LIEU, Van Tune, 1933—
VITAMIN ASSAY BY MEANS OF ULTRAVIOLET
REFLECTANCE SPECTROSCOPY.

University of Hawaii, Ph.D., 1966
Chemistry, analytical

University Microfilms, Inc., Ann Arbor, Michigan
VITAMIN ASSAY BY MEANS OF ULTRAVIOLET
REFLECTANCE SPECTROSCOPY

A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN CHEMISTRY

June, 1966

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ABSTRACT

A procedure whereby ultraviolet reflectance spectroscopy can be employed for the analysis of substances resolved on thin-layer plates was developed with the use of aspirin-salicylic acid mixtures that had been separated on silica gel plates. This system was selected for study not only because its salicylic acid-silica gel G components was relatively stable, but also because no difficulties were encountered in locating the resolved compounds. Both appeared as yellowish-brown spots when the chromatoplates were dried.

The optimum range and maximum accuracy of such analyses were then deduced by applying two graphical methods to data obtained with the use of two systems—rhodamine B adsorbed on silica gel G, which absorbs in the visible, and aspirin adsorbed on silica gel G, which absorbs in the ultraviolet. Plots of experimental data were contrasted with plots that might be expected for an ideal system that conforms to the Kubelka-Munk equation. The results seemed to indicate that the minimum error to be expected is of the order of 6% per 1% reflectance reading error, and that the optimum range for analysis can be arrived at after plotting the reflectance data according to either of the methods discussed, regardless of whether the system in question conforms to the Kubelka-Munk equation or not.

The procedure developed with the aspirin-salicylic acid mixtures was then applied to the analysis of five vitamins of the B group—thiamine hydrochloride, pyridoxine hydrochloride, nicotinic acid, nicotinamide and p-aminobenzoic acid. The suitability of two techniques
devised to locate the resolved vitamins prior to analysis was investigated. One method involved the observation, under ultraviolet light, of chromatoplates prepared with adsorbent containing fluorescent material; the other involved scanning of the plates by means of a spectrophotometer set at an appropriate wavelength. All but two of vitamins could be identified by means of their reflectance spectra, with the two having identical spectra being distinguished with the aid of their Rf values. The procedure also provided quantitative data having a standard deviation of 0.3-0.4 reflectance unit for the vitamins studied.

Finally the utility of the procedure in the analysis of relatively unstable substances was demonstrated by employing it in conjunction with the radioactive tracer technique to determine small amounts of ascorbic acid. Ascorbic acid which had been tagged with the radioactive compound was separated on thin-layer plates and then determined by reflectance spectrophotometric titration. By also measuring the radioactivity of the sample both before and after the development of the chromatoplates, it was possible to estimate the amount of acid that was originally present. Because the reflectance and radioactivity measurements were carried out on the same analytical sample, which was held in a simple, windowless cell designed for this purpose, it was unnecessary to prepare a separate sample for the radioassay.
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INTRODUCTION

Although some vitamins can be estimated biologically, bioassays are, from the standpoint of the precision they afford and the cost they entail, generally inferior to physicochemical methods. As a result, such techniques as spectrophotometry are finding increasing application for this purpose. Before such procedures can be employed, however, it is usually necessary to prevent materials usually found in extracts of plant and animal tissue, drugs and foodstuffs from interfering in the assay.

The thin-layer chromatography has proved to be an important tool in the separation and identification of vitamins. The technique offers many advantages. The required equipment is relatively inexpensive and the preparation and use of the plates are simply and easily carried out. Because of its sensitivity, the method requires only small samples. Often the separations obtained are sharper than those achieved with a similar solvent system on paper. A particular noteworthy advantage is the speed of development. In most cases, only one to two hours is required for the resolution of a mixture on thin-layer plates.

The analytical utility of the thin-layer modification of the chromatographic technique has been enhanced by employing it in conjunction with spectral reflectance. It has been shown that both qualitative and quantitative analyses can be accomplished by means of reflectance measurements carried out on various compounds resolved on thin-layer chromatograms. This research represents an attempt to extend the application of this technique to the analysis of vitamins which
absorb in the ultraviolet region of the spectrum.

Although thin-layer chromatography is still a relatively recent development, the technique has found more and more use in the study of vitamins and has been the subject of several monographs\(^1\)-\(^3\). Various fat-soluble vitamins have been resolved by means of the thin-layer technique; \(\beta\)-carotene and vitamin A by Lagoni et al.\(^4\)\(^5\); \(\alpha\)-tocopherol, \(\beta\)-carotene, vitamin K and ubiquinones by Wagner\(^6\); \(\alpha\)-, \(\beta\)- and \(\gamma\)-tocopherol by Seher\(^7\). Davidek et al.\(^8\) carried out chromatographic investigations of vitamins A, D\(_2\), E, K\(_1\), K\(_2\), K\(_3\), and \(\alpha\)- and \(\beta\)-carotene. Model mixtures were separated with the use of fourteen solvents and \(R_f\) values were reported. Thus far, relatively little has been reported concerning the water-soluble vitamins. Mixtures of nicotinic acid and nicotinamide, and mixtures of pyridoxal, pyridoxine (vitamin B\(_6\)) and pyridoxamine have been separated by Nurnberg\(^9\)-\(^10\). Strohecker\(^11\) prepared the dinitrophenylhydrazone of ascorbic acid and investigated this substance chromatographically. Using layers of silica gel G containing 2% fluorescent material, Gänshirt and Malzacher\(^12\) resolved mixtures of 6 vitamins of the B group and vitamin C.

The detection of vitamins resolved on thin-plates generally involved the use of suitable chromogenic reagents. Observation of the chromatogram under ultraviolet light, or under white light if the compounds were colored, has also been employed. An ingenious procedure devised by Gänshirt and Malzacher\(^12\) involved the observation under ultraviolet illumination of chromatograms prepared with adsorbent containing fluorescent material. This made it possible to detect colorless ultraviolet-absorbing vitamins. For evaluation of the
chromatograms, a comparison of the spots obtained with those resulting from parallel runs with a series of standards was frequently employed (5,7,9,13). This approach required a reproducible thickness of adsorbent and usually provided only a semi-quantitative estimate. Alternatively, the spot has been removed from the chromatoplate and the substance of interest has been eluted and estimated by physicochemical methods (11,14-15).

Two shortcomings of the chromatographic method, however, restrict its analytical utility. In the first place, the difficulty experienced in obtaining reproducible $R_F$ values with thin plates often makes it necessary to run standards beside the samples for comparison purposes. Secondly, the quantitative removal and the elution of individual spots from plates is a tedious process which often cannot be accomplished without decomposition occurring. Both operations are rendered superfluous by the in situ identification and analysis of the chemical species separated on thin plates by means of reflectance spectrophotometry. The use of the reflectance method thus simplified and accelerates the analysis of various mixtures.

The theoretical basis for diffuse reflectance spectroscopy was developed over thirty years ago by Kubelka and Munk (16-17). A considerable amount of information regarding practical applications of the phenomenon has been summarized by Judd and Wyszecki (18). Radiation reflected from a finely ground solid consists of two parts, a regular and a diffuse part. Specular reflection occurs to a certain extent at all surfaces which constitute the boundaries of condensed phases. The diffuse reflection is the backward multiple scattering of that portion
of the incident flux which has penetrated into the interior of the sample with attendant partial absorption as well as multiple scattering at the boundaries of the individual particles. These two types of reflection are largely complimentary and the elimination or minimization of regular reflection is important to the measurement of diffuse reflectance.

Kubelka and Munk(16-17) have shown that for special cases involving an infinitely thick, opaque layer, which can be achieved in practice with a layer thickness of a few millimeters, the diffuse reflectance is

$$F(R_\infty) = \frac{(1-R_\infty)^2}{2R_\infty} = \frac{k}{s}$$  \hspace{1cm} (1)

where $R_\infty$ is the diffuse reflectance of such a layer, relative to a nonabsorbing standard such as MgO or NaCl; $k$ is the molar absorption coefficient of the sample; and $s$ is the scattering coefficient, which is practically independent of wavelength for particles of mean diameter greater than the wavelength of the incident radiation. It has been shown by Kortüm and his co-workers(19-21) that a linear relationship between $F(R_\infty)$ and $k$ held only when $k$ was sufficiently small, as when one is dealing with weakly absorbing substances or low concentrations of an adsorbed species, and when the grain size of the powders employed is less than 1 $\mu$ in diameter. This last is in accord with the results obtained by Zeitlin and Niimoto(22), who observed that the particle size of the adsorbent can affect reflectance spectra in that absorption bands tended to broaden as particle size increased. This phenomenon was ascribed by Kortüm(20,23) to interference by regular
reflection, and he and his co-workers(24) found that such interference was diminished if samples were ground fourteen hours in a ball mill. Similar results are obtained if commercial grade thin-layer chromatography adsorbents, which consist of particles having an average diameter of 5 \( \mu \), are employed(25).

The use of spectral reflectance for analytical purposes has been suggested by various investigators. Naughton et al(26-27) pointed out the advantages afforded by this technique for studies of biochemical systems. Some of the earlier applications of this technique involved its employment in obtaining analytically useful reflectance spectra and in the determination of organic and inorganic compounds adsorbed or resolved on paper. Although various methods of graphing data were used in the preparation of calibration curves(28-33), linear plots were obtained only for the particular concentration range under consideration. Various forms of the Kubelka-Munk function were employed by early investigators in the analysis of compounds resolved on paper(34-37). In all cases, a linear or near-linear relationship between the function and concentration was found to occur over a limited range. Ingle and Minshall(38) carried out a critical comparison of the reflectance and transmission techniques for the analysis of spots on paper chromatograms and reported a precision of \( \pm 0.43 \) in percent reflectance for four replicate analyses of copper rubeanate on paper.

Other workers concerned themselves with the application of the reflectance technique to the analysis of substances adsorbed on powders. It has been demonstrated that reflectance spectra of substances concentrated on particulate adsorbents can be used for their
identification(39), and that spectral reflectance can be employed to
determine the concentration of dyes scavanged from solution by the
batchwise addition of starch(40). A critical evaluation of the direct
analysis of solid mixtures of pigments by means of reflectance measure-
ments was carried out by Lermond and Rogers(41). A method for the
determination of dyes, such as malachite green and congo red, adsorbed
on powders was devised by Fisher and Vratny(42), who observed that the
accuracy-limiting operation in the analysis was that of sample
preparation. Kortüm and Herzog(43) employed ultraviolet reflectance
spectroscopy for the analysis of rutile-anatase powder mixtures.
Recently Doyle and Forbes(44) analyzed two- and three-component solid
mixtures with the use of reflectance measurements. Kortüm et al.(24)
recommended standardized grinding of samples in a ball mill for several
hours as a means of preparing reproducible analytical samples having
uniformly flat surfaces and identical densities. This is, unfortunately,
a relatively inefficient and tedious process.

