THE SEPARATION AND QUANTITATIVE ESTIMATION OF PURINES AND PYRIMIDINES IN MINUTE AMOUNTS*

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The investigation of an entire series of compounds of great biological importance, the nucleic acids, nucleotides, and related substances, has been impeded considerably by the lack of specific methods for the characterization and estimation of their nitrogenous constituents. Many problems concerning the composition, metabolism, and biological functions of these substances cannot even be approached owing to the absence of sufficiently simple and widely applicable procedures.

The older methods for the determination of the total quantity of purines, reviewed by Jorpes (4), did not permit the identification of individual components. They were based, in the main, on the estimation of the nitrogen content of the purine mixtures precipitated with silver (5) or copper (6). A microprocedure founded on similar principles was described by Graff and Maculla (7). The introduction by Schmidt (8) of specific enzymes for analytical purposes represented a definite advance. More recently, Kalckar (9) made very elegant use of a combination of enzymatic and spectroscopic methods for the microestimation of individual purines. The attempt to determine the adenine content of purine mixtures by means of an adenine-deficient *Neurospora* mutant (10) has not yet given reliable results (11).

The pyrimidines have been neglected to an even higher degree. No specific methods seem to be available, and this has certainly handicapped our understanding of this important class of substances.

The procedure presented in this paper comprises essentially three steps: (*a*) the separation of the mixtures into individual components by means of chromatography on paper strips (12), (*b*) the demonstration of the number and position of separated compounds by their conversion into suitable metal salts, (*c*) the identification of the separated purines and pyrimidines

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through the shapes of their characteristic absorption curves in the ultra-violet and their quantitative estimation from the extinction values.
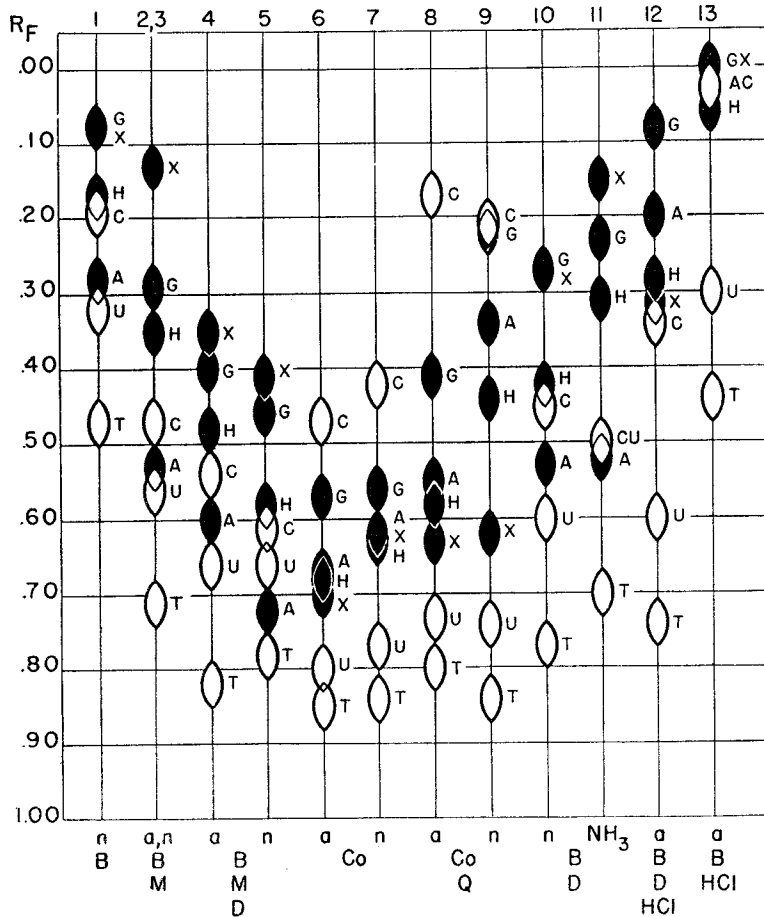
Fig. 1. Schematic representation of the position on the paper chromatogram of the purines and pyrimidines following the separation of a mixture. The numbering of the columns corresponds to the experiment numbers in Table I. *A* adenine, *G* guanine, *H* hypoxanthine, *X* xanthine, *U* uracil, *C* cytosine, *T* thymine. The conditions under which the separations were performed are indicated at the bottom. *a* acidic, *n* neutral, *B* n-butanol, *M* morpholine, *D* diethylene glycol, *Co* collidine, *Q* quinoline.

