Table I—Analysis of Procaine Penicillin and Sulfamethazine in Medicated Feeds

Sample	Procaine Penicillin, mcg./g. Added	Found	± SD	Sulfamethazine, mcg./g. Added	Found	±SD
a_1	394	380	19.7	299	290	3.0
a_1'		373	5.0		286	3.5
a_5		386	13.6		285	5.2
b_1	375	402	3.6	437	433	3.6
b_1'		395	5.0		438	3.6
b_5		390	10.0		435	5.0
c_1	161	180	11.6	288	295	1.0
c_1'		174	4.0		306	7.0
c_5		186	8.3		292	5.7
d_1	241	261	7.8	226	237	6.0
d_1'		275	3.6		237	5.0
d_5		258	7.8		231	2.2
e_1	529	541	9.0	1031	999	4.0
e_1'		552	8.0		1009	2.5
e_5		549	17.1		1017	5.7
f_1	1240	1181	12.3	746	744	7.6
f_1'		1207	16.4		749	16.4
f_5		1145	11.2		733	8.3

layer plates, developed, extracted, and estimated. Also, 10 aliquots (in duplicate) of separate standard solutions of procaine penicillin and sulfamethazine were directly determined to obtain the standard curves.

It can be seen from Fig. 2 that by the previous technique, extraction of procaine penicillin at low levels was almost in agreement with the amounts obtained with the standards; whereas at levels above 40 mcg., the recoveries were considerably poorer. This seems to indicate that with the former procedure the alumina was firmly retaining some of the drug. By the current technique, however, it is apparent that the recovery of the drug is very consistent, and better than 93% recoveries are obtained in the range 10–200 mcg.

Examination of Figs. 2 and 3 reveals that the recovery of sulfamethazine by the previous procedure (91%) was better than that of procaine penicillin. It is evident from Fig. 3 that more than 95% recovery of the sulfa drug was obtained by the current technique. By employing the present procedure, two 1-g. and one 5-g. portions of Sample *a* were analyzed and found to be homogeneous.

To test the validity of the method, six laboratory-blended samples were analyzed and the averages of the results of two duplicate analyses of each sample are given in Table I. A close examination of the data reveals that the estimated contents of procaine penicillin are slightly higher than the amount actually added. The standard deviation[6] varies from ±3.0 to 20.0 for the six samples, indicating that although the variation is large it is still acceptable. On the other hand, in the case of sulfamethazine the results are highly reproducible with exceedingly small standard deviations (within ±10.0) and are in close agreement with the amounts actually added.

A comparison of results of the two 1-g. and the 5-g. portions of each sample shows that there is complete agreement between these analyses. For routine work, a 5-g. sample extraction is recommended for the convenience in extraction and makeup of solutions. This extraction procedure can be profitably extended to many other estimations involving thin-layer chromatography.

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[6] The standard deviation was calculated using the formula $SD = \sqrt{\Sigma(x - \bar{x})^2/n}$ where x is the actual value, $\bar{x}$ the absolute arithme average, and n the number of estimations.

Factors Affecting a Fluorometric Assay of Folic Acid

JOHN R. HAZLETT and DANE O. KILDSIG

Abstract □ A fluorometric method of assaying folic acid, based on the oxidation of folic acid by potassium permanganate, has been investigated. Various factors were found to affect the relative fluorescence obtained in the assay. These include the concentration of potassium permanganate, the length of oxidation time, and the pH and temperature of the solution.

Keyphrases □ Folic acid—analysis □ Potassium permanganate effect—folic acid fluorescence □ pH effect—oxidized folic acid fluorescence □ Temperature effect—oxidized folic acid fluorescence □ Fluorometry—analysis

The oxidative degradation of folic acid by potassium permanganate yields a fluorescent pterine (1). As a result, this oxidation has been used in analytical procedures for the quantitative determination of folic acid (2, 3). The formation of the free pterine derivative provides an approximately 20-fold increase in fluorescent intensity over that of native folic acid fluorescence.

Table I—Final Fluorescent Intensity

Concentration of $KMnO_4$ (moles/l. × 10^{-5})	Fluorescent Intensity (F_∞)
4.43	80.5
6.33	78.0
9.49	75.0
31.6	80.5

In a comprehensive study, Allfrey *et al.* (2) measured directly the fluorescence of the oxidized mixture as well as that of the chromatographically isolated oxidation

