STUDIES ON VITAL STAINING

IV. India Ink and Brilliant Vital Red. Importance of Considering Liver Excretion in the Study of "Blockade of the Reticulo-Endothelial System"

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INTRODUCTION

In previous papers of this series we have presented experiments dealing with the distribution in the body of certain dyestuffs when given intravenously (1). The two dyes, brilliant vital red and Niagara sky blue, like many other acid dyestuffs are gradually taken up by phagocytic cells in various parts of the body ("reticulo-endothelial system" of Aschoff). They differ from many dyes of this group in being taken up rather slowly by these cells, so that the concentration of dye in the plasma may be followed over a period of many hours before the concentration becomes too low to be determined by colorimetric methods. At first the tissues remove either dye quite rapidly from the blood stream, but later on as the tissues become more deeply stained they become less active, and at such concentrations of dye in plasma they take up dye less readily than they normally would. This sluggishness or "inhibition" of tissue activity represents in reality an equilibrium in dye concentration between tissues and body fluids, for if more of the dye be injected the tissues will take up much of it. The dye continues to pass into the tissues until a second equilibrium point is reached at a higher level.

These two dyes are rather similar in their general physico-chemical properties and we need not be surprised that they are deposited within the same identical phagocytes (2). Our evidence shows (2) that the presence of one dye within these cells does not prevent the cells from taking up the other at the normal rate. Furthermore, the phagocytes can take up both dyes simultaneously when they are injected together. The cells respond without hesitation to this double task, with the result that each dye is removed from the plasma with normal speed, not only at first when the cells are free of dye, but later on as well, when the cells contain considerable amounts of each dye. The two dyes within the cell do not appear to exert any mutual antagonism which would slow up the rate of storing dye or hasten the onset of the equilibrium for either dye.

It is common knowledge that enormous numbers of phagocytes exist within the connective tissues and within the lymph nodes. These cells do not lie in intimate contact with the blood stream as do the Kupffer cells of the liver or certain phagocytic cells of spleen or bone marrow. It has never been clear whether or not cells of this latter type are really more actively phagocytic than the cells more distantly removed from the circulating blood. The fact that they take up foreign materials so promptly may merely be a correlary of their accessibility to the blood stream and hence to the source of the foreign materials. At a later date we shall present data to show that after removal of liver or spleen, brilliant vital red is still quite rapidly removed from the circulating blood. The removal of the liver with all of its Kupffer cells or the spleen with its numerous phagocytes does not appear to cause great impairment of the phagocytic system as a whole. We may concede that as individuals the phagocytes of liver or spleen are perhaps more highly endowed types of phagocytes, still it is our belief that the phagocytes of the connective tissues generally are so extremely numerous that much and probably most of the total phagocytic power in the body resides in them.

When India ink is injected into the blood stream the particles of carbon tend to agglutinate, and very few of them succeed in passing through the capillary walls. They are promptly taken up and stored in those phagocytes which lie in intimate contact with the blood stream. Such injections offer us an opportunity to test our ability to "block" these particular cells without in any way involving the phagocytes which lie well outside the vessel walls. To test the phagocytic response of these ink-laden cells one should inject some second foreign substance which would be taken up by these cells, and by them alone. Unfortunately most if not all substances of this class are in such an unstable state of dispersion that they precipitate out in the blood stream. Many of these particles are caught and held mechanically in the capillaries, so that speed of removal cannot be used as a measure of phagocytic activity. On the other hand most dyestuffs, like brilliant vital red, reach vast numbers of phagocytes besides those which contain ink. Our experiments with liver removal and with splenectomy have lead us to believe that these extravascular cells possess a large total phagocytic power. We could hardly expect the presence of ink in the smaller group of cells to reduce the total bodily response very markedly, even though the ink did block the cells which take it up. For these reasons we were not prepared to find that India ink does actually delay the rate at which brilliant

vital red leaves the circulating blood. At first we seemed compelled to attribute this to defective phagocytosis, but later on we found that the ink injection inhibits the excretion of dye into the bile. The longer retention of dye in the plasma can be explained by defective elimination on the part of the liver.

