## 288. *The Nucleic Acids of Normal and Tumour Tissues. The Preparation and Composition of a Pentosenucleic Acid from the Fowl Sarcoma G.R.C.H.* 15.

By R. N. Beale, R. J. C. Harris,* and E. M. F. Roe.

A pentosenucleic acid has been prepared from acetone-dried G.R.C.H. 15 fowl sarcoma tissue. The component pentose-nucleosides have been isolated and separated quantitatively by partition chromatography on a starch column. Adenine-, guanine-, cytosine-, and uracil-pentosides alone have been demonstrated and physicochemical evidence suggests that they are identical with those obtained from yeast ribosenucleic acid. The nucleic acid shows a wide divergence in composition from a " tetranucleotide " structure, and the molar ratio of purine- : pyrimidine-nucleosides is 4·83 : 3·23.

It is important that information should be gained about the structure of nucleic acids from different plant and animal sources. However, the analytical determination of components (*e.g.*, the ratio, purine : pyrimidine bases) gives limited information about the structure of a nucleic acid as it exists, in combination with proteins, in the cell (Brachet, *Growth Symposium*, 1947, **11**, 309; *Pubb. Staz. Zool. Napoli*, 1949, **21**, 77; Chantrenne, *Biochim. Biophys. Acta*, 1947, **1**, 437). Uncertainty arises because of the difficulty in assessing the extent of degradation which may occur during isolation.

Extraction of nucleic acid from tissues with alkali gives chemically degraded products. On the other hand, chemical procedures mild enough to be non-degradative *per se* provide optimum conditions for enzymic degradation. It might be expected, therefore, that pentose-nucleic acids isolated after a more or less prolonged chemical treatment, even under the mildest conditions, consist of fragments of the " native " nucleic acid of varying molecular weights and degrees of enzymic degradation.

It is well known, for example, that the ribonuclease-resistant fraction of yeast pentose-nucleic acid contains a higher proportion of purine bases than does the original acid. Moreover, there is, as yet, no evidence to justify an assumption that the nucleic acids derivable from different loci within the cell (*i.e.*, mitochondrial, microsomal, and nucleolar pentose-poly-nucleotides) are of identical structure. Pentosenucleic acids isolated by chemical methods and investigated from the point of view of the component bases, nucleosides, or nucleotides can therefore give little absolute information on the structure of " native " nucleic acids and the relationship of structure to possible specificity (both intracellular and inter-species). In the latter connection, the question of the specificity of the nucleic acids of malignant tissues needs to be examined. We propose to analyse the pentosenucleic acids of nucleoprotein–lipoprotein particles which may be isolated (morphologically intact) by physicochemical methods from filterable and non-filterable fowl tumours and from normal, non-malignant fowl and chick tissues. This, the first investigation, describes methods which may be used for the analysis of such pentosenucleic acids. The material investigated by us has been isolated by chemical extraction.

* Laura de Saliceto Student, University of London.

The requirements for the analysis of most nucleic acids from the point of view of total composition are, first, that the methods used should be applicable to very small quantities, and, secondly, that the recoveries obtained should be quantitative.

Purine and pyrimidine bases have been separated by filter-paper partition chromatography (Vischer and Chargaff, *J. Biol. Chem.*, 1947, **168**, 781; *Fed. Proc.*, 1948, **7**, 197; *J. Biol. Chem.*, 1948, **176**, 703, 715; Chargaff, Vischer, Donniger, Green, and Misani, *ibid.*, 1949, **177**, 405; Vischer, Zamenhof, and Chargaff, *ibid.*, p. 429; Hotchkiss, *ibid.*, 1948, **175**, 315; *Colloques Int. Centre Nat. Recherche Sci.*, 1948, **8**, 57; Holiday and Johnson, *Nature*, 1949, **163**, 216; Markham and Smith, *ibid.*, p. 250; *Biochem. J.*, 1949, **45**, 298). Pentose nucleosides have also been separated under similar conditions (Hotchkiss, *J. Biol. Chem.*, 1948, **175**, 315), as have yeast pentose nucleotides (Vischer, Magasanik, and Chargaff, *Fed. Proc.*, 1949, **8**, 263; Carter and Cohn, *ibid.*, p. 190).

Ion-exchange resins have been used for the chromatographic separation of pentose-nucleosides (Harris and Thomas, *Nature*, 1948, **161**, 931; *J.*, 1948, 1936) and of purines, pyrimidines, and nucleotides (Cohn, *Science*, 1949, **109**, 377; *J. Amer. Chem. Soc.*, 1949, **71**, 2275; Carter and Cohn, *loc. cit.*; Smith and Wender, *J. Amer. Chem. Soc.*, 1948, **70**, 3719).

Hammarsten and his co-workers have preferred a partition column of starch similar to that employed for the separation of amino-acids by Stein and Moore (*J. Biol. Chem.*, 1948, **176**, 337), and separations of bases (Reichard, *ibid.*, 1949, **179**, 773; *Acta Chem. Scand.*, 1949, **3**, 422; Edman, Hammarsten, Löw, and Reichard, *J. Biol. Chem.*, 1949, **178**, 395) and of nucleosides (Reichard, *Nature*, 1948, **162**, 662; *J. Biol. Chem.*, 1949, **179**, 763) have been achieved. Daly and Mirsky (*ibid.*, p. 981) have also separated purine and pyrimidine bases by this method but, as in a considerable number of the other papers cited, have confined their attention solely to model systems comprising mixtures of pure components.

It is clearly an advantage to be able to separate and identify the largest possible fragments of a nucleic acid. Oligonucleotides are not yet characterised and the recent work of Cohn *et al.* (*loc. cit.*) has cast doubt on the structural characterisation of the pentose-nucleotides which have been prepared from yeast pentosenucleic acid.

The use of paper chromatography for the separation of purines and pyrimidines (the usual procedure) has several limitations. Vischer and Chargaff (*J. Biol. Chem.*, 1948, **176**, 703) claimed that optimum separations may be obtained with 20 μg. of the bases (with an estimated accuracy of recovery of $\pm 6\%$). However, in the application of the procedures to other than model mixtures of pure components, difficulties were encountered in the quantitative hydrolysis of the nucleic acid, in the separation of the purines from the pyrimidines (an "upward correction" of 5% was applied to the pyrimidines recovered), in the tendency of cytosine to be deaminated to uracil during acid hydrolysis (see Loring and Ploeser, *ibid.*, 1949, **178**, 439), and in the evaluation of the "background correction" in the spectrophotometric determination of the bases, especially when the chromatogram had been run with solvents (such as pyridine and quinoline) which show a high ultra-violet absorption in the same region as the purine–pyrimidine bases. Moreover, as Markham and Smith (*Nature*, 1949, **166**, 1052) have again confirmed, there remains the possibility that purine bases themselves may decompose during acid hydrolysis.