As presented here, the method applies to the separation and micro-estimation of all purines and pyrimidines normally found as constituents of nucleic acids, *i.e.* adenine, guanine, uracil, cytosine, and thymine. The

separation and identification of xanthine and hypoxanthine likewise are included, but the description of the quantitative determination of these purines, which has been achieved by similar methods, will be presented later in another connection. The procedures can doubtless be extended to other substances with characteristic absorption, such as uric acid, the pterins, etc. The following paper (13) and investigations on various desoxypentose nucleic acids to be published shortly will illustrate the application of the method to the study of nucleic acid composition. Other uses, especially for the determination of individual purines and pyrimidines in tissues and body fluids, the separation of nucleotides, etc., will be discussed at subsequent occasions.

The procedures described here should, by offering a map, as it were, of the purine and pyrimidine composition of a mixture, be particularly adapted to the detection of unexpected components. The range within which the bases can be separated and determined lies between 5 γ and 40 to 50 γ. At the optimum concentration, *i.e.* with about 20 γ of each substance, the accuracy is ± 4 per cent for the purines and even better for the pyrimidines, if the averages of a large series of determinations are considered. In individual estimations the accuracy is about ± 6 per cent. Larger amounts cannot be separated satisfactorily, because then the adsorption zones tend to become diffuse. Should the necessity arise to demonstrate very small amounts of one purine or pyrimidine in the presence of relatively large quantities of the others, it would, therefore, be advantageous to resort to a preliminary fractionation of the bases by different means before the fractions are subjected to the separation procedures discussed here.

Attention may be directed to a comparison of the efficiency of different solvent systems in effecting separation, as illustrated in Fig. 1. This may be of interest, since the choice of solvents will necessarily be governed by the type of separation that is to be performed.

EXPERIMENTAL

Material

Adenine was a synthetic preparation (14), obtained through the courtesy of Dr. M. Hoffer of Hoffmann-La Roche, Inc., Nutley, New Jersey.

Guanine (Eastman Kodak Company) was three times recrystallized from HCl as the hydrochloride and then regenerated.

Hypoxanthine was a synthetic preparation.[1] *Xanthine* (Eastman Kodak Company) was twice recrystallized from water.

Uracil and *thymine* (Schwarz Laboratories, Inc., New York) were twice recrystallized from water.

[1] We are grateful to Dr. J. A. Aeschlimann, Hoffmann-La Roche, Inc., Nutley, New Jersey, for this specimen.

Cytosine was prepared from uracil by the method of Hilbert and Johnson (15).[2] It was twice recrystallized from water.

All substances used gave satisfactory elementary analyses.

The solvents employed were commercial preparations. *Morpholine* (Carbide and Carbon Chemicals Corporation) was rectified, b.p. 138°. *Quinoline* and *collidine* (crude), both supplied by the Koppers Company, Inc., were distilled.

The *filter paper* used for chromatography was Schleicher and Schüll, No. 597.

Selection of Solvent Systems for Separation

In Table I, the positions on the paper chromatogram of the several purines and pyrimidines, examined in a variety of solvent mixtures, are indicated. This is done by listing the R_F values (12), *i.e.* the proportion of the distances of the starting point from the adsorbate and from the solvent front. These values were determined at room temperature (*i.e.* at about 22°) with solutions containing only one component and were verified with mixtures.

