TABLE I. Influence of Fasting and Certain Steroids on Wt and Total Nitrogen Content of Thymus and Spleen.*

Treatment†	No. mice	Daily dose, mg	Total thymus N	Thymus wt	Total spleen N	Spleen wt
Control, fed	10	—	9.4 ± .7	250 ± 23	13.8 ± .7	410 ± 18
Fasted, 48 hr	10	—	10.0 ± .5	*153 ± 18*‡	*10.9 ± .9*	*265 ± 18*
Control, fed	10	—	10.6 ± .3	322 ± 20	13.8 ± .5	438 ± 32
Fasted, 60 hr	10	—	10.4 ± .7	*111 ± 19*	*11.4 ± 1.0*	*258 ± 32*
Control, saline	9	0.1 ml	7.3 ± .3	312 ± 10	11.7 ± .4	352 ± 14
Cortisone acetate	10	3.0	7.1 ± .7	*125 ± 7*	*7.3 ± .5*	*230 ± 21*
17-methylandrostane-3β, 17β-diol	8	1.5	*8.6 ± .2*	*158 ± 5*	*16.9 ± 1.2*	439 ± 31
Testosterone propionate	7	1.5	*5.2 ± .5*	216 ± 15	11.3 ± 1.2	365 ± 29
β-estradiol	8	1.5	*5.2 ± .3*	*159 ± 17*	13.4 ± 1.2	362 ± 36
Estrone	7	1.5	*6.1 ± .3*	*151 ± 18*	11.0 ± 1.0	293 ± 20
Desoxycorticosterone acetate	7	1.5	*6.4 ± .3*	*192 ± 13*	*8.6 ± 1.0*	302 ± 24
17-methyl-Δ⁵-androstene-3β, 17β-diol	7	1.5	7.6 ± .3	207 ± 23	13.6 ± 2.3	445 ± 74
Progesterone	7	1.5	7.8 ± .4	219 ± 22	9.4 ± .7	299 ± 19
Testosterone succinate	6	1.5	7.9 ± .5	221 ± 16	12.2 ± 1.0	383 ± 19
Control, oil	7	0.1 ml	8.0 ± .6	255 ± 15	11.9 ± .8	337 ± 21

* Thymus and spleen wt expressed in mg/100 g body wt; thymus and spleen N as mg N/total organ/100 g body wt.

† All steroids inj. subcut. daily for 3 days.

‡ Italicized values are significantly different from their controls at the 5% level of confidence. Values reported are mean ± standard error.

lished). It appears quite possible that these results may form the basis for a suitable screening procedure for protein anabolic compounds. The physiological significance of this apparent relationship between thymus nitrogen depletion and protein anabolic steroid activity is being currently investigated.

Summary. Neither the stress resulting from a 48 or 60-hour fast nor the administration of cortisone acetate has a significant effect on the total nitrogen content of the mouse thymus gland. On the other hand, it appears that the protein anabolic steroids induce a significant decrease in the thymus total nitrogen content.

Addendum. After this work was completed, the paper by Adams and White(4) came to our attention. Their data, in agreement with our results, show that a 48-hour fast has no effect on the protein content of the thymus nodes.

The authors gratefully thank Miss L. Hopkins for assistance with some of the nitrogen determinations, Mr. S. Neuwirth for the statistical computations, and Dr. E. B. Hershberg for the cortisone acetate.

3. Kochakian, C. D., *Am. J. Physiol.*, 1944, v142, 319.

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A Purified Fraction of Thioflavine-S (Vasoflavine): Valuable Agent for Visualization of Blood Vessels.* (18734)

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A method for the visualization of blood vessels and lymphatics under ultraviolet light,

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using a fluorescent dye, Thioflavine-S, was developed by one of the authors (J.U.S.)(1). This technic has proved to be a valuable adjunct to the study of vascular physiology and morphology in the mesentery, intestines, uterus, and the kidney. The original method utilized the freezing-drying technic of Gersch (2) before cutting sections for microscopic visualization. Since that time the method has been adapted by Algire and Schlegel(3) for *in vivo* visualization through a transparent chamber, and has been simplified by Schlegel and Moses(4), eliminating the necessity for freezing and drying by clearing the tissue in glycerine.