More recently, the application of spectral reflectance to thin-
layer chromatography has made it possible to effect the in situ
identification and determination of chemical species separated on thin
plates. Reliable methods have been devised by Frodyma et al. for the
in situ identification and determination of the components of dye
mixtures resolved on thin-layer plates(45), and for the analysis of
amino acids separated on chromatoplates(46-47). It was decided to
modify these methods so that they might be applied to the analysis of
vitamins, thereby combining the advantages of the thin-layer technique
with those afforded by reflectance spectroscopy. Such an in situ
analysis of vitamins resolved on thin-plates would make it unnecessary either to elute spots for physicochemical analyses or to compare spots with those resulting from parallel runs with a series of standards.
A. The application of ultraviolet reflectance spectroscopy to thin-layer chromatography (Ref. 48).

The first phase of the research concerned itself with the modification of existing experimental technique so that they could be employed with vitamins absorbing in the ultraviolet region of the spectrum. A means of locating and identifying sample spots resolved on thin plates was devised and the cell described by Frei and Frodyma(49) was modified for use in the ultraviolet region. Mixtures of aspirin (acetyl salicylic acid) and salicylic acid separated on silica gel G plates served as a pilot system for this phase of the research. These particular mixtures were selected not only because the two compounds absorb in the ultraviolet region, but also because they yielded a relatively stable system which presented no difficulties in the location of the compounds at the conclusion of the chromatographic resolution. Both appear as yellowish-brown spots when dried.

The aspirin and salicylic acid, which were of Merck U. S. P. and Chase U. S. P. purity, respectively, were dried over sulfuric acid for 24 hours before use. 0.10 M. stock solutions of the compounds in chloroform were used to prepare the dilution series employed in this study. Solutions were applied as spots, at a point approximately 3 cm. from one end of the plate and 2 cm. above the solvent level, by means of 5 and 10 μl micropitpes. The 20 x 5 x 0.35 cm. plates were coated with adsorbent by distributing a 1:2 Merck silica gel G-water mixture with a glass rod which rested on two thickness of masking tape affixed to the ends of the plates. This technique gave a uniform coating
0.5-0.7 mm. thick. After the plates had been allowed to "set" for 20 minutes at room temperature, they were dried at 110°C for 1 hour and stored in a desiccator.

After spotting, the plates were dried by letting them stand at room temperature for 10 minutes and then developed by the ascending technique with an 85:15:10 hexane, glacial acetic acid and chloroform mixture. The developing chamber was rendered air-tight by employing a rubber band in conjunction with Saran Wrap (Dow Chemical Company, Midland, Michigan), a plastic material commonly employed for the protection of foodstuffs. Approximately 90 minutes were required for the solvent front to travel the length of the plate at room temperature. The Rf values observed for salicylic acid and aspirin were 0.35 and 0.2 respectively. Although in preparative work the spots may be removed from the chromatoplates directly after their development, in the analysis of the mixtures, the plates were dried at a temperature of 90°C in an oven for 2 hours.

The reflectance spectra of the compounds were obtained with a Beckman Model DK-2 Spectrophotometer fitted with a standard reflectance attachment. All other measurements were made with a similarly equipped Beckman Model DU Spectrophotometer. The cells used to hold both the analytical samples and the reference material consisted of a circular quartz plate, which had a diameter of 22 mm., superimposed on a 40 x 40 x 1 mm. piece of white paperboard. The quartz disk was held in place by means of a 40 x 40 x 3 mm. plastic plate which affixed to the backing paper with two pieces of masking tape. A circular window, 19 mm. in diameter, in the upper surface of the plate opened into a
concentric circular well, 24 mm. in diameter, that was deep enough to accommodate the quartz disk. These data are presented schematically in Figure 1, as is a sketch of the assembled cell.

The 70 mg. which comprised the analytical samples were weighed to the nearest ± 0.2 mg. and ground in a small agate mortar for two periods of 20 seconds each to insure homogeneity and uniform particle size. The material was then introduced into the cell and carefully compressed between the quartz disk and the paperboard by rotating the former until a thin layer having an approximate diameter of 22 mm. and approximate thickness of 18 mg./cm.² were obtained. The reference standard in all cases consisted of silica gel G adsorbent from the plates under investigation.

B. Selection of the optimum range for reflectance spectrophotometric analysis.

Because there has been no attempt to define the optimum range for reflectance spectrophotometric analysis, it seemed appropriate to investigate means of selecting the optimum range for and determining the maximum accuracy of such analyses.

The dilution series employed in this study were prepared with the use of stock solutions of aspirin and rhodamine B. The former was a 0.1 M. solution of Merck U. S. P. grade aspirin in chloroform while the latter contained 50 mg. of the dye per 100 ml. of water. Aliquots of the stock solutions were added to appropriate amounts of Merck silica gel G which had been weighed to the nearest ± 0.4 mg. This amounted to 70 mg. in the case of the aspirin and 40 mg. in the case of the rhodamine B. The resulting mixtures were dried at 90°C for two hours
FIGURE 1. DIMENSIONS OF CELL ELEMENTS AND SKETCH OF ASSEMBLED CELL.
in a mechanical convection oven and then ground in a small agate mortar for two periods of 20 seconds each to insure homogeneity.

Reflectance measurements were made with a Beckman Model DU Spectrophotometer fitted with a standard reflectance attachment. The rhodamine B-silica gel and aspirin-silica gel mixtures were packed in cells employing glass(49) and quartz(48) covers, respectively. In all cases the reference standard consisted of silica gel G.

C. The detection and determination by ultraviolet reflectance spectroscopy of vitamins resolved on thin-layer plates.

Once an appropriate technique for the application of ultraviolet reflectance spectroscopy had been devised, the analysis of mixtures of vitamins that absorb in the ultraviolet was investigated. Five vitamins of the B group were included in this study; \( B_1 \) (thiamine hydrochloride), \( B_6 \) (pyridoxine hydrochloride), nicotinic acid, nicotinamide and p-aminobenzoic acid. The vitamins, from Nutritional Biochemical Corp., were used in the form of aqueous solutions. These were 0.1 M. except for the p-aminobenzoic acid which was 0.02 M. Solutions were applied as spots by means of 5 and 10 \( \mu \)l. micropipets. The use of two adsorbent systems was investigated, Merck silica gel G and Merck silica gel G to which 2% of a luminous pigment had been added. The latter was prepared by weighing out silica gel G and the luminous pigment "2S super"* in a 98:2 ratio and shaking them together in a closed flask. The 20 x 5 x 0.35 cm. plates were coated by distributing a 1:2 adsorbent water mixture with a Desaga-Brinkman Model "S II" applicator. The height of

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*Riedel-DeHaën AG. Seelze-Hannover, Federal Republic of Germany.
the applicator gate was set at 0.25 mm. for plates used in the location and identification study, and 0.50 mm. for plates used for quantitative work. A Beckman Model DK-2 Spectrophotometer fitted with a standard reflectance attachment was used to locate the components of mixtures resolved on chromatoplates and to record their reflectance spectra. Two types of cells were used. One was the type depicted in Figure 1 and was used to record the reflectance spectra. The other was a windowless cell which consisted of a 35 x 40 mm. plastic plate affixed to white paper board having similar dimensions with two pieces of masking tape, and was used to hold the analytical sample in quantitative work. As may be seen in Figure 2, which presents the dimensions of the cell elements as well as a sketch of the assembled cell, the plastic plate had a circular opening, 21 mm. in diameter, in its center. The cell was packed by introducing the sample into the opening and then compressing it with a fitted tamp made of an aluminum planchet affixed to a cork stopper.

Procedure for location and identification of components of mixtures resolved on chromatoplates

Solutions were applied as spots on 0.2 mm. thin-plates which were then dried at 45°C for 20 minutes. The plates were developed by the ascending technique with the use of a 5:5:20:70 glacial acetic acid, acetone, methanol and benzene mixture according to a procedure devised by Ganeshir et al.(12). The developed plates were dried at 45°C for 20 minutes.

In those cases where the silica gel G adsorbent included the luminous pigment the chromatoplates were examined after development by
FIGURE 2. (A) DIMENSIONS OF CELL ELEMENTS AND SKETCH OF ASSEMBLED WINDOWLESS CELL. (B) TAMM USED TO PACK CELL.
means of an ultraviolet lamp\(^{*}\) (maximum at 254 nm), with the location of the spots being revealed by the absence or diminution of fluorescence. Once the spots had been located, they were carefully removed from the plate and placed upon approximately 50 mg of adsorbent contained in the quartz window cells described earlier. The spectra were then recorded in the usual way with a Beckman Model DK-2 Spectrophotometer fitted with a standard reflectance attachment.

Where the adsorbent consisted only of silica gel G, the location of the vitamins was accomplished by scanning the thin-plates with the use of a Beckman Model DK-2 Spectrophotometer fitted with a standard reflectance attachment and set at a wavelength corresponding to an adsorption maximum for the vitamin of interest. The scanning was carried out by holding the chromatoplate, which was taped to a protective plastic plate, against the sample exit port of the reflectance attachment unit and exposing the adsorbent layer to the impinging beam of light along the path of chromatographic development. As may be seen in Figure 3, the 0.3-cm. thick plastic plate, whose outer dimensions matched those of the chromatoplates, had a 3 x 18 cm. window about which were spaced four 0.1 x 0.2 cm. strips of plastic. A sudden decrease in reflectance occurred when the beam of light impinged upon a spot containing the vitamin of interest. During the scanning, the reflectance attachment was covered with a dark cloth to eliminate outside light. Once a particular spot was located, its position was marked on the reverse side of the glass plate for \(R_f\) calculations and

FIGURE 3. ASSEMBLY EMPLOYED FOR THE SCANNING OF CHROMATOPLATES.
its reflectance was recorded in the manner described in the above paragraph. The reference standard in all cases consisted of adsorbent from the plate under investigation.

