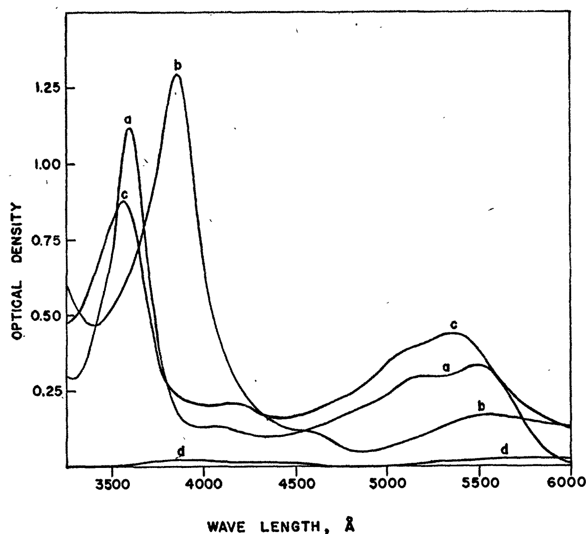

FIG. 2. Absorption spectra: *a,* vitamin B_{12}; *b,* reduced vitamin B_{12}; *c,* air-oxidized product; *d,* (Cr enta)⁻.

product form a system irreversible at the dropping mercury electrode, a situation not unexpected in view of the known loss of cyanide from cyanobalamin on reduction; furthermore, the equivalent weight of cyanocobalamin found by reductive titration corresponds to a 1-electron transfer. The brown reduction product is not oxidized by bromate ion, whereas (Cr enta)$^{=}$ is quantitatively oxidized; however, exposure to oxygen at once eliminates the anodic wave, and the solution turns red. Interestingly, when the air-oxidized material is back-titrated with chromium (II) complex, the solution becomes brown again, but no point can be reached at which an excess of (Cr enta)$^{=}$ is present; apparently the product catalyzes the reduction of water by Cr^{++}.

Diehl (*1, 2*) presents a polarogram obtained on a product termed by him vitamin B_{12r}, produced by reduction of cyanocobalamin with hydrogen on platinum catalyst. Two waves are shown at $E_{1/2} - 0.75$ and -1.37 v vs saturated calomel electrode; although their nature is not specifically defined, they are presumed to be cathodic, by comparison with a polarogram of cyanocobalamin superposed on the graph. This is surprising, because it would be anticipated that a substance having the oxygen avidity of reduced vitamin B_{12} would show anodic depolarization properties, as we indeed found.

The absorption spectra of reduced cyanocobalamin, its air oxidation product, and (Cr enta)⁻ ion at the end point of the titration are illustrated in Fig. 2, with numerical data in Table 3. Again, our reduction product differs radically from that of Diehl, for which absorption maxima are listed at 4730, 4050, and 3125 Å. Since the contribution to the absorption spectrum of reduced cyanocobalamin solution by (Cr enta)⁻ is evidently negligible, the differences are real.

TABLE 3

ABSORPTION MAXIMA OF REDUCED VITAMIN B_{12}

Wavelength (Å)	$E^{1\ cm}_{1\%}$
3853	179
4597	12.5
5537	23.6

Since Diehl reports that his vitamin B_{12r} could be back-titrated with potassium ferricyanide (consuming exactly 1 equivalent), the question is raised whether more than one reduction product of cyanocobalamin is possible. Inspection of Diehl's polarographic data yields some pertinent information. He concludes that the polarographic reduction of cyanocabalamin involves two electrons ($Co^{3+} \rightarrow Co^{+}$) and that of his vitamin B_{12r}, two stages of 1 electron each ($Co^{2+} \rightarrow Co^{+} \rightarrow Co^{0}$). However, his polarograms of cyanocabalamin and vitamin B_{12r} cover almost exactly the same voltage range, a situation difficult to reconcile with the different final valence states of cobalt which are assumed. We have obtained polarograms on various noncrystalline degradation products of vitamin B_{12} which closely resemble that presented by Diehl for vitamin B_{12r}; in this connection, we are informed by E. A. Kaczka that the maximum yield of catalytic reduction products of cyanocobalamin which are regenerable to the starting material approximates 70%. Hence the conclusions of Diehl concerning the identity of his vitamin B_{12r} do not appear justifiable on the basis of evidence which he presents. For the polarographic reduction of cyanocobalamin to a compound of univalent cobalt, no such assessment is possible unless it can be shown that reduction of the organic part of the molecule cannot occur.

In conclusion, the evidence at our disposal indicates that the valence states of cobalt in vitamin B_{12} compounds are the normal 2 and 3, and that in the divalent state the anticipated anodic depolarization properties are present.

