Cytidine Nucleotides. Part I. Isolation from Lactobacillus arabinosus.

By J. Baddiley and A. P. Mathias.

[Reprint Order No. 5233.]

Dried cells of *Lactobacillus arabinosus* have been shown to contain cytidine-5′ phosphate. Young, rapidly killed, organisms do not contain this nucleotide, but contain two new cytidine derivatives which are derivatives of cytidine-5′ phosphate containing unidentified residues attached to the phosphate group. Other nucleotides from this organism have been separated by ion-exchange chromatography and many have been identified.

It was shown by Pierpoint and Hughes (*Biochem. J.*, 1954, **56**, 130) that *Lactobacillus arabinosus* 17—5, when grown in the absence of cystine, metabolises pantothenic acid but does not convert it into coenzyme A. The metabolic product has now been identified as pantothenic acid-4′ phosphate (Baddiley, Hughes, Mathias, and Pierpoint, *ibid.*, 1954, **56**, xxii). During this work it was noticed that considerable amounts of other phosphates with high absorption in ultra-violet light were present in extracts of cystine-deficient cells. A preliminary examination of aqueous extracts of this organism by chromatography and electrophoresis on paper suggested that these substances were nucleotides. A more detailed study of the nucleotides in *L. arabinosus* was therefore undertaken.

Aqueous extracts of the dried cystine-deficient organisms (kindly supplied by Dr. D. E. Hughes) were treated with barium acetate. After removal of insoluble barium salts the solution was examined by paper chromatography. Spots were observed which corresponded in position to the 5′-phosphates of adenosine, uridine, and cytidine. The presence of adenosine-5′ phosphate was not unexpected, since it would probably arise as a precursor or decomposition product of adenosine di- and tri-phosphate and the several known coenzymes which are derived from this nucleotide. Similarly, uridine-5′ phosphate might be expected as a precursor or decomposition product of uridine-diphosphate-glucose (Caputto, Leloir, Cardini, and Paladini, *J. Biol. Chem.*, 1950, **184**, 333), uridine-diphosphate-*N*-acetylglucosamine (Cabib, Leloir, and Cardini, *ibid.*, 1953, **203**, 1055), the recently isolated uridine triphosphate (Lipton, Morell, Frieden, and Bock, *J. Amer. Chem. Soc.*, 1953, **75**, 5449; Munch-Petersen, Kalckar, Cutolo, and Smith, *Nature*, 1953, **172**, 1036), or of other less-defined uridine pyrophosphate derivatives known to occur in living cells. On the other hand neither cytidine-5′ phosphate nor any derivative of this nucleotide has been isolated hitherto from a natural source. A possible exception is ribonucleic acid, in which cytidine residues are joined to neighbouring nucleosides through 3′ : 5′-phosphate linkages.

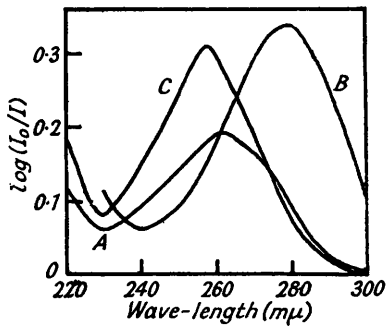
It was necessary then to establish beyond doubt that the new nucleotide was cytidine-5′ phosphate. Individual bands of the nucleotides were eluted from the paper and their ultra-violet absorption spectra were measured (Figs. 1 and 2). The spots corresponding to the 5′-phosphates of adenosine and uridine showed ultra-violet absorption spectra typical for these substances. Furthermore, the cytidine derivative showed a maximum at 279 and a minimum at 240 mμ in acid, and a maximum at 272 and minimum at 249 mμ in alkali, in good agreement with values for cytidine phosphates (Ploeser and Loring, *J. Biol. Chem.*, 1949, **178**, 431). After hydrolysis with perchloric acid (Marshak and Vogel, *ibid.*, 1951, **189**, 597; Wyatt, *Biochem. J.*, 1951, **48**, 584) the adenosine, uridine, and cytidine derivatives yielded the bases adenine, uracil, and cytosine respectively. These were identified by paper chromatography and measurement of their ultra-violet spectra.

It was found that normal cells of this organism also contain appreciable amounts of these nucleotides. However, identification was somewhat complicated in this case by the presence of large amounts of unidentified cell products which interfere with the paper-chromatographic procedure. Further confirmation of the structure of these substances was sought in a study of their behaviour on an ion-exchange resin column. The nucleotides present in an aqueous extract of the dried organisms were precipitated as their mercury salts (Cabib, Leloir, and Cardini, *loc. cit.*), inorganic phosphate was removed with barium

hydroxide, and then the soluble barium salts were separated by chromatography on Dowex-1 resin (chloride form). Separation of components was effected by elution with 0·002N-hydrochloric acid in the usual manner (Cohn and Carter, *J. Amer. Chem. Soc.*, 1950, **72**, 4276). Nucleotides emerging from the column were detected by measuring the absorption at 260 mμ of samples of eluate. From the diagram (Fig. 3) it can be seen that a strongly absorbing substance was eluted before adenosine-5′ phosphate. This is in the position expected for cytidine-5′ phosphate (Cohn and Carter, *loc. cit.*). A smaller peak corresponding to uridine-5′ phosphate followed adenosine-5′ phosphate.

