SEPARATION AND ESTIMATION OF PURINE AND PYRIMIDINE BASES FROM A HEATED SUSPENSION OF *Micrococcus flavus*

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A method has been described for the chromatographic separation of a mixture of purine and pyrimidine bases. They are identified as single substances in solution by the ultra-violet light absorption at a small number of wavelengths. Checks on the purity of the recovered bases are also described and the method is applied to the separation and estimation of purine and pyrimidine bases from the supernatant fluid of a heated suspension of *Micrococcus flavus*.

COMPLETE hydrolysis of nucleic acids (NA) yields a sugar component, phosphate and purine and pyrimidine bases. Pyrimidine derivatives found in nucleic acids are cytosine (RNA and DNA), uracil (RNA), thymine and 5-methylcytosine (DNA). A fifth pyrimidine, 5-hydroxymethylcytosine, replaces cytosine in certain strains of coliphage. Purine bases found in both types of nucleic acids are adenine and guanine. Work, particularly by Smith and Wyatt[1], has made it clear that the tetranucleotide hypothesis must be abandoned as nucleic acids vary widely in the molar proportions of bases according to the material of origin. It is known that in RNA the number of nucleotides carrying an amino group in the 6 position, adenine and cytosine, is equal to the number having a 6-oxo group, guanine and uracil. This symmetry is also found in DNA, thymine replacing uracil, but other regularities in composition are also present; (*a*) the molar sum of the purines is equivalent to the molar sum of the pyrimidines; (*b*) the molar ratio of adenine to thymine is 1; and (*c*) the molar ratio of guanine to cytosine + methylcytosine is 1. Smith and Wyatt have estimated the proportions of purine and pyrimidine in DNA from *E. coli* as follows, basing their calculations on molar proportions to a total of 4: adenine 0·90, guanine 0·98, cytosine 1·03 and thymine 1·09.

The terms RNA and DNA denote classes of compounds of varying composition. It is not known whether all molecules of RNA and DNA in an organism have the same composition, but the relative proportion of bases vary widely in nucleic acids from various sources, and even possibly in nucleic acids from the same source under varying environmental and metabolic conditions. Nucleic acids absorb ultra-violet light in the region of 260 mμ. This absorption is attributed to the conjugated double bond system of the purine and pyrimidine rings. There is no significant difference in the absorption of DNA and RNA. The ultra-violet absorption curves of the bases are illustrated in Figure 1. On hydrolysis the extinction coefficient of a nucleic acid increases significantly since the sum of the extinction of the constituent nucleotides is greater than the extinction of the polynucleotides.

Chromatographic methods have been of help in the separation of nucleic acid hydrolysis products[2–11]. The method of hydrolysis used, besides yielding quantitative cleavage of both RNA and DNA should not result in deamination of the bases. Marshak and Vogel[9] established that 12N perchloric acid cleaves nucleic acids quantitatively and permits determination of bases. After applying the usual one-dimensional chromatographic process, using some such solvent as n-butanol saturated with water, the position of the spot corresponding to the individual base