The choice of solvent will, of course, vary with the particular problem, as can be gathered from Fig. 1, which illustrates graphically the positions of the four purines and three pyrimidines following the separation of the mixture in various solvents. Adenine and guanine may be separated from each other in all solvent systems examined, with the exception of collidine (Experiments 6 and 7) and collidine-quinoline (Experiments 8 and 9). Xanthine, on the other hand, is best demonstrated in neutral solution in collidine-quinoline (Experiment 9). For hypoxanthine collidine-quinoline (in neutral solution), butanol, or butanol-diethylene glycol can be used. The separation, for qualitative purposes, of the four purines may be performed under the conditions expressed in Experiments 5 and 11 of Table I.

Almost all solvents examined may serve for the separation of the pyrimidines from each other. In the presence of purines, cytosine can be demonstrated in collidine or collidine-quinoline, uracil and thymine in butanol-HCl, both with and without the admixture of diethylene glycol. It may be mentioned that isocytosine was found to have an R_F value in butanol very similar to that of uracil (Table I, Experiment 1).

Separation and Quantitative Estimation of Adenine and Guanine

Solutions—Because of the scanty solubility of guanine at neutrality, solutions of pH 0.8 to 1.0, usually in 0.1 N sulfuric acid, were used for the separation. Their concentration was 0.1 to 0.3 per cent with respect to

[2] We are indebted to Dr. F. Misani for the synthesis of this substance.

each component.   As a rule, 10 to 30 γ of each purine were contained in 0.01 to 0.02 cc., which was the volume serving for the individual separations. For comparative purposes, it was found important to employ similar volumes of solutions of the same degree of acidity.

TABLE I

Separation of Purines and Pyrimidines in Different Solvent Systems

Experi-ment No.	Mixture for sep-aration*	Solvent system†	R_F values						
			Adenine	Guanine	Hypo-xan-thine	Xan-thine	Uracil	Cyto-sine	Thy-mine
1	n	n-Butanol (saturated with water)	0.28	0.074	0.17	0.071	0.32	0.19	0.47
2	a	n-Butanol (3), mor-pholine (1), water (4)	0.53	0.29	0.35	0.13	0.56	0.47	0.71
3	n		0.53	0.29	0.35	0.14	0.55	0.46	0.73
4	a	n-Butanol (4.5), mor-pholine (1.5), di-ethylene glycol (1), water (2)	0.60	0.40	0.48	0.35	0.66	0.54	0.82
5	n		0.72	0.46	0.58	0.41	0.66	0.61	0.78
6	a	Collidine (saturated with water)	0.67	0.57	0.68	0.70	0.80	0.47	0.85
7	n		0.62	0.56	0.63	0.63	0.77	0.42	0.84
8	a	Collidine (1), quino-line (2) (mixture saturated with 1.5 parts water)	0.55	0.41	0.58	0.63	0.73	0.17	0.80
9	n		0.34	0.22	0.44	0.62	0.74	0.21	0.84
10	n	n-Butanol (4), di-ethylene glycol (1), water (1)	0.53	0.27	0.42	0.27	0.60	0.45	0.77
11	n		0.52	0.23	0.31	0.15	0.50	0.50	0.70
12	a	n-Butanol (4), di-ethylene glycol (1), 0.1 N HCl (1)	0.20	0.08	0.29	0.31	0.60	0.34	0.74
13	a	n-Butanol (saturated with 0.1 N HCl)	0.032	0.006	0.054	0.09	0.30	0.035	0.44

* a = acidic (test mixture of purines and pyrimidines dissolved in 0.1 N H_2SO_4); n = neutral (mixture neutralized on paper with gaseous NH_3 before chromatography).   In Experiment 11 the separation was carried out in an NH_3 atmosphere.
† The figures in parentheses indicate volume proportions.

Separation—Paper sheets, 15 cm. wide and 50 cm. long, were divided, by ruling, into five 2.7 cm. wide longitudinal lanes.   A transverse line, about 8 cm. below the top of the sheet, indicated the starting points at which, in the centers of four of the lanes, known volumes of the solution were deposited.   The solutions were dispensed by means of a micro burette allowing the measurement of 0.01 cc. with an accuracy of ±1 per cent.