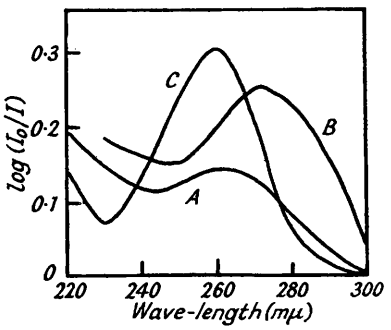
When examined on paper these nucleotides were indistinguishable from synthetic samples of the 5′-phosphates of cytidine, adenosine, and uridine (kindly supplied by Pro-

FIG. 1. *Absorption spectra of nucleotides from dried cells of* L. arabinosus *in* 0·01N-*hydrochloric acid.*



A, Uridine-5′ phosphate.
B, Cytidine-5′ phosphate.
C, Adenosine-5′ phosphate.

FIG. 2. *Absorption spectra of nucleotides from dried cells of* L. arabinosus *in* 0·01N-*sodium hydroxide.*



A, Uridine-5′ phosphate.
B, Cytidine-5′ phosphate.
C, Adenosine-5′ phosphate.

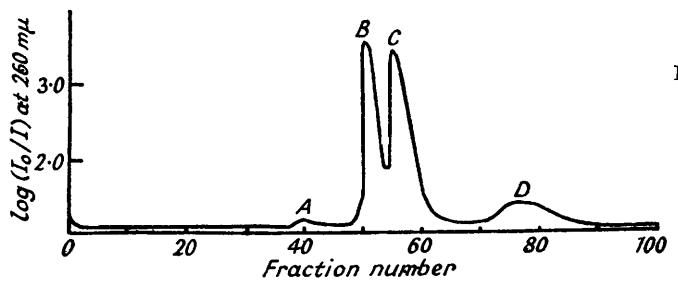


FIG. 3. *Nucleotides from dried* L. arabinosus *eluted from Dowex-1 column with* 0·002N-*hydrochloric acid.*

B, Cytidine-5′ phosphate.
C, Adenosine-5′ phosphate.
D, Uridine-5′ phosphate.

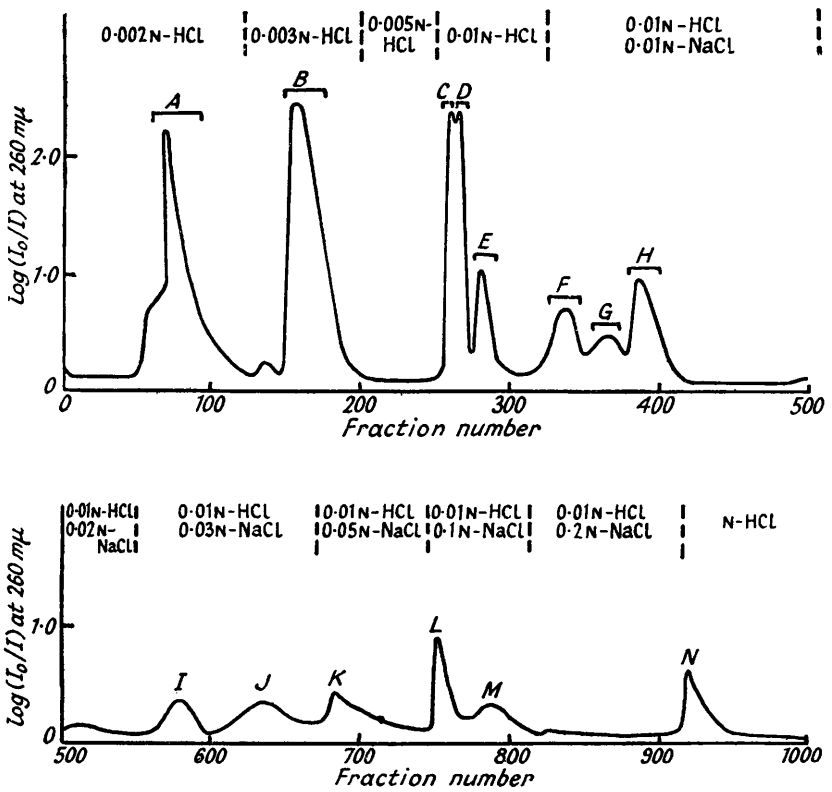
fessor A. R. Todd). They gave typical blue spots after spraying of the paper with periodate followed by Schiff's reagent (Buchanan, Dekker, and Long, *J.*, 1950, 3162), thereby indicating the absence of substituents at positions 2′ and 3′. They also differed from the corresponding nucleoside-2′ and 3′ phosphates in their position on the paper.