It appeared, therefore, that milder methods of hydrolysis should be sought which would give large but still readily identifiable fragments of the nucleic acid and could be used to provide a complete analysis on a single sample of about 10—20 mg. (Chargaff *et al.* used 5—8 mg. for purine and 15—25 mg. for pyrimidine analysis).

In the light of the above arguments and for the reasons stated below, the method of analysis selected by us has been partition chromatography of nucleosides on starch columns. First, a supply of specimen yeast ribosenucleosides was available (Harris and Thomas, *loc. cit.*) for model separations and preliminary spectrophotometric examination; secondly, model experiments, described below, showed that mixtures containing as little as 1 mg. of each nucleoside could be separated and the individual nucleosides quantitatively recovered; and finally, mild, enzymic methods of hydrolysis of the nucleic acid were possible (Schmidt, Cubiles, and Thannhauser, *Cold Spring Harbor Symp. Quant. Biol.*, 1947, **12**, 161; Reichard, *J. Biol. Chem.*, 1949, **179**, 763).

*Preparation of a Pentosenucleic Acid from Fowl Sarcoma, G.R.C.H.*15.*—The nucleic acid was prepared from acetone-dried (delipidated) sarcoma tissue by a mild method, avoiding throughout a pH >7, and was purified through the barium salt by the method used by Jorpes (*Biochem. J.*, 1934, **28**, 2102) and by Davidson and Waymouth (*ibid.*, 1944, **38**, 375). The

* A non-filterable fowl sarcoma induced with 1 : 2-5 : 6-dibenzanthracene by Dr. P. R. Peacock (Glasgow Royal Cancer Hospital) in 1939, and since maintained by serial transplantation.

material thus obtained was a protein-free, pentose-containing nucleic acid giving no Dische reaction for deoxypentose (when the test was performed on small quantities). Alkaline hydrolysis by Schmidt and Thannhauser's method (*J. Biol. Chem.*, 1945, **161**, 83) showed that the product contained 94% of pentose-nucleic acid. The ultra-violet absorption spectrum is shown in Fig. 1. The infra-red absorption of this nucleic acid closely resembles that of a protein-free purified specimen of commercial yeast ribose-nucleic acid (Harris, Orr, and Roe, unpublished) and differs from that of a commercial sodium salt of calf-thymus deoxypentose-nucleic acid. Two preparations contained respectively: N (micro-Kjeldahl), 13·9, 13·8; P (microcolorimetric), 7·7, 8·2. The second preparation was used for hydrolysis.

FIG. 1.

*Nucleic acid from G.R.C.H. 15 sarcoma.*

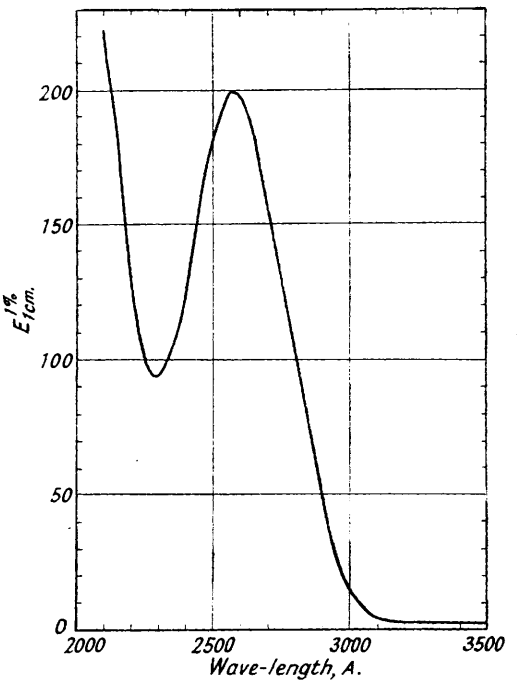
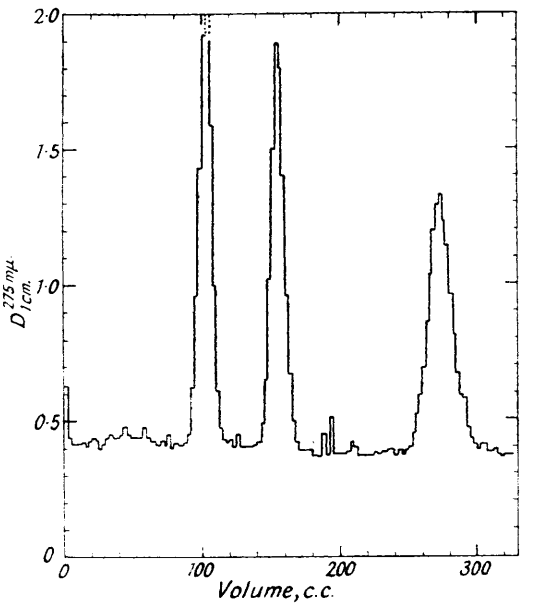



FIG. 2.

*Model separation of pure nucleosides.*




*Separation and Identification of Nucleosides.*—(a) *Model separation of pure nucleosides.* Fig. 2 shows the flow diagram for a mixture containing approximately 1 mg. each of adenosine, uridine, and guanosine. The starch column was prepared substantially by the method of Stein and Moore (*loc. cit.*). The nucleoside mixture was put on as a solution in a small amount of *n*-butanol saturated with water at room temperature (termed " saturated butanol " below). The chromatogram was developed with the same solvent and hourly fractions of eluate were collected continuously with the aid of a simple fraction-collecting machine (Cuckow, Harris, and Speed, *J. Soc. Chem. Ind.*, 1949, **68**, 208). Each fraction was examined in a 1-cm. absorption cell (distilled water as blank) in the Beckman DU spectrophotometer, and the optical density ($D = \log I_0/I$) at 275 mμ. was plotted against the volume of effluent. The fractions containing nucleosides were subsequently pooled, the solvent was removed as carefully as possible by evaporation *in vacuo*, and the nucleoside residue re-dissolved in 0·05N-hydrochloric acid for spectrophotometric identification and quantitative determination (for discussion of this, see below). The nucleosides were identified by means of their absorption spectra (aqueous solutions) and their relative and absolute *R* values (Table I) (Martin and Synge, *Biochem. J.*, 1941, **35**, 1358) where

$$R = \frac{\text{movement of position of max. concn. of solute}}{\text{simultaneous movement of surface of developing solvent in the empty column above the starch}}$$

The numerator in the above expression is equal to the length of the starch column in this

TABLE I.

