human HbO₂ in upper layer, after 0, 12, 60, 113 and 185 min. The direction of migration for the ascending patterns is towards the left, for the descending towards the right. Phosphate, pH 8; μ , 0.05; 10 V./cm.; HbO₂ concentration in upper layer, 0.05 g./100 ml.

Fig. 8. Electrophoretic patterns of oxyhaemoglobins. All experiments, with the exception of nos. 13-15, in phosphate, pH 8; μ , 0.05; 14 V./cm. Nos. 1 and 2 ascending and descending patterns of rabbit HbO₂, concentration = 1.35 g./100 ml., after 430 and 435 min. Nos. 3-15 patterns of mixtures 1:1 of human and rabbit HbO₂.

Left, ascending limb; right, descending limb. Nos. 3 and 4 = Exp. 1 of Table 4, after 320 and 322 min.; no. 5 = Exp. 2, after 299 min.; nos. 6 and 7 = Exp. 3, after 321 and 209 min.; nos. 8 and 9 = Exp. 6, after 382 and 385 min.; nos. 10-12 = Exp. 7, after 243, 360 and 364 min.; nos. 13 and 14 ascending patterns, no. 15 descending pattern of a mixture 1:1 of human and rabbit HbO₂ in phosphate pH 8, μ = 0.2; nos. 13 and 15 after 443 min. at 7 V./cm., then 160 min. at 6 V./cm.; no. 14 after 443 min. at 7 V./cm., then 173 min. at 6 V./cm., then 815 min. at 4 V./cm. Total distance migrated about 9 cm.

Chromatographic Studies of Nucleic Acids

1. A TECHNIQUE FOR THE IDENTIFICATION AND ESTIMATION OF PURINE AND PYRIMIDINE BASES, NUCLEOSIDES AND RELATED SUBSTANCES

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Until recently the isolation and identification of purine and pyrimidine bases, ribosides and nucleotides in mixtures such as nucleic acid hydrolysates were only possible by purely chemical methods of separation, necessitating the use of very large quantities (often 100 g. or more) of starting materials (Levene & Bass, 1931). Such methods are tedious, very limited in application and far from quantitative. However, the development of partition chromatography on paper (Consden, Gordon & Martin, 1944) has afforded the possibility of the separation of mixtures of purine and pyrimidine derivatives in minute amounts. The successful application of this method depends upon the location on the paper chromatogram of the areas occupied by the various substances. Once located, these areas may be cut out, extracted in a suitable solvent, and the substances estimated by ultraviolet spectrophotometry, making use of their characteristic absorption bands in the region of 260 m μ . Two methods of detecting purine and pyrimidine derivatives have been described recently. Hotchkiss (1948) cut the paper into transverse strips, 5-30 mm. wide, extracted each of these in water and, using a Beckman photoelectric spectrophotometer, determined for each extract the absorption in the ultraviolet region near 260 m μ . This method is time-consuming, does not allow of a very high resolution of adjacent spots, and for practical reasons is obviously inapplicable to two-dimensional chromatograms. In a second method, described by Vischer & Chargaff (1948*a*), the purines and the pyrimidines are converted on the chromatogram to mercury complexes. Excess

mercury is removed and the paper is treated to convert the mercury in the complex into mercuric sulphide, which being black indicates the position of the spots. While this technique allows the detection of as little as 5 μ g. of the free bases in individual spots, it has serious limitations. The method is not rapid, involving prolonged treatment of the paper in the wet state with several reagents and as described is limited to the bases. Furthermore, during the conversion of the mercury complexes into mercuric sulphide, the bases are lost so that for quantitative analysis parallel untreated strips of the chromatogram have to be used. It is thus impossible to use this technique for quantitative two-dimensional chromatograms.

To avoid these difficulties we have developed a simple and rapid method which will detect in a chromatogram all substances having a strong absorption in the ultraviolet near 260 m μ . Such substances, of course, include all purine and pyrimidine derivatives, as well as many other compounds of biological interest. The basis of this method, which has been reported briefly elsewhere (Markham & Smith, 1949) is a simple photographic method for the detection of absorbing substances.

METHODS

(a) Location of the spots

As all purine and pyrimidine derivatives have an intense ultraviolet absorption in the neighbourhood of 260 m μ , it is natural to look for methods suitable for exploiting this characteristic. Filter paper happens to be reasonably

transparent in this region, so it is possible to locate the spots of absorbing substances merely by making a contact print by filtered ultraviolet light on to sensitized paper through the dried paper chromatogram.