Thioflavine-S has been used for some time to stain the elementary bodies of the vaccinia virus(5), the potato scab(6), and as a fluorochrome stain of fixed tissue sections (7,8). Pick(9) tested several fluorescent dyes as to their efficacy as vital stains, but did not describe their ability to produce a fluorescence of the vascular walls.

He reports some experimental work in which Thioflavine in a concentration of 1:1000 was injected into the lymph sac and intracardially in the frog. It is doubtful if the blood vessels of the kidneys in his preparations would have shown any fluorescence because of the high dilution of the dye and the long period of time which elapsed before the organs were removed and inspected. (His shortest reported time between injection and observation was 5 minutes).

The technic employed in this study has been described at length in a previous publication(4), but will be briefly outlined herein. After anesthetizing the animal (in our case rabbits have been used for the most part) the abdomen is opened transversely. Hemostasis is secured and the wound is then held closed with clamps for approximately one-half hour to allow the animal to recover from the immediate effects of surgery. After this lapse of time the fluorescent dye, in a 4% solution, is injected intravenously in the amount of 1 cc per kg of body weight, and the kidneys removed within a period of 10 seconds after the onset of the injection. Sections of the fresh tissue are cut with a sharp knife and placed in glycerine (USP). The glycerine serves to clear the tissue and slow down post-mortem diffusion of the dye. It is important to use USP glycerine since excessive water promotes undesirable diffusion. The application of this method to the vessels of the rabbit kidney brings to light several interesting points. It was found that the rate of diffusion of the dye out of the kidney vessels is faster than from the vessels of the mesentery, subcutaneous tissue, and the uterus. It was also found that there was a very appreciable amount of natural fluorescence in the kidney, presumably from riboflavin and its derivatives. According to quantitative measurements by Mickelsen *et al.* (10) and Bessey *et al.*(11), the liver and kidneys of animals are uniformly higher in riboflavin content than other organs and tissues. This fluorescence was described by Ellinger (12) in the frog and the rat kidney. Attempts by him and by ourselves to eliminate this fluorescence by starvation and riboflavin deficient diets proved to be rather ineffective. This natural fluorescence appears to be greater in the dog kidney than in the rabbit kidney. In the rabbit kidney the natural fluorescence of the vessel walls is bluish-violet and of the parenchymal tissue yellow-green. The intravenous injection of Thioflavine-S makes the blood vessels fluorescent when observed in

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‡ Graduate Research Fellow in Biochemistry supported by the Biological Stain Commission.

1. Schlegel, J. U., *Anat. Rec.*, 1949, v105, 433.

2. Gersch, I., *Bull. Internat. A. M. Mus.*, 1948, v28, 179.

3. Algire, Glenn H., and Schlegel, J. U., *J. Cell and Comp. Physiol.*, 1950, v35, 95.

4. Schlegel, J. U., and Moses, J. B., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 832.

5. Levaditi, C., *Ann. Inst. Pasteur*, 1940, v64, 360.

6. Richards, O. W., *Stain. Technol.*, 1943, v18, 91.

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8. Jenkins, R., *Stain. Technol.*, 1937, v12, 167.

9. Pick, Josef, *Z. Wiss. Mikr.*, 1934-35, v51, 338.

10. Mickelsen, Olaf, Waisman, H. A., and Elvehjem, C. A., *J. Nutrition*, 1939, v18, 517.

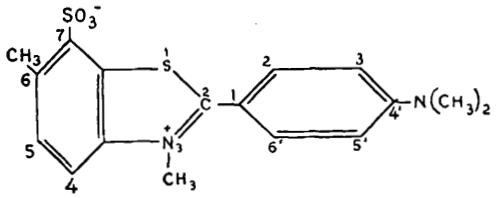
11. Bessey, O. A., Lowry, O. H., and Love, R. H., *J. Biol. Chem.*, 1949, v180, 755.

