RESEARCH PAPERS

THE ESTIMATION OF THE COMPONENT CARDIAC GLYCOSIDES IN DIGITALIS PLANT SAMPLES USING PAPER CHROMATOGRAPHY AND FLUORESCENCE PHOTOGRAPHY

By H. Silberman* and R. H. Thorp

From the Department of Pharmacology, University of Sydney, Sydney, Australia

Received March 30, 1953

Two species of Digitalis, *D. purpurea* and *D. lanata*, comprise the main source of cardiac glycosides used in therapeutics. Only very little is known of the factors necessary for the optimum production of active glycosides in these plants and this paucity of information is largely due to the absence of adequate methods for the differential analysis of the glycosidal content of such plant material.

The methods at present in use almost always depend upon a biological assay of total cardiac activity, and this comprises the resultant action of the mixture of several cardiac glycosides present in the plant. It is quite possible that similar degrees of total activity may result from differing mixtures of active glycosides and, although such biological techniques as the embryonic chick heart method of Lehman and Paff[1] are capable of giving very accurate results with microgram quantities of cardiac glycosides, such methods must be reserved for the standardisation of relatively pure samples of single glycosides using the same substance as a reference standard.

The method found most satisfactory in these laboratories comprise the separation of the mixed glycosides by paper chromatography after they have been freed from major impurities by solvent extraction processes. By appropriate chemical treatment the chromatogram is made to fluoresce under ultra-violet radiation and the fluorescence then recorded photographically. In this way permanent records are obtained for each chromatogram and the amounts of the component glycosides determined in comparison with reference standards by photoelectric densitometry measurements made upon the photographic records.

This method in our hands is straightforward and reliable although careful attention to detail is essential both in the chromatographic separation stage and in making the fluorescence photographs. The method is described and discussed in the stages indicated above.

Digitalis lanata contains three so called "genuine glycosides" digilanids A, B and C giving rise respectively by the loss of glucose and an acetyl group to digitoxin, gitoxin and digoxin. In this paper these will be referred to as "desgluco-glycosides." In *Digitalis purpurea* only digilanids A and B are found and these already lack the acetyl group and are therefore deacetyl digilanids. In this species again digitoxin, gitoxin and a third glycoside gitalin are also to be found.

* Drug Houses of Australia Research Fellow.

Since the solubility of the digilanids in water is greater than that of the desgluco-glycosides it is necessary to modify the chromatographic procedure for the analysis of either group, and both groups of substances cannot be estimated upon the same chromatogram.

A Preliminary Separation of Mixed Glycosides from Plant Material

In all cases the same method is used to prepare the mixed glycosides for chromatography and commences with the total extraction of the plant material with ethyl acetate. This extract is then reduced to a small volume by vacuum evaporation of the solvent, and the thick residue is absorbed upon diatomaceous earth which is added in sufficient quantity to produce a crumbly powder. This material contains, in addition to the glycosides; sterols, chlorophyll and other impurities which are next removed by Soxhlet extraction of the powder with light petroleum in which the cardiac glycosides are insoluble. Finally the cardiac glycosides are also removed from the adsorbent by a second extraction with a mixture of chloroform and methanol 1:1. This extract is then evaporated to dryness and the residue is dissolved in a measured volume of ethanol (80 per cent.) and used for the paper chromatography.

The separation of the glycosides from digitalis leaf by paper chromatography was described by Svendsen and Jensen[2] and by Hassel and Martin[3]. These workers quoted R_F values for the different glycosides but little mention is made of the possibility of the separation of the different components and their behaviour in mixtures.

Svendsen and Jensen describe a method to render the spots visible by spraying the developed chromatogram with a 25 per cent. solution of trichloracetic acid in chloroform followed by heat, which renders the spots fluorescent under ultra-violet radiation. This method has been used with some modifications in our work and forms the basis for quantitative estimations. Svendsen and Jensen recommended the separation of the glycosides in mixtures of chloroform, methanol and water in three mixtures of different proportions (I. 10:2:5, II. 10:4:5, III. 10:8:5) and they determined the R_F values for a considerable number of digitalis glycosides. They state also that on the basis of the appreciable differences in these values for the glycosides of *Digitalis lanata* it should be possible to achieve individual separation. In repeating this work we found that the separation of the digilanids B and C on the one hand and gitoxin and digoxin on the other was not sufficiently complete to be unambiguous and certainly not complete enough to serve as a basis for quantitative work. We found it necessary therefore to resume the study of different solvents and solvent mixtures including, in addition to chloroform and methanol, such other solvents as ethyl acetate, benzene, *cyclo*hexane and light petroleum.

