ON THE CHEMISTRY AND STAINING PROPERTIES OF CERTAIN DERIVATIVES OF THE METHYLENE BLUE GROUP WHEN COMBINED WITH EOSIN.[1]

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The only justification for writing another paper on histological stains so generally employed as eosin and methylene blue, is the fact that something new has been discovered that adapts them for a wider range of usefulness. Working along this line a stain was prepared several months ago that was so satisfactory in some work on the opsonic index[2] done by the writer that he considered it worth describing at some length.

CHEMISTRY OF EOSIN.

In 1871, Baeyer[3] found that by treating at 195° C. phthalic anhydride with resorcinol, a yellow mass was formed, slightly soluble in alcohol, which he called fluorescein, $C_{20}H_{12}O_5$, the mother substance of eosin. It is insoluble in water, ether and benzol. Its readily formed alkaline solutions show beautiful fluorescence from which can be got bright yellow star crystals; from alcohol the crystals are small dark brown. It is precipitated by acids, appearing as a brick-red powder.

The free acid eosin, tetrabrom fluorescein, $C_{20}H_8Br_4O_5$, is strongly dibasic and was first described by Baeyer[4] in 1876, although two years prior it was made commercially. The dye workers prepared it by taking one molecule of fluorescein and four molecules of acetic acid, adding thereto twenty per cent. solution of bromine. The potassium salt was next formed and the eosin precipitated by dilute sulphuric acid from which the stain was extracted by ether. The alcoholic solution on slow evaporation yields beautiful yellowish-red crystals with a formula $C_{20}H_8Br_4O_5(C_2H_5OH)$. It is precipitated from the watery solution by mineral acids, as yellowish-red amorphous eosin, the latter being more soluble in alcohol than the crystalline form. In the presence of the slightest trace of alkalies, a red-yellow color, and a beautiful yellow-green fluorescence is produced. It is almost insoluble in chloroform and benzol. On reduction with zinc

[1] Received for publication July 12, 1907.

[2] Wilson, *Amer. Jour. of Physiol.*, 1907, xix, 445.

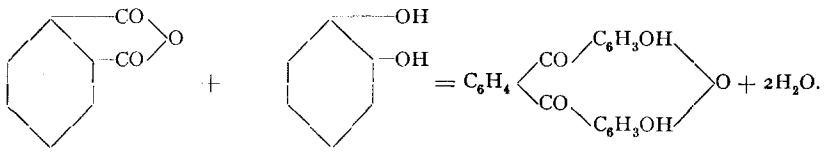
[3] Baeyer, *Berichte Deut. Chem. Gesel.*, 1871, iv, 558.

[4] Baeyer, *Liebig's Annal.*, 1876, clxxxiii, 38.

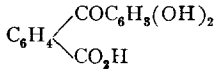
dust and alkalies it becomes colorless. Salts are readily formed by treating it with the corresponding base. The alkali salts are soluble in water, the earthy salts slightly so, while those of the heavy metals are almost insoluble. Silver eosinate, however, is somewhat soluble in water and alcohol. Water-soluble eosin is the potassium or sodium salt. The formula of the former is $C_{20}H_8Br_4O_5K_2 5H_2O$, and its molecular weight 824. It is soluble in two parts of water, and slightly soluble in absolute alcohol. Its tri-clinic crystals are red by transmitted light with a yellow-green lustre. The dilute solution is red-yellow, with strong green fluorescence. Its absorption spectrum shows a broad black band in the green.

Our knowledge of the constitution of eosin depends primarily on the manner of linking one molecule of phtalic anhydride and two molecules of resorcinol, so as to form one molecule of fluorescein and two molecules of water. E. Fischer[5] wrongly claimed that it was done as follows:

Phtalic Anhydride. Resorcinol. Fluorescein. Water.



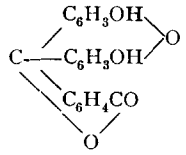
Baeyer[6] found that by heating mono-resorcin phtalein



that fluorescein and phtalic acid were formed according to the equation

$$2C_{14}H_{10}O_5 = C_{20}H_{12}O_5 + C_8H_6O_4 + H_2O,$$

and concluded that it was a derivative of phenolphtalein anhydride, representing it semi-graphically thus:

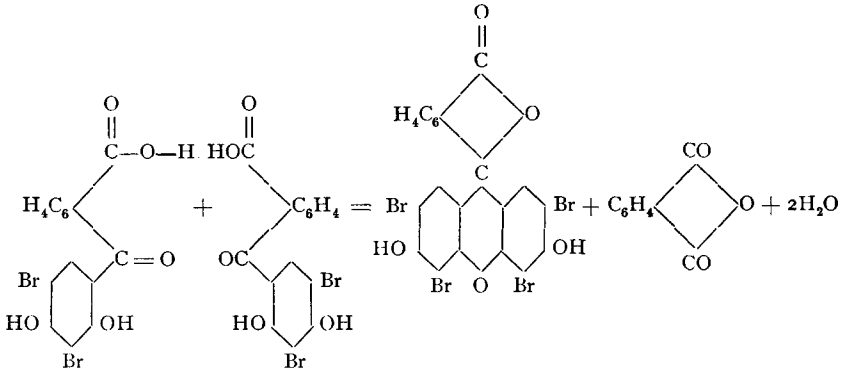


Keller[7] fixed one hydroxyl in a resorcinol radicle in the para-position with regard to its attachment to the phtalic radicle.

R. Meyer[8] obtained 3, 5, dibrom; 2, 4, dioxy-benzoyl-benzoic acid, and some phtalic anhydride by heating eosin above its melting point, and concluded that eosin was formed as graphically shown below.

[5] Fischer, *Berichte Deut. Chem. Gesel.*, 1874, vii, 1211.
[6] Baeyer, *Liebig's Annal.*, 1876, clxxxiv, 24.
[7] Keller, *Berichte Deut. Chem. Gesel.*, 1895, xxviii, 321.
[8] Meyer, R., *Berichte Deut. Chem. Gesel.*, 1896, xxix, 2624.

3, 5 Dibrom, 2, 4 Dioxy-benzoyl-benzoic Acid. Eosin. Phtalic Anhydride Water.



According to this lactone theory, eosin is tetrabrom-dioxy-fluorane. It possesses two heterocyclic rings, one five-membered, the other having six atoms. In the former is found two acid ketonic chromophores, $=C=O$. One of them is, however, replaced by an oxygen atom, $=O$, and in the six-membered ring there is another similarly substituted ketone. Linked on, as side chains, are two acid auxochromes, $O-H$, which both intensify the color and aid in the electrical dissociation. The addition of bromine atoms increases the color toward the blue end of the spectrum.

CHEMISTRY OF CERTAIN METHYLENE BLUE DERIVATIVES.

Methylene blue was first prepared in 1876 by Caro,[9] and a few years later Bernthsen[10] published two articles on the chemistry of the whole methylene blue group. Bernthsen's is still our best single source of information on this subject.