A micrometric burette of the Scholander type (16) or a "Gilmont ultra-microburet" (Emil Greiner Company, New York) was used. The fifth lane was left free.

Two solvent mixtures were employed in the quantitative determinations. One consisted of 4.5 parts (by volume) of n-butanol, 2 parts of water, 1.5 part of morpholine, and 1 part of diethylene glycol. The other mixture contained 4 parts of n-butanol and 1 part each of diethylene glycol and of water. When the latter solvent system was employed, the acidic purine solution was, after deposition on the paper, neutralized with gaseous ammonia[3] and the separation carried out in an ammonia atmosphere. The R_F values found with these solvent systems are included in Table I as Experiments 4 and 11. The experiments were carried out at room temperature in closed cylindrical Pyrex glass jars, 46 cm. high with an inside diameter of 21 cm. Two paper sheets were used simultaneously, their upper rims dipping, by means of suitable supports, into a trough filled with the solvent mixture. A beaker containing the same solvent was placed at the bottom of the jar. A second vessel contained N ammonia, if a NH_3 atmosphere was to be employed. The separation was terminated when the solvent front had almost reached the lower rim of the sheets, which ordinarily required about 20 hours.

Development—The paper sheets were first dried in air. The center column was then cut out, dried in an oven at 105° for 20 minutes, and sprayed with a 0.25 M solution of mercuric nitrate in 0.5 N nitric acid. The purines, thus fixed on the paper as Hg complexes, were made visible in the following manner. The sprayed paper strip was placed in a bath of 0.5 N nitric acid through which a slow stream of water was permitted to flow. The washing was considered as terminated when small paper strips, which, serving as controls, had also been sprayed with $Hg(NO_3)_2$ and put into the same bath, failed to blacken on treatment with ammonium sulfide. The chromatography strip was then passed through a solution of ammonium sulfide. Well defined black spots of mercuric sulfide indicated the position of the separated purines. As little as 5 γ could be demonstrated in this manner.

Extraction[4]—With butanol-morpholine-diethylene glycol-water as the

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[3] The purine samples that are neutralized on the paper before chromatographic separation must not contain more than 10 γ of guanine. Larger amounts of this difficultly soluble purine cannot, under neutral conditions, be recovered quantitatively, if contained in only 0.01 cc. of purine solution. For the recovery of larger amounts of guanine (compare Experiment 4 in Table III), it was found advisable to deposit two 0.01 cc. portions of solution side by side, in which case lanes 3.5 cm. wide were employed for the separation.

[4] The optical contamination of papers and extracts, for instance by contact with vapors of substances absorbing in the ultraviolet, must be avoided.

solvent, the four remaining untreated lanes were cut apart, kept for 15 minutes in an atmosphere of ammonia, in order to neutralize traces of acid, and dried in an oven at 105° until no more visible vapors were given off. With the developed center strip serving as a guide, small rectangles (usually 5 to 6 cm. long) were removed from the untreated strips at the positions of purine adsorption. Each rectangle was placed in a small test-tube (13 × 100 mm.) and kept anew in an NH₃ atmosphere for 15 minutes. The tubes were put in a water bath, maintained at 80°, and 1 cc. of absolute ether was introduced, in three portions, into each tube, in order to remove by volatilization the last traces of morpholine. After the ether had evaporated completely, exactly 4 cc. of 0.1 N hydrochloric acid were added to each tube containing adenine and 4 cc. of N hydrochloric acid to the tubes containing guanine, and the closed vessels were kept overnight at 37°. The extracts then were well mixed, cooled to room temperature, and centrifuged immediately before spectroscopy.

The use of butanol-diethylene glycol-water as the solvent rendered the extraction much simpler. The paper strips were dried in air for 3 to 4 hours and the paper segments, corresponding to the position of the separated purines, directly extracted, without further treatment, with 4 cc. portions of 0.1 N HCl (for adenine) or N HCl (for guanine).