Using a mixture of ethyl acetate containing a small but definite amount of ethanol, benzene and water in the proportions 86:14:50 by volume a complete and reproducible separation of digilanids A, B and C was obtained using the organic phase as the mobile one in an atmosphere

of water vapour. The percentage of ethanol in the organic phase is of great importance for the relative placement of the digilanids of the developed chromatogram. With a content of 3 to 5 per cent. of ethanol, a large increase of the R_F values can be achieved and thus good separation of the slower moving C and B components, but at the same time digilanid A travels near to the solvent front and shows a tendency to greater spreading of the spot. With a smaller content of ethanol, 0·5 to 3 per cent., the components B and C are placed closer together but digilanid A remains still well separated and forms a denser spot well apart from the desglucoglycosides or genins (if present) which travel near the solvent front. The great sensitivity of the procedure to changes of the developing mixture necessitates great care in the purification of the solvents and especially of the ethyl acetate which needs careful fractionation to free it from the ethanol usually present in the commercial material. At the same time this sensitivity is of considerable importance since it can be used to advantage to achieve the best separation of the particular digilanids in a mixture upon which the main interest is focussed at the time. In Table I are shown some R_F values of the digilanids A, B and C determined by using different concentrations of ethanol in the mixture described.

TABLE I

The change in the R_F values of the digilanids A, B and C with the increase of the ethanol content in the solvent mixture of ethyl acetate and benzene 86:14.

Ethanol per cent.	Digilanid A		B		C	
	R_{FF}	R_{FC}	R_{FF}	R_{FC}	R_{FF}	R_{FC}
1·5	0·50	0·46	0·36	0·28	0·14	0·08
2	0·54	0·51	0·395	0·355	0·15	0·14
5	0·695	0·67	0·51	0·385	0·16	0·13
7·5	0·71	0·65	0·54	0·44	0·24	0·18

The R_F values were determined in a rectangular tank 6″ × 8″ × 14″ (height) at 24° C. (± 1°); the average distance of the solvent front from the starting line was 200 mm. and the time of irrigation 1¾ to 2 hours. R_{FF} refers to the front portion. R_{FC} to the middle point of the spot.

As has been repeatedly stressed by workers in this field the R_F values of the different components are subject to variation according to the amount of the substance present but it is of particular interest to note that the position of the spots on the developed chromatogram is also dependent on the relative amounts of the glycoside components in its direct vicinity. This is best illustrated by the following example. In the separation of the 3 digilanids the compound A travels faster than B or C, which follow in that order. In a case where digilanid C is present in high concentration even a small quantity of digilanid B is displaced farther to the front as compared with a similar chromatogram with a lesser amount of the C compound.

This advantageous displacement of the different compounds by the relative concentrations of their immediate neighbours can be explained

either by a strong salting-out effect in the partition process or, more probably, by the assumption that intrinsic adsorption phenomena upon the paper material plays an important part in the separation process making it possible to separate isomerides differing only in the position of one hydroxyl group in so large a molecular structure as that found in digilanids B or C.

The separation of digoxin from gitoxin has proved of considerable difficulty and no satisfactory solution of this problem has so far been found with the various solvent mixtures used. The solvent mixtures earlier mentioned and used by Svendsen and Jensen (*loc. cit.*) as well as that normally employed by us give some separation but the spacing of the components is not sufficient for an unambiguous identification or for quantitative work. As stated by Svendsen and Jensen the R_F values of digoxin and gitoxin are very close and the figures given by them are in fact 0·76, 0·81, 0·82 for gitoxin and 0·68, 0·75, 0·76 for digoxin in different mixtures of chloroform, methanol and water. These figures have been confirmed in our experiments in contradiction to the statement of Hassel and Martin who report values of 0·0 for gitoxin in different solvents comprising mixtures of ethyl acetate and water, or chloroform and water.

It may be of interest to mention in this connection that even small quantities of digitoxin can be determined in the presence of digoxin and gitoxin using a mixture of chloroform, benzene and water in the proportions 65:35:50 and containing 5 per cent. of methanol. The digitoxin forms in these cases a well defined orange or yellow fluorescent spot well separated in front of gitoxin and digoxin. The colour of this spot is of great importance for identification and estimation since it cannot be confused with the blue fluorescing digoxin or gitoxin.