From these observations it became evident that peculiarities of liver excretion demand careful attention. It is necessary to have accurate data on this subject if we are to interpret any data on the rate at which dye leaves the blood stream. Obviously, changes in this rate cannot be ascribed to changes in phagocytic response unless we can show that liver excretion is not responsible. Much of the literature of "blockade" does not take these considerations into account. It is not sufficient to assume that liver excretion is constant or that it is proportional to dosage of the dye. In fact this latter supposition is definitely contrary to fact (3). With small doses as much as 30 per cent may be eliminated into the bile during the first day, whereas with larger doses the percentage output may not be more than 15. After chronic vital staining or after single large injections the dye may be less efficiently removed from the blood stream, but as in the case of India ink injections, this must be attributed to the inefficient liver excretion and not to "blockade" of phagocytes.

Methods

Details regarding care of the dogs and the collection and analysis of blood samples have been given elsewhere (1).

The India ink used was Higgins' American India ink. The exact composition of this ink has never been published. It contains about 10 per cent of solids. We have found that the carbon can be precipitated with dilute acids. If this black mass be resuspended in water and again precipitated with dilute acid and then washed with alcohol and dried it will be found to represent about 7 per cent of the original ink. This figure may be a trifle too high but we feel that most of the impurities have been separated from the carbon by this process of precipitation and washing. Kjeldahl determinations show that the precipitated mass contains almost no nitrogen.

The ink was injected either without dilution, or in some cases after dilution with an equal volume of normal saline. Since much of the ink is precipitated in the blood stream and is promptly filtered out by the capillaries we have not been interested in following the

rate at which the ink leaves the blood stream, for such studies give no information regarding the rate of true phagocytosis by the tissues. We may note in passing that in our experiments the blood stream was regularly free of carbon particles within an hour or less. We have taken advantage of this fact and have timed the administration of ink in such a way that ink would never be present in the plasma to interfere with the regular colorimetric determinations of dye.

Bile was collected for study in certain of the dogs.

A slight modification of the bile fistula operation of Rous and McMaster (4) has been made by Smith, Groth and Whipple (5) and their technic was used in the present experiments. By this method bile is collected by a cannula inserted into the common bile duct and is brought to the outside and collected in a rubber bag from which it can be removed daily, or oftener if desired. The rubber bag is protected from injury by suitable dressings which are placed about the dog. Bile can be collected whenever desired over a period of many weeks. The dog remains in good condition throughout unless pathogenic organisms gain entrance from the outside to the bile passages, but under favorable conditions, using aseptic technique in making the bile collections, the bile may be kept sterile for a number of weeks.

The quantitative analysis of brilliant vital red present in the bile collected offers certain difficulties. We had hoped that it would be possible to make estimations by means of the spectrophotometer, using a previously described method (6) for the quantitative analysis of colored mixtures. To investigate this possibility artificial mixtures of bile and brilliant vital red were made, but it was found that the absorption curves of the two substances were not strictly additive, though approximately so in certain portions of the spectrum. There was the further complication that the absorption bands of these two substances overlap to a very considerable extent. Both absorb light in the green and blue much more than in the red. Although the absorption curves for the two substances are by no means identical, yet the relations are such that no great precision can be expected by spectrophotometric methods. For these reasons we have adopted the well known and simple method of analysis based on the use of the "color comparator."

Two series of standard solutions are prepared, one containing bile in varying concentration from 1 to 10 to 1 to 60, the other containing brilliant vital red vary-

ing from 1 to 15 mg. per liter. The unknown is diluted to a varying degree but usually to about 1 to 30 and is poured into a test tube and placed in a rack behind another tube containing water. The light transmitted through these two tubes is then matched in quality and in intensity with that produced by placing in allignment two of the standard dye and bile tubes. By making successive trials a combination of a dye and a bile tube can be found which matches very closely the unknown, both as regards shade and intensity. Obviously, care must be taken that all of the tubes used are of the same diameter. The method is quick and simple and when dye is present in the bile in considerable quantities—200 mg. per liter or more—the error is somewhat less than 10 per cent, as can be shown by test analyses of known mixtures of dye and bile. The physiological changes which we will discuss are several times this experimental error, so that the method of analysis answers fully the present requirements.