**Procedure for quantitative work**

The same procedure was employed with both adsorbent systems. 0.10 M. aqueous solutions of nicotinic acid and nicotinamide were applied to 0.4 mm. thin-plates which were then dried at 90°C for 30 minutes. When the volume applied was 20 µl or greater, the solution was added in 10 or 15 µl increments, with the plates being dried for a few minutes at 90°C between additions. This precaution was taken to minimize the spot size so that all of the material would be included in the 80 mg. of adsorbent that comprised the analytical sample. The plates were developed for one hour with the use of a 5:5:20:70 glacial acetic acid, acetone, methanol and benzene mixture and dried for two hours at 90°C. The spots were then located by employing either of the two procedures described earlier. For quantitative analysis, the spots were scraped off along with enough adsorbent to make up a total weight of 80 ± 0.3 mg. This sample was then ground in a small agate mortar for seven sequences of thirty strokes each to insure homogeneity and uniform particle size. Between sequences, the mixture was loosened from the body of the mortar with a spatula. Finally, the sample was packed in the windowless cell described earlier and its reflectance at 264 µm was measured. The reference standard was prepared in the same manner as the analytical sample and consisted of adsorbent from the plate under investigation.
D. The determination of ascorbic acid resolved on thin-layer plates by reflectance spectrophotometric titration and radioactive tracer technique.

**Tagging, separation, and titration of ascorbic acid.**

Approximately 0.3 mg. of 14 $\mu$C./mg. $^{14}$C-ascorbic acid* was introduced into and mixed thoroughly with 1 ml of a freshly prepared 5% metaphosphoric acid solution which was 0.600 N (0.300 M.) with respect to ascorbic acid. This solution was then either treated with liquid bromine or allowed to stand at room temperature for several days. In each case analyses were carried out to determine the original ascorbic acid content. For each analysis, eight aliquots of the solution were applied as spots by means of a 10 $\mu$l. micropipet on thin-plates having 0.4 mm. thick adsorbent layer consisting of the silica gel G-luminous pigment mixture described earlier. The 20 x 20 x 0.35 cm. plates were coated by distributing a 1:2 mixture of the adsorbent and water with a Desaga-Brinkman Model "S II" applicator whose gate was set at a height of 0.50 mm. The plates were dried at 110$^\circ$C for 2 hours and stored in desicator. After spotting, the plates were dried at 50$^\circ$C for 30 minutes, developed by the ascending technique with the use of a 70:20:5:5 benzene, methanol, glacial acetic acid and acetone mixture(12), and then allowed to stand in a hood for 2-3 minutes until most of the volatile solvent had evaporated. The spots of ascorbic acid were located under ultraviolet illumination and their location marked with a pointed glass rod. To these spots, varying

*New England Nuclear Corp., 575 Albany St., Boston, Massachusetts.
increments of 0.400 N.(0.100 M.) aqueous selenious acid were added by means of micropipets. Finally, the plates were dried again at 50°C for 30 minutes. In order that decomposition of ascorbic acid might be minimized, the separation and titration procedures were carried out under red-light illumination.

Measurement of reflectance.

Reflectance spectra were recorded with a Beckman Model DK-2 Spectrophotometer fitted with a standard reflectance attachment and with the use of the cell depicted in Figure 1 to hold samples. All other measurements were made with a similarly equipped Beckman Model DU Spectrophotometer. Windowless analytical cells, similar to the one shown in Figure 2, were employed to hold the 90 mg. titrated ascorbic acid samples, which were weighed to the nearest ±0.4 mg. The samples were prepared for measurement according to the procedure outlined earlier for the use of the windowless cell. The reference standard in all cases consisted of adsorbent from the plate under investigation.

Measurement of radioactivity.

The cells used to hold the titrated samples for the measurement of reflectance were also used for the radioactivity measurements. Radioactivities of the samples were measured with a Tracerlab Model SC-81 Versa/Matic II using a Model FD-1 gas-flow, Geiger Müller counter. The counter was fitted with a ultra-thin Mono Mo1(< 150 µg./cm²) window to minimize soft beta absorption. In order to prevent variation in counting efficiency the geometry of all samples relative to the counter tube was kept constant by positioning the samples exactly in
the center of the counter planchet adapter. To this end, mutually perpendicular lines whose extensions would have intersected at the center of the cell were drawn on the plastic plate of the sample holder. Similar lines were also inscribed on the surface of the planchet adapter. The centering of the sample was thus readily accomplished by aligning the lines on the cell with those on the planchet adapter, as may be seen in Figure 4.

To measure the radioactivity of undeveloped sample spots, aliquots of the sample solution equal in volume to the aliquots used for the titration reaction were pipetted onto thin-plates and dried at 50°C for 30 minutes. The spots were then excised and prepared for the measurement of radioactivity in the same manner as the titrated samples.
FIGURE 4. THE ASSEMBLY FOR HOLDING SAMPLE FOR THE MEASUREMENT OF RADIOACTIVITY. w, WINDOWLESS ANALYTICAL CELL; c, CIRCULAR WELL FOR HOLDING SAMPLE; t, TRANSPARENT TAPE; g, GEIGER COUNTER PLANCHET ADAPTER; l, MUTUAL PERPENDICULAR LINES ON CELL; l', MUTUAL PERPENDICULAR LINES ON GEIGER COUNTER PLANCHET ADAPTER.
RESULTS AND DISCUSSION

A. The application of ultraviolet reflectance spectroscopy to thin-layer chromatography (Ref. 48).

Because a large number of substances, including some vitamins, have characteristic ultraviolet spectra that may be employed in their analysis, it was felt that the application of ultraviolet reflectance spectroscopy to thin-layer chromatography would prove to be an invaluable analytical tool, especially for those dealing with the pharmaceutical and biochemical sciences. The feasibility of this approach was ascertained by employing it to determine the composition of mixtures of aspirin (acetyl salicylic acid) and salicylic acid which had been separated on silica gel plates. This particular system was selected for study not only because the spectra of the two compounds were suited for the purpose at hand, but also because it presented no difficulties in the matter of locating the compounds at the conclusion of the resolution. Both appeared as yellowish-brown spots when the plates were dried.

Optimum conditions for drying of chromatoplates

The procedure employed in the drying of the chromatoplates following their development was decided upon after a study of the effect of drying times and temperatures upon the stabilities of salicylic acid and aspirin adsorbed on silica gel G. When samples containing 5 μmoles were prepared according to the procedure outlined in the experimental section and then subjected to different drying conditions, the reflectance spectra presented in Figures 5 and 6 were
FIGURE 5. REFLECTANCE SPECTRA OBTAINED FOR SALICYLIC ACID ADSORBED ON SILICA GEL G AT INDICATED INTERVALS AFTER SPOTTING: (1) AFTER 15 MINUTES AT ROOM TEMPERATURE; (2) AFTER AN ADDITIONAL HOUR AT 90°C; (3) AFTER STILL ANOTHER HOUR AT 90°C.
FIGURE 6. REFLECTANCE SPECTRA OBTAINED FOR ASPIRIN ADSORBED ON SILICA GEL G AT INDICATED INTERVALS AFTER SPOTTING: (1) AFTER 15 MINUTES AT ROOM TEMPERATURE; (2) AFTER 2 MORE HOURS AT ROOM TEMPERATURE; (3) AFTER AN ADDITIONAL 5 MINUTES AT 90°C; (4) AFTER AN ADDITIONAL 10 MINUTES AT 90°C; (5) AFTER STILL ANOTHER 10 MINUTES AT 90°C.
obtained. As may be seen in Figure 5, the general form of the spectrum for salicylic acid, which exhibited an absorption maximum at 302 μm, was unaffected by variations in drying time and temperature. The spectrum for aspirin, on the other hand, underwent substantial changes. A consideration of Figure 6, which shows the aspirin spectra, discloses that the position of the absorption maximum varied between 278 μm to 302 μm. Because aspirin is known to form salicylic acid upon hydrolysis and because the reflectance spectrum of the decomposition product of aspirin is identical with that obtained for salicylic acid, this shift of the absorption maximum may be ascribed to the conversion of aspirin into salicylic acid. As a matter of fact, a complete conversion of aspirin resolved on chromatoplates can be achieved by spraying the plates with water and then drying them at 90°C.

By making use of this conversion, it was possible to devise a more sensitive method for the determination of aspirin, because the decomposition product absorbs more strongly than aspirin itself. The experimental conditions under which the conversion was induced were selected after studying the effect of varying drying-times and temperatures upon the % reflectance at 302 μm—the absorption maximum for both salicylic acid and the decomposition product of aspirin—of 1 μmole of salicylic acid and 1 μmole of aspirin adsorbed on silica gel G. Three sets of samples of each compound were dried for varying periods of time. One set was dried at 65°C, the second at 90°C, and the third at 90°C, following spraying of the chromatoplates with water. The data obtained for salicylic acid are presented in Figure 7 while
FIGURE 7. % REFLECTANCE AT 302 mw. OF 1 µmole OF SALICYLIC ACID ADSORBED ON SILICA GEL G AS A FUNCTION OF DRYING TIME. ○-○ DRIED AT 65°C; □-□ DRIED AT 90°C; △-△ DRIED AT 90°C FOLLOWING SPRAYING WITH WATER.
those for aspirin may be found in Figure 8. As shown therein, all three drying procedures eventually resulted in a constant per cent reflectance reading. A drying period of 2 hours at 90°C was selected since it was most convenient and expeditious.

**Optimum sample thickness**

The relationship between sample thickness and reflectance in the visible region of the spectrum has been studied by Frei and Frodyma(49), who recommended an optimum sample size of 40 mg. for use with silica gel G adsorbent and white paperboard as backing material. Since the diameter of the samples was approximately 1.8 cm., this amounted to a sample thickness of 16 mg./cm². The matter was re-examined from the standpoint of employing ultraviolet radiation by measuring the per cent reflectance at 302 μm of samples consisting of 3 μmoles of salicylic acid per 70 mg. of adsorbent but of different thicknesses. In Table I, which summarizes the results of this study, the per cent reflectance readings which are listed for the different thicknesses and backing materials are mean values of six measurements made with single samples that were repacked prior to each measurement. As indicated, constant reflectance readings were obtained when the sample thickness exceeded 16 mg./cm² with white backing paper, and 22 mg./cm² with brown, plastic backing material. For values less than these, a gradual increase in per cent reflectance accompanied each increase in sample thickness. It was decided to employ a thickness of 18 mg./cm² in conjunction with white paperboard as this would allow for possible losses of material that might occur during the packing of the analytical sample in the cell.
FIGURE 8. % REFLECTANCE AT 302 μm OF 1 μmole OF ASPIRIN ADSORBED ON SILICA GEL G AS A FUNCTION OF DRYING TIME. □-□ DRIED AT 65°C; ○-○ DRIED AT 90°C; △-△ DRIED AT 90°C FOLLOWING SPRAYING WITH WATER.
TABLE I. % REFLECTANCE AT 302 μm OF SALICYLIC ACID ADSORBED ON SILICA GEL G AS A FUNCTION OF SAMPLE THICKNESS AND NATURE OF BACKING MATERIAL.