Methylene blue is prepared by oxidizing dimethyl-phenylene diamine with ferric chloride in the presence of hydrogen sulphide. Its formula is $C_{16}H_{18}N_3SCl + 3H_2O$ and its molecular weight 373. At 100° C. it possesses three molecules of water, but loses these at 150° C. It crystallizes, by means of sodium chloride and hydrochloric acid, in small glittering microscopic leaflets, which have either a copper or bronze-colored lustre which, on cleavage, shows a cantharides gloss. It is soluble in water and alcohol. Its cold blue aqueous solution is not destroyed by strong hydrochloric acid, weak nitric acid or by alkalies. It is not destroyed by boiling its aqueous solutions. It forms a base in the cold with fresh silver oxide. By long boiling with weak alkalies or silver oxide, it yields methylene violet, methylene azure, and their leuco-bases, besides other lower derivatives, such as dimethyl-amine. Its spectrum in dilute solution gives a dark band between B and C, and a dim one to the left of D, with the maxima at λ 670 and λ 610, respectively.[11]

[9] Caro, *Berichte. Deut. Chem. Gesel.*, 1876, xi, 360.

[10] Bernthsen, *Liebig's Annal.*, 1885, ccxxx, 137; also 1889, ccli, 1.

[11] λ equals a thousandth part of a μ.

Methylene azure,[12] $C_{16}H_{18}N_3SO_2$, is an oxidation product formed by boiling weak alkalies or silver oxide with methylene blue. Its direct separation in pure form from methylene violet has not been accomplished, but by converting these bodies into their leuco-compounds the azure can, by alkalies, be separated from the leuco-methylene violet. After this process, the leuco-azure can, by oxidation, be readily reconverted into the methylene azure. It is soluble in ether, with raspberry color; in water, with blue color; while in alcoholic and chloroform solution it is violet red, and in benzolic and xylolic, pure red. The leuco-compound obtained by alkalies and stannous chloride is insoluble in alkalies.

Methylene violet, $C_{14}H_{12}N_2SO$, is also formed in the boiling of methylene blue with an excess of silver oxide. Its crystals separate out, and can be purified by dissolving them in hot alcohol, to which it gives a violet color and red-brown fluorescence. It is slightly soluble in ether, cold or hot water, and more soluble in chloroform and acetone. It forms long black greenish glittering needles.. The hydrochloride of the violet is obtained by crystallizing the base with dilute hydrochloric acid, when beautiful centimeter-long crystals appear.

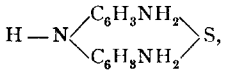
Leuco-methylene blue present in the precipitate formed by boiling silver oxide with methylene blue is soluble in ether, cold alcohol, and slightly in water. It crystallizes in broad atlas glittering needles and oxidizes in the moist condition very easily, forming methylene blue. It is insoluble in strong potassium hydrate. Like other leuco-derivatives of methylene blue, it is colorless, due to the loss of its double nitrogen bond, and the introduction, therefore, of hydrogen.

Methylene red is always present in the mother liquid from which methylene blue is prepared. It is soluble in water, and alcohol, giving thereto a violet-purple color. It is not soluble in ether. The formula for the chloride was given as $C_{16}H_{18}N_4S_42(HCl)$, which later research has shown should be halved.

A last compound of interest is the free base of methylene blue, $C_{16}H_{18}N_3SOH$, which is obtained from the chloride by treating it several days with luke-warm, freshly prepared silver oxide. On evaporating this solution over sulphuric acid, a green amorphous mass with metallic lustre remains. It is insoluble in ether, but very soluble in water and alcohol.

The Structure of Methylene Blue.

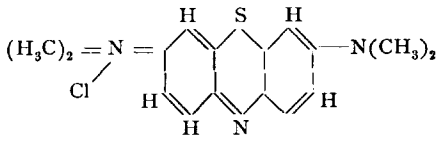
The different methods of preparing thionin, or Lauth's violet,



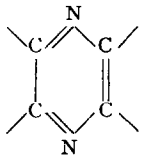
from (*a*) p-phenylene diamine, (*b*) thio-diphenyl amine, (*c*) p-diamido-diphenyl-amine, show that its real structural name should be diamido-diphenthiazine, and since it is the mother substance of methylene blue, it furnishes the starting point for determining the constitution of the latter body.

Methylene blue, according to the quinone theory, is the chloride of tetra-methyl-diamido-diphenthiazine.

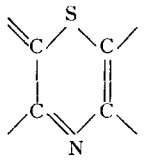
[12] Michaelis, *Chem. Cent.*, 1901, ii, 708.



The termination "azine," also called azinium, indicates the substitution of one or more methin groups by nitrogen atoms in a six-membered mono-hetero-



atomic ring. For example, the radicle would be a p-diazine. If, now, one of the nitrogen atoms in it were replaced by a sulphur atom, the group would then be called p-thiazine.



It would consist of one sulphur, one nitrogen and four carbon atoms. It would still be a stable body, since the replacement of methin groups by nitrogen, or the second replacement of nitrogen by sulphur, affects the stability of a six-membered ring very slightly, as shown by the high melting and boiling points of many compounds in which this ring occurs. The strong basic chromophore indamin $-N=$ occurring as part of the thiazine ring has two of its bonds united to one benzene ring, while the third is linked to the other benzene. The presence of the sulphur atom in the thiazine group might be regarded as a substituted ketonic acid chromophore, $=C=O$ becoming $=S$, lessening, however, the strong basicity of the indamin, but still leaving p-thiazine decidedly chromogenic. This group linked with the two benzene rings forms dipenthiazine.

A glance at the graphic formula of methylene blue will show that there is a substituted hydrogen atom in each of the two benzene nuclei which occupies the para- position with regard to the nitrogen in the thiazine group. If, then, one of these hydrogens is replaced by a dimethylamine and the other hydrogen by a double-bonded nitrogen, carrying two methyl groups and one chlorine atom, the resulting compound will be methylene blue. In Bernthsen's theory, the nitrogen in the heterocyclic ring is united to the nitrogen carrying the chlorine atom. The strongly basic auxochrome, NH_2, attached to one benzene ring does not influence the dissociation of chromogen, as do the other salt formers. It, however, intensifies the color of the chromophores and gives the dye a basic character. It never dissociates electrically, but it influences other nitrogen atoms which may be present and gives them power to unite with the tissues. The

center of each spectrum band is moved λ 15 for each methyl group introduced. The substitution of the two methyl groups in the amidogen side chain renders the stain darker and slightly less basic. Why these phenomena occur is still unknown. In the other benzene ring there is present the group $=N(CH_3)_2Cl$ holding on to its benzene ring by a double bond. It may, therefore, be considered as a substitution for the oxygen in the acid chromophore, $=C=O$, in which the oxygen atom is replaced by two of the bonds of a pentad nitrogen. This replacement not only annuls the acid power of the chromophore but renders it decidedly basic. Moreover, the presence of this pentad nitrogen renders the whole dye a chloride of an ammonium base which dissociates into the colorless anion Cl and the remainder, the colored kation. Thus the presence of the three basic chromophores more than counterbalances the single acid one and so the compound as a whole is basic.