For all the work described we have used Whatman No. 1 paper and the ascending method of irrigation. The descending method has been tried in some experiments but gave less reproducible results. In the case of chloroform, methanol, water mixtures we sometimes experienced, for unexplained reasons, some difficulty from a tendency of the mobile phase to change the concentration of the irrigating liquid as evidenced by bands of different transparency of different heights in the paper.

The time required for development using ethyl acetate-benzene-water mixtures was 2 to 2¼ hours and the distance travelled was approximately 22 to 24 cm. The time required for the slower moving chloroform-methanol-water mixtures was about 3½ hours for the solvent front to travel the same distance from the starting line. The temperature during a single experiment remained constant to within 0·5 to 1·0° C. and most experiments were performed at a temperature of 22 to 24° C. After removal of the paper from the chromatograph chamber it was dried in air for 2 to 3 hours at 25° to 30° C. and then sprayed abundantly with a 25 per cent. solution of trichloracetic acid in chloroform using an Aerograph MP spray gun with a No. 2 nozzle and air at 5 to 10 lbs. pressure. The trichloracetic acid solution was prepared freshly each week, with chloroform purified and distilled before use and kept protected

from moisture and light. The sprayed paper is then heated in a drying oven at 105° to 110° C. keeping it free of contact with any other material by clamping it vertically between glass rods at each end. After 4 to 5 minutes the paper is removed and given a quick airing to free it of acid vapours. It is then replaced and heated in the oven for a further 4 to 5 minutes. The chromatogram so treated should fluoresce brightly in ultra-violet light and does not fade appreciably over periods of less than one week. To achieve the brightest fluorescence with digilanid A the heating of the sprayed chromatogram should be extended to 8 to 10 minutes before airing and then heated for a further period of 4 to 5 minutes.

The paper chromatogram is then used to prepare a photographic record for qualitative and quantitative examination by a modified method of fluorescence photography, the basic principles of which are described by Henney and Dudley[4] and by Auerbach and Auerbach[5]. This procedure must be carried out in a photographic darkroom illuminated with a safe light suitable for bromide paper.

The chromatogram is illuminated by ultra-violet light produced by a mercury vapour lamp in Wood's glass envelope (Mazda MBW/V 125 Watt. Mercury vapour) which is further filtered by a sheet of Corning glass "Violet ultra No. 586" attached to the front of the light-tight lamp house. The photographic exposure is made by brief controlled illumination from this lamp which, since it does not restart when hot, cannot be switched on and off for this purpose. A suitable shutter is therefore arranged to cut off the ultra-violet light completely when closed. The device we have used consists of a series of slats of thin brass resembling a venetian blind arranged just behind the Corning Glass filter. These slats are approximately 1 in. wide and $\frac{5}{8}$ in. apart and are coupled together and operated by an electromagnet. When open the passage of light through the filter, approximately 5 in. $\times$ 4 in. is almost completely unobstructed and when closed no ultra-violet light leaves the lamphouse.

The lamphouse is mounted with the Corning glass filter and shutter horizontal on the under surface approximately 25 in. above a bench so that the surface below is uniformly irradiated with ultra-violet.

The chromatogram is placed upon the bench beneath a sheet of $\frac{1}{4}$ in. plate glass to keep it flat and centred beneath the ultra-violet source. The chromatogram in addition to fluorescing transmits and reflects ultra-violet and hence a sheet of special filter glass is placed beneath the chromatogram in order to cut off all transmitted ultra-violet but allow the visual fluorescent light to be as little reduced as possible. Finally beneath this filter is placed a sheet of bromide paper of sufficient size to include all the fluorescent areas on the chromatogram. Using a chromatograph paper 8 in. wide, standard 10 in. $\times$ 8 in. sheets of bromide paper are satisfactory. The bromide paper used should have sufficient contrast to enable the fluorescent spots to produce a good black image against a very pale grey background. The most suitable material in our experience is Kodak contrast No. 3 glossy bromide paper of single weight thickness. The arrangement of the apparatus for this process is illustrated diagramatically in Figure 1.