EXPERIMENTAL

Brilliant vital red injected into a dog by the intravenous route disappears from the circulation at a rate which varies slightly with different individuals, but we have numerous experiments to show that for a given dog the experiment may be repeated at intervals of weeks or months with almost identical results. The elimination rate for the dog may be determined by a preliminary test dose of dye, and after several weeks the tissues will be almost entirely free of the latter and we are at liberty to repeat the experiment either with or without the introduction of procedures calculated to influence this established disappearance rate of dye.

Dog 24-74. Shepherd. Weight 32 kg. (See Table 41.)

February 3, 1928. Control experiment to determine normal elimination from the blood stream of brilliant vital red. Twenty cubic centimeters of an aqueous 2 per cent solution of brilliant vital red were injected into the jugular vein. At varying intervals thereafter samples of blood from the opposite jugular vein were taken into oxalate solution, and after centrifugalization the concentration of dye in the supernatant plasma was determined by means of the spectrophotometer. The results are given in the second column of Table 41.

March 25, 1928. Experiment to determine the effect of ink injection on the disappearance rate of dye. At 9:52 A.M. 20 cubic centimeters of 2 per cent brilliant vital red were injected. The 5 minute sample was taken at 9:57 A.M. and immediately following this, 10 cc. of Higgins' American India ink were injected into the jugular vein. The ink disappeared rather rapidly from circulation and the blood samples taken 1 hour later and subsequently contained no ink whatever. The amount of dye in the various samples of plasma taken during this

elimination period of 4 days is shown in the third column of Table 41. At no time during or following the injection of the dye or ink was there any evidence of sickness on the part of the dog.

May 20, 1928. Dye injection to test elimination rate 2 and 6½ months after injection of India ink. The experiment was carried out as on February 3. The results are shown in the fourth and fifth columns of Table 41.

TABLE 41

Dog 24-74. Effect of Ink on Disappearance of Dye from Plasma

Time after dye injection	Brilliant vital red in 1000 cc. plasma			
	Dye without ink	Dye with ink*	Dye 2 months after ink	Dye 6½ months after ink
	mg.	*mg.*	*mg.*	*mg.*
5 min.	260	260	240	255
1 hr.	225	225	225	215
6 hrs.	145	160	160	155
24 hrs.	37	114	72	43
48 hrs.	18	55	26	17
72 hrs.	8	32	16	13
96 hrs.	4	11	5	3

* Ink 5 mins. after dye injection.

TABLE 42

Dog 24-96. Effect of Ink on Disappearance of Dye from Plasma

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink	Dye with ink*	Dye 4½ months after ink
	mg.	*mg.*	*mg.*
5 mins.	480	460	460
1 hr.	335	460	390
6 hrs.	310	350	315
24 hrs.	160	240	180
48 hrs.	74	140	98
72 hrs.	57	91	54
96 hrs.	31	81	48

* Ink 5 mins. after dye injection.

Dog 24-96. Shepherd. Weight 15 kg. (See Table 42.)

February 17, 1928. Control to determine rate at which brilliant vital red normally leaves the blood stream. Twenty cubic centimeters of 2 per cent dye injected into jugular vein. Samples of blood taken at intervals during the next 4 days

were received into isotonic sodium oxalate (1.6 per cent) solution. Each sample was promptly centrifuged. The amount of dye in the supernatant plasma in each case is shown in the second column of Table 42.

May 27, 1928. The effect of an injection of India ink upon the disappearance rate of the dye. As in the case of dog 24-74, 20 cc. of brilliant vital red were injected and 5 minutes were allowed for admixture with the circulating blood. Immediately after the 5 minute sample of blood had been taken for dye analysis, an injection of 10 cc. Higgins' American India ink was made. That the ink particles were rapidly swept out of the blood stream is shown by the fact that a blood sample taken an hour later contained no trace of carbon. The rate of dye elimination from the blood is indicated by the figures shown in column 3 of Table 42. There were no signs that either dye or ink were toxic to the animal.

TABLE 43

Dog 25-29. Effect of Ink on Disappearance of Dye from Plasma

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink	Dye with ink*	Dye 4½ months after ink
	mg.	*mg.*	*mg.*
5 mins.	370	385	320
1 hr.	325	350	275
6 hrs.	205	320	215
24 hrs.	102	150	109
48 hrs.	50	94	61
72 hrs.	33	65	37
96 hrs.	17	48	20

* Ink 6 hours before dye injection.