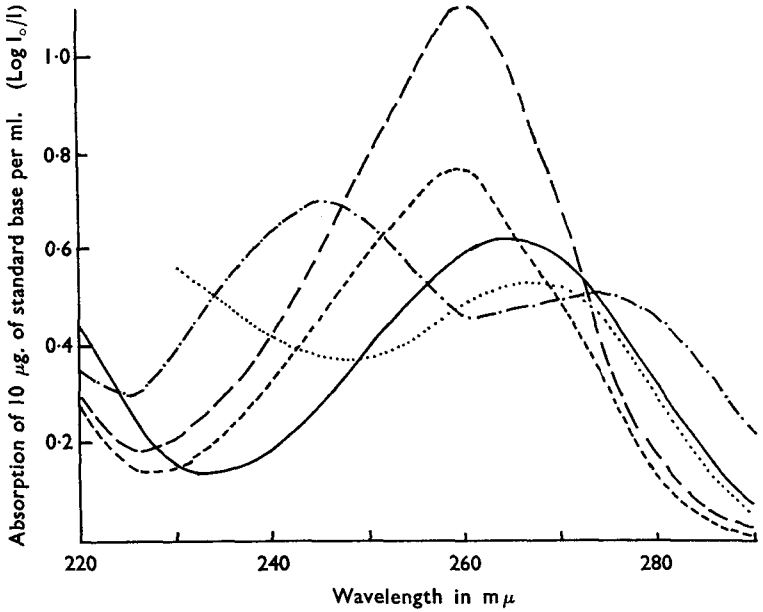

FIG. 1. Ultra-violet absorption curves for purine and pyrimidine bases at pH 7.
—— Thymine; – – – adenine; - - - uracil; –·–·– guanine; . . . . . cytosine.

or nucleotide is determined. The quickest and most useful method has been devised by Holiday and Johnson[12], in which the light transmitted by a Corning 9863 (230–400 mμ) filter shows the spots as dark patches against the background of paper fluorescence. The most suitable light source was found to be a low-pressure mercury resonance lamp. The spots are visible at concentrations of 0·5 μg./cm². Markham and Smith[2–5] have developed a method in which a permanent record may be kept of the chromatogram on photographic document paper exposed to ultra-violet light under suitable conditions. The print shows the absorbing substances as light areas on a black background. Other workers have developed a large number of methods and modifications for the isolation and identification of purines, pyrimidines and nucleosides[13–24]. These methods are complicated since they involve determinations of ultra-violet light absorption along a large range of wavelengths, and are made on mixtures of bases.

Experimental Methods

Purine and pyrimidine bases. The purity of commercial samples of cytosine, adenine, guanine, uracil and thymine was confirmed by spectrophotometric measurement of their solutions. Molecular extinction coefficients are given in Table I, together with figures obtained by other workers. Guanine showed 98·60 per cent of the theoretical weight of nitrogen by Kjeldahl determination.

Spectrophotometric measurements was by Unicam S.P.500 Ultra-violet spectrophotometer with 1 cm. matched silica cells.

Chromatographic methods. Whatman No. 1 papers were developed by the ascending technique for 24 to 30 hours at 19—20° after equilibration with n-butanol: water for 10 hours.

A Hanovia Chromatolite portable ultra-violet lamp fitted with suitable filters was used to examine the 260 mμ absorbing substances. The

TABLE I

Ultra-violet absorption data

Base	Extinction coefficient (ε)		Wavelength (mμ)	pH	Log I_0/I at 260 mμ (10 μg./ml. at neutral pH)
	Standard	Reference value*			
Uracil	8,512	8,600 (30)	258	7	0·73
Thymine ..	7,560	7,800 (30)	264	7	0·59
Cytosine ..	5,880	6,100 (11)	266	7	0·49
Adenine ..	12,725	13,000 (14)	260	0·1 N HCl	1·05
Guanine ..	10,180	11,000 (14)†	250	0·1 N HCl	0·465

* Figures in parentheses denote references in literature.
† Markham and Smith[9] and Wyatt[10] obtained a value of 10,500.

dark areas were marked in pencil outline and cut out. Blanks were prepared by tracing identical areas. Each spot and blank was eluted overnight in a test tube containing 4 ml. of water, and the absorption at 260 mμ measured spectrophotometrically. Five minutes was allowed to elapse with the sample in the cuvette, to allow fibres to settle.

The culture medium (Agar slopes). A commercially available dehydrated form of nutrient agar was used, prepared by Baltimore Biological Laboratories.

Organism. Micrococcus flavus MCI. NCIB 8134, obtained from the South African Bureau of Standards, Pretoria.

Preparation of the bacterial suspension. The slopes were innoculated and grown at 37° for one day, the organisms were washed off, suspended in distilled water, and then centrifuged at 2,000 *g* for 1 hour. After resuspension they were again centrifuged at 2,000 *g* for 1 hour. The supernatant liquid was again removed and the bacteria were resuspended in distilled water.

