VII. THE NEPHELOMETRIC ESTIMATION OF QUININE IN BLOOD.

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IN a group of papers, Ramsden and Lipkin [1918], Ramsden, Lipkin and Whitley [1918] and Lipkin [1919] have described the chemical assay by a nephelometric method of traces of quinine when present in tissues and body fluids. The method consists essentially in matching the turbidity produced in the quinine solution of unknown strength by the addition of Tanret's reagent (potassium mercuric iodide) with the turbidity produced in a series of standard solutions. The sensitiveness of the test is enhanced by producing the turbidity in saturated ammonium sulphate solutions, preliminary trial having indicated the dilution necessary to bring the solution of unknown strength within the range of the standard solutions, usually between 1 in 200,000 and 1 in 300,000.

One of us [Acton, 1920] having found other cinchona alkaloids of great value clinically in the treatment of malaria, it was thought that some light might be thrown on the mode of action of quinine in malaria by the interpretation of the results obtained by using the afore-mentioned method for determining the relative distribution of the various cinchona alkaloids in the body.

That results of practical significance might be obtained by such an investigation seemed to be suggested not only by Ramsden and Lipkin's results but more particularly by the recent work of Morgenroth [1918], who has based an ingenious theory of the action of quinine, on the distribution of this alkaloid and of the closely related alkaloid optoquine (ethylhydrocupreine) between the corpuscles and plasma as determined by himself by physiological methods—production of anaesthesia in the rabbit's cornea and inhibition of the growth of bacteria. According to Morgenroth's determinations by these methods quinine and the related alkaloids, when added to blood, accumulate in or on the red corpuscles, the serum or plasma retaining only a low proportion.

This statement of Morgenroth is in pronounced disagreement with the various determinations of the distribution of quinine in blood which have been made by chemical methods. Hartmann and Zila [1918] found a distribution between plasma and corpuscles in the ratio of 8 to 5. Halberkann

[1919], using larger quantities of quinine, found 35 to 48 %, and in one case as much as 54 % in the corpuscles; but neither of these observers showed a predominance in the corpuscles such as that suggested by Morgenroth's results. Even more definitely in disagreement with Morgenroth's findings were those of Ramsden, Lipkin and Whitley, who found a distribution between serum and corpuscles in favour of the serum in the proportion of from 3 : 1 to 2 : 1. More recent determinations by Lipkin [Ramsden, 1920] have again shown a proportion of 3 : 1 in favour of the serum.

It seemed desirable to make an effort to clear up this discrepancy as regards quinine and to apply to a study of the distribution of the other cinchona alkaloids methods found to give good results with quinine. The method described by Ramsden and Lipkin seemed to be the best adapted to our purpose, since in the hands of its authors, it appeared to have made possible the estimation of very small quantities of quinine with an error of not more than 5 %. We met with unexpected difficulties in the application of the method, and, after months of effort, we were forced to the conclusion that a strict adherence to the written description of the method would not give us results to which we could attach any quantitative significance. That description seems to us to err in greatly underestimating the difficulty of extracting quinine from blood: with the volume of fluid and amount of extraction which it indicates we could never recover more than 30 to 40 % of the quinine which we added to samples of blood from a number of different species. We found in it, further, no adequate indication of the extreme care which is necessary to avoid the contamination of the extracted quinine with traces of other substances, which affect the turbidity with Tanret's reagent upon which the final determination depends. By increasing the volume of fluid used in extracting the blood coagulum, and introducing as the result of experience various additional precautions against contamination, we have succeeded in obtaining in clean condition practically constant proportions of the quinine added to samples of blood, corpuscles, plasma or serum. With due allowance for the proportion of quinine which, in each case, is, in our experience, irrecoverable by any amount of extraction, a fairly accurate estimate of distribution can be obtained and the rate of absorption and disappearance of quinine from the circulating blood can be followed. Under such conditions it may be possible to obtain comparative figures of moderate accuracy with the other alkaloids. Our purpose here is to indicate the modifications of the Ramsden and Lipkin technique which we have found it necessary to adopt, and the conclusions as regards the distribution of quinine in blood to which we have been led.

EXPERIMENTAL DETAILS.

Standard Reagents.

1. *Aqueous quinine solution* made by dissolving 0.25 g. of anhydrous quinine base in water containing 7.71 cc. of *N*/10 sulphuric acid (one equi-

valent) plus 0.77 cc. of acid to facilitate solution, and diluting to 250 cc. 1 cc. of final solution contains 1 mg. of quinine base.

2. 1 in 100,000 *quinine in ammonium sulphate solution*, made by adding 5 cc. of solution 1 to a saturated solution of pure recrystallised ammonium sulphate and diluting to 500 cc. with the same saturated solution.

Solutions 1 and 2 were kept in the dark as there is a marked deterioration of quinine content of 2 on prolonged exposure to sunlight.

3. 0.6 % *sulphuric acid solution of saturated ammonium sulphate* made by adding 12 g. of sulphuric acid to a saturated solution of pure recrystallised ammonium sulphate and diluting with the same saturated solution to 2000 cc.

