Milligrams per Milliliter	Klett Readings			
		Zero	15-Min.	30-Min.	60-Min.
Control	(No Drug)	67	60	53	45
"	"	66	55	50	41
"	"	66	56	53	41
"	"	68	59	54	45
"	"	68	59	53	43
"	"	63	55	58	40
"	"	64	54	48	41
"	"	62	54	49	40
"	"	62	53	47	40
"	"	62	53	48	39
"	"	63	53	48	38
"	"	61	54	45	37
"	"	62	53	46	38
"	"	61	53	46	39
"	"	61	54	46	37

APPENDIX F

Data Relative to Table VIII

Drug Used	Concentration of Drug in Milligrams per Milliliter	Klett Readings			
		Zero	15-Min.	30-Min.	60-Min.
Quinine	0.5	57	50	45	40
"	"	58	49	44	42
"	"	60	51	47	44
"	"	60	52	47	44
"	1.0	64	55	50	48
"	"	60	52	46	43
"	"	50	42	37	34
"	"	51	43	36	35
Strychnine	0.5	59	52	46	43
"	"	59	50	46	43
"	"	60	52	48	45
"	"	59	51	43	43
"	1.0	62	53	48	46
"	"	61	53	46	43
"	"	51	45	40	37
"	"	51	44	39	36
Tubocurarine	0.5	58	50	45	41
"	"	58	51	46	44
"	"	61	52	47	46
"	"	60	56	49	47

APPENDIX F (Continued)

Drug Used	Concentration of Drug in Milligrams per Milliliter	Klett Readings			
		Zero	15-Min.	30-Min.	60-Min.
Tubocurarine (Continued)	0.75	60	51	46	43
"	"	62	50	47	45
"	"	52	45	43	39
"	"	53	44	40	39
Atropine	0.5	60	50	45	42
"	"	61	51	46	44
"	"	52	44	37	36
"	"	51	42	39	37
"	1.0	61	52	43	42
"	"	60	52	46	45
"	"	52	45	37	36
"	"	53	44	38	37
Trimethaphan	0.5	62	52	46	41
"	"	61	53	47	44
"	"	53	42	39	38
"	"	50	41	34	33
"	1.0	60	54	45	42
"	"	62	51	46	45
"	"	54	43	39	38
"	"	52	43	37	36
Control	(No Drug)	59	50	46	44
"	"	58	52	47	45
"	"	60	52	44	43
"	"	61	50	45	43
"	"	58	51	44	44
"	"	58	51	43	43
"	"	54	48	39	37
"	"	50	41	35	34

APPENDIX F (Continued)

Drug Used	Concentration of Drug in Milligrams per Milliliter	Klett Readings			
		Zero	15-Min.	30-Min.	60-Min.
Control (Cont'd.)	(No Drug)	50	42	36	34
"	"	49	41	37	37
"	"	49	41	35	34
"	"	54	45	39	39
"	"	53	43	36	38
"	"	54	46	38	38
"	"	50	40	34	33
"	"	51	42	37	35