HISTORY OF METHYLENE BLUE AND EOSIN STAINING.

Although eosin was first made in 1874, and methylene blue two years later, it was not till 1891 that Romanowsky[13] first employed a combination of the two for histological purposes. In staining for Plasmodium malariæ, he sought to obtain an aqueous solution of a neutral compound of the two ingredients. He, therefore, took one part of a saturated solution of methylene blue and two parts of one per cent. water-soluble eosin. His sections fixed by heating for one hour at 105° C. sometimes required several hours to bring about the desired staining effect. His successful results with human blood which did not always occur, are given in Table I.

Unna[14] founded the ingenious method of special contrast staining of certain tissue cells by using polychrome methylene blue. He obtained metachromatic staining of Bacillus tuberculosis, *mast*, and plasma cells by alkalinizing ordinary methylene blue, and thus obtaining some methylene violet and methylene azure, which latter substance he wrongly called methylene red.

Mathews[15] was the first to bring out the fact that staining with methylene blue, as with any other basic dye, depends upon the alkaline reaction of the tissues.

Ziemann,[16] who made use of the same stain as Romanowsky, observed that the precipitate formed upon the union of eosin with methylene blue was essential for the success of the stain, and was soluble in an excess of either constituent. By adding borax, potassium hydrate or sodium carbonate, he could always get a body capable of staining plasmodia. This body he supposed to be a compound of eosin. He employed it in staining flagella, fungi and various bacteria. It gave the same results as a good Romanowsky stain, and was more certain, for reasons to be shown later.

Laurent[17] united the dyes according to their valencies. Methylene blue being

[13] Romanowsky, *St. Petersburg. med. Woch.*, 1891, viii, 297.
[14] Unna, *Zeit. f. wissensch. Mikros.*, 1892, viii, 475.
[15] Mathews, A. P., *Amer. Jour. of Physiol.*, 1898, i, 449.
[16] Ziemann, *Cent. f. Bakt.*, 1898, xxiv, 945.
[17] Laurent, *Cent. f. allg. Path. u. Anat.*, 1898, ix, 86.

monobasic requires twice its molecular weight to neutralize one volume of the dibasic acid, eosin. After thus forming the precipitate, it was kept in well-stoppered bottles, and before using, was shaken and a portion diluted four times. It was then boiled for half an hour, thus dissociating the neutral salt. The colored ions remain in this dissociated condition for five or six hours. The time required for staining with the cold liquid was from thirty minutes to five or six hours. The specimens were differentiated in absolute alcohol.

Rosin[18] points out that when methylene azure solution is evaporated it forms a red-violet body. This is the essential ingredient in Unna's polychromatic stain, and forms in long-standing methylene blue. The variability[19] of the staining reaction of methylene blue and eosin led to his finding five different bodies soluble in alcohol and water, but differently soluble in chloroform. This latter fact enabled them to be separated. One body he designated methylene blue eosin. Its blue violet needle-shape crystals[20] have a metallic lustre and decompose on exposure, becoming dark brown. They are insoluble in cold water, but the washings will continue for days to show a bright rose tinge with green fluorescence. In hot water they are slightly soluble, with strong fluorescence. The body is difficultly soluble in alcohol, with blue violet-green fluorescence. Mineral acids change this color, which fails to return on neutralization. With organic acids, the color of alcoholic solutions varies from pure blue to blue green. Alkalies change the solution to red, and violet blue returns on neutralization. It is slightly soluble in chloroform, with red violet color, and no fluorescence; but absolutely insoluble in ether. It stains celloidin, mucin, nuclei, and Nissl granules blue, while protoplasm of nerve cells, proteids, fibrin, and even glass become rose-red. Cytoplasm strikes a red tint, and neutral granules become violet. He claims that this stain, like all others formed by the union of basic and acid dyes, differentiates the tissues into their component parts, staining the nuclei blue, and the cytoplasm red. He also claims that methylene azure and methylene violet are two other bodies formed in uniting methylene blue and eosin. This statement has been indirectly dealt with in discussing the work of Bernthsen, and will be referred to later. Methylene orange, a fourth compound, is very soluble in ether and appears to be nothing but tetrabrom fluorescein derived from the water-soluble eosin. The remaining substance is a black dye, insoluble in ether, but soluble in chloroform.

Zettnow[21] shows how to treat the methylene blues manufactured by the different German firms, with alkalies to get the best results in staining bacteria and blood. In differentiation, he used 0.2 per cent. eosin, and 0.1 per cent. methylene blue.

Nocht[22] showed that neither polychrome methylene blue nor eosinate of methylene blue taken singly, but a combination of the two was essential for the staining of malarial parasites. Hence it was that Unna's polychrome added to Romanowsky's mixture increased the intensity of that stain. Nocht's directions

[18] Rosin, *Deut. med. Woch.*, 1898, xxiv, 616.
[19] *Idem., Cent. f. Physiol.*, 1899, xiii, 561.
[20] *Idem., Berl. klin. Woch.*, 1899, xxxvi, 251.
[21] Zettnow, *Zeit. f. wissensch. Mikros.*, 1899, xvi, 254.
[22] Nocht, *Cent. f. Bakt.*, 1898, xxiv, 839.

required 1 c.c. of neutralized polychrome methylene blue (litmus paper as an indicator) and 1 c.c. of water, then ordinary concentrated solution of methylene blue till the polychrome color had changed to a clear blue. This mixture is now added to 0.1 per cent. water-soluble eosin. The specimens took up this stain in from three to twenty-four hours, and were differentiated by very dilute acetic acid. In his second paper,[23] Nocht modified the stain by treating at 50° to 60° C. ordinary one per cent. methylene blue with 0.5 per cent. sodium carbonate, cooling and adding it to one per cent. eosin, until the resulting color shows no red. A slight excess of either does not affect the stain. Nocht avoids committing himself as to the real nature of the ingredient essential for staining malaria parasites by calling it "*Rot aus Methylenblau.*" He claims it is neither methylene red nor methylene violet. A water solution of fresh methylene blue gives to chloroform a blue tint, while a polychrome solution gives to it a red color. A study of Bernthsen would have thrown light on this color-producing material.

Laveran,[24] who discovered the parasite of malaria in 1881, worked on this compound stain. He employed the free base of methylene blue, called by him *Bleu Borrel,* after Dr. Borrel, who prepared it by adding fresh silver oxide to any methylene blue, and letting it stand for several days. 1 c.c. of this base was added to 5 c.c. of 0.1 per cent. eosin and 4 c.c. of distilled water. This stained the nuclei of the hæmatozoa violet and their protoplasm blue.