12. Ellinger, P., *Biochem. J.*, 1938, v32, 376.

ultraviolet light, but the above factors tend to reduce the contrast between the vessels and the surrounding tissue. Since, as we have seen, there is little that can be done to influence the natural fluorescence, the problem of improving the contrast of the preparations was, of necessity, approached from the standpoint of the dye.

The commercial grade of Thioflavine-S shows both a yellow-green and blue-violet fluorescence. As seen in the kidney preparations from injected animals, the vessels and glomeruli fluoresce in the yellow-green and the surrounding tissues fluoresce in the blue-violet from the dye which has diffused out of the vessels. This is the exact opposite of the natural fluorescence. Keller(13) attributed this difference in fluorescence of Thioflavine-S to differences in "electric charge" of the tissues. Thioflavine-S, according to the Colour Index(14), is presumed to be composed of methylated and sulfonated derivatives of dehydrothio-p-toluidine. Synthesis is carried out with the primulin base as the starting material, successively methylated and sulfonated. This method can, understandably, produce a series of homologues, varying in degree of methylation and sulfonation, rather than any single pure dye. There is a strong possibility, however, that the major components are members of a limited series of the lower molecular weight homologues. Since it seemed probable that this mixture contained one or several compounds which would diffuse particularly slowly through the vessel walls, and since the yellow-green fluorescence was typical for the dye retained in the walls of the vessels, we were especially interested in separating this or these compounds from those with a higher rate of diffusion.

We took our problem to the research laboratory of the Eastman Kodak Co., where it was received with great interest and cooperation. It seems that the principal components of Thioflavine-S are the lower homologues of quaternarized, methylated, and sulfonated dehydrothio-p-toluidine, derived from the following basic structure.



The following compounds which are believed to be closely related to the components of commercial Thioflavine-S were prepared for our use:

A. Thioflavine-S, E-853-01 disulfonate. Anhydro-2-(3'-sulfo-4'-dimethyl aminophenyl)-3,6-dimethyl-7-sulfobenzothiazolium hydroxide.

B. Thioflavine-S, E-839-95A-monosulfonate. Anhydro-2-(4'-dimethyl aminophenyl)-3,6-dimethyl-7-sulfobenzothiazolium hydroxide.

C. Thioflavine-T, E-839-90A. 2-(4'-dimethyl aminophenyl)-3,6-dimethyl benzothiazolium chloride.

D. 2-(4'-aminophenyl)-6-methyl-7-sulfobenzothiazole, ammonium salt.

E. 2-(4'-amino-3'-sulfophenyl)-6-methyl-7-sulfobenzothiazole, disodium salt.

F. Primuline, recrystallized.

None of these substances when tested by our injection technic were as satisfactory from the standpoint of slowness of diffusion, or fluorescence, as the technical grade Thioflavine-S.

A more highly purified Thioflavine-S (Erie-Flavine-S conc. 130%)§ was obtained. A water extract of this sample was made by extracting 10 g of the dye with 50 cc of distilled water at room temperature. A 16% solution was obtained by this method and upon evaporation yielded approximately 8 g of dye. This water extract is supposedly composed of the more highly sulfonated fractions of Erie-flavine-S. We have named the preparation Vasoflavine.¶ When injected intravenously into rabbits in the same concentration and over the same period of time, Vasoflavine proved to be more fluorescent and to diffuse less rapidly than Thioflavine-S. The contrast

13. Keller, R., *Anat. Rec.*, 1949, v103, 133.

14. Colour Index, Ist Edition, Jan. 1924, Society of Dyers and Colourists, Bradford, Yorkshire, England.

§ Nat. Aniline Div., Allied Chemical and Dye Corp.