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Effect of Toluidine Blue on the Coagulation of Fibrinogen by Thrombin[1]

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Haley and Stolarsky (*1*) pointed out that, although Toluidine Blue was capable of inactivating heparin and decreasing coagulation time *in vitro,* the range

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within which this action occurred was narrow. Furthermore, beyond a concentration of 25 γ/0.1 ml Toluidine Blue exerted an increasing anticoagulant effect. The complexity of the reactions occurring during coagulation of rabbit plasma did not allow an assessment of the role played by Toluidine Blue in increasing the coagulation time. In an attempt to obtain more information about the influence of Toluidine Blue on the coagulation process, we have investigated its effect on the coagulation of fibrinogen by thrombin. Inasmuch as the results obtained indicate that the dye has a different effect on the purified system than on plasma, it appears that the anticoagulant effect of Toluidine Blue previously reported is not a direct effect on either fibrinogen or thrombin, but it is related to some of the other factors in the coagulation process.

A 1% solution of bovine fibrinogen (Armours, Fraction I, containing 40–50% sodium citrate) in imidazol buffer was prepared as directed by Ware and Seegers (*2*). This solution was kept in a refrigerator overnight and centrifuged before use. Fresh solutions were prepared every 2 days. A stock solution of bovine thrombin (Parke-Davis & Co) containing 100 u/ml was prepared as directed by Ware and Seegers (*2*). A 1:10 dilution of this stock solution was kept in an ice bath before it was made into a working dilution of 1:200. The concentrations of Toluidine Blue in saline were 5, 10, 15, 25, 50, 75, and 100 γ/0.1 ml.

Triplicate tubes containing 0.1 ml of dye and 0.2 ml of fibrinogen solution were set aside for 0, 1, 2, 5, and 10 min prior to the addition of 0.2 ml of 1:200 thrombin. In the second series of experiments the dye and the thrombin were mixed and set aside for 0, 2, 5, and 10 min prior to the addition of the mixture to the fibrinogen solution. Control evaluations employed 0.1 ml of physiological saline instead of the dye solution. The endpoint was the time of first appearance of fibrin threads.

In order to determine if the acidic groups in the fibrinogen reacted with the free primary amine group of the dye, spectrophotometric analyses using the 0.5-cm cell in the Cary Recording Spectrophotometer throughout the range 700 to 220 mμ were made at dye concentrations of 5, 10, 15, 25, and 50 γ/0.1 ml and time intervals of 0, 2, 5, and 10 min. pH determinations of the Toluidine Blue, fibrinogen-Toluidine Blue, and fibrinogen-Toluidine Blue-thrombin solutions were made with the Beckman pH meter.

Figure 1 shows the results obtained at different time intervals when the dye and fibrinogen were mixed first. These results are based upon 9–12 determinations for each point on the curve, and the vertical lines represent the standard deviation. The results obtained when the dye and thrombin were mixed first were practically identical with those obtained when the dye and fibrinogen were mixed first, except that at the 10-min interval all dye concentrations completely inactivated the thrombin and no coagulation occurred. Comparison of the various curves in Fig. 1 shows that those for the 0, 5, and 10 min have a similar shape, with the dye increasing the coagulation time up to the 10- or 15-γ concentrations and then acting as a coagulation accelerator thereafter. The results obtained were not due to changes in pH values because the Toluidine Blue solutions, at the concentrations employed, had values of 6.98–7.0 before mixing with the fibrinogen solution, and a value of 7.0 after mixing. The addi-