The insoluble barium salts obtained from the dried organisms contained much inorganic phosphate. However, after hydrolysis with acid followed by chromatography on paper a rather weak spot was observed which corresponded in position and ultra-violet spectrum with guanine. The nature of the substance giving rise to this purine was not examined further, but it is probable that it was present originally as a guanosine phosphate. It is noteworthy in this connection that guanosine-5′ triphosphate has been isolated recently from natural sources (Bergkvist and Deutsch, *Acta Chem. Scand.*, 1953, **7**, 1307).

It appears from Fig. 3 that relatively large amounts of cytidine-5′ phosphate are present, either free or in some labile combined form, in *L. arabinosus*. At present the only known natural derivative of cytidine is ribonucleic acid. It is true that cytidine-5′ phosphate has been obtained, along with the corresponding 2′- and 3′-phosphate, by ribonuclease

digestion of ribonucleic acid (Cohn and Volkin, *Nature*, 1951, **167**, 483). However, special conditions are required for this type of hydrolysis in order to prevent the action of 5′-phosphatases which occur in many cells. For this and other reasons, we considered it unlikely that the cytidine-5′ phosphate in our preparations had been formed by degradation of ribonucleic acid. Furthermore, no 2′- or 3′-phosphates were present in the extracts, and these would be expected if ribonucleic acid was being hydrolysed during the preparation of the dry cells. It seemed probable that the nucleotide was either a nucleic acid precursor or a hydrolysis product of hitherto unknown cytidine-containing intermediates or co-enzymes.

FIG. 4. *Elution of nucleotides from fresh cells on Dowex-1.*



A, DPN. *B*, *Adenosine-5′ phosphate.* *C and D*, CPX *and* CPY. *E*, *Uridine-5′ phosphate.* *F*, *Guanine derivative.* *G*, *Adenine derivative.* *H*, ADP. *I*, *J*, *K*, *L*, *Uridine derivatives.* M, N, *Adenine derivatives.*

The nucleotides present in the dried organisms were probably formed by the action of phosphatases on various more complex coenzymes. Consequently, a more detailed examination of heat-killed organisms was undertaken. It was considered that phosphatases would be destroyed by this treatment. Rapidly growing cells were harvested and immediately extracted with boiling aqueous alcohol. The nucleotides were precipitated as their mercury salts which were then converted into ammonium salts, and the mixture was resolved on Dowex-1. As expected, the elution diagram (Fig. 4) indicated that several pyrophosphates were present in rapidly killed cells which were absent from the dried organisms.

Substance *A* was eluted with 0·002N-acid. It was identified as diphosphopyridine nucleotide (DPN) by its position on the diagram, its absorption spectrum, and the characteristic spectral changes shown in the presence of potassium cyanide. Substance *B*, eluted with 0·003N-acid, was identified as adenosine-5′ phosphate. It showed maximum

absorption at 260 mμ, yielded adenine on acid hydrolysis, and was indistinguishable from the 5′-phosphate on paper. It gave a positive periodate–Schiff test and contained adenine and phosphorus in the ratio approx. 1 : 1. It is interesting to note that no cytidine-5′ phosphate was eluted up to this point. If this nucleotide were present in these extracts it should have appeared between DPN and adenosine-5′ phosphate. Peaks *C* and *D*, although incompletely resolved, represent two substances with typical cytidine spectra. These substances, which have been called CPX and CPY, are discussed in more detail below. They probably represent the source of cytidine-5′ phosphate present in the dried organisms. *E* followed *C* and *D* rather closely. It was identified as uridine-5′ phosphate by the absorption spectrum, comparison with synthetic uridine-5′ phosphate on paper, and by the positive periodate–Schiff reaction; the substance gave uracil after acid hydrolysis and had a uridine : phosphate ratio of 1 : 0·9. The 5′-phosphates of adenosine and uridine, diphosphopyridine nucleotide, CPX and CPY, and adenosine diphosphate (peak H) were isolated from the appropriate fractions of eluate by precipitation of the mercury salts and conversion into ammonium salts. Other nucleotides were not present in large enough amounts to permit their isolation.