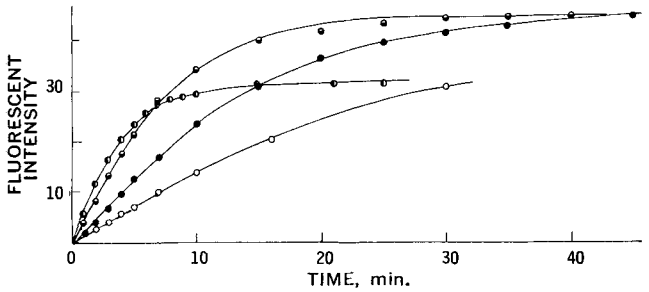

Figure 1—*Effect of $KMnO_4$ concentration on the fluorescent intensity of folic acid solutions. Key: ○, 2.54 × 10^{-5} moles/l.; ●, 4.43 × 10^{-5} moles/l.; ◒, 9.49 × 10^{-5} moles/l.; and ◐, 3.16 × 10^{-4} moles/l.*

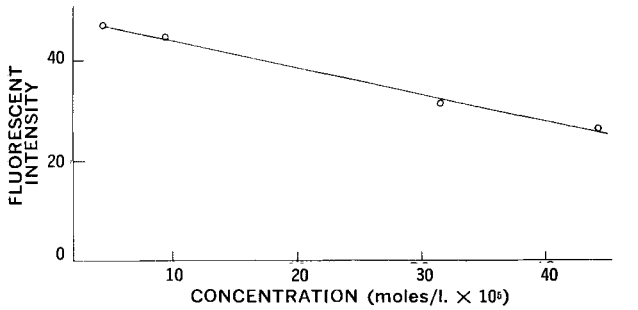

Figure 2—*Decrease in maximum fluorescence with increasing $KMnO_4$ concentration.*

product. Their simplest direct procedure was valid only over the narrow pH range of 3.9 to 4.4, and there was no evidence of fluorescence dependence on potassium permanganate concentration. In the present investigation, this direct method is more thoroughly investigated with respect to the influence of the potassium permanganate concentration on fluorescent intensity.

EXPERIMENTAL

The fluorescence measurements were made on an Aminco-Bowman spectrophotofluorometer. Excitation maxima were found at 275 and 375 mμ with maximum fluorescence observed at 445 mμ.

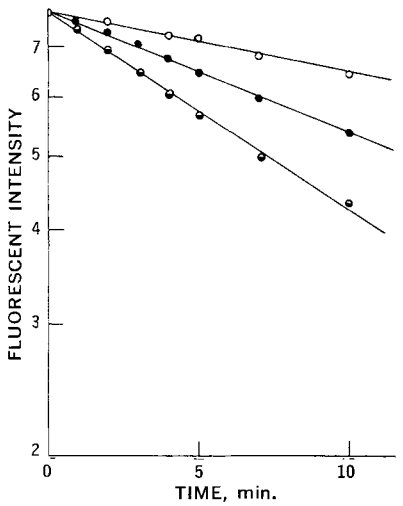

Figure 3—*First-order plots of the oxidation of folic acid with varying concentration of $KMnO_4$. Key: ○, 2.54 × 10^{-5} moles/l.; ●, 6.33 × 10^{-5} moles/l.; and ◒, 9.49 × 10^{-5} moles/l.*

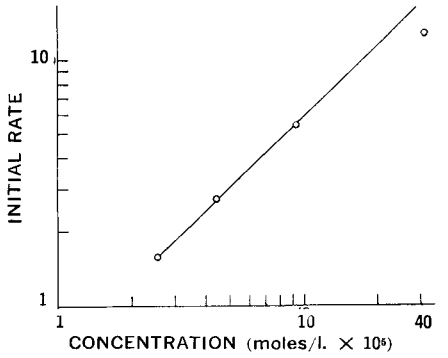

Figure 4—*Log-log plot of the initial rate of the oxidation reaction as affected by the concentration of $KMnO_4$.*

To determine the effect of $KMnO_4$ concentration on the fluorescent intensity of folic acid solutions, the concentration of $KMnO_4$ was varied from 2.54 × 10^{-5} to 4.43 × 10^{-4} moles/l. All solutions contained 4.53 × 10^{-6} moles/l., of folic acid and were buffered at pH 8.12 with a borate buffer. The spectrophotofluorometer was standardized and the fluorescence measured at 25°. The standard solution was obtained by filtering the oxidized folic acid solution to remove the residual manganese dioxide, resulting in a clear colorless solution. If necessary, 1.0 ml. of 3% hydrogen peroxide was added to reduce the remaining $KMnO_4$.