The ammonium sulphate used throughout the results recorded below was an unusually pure sample containing only the faintest trace of chloride. We find that commercial ammonium sulphate "recryst" is pure enough for the solutions recorded here, after removal of adventitious matter by filtration.

4. *Pure ether, free from aldehydes* prepared as described by Ramsden and Lipkin.

5. *Pure ammonia solution* prepared by distilling 0.880 ammonia through an all glass apparatus and collecting the gas in distilled water at 0°. This precaution is essential owing to the solvent action of strong ammonia on organic matter, grease, etc. and a separation, moreover, is effected from less volatile tar-bases present in traces in commercial ammonia which might affect the subsequent estimation of quinine.

Method of estimating Quinine in Blood.

This is based on the Ramsden Lipkin process with important modifications which will appear in the sequel.

5 cc. of fresh defibrinated blood were added to 1/20 mg. of quinine in a beaker, well stirred and then laked with about 5 cc. of distilled water. 20 cc. of saturated ammonium sulphate solution containing 0.6 % of sulphuric acid were added and 5 g. of solid pure recrystallised ammonium sulphate. The solution was brought to the boil over a free flame, after a preliminary coagulation of the contents on a boiling water-bath, with constant rotation of the contents and filtered through a Gooch crucible packed with a layer of asbestos, covered with a porcelain filter disc and over the latter a filter paper so as to prevent the coagulum passing into the asbestos. The entire coagulum on the filter paper was returned to the beaker, treated with a second 20 cc. of acidified ammonium sulphate solution, disintegrated and the solution again brought to the boil and filtered as before, the coagulum being in each case well pressed down. The same process was repeated ten times in all, the entire coagulum being each time returned to the beaker. The combined filtrate, about 200 cc., was transferred to a well-stoppered 250 cc. measuring cylinder, and 10 cc. of pure ammonia solution added, followed by 20 cc. of pure ether. On shaking with further successive quantities of 15, 15 and 10 cc. of ether, practically the whole

of the quinine present passed into the ether, which was in each case pipetted off by means of a 1 cc. pipette fitted with a rubber teat, and transferred to a boiling tube dipping into a gently boiling water-bath. When every trace of ether had boiled away the quinine was left as a clear colourless varnish which was dissolved in 10 cc. of pure saturated ammonium sulphate solution by just bringing to the boil. The solution was rapidly cooled in water and 5 cc. transferred to one of a series of standard tubes, the remainder of which contained 5 cc. each of various standard solutions of quinine made by mixing the requisite quantities of 1 in 100,000 quinine in saturated ammonium sulphate solution with saturated ammonium sulphate solution. 0.05 cc. of Tanret's reagent was rapidly added to each and the contents mixed. The whole series of tubes was transferred simultaneously to a boiling brine-bath for a few minutes and then rapidly cooled in running water. By comparing the turbidities in uniform daylight (not direct sunlight) against a dark background, the standard solutions differing in strength by as little as 7 % can be invariably placed in the correct sequence of strengths and the solution of unknown strength is also placed without difficulty and is qualitatively indistinguishable from the standards. We have tried the Ramsden nephelometer but in our opinion it fails to offer any advantage over direct observation in daylight.

The points of importance¹, which Ramsden and co-workers have not laid stress on, are that the most scrupulous care must be exercised throughout the experiment so as to ensure complete freedom from all traces of grease of all surfaces with which the solutions, whose turbidities are going to be compared, come in contact. The standard tubes cannot even be washed out with organic solvents to accelerate drying, but must be washed with several changes of distilled water and then dried by draining or by blowing out in a current of warm air.

Mere contact of the final solutions with the fingers is sufficient to vitiate the results and in addition there is the importance of the use of pure ammonia referred to above. Failure to take these precautions causes the turbidities to be qualitatively abnormal and renders quantitative estimation impossible.

Results with the Method.

Using the method as just described the following table shows to what extent quinine can be extracted from blood or its constituents.

Treatment		Volume cc.	Quinine added mg.	Quinine recovered %	Mean %
Serum		5	0.05	(a) 84, (b) 84	84
Corpuscles	Laked	5	0.05	(a) 77, (b) 69	73
Whole blood	Not laked	5	0.05	(a) 62, (b) 69	65
Whole blood	Not laked (15 boilings)	5	0.05	71	—
Whole blood	Laked	5	0.05	(a) 74, (b) 74	74

¹ Professor Ramsden informs us that, with the exception of the dangers arising from impurities in the ammonia, they were themselves well aware of them.

It is evident that laking the blood materially assists the extraction of quinine and that recovery from corpuscles is more difficult than from serum.

The effect of increasing the number of changes of boiling fluid and of increasing the volume of the fluid is shown by the following table, 5 cc. of freshly defibrinated goat's blood being used in each case and the quinine added dissolved in saturated ammonium sulphate solution.