The remaining peaks shown in Fig. 4 were not fully identified. However, the nature of the base present in each case was established by measurement of absorption spectra. With this information, tentative identification of most fractions was possible by direct

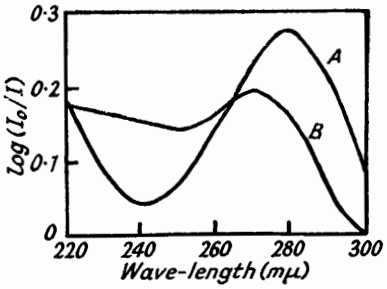


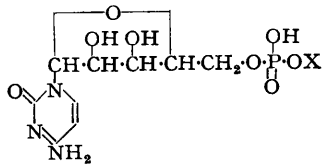
FIG. 5. *Absorption spectra of* CPX *and* CPY.
A, In 0·01N-*hydrochloric acid.*
B, In 0·01N-*sodium hydroxide.*

comparison with the similar elution diagram of yeast extracts (Cabib *et al.*, *loc. cit.*). *F* is due to an unknown derivative of guanine. *G* is due to probably the " ADP-ribose " of Cabib *et al.*, and *H* to adenosine diphosphate. *I*, *J*, *K*, and *L* represent uridine derivatives, *I* and *J* possibly being uridine diphosphate-*N*-acetylglucosamine and uridine-diphosphate-glucose respectively. Similarly, *L* would represent uridine diphosphate, *K* an unknown uridine derivative, and *M* probably adenosine triphosphate (ATP). The peak *N* is due to an adenine derivative which, in view of the high concentration of acid required to elute it from the column, is probably a stronger acid than ATP. The characteristics of this substance on the column are approximately consistent with those described recently for adenosine tetraphosphate (Marrian, *Biochem. Biophys. Acta*, 1954, **13**, 278) but insufficient material corresponding to this peak has been available to enable reliable characterisation.

In a preliminary report of this work (Baddiley and Mathias, *Chem. and Ind.*, 1954, 277) we had not recognised the dual nature of peaks *C* and *D*. Consequently, early experiments on the nature of CPX and CPY were performed on a mixture of the two substances. This is unimportant as far as the identification of the pyrimidine nucleotide part of the molecule is concerned since both yield cytidine-5′ phosphate on hydrolysis in N-hydrochloric acid for 10 min. at 100°. No other nucleotide was present in the hydrolysate. Cytidine-5′ phosphate was identified by comparison with the authentic nucleotide on paper chromatography in two solvents, by its spectrum, and by hydrolysis to cytosine. The materials CPX and CPY, and the cytidine phosphate obtained from them by acid hydrolysis, gave positive periodate–Schiff tests on paper. Only one spot, corresponding to cytidine-5′ phosphate, was observed by this technique on chromatograms of the acid-hydrolysis products of CPX and CPY. This indicates that positions 2′ and 3′ are unsubstituted in both CPX and CPY. The ultra-violet absorption spectra, determined on fractions corresponding to the maxima *C* and *D*, were identical. The positions of maxima and minima

in acid and alkali (Fig. 5) correspond closely with those for cytidine and its phosphates. It is unlikely then that the residues X and Y are directly attached to the pyrimidine ring.

The two cytidine derivatives accompanied inorganic phosphate when eluted from the column. However, as paper chromatography indicated the absence of other organic phosphates, it was possible to determine the ratio of cytidine to organic phosphate in the combined substances. A value of almost exactly 1 : 1 was obtained. Very similar values were observed from analyses of spots of CPX and CPY eluted from a paper chromatogram in *n*-propyl alcohol–ammonia. In this solvent mixture inorganic phosphate was separated from the phosphoric esters. No inorganic phosphate was liberated on hydrolysis of these substances in N-hydrochloric acid at 100° for 15 min. It is clear then that CPX is a derivative of cytidine-5′ phosphate in which a group X is joined in ester linkage with the phosphate residue according to the annexed formula. Similarly, CPY must be represented by a corresponding formula in which X has been replaced by Y. These substances are unusual in that, although from their nature it is probable that they are coenzymes or intermediates, all known nucleotide coenzymes are esters of pyrophosphoric acid.



It has not yet been possible on the very small amounts of material available to determine the nature of X and Y or of the type of linkage involved with the rest of the molecule. It is likely that the two groups are chemically similar, since CPX and CPY run as a single spot on paper in most solvent systems and are incompletely separated on the Dowex-1 column. The linkage between cytidine-5′ phosphate and X or Y is very labile, since both are completely hydrolysed to cytidine-5′ phosphate in N-hydrochloric acid at 100° in 10 min. Furthermore, almost complete hydrolysis to the 5′-phosphate occurred during attempts to remove inorganic phosphate from these substances by chromatography on Dowex-1 in the presence of borate (Khym and Cohn, *J. Amer. Chem. Soc.*, 1953, **75**, 1153). It appears that the rather prolonged contact with dilute alkali which this method entails removes both X and Y : this was confirmed by paper chromatography in *n*-propyl alcohol–ammonia. Although the main spot on these chromatograms represented the mixed esters, some cytidine-5′ phosphate was produced. It follows that they are not simple alkyl esters. Also X and Y contain no phosphorus and do not show marked absorption in ultra-violet light.