The filter used to prevent the transmission of ultra-violet to the bromide paper is of paramount importance and should be of sufficient size to cover the sheet of bromide paper. The radiation from the filtered ultra-violet source is almost entirely from the 365 mμ Hg line and this filter must be opaque at this wavelength and have a high transmission in the visual region. It is also essential that it shall not fluoresce itself as so many dyed gelatine filters do. The literature indicates that the Wratten No. 2A filter should be satisfactory but we have not tried this and would anticipate difficulty in obtaining a large sheet. The filter used in these experiments was a sheet of glass 15 in. × 12 in. × ¼ in. known commercially as "champagne plate" and manufactured in England. This was found as a result of experiments with various pale yellow glasses most of which are quite unsuitable.

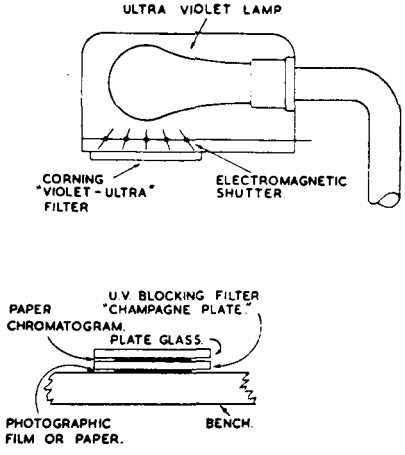


FIG. 1. General arrangement of the apparatus for the photographic recording of the fluorescence chromatogram.

In Figure 2 is given a transmission curve for this glass in air in order that comparisons with published transmission curves for other glasses can be made. Although there is rather a sharper cut off at 410 mμ with the Wratten No. 2A filter the champagne plate glass is readily available, inexpensive and quite satisfactory.

A fluorescence photograph of a chromatogram of digilanids B and C is reproduced in Figure 3. Digilanid A and digitoxin fluoresce with an orange light which is non-actinic to bromide paper. By this means, therefore, these substances are effectively excluded from the analysis and the photographic process is modified accordingly to detect these substances.

The edge of the chromatogram is notched in two or three places with a ticket punch to enable exact identification to be made between any chromatogram and the corresponding photograph on the assumption that an identical arrangement of notches is never likely to occur twice.

When the yellow fluorescence of digitoxin or digilanid A can be seen on a chromatogram a second photographic record is made after the bromide paper print since this excludes these substances.

For the second photograph the "champagne glass" filter is exchanged for a sheet of photographic amber glass in order to further reduce the intensity of the bright blue fluorescence already recorded and allow the yellow-orange light to have a proportionately larger effect. Instead of bromide paper Kodak Process Panchromatic cut film is used in these

cases. The spectral transmission curve for the amber glass is also reproduced in Figure 2.

The problem of the most suitable filters to cut off the excess of ultra-violet radiation is by no means a simple one. It would be possible if ideal filters could be obtained to expose the photographic material indefinitely without producing fogging of the background since the

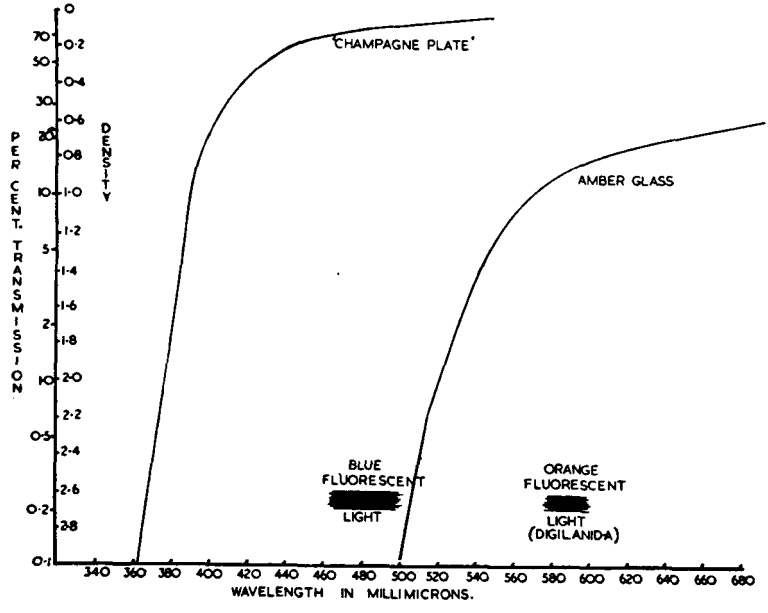


FIG. 2. Transmission curves for filters used above photographic materials in preparing fluorescence photographs.

fluorescence alone would provide the illumination. Using the "champagne plate" glass the length of exposure is limited to approximately 10 sec. under our conditions before background fog becomes appreciable. Under these circumstances it is possible to obtain clearly exposed spots with as little as 0·5 to 1·0 μg of digilanids B or C but if filters could be obtained which would enable longer exposures to be made this lower limit might well be much smaller.