October 7, 1928. Disappearance rate of dye 4½ months after injection of India ink. The experiment is identical to that carried out on February 17. The concentration of dye in the plasma in each of the samples taken is shown in column 4 of Table 42.

Dog 25-29. Collie. Weight 28 kg. (See Table 43.)

March 2, 1928. Control to determine the rate at which brilliant vital red normally leaves the blood stream. Twenty-four cubic centimeters of 2 per cent brilliant vital red injected into the jugular vein. In the second column of Table 43 is shown the concentration of dye in each of a number of samples of plasma collected during the next 4 days.

May 30, 1928. Effect of an injection of India ink upon the disappearance rate of the dye. Unlike the experiments on dogs 24-96 and 24-74 the regular dose of 10 cc. Higgins' American India ink was given 6 hours *before* making the dye

injection. The latter consisted of 24 cc. of 2 per cent brilliant vital red, also given intravenously. No evidence of toxicity either on the part of ink or dye. Plasma collected at varying intervals following the injection of dye was analyzed for its dye content. The results are shown in the third column of Table 43.

October 6, 1928. Disappearance rate of dye 4½ months after injection of India ink. The experiment is similar to that performed on this dog on March 2. The concentration of dye in each plasma sample collected is shown in the fourth column of Table 43.

Dog 27-243. Male collie. Weight 24 kg. (See Table 44.)

April 29, 1928. Control to determine the rate at which brilliant vital red normally leaves the blood stream. Twenty cubic centimeters of 2 per cent aqueous brilliant vital red injected intravenously. In column 2 of Table 44 are given data showing the concentration of the dye in the plasma in various samples subsequently taken.

TABLE 44

Dog 27-243. Effect of Ink on Disappearance of Dye from Plasma

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink	Dye with ink*	Dye 4 months after ink
	mg.	*mg.*	*mg.*
5 mins.	250	300	335
1 hr.	225	280	295
6 hrs.	155	205	185
24 hrs.	70	105	76
48 hrs.	30	74	26
72 hrs.	22	44	17
96 hrs.	11	37	6

* Ink 24 hours before dye injection.

May 30, 1928. Effect of an injection of India ink upon the disappearance rate of the dye. Unlike the other cases the 10 cc. dose of American India ink was given intravenously *24 hours before* the dye was given. As in the control experiment one month previously, 20 cc. of the 2 per cent aqueous brilliant vital red were given. The plasma collected at varying intervals following the injection of dye was analyzed for its dye content. The results are shown in column 3 of Table 44. There was a short period of deep breathing and some nystagmus following the injection of ink. Prompt and complete recovery.

October 7, 1928. Disappearance rate of dye 4 months after injection of India ink. The experiment is identical to the control carried out on April 29. The results are shown in the fourth column of Table 44.

We have a number of experiments, all of which go to show that the intravenous injection of India ink slows up very markedly the rate at which brilliant vital red leaves the blood stream. We submit four such experiments (Tables 41–44 inclusive). The results of these experiments are in agreement and the outstanding features are averaged and shown graphically in Chart 4A. It will be observed that the initial blood sample taken 5 minutes after the injection of dye shows the plasma to contain nearly 350 mg. dye per liter plasma. In all cases the greater part of the dye leaves the blood stream during