Some degree of separation of CPX and CPY has been obtained by paper chromatography in *iso*butyric acid–ammonium *iso*butyrate–water. However, it has not yet been possible to decide which of the two spots produced corresponds to a particular peak on the elution diagram. Further work on the nature of these nucleotides is in progress.

Experimental

Whatman No. 4 paper, washed with 2N-acetic acid, was used throughout this work.

Preliminary Examination of Cystine-deficient Cells.—Washed cells (1·06 g.) which had been dried over phosphoric oxide were suspended in water (10 c.c.), kept at 0° for 2 hr. with occasional stirring, and finally heated at 100° for 5 min. The cell debris was removed by centrifugation and extracted twice with water. The combined supernatant liquors were freeze-dried. A portion of the extract (95 mg.) was dissolved in water (1 c.c.), and saturated barium hydroxide solution was added cautiously until precipitation was complete. The precipitate was centrifuged off and washed with water. The combined supernatant liquid and washings were passed through a column of Amberlite IRC–50 resin (H^+ form) (0·6 cm.^{2} × 17 cm.). The acidic effluent was neutralised with ammonia and evaporated to dryness under reduced pressure. The residue was dissolved in water (0·1 c.c.) and applied to the base-line of a paper chromatogram as a band (16 cm. long). Appropriate authentic nucleotides were applied at each end of the band. The chromatogram was developed with *iso*butyric acid–ammonium *iso*butyrate–water (Magasanik, Vischer, Doniger, Elson, and Chargaff, *J. Biol. Chem.*, 1950, **186**, 37), dried in warm air, and examined under a Mineralight lamp. Two narrow vertical strips were cut out and examined with the periodate–Schiff spray and with a phosphate spray reagent (Hanes and Isherwood, *Nature*, 1949, **164**, 1107). Several bands were observed which absorbed ultra-

violet light, contained phosphorus, and gave positive periodate–Schiff reactions. Three of these corresponded in position with uridine-5′ phosphate (R_F 0·35), cytidine-5′ phosphate (R_F 0·41), and adenosine-5′ phosphate (R_F 0·51).

Identification of Uridine-5′ Phosphate.—The band (R_F 0·35) was cut out and eluted from the paper by steeping it in water (5 c.c.) for 2 hr. Paper was removed and the solution was evaporated to dryness in a desiccator. The residue was dissolved in water (0·1 c.c.) and a portion diluted to 2 c.c. with 0·01N-hydrochloric acid. The ultra-violet absorption spectrum (Fig. 1) was measured in a 1 cm. cell in a Hilger "Uvispek," ($\lambda_{max.}$ 262 mμ; $\lambda_{min.}$ 231 mμ). Sufficient N-sodium hydroxide was added to give a solution 0·01N with respect to sodium hydroxide, and the spectrum was re-examined ($\lambda_{max.}$ 261 mμ; $\lambda_{min.}$ 242 mμ) (Fig. 2). The spectra closely resemble those reported for uridylic acid (Ploeser and Loring, *loc. cit.*).

Only one absorbing spot was observed when this fraction was run on paper in *iso*propanol–hydrochloric acid–water (Wyatt, *loc. cit.*) and in *n*-propanol–ammonia. This spot gave positive reactions for organic phosphate and with the periodate–Schiff spray, and was indistinguishable from uridine-5′ phosphate. Uridylic acid from yeast did not give the latter reaction and moved faster than the 5′-phosphate in both solvent systems. After hydrolysis with 72% perchloric acid it gave uracil, identified by its R_F in *iso*propanol–hydrochloric acid and by its absorption spectrum ($\lambda_{max.}$ 259 mμ, $\lambda_{min.}$ 230 mμ in 0·01N-hydrochloric acid; $\lambda_{max.}$ 282 mμ, $\lambda_{min.}$ 242 mμ in 0·01N-sodium hydroxide).

Identification of Cytidine-5′ Phosphate.—The band (R_F 0·41) was eluted and the absorption spectrum measured in the same way as for uridine-5′ phosphate. It corresponded closely with that of cytidylic acid. The substance was indistinguishable from cytidine-5′ phosphate on paper chromatography in *iso*butyric acid–ammonium *iso*butyrate–water, *n*-propanol–ammonia, and *iso*propanol–hydrochloric acid, in all of which it gave a single spot containing phosphorus and giving a positive reaction with the periodate–Schiff reagent. Cytidylic acid moved faster than the 5′-phosphate in these solvents (see Table). After hydrolysis with perchloric acid it yielded cytosine, identified by paper chromatography in *iso*propanol–hydrochloric acid and by its absorption spectrum ($\lambda_{max.}$ 274 mμ, $\lambda_{min.}$ 239 mμ in 0·01N-hydrochloric acid; $\lambda_{max.}$ 271 mμ, $\lambda_{min.}$ 249 mμ in 0·01N-sodium hydroxide).

