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Isolation and Identification of the Acid-soluble Phosphorus Compounds of Yeast

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Food yeast (*Candida utilis*) was grown in a nutrient solution containing ^{32}P-labelled orthophosphate, and the fraction of the acid-soluble phosphates was isolated, fractionated by Dowex-1 ion exchange chromatography using mixtures of formic acid and ammonium formate as eluent, and the fractions obtained, containing several compounds, were further resolved into single compounds by paper chromatography. The compounds were identified by UV-spectra, elementary analysis, spot tests, hydrolysis, and co-chromatography on paper. In addition to the common sugar phosphates and the mono-, di-, and triphosphates of the common nucleosides, a number of nucleotide derivatives, *e.g.* CDP-glycerol, CDP-choline, CDP-ethanolamine, probably CMP-ethanolamine, CDP-mannose, ADP-ribose, UDP-acetylglucosamine and adenosinephosphosulphate and its succinyl derivative were identified and their location on the Dowex chromatogram and 2-dimensional paper chromatogram shown. The latter method can be used to follow changes in the amounts of these compounds in yeast, if it is labelled with radiophosphorus.

The significance of organic phosphorus compounds in the life-processes of all living cells has proved to be the greater the more deeply investigators have delved into the subject. For a long time lack of suitable methods hindered the investigations. For optimum usefulness detection methods should be valid even at concentrations that are barely detectable, and the separation methods should give sufficient separation of very similar compounds from complex mixtures. The classical methods were based mainly on precipitation, which, because of solubility limits and coprecipitation, does not give good enough results at very low concentrations.

The application of ion exchange chromatography and paper chromatography opened up unlimited possibilities for the investigation of biologically important phosphate compounds. In consequence, there was a deluge of investigations and the number of new compounds found increased in a few years by factors of ten.

Cohn [1] was the first to apply the ion exchange methods to the investigation of phosphate compounds. However, the method presented by Hurlbert and

co-workers,[2] applying gradient elution to the isolation of phosphate compounds, was the first to meet with general approval, and it is still, with small variations, the best one in use. Soluble nucleotides of yeast have been studied by ion exchange chromatography by, *e.g.*, Saukkonen,[3] Pontis,[4] Gilbert and Yemm,[5] Ingle,[6] and, on a pilot plant scale, by Munden *et al.*[7]

The aim of our study has been to develop paper chromatography into a reliable and easy method for studying the soluble phosphates present in yeast extracts. Ordinary paper chromatograms cannot be loaded with enough material to give detectable spots of the nucleotides present in smallest amounts, but combination with ^{32}P labelling, for example, greatly increases their sensitivity. The usefulness of the technique was shown in an earlier paper by one of us,[8] in which the location of the 32 then commercially available phosphate esters on two-dimensional paper chromatograms was given and most of them were shown to be present in yeast. Several of the spots labelled remained unidentified, however. It was the purpose of this study to determine the position of these compounds in both ion exchange and paper chromatography and to elucidate their structures.

EXPERIMENTAL

Material. Candida utilis (from Kluyver's laboratory) was cultivated aseptically at 30°C on wort agar in a test tube for 24 h, then transferred to 4 to 6 Petri dishes on wort agar for another 24 h. Finally, the combined harvest was suspended in a Kluyver flask containing 2 l of "complete" nutrient solution "A" (50 g saccharose, 3 g $(NH_4)_2HPO_4$, 1.5 g K_2SO_4, 1.0 g $MgSO_4 \cdot 7H_2O$ and 0.5 g $CaCl_2$ per litre of tap water) and aerated at 30°C, keeping the pH automatically between 4.6 and 5.4. The yield after 15 h is 50 to 60 g of aseptic "normal" yeast with about 2 % P, 6 % N of dry weight. If more yeast is needed, this lot is suspended in 50 to 70 l of medium "A" and aerated for a further 15 h, the yield being about 0.5 to 1 kg of fresh cells. To obtain phosphorus-deficient cells a 5 % suspension of normal cells is made in a nutrient medium "B", which is otherwise like medium "A" but contains only 0.3 g $(NH_4)_2HPO_4$ per l. The yeast mass doubles in 2 to 3 h. These phosphorus-deficient cells have about 1.5 % P, 4.5 to 5 % N, of dry weight.