<table>
<thead>
<tr>
<th>White Paperboard Thickness (mg./cm²)</th>
<th>Reflectance (%R)</th>
<th>Brown Plastic Thickness (mg./cm²)</th>
<th>Reflectance (%R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>45.0</td>
<td>7</td>
<td>40.1</td>
</tr>
<tr>
<td>7</td>
<td>45.6</td>
<td>11</td>
<td>40.8</td>
</tr>
<tr>
<td>12</td>
<td>45.7</td>
<td>17</td>
<td>41.3</td>
</tr>
<tr>
<td>16</td>
<td>46.0</td>
<td>20</td>
<td>41.7</td>
</tr>
<tr>
<td>19</td>
<td>45.9</td>
<td>22</td>
<td>41.8</td>
</tr>
<tr>
<td>23</td>
<td>46.0</td>
<td>28</td>
<td>41.8</td>
</tr>
<tr>
<td>30</td>
<td>46.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Inasmuch as the samples used in this study had a diameter of 22 mm.,
this thickness corresponded to an optimum sample size of 70 mg.
Grinding and packing of samples

The standardized grinding procedure outlined in the experimental section had to be adhered to as variations in the grinding operation resulted in measurable changes in the apparent reflectance. Similar observations were reported by Frodyma et al. (45) for measurements made with visible light. Such fluctuations can be attributed, at least in part, to the variation in particle size caused by deviations from a standardized grinding procedure, and Kortüm (21) has found that the apparent reflectance increases with decreasing particle size. This increase Kortüm ascribed to a decrease in the average thickness of the layer of material being penetrated by the light.

The greatest difficulty encountered in the attainment of an acceptable degree of precision was associated with the packing of the sample in the cell. In an earlier investigation of the packing of samples carried out by Frodyma et al. (45) in connection with work in the visible region it was found that maximum reproducibility was attained with samples of uniform thickness and of constant dimensions. Except for the fact that uniformity in thickness was not as critical, the same requirements held for measurements made with ultraviolet light. That the uniformity of sample thickness was not as critical is probably due to the fact that the thickness was 2 mg/cm² in excess of the minimum needed for constancy of reflectance. Another variable in the preparation of the sample was the extent of the pressure that was applied when the material was compressed between the quartz disk and the backing paper. When the pressure exerted was excessive, a decrease in the apparent reflectance of a salicylic acid-silica gel G sample
amounting to 1 to 2% was noted. For pressures less than excess, any deviations observed were within instrumental limitations.

To determine the reproducibility of packing the reflectance cell, the per cent reflectance of three samples of different concentrations of salicylic acid adsorbed on silica gel was measured after each of three separate packings of each sample. The results of this study, which are set forth in Table II, are almost identical with data obtained during an earlier study dealing with visible light(45). The differences observed between pairs of readings for the same sample were of the same order as those obtained for replicate samples, and in no case exceeded 0.6 reflectance units. It is therefore apparent that the factor limiting the precision of the technique is associated with the packing of the sample in the cell.

The determination of aspirin and salicylic acid

Calibration curves were prepared for both aspirin and salicylic acid by applying different concentrations of the two compounds on thin-layer plates which were then developed, dried, and readied for the measurement of reflectance according to the method outlined in the experimental section. In both cases the per cent reflectance at 302 μm was determined and then plotted as a function of concentration to give a smooth curve. Three of the curves obtained with salicylic acid and two obtained with aspirin are presented in Figure 9. Although the data graphed there were gathered at different times and with the use of different dilution series of the two compounds, the five curves are remarkably similar and almost coincide in the concentration range
TABLE II. TEST OF REPRODUCIBILITY OF PACKING REFLECTANCE CELL.
% REFLECTANCE AT 302 μm. OF SALICYLIC ACID ADSORBED
ON SILICA GEL G.

<table>
<thead>
<tr>
<th>Concentration (μmole/70 mg. silica gel G)</th>
<th>1st Packing</th>
<th>2nd Packing</th>
<th>3rd Packing</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>54.3</td>
<td>54.4</td>
<td>54.3</td>
</tr>
<tr>
<td>1.0</td>
<td>70.2</td>
<td>70.8</td>
<td>70.4</td>
</tr>
<tr>
<td>0.5</td>
<td>86.2</td>
<td>86.1</td>
<td>86.8</td>
</tr>
</tbody>
</table>
FIGURE 9. % REFLECTANCE AT 302 μm. OF SALICYLIC ACID AND ASPIRIN ADSORBED ON SILICA GEL G AS A FUNCTION OF CONCENTRATION.
below 1.4 μmoles. This suggests the possibility of employing a single calibration curve for the determination of both substances. The close coincidence of the curves obtained with a single compound also indicates that it is unnecessary to run a set of standards beside each unknown in routine analysis. A more useful form of the data results when absorbance is plotted against the square root of the concentration, as is done in Figure 10, since the linear or near-linear relationship which is barely evident in Figure 9 and then only at low concentration levels is extended to an upper limit of approximately three μmoles for both salicylic acid and aspirin.

The precision that can be anticipated when aspirin and salicylic acid are resolved on chromatoplates and then analysed by means of ultraviolet reflectance spectroscopy was determined by running through the procedure outlined above with four one μmole replicates of each compound. The standard deviation of the reflectance readings obtained for these different samples and listed on Table III was found to be 0.37 reflectance units for salicylic acid and 0.47 reflectance units for aspirin. There was no difference between any pair of readings which exceeded 0.9 reflectance units with salicylic acid and 1.1 reflectance units with aspirin. Finally, by making use of the precision data given in Table III and the calibration curves presented in Figure 9, the probable errors in the measurement of concentrations of salicylic acid and aspirin were determined to be 2.2% and 2.8%, respectively.

From the above it is obvious that the components of salicylic acid-aspirin mixtures resolved on thin-layer plates can be determined
FIGURE 10. ABSORBANCE AT 302 μm OF SALICYLIC ACID AND ASPIRIN ADSORBED ON SILICA GEL G AS A FUNCTION OF THE SQUARE ROOT OF CONCENTRATION.
TABLE III. REPRODUCIBILITY OBTAINED FOR DIFFERENT SAMPLES OF IDENTICAL CONCENTRATIONS (1 μmole) OF SALICYLIC ACID AND ASPIRIN ADSORBED ON SILICA GEL G. % REFLECTANCE AT 302 μm.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Salicylic Acid (%R)</th>
<th>Aspirin (%R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>67.6</td>
<td>68.0</td>
</tr>
<tr>
<td>2</td>
<td>66.7</td>
<td>67.6</td>
</tr>
<tr>
<td>3</td>
<td>67.3</td>
<td>66.9</td>
</tr>
<tr>
<td>4</td>
<td>67.2</td>
<td>67.8</td>
</tr>
<tr>
<td>Mean and Std. Dev.</td>
<td>67.2 ± 0.37</td>
<td>67.6 ± 0.47</td>
</tr>
</tbody>
</table>
by carrying out ultraviolet spectral reflectance measurements on spots removed from the dried plates and packed in an appropriate cell. Calibration curves obtained for the two compounds by means of this procedure were so similar and so reproducible that for routine analyses a single curve that needed to be checked only periodically sufficed for the analysis of both salicylic acid and aspirin. The method provided quantitative data having a standard deviation amounting to 0.37 reflectance units for salicylic acid and 0.47 reflectance units for aspirin. A linear or near-linear relationship between absorbance and the square root of the concentration was observed with spots containing up to 3.0 μmoles of either compound.

This technique should be applicable to the analysis of a large number of complex organic compounds which absorb in the ultraviolet and which are resolvable on thin-layer plates. Especially promising is the employment of this technique in conjunction with another involving the incorporation of fluorescent and phosphorescent materials in the adsorbent layer(12, 50-52).

B. Selection of the optimum range for reflectance spectrophotometric analysis.

For the purpose at hand probably the most appropriate theory treating diffuse reflection and the transmission of light-scattering layers also happens to be the most general theory developed by Kubelka and Munk(16-17). For cases involving an infinitely thick, opaque layer (achieved in practice with a layer thickness of a few millimeters), the Kubelka-Munk equation may be written as
\[
F(R_{\infty}) = \frac{(1 - R_{\infty})^2}{2R_{\infty}} = \frac{k}{s}
\]

Where \( R_{\infty} \) is the diffuse reflectance of the layer relative to a nonabsorbing standard such as magnesium oxide, \( k \) is the molar absorption coefficient of the sample, and \( s \) is the scattering coefficient. Provided \( s \) remains constant, a linear relationship should be observed between \( F(R_{\infty}) \) and \( k \). This has been confirmed for weakly absorbing materials where the contribution of regular reflection is small(53).

When the reflectance of a sample diluted with a non- or low-absorbing powder is measured against the pure powder, the absorption coefficient, \( k \), may be replaced by the product \( 2.303 \, e \, c \), where \( e \) is the extinction coefficient and \( c \) is the molar concentration(54). Since \( F(R_{\infty}) \) is proportional to the molar concentration under constant experimental conditions, it should be possible to employ the Kubelka-Munk relationship for purpose of analysis in the same way that the Beer-Lambert law is being used. At high enough dilutions, the regular reflection from the sample approximates that from the standard and is thus cancelled out in any comparison measurement.