Ultraviolet Spectroscopy—The absorption in the ultraviolet of the extracts was read, in 1 cm. quartz cells, in a Beckman photoelectric quartz spectrophotometer. The HCl extract of the corresponding paper rectangles removed from the fifth lane that had been left free, as explained above, served as the blank. Acidic extracts of filter paper themselves exhibit a low, but neither constant nor regular, absorption in the ultraviolet. For this reason, it was preferable, rather than taking the absolute extinction values at the absorption maxima (adenine at 262.5 mμ, guanine at 249 mμ) as the bases of calculation, to estimate the purine contents of the extracts from the difference in the extinction values read at the absorption maximum and at 290 mμ. For 0.001 per cent test solutions in 0.1 N HCl, *i.e.* for 10 γ of purine per cc., the difference Δ was determined as follows.

Adenine, $E_{262.5}$ 0.930; E_{290} 0.030; $\Delta = 0.900$
Guanine, E_{249} 0.737; E_{290} 0.262; $\Delta = 0.475$

In order to verify the position of the maximum, the ultraviolet absorption of the extracts was invariably also determined at 5 mμ above and below the characteristic absorption maximum of the purine in question, *i.e.* at 267.5 and 257.5 mμ for adenine, at 254 and 244 mμ for guanine. In addition, the extinction of the extracts also was measured at 300 mμ, at which wave-length the purines absorb very little light. The extinction values

found at 300 mμ should, therefore, be very low, usually between −0.010 and +0.040. Readings outside this range were indicative of contamination, and such extracts were discarded.

The recovery of adenine with butanol-morpholine-diethylene glycol-water as the solvent system and the readings recorded in a series of such determinations are exemplified in Table II.

The results of a typical separation of adenine and guanine by means of butanol-diethylene glycol-water are presented here as an example. A mixture of 13.6 γ of adenine and 10.08 γ of guanine was subjected to

TABLE II

*Recovery of Adenine with Butanol-Morpholine-Diethylene Glycol-Water As Solvent**

Experiment No.	Adenine subjected to chromatography	Extinction at wave-length					Δ_x	$\dfrac{10\Delta_x}{\Delta}$	Adenine recovered	
		300 mμ	290 mμ	267.5 mμ	262.5 mμ	257.5 mμ				
	γ							γ	γ	*per cent*
1	19.64	0.023	0.039	0.429	0.458	0.439	0.419	4.66	18.6	95
2	19.47	0.008	0.024	0.418	0.448	0.428	0.424	4.71	18.8	97
3	19.96	0.007	0.023	0.412	0.442	0.428	0.419	4.66	18.6	93
4	19.47	0.013	0.026	0.408	0.428	0.411	0.402	4.47	17.9	92
5	19.64	0.019	0.036	0.434	0.460	0.440	0.424	4.71	18.8	96
6	19.64	0.016	0.031	0.429	0.455	0.438	0.424	4.71	18.8	96

* Δ_x is the difference in the extinction of the unknown at 262.5 and at 290 mμ, Δ the same difference for a standard solution containing 10 γ of adenine per cc. The expression $10\Delta_x/\Delta$ corresponds to the adenine concentration in 1 cc. of the unknown and, therefore, to one-fourth of the total recovered adenine.

separation. The following extinction values were recorded at different wave-lengths.

Adenine		Guanine	
300 mμ	0.005	300 mμ	0.005
290 "	0.017	290 "	0.047
267.5 mμ	0.293	254 "	0.150
262.5 "	0.311	249 "	0.164
257.5 "	0.300	244 "	0.156

The computations which follow were based on the proportion between the Δ_x values found for the unknown and the Δ values determined, as explained above, with purine solutions containing 10 γ per cc.

Adenine, $\Delta_x = 0.311 - 0.017 = 0.294$; recovered in 1 cc. (10 $\Delta_x/\Delta = 2.94/0.900$), 3.27 γ; total recovered in 4 cc., 13.1 γ; recovery, 96 per cent.