Preparation of the supernatant liquid of a heated suspension of M. flavus. The suspension previously described was heated for 1 hour in a water bath, the bacteria removed by centrifugation, and the supernatant was further heated to concentrate the solutes. This solution was evaporated *in vacuo*, and the solids obtained were hydrolysed by dissolving in 12N perchloric acid. This liquid was filled into neutral glass ampoules,

which were sealed and boiled for 1 hour. The acid was neutralised with ammonium hydroxide.

RESULTS

Ultra-violet Absorption of Standard Purine and Pyrimidine Bases in Neutral Solutions

Solutions of each of the five bases, adenine, guanine, cytosine, uracil and thymine, were prepared, containing 10 μg./ml. Absorption curves

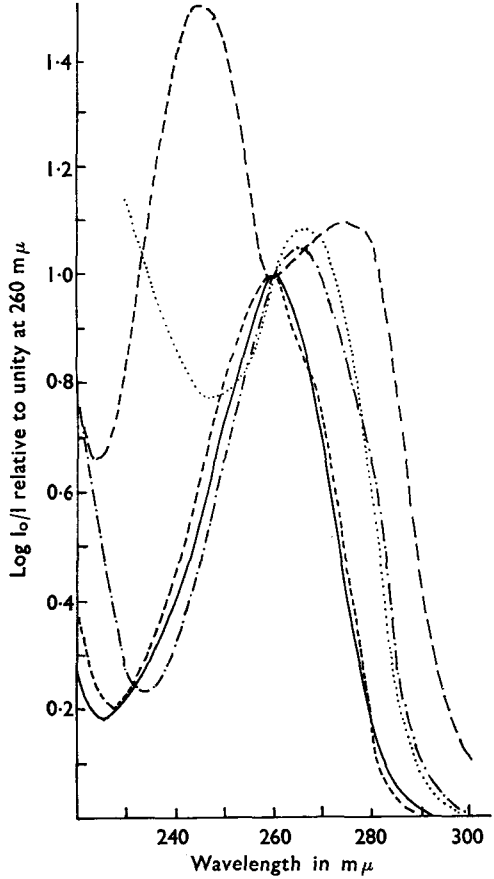

FIG. 2. Ultra-violet absorption curves for purine and pyrimidine bases at pH 7, derived from the corresponding curves on Figure 1.
—— Adenine; – – – guanine; - - - - uracil; –·–·– thymine; . . . . . cytosine.

are shown for each of these bases (Fig. 1). Guanine and adenine needed the addition of a small quantity of N NaOH to aid solution. This solution was then adjusted to neutrality with 0·1N HCl. Each of the other three solutions were also adjusted to pH 7, where necessary.

Identification of Purine and Pyrimidine Bases in Solution

A scheme, based on the results shown in Figure 1, was worked out which enabled the rapid identification of these bases in solution, when

present singly. Each result was recalculated relative to an optical density of unity at 260 mμ. The curves obtained are shown in Figure 2. From these curves, the ratios of the optical density at 240 mμ and 265 mμ to the optical density at 260 mμ were calculated. These ratios are denoted as N240/N260 and N265/N260 respectively.

TABLE II

ULTRA-VIOLET ABSORPTION OF PURINE AND PYRIMIDINE STANDARD BASES

Ratios of optical densities at various wavelengths

Base	$\frac{N240}{N260}$	$\frac{N265}{N260}$	$\frac{OH285}{OH270}$
Guanine ..	1·38	1·02	—
Cytosine ..	0·86	1·08	—
Uracil ..	0·44	0·88	1·13
Adenine ..	0·40	0·89	0·11
Thymine ..	0·32	1·05	—

N and OH refer to neutral solutions and to solutions containing 10 per cent N NaOH respectively. The figures denote the wavelength in mμ.