For systems exhibiting no deviation from the Kubelka-Munk equation, the optimum conditions for maximum accuracy can be deduced by computing the relative error, \( \frac{dc}{c} \). Thus, if the absorption coefficient \( k \) is replaced by the product \( 2.303 \, e \, c \), the Kubelka-Munk equation, (1), can be written in the form
where $k'$ is a constant equal to $s/2.303e$. The error in $c$ is

$$dc = \frac{k' (R_\infty^2 - 1)}{2 R_\infty^2} \, dR_\infty,$$  \hspace{1cm} (3)

and the relative error in $c$ is

$$\frac{dc}{c} = \frac{(R_\infty + 1)dR_\infty}{(R_\infty - 1)R_\infty}.$$  \hspace{1cm} (4)

Assuming a reading error amounting to one reflectance unit, this is $dR_\infty = 0.01$,

$$\frac{dc}{c} \times 100 = \frac{(R_\infty + 1)}{(R_\infty - 1)R_\infty} = \% \text{ error in } c.$$  \hspace{1cm} (5)

To determine the value or values of $R_\infty$ which will render the percent error in $c$ a minimum, $d(\% \text{ error in } c)/dR_\infty$ is then equated to zero. The positive solution of the resulting equation,

$$R_\infty^2 + 2R_\infty - 1 = 0,$$  \hspace{1cm} (6)

indicates that the minimum percent error in $c$ occurs at a reflectance value of 0.414. This corresponds to a reflectance reading of 41.4% $R$. This is presented graphically in Figure 11, in which the percent error, computed with the use of equation (5), is plotted as a function of the percent reflectance. As is shown there, the minimum in the resulting curve corresponds to the 41.4% $R$ value obtained with the use of equation (6).

Reflectance spectrophotometric methods of analysis usually involve the use of a calibration curve prepared by measuring the reflectance of samples containing known amounts of the substance of interest. Conformity to the Kubelka-Munk equation is indicated if the data, when
Figure 12.oretical curve, computed from the use of equation (5), as a function of percent reduction.
plotted in the form $F(R_\infty)$ \textit{versus} concentration, take the form of a straight line. From the standpoint of utility in analysis, however, it is immaterial whether the system in question conforms to the Kubelka-Munk equation or not. Provided some sort of near-linear relationship is found to exist between reflectance and concentration, it is more important, if the method is to be used for analysis, to select a suitable concentration range for the analysis and to evaluate its accuracy.

To illustrate how this might be done in actual practise, two systems were selected. These were rhodamine B adsorbed on silica gel G, which absorbs in the visible, and aspirin adsorbed on silica gel G, which absorbs in the ultraviolet. The experimental data obtained with the first are presented as curve (1) in Figure 12 with the second as curve (1) in Figure 13. In both cases percent reflectance is plotted as a function of concentration. When these data are graphed in the form $F(R_\infty)$ \textit{versus} concentration, curve (1) of Figure 14 results for rhodamine B and curve (2) of Figure 13 results for aspirin. Although both curves are linear over a considerable portion of the concentration ranges investigated, it is readily apparent that neither system conforms to the Kubelka-Munk equation. This becomes more evident when the pair of curves obtained with rhodamine B - curve (1) of Figure 12 and curve (1) of Figure 14 - are contrasted with the pair of hypothetical curves - curve (2) of Figure 12 and curve (2) of Figure 14 - that would have been obtained had the system behaved ideally. The departure from linearity observed with both rhodamine B and aspirin at higher concentrations may be ascribed to the approaching
FIGURE 12. PERCENT REFLECTANCE AT 545 μm. OF RHODAMINE B ADSORBED ON SILICA GEL G AS A FUNCTION OF CONCENTRATION. CURVE (1): 0-0 EXPERIMENTAL VALUES. CURVE (2): 0-0 THEORETICAL VALUES.
FIGURE 13. EXPERIMENTAL VALUES OBTAINED AT 302 m\(\mu\) FOR ASPIRIN ADSORBED ON SILICA GEL G. CURVE (1): o-o PERCENT REFLECTANCE AS A FUNCTION OF CONCENTRATION. CURVE (2): ----- KUBELKA-MUNK VALUES AS A FUNCTION OF CONCENTRATION.
saturation of the adsorbent surface by the first monomolecular layer of the adsorbed species\(^{(54)}\). Blank values were at least partially responsible for the nonlinearity that was also observed at lower concentrations in the case of the aspirin.

The relative error in an analysis can, perhaps, be most readily visualized by graphical methods. As may be seen from a consideration of any of the three plots showing percent reflectance as a function of concentration, i.e. experimental rhodamine B, theoretical rhodamine B or experimental aspirin, the largest absolute errors in concentration resulting from a reading error amounting to 1\% R would be found in the region of lowest reflectance. These would, in turn, result in large percent errors. At the other extreme a similar reading error would result in much smaller absolute errors in concentration. These would, however, comprise a larger fraction of the total concentration, and so again give rise to a large percent error. It is evident that the percent error must be minimal at some intermediate point. The location of this point may be approximated by plotting the percent error arising from a reading error of 1\% R as a function of percent reflectance, as is done in Figure 15 with the experimental values obtained for rhodamine B and aspirin and the hypothetical values computed for rhodamine B. The errors in concentration resulting from a one percent reading error were estimated graphically with the use of the appropriate percent reflectance-concentration plots, i.e. curves (1) and (2) of Figure 12 and curve (1) of Figure 13. There is close agreement between the percent error-percent reflectance plot that is obtained for the hypothetical values of rhodamine B and
FIGURE 15. PERCENT ERROR ARISING FROM A READING ERROR OF 1% R (ESTIMATED GRAPHICALLY) AS A FUNCTION OF PERCENT REFLECTANCE. CURVE (1): o-o EXPERIMENTAL RHODAMINE B. CURVE (2): •• HYPOTHETICAL RHODAMINE B. CURVE (3): ▲-▲ EXPERIMENTAL ASPIRIN.
Figure 11, in which the percent error that might be expected with an ideal system is computed with the use of equation (5). Not surprisingly, there is much less agreement between Figure 11 and the percent error-percent reflectance plots obtained for the experimental values of rhodamine B and aspirin, neither of which conforms to the Kubelka-Munk equation. Despite this, both curves exhibit a wide range of reflectance values within which analyses should be characterized by relatively good accuracy.

Another approach to the selection of the optimum range for reflectance spectrophotometric analyses is one suggested by Ringbom(55), and later Ayres (56), who evaluated relative error and defined the suitable range for absorption spectrophotometric analysis by plotting absorptancy (l-transmittance) against the logarithm of concentration. When the reflectance data obtained with rhodamine B and aspirin and the hypothetical data computed for rhodamine B are graphed in the form percent reflectance versus the logarithm of concentration, as is done in Figure 16, the advantages of this method become manifest. The optimum range for purposes of analysis corresponds to that portion of each curve exhibiting the greatest slope. Since a considerable portion of each of the curve shown in Figure 16 is also reasonably linear about this point, it is apparent that good accuracy can be expected over a not inconsiderable range. The maximum accuracy can be estimated with the use of an equation derived from the definition of percent error, or

$$\frac{dc}{c} \times 100 = 2.303 \frac{d \log c}{c} \times 100 = 2.303 \frac{d \log c}{dR} \times 100 \quad (7)$$

Assuming a constant reading error amounting to 1% R, i.e. $dR = 0.01$, 

% error = \frac{2.303 \, d \, \log c}{dR} \tag{8}

For the three systems under consideration the optimum range for analysis can be arrived at by a consideration of the curves in Figure 16, and the percent error resulting from a reading error of 1% R can be computed with the use of equation (8) and the slope of the appropriate curve. When this is done, the data obtained, which are presented in Table IV, are found to be in accordance with those resulting from the application of the graphical method. Values for the hypothetical rhodamine B also agree with the figures obtained for an ideal system and computed with the use of equation (5).

It would seem, therefore, that the minimum error in reflectance spectrophotometric analysis is of the order of 6% per 1% R reading error, regardless of whether the system in question conforms to the Kubelka-Munk equation or not. This value can obviously be decreased by reducing the reading error. Although a reading error amounting to 0.5% R should not be too difficult to attain, it would be unrealistic to expect a precision better than 0.1-0.2% R, and, therefore, a smaller minimum percent error than 1 to 2%.

Similarly, regardless of whether a system conforms to the Kubelka-Munk equation or not, the optimum range for analysis can be arrived at after plotting the reflectance data according to either of the two procedures described herein. The Ringbom method has the advantage of not only making available the optimum range and maximum accuracy, but also providing a plot capable of being employed as a calibration curve. The rather large difference between the optimum range computed for an
<table>
<thead>
<tr>
<th>Curve</th>
<th>System</th>
<th>Optimum Range (%R)</th>
<th>%Analysis error/1% reading error</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Experimental rhodamine B</td>
<td>25-65</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Hypothetical rhodamine B</td>
<td>20-65</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Experimental aspirin</td>
<td>55-85</td>
<td>6</td>
</tr>
</tbody>
</table>
ideal system (20-65% R) and that found for aspirin adsorbed on silica gel (55-85% R) is worth noting. On the basis of the limited data available, it would seem that this shift of optimum range to higher reflectance values is characteristic of substances having a relatively small molar absorptivity. Because of this property of the adsorbed species, the surface of the adsorbent approaches saturation with a monomolecular layer of the adsorbate at a much higher reflectance value than one would expect. For this reason, it is suggested that, when the optimum range is not known, reflectance values lying in the upper portion of the optimum range computed for an ideal system be used for analysis.

C. The detection and determination by ultraviolet reflectance spectroscopy of vitamins resolved on thin-layer plates.

Because most vitamins have characteristic ultraviolet spectra, it was felt that the application of ultraviolet reflectance spectroscopy to thin-layer chromatography would prove to be invaluable in their analysis. The feasibility of this approach was demonstrated by the successful determination of mixtures of aspirin and salicylic acid described earlier. In that instance, however, the location of the resolved components depended on the formation of colored substances which resulted from the heating of the chromatoplates following their development. While such a procedure may be applicable in some situations, it is not only destructive in nature but is also unsuited for compounds which are stable or which cannot be converted to colored substances. Ideally the ultraviolet reflectance technique should be capable of analyzing adsorbed species directly without recourse to any
preliminary operations.

In this study, the ultraviolet reflectance technique was applied to the nondestructive analysis of five B group-vitamins -- vitamin B₁, vitamin B₆, nicotinic acid, nicotinamide and p-aminobenzoic acid -- in the ultraviolet region of the spectrum. Because these vitamins are colorless, it was first necessary to develop a means of locating them once they had been resolved on thin-plates. Two methods of location were investigated. One involved the addition of a luminous pigment to the silica gel G adsorbent and examination of the chromatoplates under ultraviolet illumination. This approach was originally developed by Brockman et al. (57) and Sease (58-59) for the location of colorless, ultraviolet-absorbing material in column chromatography, and has subsequently been used by others in thin-layer chromatography (12, 50-51). The other method employed direct scanning of silica gel chromatoplates by means of a spectrophotometer fitted with a standard reflectance attachment and set at a wavelength corresponding to the absorption maximum of the vitamin of interest.