Identification of Adenosine-5′ Phosphate.—The band (R_F 0·51) had an absorption spectrum typical of adenosine phosphates ($\lambda_{max.}$ 259 mμ, $\lambda_{min.}$ 232 mμ in 0·01N-hydrochloric acid; $\lambda_{max.}$ 260 mμ, $\lambda_{min.}$ 232 mμ in 0·01N-sodium hydroxide). The substance gave a single spot in the above-mentioned solvent systems and was indistinguishable from adenosine-5′ phosphate. After hydrolysis with perchloric acid it gave adenine ($\lambda_{max.}$ 263 mμ, $\lambda_{min.}$ 230 mμ in 0·01N-hydrochloric acid; $\lambda_{max.}$ 262 mμ, $\lambda_{min.}$ 231 mμ in 0·01N-sodium hydroxide).

Examination of Dried Normal Cells of L. arabinosus.—The dried cells were extracted by a method similar to that adopted for the cystine-deficient organisms. Direct paper chromatography of this extract was not satisfactory, so nucleotides were precipitated before examination in the following manner. A 5% solution of the extract in aqueous alcohol (50%) was adjusted to pH 5 with dilute nitric acid, and a solution of mercuric acetate in acetic acid (Caputto, Leloir, Cardini, and Paladini, *loc. cit.*) was added dropwise until no further precipitation occurred. The precipitate was centrifuged off, washed with aqueous alcohol, suspended in water, and decomposed with hydrogen sulphide. Mercuric sulphide was removed by centrifugation and washed with water. The supernatant liquid and washings were combined, aerated, adjusted to pH 7 with aqueous ammonia, and freeze-dried. Identification of the nucleotides was carried out by paper chromatography and ultra-violet spectroscopy in the same manner as for those from cystine-deficient cells. The 5′-phosphates of uridine, cytidine, and adenosine were present. No other nucleotides were observed.

Ion-exchange Chromatography of Nucleotides from Dried Normal Cells (see Fig. 3).—The freeze-dried ammonium salts obtained from the insoluble mercury salts (*ca.* 0·1 g.) described above were dissolved in water (2 c.c.), and saturated barium hydroxide solution was added until precipitation ceased. The precipitate was removed by centrifugation and washed with water (2 × 2 c.c.). The combined supernatant liquid and washings were adjusted to pH 7·5 with acetic acid and passed through a column (0·5 cm.^{2} × 7 cm.) of Dowex-1 resin (chloride form, 200—400 mesh). After thorough washing with water, elution was commenced with 0·002N-hydrochloric acid at 0·3 c.c./min. The eluate was collected in 6-c.c. fractions on an automatic fraction-collector, and the extinction coefficient at 260 mμ of each fraction was measured. Three main peaks, designated *B*, *C*, and *D* in order of elution, were observed (Fig. 3). The fractions corresponding to the peaks were combined and adjusted to pH 5 with ammonia solution. Several drops of the mercuric acetate reagent were added to each, and the solutions were kept

at 0° overnight. The precipitates were centrifuged off and washed with 50% alcohol, and individual nucleotides were regenerated as before. The resulting solutions were freeze-dried as ammonium salts. The spectra of " peak tubes " (diluted appropriately) and the R_F values (see Table) indicated that *B*, *C*, and *D* represented the 5′-phosphates of cytidine, adenosine, and uridine respectively.

Growth and Extraction of Fresh Cells.—*L. arabinosus* 17–5 was grown at 37° on the following medium: glucose 2%, " Pronutrin " (Herts Pharmaceuticals Ltd.) 2%, hydrated sodium acetate 1%, yeast extract (1 part of yeast, 4 parts of water; boiled for 10 min. and filtered) 200 c.c./l., potassium dihydrogen phosphate 0·45%, N-sodium hydroxide 26·0 c.c./l., and inorganic salts " B " (Barton-Wright, " Practical Methods for the Microbiological Assay of Vitamin B Complex and Essential Amino Acids," London, Ashe Laboratories, 1946) 5·0 c.c./l. The medium was sterilised in the autoclave. The organism, supplied as a stab-culture, was sub-cultured on the above medium for 7 days with daily transfer. An inoculum (500 c.c.) was grown for 24 hr. and used to inoculate 3 flasks each containing 4 l. of medium. After growing for 24 hr. the cells were harvested in a Sharples centrifuge. The wet cells (65—70 g.) were heated immediately with constant stirring in a mixture of 96% alcohol (70 c.c.) and 50% alcohol (140 c.c.) until the suspension boiled. The mixture was boiled for 5 min., then cooled to room temperature, and cell debris were removed by centrifugation. The precipitate was re-extracted with 30% alcohol (140 c.c.), and the combined extracts were submitted to mercury-salt precipitation as described before. The nucleotides were obtained as freeze-dried ammonium salts (*ca.* 1·25 g.).