*Absolute and Relative* R *values.*

| Procedure. | Starch column (cm.). | Flow (c.c./hr.). | Volume (c.c.) at which band maximum occurred : adenosine. | uridine. | guanosine. | cytidine. |
|---|---|---|---|---|---|---|
| Model separation (Fig. 2). | 20 | 2·5±0·2 | 103 | 153 | 272 | — |
| Hydrolysate aliquot from yeast pentose-nucleic acid (Fig. 4). | 19 | 3·6±0·2 | 90 | 135 | 227 | 307 |
| Pyridine hydrolysate of yeast pentose-nucleic acid (Fig. 5). | 19 | 3·2±0·2 | 91·5 | 153 | 303 | 380 |
| G.R.C.H.15 pentosenucleic acid (Fig. 6). | 20 | 3·2±0·2 | 98 | 147 | 225 | 299 |
| Model separation (Reichard, *loc. cit.*). | — | — | — | — | — | — |

| | *R* (absolute). | | | | *R* (relative). | | |
|---|---|---|---|---|---|---|---|
| Procedure. | Adeno-sine. | Urid-ine. | Guanos-ine. | Cytid-ine. | $R_U : R_A$ | $R_G : R_A$ | $R_C : R_A$ |
| Model separation (Fig. 2). | 0·61 | 0·41 | 0·23 | — | 0·67 | 0·38 | — |
| Hydrolysate aliquot from yeast pentose-nucleic acid (Fig. 4). | 0·66 | 0·44 | 0·26 | 0·20 | 0·66 | 0·39 | 0·30 |
| Pyridine hydrolysate of yeast pentose-nucleic acid (Fig. 5). | 0·65 | 0·39 | 0·20 | 0·16 | 0·60 | 0·31 | 0·25 |
| G.R.C.H.15 pentosenucleic acid (Fig. 6). | 0·64 | 0·43 | 0·28 | 0·21 | 0·67 | 0·43 | 0·33 |
| Model separation (Reichard, *loc. cit.*). | 0·75 | 0·48 | 0·29 | 0·21 | 0·64 | 0·39 | 0·28 |

case and the denominator to $V/\pi r^2$, where $V$ is the volume of effluent and $r$ is the radius of the column. Two problems arose in connection with this model separation : (1) the necessity for using a solvent with a low absorption at the wave-length characteristic of purines and pyrimidines; and (2) evaluation of methods for the determination of the nucleosides.

(1) Purification of *n*-butanol. Normally purified *n*-butanol (supplied by Messrs. A. Boake Roberts and Co.) has a high and variable optical density at 260—270 mμ. Various purification procedures were tried in attempts to reduce this absorption, none of which was really satisfactory. Edman *et al.* (*loc. cit.*) reported that their solvent had been refluxed with zinc dust and alkali, followed by two redistillations *in vacuo*. We have since found that shaking *n*-butanol with freshly prepared Raney nickel catalyst produced a solvent of sufficiently low optical density in the region of 260 mμ. (with distilled water as blank). Since these experiments were not completed before the end of the present investigations, a wave-length was chosen such that a " background density " of about 0·4 was obtained on using a 1-cm. or 0·5-cm. absorption cell. Fig. 3 shows the absorption curves for a typical sample of *n*-butanol before and after treatment with Raney nickel, and storage over the catalyst.

(2) Evaluation of methods of spectrophotometric determination. In order to determine quantitatively the recovery of nucleosides after chromatographic separation two questions must be considered : (i) whether the appropriate fractions for one nucleoside should be pooled, evaporated to dryness, re-dissolved in a solvent, and determined by comparison with the absorption spectrum of the pure material in the same solvent; or whether the quantities found in each fraction of one chromatographic band (determined, as before, by comparison of optical densities with a standard absorption spectrum) should be summed, thus avoiding possible loss of material during evaporation; and (ii) by what means the contribution of " background absorption " due to solvent and/or starch may be evaluated.

Reichard (*loc. cit.*), who identified and determined individual nucleosides from evaporated, combined fractions, dissolved each residue in 10 c.c. of 0·1N-hydrochloric acid, and after dilution, measured the absorption between 240 and 310 mμ. The ratio $E_{max.}$ : μg. of N per c.c. was determined for each nucleoside and compared with similar data for the standard compound. No correction was applied for " background absorption " caused by contamination with solvent or starch. This procedure appears to be sufficiently accurate for the comparatively large amounts (3·5—5·0 mg.) of nucleosides used in these model analyses, but it is possible that significant errors would be introduced in the analysis of smaller quantities owing to the increase in the proportion of " background absorption."

Daly and Mirsky (*loc. cit.*) separated purine and pyrimidine bases on columns of starch. Determination was carried out (*a*) by evaporation of combined fractions, dissolution in 0·1N-hydrochloric acid, and comparison of spectra with standards without correction for " background absorption," and (*b*) by summation of the weight of material corresponding to each fraction from one chromatographic band. They indicate that an average fraction before or behind the band should be used as a blank to correct for " background absorption."

Vischer and Chargaff used a different correction in experiments with paper chromatograms of bases. The difference between the optical densities at the relevant maximum and at 290 mμ. was taken as a basis for determination of the amounts of purine and pyrimidine present (dissolved out of a paper segment). This procedure is an attempt to eliminate a variable " background absorption " arising from contamination. It assumes, however, that this is constant in value from 290 mμ. throughout lower wave-lengths and, although a blank extract of paper was used in the comparison cell, this procedure is certainly not valid for our study of " background absorption " from starch columns.

FIG. 3.

*n-Butanol.*

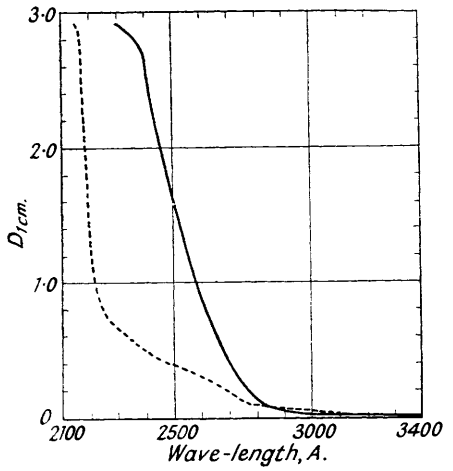
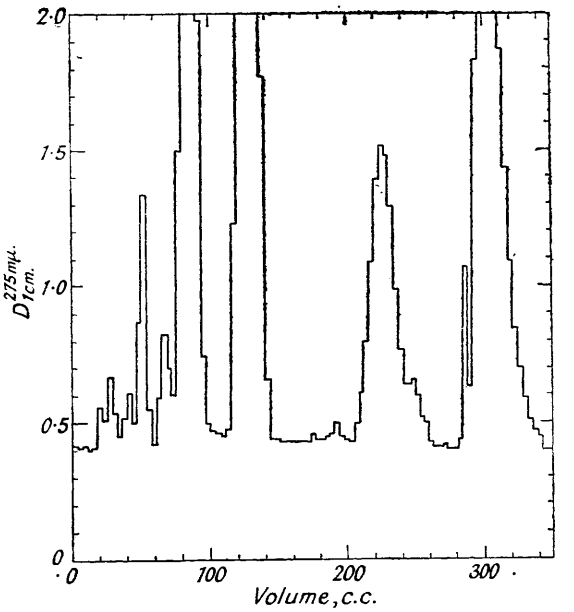



FIG. 4.