**Location and identification of spots on chromatoplates**

As may be seen in Figures 17 and 18, each of the vitamins, with the exception of nicotinic acid and nicotinamide, has a unique reflectance spectrum when adsorbed on either silica gel G or the silica gel G-luminous pigment mixture. In the case of the exceptions, whose spectra are almost identical, the R_f values differ enough, as shown in Table V, to permit their identification. It is therefore possible, making use of both reflectance spectra and R_f values, to
FIGURE 17A. REFLECTANCE SPECTRA OF (A) VITAMIN B1; (B) VITAMIN B6; AND (C) NICOTINIC ACID ADSORBED ON SILICA GEL G.
FIGURE 17B. REFLECTANCE SPECTRA OF (D) NICOTINAMIDE; AND (E) p-AMINOBENZOIC ACID ADSORBED ON SILICA GEL G; (F) SILICA GEL G CONTAINING 2% LUMINOUS PIGMENT USING SILICA GEL G AS A REFERENCE STANDARD.
FIGURE 18A. REFLECTANCE SPECTRA OF (A) VITAMIN B₁; (B) VITAMIN B₆; AND (C) NICOTINIC ACID ADSORBED ON SILICA GEL G CONTAINING 2% LUMINOUS PIGMENT.
FIGURE 18B. REFLECTANCE OF (D) NICOTINAMIDE; AND (E) p-AMINOBENZOIC ACID ADSORBED ON SILICA GEL G CONTAINING 2% LUMINOUS PIGMENT.
<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Absorption Maximum (μλ.)</th>
<th>( R_f ) Value</th>
<th>Sensitivity (μmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Silica gel G- lum. pig.</td>
<td>Silica gel G</td>
<td>Silica gel G- lum. pig.</td>
</tr>
<tr>
<td>B₁</td>
<td>278</td>
<td>278</td>
<td>0.00</td>
</tr>
<tr>
<td>B₆</td>
<td>298</td>
<td>298</td>
<td>0.17</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>262-268</td>
<td>264</td>
<td>0.66</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>263-268</td>
<td>264</td>
<td>0.51</td>
</tr>
<tr>
<td>p-amino-benzoic acid</td>
<td>295</td>
<td>295</td>
<td>0.85</td>
</tr>
</tbody>
</table>
make an unequivocal identification of each of the vitamins investigated. The effect that might be produced upon the reflectance spectra of the vitamins by incorporating the luminous pigment in the silica gel G adsorbent was studied by obtaining a spectrum of the mixture relative to the silica gel G. The resulting spectrum, which is also depicted in Figure 17 is such that one would expect little or no effect upon the absorption maxima of the vitamins. That this is actually the case becomes evident when one considers the spectra shown in Figures 17 and 18 and the data presented in Table V, which lists the absorption maxima, \( R_F \) values and sensitivities of the vitamins adsorbed on silica gel and on the silica gel G-luminous pigment mixture. The spectra and \( R_F \) values obtained with the two adsorbent systems are in such close agreement that it should be possible to use them interchangeably for most purposes. The sensitivities obtained with the silica gel G-luminous pigment mixture range from 0.01 to 0.05 \( \mu \)mole, and are in general agreement with values reported by Gühnshirt and Malzacher(12). With silica gel alone, the sensitivities are greater and range from 0.005 to 0.01 \( \mu \)mole.

Of the two methods, the one employing only silica gel obviously provides greater sensitivity. The other, however, is somewhat less time-consuming as it does not require the use of a spectrophotometer to locate the spots on the developed chromatoplates. Both methods are rapid, nondestructive, and have been successfully applied to the location and identification in multi-vitamin preparation. They should also be applicable to the location and identification of a large number of other colorless, ultraviolet-absorbing substances.
Determination of vitamins resolved on thin-plates.

Once a procedure had been developed for the location and identification of vitamins resolved on thin-plates, an effort was made to devise a method whereby they might be determined by means of the ultraviolet reflectance technique. Since the factors limiting the precision of the technique usually are those associated with the packing of the sample in the cell (45, 48), it was felt that the reproducibility of packing of the windowless cell, which was employed for the first time, should be determined. This was done by measuring the percent reflectance of three samples of different concentrations of nicotinic acid adsorbed on silica gel G after each of four separate packings of these samples. The readings obtained, recorded in Table VI, had an average standard deviation of 0.1 %R which is substantially better than the 0.3 %R standard deviation achieved with the use of the cells employed in the salicylic acid-aspirin determinations. This improvement in precision is due, at least in part, to the greater care that was exercised in this study in the preparation of the analytical sample. It was found that the standard deviation obtained decreased with each increase in the number of 30 stroke-grinding periods employed, reaching a relatively constant value after seven periods. For this reason, seven 30-stroke grinding periods were used in preparing samples for analysis.

Calibration plots for nicotinic acid and nicotinamide adsorbed on pure silica gel G and on the silica gel G-luminous pigment mixture were made by applying different concentrations of the vitamins to thin-layer plates, which were then developed, dried and readied for the
TABLE VI. TEST OF REPRODUCIBILITY OF PACKING WINDOWLESS REFLECTANCE CELL. % REFLECTANCE AT 264 μm OF NICOTINIC ACID ADSORBED ON SILICA GEL G.

<table>
<thead>
<tr>
<th>Concentration (μmole/80 mg. silica gel G)</th>
<th>1st packing</th>
<th>2nd packing</th>
<th>3rd packing</th>
<th>4th packing</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>87.5</td>
<td>87.7</td>
<td>87.6</td>
<td>87.8</td>
<td>± 0.1</td>
</tr>
<tr>
<td>1.0</td>
<td>76.3</td>
<td>76.3</td>
<td>76.5</td>
<td>76.5</td>
<td>± 0.1</td>
</tr>
<tr>
<td>2.5</td>
<td>60.0</td>
<td>60.1</td>
<td>60.2</td>
<td>60.2</td>
<td>± 0.1</td>
</tr>
</tbody>
</table>
measurement of reflectance according to the method outlined in the experimental section. A study of the effect of drying time upon the reflectance samples containing nicotinic acid and nicotinamide disclosed that drying the plates at 90°C for two hours was not only convenient but most expeditious. In all four cases, the percent reflectance at 264 μ was determined and then graphed as a function of concentration. The plots obtained for nicotinic acid and nicotinamide adsorbed on the silica gel G-luminous pigment mixture are presented in Figure 19, while those for the two vitamins adsorbed on silica gel G are shown in Figure 20. Each plot incorporates data obtained with the use of two sets of dilution series and gathered at different times. The degree of precision achieved is indicated by the fact that in practically every instance reflectance values obtained for comparable concentrations did not deviate from each other by more than two reflectance units. The close agreement among the four plots suggests the feasibility of employing a single calibration curve for the determination of both compounds with either adsorbent system.

A more useful form of the data results when they are presented as Ringbom Plots, as is done in Figures 21 and 22. Not only is the linear portion of the curve extended in this manner, but, more important, the optimum range for analysis, which corresponds to the region of greatest slope, becomes more apparent. By employing the slope of this curve in conjunction with the equation \( % \text{error} = 2.303 \ \text{d log} \ c/\text{dR} \) as described earlier, it is also possible to estimate the percent error resulting from a reading error of 1 %R. The optimum range for analysis and the percent error deduced with the use of the Ringbom plots in general agree
FIGURE 19. % REFLECTANCE AT 264 μm. OF NICOTINIC ACID (○ - ○) AND NICOTINAMIDE (△ - △) ADSORBED ON SILICA GEL G-LUMINOUS PIGMENT MIXTURE AS A FUNCTION OF CONCENTRATION.
FIGURE 20. % REFLECTANCE AT 264 mp. OF NICOTINIC ACID (o-o) AND NICOTINAMIDE (o-o) ADSORBED ON SILICA GEL G AS A FUNCTION OF CONCENTRATION.
FIGURE 21. % REFLECTANCE AT 264 mp. OF NICOTINIC ACID (o-o) AND NICOTINAMIDE (■-■) ADSORBED ON THE SILICA GEL G-LUMINOUS PIGMENT MIXTURE AS A FUNCTION OF THE LOGARITHM OF CONCENTRATION.
FIGURE 22. % REFLECTANCE AT 264 mp. OF NICOTINIC ACID (o – o) AND NICOTINAMIDE (□ – □) ADSORBED ON SILICA GEL G AS A FUNCTION OF THE LOGARITHM OF CONCENTRATION.
with the range and error arrived at by employing the graphical method, which involves plotting percent error as a function of percent reflectance in the manner depicted in Figures 23 and 24. As indicated in Table VII, which summarizes the values obtained for nicotinic and nicotinamide adsorbed on silica gel G and on the silica gel G-luminous pigment mixture, both methods indicated, in practically all cases, that the optimum range for analysis was 60-80 %R and that the probable percent error arising from a reading error of 1 %R would be 5-6 %.