A sample (*ca.* 5 mg.) was hydrolysed with 72% perchloric acid, and the hydrolysate run on paper in *iso*propanol–hydrochloric acid. Four spots were detected in ultra-violet light. These were identified by their R_F values and absorption spectra as adenine, uracil, cytosine, and guanine. From their extinction coefficients they were shown to be present in the approximate proportions 15 : 9 : 5 : 1 respectively.

Chromatography of Nucleotides from Fresh Cells on Dowex-1.—A sample of the ammonium salts (500 mg.) in water (350 c.c.) was adsorbed on a column of Dowex-1 (chloride form, 200—400 mesh, 1·4 cm.^{2} × 21 cm.). The column was washed with water, until the absorption of the eluate had fallen to 0·1 optical-density unit. Elution with 0·002N-hydrochloric acid was commenced and the eluate was collected in 6·6-c.c. fractions at 0·28 c.c./min. The absorption spectra of the peak solutions were measured on samples from the peak tubes. The nucleotides corresponding to the peaks were isolated through their mercury and ammonium salts in the manner described before. This technique was unsuccessful for peaks *I* onwards, probably owing to the extreme dilution of these nucleotides together with the high salt concentration.

Identification of Peaks from Column (see Fig. 4).—*Peak A* (*diphosphopyridine-nucleotide*). This substance had a typical adenine spectrum. On the addition of potassium cyanide there was a marked fall in the absorption at 260 mμ and appearance of a peak at 325 mμ. This behaviour is typical of a pyridine-nucleotide (Colowick, Kaplan, and Ciotti, *J. Biol. Chem.*, 1951, **191**, 447). Paper chromatography in ethanol–ammonium acetate–water (pH 7·5) (Paladini and Leloir, *Biochem. J.*, 1952, **51**, 426) and in *iso*butyric acid–ammonium *iso*butyrate–water (R_F 0·4) indicated that a minor component was present which also contained phosphorus. From the spectral and ion-exchange characteristics of the main component of peak *A* it is concluded that this is diphosphopyridine-nucleotide. This, and all other substances from the column which were isolated through their mercury salts (peaks *A*—*E*, and *H*), absorbed ultra-violet light, contained phosphorus and showed a positive periodate–Schiff reaction on paper.

Peak B (*adenosine-5′ phosphate*). This had an adenine spectrum. It was indistinguishable from synthetic adenosine-5′ phosphate on paper chromatography (see table of R_F values). A minor non-absorbing organic phosphate was also present (R_F 0·23 in the *iso*butyric acid solvent). The main spot was cut out and eluted from the paper. A blank spot of corresponding position on the paper was treated similarly. The " adenine " content of the eluate was determined from the extinction coefficient and organic phosphate was determined by Allen's method (*Biochem. J.*, 1940, **34**, 858). A ratio adenine : phosphorus of 1 : 0·9 was found, in good agreement with the expected value for adenosine-5′ phosphate.

Peaks C and D (CPX *and* CPY). These peaks were poorly separated. The substances showed identical spectra ($\lambda_{max.}$ 280 mμ, $\lambda_{min.}$ 241 mμ in 0·01N-hydrochloric acid; $\lambda_{max.}$ 272 mμ, $\lambda_{min.}$ 250 mμ in 0·01N-sodium hydroxide), typical for cytidine derivatives. In the early chromatograms in *iso*butyric acid–ammonium *iso*butyrate–water the substances appeared to give a single spot (R_F 0·23), moving at approximately half the speed of cytidine-5′ phosphate (R_F 0·41), and at the same speed as inorganic phosphate, which was readily detected in both fractions

by the yellow colour produced immediately when the paper was sprayed with the ammonium molybdate reagent. Spots from *C* and *D*, and spots of corresponding area and R_F from blank tracks, were cut out and eluted from such a paper with 0·01N-hydrochloric acid. The cytidine content of the eluate was determined from its absorption at 280 mμ; also the total and inorganic phosphate content was measured. For *C* a ratio of cytidine : organic phosphate of 1 : 1·005 was found, and for *D* the ratio was 1 : 1·04. Samples of a similar eluate in N-hydrochloric acid were heated at 100° for 10 min. and inorganic phosphate was determined. A ratio of cytidine : labile phosphate of 1 : 0·03 for *C*, and 1 : 0·08 for *D* was obtained. Unheated samples were used as controls. This indicates that no inorganic phosphate is liberated by such treatment. Similar results were obtained from analyses on the substances from *C* and *D*. Samples were dissolved in 0·01N-hydrochloric acid (3 c.c.). Samples (0·5 c.c.) of each solution were diluted to 2 c.c. with 0·01N-hydrochloric acid and the absorptions at 280 mμ were measured. Aliquot portions (0·7 c.c.) were used for total, inorganic, and labile phosphate determinations. Labile phosphate was determined by adding concentrated hydrochloric acid (0·07 c.c.) and then heating at 100° for 15 min. For *C* the ratio cytidine : organic phosphate : labile phosphate was 1 : 1·02 : 0·1 and for *D* 1 : 1·02 : 0·01. Very similar values were found when the method of Lowrey and Lopez (*J. Biol. Chem.*, 1946, **102**, 41) was used for inorganic and labile phosphate determinations. This eliminates the possibility that hydrolysis of a very labile phosphate had occurred before or during the inorganic phosphate determinations by Allen's method.