Hotchkiss[14] made use of pH absorption shifts to aid the determination of nucleic acid bases and a similar method is used here for uracil and adenine. Samples of these solutions were mixed with 10 per cent v/v of N NaOH and the absorption determined at 285 and 270 mμ. The ratio of the first reading to the second reading was calcultaed and was denoted OH285/OH270. The results are given in Table II. By the use of the values presented in this Table, it is possible to identify the base present in

TABLE III

SCHEME FOR THE IDENTIFICATION OF PURINE AND PYRIMIDINE BASES WHEN PRESENT AS SINGLE SUBSTANCES IN SOLUTION

Ratio	Result	Deduction
$\frac{N240}{N260}$	A Greater than 1·38	Guanine
	B Greater than 0·86, but smaller than 1·38	Cytosine
	C Smaller than 0·86	Uracil, adenine or thymine
$\frac{N265}{N260}$	D Greater than 0·95	Guanine, cytosine or thymine
	E Smaller than 0·95	Uracil or adenine
$\frac{OH285}{OH270}$	F Value greater than 1	Uracil
	G Value considerably less than 1	Adenine

a solution by neutralising the solution and measuring the absorption at 240, 260 and 265 mμ. The scheme adopted is represented in Table III.

Combination of results C and D will determine whether the base is thymine. From results A to E, all bases can be identified with the exception of uracil and adenine. If one or other of these bases are present, 10 per cent N NaOH is added and the absorption determined at 270 mμ and 285 mμ. If the ratio OH285/OH270 is greater than 1·0, the substance is uracil. If the ratio is smaller than 1·0, the base is adenine. Small variations in acidity may cause large deviations from the figures given. Where results are incompatible with those expected from R_F values then further experiments must be performed, by varying the pH

value of the sample. Results for the ratio N240/N260 from solutions recovered by chromatography sometimes showed a variation of as much as 0·3 higher than the theoretical. This discrepancy may be due to the presence of impurities with absorption at low wavelengths. Results for the ratio N265/N260 should agree to within 10 per cent.

As a confirmation to these tests, the relative R_F values of the spots are valuable[4,7,10]. R_F values have been found to vary considerably, thus they are not of value as absolute measurements. The variations encountered in R_F value could be accounted for by slight changes in temperature and solvent composition. The presence of acids or salts in

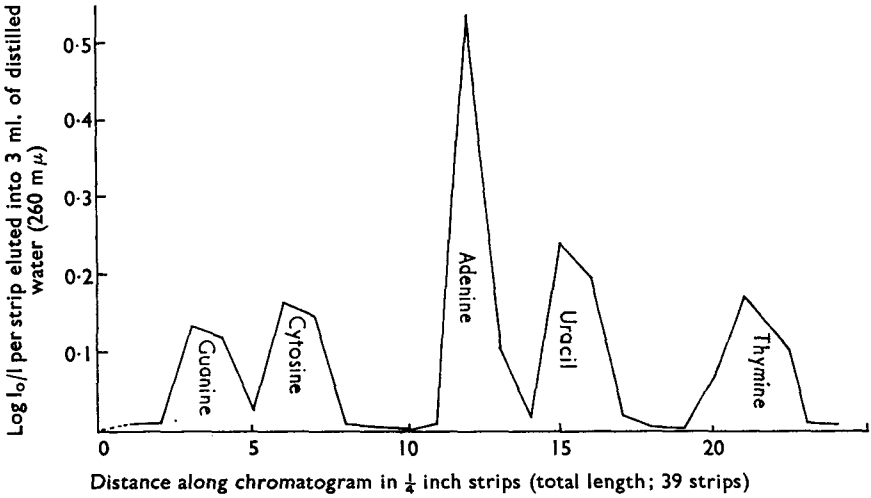

FIG. 3. Paper chromatogram of known bases. Sample contained 20 μg. of each of the five bases.

the solutions of bases, would also cause these variations. The pH of the solvent is known to have a marked effect on the movement of the bases. Increasing acidity causes purines to be held back more than pyrimidines. This effect is the apparent cause of the reversal of the position of adenine and cytosine noted by Wyatt[10] using an acidic solvent. Hotchkiss[14] using an alkaline system obtained a chromatogram in which the positions of uracil and adenine are in reverse to those obtained in the present paper.