Assimilation experiments. The yeast was suspended in a third nutrient solution "C" (= nutrient solution A without any $(NH_4)_2HPO_4$ to obtain a 10 % suspension and aerated for 5—10 min at 30°C. Following this, the isotope was added and with it as a carrier enough KH_2PO_4 to obtain a phosphate concentration of 10^{-4} M. The samples were pipetted into centrifuge tubes and centrifuged quickly at +5°. A TCA extract was made from the yeast separated by first adding as many millilitres of 16 % TCA solution as there were grams of yeast in the sample and then repeating the extraction, using half the amount of 8 % TCA solution used earlier. TCA was removed from the extract by agitating with ether. Agitation, when repeated 3 times (v/v = 1:1), removes all undecomposed TCA from the extract. About 5—10 % of the TCA decomposes during the treatment; the HCl split lowers the pH.

The fractionation of the TCA extract with Dowex-1 resin. The resin used was Dowex 1 × 8 formate, 200—400 mesh. The transformation of the resin into formate and the construction of the column was according to the method of Hurlbert.[2] For elution, the eluents used by Yemm and Gilbert were employed.[5] The UV absorption of the eluate at 262 mμ was measured either automatically with a flow arrangement, an automatic recorder being connected to the spectrophotometer, or manually, measuring the absorption spectrum of the fractions between 250 and 300 mμ with a Beckman quartz spectrophotometer. The radioactivity was counted during the fractionation, using a Frieseke and Hoepfner FHZ 40 flow counter tube, surrounded with 0.07 mm aluminium foil around which was coiled enough polyethylene tubing of 1.5 mm inside diameter to ensure that the volume of liquid in the tube was about 1 ml. The relatively strong formic acid eluent

has a hydrolysing effect, and the ammonium formate of the eluant is difficult to remove. These are two drawbacks to the method. The chromatography has to be carried out in the cold (0° to + 5°C) and the fractions have to be lyophilized as rapidly as possible. Even though the fractions have been stored at −20°C before lyophilization, signs of hydrolysis have been observed after as little as one week. UDP-sugar compounds are especially sensitive to acidic hydrolysis and hence their isolation with Dowex chromatography cannot be accomplished without losses.

To eliminate ammonium formate, the lyophilized fractions have been treated with a strong cation exchange resin (Amberlite IR 120 or Dowex 50) in the ionized (H^+) form, and the effluent lyophilized again. Using a resin column of ample proportions ammonium formate can be changed to formic acid, which is eliminated by repeating the lyophilization. The treatment is long and causes considerable losses. Hence, quantitative results cannot be obtained.

Paper chromatography. As the first solvent t-butanol-formic acid-water in the proportions 8:3:4 [8] and in the second dimension propanol-conc. ammonia-water (6:3:1) were used. Whatman No. 4 was used as the paper. The nucleotides were located in UV light, the other phosphorus compounds with the Hanes and Isherwood reagent.[9] A chromatographic map is presented in Fig. 1.

In co-chromatography the purest commercial preparations obtained from the following firms were used: Pabst Laboratories and California Foundation for Biochemical Research (nucleotides and nucleotide derivatives), Schwarz Laboratories (nucleotides and sugar phosphates), Nutritional Biochemical Center (sugar phosphates, glycerophosphates, pyridoxine phosphate, propandiol phosphate), Sigma Chemical Co. (GDPM and sugar phosphates)[¶] and Boehring and Soehne (phosphoenolpyruvate and triosephosphate mixture).

The *chemical determinations.* Phosphorus determinations were done according to the method of Berenblum and Chain, modified by Martin and Doty and following the description of Lindberg and Ernster.[10]

Ribose determinations have been done by the orsinol reaction of Mejbaum.[11] Since the ribose of pyrimidine nucleotides is released extremely slowly, the samples containing pyrimidine nucleotides have been treated before determination with Na amalgam. (*Ca.* 50 μg of nucleotides + 0.25 ml water or 50 μg of nucleoside + 1.0 ml water and 50 mg Na amalgam was agitated for 3—4 h in a test tube at room temperature. Following the treatment, the solutions were made up to 1.0 ml. From this 0.8 ml was taken and the normal ribose determination carried out.)