Jenner[25] simplified this stain greatly. He united equal volumes of 1.5 per cent. eosin and one per cent. methylene blue and collected the precipitate, which was dried at 55° C., and dissolved in pure methyl alcohol. The air-dried smear of blood was flooded with the stain for one to three minutes which was then poured off; the film was rinsed in distilled water, dried in the air, and mounted in balsam. He claims that good results can be obtained by making separately alcoholic solutions of methylene blue, and eosin, and then mixing them.

Leishman[26] made a solution of 0.5 per cent. of sodium carbonate in one per cent. methylene blue, heated it at 65° C. for twelve hours, and let it stand for ten days. To this he added an equal volume of 0.1 per cent. eosin and dissolved the precipitate in pure methyl alcohol. He applied the stain in the same manner as Jenner, leaving it undiluted on a blood specimen one and one half minutes, then diluting it with double the volume of distilled water and allowing it to act for from five to ten minutes.

Wright[27] steams at 100° C. one per cent. sodium bicarbonate for one hour, and after cooling adds 0.1 per cent. eosin till a purple color is formed with a metallic scum. He states the proportions as two of methylene blue solution to one of eosin. The collected precipitate is dried and dissolved in pure methyl alcohol to make an 0.5 per cent. solution. He applies it by nearly flooding the slide containing the air-dried specimen for one minute with the stain. Water, drop by drop, is next applied until a scum forms, but not enough to make the fluid transparent. In this condition it remains from two to three minutes. He

[23] Nocht, *Cent. f. Bakt.,* 1899, xxv, 764.

[24] Laveran, *Compt. rend. de la Soc. de Biol.,* 1899, li, 249.

[25] Jenner, *Lancet,* 1899, i, 370.

[26] Leishman, *Brit. Med. Jour.,* 1901, i, 635; 1901, ii, 757.

[27] Mallory and Wright, Pathological Technique, 1904, 3d edition, p. 370.

TABLE I.

STAINING EFFECTS.

	Romanowsky.[28]	Nocht.	Jenner.	Leishman.	Wright.	Eosinate of Thionin.
Red corpuscles.	Rose red.	Rose to brown red.	Terra cotta.	Pale pink or greenish tint.	Orange or pink.	Pale blue.
Platelets.			Mauve.	Red.	Dark lilac.	
Nucleated reds.	Deep violet.			Ruby red (fine red granules).		
Lymphocytes.	Blue.				Purplish blue nuclei; robin egg blue cytoplasm.	Purplish blue.
Large monon. leuc.						Lavender.
Nuclei of polym. leuc.		Carmin violet.	Pink.			Purple.
Granules of polym. leuc.	Bright violet.				Red lilac.	Pale red.
Granules of eosinophiles.			Red.	Pale pink.	Eosin.	Reddish.
Granules of basophiles.			Violet.	Greenish purple or black.	Purplish.	Purple.
Nuclei of plasmodia.	Carmin violet or bright red.	Bright red.		Ruby red.	Lilac red to black.	
Protoplasm of plasmodia.	(Granules black) clear blue.	Pure blue.			Blue.	
Protoplasm of white corpuscles.	Blue.	Blue or eosin.				Pale blue.
Bacteria.			Blue.	Blue.		Dark purple.

[28] Jackson, Tropical Medicine, 1907, p. 320.

differentiates by means of water, examining microscopically until the red corpuscles become pinkish. When the proper color develops, the specimen is quickly dried between layers of filter paper and mounted in Canada balsam.

The staining results of the different investigators are given in Table I., and along with them, those brought out by using eosinate of thionin.

This survey of the literature and examination of the chemistry shows that eosin and certain derivatives of the methylene blue group are essential to obtain certain effects in the staining of blood. It was first pointed out by Nocht that eosinate of methylene blue stains the basic cytoplasm and the acid chromoplasm, and the presence of "*Rot aus Methylenblau*" produces the characteristic malarial parasite staining. Nocht, however, failed to state what that specific staining body really was chemically. By Rosin and others it was assumed to be methylene azure, although this was not demonstrated. As to the staining functions of other derivatives of methylene blue which are invariably present, nothing is recorded.

For the purpose of throwing light on these problems, and, if possible, making the preparation of these stains less empirical, an attempt was made to isolate these derivatives and study their staining reactions for normal and abnormal elements in the blood, and also, to ascertain what bodies are formed by the action of the silver oxide or dilute alkalies, and finally, by using the facts acquired, to treat a specimen of methylene blue so as to obtain a known mixture of its chief derivatives, and then to combine them under proper conditions with eosin.

THE ISOLATION OF CERTAIN METHYLENE BLUE DERIVATIVES.

Preparation of Methylene Violet.

Methylene violet was prepared by boiling 0.5 per cent. methylene blue with 0.5 per cent., silver oxide for about six hours. A drop placed on the slide showed microscopically, besides the silver debris, a purple flocculent precipitate, soluble in ether, and crystals in the form of sea urchins, whose rays were dark brown by transmitted light, and when dried, dark green by reflected light. These latter forms were insoluble in both cold water and ether, but soluble in hot alcohol. The boiled methylene blue was extracted with ether

and the filtered residue first washed with cold water, and then treated with hot alcohol. The resulting solution gave on standing in an atmosphere of hydrogen almost pure crystals of methylene violet. Suspended in dilute hydrochloric acid, one sees the crystals disappear, surrounded by a purplish halo. The resulting small star crystals of the hydrochloride were found insoluble in 0.5 per cent. hydrochloric acid, soluble in cold 95 per cent., insoluble in 10 per cent. alcohol and insoluble in distilled water, dilute alkalies and ether. The purplish alcoholic solution gives with sulphuric acid, a port wine color. Chloroform dissolves the crystals, with red brown fluorescence. Tar soap gave with the alcoholic solution a pretty green color. The methylene violet prepared as described above was soluble in xylol, benzol, and toluol, with red color. Potassium bichromate gave a dark carmin brown precipitate, made up of star crystals about seven μ in diameter. Zinc chloride, and hydrochloric acid gave violet fluffy precipitate. A solution of the methylene violet in methylated spirits stained the nuclei purple, and the red corpuscles greenish blue. Methylene violet will not unite with eosin in alkali or in neutral solutions. The hydrochloride, however, of the methylene blue readily forms a purplish compound with eosin which stains blood smears faintly.