Determination of Purity of Bases Recovered in Chromatography

The chromatographic techniques depend upon the complete separation of the bases. Readings below 240 mμ are unreliable in that the recovered bases yield high values; these may be derived from quantities of soluble extractive from the paper or the materials or from fluctuations in pH. Hotchkiss[14] has suggested that undue weight should not be placed upon comparisons made in this portion of the spectrum. Thus for practical purposes, it is better to rely on a small number of readings as described in the scheme for identification.

Separation and Estimation of Purine and Pyrimidine Bases from Solutions of Known Composition

A solution was made containing 0·1 per cent of each of the five bases. Spots were prepared on the chromatographic paper, from a short capillary tube of known capacity. The chromatogram was developed as described previously. By running the chromatogram for 24 to 30 hours it was possible to separate the spots completely. By applying the data given in Tables I–III, the amount of each base recovered from the mixture was calculated. Figure 3 shows a chromatogram that was obtained by cutting the chromatogram into strips. Table IV shows the quantitative results obtained in two experiments. Recoveries are quantitative, with

TABLE IV

THE SEPARATION, IDENTIFICATION AND ESTIMATION OF BASES

	1st expt.	2nd expt.	1st expt.	2nd expt.	1st expt.	2nd expt.	1st expt.	2nd expt.	1st expt.	2nd expt.
RF value	0·05	0·08	0·13	0·15	0·26	0·29	0·36	0·38	0·54	0·54
N240/N260	1·44	—	1·07	—	0·69	—	0·65	—	0·56	—
N240/N260 for standard solution	1·38	—	0·86	—	0·40	—	0·44	—	0·32	—
N265/N260	1·01	—	1·09	—	0·91	—	0·90	—	1·03	—
N265/N260 for standard solution	1·02	—	1·08	—	0·89	—	0·88	—	1·05	—
OH285/OH270	—	—	—	—	0·16	—	1·08	—	—	—
OH285/OH270 for standard solution	—	—	—	—	0·11	—	1·13	—	—	—
Base present	Guanine		Cytosine		Adenine		Uracil		Thymine	
μg. of base (recovered)	32·85	17·30	19·57	19·95	19·89	18·45	36·75	18·65	37·17	18·00
μg. of base (actual)	38·95	18·86	19·55	19·70	19·73	19·34	39·01	19·38	39·00	19·14
Recovery (per cent)	84·5	91·8	100·0	101·2	100·1	95·5	94·2	96·3	95·5	94·2

an error less than 5 per cent. Guanine alone yields a poor recovery, possibly because its extinction coefficient has a critical relationship to the pH. It should be noted that guanine loses its maximum absorption in a short waveband (245 mμ) as the pH moves in the alkaline direction. On the other hand, it loses its maximum absorption in its long waveband (275 mμ) as the pH moves in the direction of acidity. The results obtained in this experiment show that guanine does not move appreciably in the solvent used, which is contrary to the results found by Hotchkiss[14].

Separation and Estimation of Purine and Pyrimidine Bases from the Supernatant of a Heated Suspension of M. flavus

A suspension of *M. flavus* was filled into neutral glass ampoules and sealed. Some were autoclaved at 115° for 30 minutes and the others stored at room temperature. After autoclaving, the heat-treated ampoules were stored at room temperature with the untreated ampoules. After approximately 24 hours, the supernatant fluids were examined spectrophotometrically (Fig. 4). The data for the purine and pyrimidine contents of the supernatant liquid are given in Table V.

After clarification of the supernatant liquid, the unautoclaved suspension exhibited the normal absorption. The autoclaved solutions showed greater absorption with a peak at 255 mμ.