The source of ultraviolet light used in these experiments was a Hanovia 'Homesun' lamp, a 450 W., 200 V., mercury arc lamp. This lamp has a high emission at $253.7\text{ m}\mu$, a wavelength which, though not ideal, is satisfactory for most purposes. In order to make the method as sensitive as possible it is desirable to use filters in order to obtain light of this wavelength as nearly monochromatic as possible, particularly as photographic materials are very sensitive to the longer wavelength ultraviolet light emitted by this lamp. Several filters are available for this purpose, but for convenience a liquid filter containing CoSO_4 and NiSO_4 ,

Fig. 1. *a*, Longitudinal section of the chlorine filter (*A*) and liquid filter (*B*) showing their design. *C* and *D* are diaphragms and *E* is the mercury vapour lamp. *b*, Partial transverse section of chlorine filter showing taps used for filling and, *F*, calcium chloride for keeping the gas dry.

followed by a gaseous chlorine filter, was chosen (Bowen, 1946). The filters are of glass with fused quartz windows cemented on. For the liquid filter a resinous cement is used, but for the chlorine filter vaseline is used, a few dabs of Picein wax round the outside holding the assembly firm. The liquid filter, which contains an aqueous solution of $\text{NiSO}_4 \cdot 7\text{H}_2\text{O}$, 350 g. and $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$, 100 g./l., is 30 mm. thick and the gas filter containing chlorine at atmospheric pressure dried over CaCl_2 is 35 mm. thick. The cells have a free aperture of 22 mm. and their design is shown in Fig. 1. It seems to be important to have the liquid filter nearer the lamp, as otherwise the chlorine gas will need renewing frequently, whereas if they are arranged as recommended the gas persists indefinitely. A useful check on the efficiency of the filters is given by observing the spectrum of the light transmitted with a Beck ultraviolet spectroscope. The only lines visible should be at 253.7 and $265\text{ m}\mu$, the latter being much less intense. If such an instrument is not available, it is possible to test the light source by printing out a few test spots of adenine on Whatman no. 1 paper. It should be

possible to detect $1\text{ }\mu\text{g.}$ of adenine in a $10\text{ }\mu\text{l.}$ spot quite easily, whilst $5\text{ }\mu\text{g.}$ in the same volume should prove almost completely opaque. It is, in fact, possible to detect quantities of materials which are much too small to estimate accurately by means of a spectrophotometer.

The photographic material used is Ilford Reflex Document Paper, no. 50 (similar material is also manufactured by other firms). This material is comparatively cheap, and has the advantage of being extremely contrasting; and it may, if necessary, be handled in subdued artificial light. It is not affected by the yellow light transmitted by the filter system. (Other papers, though faster, have not proved so sensitive to small amounts of absorbing material.) We have not found that the developers used modify the results greatly and either the maker's formula I.D. 36 or a process developer (Kodak D 153) seems quite satisfactory.

Fig. 2. Printing the chromatogram. The photographic paper is held flat by the filter-paper chromatogram which is pinned over it on the convex surface of the plywood.

Exposure. Under our conditions a suitable exposure is 1.5 min. at a distance of 1.2 m. (which covers an area of about $15 \times 40\text{ cm.}$). For larger areas such as are needed for two-dimensional work, the light source has to be farther away, and the exposure may be estimated approximately by applying the inverse square law. The exposures are not very critical.

For making the prints it would be desirable to have a printing frame of sufficient size with a fused quartz window, but for all practical purposes we find that pinning the chromatogram paper over the photographic paper on a plano-convex surface (a piece of plywood fastened to a board with a small strip of wood under the centre to bow the former out a little (Fig. 2)) is entirely satisfactory.

After development the paper is fixed and washed in the usual way. If it is necessary to dry the paper rapidly it should be bathed after washing in ethanol or acetone and then dabbed with a clean cloth. Dimensional changes in the print appear to be negligible, but if they should be a source of trouble similar emulsions may be obtained coated on water-proof paper, cellulose acetate or glass.

The positions of absorbing substances may be seen on the developed papers as light areas (Fig. 3) and these prints serve as a permanent record of each experiment.

For many purposes the photographic image alone will suffice, but if the spots need to be extracted, their positions may be found by shining a light through the photographic paper and drawing their outlines on the filter paper, which is registered by means of pencil lines or other reference marks made on the latter and which show up on the print. As a general routine we write all the necessary data in pencil on

investigated (excluding certain methylated derivatives) were less than 0.5, the lower edge of the paper was cut to a point and the solvent front was run well beyond the end of the paper and a substance of known R_F value, usually adenine, was used as a marker. Two-dimensional chromatograms were run on standard sheets of filter paper in large glass-sided boxes. Solutions were placed on the paper in small areas 6 cm. from the upper edge, either with small glass capillary tubes, or, for quantitative work, with a Burroughs Wellcome Agla syringe. Volumes of 10–20 μ l. of solutions containing less than 50 μ g. of individual substances were used.