The precision that can be attained when the reflectance technique is employed in the assay of nicotinic acid and nicotinamide was determined by running through the analysis procedure with four 2-μmole replicate samples of each vitamin as may be seen in Table VIII, the reflectance readings obtained for samples of nicotinic acid and nicotinamide adsorbed on silica gel G-luminous pigment mixture were found to have a standard deviation of 0.36 and 0.39 reflectance units, respectively. Corresponding values for the vitamins adsorbed on silica gel G alone were found to be 0.33 and 0.39 reflectance units, respectively. By making use of data included in Table VII, the corresponding probable errors in the measurement of concentrations of nicotinic acid and nicotinamide were determined to be 2-2.5 % with either adsorbent system. Although this study was restricted to nicotinic acid and nicotinamide, there is no reason why the analysis procedure, with slight or no modification, cannot also be applied to the assay of vitamin B₁, vitamin B₆ and p-aminobenzoic acid.
FIGURE 23. PERCENT ERROR ARISING FROM A READING ERROR OF 1 %R (ESTIMATED GRAPHICALLY) AS A FUNCTION OF PERCENT REFLECTANCE. CURVE (1): NICOTINIC ACID ADSORBED ON THE SILICA GEL G-LUMINOUS PIGMENT MIXTURE. CURVE (2): NICOTINAMIDE ADSORBED ON THE SILICA GEL G-LUMINOUS PIGMENT MIXTURE.
FIGURE 24. PERCENT ERROR ARISING FROM A READING ERROR OF 1 %R (ESTIMATED GRAPHICALLY) AS A FUNCTION OF PERCENT REFLECTANCE. CURVE (1): NICOTINIC ACID ADSORBED ON SILICA GEL G. CURVE (2): NICOTINAMIDE ADSORBED ON SILICA GEL G.
### TABLE VII. OPTIMUM RANGE FOR ANALYSIS AND PERCENT ERROR

<table>
<thead>
<tr>
<th></th>
<th>Silica gel G-luminous pigment mixture</th>
<th>Silica gel G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nicotinic acid</td>
<td>Nicotinamide</td>
</tr>
<tr>
<td></td>
<td>Graphical</td>
<td>Ringbom</td>
</tr>
<tr>
<td>Optimum range (%R)</td>
<td>60-80</td>
<td>60-80</td>
</tr>
<tr>
<td>% Analysis error/1% reading error</td>
<td>6</td>
<td>5</td>
</tr>
</tbody>
</table>
TABLE VIII. REPRODUCIBILITY OF % REFLECTANCE AT 264 mp. OBTAINED FOR DIFFERENT SAMPLES OF IDENTICAL CONCENTRATIONS (2.0 µmole/80 mg. of adsorbent) OF NICOTINIC ACID AND NICOTINAMIDE ADSORBED ON THE SILICA GEL G-LUMINOUS PIGMENT MIXTURE AND ON SILICA GEL G.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Silica gel G-luminous pigment mixture</th>
<th>Silica gel G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nicotinic acid ( %R )</td>
<td>Nicotinamide ( %R )</td>
</tr>
<tr>
<td>1</td>
<td>65.0</td>
<td>64.9</td>
</tr>
<tr>
<td>2</td>
<td>65.6</td>
<td>63.8</td>
</tr>
<tr>
<td>3</td>
<td>65.8</td>
<td>64.2</td>
</tr>
<tr>
<td>4</td>
<td>65.2</td>
<td>64.5</td>
</tr>
<tr>
<td>Mean and std. deviation</td>
<td>65.4 ± 0.36</td>
<td>64.4 ± 0.39</td>
</tr>
</tbody>
</table>
D. The determination of ascorbic acid resolved on thin-layer plates by reflectance spectrophotometric titration and radioactive tracer technique.

One of the most difficult problems encountered in vitamin assay is the analysis of those vitamins which are sensitive to atmospheric oxidation, light, heat, and solvent action. This study represents an attempt to utilize the spectral reflectance technique in conjunction with thin-layer chromatography in the analysis of such an unstable vitamin, namely ascorbic acid.

The analytical scheme employed depends on the use of radioactive ascorbic acid to provide a means of determining the extent of the chemical change that occurs during the preparation of the analytical sample. The ascorbic acid in tagged samples was isolated from its oxidation and decomposition products by means of thin-layer chromatography, and the amount of acid isolated determined by reflectance spectrophotometric titration. This was accomplished by adding increasing increments of selenious acid titrant to spots containing equivalent amounts of isolated ascorbic acid, measuring the reflectance of the resulting mixture and plotting the percent reflectance as a function of the amount of titrant added. It was then a simple matter to estimate the quantity of ascorbic acid that had been isolated. By measuring the radioactivity of samples prepared from both developed and undeveloped spots, it was possible to take into account the changes that occurred after the addition of the radioactive ascorbic acid. Both reflectance and radioactivity measurements were carried out on the same samples packed in the windowless analytical
cell described earlier which had been appropriately modified.

**Reflectance spectrophotometric titration.**

The titration makes use of the intense salmon color associated with the metallic selenium that is formed when the selenious acid reacts with the ascorbic acid adsorbed on the silica gel G-luminous pigment mixture. Although the principal reaction that occurs is

\[
\text{H}_2\text{SeO}_3 + 2 \text{HOCH}_2\text{CHOHCH}=\text{C}=\text{O} \rightarrow \text{Se}^{\circ} + 2 \text{HOCH}_2\text{CHOHCH}=\text{C}=\text{O} + 3 \text{H}_2\text{O}
\]

there is reason to believe that the dehydroascorbic acid undergoes further chemical change. A wavelength of 410 \(\mu\)m was selected for the titration after a consideration of the reflectance spectra obtained with ascorbic acid, selenious acid, selenious acid which had been mixed with excess ascorbic acid, and ascorbic acid which had been mixed with excess selenious acid. As may be seen in Figure 25, the spectra obtained with the ascorbic-selenious acid mixtures are characterized by strong absorption at 410 \(\mu\)m whereas those obtained with the use of only ascorbic acid or selenious acid are not.

Since a reflectance spectrophotometric titration would be possible only if a linear or near-linear relationship existed between the concentration of the species being determined and the reflectance of the analytical sample, it was next necessary to ascertain whether such a relation did exist between the percent reflectance at 410 \(\mu\)m for selenious acid added to excess ascorbic adsorbed on silica gel G-luminous pigment mixture and the amount of selenious acid added. To this end, 12 \(\mu\)equivalent- aliquots of ascorbic acid were applied to
FIGURE 25. REFLECTANCE SPECTRA OF (A) ASCORBIC ACID (10 µequiv.); (B) SELENIOUS ACID (10 µequiv.); (C) EXCESS ASCORBIC ACID (10 µequiv.) WITH SELENIOUS ACID (4 µequiv.); (D) EXCESS SELENIOUS ACID (10 µequiv.) WITH ASCORBIC ACID (4 µequiv.) ADSORBED ON SILICA GEL G-LUMINOUS PIGMENT MIXTURE.
thin-plates, which were then developed according to the procedure outlined in the experimental section. After increasing increments of the selenious acid titrant had been added to the isolated ascorbic acid, each spot was incorporated into an analytical sample whose reflectance was measured. When the Kubelka-Munk values obtained were plotted as a function of the amount of selenious acid added, as is done in Figure 26, a smooth curve resulted. A more satisfactory plot resulted when percent reflectance was graphed as a function of the square root of the amount of selenious acid added, as shown in Figure 27. Because the curve was nearly linear over the entire concentration range investigated, it was decided to present the titration data in this form.

Radioassay

So that it would be unnecessary to expend an undue amount of effort preparing analytical samples having a specified thickness, it was decided to employ samples of such thickness for the radioassay that the dependence of the count rate upon the sample thickness would not be critical. This optimum sample size was determined with the use of samples of varying weight which contained the same concentration of C\textsuperscript{14}-ascorbic acid per unit weight of the silica gel G-luminous pigment adsorbent mixture\(\mu\text{C./mg. adsorbent}\). These samples were prepared by adding approximately 100 \(\mu\text{l.}\) of 4 \(\mu\text{C./ml.}-\text{C}^{14}\)-ascorbic acid solution to 1.5 grams of the adsorbent, mixing the two thoroughly, drying at 50\(^{\circ}\)C for 30 minutes, and then weighing out appropriate amounts of the mixture into the windowless cell described earlier. When the count rate determined for these samples was plotted as a function of the
CONCENTRATION (μequiv./90 mg. of adsorbent)

FIGURE 26. KUBELKA-MUNK VALUES AT 410 μ. FOR SELENIOUS ACID ADDED TO EXCESS ASCORBIC ACID (12 μequiv.) ADSORBED ON SILICA GEL G-LUMINOUS PIGMENT MIXTURE AS A FUNCTION OF THE AMOUNT OF SELENIOUS ACID ADDED.
FIGURE 27. % REFLECTANCE AT 410 μm. FOR SELENIOUS ACID ADDED TO EXCESS ASCORBIC ACID (12 μequiv.) ADSORBED ON SILICA GEL G-LUMINOUS PIGMENT MIXTURE AS A FUNCTION OF THE AMOUNT OF SELENIOUS ACID ADDED.
sample thickness, as is done in Figure 28, it became apparent that almost any thickness greater than 17 mg./cm.\(^2\), which corresponded to the smallest sample size that provided a continuous surface, would serve the purpose. A sample size of 90 mg., which provided a thickness of 26 mg./cm.\(^2\), was selected for use in this study because it easily accommodated all of the material which had to be included in the analytical sample.

The reproducibility achieved in the measurement of the radioactivity was determined by carrying out a radioassay on five replicates. These were prepared by applying five 10 μl.-spots of a solution consisting of approximately 0.3 mg. of 14 μC./mg. of C\(^{14}\)-ascorbic acid dissolved in 1 ml. of 0.6 N. ascorbic acid to a thin-plate which was then dried at 50°C for 30 minutes. Following this, the spots were removed from the plate with a spatula and each was made up into a 90 ± 0.4 mg. sample which was prepared for radioassay according to the procedure outlined in the experimental section. The count rates obtained for the five replicates, which are listed in Table IX, had a standard deviation of ± 2.3%. When the radioactivity of a single sample was measured repeatedly without altering its position, the count rate was found to have a standard deviation of ± 0.8%. This would seem to indicate that the precision of the technique is limited by the factors associated with the preparation of the analytical sample.

Although one would expect the selenious acid and the nonradioactive ascorbic acid to have little or no effect upon the measured count rate, it was decided to ascertain whether such was actually the
FIGURE 28. COUNT RATE FOR SAMPLES OF VARYING WEIGHT CONTAINING THE SAME CONCENTRATION OF $^{14}$-ASCORBIC ACID PER UNIT WEIGHT OF SILICA GEL-G-LUMINOUS PIGMENT MIXTURE ($\mu$C./mg. adsorbent) AS A FUNCTION OF SAMPLE THICKNESS.
TABLE IX. REPRODUCIBILITY OF RADIOACTIVITY MEASUREMENTS OBTAINED FOR DIFFERENT SAMPLES CONTAINING IDENTICAL AMOUNTS OF RADIOACTIVE ASCORBIC ACID ADSORBED ON SILICA GEL G-LUMINOUS PIGMENT MIXTURE

<table>
<thead>
<tr>
<th>Sample</th>
<th>Count rate (C.P.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3490</td>
</tr>
<tr>
<td>2</td>
<td>3639</td>
</tr>
<tr>
<td>3</td>
<td>3630</td>
</tr>
<tr>
<td>4</td>
<td>3450</td>
</tr>
<tr>
<td>5</td>
<td>3507</td>
</tr>
</tbody>
</table>

Mean: 3543

% Std. Dev.: ±2.3%
case. This was done by adding varying amounts of ascorbic and selenious acid to ten spots which had been made by applying 10 μl. increments of a solution consisting of approximately 0.3 mg. of 14 μC./mg. of \textsuperscript{14}C-ascorbic acid in 1 ml. of distilled water to a thin plate. From this point on the procedure followed was identical to that outlined in the above paragraph. As shown in Table X, which summarizes the results of this study, the standard deviation of the counting rates observed with the series to which selenious acid was added was 2.5% and the standard deviation of the counting rates observed with the series to which nonradioactive ascorbic acid was added was 2.2%. Since these approximate the 2.3% deviation that was obtained in the reproducibility study, it can be concluded that within experimental limitations neither acid has any effect upon the measured count rate.