	No. of boilings	No. of changes of fluid	Total volume of fluid cc.	Quinine recovered %
(a)	10	4	50	44
(b)	10	10	100	69
(c)	10	10	200	87

That the chief loss lies in the difficulty of extracting the quinine from the coagulum is shown by omitting the blood and adding the quinine to the acidified ammonium sulphate, making alkaline and extracting as described for the remainder of the process. A duplicate estimation was made giving the same result in both. Out of 0.05 mg. added to 200 cc. of acidified saturated ammonium sulphate solution, the quantity recovered lay between 0.05 mg. and 0.045 mg. As an approximation we put it at 95 % recovery.

The substitution of 0.6 % sulphuric acid by weight by 2 % sulphuric acid by volume, or the substitution of saturated ammonium sulphate by 30 % ammonium sulphate in the boiling fluid¹, made no difference to the amount of quinine extracted, as is shown by a comparison of the following table with the first table.

	Volume cc.	H ₂ SO ₄ %	(NH ₄) ₂ SO ₄ %	Quinine added mg.	Quinine recovered %
Blood	5	2	30	0.05	66
Serum	5	2	Saturated	0.05	80
Corpuscles	5	2	Saturated	0.05	67 and 70

Distribution of Quinine between Serum and Corpuscles.

The distribution of quinine as found by us is shown by the following:

1. 0.10 mg. of quinine was added to 10 cc. of fresh defibrinated goat's blood of which the serum-corpuscle ratio as determined in duplicate was 65/35 by volume.

Quinine found in total serum	0.045 mg.
Quinine found in laked corpuscles	0.027 mg.
Quinine recovered	0.072 mg.

whence it can be calculated that for equal volumes of the two fluids

$$\frac{\text{Quinine in corpuscles}}{\text{Quinine in serum}} = \frac{53}{47}.$$

2. 0.10 mg. of quinine was added to 17 cc. of fresh defibrinated sheep's blood of which the serum-corpuscle volume ratio had been artificially adjusted by removal of serum at 50/50.

¹ These modifications were suggested to us by Professor Ramsden as being more expeditious.

Quinine found in total serum	0.041 mg.
Quinine found in laked corpuscles	0.035 mg.
Quinine recovered	0.076 mg.

$$\text{whence} \quad \frac{\text{Quinine in corpuscles}}{\text{Quinine in serum}} = \frac{46}{54}.$$

3. 0.17 mg. of quinine was added to 14 cc. of fresh defibrinated goat's blood of which the serum-corpuscle volume ratio had been artificially adjusted by removal of serum at 50/50.

Quinine found in total serum	0.047 mg.
Quinine found in laked corpuscles	0.043 mg.
Quinine recovered	0.090 mg.

$$\text{whence} \quad \frac{\text{Quinine in corpuscles}}{\text{Quinine in serum}} = \frac{48}{52}.$$

Bearing in mind the greater difficulty of extracting quinine from laked corpuscles than from serum, and the impossibility of completely removing serum from corpuscles, these three experiments point to an approximately uniform concentration of quinine in corpuscles and serum.

Quinine and Hydroquinine Content of Human Blood.

One of us took 1 g. of quinine base and on another occasion 1 g. of hydroquinine base (both as bisulphate in aqueous solution) by mouth at 10 a.m. 5 cc. of blood were removed at hourly intervals and the quinine or hydroquinine content estimated. Previous experience with laked blood has shown that as a rule about three-quarters only of the alkaloid can be estimated. By calculating on the basis of a 75 % recovery of quinine from blood the following concentrations of quinine and hydroquinine were found, and if the total volume of blood be put at 5 litres, the percentage of the quinine taken orally which is subsequently found in the blood is determined.

Interval after administration hours	Concentration of quinine in blood	Amount in blood mg.	% of dose taken
1	1 in 150,000	33	3.3
2	1 in 187,000	27	2.7
3	1 in 225,000	22	2.2

For Hydroquinine.

1	1 in 250,000	20	2.0
3	1 in 280,000	18	1.8

As a point of interest it can readily be shown by calculating from these figures that at the end of one hour the number of molecules of quinine per corpuscle is about 20 millions.

The concentrations reached in the blood after oral administration of smaller doses of quinine are shown in the following table:

Dose	Concentration after 1 hour	Concentration after 2 hours
0.5 g.	1 in 375,000	1 in 500,000
0.25	Below 1 in 375,000	Below 1 in 750,000

As a practical method for estimating traces of quinine in blood we cannot recommend the process. Apart from the non-quantitative recovery of the quinine, a single complete estimation takes three to four hours, the scrupulous care required to be exercised over this period involving a considerable strain on the operator.

In conclusion our results show that neither the preponderance of quinine in corpuscles as maintained by Morgenroth nor the preponderance in plasma as held by Ramsden and Lipkin can be correct. On the contrary, like other observers, we find approximately equal or uniform distribution of quinine between corpuscles and serum. On such evidence it is clearly undesirable to found any theory of the mode of action of quinine in malaria, such as that propounded by Morgenroth.

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