Discussion

The supernatant solution from a heated suspension of *M. flavus* appears to have the peak of absorption at 255 mμ. We have found *Eschericia coli* and *Bacillus subtilis*, when treated in a similar manner, to have the peak absorption of the supernatant fluid at 260 mμ, corresponding to the absorption of nucleic acid. Others have reported

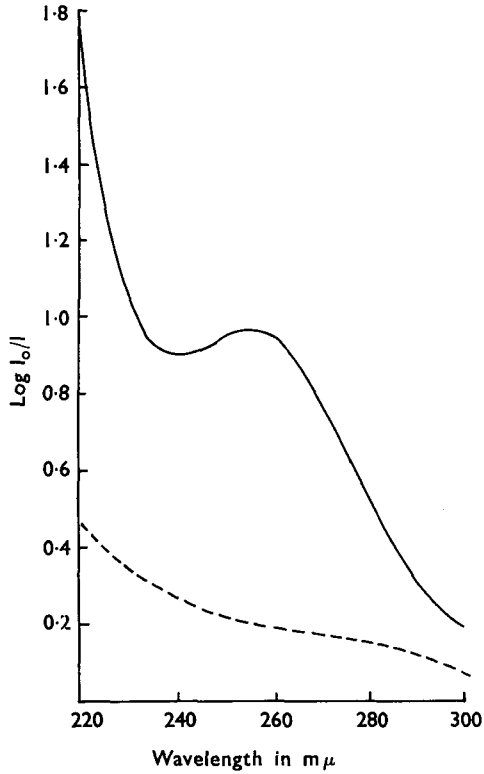

FIG. 4. Ultra-violet absorption of supernatant fluid from *M. flavus*.
- - - - Before autoclaving.
—— After autoclaving.
Bacterial concentration arbitrary.

the release of bacterial exudate under various conditions with maximum absorption at 260 mμ([25–28]). It appears that the locations of the absorption peaks of the supernatant solutions are dependent upon such factors as the age of the culture and the temperature at which they were stored. In this work, however, these factors remained constant.

In the supernatant of the organism examined, we find the sum of the cytosine and adenine to be equal to the sum of the guanine, uracil and thymine molecules, as did Elson and Chargaff[29]. *M. flavus* has been shown to have an exceptionally low thymine content and a high uracil and adenine content, which may account for the peak absorption at

255 mμ in the supernatant, rather than at the expected 260 mμ. The peak absorption of thymine solutions is at 265 mμ, thus in a mixture of the five bases, a paucity of this compound would yield a mean reading at a wavelength somewhat lower than normal. The low value obtained for thymine also indicates an unusually high predominance of RNA over DNA.

The purpose of the chromatographic study was not so much to investigate the mechanism of the release of cell exudate, as to attempt an insight into the distribution within the cell of nucleic acid. The results show

TABLE V

SEPARATION AND ESTIMATION OF PURINE AND PYRIMIDINE BASES FROM THE SUPERNATANT OF A HEATED SUSPENSION OF *M. flavus*

RF value	N265/N260	N240/N260	OH285/OH270	Base present	Log I_0/I*	μg. of base per spot	Molar ratio of bases
0·07	1·057	1·4	—	Guanine	0·087	7·49	5·0
0·17	1·01	1·09	—	Cytosine	0·128	10·45	9·4
0·27	0·78	0·86	0·29	Adenine	0·211	8·05	15·5
0·37	0·91	0·86	1·09	Uracil	0·399	21·90	19·5
0·47	1·00	0·86	—	Thymine	0·012	0·82	0·7

* Per spot eluted into 4 ml. of distilled water.

that most of the nucleic acid-containing tissues are involved in the release of cell exudate. This suggestion is prompted by the fact that the bases found in the supernatant are in the proportions expected when extracting DNA and RNA from intact cells. This postulation has a serious criticism, since it is based on the assumption that nucleic acids in different sites of a particular cell are not constant in their content of bases. Davidson[11] however, cites evidence obtained by several workers leading to the conclusion that the DNA complement of any one nucleus may be a mixture of DNA molecules of different composition of bases, but that the same mixture of DNA molecules may be present in all nuclei of one species. In any event, it is reasonable to assume that the RNA and DNA molecular complement of an individual cell must vary either in relation to the proportion or sequence (or both) of bases.

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After Dr. Levius presented the paper there was a DISCUSSION.