The relationship between count rate and the concentration of the adsorbed radioactive ascorbic acid was determined with the use of a dilution series. This was prepared by applying to thin-plates increments of 0.600 N. radioactive ascorbic acid(approximately 4.5 μC./ml.) ranging from 2 to 17 μl. After the plates had been dried at 50°C for 30 minutes, the spots were made ready for the radioassay according to the method utilized in the preceding two studies. As may be seen in Figure 29, which presents the data that were obtained, a linear relationship was observed between the count rate and the volume of radioactive ascorbic acid that had been added.

**Determination of ascorbic acid**

Following the addition of known amounts of \textsuperscript{14}C-ascorbic acid,
TABLE X. EFFECT OF VARYING AMOUNTS OF ASCORBIC AND SELENIOUS ACIDS UPON THE COUNT RATE OBTAINED FOR DIFFERENT SAMPLES CONTAINING IDENTICAL AMOUNTS OF RADIOACTIVE ASCORBIC ACID ADSORBED ON SILICA GEL G-LUMINOUS PIGMENT MIXTURE.

<table>
<thead>
<tr>
<th>Amount of adsorbate (μequiv.)</th>
<th>Count rate</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ascorbic acid (C.P.M.)</td>
<td>Selenious acid (C.P.M.)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3637</td>
<td>3362</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3500</td>
<td>3560</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>3631</td>
<td>3411</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>3460</td>
<td>3550</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>3528</td>
<td>3428</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>3551</td>
<td>3460</td>
<td></td>
</tr>
<tr>
<td>% Std. Dev.</td>
<td>±2.2%</td>
<td>±2.5%</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 29. COUNT RATE OBTAINED FOR C\textsuperscript{14}-ASCORBIC ACID ADSORBED ON SILICA GEL G-LUMINOUS PIGMENT MIXTURE AS A FUNCTION OF THE VOLUME OF 0.600 N. C\textsuperscript{14}-ASCORBIC ACID ADDED.
aliquots of freshly prepared 0.600 N. ascorbic acid were either treated with measured quantities of liquid bromine which quantitatively oxidized part of the ascorbic acid; or permitted to slowly decompose at room temperature for periods extending up to few days. The solution treated with bromine were analyzed immediately afterward according to the procedure described in the experimental section. The solution permitted to slowly decompose at room temperature was analyzed periodically.

Typical of the curves that were obtained for the reflectance spectrophotometric titration are the two depicted in Figure 30. It is possible to estimate the volume of standard selenious acid required to react with the adsorbed ascorbic acid from such curves and then to use this in conjunction with the concentration of the selenious acid to calculate the equivalent concentration of the ascorbic acid, \( C_a \), in each spot, i.e. the concentration that would be obtained if the acid were to remain in solution. The concentration \( C_a \) can be computed with the use of the expression

\[
C_a = \frac{C_s \times V_s}{V_a}
\]

where \( C_s \) is the concentration of the standard selenious acid in normality, \( V_s \) is the volume of that acid required to reach the titration end point in \( \mu l \), and \( V_a \) is the volume, also in \( \mu l \), of original ascorbic acid solution applied per spot on the chromatoplate.

To arrive at the concentration of ascorbic acid actually present at the time when the radioactive ascorbic acid was added, it is necessary to multiply \( C_a \) by a correction factor, \( S_2/S_1 \), where \( S_1 \) is the
FIGURE 30. TYPICAL CURVES OBTAINED FOR THE REFLECTANCE SPECTROPHOTOMETRIC TITRATION OF ASCORBIC ACID WITH SELENIOUS ACID. % REFLECTANCE PLOTTED AS A FUNCTION OF THE SQUARE ROOT OF THE VOLUME OF 0.400 N. SELENIOUS ACID ADDED.
average count rate in c.p.m. obtained for the titrated samples corrected for background, and \( S_2 \) is the average count rate in c.p.m. obtained for undeveloped spots corrected for background.

The results of this study are presented as Tables XI and XII. As may be seen there, an average error of 5.4% was observed in the analyses carried out with the ascorbic acid solutions that had been treated with a known quantity of liquid bromine, while a 5.3% average error was noted in the periodic analyses that were made on the ascorbic acid solution which had been allowed to stand at room temperature. In the computation of the percent error, a value of 0.603 was used for the true normality of the ascorbic acid solution to correct for the increase in concentration resulting from the addition of the radioactive ascorbic acid, which contained approximately 0.3 mg. of acid per milliliter of solution.

For those analyses which were carried out on solutions that had been treated with a known quantity of liquid bromine, the values obtained for the concentration of the ascorbic acid by means of the reflectance spectrophotometric titration were 6 to 7% lower than those arrived at by taking into account the ascorbic acid that was oxidized by treatment with a known quantity of liquid bromine. These discrepancies can be attributed to the decomposition of the ascorbic acid that occurs during the drying of the chromatoplates and their subsequent development. Even where freshly prepared solutions were employed, it was usually possible to observe on the thin plates, in addition to the ascorbic acid spot, a second, light-yellow spot located at the point of application of the ascorbic acid solution as well as
### TABLE XI. ANALYSES OF 0.600 N. ASCORBIC ACID SOLUTIONS TREATED WITH KNOWN QUANTITIES OF LIQUID BROMINE

<table>
<thead>
<tr>
<th>Solution</th>
<th>Conc. calc. following treatment with Br₂(^*) (N.)</th>
<th>Conc. det'd by titration (N.)</th>
<th>% Decomposition during drying &amp; development (%)</th>
<th>Correction factor (\frac{S_2}{S_1})</th>
<th>Original conc. (C_a \times \frac{S_2}{S_1}) (N.)</th>
<th>% Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.460</td>
<td>0.428</td>
<td>7.0</td>
<td>1.32</td>
<td>0.566</td>
<td>6.1</td>
</tr>
<tr>
<td>2</td>
<td>0.358</td>
<td>0.336</td>
<td>6.2</td>
<td>1.68</td>
<td>0.564</td>
<td>6.5</td>
</tr>
<tr>
<td>3</td>
<td>0.253</td>
<td>0.238</td>
<td>6.0</td>
<td>2.62</td>
<td>0.624</td>
<td>3.5</td>
</tr>
</tbody>
</table>

\*Values computed by substraction of ascorbic acid oxidized by known amounts of bromine from original concentration (0.603 N).
<table>
<thead>
<tr>
<th>Days</th>
<th>Conc. determined by titration, $C_a$ (N.)</th>
<th>Correction Factor $(S_2/S_1)$</th>
<th>Original Conc. $C_a \times S_2/S_1$ (N.)</th>
<th>% Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.575</td>
<td>1.10</td>
<td>0.633</td>
<td>5.0</td>
</tr>
<tr>
<td>2</td>
<td>0.518</td>
<td>1.10</td>
<td>0.571</td>
<td>5.3</td>
</tr>
<tr>
<td>4</td>
<td>0.475</td>
<td>1.20</td>
<td>0.570</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Mean 5.3
some tailing.
SUMMARY

A procedure whereby ultraviolet reflectance spectroscopy can be employed for the analysis of substances resolved on thin-layer plates was developed with the use of aspirin-salicylic acid mixtures that had been separated on silica gel G plates. This system was selected for study not only because its salicylic acid-silica gel G component was relatively stable, but also because no difficulties were encountered in locating the resolved compounds. Both appeared as yellowish-brown spots when the chromatoplates were dried.

The optimum range and maximum accuracy of such analyses were then deduced by applying two graphical methods to data obtained with the use of two systems—rhodamine B adsorbed on silica gel G, which absorbs in the visible, and aspirin adsorbed on silica gel G, which absorbs in the ultraviolet. Plots of experimental data were contrasted with plots that might be expected for an ideal system that conforms to the Kubelka-Munk equation. The results seemed to indicate that the minimum error to be expected is of the order of 6% per 1% reflectance reading error, and that the optimum range for analysis can be arrived at after plotting the reflectance data according to either of the methods discussed, regardless of whether the system in question conforms to the Kubelka-Munk equation or not.

The procedure developed with the aspirin-salicylic acid mixtures was then applied to the analysis of five vitamins of the B group—thiamine hydrochloride, pyridoxine hydrochloride, nicotinic acid, nicotinamide and p-aminobenzoic acid. The suitability of two techniques
devised to locate the resolved vitamins prior to analysis was investigated. One method involved the observation, under ultraviolet light, of chromatoplates prepared with adsorbent containing fluorescent material; the other involved scanning of the plates by means of a spectrophotometer set at appropriate wavelength. All but two of the vitamins could be identified by means of their reflectance spectra, with the two having identical spectra being distinguished with the aid of their Rf values. The procedure also provided quantitative data having a standard deviation of 0.3-0.4 reflectance unit for the vitamins studied.

Finally the utility of the procedure in the analysis of relatively unstable substances was demonstrated by employing it in conjunction with the radioactive tracer technique to determine small amounts of ascorbic acid. Ascorbic acid which had been tagged with the radioactive compound was separated on thin-layer plates and then determined by reflectance spectrophotometric titration. By also measuring the radioactivity of the sample both before and after the development of the chromatoplates, it was possible to estimate the amount of acid that was originally present. Because the reflectance and radioactivity measurements were carried out on the same analytical sample, which was held in a simple, windowless cell designed for this purpose, it was unnecessary to prepare a separate sample for the radioassay.
BIBLIOGRAPHY


11. Ref. 1, p.246.


50. H. Günshirt and A. Malzacher, Arch. Pharm. 293/65, 925 (1960).