¶ May be obtained from the Pharmaceutical Laboratories of the National Aniline Division, Allied Chemical and Dye Corp., New York City.

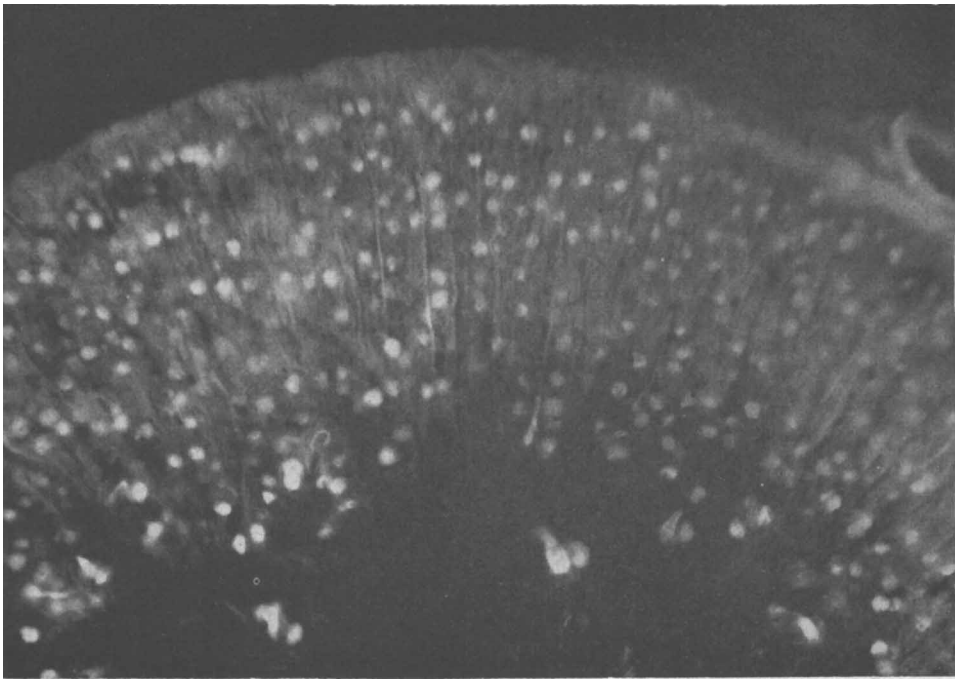


FIG. 1. Photomicrograph taken in incident ultraviolet light of a kidney from a rabbit injected intravenously with Thioflavine S. Note lack of contrast due to diffusion of the dye. 16.5×.