RESULTS AND DISCUSSION

Oxidation of Folic Acid—The oxidation of folic acid may be followed by determining the increase in fluorescence of the solution due to the formation of a pterine.[1] The increase in fluorescent intensity as related to the concentration of $KMnO_4$ is shown in Fig. 1. The rate of fluorophor formation is seen to increase with increasing concentration of $KMnO_4$. However, as the concentration is increased above 4.43 × 10^{-5} moles/l., the total magnitude of the fluorescence decreases. The decrease in maximum fluorescence with increasing $KMnO_4$ concentration is shown in Fig. 2. This decrease in

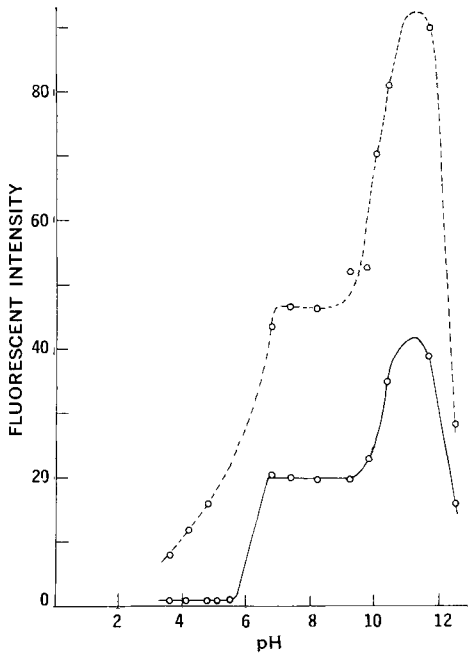

Figure 5—*Effect of pH on the fluorescent intensity of the folic acid oxidation product. Key: - - -, excitation 375 mμ; and ——, excitation 275 mμ.*

[1] 2-Amino-4-hydroxy-6-carboxypteridine (*Reference 1*).

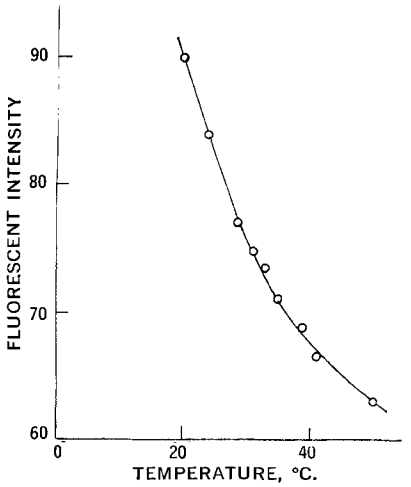

Figure 6—*Effect of temperature on the fluorescent intensity of the folic acid oxidation product.*

fluorescence is probably due to quenching by the MnO_2 formed during the reaction. This effect of $KMnO_4$ concentration probably was not observed by Allfrey *et al.* (2) because of the magnitude of the $KMnO_4$ concentration used (1.23×10^{-3} moles/l.).

A plot of the logarithm of the difference $F_\infty - F_t$, where F_∞ is the final fluorescence and F_t is the fluorescence at time t, is shown in Fig. 3. The linearity of the plot indicates the reaction is first order with respect to folic acid. The value of F_∞ used in this plot was obtained by allowing the solutions to equilibrate for 48 hr. and filtering the solutions to remove the MnO_2. In some cases, 1.0 ml. of 3% hydrogen peroxide was added to reduce any remaining $KMnO_4$. The resulting divalent manganese ions do not appear to quench the fluorescence of the oxidation product. The values obtained for F_∞ are shown in Table I. The close agreement of the final values, essentially independent of $KMnO_4$ concentration, justifies the use of the expression $F_\infty - F_t$ in the logarithmic plot.

The order of the reaction with respect to $KMnO_4$ can be determined from the slope of a plot of log initial rate of reaction *versus* log $KMnO_4$ concentration as shown in Fig. 4. The slope of this line, 0.986, indicates a first-order reaction with respect to $KMnO_4$. The effect of quenching is seen at the highest concentration. The mechanism of this oxidation reaction, which is apparently second order, is being investigated.

Fluorescence *versus* pH—The effect of pH on the fluorescence of the oxidized folic acid is shown in Fig. 5. The existence of a plateau between pH 7 and 9 differs from the narrow plateau between pH 3.9 and 4.4 previously reported (2). This again could be due to the greater concentration of $KMnO_4$ used in the earlier investigation, 1.23×10^{-3} moles/l., which as seen in Fig. 2 would have resulted in a considerable decrease in the fluorescent intensity. The increase in fluorescence near pH 11 may be due to the ionization of the lactim hydrogen of the pterine.

Fluorescence *versus* Temperature—As discussed by Udenfriend (4), fluorescence can be very sensitive to temperature. The effect of temperature on the fluorescence of the oxidation product is shown in Fig. 6. The greatest temperature dependence occurs between 20 and 28° and represents an error of approximately 2% per degree.

CONCLUSIONS

The oxidation of folic acid with $KMnO_4$ has been found to produce a fluorescence whose magnitude is dependent on the concentration of $KMnO_4$. An optimum concentration of $KMnO_4$ was found, above which a decrease in fluorescence occurred due to quenching. The time required for maximum fluorescence also varied with the concentration of $KMnO_4$. As expected, both pH and temperature markedly influenced the fluorescence obtained, the optimum pH being between 6 and 9.

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