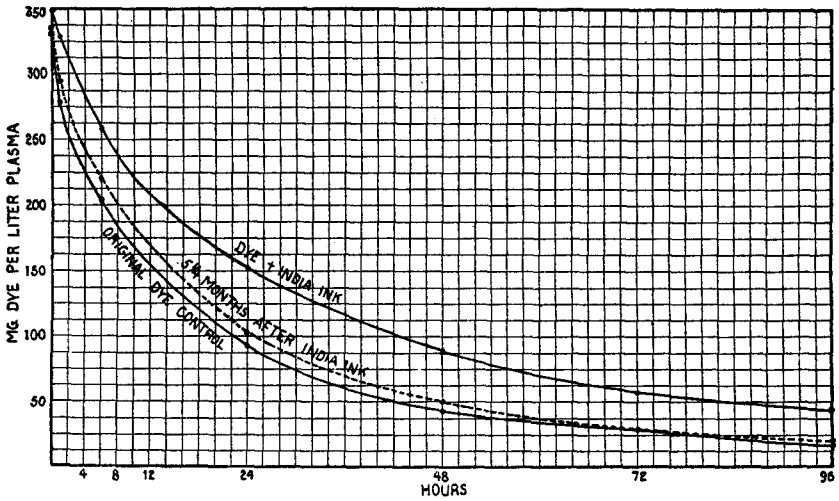

CHART 4A. Elimination of brilliant vital red from plasma. Effect of India ink. (Curves represent the averages of four dogs—Tables 41–44 included.)

the course of the next four days, but there is a most striking retardation in the elimination rate when a small dose of ink is injected at the beginning of the experiment. This retardation begins almost at once and at the end of 48 hours there is twice as much dye in circulation as in the normal control determinations, and later on the discrepancy is even somewhat greater. The change is several times what we know to be the maximum experimental error.

Large doses of India ink are somewhat toxic but the small doses used by us did not disturb the animal. In all cases they ran about in a normal manner and ate the regular ration of hospital scraps.

We have noted occasionally that there is some retardation in coagulation of blood following injection of ink, but hemorrhages into skin, mucous membranes or intestinal tract were never noted. We have abundant proof that the elevation of the dye elimination curve following ink is not due to a decrease in the blood volume, for the centrifuged samples of blood showed a constant ratio of cells to plasma throughout the experiment. Furthermore in two of the experiments, (Tables 43 and 44), the ink was given a number of hours before the dye was injected, and in neither case was the initial dye reading greatly elevated as one would expect if the ink had caused a reduction

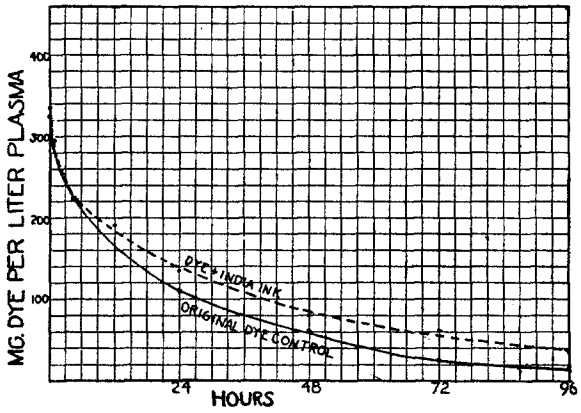

CHART 4B. Dog 27-229. Elimination of brilliant vital red from plasma. Normal control contrasted to curve showing ink given six hours after dye.

in blood volume. There can be no doubt whatever that the small dose of ink has brought about a true retention of dye in the blood stream. Another experiment showing this phenomenon is shown in Chart 4B. Here the administration of ink was delayed until 6 hours after the dye had been introduced into the blood stream. We note that during these first 6 hours the dye curve falls off at a rate which is almost identical to the control done 5 weeks previously, but from the moment the ink was injected we note that the two curves separate, indicating that the ink has acted very promptly in slowing up the disappearance rate of the dye.

Dog 27-229. Weight 22.5 kg. (See Chart 4B.)

April 29, 1928. Control to determine the rate at which brilliant vital red normally leaves the blood stream. Twenty cubic centimeters of 2 per cent dye were injected intravenously. There were no untoward symptoms following this procedure. The concentration of the dye in the plasma at different intervals is recorded in Chart 4B.

June 2, 1928. Effect of an injection of India ink upon the disappearance rate of the dye. Twenty cubic centimeters of 2 per cent dye were injected intravenously, but *6 hours after the dye,* 10 cc. of American India ink were given. During the ten minutes following the ink the animal was dyspneic, his muscle tone diminished and for the rest of the day he seemed somewhat depressed. However, the next day he appeared quite well.

The concentration of the dye in the circulation at different intervals is shown by the dotted line in Chart 4B.