Hydrolysis of material from *C* and *D* with perchloric acid gave cytosine, identified by paper chromatography and from its absorption spectrum. With N-hydrochloric acid at 100° (10 min.) both substances were converted into cytidine-5′ phosphate, identified by paper chromatography in the solvents shown in the Table. No other phosphates or substances absorbing ultra-violet light were observed on the papers.

R_F values.

The values given are averages. Individual values varied because of lack of temperature control. In all cases authentic nucleotides were run as controls. Ascending-front chromatography was used throughout, and solvent mixtures were freshly prepared.

	Pr^{i}·CO_2H–NH_3–H_2O	$Pr^{n}OH$–NH_3–H_2O (6 : 3 : 1)	EtOH–$AcONH_4$–H_2O, pH 7·5
Cytidine-5′ phosphate	0·41	0·27	—
Cytidine-3′ phosphate (*b*)	0·47	0·34	—
CPX, CPY	0·18, 0·22	0·38	0·17
Adenosine-5′ phosphate	0·51	0·33	—
Adenosine-3′ phosphate (*b*)	0·58	0·39	—
ADP	0·42	—	—
Uridine-5′ phosphate	0·30	0·23	—
Uridine-3′ phosphate (*b*)	0·35	0·30	—

Substances from *C* and *D* were separated from inorganic phosphate by chromatography in *n*-propanol–ammonia–water. The main spot and a blank area were cut out and eluted with 0·01N-hydrochloric acid. Analysis as before gave a ratio of cytidine : organic phosphate : labile phosphate 1 : 1·08 : 0·1. Re-chromatography of the main spot in the *iso*butyric acid solvent indicated that some hydrolysis to cytidine-5′ phosphate had occurred.

Peak E (*uridine-5′ phosphate*). This material gave a uridine spectrum. Paper chromatography (see Table) indicated that only one substance was present which was indistinguishable from uridine-5′ phosphate. The ratio uridine : organic phosphate was 1 : 0·9, in good agreement for a uridine monophosphate.

Peak F. The absorption spectrum of this material was very similar to that of guanylic acid. However, it was not precipitated by mercuric acetate and has not been examined further.

Peak G. An adenine spectrum was given by this material. From its position on the elution diagram it corresponds to the " ADP-ribose " of Cabib *et al.* (*loc. cit.*), but complete identification is still required.

Peak H (*adenosine-5′ pyrophosphate*, ADP). This material also gave an adenine spectrum. The ratio adenine : organic phosphate was 1 : 1·8 and the substance was indistinguishable from synthetic adenosine-5′ pyrophosphate (ADP) on paper chromatography in the *iso*butyric acid system and in disodium hydrogen phosphate–*iso*amyl alcohol–water (Carter, *J. Amer. Chem. Soc.*, 1950, **72**, 4274).

Peaks I, J, K, and L. These materials had uridine spectra. They were not characterised further.

Peaks M and N. These materials were adenine derivatives.

Examination of CPX *and* CPY *on Dowex*-1 *in the Presence of Borate.*—A solution of the mixed substances (30 mg.) in water (25 c.c.) was passed through a column (1·4 cm.^{2} × 21 cm.) of Dowex-1 (chloride form, 200—400 mesh). After washing with water (50 c.c.) elution with 0·025M-ammonium chloride and 0·01M-sodium borate was commenced. Fractions of eluate were examined for absorption at 260 mμ and for the presence of inorganic phosphate. The latter emerged as a fairly sharp peak (vol. to max., 1270 c.c.). The borate washing lasted for 4 days, during which the optical density remained below 0·05 unit. After removal of inorganic phosphate nucleotides were eluted with 0·005N-hydrochloric acid. Two peaks were detected. Paper chromatography of the first, strong peak indicated the presence of cytidine-5′ phosphate, whereas the second, weak peak was due to the CPX and CPY mixture.

Thanks are extended to Dr. D. E. Hughes for a culture of *L. arabinosus* and for information about its growth. This work was supported by a grant from the Nuffield Foundation.

THE LISTER INSTITUTE OF PREVENTIVE MEDICINE,
LONDON, S.W.1.

[*Received, March* 23*rd*, 1954.]