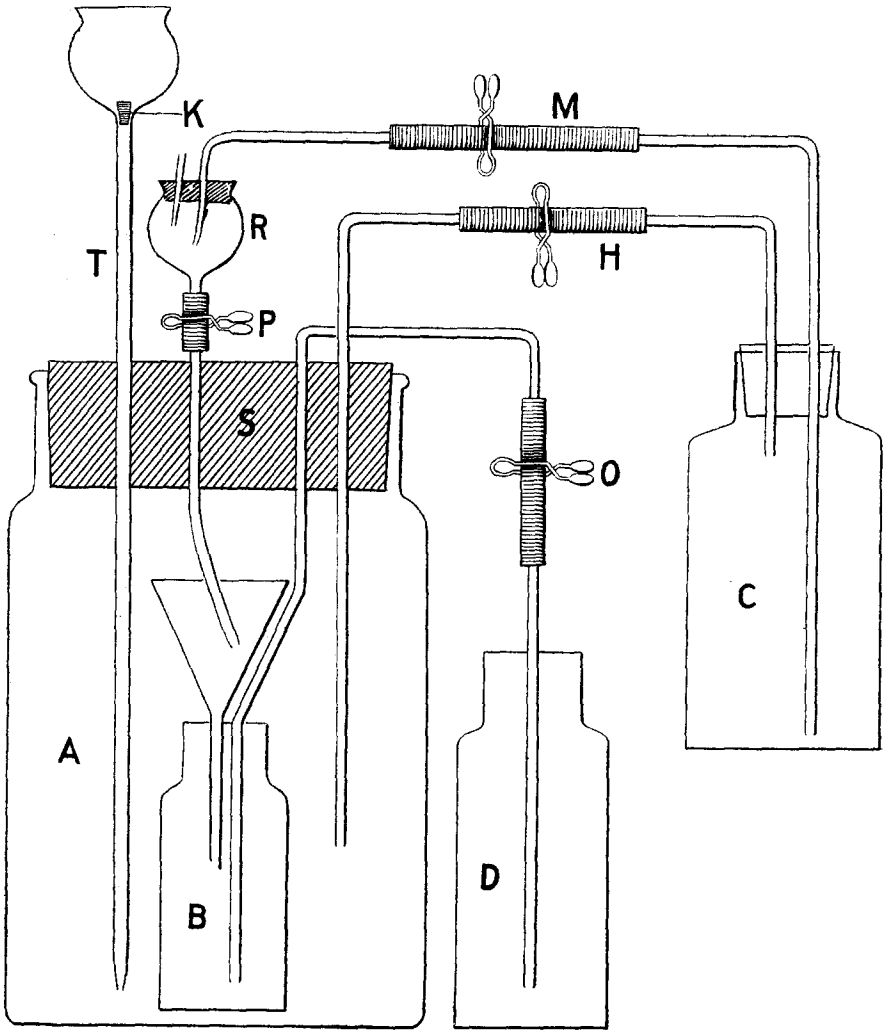
Preparation of Methylene Azure.

The isolation of methylene azure was more difficult. Ether as its solvent was discarded, because methylene violet was also found in the extract. Partial precipitation of a chloroform extract also proved unsatisfactory, since the high concentration at which separation occurred was so great that, although the filtration was performed in an atmosphere of chloroform, no reliance could be placed on the purity of the product. It was found, however, that leuco-methylene blue and leuco-methylene azure are insoluble in sodium hydrate, whereas leuco-methylene violet is soluble. If, then, the boiling with silver oxide were continued till all the methylene blue had disappeared, the other two bodies could be separated. For the purpose of ascertaining when this disappearance occurred, 1 per cent. methylene blue, 1.5 per cent. silver oxide, and 0.5

per cent. sodium bicarbonate were boiled together. Extracts from the specimens slightly acidulated with hydrochloric acid were made with chloroform at intervals of ten minutes. This process removed the methylene azure and the violet, from the other bodies insoluble in chloroform. The filtrate from the residue was treated with hydrochloric acid. The persistent blue color, even when extremely diluted and treated with ammonia water proved the presence of methylene blue. In this way it was found that in forty-five minutes all the methylene blue had disappeared from the material. A similar specimen, boiled forty-five minutes, was filtered so as to remove the debris, and most of the methylene violet and the filtrate reduced by means of a hot strongly alkaline stannous chloride solution. To it was added half its volume of alcohol, and the resulting leuco-methylene azure was allowed to crystallize. The product was filtered and washed in an atmosphere of hydrogen. The leuco-base was oxidized into the hydrochloride and then studied. It was found to stain the red corpuscles green, the lymphocytes blue, and the nuclei of the polymorphonuclear leucocytes deep purple. These staining effects were found to be identical with those from the same methylene blue mixture when boiled thirty minutes with hydrochloric acid and then neutralized.

Several experiments led to the inference that methylene azure does not unite with eosin when the latter is added to a methylene blue mixture containing azure. Ether is a solvent for methylene azure. When an extract of the latter is evaporated, the residue is very slightly soluble in water or dilute alkalies, and no precipitate is detected microscopically when eosin is added to the saturated blue aqueous solution. If, however, the extract is evaporated in an atmosphere of hydrogen, or if some acid is first added, then a fine pale purplish precipitate is visible under medium magnification. Care in the latter case, however, must be exercised to avoid so much acid as to precipitate the yellowish red tetrabrom-fluorescein from the water-soluble eosin. Proof of the correctness of the inference was further secured by observing that as much matter soluble in ether was obtained from the mixture treated with eosin as from that untreated with it.

The apparatus used in the filtration of leuco-methylene azure and of other bodies to be dealt with later is shown in the diagram which follows.



The small bottle, *B*, with funnel and filter was placed in the large bottle, *A*, containing some water and zinc. Through its large rubber stopper, *S*, pass four tubes fitting snugly to prevent leakage. The tube, *T*, dipping under the water in *A* is closed by a small rubber cork, *K*, which is only removed when acid is being

added for the generation of hydrogen. *R* is a thistle tube with rubber connection and attached clamp to permit the entrance of the fluid to be filtered. On opening the clamp at *O* the pressure of the hydrogen forces the filtrate into the bottle *D*. When the clamp on *H* is open, hydrogen passes into the corked bottle *C* containing the material for filtration. On closing the outlets from *A* the pressure drives the solution through the open tube, *M*, into the receptacle of the thistle tube, *R*. As soon as the latter is filled, *S* is clamped, *O* is opened, and after the pressure has gone down, the pinch cock, *P*, is opened, and the material passes on to the filter paper.

The Examination of Thionin, Thionolin and Thionol.

These three bodies should receive a passing notice, since their presence was strongly suspected in the boiled methylene blue solution along with the two bodies already identified. Thionol was prepared from thionin by boiling it with dilute sodium hydrate, and the product, supposedly thionolin, formed by boiling thionin for one hour with 500 times its volume of water. The three substances were examined chemically, and the results are recorded in Table II, along with those of the two other products derived from methylene blue.

On Certain Bodies Obtained from Methylene Blue when Boiled with Dilute Alkalies.

I next sought a rapid simple method of identifying the methylene violet and methylene azure already isolated, and estimating approximately the quantity of each produced by the ordinary treatment of methylene blue with dilute sodium bicarbonate.