*Hydrolysate aliquot from yeast pentose-nucleic acid.*




In the following work, three methods have been used for determination of the nucleosides.

I. The combined fractions from one band were evaporated to dryness *in vacuo* and then dissolved in 5 or 10 c.c. of a suitable solvent (see below), and the maximum optical density, $D_{max.}$, of the solution was compared with that of a standard solution. No correction was applied for " background absorption."

II. In order to avoid possible losses during evaporation, calculations were made employing only the data from the flow diagram. From Figs. 4, 5, and 6 it may be seen that the " background absorption " before and behind a band is almost constant. For any particular fraction, therefore, the amount of nucleoside present may be calculated from the equation :

$$\text{Amount of nucleoside (mg.)} = MV(D - D_b)/\varepsilon l$$

where $M$ = molecular weight of the nucleoside, $D$ = observed optical density of the fraction, $D_b$ = average of optical density of fractions before and behind the band, $V$ = volume of fraction in c.c., $l$ = cell length in cm., and $\varepsilon$ = molecular extinction coefficient for the nucleoside.

These amounts may then be summed over the whole band. For the purposes of this calculation it is necessary to assume that the absorption spectrum of a nucleoside in saturated butanol is the same as that in water. This has been found to be true for adenosine, within 2% in the region of the maximum.

III. The combined fractions from one band were evaporated to dryness *in vacuo*, dissolved in 0·05N-hydrochloric acid, suitably diluted with the same solvent, and examined spectrophotometrically. " Background absorption " was determined from similarly treated effluent at the end of the chromatogram (fractions A–B, Fig. 6), and its spectrum is shown in Fig. 7. Interband fractions were also examined and found to contain very small amounts of nucleosides. These were also observed in minute amounts in fractions A–B. An interpolated correction

FIG. 5.

*Pyridine hydrolysate of yeast pentosenucleic acid.*

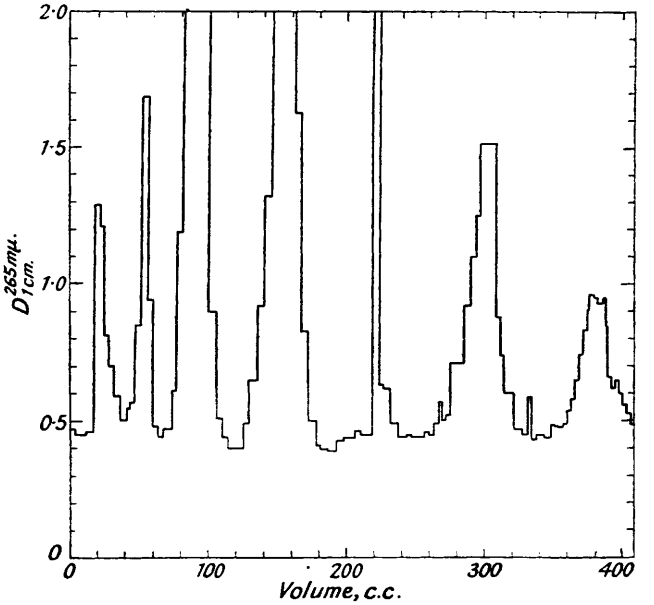
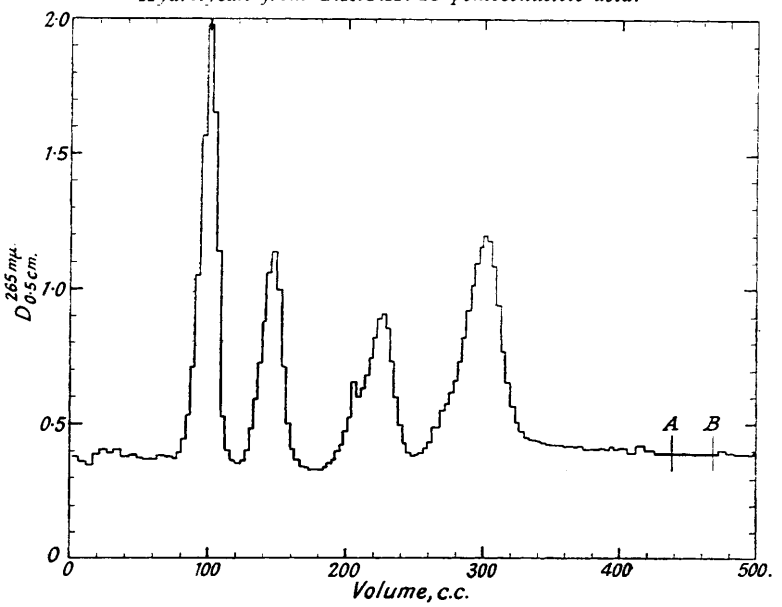



FIG. 6.

*Hydrolysate from G.R.C.H. 15 pentosenucleic acid.*




curve was therefore employed (cf. Fig. 7). This correction curve was used to eliminate " background absorption " in the following way :

Let $e_1$ = extinction coefficient of recovered material at $\lambda_1$ (measured as $E^{x}_{1\,cm.}$, $x$ being mg. per 100 c.c.),

$e_2$ = do. at $\lambda_2$,

$E_1$ = extinction coefficient of pure material at $\lambda_1$ (measured as $E^{x}_{1\,cm.}$, $x$ being as above),

$E_2$ = do. at $\lambda_2$,

$\rho$ = ratio of " background absorption " from correction curve at $\lambda_1$ and $\lambda_2$.

From Fig. 8, $e_1 = AC$; $e_2 = DF$;
$E_1 = AB$; $E_2 = DE$;

Now $BC/EF = (AC - AB)/(DF - DE) = (e_1 - xE_1)/(e_2 - xE_2) = \rho$.

Thus $x = (e_1 - \rho e_2)/(E_1 - \rho E_2)$ mg.

In the application of this equation, any two wave-lengths may be used but it is preferable to work near the maximum and minimum of the nucleoside curve, so that $E_1 \gg E_2$.