We call especial attention to the results shown in Table 44. In this case the ink was injected 24 hours before the dye was given. The ink caused only slight and very transient clinical disturbance. Of particular significance is the fact that we note well marked delay in the rate at which the dye leaves the blood stream. In fact the delay seems to be as great as when ink and dye are given on the same day. It is clear that the action of ink in retarding dye elimination persists for several days at least. When a test dose of dye was injected 5 months after ink injection (Chart 4A) the rate of dye elimination appears to be almost if not entirely normal. In fact we believe that recovery is practically complete in 3 weeks (Chart 4C) although the departure from normal at that time seems to be slightly more than we can charge to exp nental error. We have other experiments which show retardation 3 weeks after ink even more distinctly than this; still we feel that the effect of the ink largely wears off after the first week.

Dog 28-165. Mongrel. Weight 15.5 kg. (See Table 45.)

May 11, 1929. Control to determine rate at which brilliant vital red normally leaves the blood stream. Fifteen cubic centimeters of 2 per cent dye injected into jugular vein. At intervals during the next 4 days samples of blood were drawn into isotonic sodium oxalate and after centrifugalization the amount of dye in the plasma was determined. The results are shown in the second column of Table 45.

June 1, 1929. The effect of an injection of India ink upon the disappearance rate of the dye. As on May 11, 15 cc. of 2 per cent brilliant vital red were injected

into the jugular vein. Five minutes were allowed for complete admixture of the dye with the circulating blood and at the end of this time a sample was taken for analysis. Immediately thereafter 8 cc. of Higgins' American India ink were injected. Dog was very slightly depressed for about an hour only. No vomiting.

TABLE 45

Dog 28-165. Effect of Ink on Elimination of Dye from Plasma

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink	Dye with ink*	Dye 3 weeks after ink
	mg.	*mg.*	*mg.*
5 mins.	520	455	495
1 hr.	430	390	395
6 hrs.	245	300	305
24 hrs.	160	165	160
48 hrs.	69	110	68
72 hrs.	43	69	43
96 hrs.	28	43	30

* Ink 5 mins. after dye injection.

TABLE 46

Dog 28-184. Effect of Ink on Elimination of Dye from Plasma

Time after dye injection	Brilliant vital red in 1000 cc. plasma		
	Dye without ink	Dye with ink*	Dye 3 weeks after ink
	mg.	*mg.*	*mg.*
5 mins.	410	380	375
1 hr.	340	370	325
6 hrs.	210†	275	230
24 hrs.	96	165	109
48 hrs.	45	86	49
72 hrs.	39	45	24
96 hrs.	39	28	16

* Ink 5 mins. after dye injection.

† By interpolation.

Samples of blood taken an hour after these injections as well as those taken later contained no dye in the plasma though smears showed some carbon in the white cells. The dye concentration was determined at intervals during the following 4-day interval. The results are shown in the third column of Table 45.

June 22, 1929. Disappearance rate of dye 3 weeks after injection of India ink.

The experiment is identical in technical details to that carried out on May 11. The concentration of dye in the samples taken for analysis is shown in the fourth column of Table 45.

Dog 28-184. Mongrel, male. Weight 16.5 kg. (See Table 46.)

As regards dates, dosage and the taking of samples this experiment is in every way identical to that on dog 28-165. The details are given in the protocol of that experiment. As in that experiment there was slight transitory clinical disturbance following the injection, but later on in the day the dog ate well and was normally active. The dye readings in the plasma are given in Table 46.

The fact that an injection of India ink will retard the rate at which brilliant vital red leaves the blood stream might be thought to prove

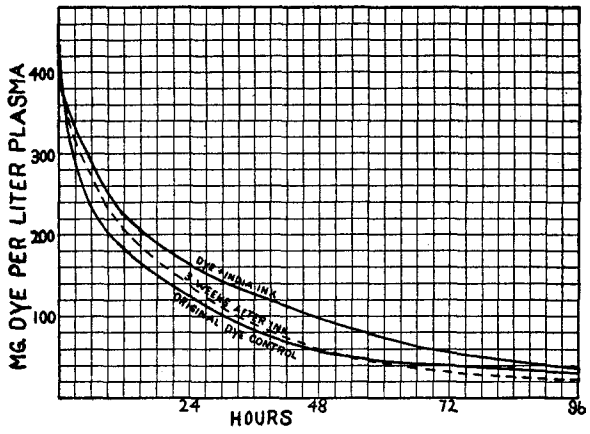

CHART 4C. Elimination of brilliant vital red from plasma. Effect of India ink. Almost complete recovery shown within three weeks. (Curves represent the averages of two dogs—Tables 45 and 46.)