The spectrum of a dilute solution in alcohol obtained from the dry ether extract gave an absorption band, reaching from 665λ to 541λ, and darkest at 628λ. It occurred towards the red end of the spectrum and terminated abruptly, while towards the portion of the spectrum where the rays were least refrangible, the band was dim and gradually faded away. An alcoholic solution from a second chloroform extract gave a band from 651λ to 550λ, darkest at 594λ. This analysis indicates that ether and chloroform extract different bodies, hence these solvents were considered best adapted for my purpose. Ether dissolves chiefly methylene azure, while chloroform extracts both the azure and the violet, and

TABLE II.

Reagent.	Methylene Blue Chloride.	Methylene Violet.	Methylene Azure.	Thionin.	Thionolin.	Thionol.
Crystals.	Long, thin, bluish needles. Small glittering leaflets. Blue violet.	Rhomboids deep brown, sea urchins, sheaves, needles. May be purplish brown with greenish color on reflected light.	Dirty brown. Occasionally needles or hair-like forms.	Forms yellow precipitate. Microscopic needles. Sometimes black crystalline powder. Weak green shining crystals.	Metallic green powder.	Brown red, fine, dirty precipitate. Metallic green.
Cold water.	Blue. Soluble.	Insoluble.	Nearly insoluble.	Almost insoluble.	Slightly soluble, with red violet color in all dilutions.	Insoluble.
Hot water.	Blue. Soluble.	Soluble.	Purplish blue. No. fluorescence.	Soluble.	Violet color.	Slightly soluble. Reddish color.
Alcohol.	Blue. Soluble.	Soluble in hot 10 per cent. alcohol	Bluish red. Very faint fluorescence, purple on reflected light.	Difficultly soluble. Red brown fluorescence.	Violet. Soluble.	Deep red with violet-red fluorescence.
Ether.	Insoluble.	Insoluble as shown by crystals.	Fiery red. Very slight fluorescence.	Insoluble.	Almost insoluble. Slight tinge of red.	Very slightly soluble. Yellow red. Slight fluorescence.
Chloroform.	Insoluble. Slight blue tinge.	Very soluble.	Purple color. Slight fluorescence.	Insoluble.	Difficultly soluble.	Red yellow. Slightly soluble.
Carbon bisulphate	Insoluble.	Soluble.		Slight trace. Insoluble.	Insoluble.	Insoluble.
Toluol.	Insoluble.	Red color.	Red color.	Insoluble.	Insoluble.	Insoluble.
Ligron.	Insoluble.	Soluble.		Insoluble.	Insoluble.	Insoluble.
Benzol.	Insoluble.		Red solution.	Insoluble.	Difficultly soluble.	Red. Slightly soluble.
Hydrochloric acid	Blue.	Soluble, but the crystals are insoluble in dilute HCl. Sea urchin forms.	No precipitate. Greenish colored solution.	Slight red color. Forms dark purple precipitate with $HgCl_2$ insol. in cold; sol. in hot.	Deep red, strong blue, then violet.	Fine, green, glittering, straight or needles.

TABLE II.—*Continued.*

Reagent.	Methylene Blue Ch oride.	Methylene Violet.	Methylene Azure.	Thionin	Thionlin.	Thionol.
Potassium bichromate.	Needles.	Forms fine pale carmin-brown precipitate. Star crystals, of R. C. size	Purplish precipitate.	Brown precipitate.	Deep red precipitate.	Slight precipitate.
Zylol.	Insoluble.	Red.	Red.	Insoluble.	Insoluble.	Insoluble.
Sulphuric acid.	Green.	Blue. Soluble.	Green in solution	Green color.	Decomposes by heating. Forms Thionol.	Blue in strong acid. Violet reflex. In dilute acid, violet-red to red.
Zinc, chloride and sodium hydrate.	Purple.		Deep red brown.	No precipitate.		
Dilute alkalies	Blue.	Insoluble.	Slightly red.	Deep red color, then precipitate.	Deep red.	Violet red.
Eosin.	Dark blue precipitate.	No precipitate.	Precipitate pale purple ; slight.	Purple precipitate.	Fine purple star, burr formed crystals.	Deep purple-red precipitate.
Potassium iodide.	Bluish precipitate.		Light brown, fluffy precipitate.	Dark purple.	Light violet.	Brown precipitate.
Aniline.	Green.			Green, soluble which become yellow.	Greenish color.	Red-brown color. Soluble.
Alcohol and sulphuric acid.	Green. Soluble.	Yellowish.	Yellow. Turns brown.	Green. Soluble.	Insoluble.	Eosin color. Soluble, afterwards becoming brown.
Sodium hydrate. Strong.	Blue.	Deep red.	Deep red.	Forms red-brown precipitate.	Forms yellow precipitate of thionol.	Violet color.

methylene blue remains insoluble in both. An ether extract, therefore, contains chiefly the azure, and the residue will have lost its red color, while the methylene violet will be obtained in the second extraction by chloroform. It was therefore proposed to treat different samples successively with ether and chloroform, and estimate by colorimetric methods the amount of the solutes present. A quantity of one per cent. methylene blue, containing 0.5 per cent. sodium bicarbonate, was boiled in a graduated beaker and from it at definite intervals, ten cubic centimeters were transferred into labelled bottles, care being taken to add distilled water to make up for evaporation between the intervals of extraction. From the five minute specimen bottle, four cubic centimeters were transferred to a clean dry test tube to which was added 2.5 times the amount of ether. The mixture was then shaken gently (not vigorously), so as to avoid, as far as possible, carbon dioxide uniting with the unstable azure. Of this extract, eight cubic centimeters, freed from water and from any methylene blue, were transferred to another test tube, which was afterwards filled with coal gas and tightly corked. This pale red solution was used as a color unit. The other tubes were extracted with ether and records kept of the number of dilutions necessary to yield the same tint as the unit. The result, divided by 2.5 gave the quantity of the azure in the specimen as compared with the original. The methylene violet determinations were similar, but on account of the more intense color, much smaller quantities of the specimens were employed for extraction. The standard color unit in this case was obtained by diluting 0.5 cubic centimeter of the ten-minute specimen, twenty times, that is, by adding ten cubic centimeters of chloroform. The findings of another similar experiment wherein 0.2 per cent. sodium bicarbonate was employed instead of the 0.5 per cent., where the same color units of the former series of experiments were retained as units in this shorter series, showed a much smaller increase of material soluble in ether and chloroform, during the sixty minutes' boiling.

The results of both series given in Table III show that the amount of material in the ether and chloroform extracts depends

on the duration of the boiling and the strength of the alkali; and further that the substances soluble in each solvent do not increase in the same proportion.

TABLE III.

TIME IN MINUTES.

	0 m.	5 m.	10 m.	20 m.	30 m.	40 m.	50 m.	60 m.	75 m.	105 m.
One per cent. MbCl boiled with 0.5 per cent. $NaHCO_3$ extracted with ether.	0	1	5	40	80	110	180	194	660	480
The same extracted with chloroform.	0		1	3.5	5	6	6	7.2	14	18.6
One per cent. MbCl boiled with 0.2 per cent. $NaHCO_3$ extracted with ether.	0	1	2	3	3.6			6		
The same extracted with chloroform.	0		0.2	0.4	0.5			2.4		

It was noticed microscopically that at least two distinct forms of colored crystals resulted from methylene blue when boiled with dilute alkalies, but since the literature mentions only one red colored crystalline form, viz., methylene violet, as being thus formed, it was assumed that another compound was present.