(c) Choice of solvent

Naturally in this work solvents such as phenol, quinoline, collidine, etc., which absorb heavily in the ultraviolet and are difficult to remove from the paper must be avoided if possible. Several aliphatic alcohols and ketones were tried, but *n*-butanol saturated with water (14% water in butanol (v/v); Hotchkiss, 1948) proved the most useful basic solvent for the separation of the bases and the nucleosides. For a satisfactory differentiation between hydroxy- and amino-purines and pyrimidines (Fig. 3), either ammonia was added to the vapour phase or the solvent was acidified. The systems used most frequently were: (1) a mixture containing 90% *n*-butanol saturated with water and 10% glacial formic acid (v/v); (2) *n*-butanol saturated with water in the presence of an atmosphere containing NH_3 ; (3) *n*-butanol saturated with water. For two-dimensional chromatograms a combination of (1) and (2) will separate all the substances likely to be encountered in nucleic acid hydrolysates (including xanthine and hypoxanthine, traces of which are usually present).

(d) Materials

Cytidine and uridine were obtained from Dr J. Davoll and yeast adenylic acid from Dr T. Mann. The methylated xanthines and 6:8-dihydroxypurine were from Prof. D. Keilin, F.R.S. Cytosine was obtained by hydrolysis of cytidine at 145° in 6*N*-HCl. Though hydrolysis was practically complete no trace of uracil was found, although we have confirmed the observation of Vischer & Chargaff (1948*b*) that during the acid hydrolysis of nucleic acids some uracil is formed from cytosine. Thymine was obtained from thymus nucleic acid by hydrolysis. The other substances were commercial products.

RESULTS

Table 1 gives the positions on the chromatograms of purines and pyrimidines and some of their derivatives in various solvents. This table is only intended as a guide to the relative positions of these substances, as we find a certain amount of variation from time to time. For instance, particularly in butanol-ammonia systems, the pyrimidines, uracil and cytosine (and their corresponding ribosides) are very sensitive to changes in the composition of the vapour phase and they may change their relative positions. In such chromatograms it is necessary to use adenine and uracil as markers. In the butanol-formic acid system, adenine and hypoxanthine may undergo a similar displacement. When running two-dimensional chromatograms it is essential to use

Fig. 3. Print of chromatogram run for 9 hr. in *n*-butanol-formic acid to illustrate points in text. Mixtures were put on at spots 1–5 and each column reads from the top downwards. (1) Uric acid, 6:8-dihydroxypurine and hypoxanthine, and (2) guanine and adenine, both showing effect of OH groups; (3) adenosine and adenine, and (4) cytidine and cytosine, showing effect of ribose; (5) xanthine, 3-methylxanthine, 3:7-dimethylxanthine (theobromine) and 1:3:7-trimethylxanthine (caffeine) showing effect of methylation. (Note. To resolve all these substances when present together several solvents would have to be used and the chromatograms run for longer times.)

the filter paper so that it is recorded on the print as well. The areas of filter paper corresponding with the spots on the photographic print are cut out and extracted in 5 ml. of water or 0.1*N*-HCl for estimation in a Beckman photoelectric spectrophotometer. Areas identical in size are cut from adjacent strips for use as blanks (see Hotchkiss, 1948).

(b) Chromatographic technique

All chromatograms were run on Whatman no. 1 paper. For single-dimensional work sheets 13 × 33 cm. were run in glass battery jars. As the R_F values of most of the substances

Table 1. R_F values of purines, pyrimidines and ribosides in various solvents

Solvent system* ...	<i>n</i> -Butanol 86%:water 14%	<i>n</i> -Butanol 86%:water 14%: + NH ₃ †	<i>n</i> -Butanol 77%:water 13%:formic acid 10%	<i>n</i> -Butanol 50%:ethanol 15%:water 35%	Amyl alcohol saturated with water	Amyl alcohol saturated with water + NH ₃ †	Amyl alcohol saturated with water 90%:formic acid 10%
Purines:							
Hypoxanthine	0.26	0.12	0.30	—	0.15	0.02	0.19
Xanthine	0.18	0.05	0.24	—	0.10	0	0.16
6:8-Dihydroxypurine	0.18	0.04	0.24	—	0.07	0	0.11
Uric acid	0.01	0	0.14	—	0	0	0.06
3-Methylxanthine	0.29	0.13	0.32	—	0.19	—	—
1:3-Dimethylxanthine (theophyllin)	0.52	0.22	0.64	—	0.54	0.12	0.55
3:7-Dimethylxanthine (theobromine)	0.42	0.27	0.47	—	0.28	0.15	0.36
3:8-Dimethylxanthine	0.42	0.18	0.48	—	0.34	—	—
1:3:7-Trimethylxanthine (caffeine)	0.63	0.65	0.71	—	0.56	0.50	0.67
Adenine	0.38	0.28	0.33	0.55	0.28	0.16	0.12
Guanine	0.15	0.11	0.13	0.37	0.05	—	0.04
Purine ribosides:							
Adenosine	0.20	0.22	0.12	0.50	0.11	0.09	0.04
Guanosine	0.15	0.03	0.17	0.40	0.02	0	0.04
Pyrimidines:							
Cytosine	0.22	0.24	0.26	0.53	0.09	0.09	0.07
Uracil	0.31	0.19	0.39	0.46	0.22	0.08	0.23
Thymine	0.52	0.35	0.56	—	0.40	—	—
Pyrimidine ribosides:							
Cytidine	0.12	0.11	0.18	0.42	0.02	0.03	0.03
Uridine	0.17	0.08	0.25	0.49	0.05	0.03	0.07