Strong hydrolyses. Peptides and presumed nucleotide peptides were hydrolysed in 6 N hydrochloric acid for 20 h at 108°C. Purine and pyrimidine bases were liberated from nucleotides by hydrolysing with conc. formic acid for 2 h at 170°C. Uridine nucleotides are hydrolysed incompletely in this process giving uridine besides uracil.[12]

Weak hydrolyses. Purine bases are split from nucleotides when these are hydrolysed with 1 N hydrochloric acid for 1 h at 100°C. Pyrimidine nucleotides are not hydrolysed, but cytosine is deaminated to uracil.[13]

To split phosphoryl choline and phosphoryl ethanolamine from UDP-choline and CDP-ethanolamine, the compound was hydrolysed with 1 N hydrochloric acid for 40 min at 100°C.[14]

Other nucleotide diphosphate derivatives are hydrolysed with 0.01 N hydrochloric acid in 5—15 min at 100°C.[15]

Spot reactions. Sulphate was detected qualitatively with Feigl's permanganate-$BaCl_2$ test [16] and glycerol with Feigl's periodate test.[16] When the glycerol test is done on a compound isolated by paper chromatography it should be noted that ammoniacal eluents may also cause the formation of a colour. This error can be eliminated by evaporating the extract obtained from the spot to dryness several times before the determination. (A "blind test" of a spot cut from the same chromatogram was also carried out.)

Acid anhydrate determination. The presence of possible nucleotidepeptide compounds was investigated with Lippman and Tuttle's [17] hydroxamic acid method, succinic acid being used as the standard.

UV absorption spectra. The UV absorption spectra of the compounds were measured with a Beckman DU or DK spectrophotometer, usually at three pH's, 1, 7, and 10. The identity of DPN was always confirmed by measuring the spectrum of its cyanide complex.

The spectra of the adenosine and uridine nucleotides at different pH's vary only slightly. These compounds can be identified by spectrophotometry in the following way. Following the measurement of the UV-absorption of an acidic solution, a drop of bromine water is added. The mixture is stirred, and the excess of bromine removed after 10 min with an air stream. The ring of pyrimidine compounds is reduced (double bond at 4′—5′ position opens up) and the UV-absorption spectrum disappears completely. Of the purine compounds the spectra of adenosine and hypoxanthine nucleotides remain unchanged, and the guanosine spectrum also disappears almost completely.

RESULTS

In Fig. 2 a Dowex chromatogram of a TCA extract of the yeast *Candida utilis* is presented. UV-absorption of the fractions at 262 mμ, radioactivity, the solvents used, the effective solvent concentrations, and the way of combining the fractions are indicated in the figure. The most important compounds in the different peaks are also marked on the figure. The fractions were in reality still very complex. However, by rechromatography of the Dowex fractions with 2-dimensional paper chromatography, the compounds could be isolated in a sufficiently pure state for further structural studies. The peaks were analysed in the following way:

CDP-glycerol. The compound which in Fig. 2 is denoted CMPX, emerged from the Dowex column in the first fractions. When investigated by paper chromatography it was run in the first direction between ATP and ADP (Fig. 1) and in the second direction faster than either of these. When isolated from the paper chromatogram, the compound had a typical cytidine spectrum, and gave a molar ratio base:phosphate of 1:2. Hydrolysis with 1 N hydrochloric acid for 40 min at 100°C gave two phosphorus compounds, which in paper chromatography moved as CMP and β-glycerophosphate.

According to Feigl's periodate test for multivalent alcohols,[16] the original spot contained glycerol in the same molar proportion as cytidine (determined by UV-absorption). The compound is hence CDP-glycerol. It decomposes into CMP and β-glycerophosphate when stored without lyophilization at −20°C.

CDP-choline, CDPCHO. This compound, too, can be eluted from the Dowex column with almost plain water. On a paper chromatogram it moves in the first direction a little slower, in the second direction a little faster than AMP (Fig. 1). The spot cut from the paper chromatogram gave a cytidine spectrum and the molar ratio of base:ribose:phosphate 1:1:2. When hydrolysed with 1 N hydrochloric acid for 40 min at 100°C, the compound splits in two and the new compounds move on a paper chromatogram like CMP and PCHO. The original compound moved on paper exactly like the commercial CDPCHO. Thus, the compound was identified as CDP-choline. In most extracts it has been only quite weakly radioactive.