The amber glass filter has an appreciable absorption of the yellow fluorescence of the digilanid A or digitoxin spots and hence these tend to be rather poorly recorded but it does cut off residual radiation from the lamp so that exposures up to 5 minutes in duration do not produce background fog. When these glycosides are to be measured the exposure we have used is 3 minutes with the ultra-violet source brought to within 18 in. of the photographic material.

Using the amber filter it is possible to record all glycosides since the blue fluorescence is still transmitted sufficiently to give a good record on panchromatic film but in view of the greater expense of this material and when interest is centred on the blue-fluorescing glycosides bromide paper

gives an excellent record. The "champagne plate" filter presents one disadvantage since it is $\frac{1}{4}$ in. thick and separates the fluorescing chromatogram from the sensitive material thus causing some slight diffusion of the spots. The thinner these filters are the less the spots will tent to merge together. The amber glass is more satisfactory from this point of view since it is only approximately 0·1 in. thick.

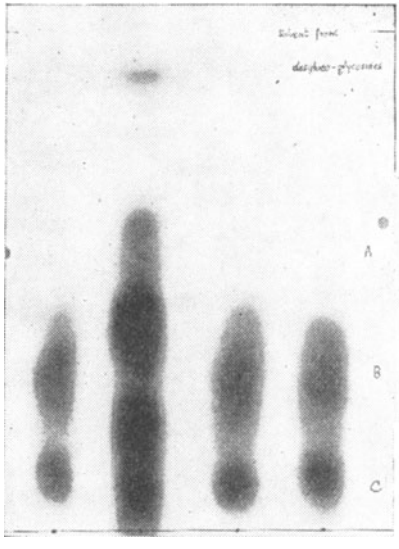


FIG. 3*a*. Fluorescence photograph of a chromatogram with known amounts of digilanids A, B and C. The yellow fluorescence of digilanid A is not seen in this record.

I. Digilanids:
A 4 μg.
B 2 μg.
C 6 μg.

II. Digilanids of plant sample 19z: 0·01 ml.

III. Digilanids:
A 6 μg.
B 4 μg.
C 8 μg.

IV. Digilanids:
A 6 μg.
B 4 μg.
C 10 μg.

FIG. 3*b*. Record on panchromatic film of part of the same chromatogram as Fig. 3*a* to show the fluorescence of digilanid A.

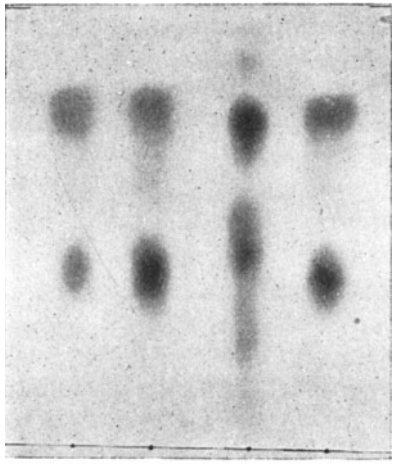
In all cases the developer used in the Kodak D 19 formula and development under standardised conditions is of 2 minutes duration for the bromide paper and 3 minutes for the film at 22° C.

On the chromatogram of Figure 3A some digilanid A was also present and in Figure 3B is seen a reproduction of a photograph of the panchromatic film obtained, which shows the content of this third digilanid and also the registration marks.

In Figure 4 is seen an analysis of an unknown mixture of glycosides from plant material using the mixture of solvents to show the separation of the digilanids and in this case the digitoxin, gitoxin and digoxin have travelled completely to the solvent front and it will be seen that

there is a substantial amount of digilanid C and digilanid B, the former corresponds to approximately 10 μg. and the latter rather more than 6 μg. of digilanid B in 0·01 ml. of extract.