Guanine, $\Delta_x = 0.164 - 0.047 = 0.117$; recovered in 1 cc. (10 $\Delta_x/\Delta = 1.17/0.475$), 2.46 γ; total recovered in 4 cc., 9.84 γ; recovery, 98 per cent.

The results of several similar separation experiments are listed in Table III.

Separation and Identification of Adenine, Guanine, Hypoxanthine, and Xanthine

It will suffice to mention only those points in which the procedures differed from the quantitative method described above. The solutions employed (in 0.1 N H_2SO_4) were 0.1 to 0.3 per cent with respect to each of the four purines. The solvent systems used for the separation are listed in Table I.

The development of the guide strips was in all experiments carried out as described above for the quantitative estimations, with the exception of Experiments 8 and 9 (Table I) in which the paper strips were briefly washed

TABLE III

Separation of Mixtures of Adenine and Guanine by Means of n-Butanol-Diethylene Glycol-Water in NH_3 Atmosphere

Experiment No.	Adenine			Guanine		
	Amount in mixture	Amount recovered		Amount in mixture	Amount recovered	
	γ	γ	per cent	γ	γ	per cent
1	13.6	13.8	101	10.1	10.1	100
2	13.6	12.9	95	10.1	10.4	103
3	13.6	12.9	95	10.1	9.9	98
4	27.2	25.4	93	20.1	19.0	95

with ether before being sprayed with mercuric nitrate, since quinoline interfered with the development.

For the extraction of the adsorbates, the strips were, in Experiments 1 and 10 to 13 (Table I), dried in air for 4 hours, and then divided into segments and extracted as described above. In Experiments 2 to 5 the procedures employed for the quantitative estimation with butanol-morpholine-diethylene glycol as the solvent system were followed.

In Experiments 6 to 9 (Table I), *i.e.* with collidine or collidine-quinoline,[5] it was necessary to remove the last traces of these solvents which absorb strongly in the ultraviolet. This was done by steam distillation. The paper segment was placed in a test-tube and wetted with 2 N sodium carbonate, in order to liberate the solvent. Two 0.5 cc. portions of water were then permitted to evaporate from the tube in a bath of 110°. The

[5] The use of a quartz lamp made it possible to ascertain the extent to which the strongly fluorescent quinoline sulfate had spread during the chromatography, since the separation was more complete when all purines had migrated below the acid zone.

subsequent extraction with 0.1 N hydrochloric acid was carried out in the same tubes.

In many of the separation experiments the complete absorption curves of the HCl extracts were determined, in order to check the purity of the extracted bases.

Separation and Quantitative Estimation of Uracil, Cytosine, and Thymine

Solutions—Aqueous solutions of the pyrimidines served for the separation experiments. The volumes deposited on the paper were 0.01 to 0.02 cc., containing 10 to 30 γ of each component.

Separation—*n*-Butanol, saturated with water, was employed for the separation, which required about 12 hours. Otherwise, the procedures were identical with those followed in the quantitative purine separation. The R_F values of the separated pyrimidines in several solvent mixtures are included in Table I.

Development—The paper sheet was spread out and dried in air and the center column cut out and dried in an oven at 105° for 20 minutes. It then was placed for about 30 seconds in a buffered 0.01 M mercuric acetate solution of pH 6.2. This solution was freshly prepared by mixing 1 part of 0.1 M mercuric acetate solution with 3 parts of M sodium acetate solution and 6 parts of water. The strip, after having been bathed for exactly 20 seconds in slowly renewed water, was passed through an ammonium sulfide solution. Compact spots of mercuric sulfide denoted the positions of the separated pyrimidines. The identification limits were about 5 γ for uracil and cytosine, 10 γ for thymine.

Extraction—The paper strips were dried in air for 4 hours and the 3.5 to 5 cm. long paper rectangles, cut out with the guidance of the developed strip, were each extracted with exactly 4 cc. of water in closed tubes that were kept overnight at 37°. The well mixed extracts were centrifuged before spectroscopy.