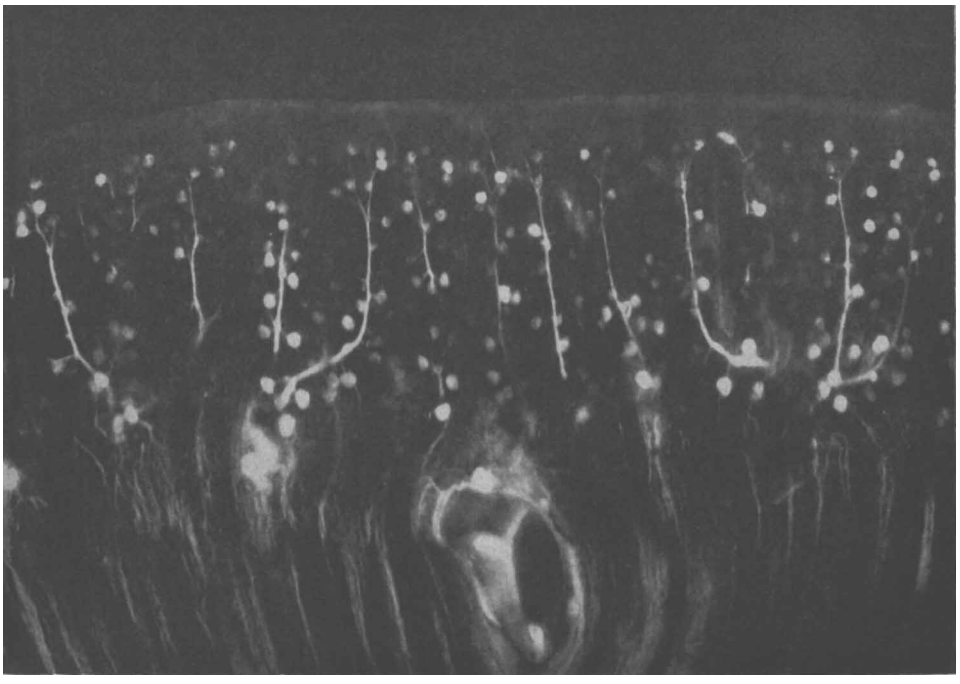


FIG. 2. Photomicrograph taken in incident ultraviolet light of a kidney from a rabbit injected intravenously with Vasoflavine. Note the excellent contrast, lack of diffusion of the dye, and the good injection of the smaller arterioles and vasa rectae. 16.5×.

provided with this preparation was thus very much improved. Small vessels, which with Thioflavine-S had been obscured by the natural fluorescence and by the diffused dye, were clearly visible with Vasoflavine. A comparison between the two dyes is shown in Fig. 1 and 2.

Since Vasoflavine more nearly approached the ideal dye for our purposes, it was of interest to determine the nature and character of this substance. Using the purified water extract as the experimental material, several studies were conducted to attempt more complete characterization of this dye.

Paper partition chromatographic studies. Paper partition chromatography was applied and many solvent systems were tested. These consisted of the more common ones such as: n-butanol-water; n-butanol with 10% acetic acid-water; n-butanol with 20% NH_4OH-water; phenol-water; ether-water; benzene-water; and collidine-water. None of these solvent systems produced good separation of any fractions, but most of them showed similar results which consisted of a slightly bluish fluorescent zone from the region of spotting on the chromatograph to the solvent front, while at the original spot or in the nearby region there was a highly fluorescent yellowish substance. It is doubtful that this was a result of the dye being fixed to the cellulose of the paper (this dye is known to be a direct cotton dye) for, by washing with water after development and drying of the chromatograph, the yellow fluorescent region was capable of further movement, and could be, at least partially eluted from the paper. The paper partition chromatograph did not give conclusive evidence as to whether more than one dye fraction was present, but rather it indirectly seemed to indicate that the dye may be found to exist in different forms which may account for the difference in intensity and color of the fluorescence under ultraviolet light.

It is a well known fact that practically all dyes readily form dimers and polymers especially in more concentrated solutions. Michaelis(15), Corrin(16), Rabinowitsch (17), and Sheppard(18,19) and others have conducted extensive studies which substantiate this. It seemed possible that the dimer or the higher polymeric forms are responsible for the yellow, highly fluorescent region near the original spot, while the monomeric form, which according to previous work should be the only form present in an alcohol solution, is the cause of the blue, slightly fluorescent region that moves with the n-butanol. Dialysis of the purified water extract of Erieflavine-S and spectra of the dye at various dilutions were studied to yield further evidence for the existence of polymeric forms.

Dialysis and spectrophotometric studies. Solutions of approximately 1% were continuously dialyzed for a week against distilled water which was changed 3 to 4 times daily. Viscose tubing (Visking Corp.) was used for dialysis. This retains compounds of molecular weight greater than 10,000-15,000. It was found that only a blue, slightly fluorescent substance was dialyzed, while the solution remaining within the tubing fluoresced yellowish-green under ultraviolet light. It is difficult to conceive why any of the dye should be retained unless it is partially composed of highly polymerized components. The dialysate and non-dialyzable material (hereafter called the non-dialysate) were evaporated to dryness on a steam bath. Fifty mg of each product was dissolved in 200 ml of distilled water. These two solutions were subjected to spectrophotometric analysis.‖ At the outset it was observed that the dialysate preparation had a considerably greater absorption in the region studied than the non-dialysate. Hence in the curves presented in Fig. 3, that for the dialysate represents a concentration one-quarter that for the non-dialysate. The spectral curves for the two fractions (Fig. 3) were fairly similar indicating that they were the

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16. Corrin, M. L. and Harkins, W. D., *J. Am. Chem. Soc.*, 1947, v69, 679.