Direct visual comparison from these photographs is of great value in the quantitative field since standard mixtures of glycosides are always included on each paper at more than one concentration of each. It is essential to note that since the photographic record is the result of a prolonged exposure to the fluorescence small amounts of fluorescence not easily seen on the chromatogram cause an integration of silver deposition in the photograph and thus become clearly visible.

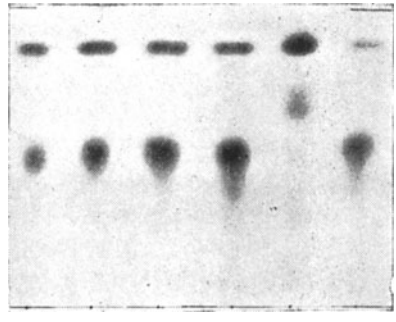


I II III IV

FIG. 4. The analysis of a plant sample by visual comparison from the photographic record.

N.B.—The several processes of reproduction have inevitably impaired the quality of the records which are somewhat more easily studied in the original.

I. Digilanids: B 4 μg. C 6 μg.
II. Digilanids: B 4 μg. C 10 μg.
III. Digilanids of plant sample 19z: 0·005 ml.
IV. Digilanids: B 6 μg. C 10 μg.



I II III IV V VI

FIG. 5. Arrangement of chromatogram for the analysis of plant samples by photographic densitometry. In this case the amounts of standard glycosides are carefully chosen to give a graduation of fluorescence above and below that of the plant sample under examination. In this record the digilanid C is suitable for accurate analysis but the spots of digilanid B are rather too close to the solvent front.

I. Digilanids: B 2 μg. C 4 μg.
II. Digilanids: B 4 μg. C 6 μg.
III. Digilanids: B 6 μg. C 8 μg.
IV. Digilanids: B 4 μg. C 10 μg.
V. Digilanids of plant sample 19z: 0·0025 ml.
VI. Digilanids: B 2 μg. C 10 μg.

Where a greater degree of accuracy is required the process is further elaborated by making negatives from the chromatogram photographs which then show a dense black background with variable clear areas corresponding to the spots on the original prints. The size and density of these spots is then measured by a simple photoelectric densitometer. For such densitometry measurements it is desirable to have the spot represented as a clear area in a black background since problems of the area covered by the photocell system do not arise. These measurements

are made upon Kodak Process cut-film by exposing this directly under the bromide paper print or panchromatic film using these as negatives and placing the emulsion surfaces of the two materials in contact. Exposure is carried out in this case under a sheet of plain glass to a low intensity tungsten lamp and is of such duration that the transmission of the background does not exceed 2 per cent. of that of the clear film.

The photoelectric densitometer is a simple arrangement of a selenium photocell arranged over the clear area of film and a condenser lens below. The light source is in this case a motor car headlamp bulb operating from an accumulator. The amount of light transmitted being measured by means of a Cambridge Spot galvanometer. In Figure 5 is shown a photographic print from a chromatogram of digilanids B and C suitable for more exact examination in this way. By arranging the largest amount of the glycoside to correspond to 100 per cent. transmission in the negative film there is a linear relationship between spot size and density integrated in this way and the amount of the glycoside on the paper chromatogram.

The authors are indebted to Messrs. Sandoz of Basle for gifts of pure digilanids A, B and C; Messrs. Burroughs Wellcome & Co. (Australia) Ltd. for gifts of digoxin and digitoxin and Dr. D. W. Adamson of the Wellcome Research Laboratories, Beckenham, Kent, England, for a gift of gitoxin. For much valuable advice upon the selection of light filters we should like to thank Dr. R. G. Giovanelli of the Department of Optics, National Standards Laboratory, Sydney. A grant in support of this work from the National Health and Medical Research Council of Australia provided technical assistance and we wish to take this opportunity to express our thanks in this regard. To the Directors of Drug Houses of Australia we are greatly indebted for the support of one of us (H. S.) throughout this work.

REFERENCES

1. Lehman and Paff, *J. Pharmacol.*, 1942, **75**, 207.
2. Svendsen and Jensen, *Pharm. Acta Helvet.*, 1950, **25**, 241.
3. Hassel and Martin, *J. chem. Soc.*, 1951, 2766.
4. Henney and Dudley, 1939, *Handbook of Photography*, McGraw-Hill, 597.
5. Auerbach and Auerbach, *Photo Technique*, 1941, **3**, No. 6, 48.