Ultraviolet Spectroscopy—The principles discussed above with respect to the estimation of the purines apply here too. The concentrations of cytosine and thymine were determined, as for the purines, from the difference in the extinction values found at the respective absorption maxima and at 290 mμ. For uracil the difference between the absorption maximum and the reading obtained at 280 mμ was used. The absorption maxima recorded for the preparations were uracil 259, cytosine 267.5, and thymine 264.5 mμ. The following values for the difference Δ were found with 0.001 per cent solutions of the pyrimidines in distilled water.

Uracil, E_{259} 0.738; E_{280} 0.148; $\Delta = 0.590$
Cytosine, $E_{267.5}$ 0.598; E_{290} 0.053; $\Delta = 0.545$
Thymine, $E_{264.5}$ 0.626; E_{290} 0.081; $\Delta = 0.545$

As was explained before with regard to the purines, additional absorption readings at 5 mμ above and below the respective maxima and also at 300 mμ served to ascertain the purity of the extracts. The readings recorded in a typical separation of uracil, cytosine, and thymine are exemplified below. Table IV summarizes the results of five separation experiments.

TABLE IV

Separation of Mixtures of Uracil, Cytosine, and Thymine

Experiment No.	Uracil			Cytosine			Thymine		
	Amount in mixture	Amount recovered		Amount in mixture	Amount recovered		Amount in mixture	Amount recovered	
	γ	γ	*per cent*	γ	γ	*per cent*	γ	γ	*per cent*
1	24.3	23.1	95	24.8	23.9	96	24.8	24.5	99
2	25.0	23.7	95	25.0	24.5	98	25.0	23.6	95
3	24.5	24.5	100	24.3	24.6	101	24.5	23.1	94
4	11.9	11.9	100	10.7	10.5	98	11.9	11.9	100
5	11.8	11.4	97	10.5	9.9	94	11.8	11.2	95

TABLE V

Extinction Values for Eluates of Separated Pyrimidines

Uracil		Cytosine		Thymine	
Wave-length	E	Wave-length	E	Wave-length	E
mμ		*mμ*		*mμ*	
300	0.004	300	0.003	300	−0.003
280	0.046	290	0.014	290	0.016
264	0.210	272.5	0.150	269.5	0.172
259	0.222	267.5	0.157	264.5	0.178
254	0.212	262.5	0.153	259.5	0.174

A mixture containing 11.9 γ of uracil, 10.7 γ of cytosine, and 11.9 γ of thymine was subjected to a chromatographic separation. The readings of the extinction values recorded for the eluates (4 cc.) of the separated pyrimidines are shown in Table V.

The computations, based on the proportion between the Δ_x values found for the unknown and the Δ values found for pyrimidine solutions containing 10 γ per cc., follow here.

Uracil, $\Delta_x = 0.222 - 0.046 = 0.176$; recovered in 1 cc. (10 $\Delta_x/\Delta = 1.76/0.590$), 2.98 γ; total recovered in 4 cc., 11.9 γ; recovery, 100 per cent.

Cytosine, $\Delta_x = 0.157 - 0.014 = 0.143$; recovered in 1 cc. (10 $\Delta_x/\Delta = 1.43/0.545$), 2.62 γ; total recovered in 4 cc., 10.5 γ; recovery, 98 per cent.

Thymine, $\Delta_x = 0.178 - 0.016 = 0.162$; recovered in 1 cc. (10 $\Delta_x/\Delta = 1.62/0.545$), 2.97 γ; total recovered in 4 cc., 11.9 γ; recovery, 100 per cent.

SUMMARY

Mixtures containing minute amounts of purines (adenine, guanine, hypoxanthine, xanthine) and pyrimidines (uracil, cytosine, thymine) were separated in a variety of solvent systems. The method developed for this purpose, which makes use of paper chromatography, permits not only the demonstration of the individual components by their conversion into mercury salts, but also their identification and quantitative estimation by means of ultraviolet spectroscopy. Amounts ranging from 5 to 40 γ of adenine, guanine, uracil, cytosine, and thymine thus were separated and determined quantitatively.

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