In the model separations of pure nucleosides, methods I and III were used for the determination of recovery. The results are given in Table III. Table II shows the standard data employed, which were obtained from purified nucleosides (Harris and Thomas, *loc. cit.*).

FIG. 7.

*"Background absorption" spectrum.*

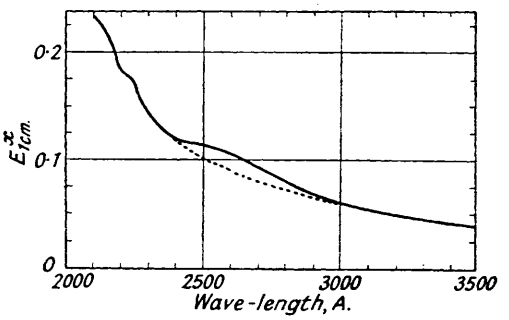



FIG. 8.

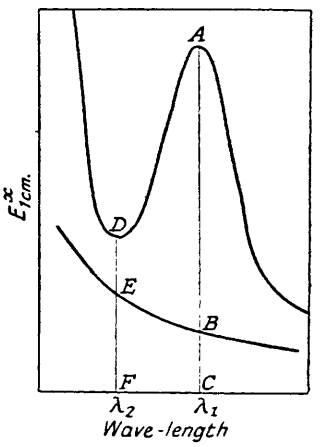



TABLE II.

*Spectrophotometric data for pure nucleosides.*

| | 0·05N-HCl. | | | | 0·05N-NaOH. | | | |
|---|---|---|---|---|---|---|---|---|
| | Max., | | Min., | | Max., | | Min., | |
| | λ (mμ.). | ε. | λ (mμ.). | ε. | λ (mμ.). | ε. | λ (mμ.). | ε. |
| Adenosine ... | 257 | 14,240 | 230 | 3360 | 259·5—260 | 14,550 | 229 | 2965 |
| Guanosine ... | 256 | 12,870 | 227·5 | 2720 | 266 | 11,480 | 231—231·5 | 4370 |
| Uridine ..... | 262 | 10,170 | 230—230·5 | 2110 | 262 | 7,330 | 242 | 5300 |
| Cytidine ..... | 279·5 | 13,210 | 240·5 | 1550 | 271·5 | 9,040 | 250 | 6300 |

In this table ε is the molar extinction coefficient given by

$\varepsilon = D/cl$, where $D = \log I_0/I$, $c$ = concn. in g.-mol./l., and $l$ = cell-length in cm.

TABLE III.

*Comparison of recoveries of nucleosides in model experiments.*

| Recovery by : | method I (mg.). | method III (mg.). | Theor. (mg.). |
|---|---|---|---|
| Adenosine ..... | 1·20 | 1·12 | 1·05 |
| Guanosine ..... | 0·99 | 0·85 | 1·03 |
| Uridine ..... | 0·95 | 0·91 | 1·00 |

The recoveries from this model separation were considered to be satisfactory and the separation of nucleosides from hydrolysates of yeast ribosenucleic acid was next attempted.

(b) *Separation of the nucleosides derived from yeast ribose nucleic acid by hydrolysis with aqueous pyridine.* The nucleoside mixture was derived from an aliquot of an aqueous-pyridine hydrolysate (Bredereck, Martini, and Richter, *Ber.*, 1941, **74**, 694) of a very large quantity of commercial yeast nucleic acid. Guanosine had largely been removed by crystallisation and free phosphate by precipitation with barium hydroxide. Pyridine was absent. The chromatography was conducted in the usual way and the flow diagram is given in Fig. 4. No quantitative determinations were made, the nucleosides being merely identified by their absolute and relative $R$ values (Table I). Qualitatively the separation was excellent, and a small quantity

(50 mg.) of purified commercial yeast nucleic acid was accordingly hydrolysed by this method and an aliquot (20 mg.) of the *total* hydrolysate prepared and chromatographed. The flow diagram obtained is illustrated in Fig. 5 and the corresponding $R$ values in Table I. The four nucleosides were present as before, together however with other purine- and pyrimidine-containing substances such as adenine [$R = 1{\cdot}10$; Reichard (*loc. cit.*) gives $R = 1{\cdot}19$]. Spectrophotometric determination using method III showed that the recoveries were extremely small (Table IV).

TABLE IV.

*Recoveries of nucleosides from aqueous-pyridine hydrolysate by method* III.

| | Mg. | | Mg. |
|---|---|---|---|
| Adenine | 0·08 | Uridine | 0·64 |
| Adenosine | 0·27 | Cytidine | 0·27 |
| Guanosine | 0·28 | | |

(c) *Preparation of nucleosides from G.R.C.H.*15 *pentosenucleic acid by enzymic hydrolysis.* Nucleotides may be obtained from pentosenucleic acids in almost quantitative yield by alkaline hydrolysis. Schmidt, Cubiles, and Thannhauser (*loc. cit.*) have shown that nucleotides may be quantitatively dephosphorylated to nucleosides by incubation at 37° with an acid phosphatase obtained from hypertrophic human prostate gland. Reichard (*loc. cit.*) used a similarly derived enzyme preparation. The two groups of workers differ considerably in their estimates both of the stability of the enzyme in a crude gland extract and of the time of incubation required for dephosphorylation. Schmidt *et al.* recorded over 90% dephosphorylation of yeast adenylic acid in 3 hours (1 c.c. of 1 : 500 aqueous gland extract to 12 c.c. of 0·1N-sodium acetate buffer, pH 5·3, containing about 4 mg. of the substrate per c.c.). Reichard found about 85% dephosphorylation of a similar substrate in 20 hours (1 c.c. of 1 : 500 aqueous gland extract to 10 c.c. solution, pH 4·1, containing 1 mg. of the substrate per c.c.). In practice, Reichard used a fresh 1 : 100 extract and incubated the mixture for 24 hours at 37°. Model experiments with 1 : 100 extracts of human prostate gland confirmed the results of Schmidt *et al.* in that, at pH 4·5—5·0, yeast adenylic acid could be quantitatively hydrolysed to adenosine in 3 hours. To avoid the difficulty of obtaining fresh prostate tissue in view of the impossibility of storing an aqueous extract, the enzyme solution was prepared each time from an initial extract which had been dried from the frozen state.

Preliminary recovery experiments indicated that it was unwise to attempt to extract the nucleosides from the residue produced by the direct evaporation *in vacuo* of the nucleotide hydrolysate. Such a hydrolysate is acid and hydrolysis of purine nucleosides must occur on concentration. This may explain why Reichard obtained low overall yields of nucleosides from both adenylic and guanylic acids in his model experiments, and, in fact, adenine was obtained in the chromatogram.