17. Rabinowitsch, E. and Epstein, L. F., *J. Am. Chem. Soc.*, 1941, v63, 69.

18. Sheppard, S. E., *Rev. Mod. Physics*, 1942, v14, 303.

19. Sheppard, S. E., *J. Am. Chem. Soc.*, 1944, v66, 1995.

‖ Beckman, Model DU Spectrophotometer.

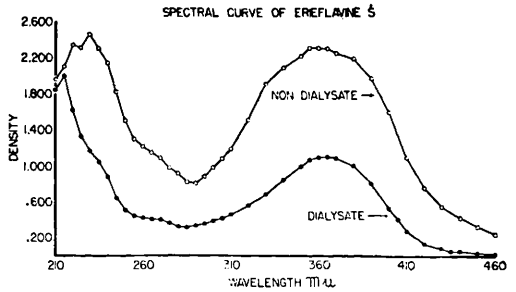

FIG. 3. Spectral curves of the dialysis products of Erieflavine-S purified, (Vasoflavine). Dialysate concentration 6.25 mg %, non-dialysate concentration 25 mg %.

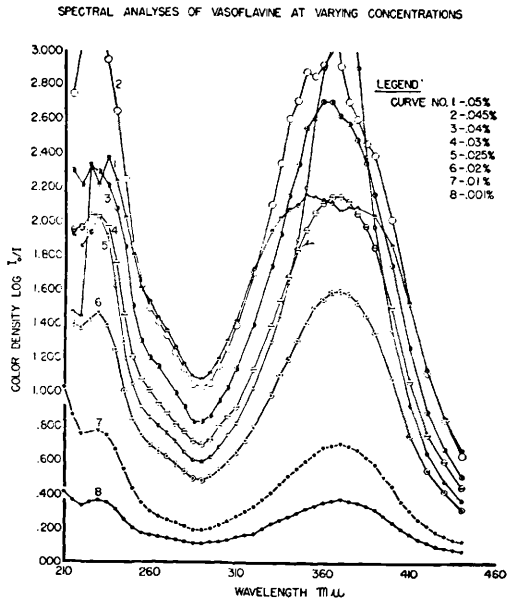

FIG. 4.

same dye (which was apparently quite homogeneous as concerns homologues), but there were slight differences in the 2 curves. There was a double peak for the non-dialysate (220 and 230 mμ) in the lower ultraviolet as compared with dialysate (215 mμ), and the major peak (360-365 mμ) was also flattened somewhat. The slight hump (330-340 mμ) on the side of the peak may be indicative of a dimer as illustrated by Michaelis(15) for other dyes. The curves do indicate that Vasoflavine is fairly homogeneous and does not consist of a series of homologues, since the curves show only two distinct principal absorption peaks which is common for many pure dyes. However, the difference in absorption coefficients of the dialysate and non-dialysate, and the variations of the spectral curves indicated that spectrophotometric analyses at varying concentrations of Vasoflavine were needed.

Spectrophotometric studies. A thorough study of spectrophotometric curves at various dye concentrations was conducted to determine whether the purified water extract, Vasoflavine, formed polymers in solution. A series of dilutions in distilled water ranging from 0.001% to 0.05% of Vasoflavine was made and spectral analyses were made on each from 210 to 440 mμ, using quartz cuvettes having a light path of 2 mm.