Evidence that at Least Four Bodies are Formed.

Various reagents insoluble in water, suitable for making colored extracts from the treated methylene blue such as xylol, toluol, benzol, turpentine, ether and chloroform were tried. Of those investigated, the best solvent for the derivatives of methylene blue was chloroform, which took twenty per cent. of the 105-minute purple specimen. Toluol was next best, ether third, and carbon bisulphide fourth. It was noted that when a chloroform extract was first made, no further extraction could be made with either toluol or ether. Utilizing these findings, four successive extracts (first, ether; second, toluol; third, chloroform; and lastly, carbon bisulphide) were made of a specimen containing one per cent. methylene blue, and 0.5 per cent. sodium bicarbonate, boiled for ten minutes. Similar extracts were made from the same substance, boiled 105 minutes, and the increased color in each extract compared by colorimetric measurements with the corresponding one of the first series. Care was taken in the second series to make the

last washing of each extraction below the standard color before the next solvent was employed. The colored material in the ether extract increased ninety times, that in toluol, fifty-two times, chloroform, ten times, and carbon bisulphide, three times.

It is difficult to explain these results except on the assumption that there were present in certain extracts at least four color-producing compounds, and that they are formed in different ratios by the above methods. The results harmonize with the further fact that the dried extracts when dissolved in boiling water, formed four different crystals, together with considerable amorphous material.

Probable Formation of Thionin or Thionol.

In another experiment, it was noted that very little matter soluble in ether and chloroform was present in the filtrate of a methylene blue specimen boiled for eight hours with an excess of silver oxide. The substance in the residue was red and might be supposed to be the free base of methylene blue, which gives a reddish color and is insoluble in ether and chloroform, but its acidification with hydrochloric acid and the subsequent addition of alcohol gave a brown ring with sulphuric acid, not a green one, as would be the case, had the substance been the free base. On further examination the eight-hour filtrate gave some reactions very similar to thionol, among these being its ability of uniting with eosin and of forming a metallic purplish precipitate. This fluffy product could not be the eosinate of methylene blue, since the latter is dark blue, not purple. It could not be methylene violet, since methylene violet remains along with the original precipitate in its unaltered crystalline form. The filtrate from it contains almost as much material soluble in ether [29] as does the original methylene blue mixture untreated by eosin, and therefore it could not be methylene azure. This purple compound of eosin was soluble in methylated spirits, gave staining reactions very much like those resulting when thionin was similarly treated with eosin, and was, therefore, considered to be thionin or thionol.

[29] Ether will not dissolve eosin, methylene violet, or the eosinates of methylene blue, thionol, or its closely allied bodies.

SOME CONSIDERATIONS REGARDING THE MAKING OF A METHYLENE BLUE-EOSIN STAIN.

From the above, it is evident that the derivatives of methylene blue essential for a good stain may be different in their composition and varied in their mode of production. A very fine stain is secured by boiling for twenty minutes a quantity of one per cent. methylene blue, containing 0.5 per cent. sodium bicarbonate, and at least 0.5 per cent. freshly precipitated silver oxide. One-third is then removed, and after another twenty minutes, half of the remainder is withdrawn. The process is continued with the rest of the mixture for the remainder of the hour. The three products are reunited, distilled water added to compensate for the loss by evaporation, and the mixture allowed sufficient time for the precipitate to settle. The silver oxide in the above process being 0.03 per cent. soluble in water, and ionizing into Ag and OH, reacts on the chloride of methylene blue, forming the free base, according to the equation $Mb - Cl + Ag - O - H = Mb - O - H + AgCl$.

Depending on the chemical nature of derivatives of methylene blue, the action of sodium bicarbonate may be one of oxidation or of reduction. The $Mb - O - H$ in the presence of these reagents readily forms derivatives, among which are methylene azure and methylene violet, the former being an oxidation process, and the latter, a reduction process. The reduced body, possibly thionin, or its highly oxidized body, thionol, might be formed partly by the interaction of other derivatives. One-half per cent. filtered eosin was next added to an equal volume of the prepared mixture. Theoretically, the weights of the untreated dyes should be taken in the ratio of 824 to 746 or one gram of eosin to 0.92 gram of methylene blue, but on account of the free bases of methylene violet and methylene azure not uniting with eosin, the weight of the latter taken was one half that of the methylene blue. An excess of either eosin or methylene blue must be avoided, since it was found that not only the precipitates formed are soluble in the excess of either reacting body, but also all the crystalline material occurring in the treated mixture is likewise soluble. The reaction of the free base

of methylene blue with eosin is represented thus: $2Mb - O - H + Eo.K_2 = Eo.Mb_2 + 2KO - H$. Whenever it is desired to form eosinates of methylene violet and methylene azure the slight alkalinity of the mixture should be reduced by almost neutralizing it with very dilute hydrochloric acid before the eosin is added. There is no good reason for diluting the reacting bodies, since the eosinates are less soluble in neutral salts than in distilled water. Moreover, the reaction occurs equally well, whether eosin is added to the mixture or the mixture to the eosin. It might appear that the dark red amorphous silver eosinate, previously referred to, might be formed as one of the products in the above manipulation. This salt, however, could not be detected microscopically after an hour's treatment of eosin with silver oxide, and .05 per cent. sodium bicarbonate.

The Use of Methylated Spirits as a Solvent.

The eosin combinations and other precipitates which may be mingled with them, after standing for an hour or so, are thrice filtered and washed with distilled water or preferably by a solution of sodium chloride to remove any free alkalies which in subsequent staining would injure the tissues and likewise give great prominence to the basic part of the stain. Methylated spirits has an economic advantage over pure methyl alcohol, and was found better adapted for the staining technique which was employed. It was, therefore, used in dissolving the precipitate above described, as it had been previously employed in studying the staining reactions of the other eosin compounds, including those of thionin, thionolin and thionol. The stain was made half saturated by adding the filter and its contents to the alcohol and bottling the same for several hours. The saturated solution thus formed was filtered and to the filtrate was added an equal volume of methylated spirits.

In this stain there are present at least seven compounds, the eosinate of methylene blue, a small amount of methylene azure, considerable methylene violet, the probable eosinates of thionin, thionolin, and thionol, and, according to Rosin, some methylene orange, and a black precipitate, insoluble in ether.

Technique and Staining Results.

Modified methods were employed in making smears for testing the above stain. Strips of tissue or cigarette paper having a width of the cover glass were cut, and in work with undiluted blood, one edge slightly moistened with physiological salt solution. Over this moist edge were passed two cubic millimeters of blood, measured from a medicine dropper of very fine bore. The strip containing blood was then quickly drawn across the cover glass at an angle of 60° with the slip. The smear was quickly air-dried, and was then thrice passed through the flame.