It has been found that quantitative recoveries could be obtained if the pH of the hydrolysate was first raised to 7, the mixture was dried from the frozen state, and the nucleosides were extracted from the tractable residue with hot half-saturated butanol. Even under optimum conditions, however, guanosine is extremely difficult to extract and re-extraction of the residue with 0·05N-hydrochloric acid yielded a considerable quantity of guanosine.

Application of these hydrolytic procedures to the analysis of 11·7 mg. of the pentose-nucleic acid from G.R.C.H.15, and chromatography of the nucleoside mixture under the standard conditions, gave the flow diagram shown in Fig. 6. Comparison of the $R$ values with those obtained from mixtures of pure nucleosides, and from hydrolysates of yeast pentose-nucleic acid, are given in Table I. Table V gives the quantitative determinations of the four nucleosides by the three spectrophotometric methods. It may be seen that methods II and III give results in close agreement, whereas method I gives higher values. Since no allowance is made in method I for " background absorption," this is to be expected. The absorption spectra of the various fractions in both 0·05N-hydrochloric acid and 0·05N-sodium hydroxide were compared with the standard data in Table II. The characteristic spectral shifts on change of pH (noted also by Hotchkiss, *loc. cit.*), together with the $R$ values given in Table I, served to identify the four nucleosides.

The total recovery based on the nucleotide nitrogen of the nucleic acid was 85% (see Table VI).

The identity in behaviour, both chromatographically and spectrophotometrically, of the four nucleosides with the four nucleosides of yeast ribosenucleic acid renders it highly probable that these compounds are adenosine, guanosine, cytidine, and uridine.

TABLE V.

*Comparison of recoveries (mg.) of nucleosides from G.R.C.H.15 by methods* I, II, *and* III.

| | Method I. | | Method II. | | Method III. | |
|---|---|---|---|---|---|---|
| Adenosine | — | 0·92 | — | 0·89 | — | 0·83 |
| Guanosine : fraction from chromatogram | 0·93 | — | 0·73 | — | 0·81 | — |
| extracted residue | 2·18 | — | 2·13 | — | 2·13 | — |
| total | — | 3·11 | — | 2·86 | — | 2·94 |
| Uridine | — | 0·73 | — | 0·66 | — | 0·68 |
| Cytidine | — | 1·62 | — | 1·49 | — | 1·52 |
| Total nucleoside recoveries | — | 6·38 | — | 5·90 | — | 5·97 |

TABLE VI.

*Analysis of G.R.C.H.15 pentose-nucleic acid.*

| Nucleoside calc. as | Mg. (method III). | % of sample. | % of hydrolysate (94% of sample). | N, % of hydrolysate-N. | P, % of hydrolysate-P. | $\frac{\text{Pur.-N}}{\text{Pyr.-N}}$ | Molar ratio. |
|---|---|---|---|---|---|---|---|
| Adenosine ... | 0·83 | 7·1 | 7·55 | 14·3 | 10·7 | 62·2 | 1·11 |
| Guanosine *... | 2·94 | 25·1 | 26·7 | 47·9 | 35·8 | | 3·72 |
| Uridine ..... | 0·68 | 5·8 | 6·2 | 5·15 | 9·6 | 22·45 | 1·0 |
| Cytidine ..... | 1·52 | 13·0 | 13·80 | 17·3 | 21·5 | | 2·23 |
| Totals ..... | 5·97 | — | — | 84·65 | 77·6 | 2·8 | — |

* 0·81 Mg. of guanosine was recovered from the chromatogram, and the remainder by further extraction of the hydrolysate residue with 0·05N-hydrochloric acid. This solution had the spectrophotometric and chemical properties of guanosine.

*Discussion.*—At least 85% of the total nitrogen of the sample of tumour nucleic acid could be accounted for as the pentosides of adenine, guanine, cytosine, and uracil. The purine-N : pyrimidine-N ratio thus obtained (2·8) agrees with the value (2·8) found directly on a sample (of almost equal weight) by the method of Kerr and Blish (*J. Biol. Chem.*, 1940, **132**, **147**), namely, hydrolysis of the nucleic acid with N-sulphuric acid and precipitation of the purines with copper hydroxide and sodium hydrogen sulphite. The composition of this nucleic acid, the first tumour nucleic acid to be examined in this way, differs from that of yeast ribosenucleic acid (deduced from paper chromatography of the bases by Chargaff *et al.*), from liver ribonucleic acid (Davidson and Waymouth, *loc. cit.*; evidence—purine : pyrimidine ratio)

TABLE VII.

| | N, %. | P, %. | N : P. | $\frac{\text{Pur.-N}}{\text{Pyr.-N}}$ | Adenine : Guanine : Cytosine : Uracil. |
|---|---|---|---|---|---|
| Yeast pentosenucleic acid ..... | 15·3 | 8·0 | 1·9 | 2·9 | 3·2 : 3·1 : 3·0 : 1·0 |
| Pig-pancreas pentose-nucleic acid ..... | 15·4 | 7·9 | 1·95 | 4·0 | 3·6 : 8·8 : 4·5 : 1·0 |
| Liver ribonucleic acid | 15·0 | 6·7—7·6 | 2·1 | 1·4 | — — — — |
| G.R.C.H.15 pentose-nucleic acid ..... | 13·8 | 8·2 | 1·7 | 2·8 | 1·11 : 3·72 : 2·23 : 1·0 |

and from pig-pancreas pentose-nucleic acid (Chargaff *et al.*, *loc. cit.*—analysed by paper chromatography of the bases). These differences are tabulated in Table VII. The preponderance of guanosine in the tumour nucleic acid may be due to the fact that the material isolated is a nuclease-degraded fraction (as Chargaff has suggested for his pig-pancreas pentose-nucleic acid) but it may also be significant that the nucleic acid isolated by Claude (*Science*, 1939, **90**, 213) from a particulate cell fraction from another fowl sarcoma was stated to have an increased guanine content (see also Shemin, Sproul, and Jobling, *J. Exp. Med.*, 1940, **72**, 697). This is under investigation.

## EXPERIMENTAL.

[With the assistance of J. F. THOMAS.]