The results of these analyses are shown in Fig. 4. They indicate clearly that polymerization does occur to varying degrees, depending on the concentration of the dye. Upon analysis of the results it is seen that the curves are similar in shape at the lower concentrations (0.001-0.025%), having their major peak at 370 mμ and their minor peak at 230 mμ (Fig. 4, curves 5, 6, 7, and 8). According to Beer's law the density at these peaks increases proportionally as the concentration increases. This, however, is found to hold for this substance only in the lowest concentrations up to approximately 0.01%. The similarity of these curves is indicative that they represent spectral curves of the monomeric form of the dye. At higher concentrations (0.03%) it is noted that the density at 370 mμ decreases in spite of an increase in concentration (Fig. 4, curve 4). There is also a possible slight shift of the peaks toward the lower wave lengths. According to Michaelis and others(15-19), this is typical of the formation of a dimer or a higher polymeric form. When the concentration of the dye is increased to 0.04% (Fig. 4, curve 3) it is seen that the major and minor peaks shift further to lower wave lengths and the density is then increased with the increase in concentration. This is indicative of an increase in concentration of a higher polymeric form. At a still higher concentration of 0.045% (Fig. 4, curve 2) the amount of this form appears to be increased, but the appearance of another small peak at 350 mμ seems to indicate the

beginning of a still higher polymer. When the concentration is increased even more to 0.05% the curve shows two peaks (Fig. 4, curve 1), which are greatly flattened and shifted from those of the monomeric and other lower forms in both the major and minor peak regions. The density is reduced from that of the 0.045% solution, indicating the formation of one or more new polymeric forms with lower absorption coefficients.

The data obtained from this study clearly indicate that Vasoflavine is capable of forming polymers, dependent upon the dye concentration. The dye apparently exists in polymeric forms at concentrations greater than 0.025%, and even higher polymers appear to exist above 0.04%.

Upon calculation of the possible concentration of Vasoflavine in the blood of injected rabbits according to our regular technic, it was found that the best results, with less diffusion out of the vessels, were obtained when the dye concentration in the blood was greater than 0.03%, assuming that the dye dispersed rapidly throughout the blood stream. It seems therefore reasonable to believe that the dimeric or polymeric form of the dye is the more desirable, for it diffuses from the blood vessels less readily and apparently imparts a more brilliant yellow-green fluorescence to the blood vessel walls.

Summary and conclusions. A more fluorescent and less rapidly diffusible dye with a strong affinity for the walls of the blood vessels has been described. This dye is a purified, water soluble fraction of Thioflavine-S and has been named Vasoflavine. Studies leading to a more accurate characterization of this dye are described. These include paper chromatography, dialysis, and spectrophotometry.

The following conclusions are offered: 1. Vasoflavine approaches the ideal dye for the visualization of blood vessels under ultraviolet light and is much superior to the Thioflavine-S previously used. 2. The solution of dye probably exists as an equilibrium between monomeric and various polymeric forms. 3. The higher the concentration of an aqueous solution of the dye the more of the polymeric forms exist. 4. The yellow-green fluorescence is apparently produced by the higher form and the bluish by the monomeric form. 5. The polymers are probably too large in molecular size to pass the barrier of the vessel walls readily, while the monomeric form quickly diffuses out of the vessels, thus explaining the yellow-green fluorescence of vessels and the bluish fluorescence of the surrounding tissue. It should be noted that Vasoflavine exhibits no apparent toxic effects in experimental animals (rats, rabbits, and dogs) in blood concentrations as high as 275 mg %, but extensive toxicity studies have not been carried out.

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Mechanism of Action of Mercurial Diuretics. (18735)

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Gremels(1) observed that mercurial and xanthine diuretics increase the oxygen consumption of the isolated dog kidney perfused with blood from a heart-lung preparation.

1. Gremels, H., *Arch. Exp. Path. u. Pharmakol.*, 1929, v140, 205.