that the ink-laden phagocytes were partially blocked against the entrance of the dyestuff. Indeed, this was our original view. The evidence seems more convincing than much of that commonly offered to support the blockade theory. Our doubts were aroused in the course of studies which we were carrying out on bile fistula dogs. In the course of these experiments it was found that India ink possesses a remarkable ability to slow up or prevent the passage of the dye into the bile. In some cases this effect was associated with considerable diminution in the total volume of bile output per day,

but in many cases the normal amount of bile was excreted. Ordinarily 5 or 10 cc. of ink were injected, and as a rule about twice this amount of two per cent brilliant vital red. In all cases it was clearly seen that for 24 or 48 hours the bile recovered from the animal was of the normal greenish yellow color, quite in contrast to the orange or cherry red colored bile obtained when dye alone was injected. Obviously, such dye retention within the body may well account for the longer retention of dye in the plasma.

In Tables 47 and 48 we present the results of two experiments of the sort just described. In the first, only 100 mg. of dye were injected but we noted that in the normal control period the bile was brightly colored for some days. More dye was eliminated in the first 24 hours than later on, but considerable amounts are present for some time. In all, about half of the dye was recovered in the course of the first 6 days. In the second half of the experiment ink was given along with the dye, and here we noted almost complete failure of dye excretion during the first 24 hours; nor is the inhibition entirely transitory, for the total excreted during the first 4 days is less than half the amount normally excreted in the first 24 hours. Despite this remarkable inhibition in dye excretion we observed no evidence of toxicity on the part of the ink or dye; in fact the dog ate normally throughout the experimental period and the normal amount of bile was excreted. We have analyses to show that in such cases the total output of bile pigments and bile salts also approximates the normal, though the results are somewhat variable. These facts suggest that there is no overwhelming injury to the liver. It would appear that there is a dissociation of functions, for ink seems to inhibit dye excretion without necessarily affecting certain other well established functions. Experiment 32 (Table 48) differs from this experiment in minor respects only. The amount of dye given was three times as great, and as a result we note rather large amounts of dye excreted in the control period. Again, the effect of ink is to suppress the elimination of dye into the bile. The suppression is practically complete for 48 hours, and along with this we note that the daily volume of bile is markedly reduced. Later on the volume of bile returns to normal or slightly above normal, and we observe that some dye is being eliminated, though this activity is rather transitory. In this

case nearly 85 per cent was eliminated during the 9 day control period, but influenced by ink, the liver secreted only one-fourth as much in a similar period.

The dye thus retained within the body becomes distributed between plasma and tissues, coloring each more intensely than normal. We see in these observations a ready explanation for the fact that following an ink injection the dye leaves the blood stream much more slowly than normal, and we feel it unnecessary to suppose that ink has impaired the capacity of the phagocytic system for storing dye.

TABLE 47

Experiment 31. Effect of 5 Cc. India Ink on Excretion of Brilliant Vital Red

	Volume of bile eliminated	Dye per 1000 cc. bile	Total 24 hour dye output
	cc.	*mg.*	*mg.*
Dye (100 mg.) injected without ink			
1st day	85	240	20
2nd day	75	140	11
3rd day	80	120	10
4th day	75	40	3
5th day	60	30	2
6th day	80	15	1
Dye (100 mg.) injected along with ink (5 cc.)			
1st day	85	0	0
2nd day	85	40	3
3rd day	85	40	3
4th day	75	40	3

Dog is a 13 kg. female terrier.