*Preparation of G.R.C.H.15 Pentosenucleic Acid.*—Acetone-dried sarcoma tissue (30 g.) was mixed with 0·9% sodium chloride solution (1·5 l.) in a Waring blendor for 1—2 minutes and allowed to be extracted for 3 days at 0°. The suspension was centrifuged and the deposit re-extracted under the same conditions for 24 hours with a further 750 c.c. of sodium chloride solution. The combined supernatant liquids were treated with alcohol (4·5 l.), and the precipitate was collected by centrifugation. The deposit was dissolved in 0·9% aqueous sodium chloride (350 c.c.), the pH adjusted to 7·5 (glass electrode), and crystalline trypsin (25 mg.; containing 50% of magnesium sulphate) added. The flask was " layered "

with toluene and incubated at room temperature for 36 hours. The suspension was filtered (with the aid of "Hyflo Super-cel") and alcohol (2 volumes) added to the clear filtrate. The precipitate was collected as before, and dissolved in water (15 c.c.; final volume, 30 c.c.). The pH was adjusted to 7·2, and 20% barium acetate solution (30 c.c.; pH 6·8) added. The suspension was cooled to 0° and then centrifuged, the supernatant liquor rejected, and the deposit washed with 5% barium acetate solution (3 × 15 c.c.). The final deposit was extracted rapidly with ice-cold N-hydrochloric acid (10 c.c.), centrifuged, and washed with cold, distilled water (4 × 5 c.c.). The washed deposit was re-suspended in water (10 c.c.) and dissolved by the very cautious addition of 0·1N-sodium hydroxide to a final pH of 6·8. Care was taken to ensure that the solution never became alkaline. Glacial acetic acid was then added to bring the pH to 5·5. The solution was kept at 0° overnight, and a slight deposit centrifuged off. The pH was then brought to 1·0—2·0 with N-hydrochloric acid, and the nucleic acid precipitated immediately by the addition of alcohol (2 volumes). The colourless precipitate was collected by centrifugation and dried by being washed successively with 70%, 80%, 95%, and absolute alcohol and, finally, with dry ether. The product was then dried over phosphoric oxide *in vacuo*. Two preparations were made by this method. Preparation A (103 mg.) gave: N (micro-Kjeldahl), 13·9; P, 7·7%. Preparation B, (186·5 mg.), gave: N, 13·8; P, 8·2%. Both preparations were hygroscopic, gave strongly positive Bial reactions for pentose, and were qualitatively free from protein and deoxypentose (Dische reaction).

*Deproteinization by the Sevag procedure.*—Preparation A (49·1 mg.) was dissolved in water (25 c.c.) by addition of 0·1N-sodium hydroxide to pH 7·0. The solution was shaken with successive 5-c.c. portions of 4 : 1 chloroform–amyl alcohol. The layers were separated by centrifugation. After several such treatments, 20·7 mg. of nucleic acid were recovered from the aqueous phase and had the same N : P ratio (1·8) as the starting material.

*Preparation of Starch for Chromatography.*—Commercial potato starch ("Farina," Corn Products Ltd., Strand, W.C.2) was freed from a yellow gummy contaminant by extraction with boiling methanol for 24 hours in a large Soxhlet extractor. The starch was then dried in a desiccator. The product was free-flowing and had a residual water content of about 10%, which must be taken into consideration in the preparation of the column.

*Model Chromatogram with Pure Nucleosides.*—Purified starch (40 g.) was made into a slurry with a mixture of *n*-butanol (70 c.c.) and water (12 c.c.). The slurry was poured into the conventional chromatographic column (25 cm. long × 2·0 cm. in diameter). The column was allowed to pack under gravity and was washed overnight with saturated *n*-butanol. The surplus head of solvent was then removed. Adenosine (1·05 mg.), guanosine (1·03 mg.), and uridine (1·0 mg.) were dissolved separately in the minimum quantity of cold, saturated *n*-butanol, mixed, and added to the column. Slight suction was applied and, when the solution had been almost completely absorbed, the column was washed again with the same solvent and the washing continued for 139 hours. Fractions were collected over hourly intervals with the aid of the automatic fraction collector. The rate of flow was 2·5 ± 0·2 c.c./hr. The recoveries are shown in Table III.

*Chromatogram with an Aliquot of Aqueous-pyridine Hydrolysate.*—The column was made from starch (40 g.) in the usual way. The nucleoside solution was derived from an aliquot of an aqueous-pyridine hydrolysate of a large quantity of yeast ribosenucleic acid by the procedure of Bredereck *et al.* (*loc. cit.*). Guanosine was largely removed by crystallisation and the free phosphate with barium hydroxide. The filtrate was freed from a slight excess of barium with sulphuric acid, and the precipitated barium sulphate was collected by centrifugation and washed once with hot water. The combined supernatant liquors were concentrated by evaporation *in vacuo* and finally dried in a vacuum-desiccator. 20 Mg. were dissolved in 3 c.c. of cold, saturated *n*-butanol and chromatographed in the usual way, 119 hourly fractions being collected. In view of the unknown quantitative constitution of the original hydrolysate, no attempt was made to estimate the nucleosides.

*Chromatogram of Complete Aqueous-pyridine Hydrolysate.*—Deproteinized (Sevag procedure) commercial yeast ribosenucleic acid (50 mg.) was refluxed for 108 hours with 50% aqueous pyridine (3 c.c.). The solution was then diluted with water to 10 c.c. and evaporated *in vacuo* to dryness. The residue was re-suspended in water (10 c.c.) and re-evaporated, and this process was repeated once more. The final residue was dissolved in water (30 c.c.), and saturated barium hydroxide solution was added to pH 9·0. The barium phosphate precipitate was removed by centrifugation and washed twice with cold water. N-Sulphuric acid was added to the combined supernatant liquor and washings to bring the pH to 7·4. The slight precipitate of barium sulphate was centrifuged off and the supernatant liquor evaporated to dryness *in vacuo*. The residue was then dissolved in saturated *n*-butanol (10 c.c.) and an aliquot (4 c.c., equivalent to 20 mg.) was then chromatographed in the usual way. Although qualitatively satisfactory, the separation was useless for quantitative analysis. (The recoveries are given in Table IV.)

*Preparation of Crude Acid Phosphatase from Prostatic Tissue.*—Hypertrophic human prostatic tissue (73 g.), which had been frozen in solid carbon dioxide within ½ hour of removal, was partly thawed, and disintegrated in a Waring blendor with water (250 c.c.). The cold mixture was filtered rapidly through surgical gauze to remove large fragments, and the filtrate rapidly dispensed in 10-c.c. aliquots into McCartney bottles and immediately dried from the frozen state in an Edwards Type 3 centrifugal freeze-drier. The dried contents of each bottle corresponded to 3 g. of wet tissue. The enzyme solution was prepared by shaking 50 mg. of the dry material with 10 c.c. of water until re-suspension had occurred. The mixture was then filtered with the aid of "Hyflo Super-cel," and the filtrate used immediately.