The manner of using the stain is simple. Add a few drops to the specimen; wait until a scum forms on the surface (its appearance depending upon the amount of the stain used), after which a few more drops are momentarily poured on the smear, so as to dissolve any precipitate that may have formed among the corpuscles. Wash with distilled water; dry thoroughly and mount in thick Canada balsam. In staining the malaria parasite especially, thin balsam should be avoided, as the xylol in it dissolves out the methylene azure and methylene violet, and thus mars the permanency of the preparation.

The staining as above described showed red corpuscles colored terra cotta to pink, the granules of the polymorphous neutrophiles pink, of the eosinophiles red, those of the basophiles purple, the bacteria dark blue and the nuclei of the polymorphous cells pale blue, the lymphocytes deep blue with a halo of pale red sometimes containing faint purple granules, and the platelets pink, while there was a total absence of the amorphous precipitates so frequently marring blood stains. A deeper blue staining effect may be secured when desired by adding to the stain one per cent. methylene blue dissolved in methylated spirits previously freed from acids by shaking with calcium hydrate.

Where the stain is desired for detecting malaria parasites, the dried red ether extract from a thirty-minute specimen can be dissolved in methylated spirits and added to the original stain.

SOME THEORETICAL CONSIDERATIONS IN STAINING.

It was noted that the extracts of the mixtures in ether, xylol, benzol, toluol, chloroform, and acetone were always red. This color seemed due to the power these solvents had of taking up red compounds in their unaltered form. This view was sustained by the mixtures becoming less red and more bluish after the extracts were made. In water, the dried red ether extract is very slightly soluble with a blue color which persists in an atmosphere of hydrogen or of carbon dioxide. From what Heidenhain[30] has found regarding Nile blue base, we should suspect that the carbon dioxide of the water had united with the base and formed the blue colored salt, when the extract had been evaporated in the air. This suspicion proved to be correct, since a considerable quantity of the dried extract gave bubbles of gas when treated with hydrochloric acid. When the same extract was dissolved in alcohol, previously treated with calcium hydrate, the liquid had a reddish tinge. The same extract suspended in water, treated with dilute sodium hydrate gave microscopically a reddish precipitate which, when redissolved, in ether produced a fiery red color. All these facts point to the base or bases as being red, and that the six solvents above mentioned dissolve them unchanged. In water and in alcohol the products become bluish or purplish, because salts were formed from the base uniting with the carbon dioxide of the atmosphere on the one hand, or some acid body in the alcohol on the other. On theoretical grounds, the color produced could not be due to the formation of different ions, since the colored ions of the free base occurring in treated methylene blue, would still remain the positive colored ions in the newly formed salts, while the negative ions of these salts would be colorless. From this the conclusion can be drawn that the blue color must be due to the color of undissociated salts.

The staining effects already described in this paper require some explanation. It was mentioned that the red corpuscles were sometimes stained green or blue, sometimes red or brown. Where the

[30] Heidenhain, *Pflüger's Archiv f. d. gesammte Physiologie*, 1903, c, 217.

corpuscles were bluish, the base of the methylene blue derivative united with an acid radical of the cell proteids and formed a blue or green colored salt. Where a red color appeared, the acid radical of eosin had united with the basic radical of the proteid and formed a blue colored salt. The nuclei always contained nucleic acid and hence were always able to form the blue or purplish salts with methylene blue or its derivatives. Even the stain itself, was of a violet hue, as a summation of the eosin and methylene blue colored chromogens.

It was noted that the staining of a specimen occurred slowly when the evaporation of the alcohol was prevented, but when the alcohol was freely allowed to volatilize, the characteristic colors developed quickly. A couple of tentative experiments were made on the conductivity of the stain, diluted with distilled water, with a view to possible explanation of this and certain other phenomena. The conductivities of the solutions examined expressed in reciprocal ohms at 5° C. multiplied by 10^8 are here given for two different experiments.

First Example.		Second Example.	
Undiluted stain	1.58	Undiluted stain	1.85
Distilled water	0.28	Distilled water	0.37
Equal parts of stain and distilled water	0.44	Equal parts of stain and distilled water	0.62
Two parts of stain and one part distilled water	1.07		

The gram equivalent in terms of sodium chloride was found to be .0018, as calculated from my chart,[31] and hence, would require the same gram equivalent of the completely ionized eosinate of methylene blue to give the same conductivity. This latter value would correspond to 0.28 per cent. of the compound dye, and is much above the medium, since a large portion of the conductivity of the stain must be due to inorganic ions. Instead of the conductivity of a mixture of equal parts of the stain and water being the average between the water and the stain, when separately examined, its value in the above examples was only about half this

[31] Wilson, *Amer. Jour. of Physiol.*, 1906, xvi, 447.

average. The change in the conductivities of the stain was found to be due to the formation of a microscopic precipitate.

The addition of water to the stain is comparable to the loss of alcohol by the evaporation which occurs in the method of staining above described. The residue in the latter case becomes more and more aqueous as the volatilization proceeds. In both processes there is a precipitation of the dye and a reduction in the number of ions. The more rapid staining when free evaporation is permitted may be due to the dye molecules or ions being more rapidly brought within the absorption sphere (0.05 μ according to Quincke), or within the chemical sphere of the cell elements concerned.

The staining effects in either case cannot be explained according to the physical or solution theory of Witt and Michaelis[32] since methylene violet and methylene azure are more soluble in methylated spirits than in any of the solvents studied. It seems wellnigh impossible that the dye molecules or ions should be relatively so soluble in the cytoplasm and in the nuclei of the cells as to enable the stain to be so condensed in these cells, as certainly occurs in the staining act. In order to realize the truth of the latter statement, one has only to examine the stain microscopically to see how weak in color it is as compared with the same thickness of stained material. Moreover, the stain is largely colloidal, or at least diphasic[33] and, therefore, diffusion would be too slow to produce the staining effect in the short time ordinarily required for the staining act.

SUMMARY.

1. Eosinate of thionin gives very satisfactory staining for blood smears. It is easily prepared and dissolves readily in methylated spirits.

2. In the methods heretofore employed for making mixtures of eosin and methylene blue derivatives, the eosinates of methylene violet and methylene azure are present in very small quantities or are altogether absent.

[32] Michaelis, *Pflüger's Archiv,* 1903, xcvii, 634.
[33] Barratt, *British Bio-Chem. Jour.,* 1906, i, 424.

3. Thionol and thionin are probably formed in methylene blue which has been long boiled with dilute alkalies and silver oxide.

4. Good stains of eosin and methylene blue derivatives can be obtained by a variety of manipulations.

5. Methylated spirits is more economical as a solvent for this stain and better adapted for the simple technique above described than is methyl alcohol. There is some evidence that the staining act is of a chemical nature.