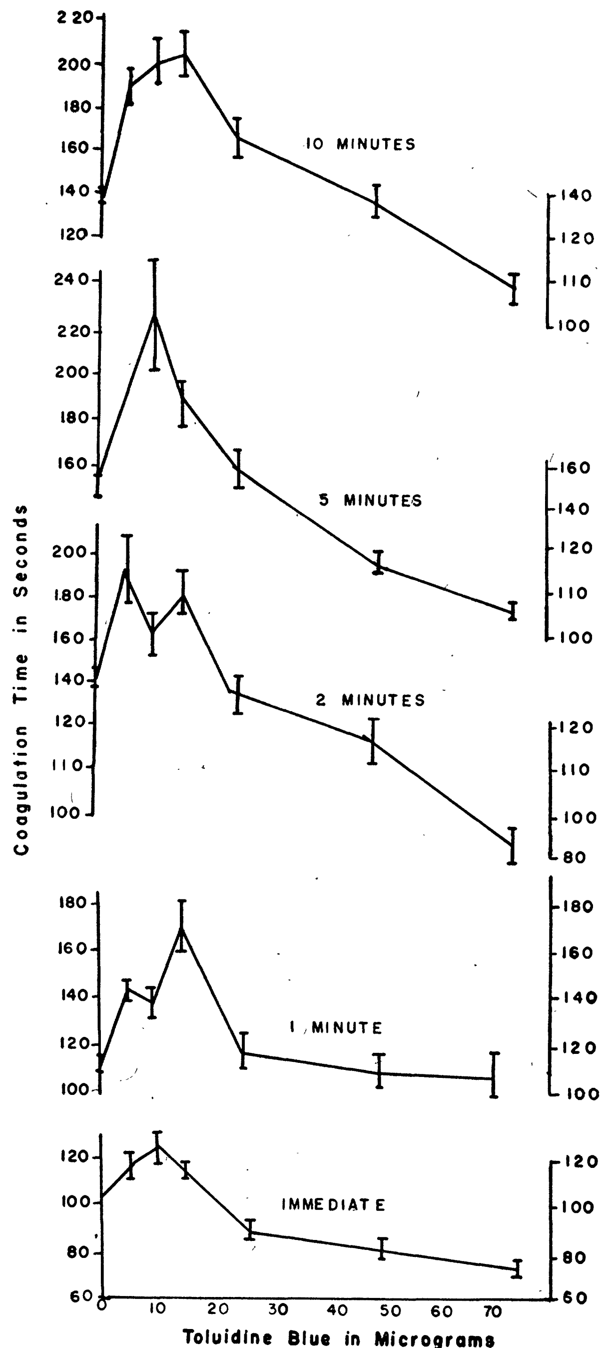

FIG. 1. Effect of Toluidine Blue on coagulation of fibrinogen.

tion of thrombin did not change the pH value of the Toluidine Blue-fibrinogen mixture.

Visible absorption spectra were obtained at all dye concentrations. Absorption spectra were also obtained in the ultraviolet region, but the absorption contribution of fibrinogen was so great at the concentration used in the coagulation studies that accurate measurements were not possible. Toluidine Blue gave a peak at 285 mμ and fibrinogen one at 280 mμ; the combination gave a peak at 282 mμ. The fibrinogen maximum is the same as was previously reported by Waugh and Livingstone (*3*).

The curves for the 1- and 2-min intervals have a similar shape but differ from those of the other time intervals at the 10-γ concentration, where the dye produced a definite acceleration in coagulation. This effect was lost, however, at the 15-γ concentration, where an increased coagulation time was observed. The accelerator effect seen at the 1- and 2-min intervals and at the 10-γ dye concentration cannot be explained on the basis of any effect on the thrombin because, although the dye-fibrinogen and dye-thrombin curves at the 2-min interval and at the 10-γ dye concentration were identical, the thrombin was still active in causing fibrin formations at the other dye concentrations and time intervals. If the thrombin had been inactivated, no fibrin formation would have been detected beyond the 10-γ dye concentration, but such interference with the reaction did not occur except at the 100-γ dye concentration or when the time interval of interaction between the dye and the thrombin was increased to 10 min.

On the other hand, it may have been possible that Toluidine Blue and fibrinogen reacted to form a complex similar to those observed by Michaelis (*4*) for the Toluidine Blue-nucleic acid system. If such were the case, however, definite differences in the absorption spectra of the dye-fibrinogen system would have been apparent in the visible region, and there might have been a definite downward shift in the curve, indicating the presence of the dimeric and polymeric forms of Toluidine Blue. There were, however, no obvious changes in the absorption curve in either the presence or absence of fibrinogen. Furthermore, the curves were identical with the Michaelis (*4*) curves for the monomeric form of Toluidine Blue. These data indicate an additive effect rather than a combination of the two substances. Both the visible and ultraviolet absorption data indicate that the dye and fibrinogen do not form a complex, or, if such a complex is formed, it is a very loose one which does not prevent thrombin from converting fibrinogen into fibrin. Moreover, the coagulation-accelerating action of the dye at the higher dye concentrations might be considered further evidence of no rigid complex formation between the dye and the fibrinogen. Furthermore, the endpoint, fibrin thread formation, cannot be confused with isoelectric precipitation of the fibrinogen because the isoelectric point of fibrinogen is pH 5.4 (*5*), whereas our evaluations were made at pH 7.0. The great differences in the molecular weights of Toluidine Blue (305) and fibrinogen (350,000) (*6*) make it doubtful that additional work using electrophoretic or ultracentrifuge techniques would increase our total knowledge of the reaction taking place. Further investigation must be centered on the other purified components of the blood coagulation system; work on these factors will be reported in detail at a later date.

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The Part Played by Chlorophyll in Plant Transpiration Studied by a New Method: Hygrophotography

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It is known that the transpiration of leaves increases under the effect of light, the first action of which is to cause an increase of the stomatal aperture. The effects of the various spectrum radiations on green leaves are not the same; blue and red radiations, which are precisely those absorbed by chlorophyll, prove to be the most efficient for increasing transpiration. The conclusion arrived at was therefore that light acting upon chlorophyll would thus play an important part in plant transpiration. Van Tieghem (*1*) was even led to assume that in addition to the transpiration function proper of the leaf, as shown by its cells not containing chlorophyll, there also occurred a release of water vapor due to chlorophyll activity, to which he gave the name "chlorovaporization."

This notion of chlorovaporization was not accepted by all botanists, some of whom considered that the phenomenon of chlorovaporization does not occur or if it does occur, causes the vaporization of a very small amount of water which cannot be demonstrated experimentally (*2*).

The hygrophotographic method, which has been described elsewhere (*3*), offers a very sensitive and extremely simple means for definitely solving this problem. This method is based on the use of mercury and silver iodide gelatin photograph films or plates whose preparation has been described and whose properties have been shown (*4*). These plates, which are sensitive to light and normally yellow, blacken rapidly when exposed to light. They are also extremely sensitive to moisture and to water, which instantaneously discolors the plate blackened through exposure. This