* Composition of solvents is given in terms of volume percentages.

† 5% by vol. of NH₃ solution (sp.gr. 0.880) was added to the solvent mixture in the bottom of the container. Fig. 4. The variation of R_F values of adenylic acid (open circles) and adenine (solid spots) in solvents of varying water content.

separate markers in each dimension. A print is then taken of the markers after running in each solvent. The markers are, of course, placed on the other end of the top edge of the filter-paper sheet and thus do not interfere.

Solvents which contain little water do not bring about much movement of the phosphorylated derivatives (nucleotides, dinucleotides, etc.) owing to the much greater relative solubility of these substances in water, and consequently they are to be found at the position of the original spot in butanol-water chromatograms. Some separation of these substances may be obtained by the use of solvents based on ternary mixtures such as butanol-ethanol-water and on completely miscible solvents such as propanol-water (Fig. 4).

DISCUSSION

From Table 1 it appears that certain generalizations as to the movement of substances containing certain groupings may be made, and we enumerate them as follows:

(1) Pyrimidines move faster than the corresponding purines. For example, uracil runs faster than xanthine and cytosine faster than guanine

(2) In the presence of ammonia the amino derivatives move much faster than the corresponding hydroxy compounds, but on changing to an acidic solvent this difference is greatly decreased and the relative positions may even be reversed. This is particularly evident in the case of the pyrimidines cytosine and uracil (and their ribosides) in which the reversal takes place at neutrality.

(3) Increase in the number of hydroxyl groups possessed by a substance decreases its rate of movement in all the solvents. This is illustrated by a comparison of the R_f values of hypoxanthine, 6:8-dihydroxypurine and uric acid, and those of adenine and guanine, adenosine and guanosine.

(4) Ribosides move more slowly than the free bases but maintain the same relative positions.

(5) Increasing methylation tends to increase the movement of the substances in all the solvents.

Comparing these results with those of other workers some points of interest may be noted. We find, contrary to Hotchkiss (1948), that, when present in amounts small enough to be completely in solution in the aqueous phase, guanine moves at an appreciable rate in butanol mixtures, and probably corresponds with the 'epiguanine' to which

he refers. This is probably due to the fact that his method has too poor a resolution to give a satisfactory picture of the chromatogram as a whole.

Except in acidic solvents, uracil always runs in our chromatograms more slowly than adenine, contrary to the observations of Vischer & Chargaff (1948*a*). These and other minor discrepancies would appear to be due, in part at least, to the different filter paper used by these authors.

SUMMARY

1. A micromethod for the detection and estimation of purines, pyrimidines and related substances is described. It is based upon the detection of spots of these compounds on paper chromatograms by means of a simple contact-printing technique using photographic paper and filtered ultraviolet light, and the sensitivity is such that a few micrograms may be detected.

2. The R_f values of a number of compounds in several solvents are tabulated.

3. Certain generalizations are made correlating chemical structure with the chromatographic behaviour of these substances.

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Studies in Vitamin A

10. VITAMIN A₁ AND RETINENE₁ IN RELATION TO PHOTOPIC VISION

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Scotopic (or low intensity) vision is related to the activity of rods, and photopic (or daylight) vision operates by means of cone receptors. When light falls only on rods there is no sensation of colour, and the spectral distribution of sensitivity in scotopic vision can be interpreted in terms of one receptor substance, rhodopsin. For photopic vision, however, the phenomena of colour sensation cannot be explained on the basis of a single absorbing entity with a spectrum corresponding to the photopic sensitivity curve. Nevertheless, the full cone response curve obtained by electrophysiological

methods is very similar to the normal photopic sensitivity curve.

One interpretation of the Young-Helmholtz theory is that it implies the existence of three selectively absorbing substances sensitive to different spectral ranges. Many workers have attempted to measure the responses of different receptors to different wavelengths. The usual procedure was first to expose the eye to filtered (or better monochromatic) light in the hope that the receptor affected would be put out of action by photochemical exhaustion. The response of another receptor to