We have been concerned with the question of how it is that ink so completely prevents the excretion of a dye by the liver. There is little to indicate that ink is a liver poison, for we often see apparently normal elimination of bile pigments and bile salts despite the inability of the liver to excrete dye for several days, and with these small doses of ink the dog gives little or no external evidence that the ink is toxic. Work still in progress seems to negate our earlier supposition that the particles of carbon are responsible. We hope to report our

findings more fully in the future, but the evidence seems to indicate that other substances within the ink may be to blame. Finely ground graphite suspended in acacia may be injected without impairing the ability of the liver to excrete dye. Unless such mixtures are made rather strongly alkaline they are quite unstable and the particles are

TABLE 48

Experiment 32. Effect of 7.5 Cc. India Ink on Excretion of Brilliant Vital Red

	Volume of bile eliminated	Dye per 1000 cc. bile	Total 24 hour dye output
	cc.	*mg.*	*mg.*
Dye (300 mg.) injected without ink			
1st day	100	400	40
2nd day	120	400	48
3rd day	110	360	40
4th day	125	240	30
5th day	110	240	26
6th day	130	200	26
7th day	115	160	18
8th day	130	120	16
9th day	120	80	10
Dye (300 mg.) injected along with ink (7.5 cc.)			
1st day	31	0	0
2nd day	23	0	0
3rd day	56	20	1
4th day	140	40	6
5th day	140	120	17
6th day	140	80	11
7th day	140	80	11
8th day	140	80	11
9th day	130	40	6

Dog is a 13 kg. female terrier.

rather large, but we have observed that the particles are distributed about the body in the same manner observed after India ink injections and in each case the Kupffer cells of the liver are heavily laden with carbon particles. These experiments suggest strongly that the inhibitory effects of India ink must be sought in elements other than in the carbon.

This conclusion is reinforced by several experiments in which we have precipitated the carbon from India ink and have observed that the brownish colored fluid so obtained was effective in inhibiting dye excretion by the liver. The carbon of the ink was precipitated by the addition of a known amount of dilute hydrochloric acid and after centrifugalization the supernatant fluid was kept and later combined with certain brownish soluble ingredients which can be extracted from the mass of carbon with the aid of alcohol and of dilute acid. The alcohol was evaporated off and the various carbon-free fractions combined, and enough standard sodium hydroxide added to restore the original alkalinity. As in the case of India ink this solution may be given intravenously without producing any external manifestations of toxicity, but dye elimination by the liver is almost as completely inhibited as in the case of the original untreated ink. It is well known that India ink is rather strongly alkaline. Titration shows that it is neutralized by an approximately equal volume of N/14 hydrochloric acid. We have injected 10 cc. of N/14 sodium hydroxide into the blood stream without observing any decrease in the rate at which the liver will excrete brilliant vital red, simultaneously injected. We do not believe that the alkalinity of the ink is responsible for the inhibition observed with ink injections.

SUMMARY

When brilliant vital red is injected into the blood stream of dogs much of it is slowly taken up into numerous phagocytes scattered throughout the tissues ("reticulo-endothelial system" of Aschoff).

The rate at which the dye leaves the blood stream is determined in large part by the action of these phagocytic cells, but the excretion of dye into the bile is also in part responsible for the loss of dye from the plasma.

The injection of a small amount of India ink into the blood stream results in lowering the rate at which the dye disappears from circulation. The fact that much of the carbon of the ink is promptly taken up by the phagocytes would lead one to suspect that they were saturated with foreign materials, or "blocked" against the entrance of dye, but it is shown that the ink causes a remarkable inhibition of the excretion of dye into the bile, and this alone seems to account for

the longer retention of dye in the blood stream. There is no evidence that any of the retention is due to defective activity on the part of the phagocytes.

Thus, prolonged retention of foreign materials in the blood stream cannot be cited to prove "blockade of the reticulo-endothelial system" unless one can rule out such peculiar reactions on the part of excretory organs. It is felt that the literature of "blockade" should be studied with such sources of error in mind.

Preliminary studies indicate that the suppression of dye excretion by the liver is not due to the carbon content of the ink. Studies of other components of the ink are now in progress.

BIBLIOGRAPHY

1. Smith, *Bull. Johns Hopkins Hosp.*, 1925, **36,** 413; *J. Exp. Med.*, 1930, **51,** 379, 395.
2. Davies, Wadsworth and Smith, *J. Exp. Med.*, 1930, **51,** 549.
3. Smith, *J. Exp. Med.*, 1930, **51,** 379.
4. Rous and McMaster, *J. Exp. Med.*, 1923, **37,** 11.
5. Smith, Groth and Whipple, *J. Biol. Chem.*, 1928, **80,** 659.
6. Smith, *J. Exp. Med.*, 1930, **51,** 369.