*Model Experiments with Acid Phosphatase from Prostatic Tissue.*—Samples of yeast adenylic acid were weighed into three tubes, A (23·3 mg.), B (25·5 mg.), and C (24·8 mg.). Each was suspended in 9 c.c. of water and brought to pH 5·0 (glass electrode) by the addition of 0·1N-sodium hydroxide (about 0·18 c.c.). The tubes were then equilibrated at 37° (thermostat). To A were added 2·8 c.c. of water and to B and C 1·8 c.c. of water and 1 c.c. of a filtered 1 : 100 extract of *fresh* human prostate. Aliquots of each were removed for determination of inorganic phosphorus after 5, 60, 120, and 180 minutes. The results are given in the table.

| Time (mins.). | P liberated (mg.). A. | B. | C. | Time (mins.). | P liberated (mg.). A. | B. | C. |
|---|---|---|---|---|---|---|---|
| 5 | 0·126 | 1·11 | 1·2 | 120 | 0·375 | 2·28 | 2·31 |
| 60 | 0·420 | 2·22 | 2·21 | 180 | — | 2·34 | 2·31 |

*Extraction of Nucleosides from Enzymic Hydrolysates of Nucleotides.*—Two mixtures of yeast ribosenucleotides were prepared with the composition : (i) adenylic acid 5·7, guanylic acid 4·9, cytidylic acid 5·7, and disodium uridylate 5·4 mg.; and (ii) 5·3, 4·8, 6·3, and 5·6 mg., respectively. Each mixture was dissolved in 5 c.c. of 0·2N-sodium hydroxide, and the pH adjusted to 4·5 by addition of N-hydrochloric acid. 0·5 C.c. of filtered 1 : 100 extract of *fresh* prostate was added and the tubes were incubated at 37° for 3 hours. Aliquots were removed for determination of inorganic phosphate and indicated that 90 and 92·5% respectively of the total phosphorus had been split off. The contents of both tubes were then brought to pH 7 by addition of 0·2N-sodium hydroxide (about 0·15 c.c.). The residual content of each tube [(i) 8 c.c.; (ii) 9 c.c.] was halved and frozen-dried in McCartney bottles. One bottle of each was extracted with 2 × 5 c.c. of saturated *n*-butanol. Separation of an aqueous phase occurred and only 70% of the total nucleoside present was extracted (spectrophotometric determination of an equivalent mixture of the four nucleosides as standard). However, extraction of the material with 2 × 5 c.c. of warm aqueous *n*-butanol (half-saturated with water at room temperature) gave no phase separation and a 90% extraction.

*Hydrolysis and Chromatography of the Pentosenucleic Acid of G.R.C.H.*15.—24·25 Mg. of the pentosenucleic acid of G.R.C.H.15 were dissolved in 5 c.c. of 0·2N-sodium hydroxide and incubated at 37° for 18 hours. The pH was then adjusted to 5·0 with N-hydrochloric acid, 0·5 c.c. of prostate extract (from frozen-dried prostate) was added, and the incubation continued for a further 3 hours. The solution was then cooled to room temperature and brought to pH 6·9 with N-sodium hydroxide. The volume was adjusted to 15 c.c., and a 0·5 c.c. aliquot removed for determination of the liberated inorganic phosphate. 94% of the initial nucleic acid phosphorus was found in this fraction. The residual 14·5 c.c. were divided into two equal portions and frozen-dried. Each half thus contained 11·7 mg. of the original nucleic acid. The contents of one bottle were extracted with 2 × 5 c.c. of hot half-saturated *n*-butanol in the usual manner and the insoluble material was separated by centrifugation. The combined supernatant liquids were cooled to room temperature and carefully saturated by addition of water. This solution was then applied to a washed chromatographic column (40 g. of starch) and the chromatogram developed in the usual way. At the 13th hour the rate of flow was 3·5 c.c./hr. which decreased to 3·2 c.c./hr. at the 33rd hour and was maintained at this rate (±0·2 c.c.) for the duration of the separation (160 hours). The head of solvent over the column was 17·5 ± 1·5 cm. The flow diagram is illustrated in Fig. 6 and the recoveries are given in Table V. In view of the great insolubility of guanosine, the hydrolysate residue was re-extracted with 10 c.c. of 0·05N-hydrochloric acid and the resulting solution, containing pentose, gave ultra-violet absorption results characteristic of guanosine.

*Estimation of Purine Bases in G.R.C.H.*15 *Pentosenucleic Acid.*—The method used was a modification of that developed by Kerr *et al.* (*loc. cit.*) and is due to Dr. K. A. Smith of this Institute.

An aliquot, in a 15-c.c. centrifuge tube, of a solution of the nucleic acid at pH 7·0 containing 0·2—0·3 mg. of purine-nitrogen was adjusted to acid normality by addition of 4N-sulphuric acid and hydrolysed at 100° (water-bath) for 1 hour. The solution was then cooled to room temperature and neutralised (phenolphthalein) with 4% (w/v) sodium hydroxide solution, and 1 c.c. of 10% (w/v) cupric sulphate ($CuSO_4,5H_2O$) was added, together with more 4% sodium hydroxide solution until the suspension changed in colour from bluish-green to dark blue. The mixture was left for 30 minutes at room temperature and then centrifuged, the supernatant liquor being then decanted. The deposit was re-dissolved in 1 c.c. of 5% (v/v) sulphuric acid, and 4% sodium hydroxide solution was added until a small quantity of cupric hydroxide remained in suspension. This was re-dissolved by addition of one drop of glacial acetic acid. The tube was placed in a boiling water-bath and, when the contents had reached the bath temperature, 1 c.c. of 40% (w/v) sodium hydrogen sulphite solution was added and the suspension vigorously stirred. The tube was removed from the bath after 5 minutes and the stirring-rod and the sides of the tube were washed with 5% (w/v) sodium sulphate solution. The deposit was collected by centrifugation, the supernatant liquid discarded, and the deposit re-dissolved in 1 c.c. of 25% (v/v) hydrochloric acid. This solution (and any insoluble residue) was transferred to a micro-Kjeldahl digestion flask and digested in the usual way with sulphuric acid and hydrogen peroxide. This procedure was substantially that of Ma and Zuazaga (*Ind. Eng. Chem., Anal.*, 1942, **14**, 280) but no special digestion mixture was used and the ammonia was distilled in a Markham-type apparatus (*Biochem. J.*, 1942, **36**, 790). The total nitrogen content of a similar aliquot of the nucleic acid solution was determined by the same micro-Kjeldahl procedure. The determinations were performed in triplicate. 0·925 Mg. of purine-nitrogen was recovered from a total nitrogen content of 1·256 mg. The ratio of purine- : pyrimidine-nitrogen is thus 2·8 : 1. Model experiments with adenosine solution gave recoveries of purine nitrogen of 95—97% of the total.

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CHESTER BEATTY RESEARCH INSTITUTE, ROYAL CANCER HOSPITAL,
FULHAM ROAD, LONDON, S.W.3. [*Received, January 23rd*, 1950.]