CDP-ethanolamine, CDPEA (CMPEA). Immediately after the former peak another compound with a cytidine spectrum is eluted from the Dowex column. In the first direction it moves noticeably slower than CDPCHO, but in the second direction at the same speed (Fig. 1). The molar ratio base: ribose:phosphorus is 1:1:2. The same fraction contains a very low concentration of a compound which with the second paper chromatography solvent

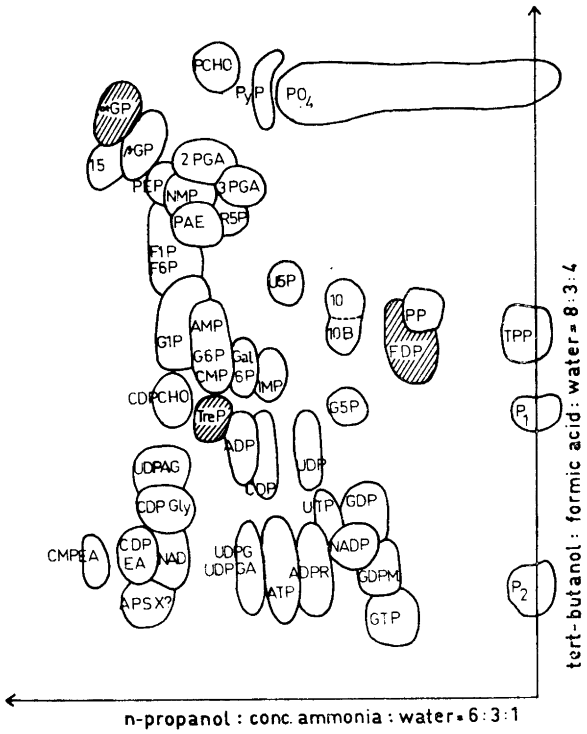

Fig. 1. A map of the soluble phosphate esters of the yeast *Candida utilis* separated by 2-dimensional paper chromatography with the solvent system indicated. The solvent containing tert. butanol run first.

The following abbreviations are used:
AMP, CMP, GMP, IMP, UMP 5′-Monophosphates of adenosine, cytidine, guanosine, inosine, and uridine. ADP, CDP, GDP, UDP 5′-Pyrophosphates of adenosine, cytidine, guanosine, and uridine. ADPR Adenosine diphosphoribose. APSX A derivative of adenosine 5′-Phospho-sulphate. ATP, CTP, GTP, UTP 5′-Triphosphates of adenosine, cytidine, guanosine, and uridine. CDPCHO Cytidine diphosphocholine. CDPEA Cytidine diphosphoethanolamine. CDPGLY Cytidine diphosphoglycerol. CMPEA Cytidine monophosphoethanolamine. F1P Fructose-1-phosphate. F6P Fructose-6-phosphate. FDP Fructose-1,6-diphosphate. Ga16P Galactose-6-phosphate. GDPM Guanosine diphosphate mannose. α-GP α-Glyceryl phosphate. β-GP β-Glyceryl phosphate. G1P Glucose-1-phosphate. G6P Glucose-6-phosphate. NAD Nicotinamide-adenine-dinucleotide. NMP Nicotinamide mononucleotide. NADP Nicotinamide-adenine-dinucleotide phosphate. PAE Phosphorylaminoethanol. PEP Phosphoenolpyruvate. PCHO Phosphorylcholine. 2-PGA 2-Phosphoglyceric acid. 3-PGA 3-Phosphoglyceric acid. PP Pyrophosphate. P_1 and P_2 Polymetaphosphates (?). PO_4 Inorganic phosphate. PyP Pyridoxal phosphate. R5P Ribose-5-phosphate. TPP Thiamine pyrophosphate; cocarboxylase. TreP Trehalose-6-phosphate. UDPAG Uridine diphosphate acetylglucosamine. UDPG Uridine diphosphate glucose. UDPGA Uridine diphosphate glucuronic acid. 10, 10 B Unknown compounds (10 possibly succinyl AMP). 15 Unknown non-nucleotide.

moves faster than the principal compound of the fraction. It is also a cytidine compound and its molar ratio base:ribose:phosphorus is 1:1:1. Ethanolamine and phosphorylethanolamine are released from both these compounds when hydrolysed with 1 N hydrochloric acid for 40 min at 100°C. These latter compounds have been identified paper chromatographically by comparison with commercial reference compounds. The main component of the fraction is CDP-ethanolamine. The compound present in minor proportions is evidently the corresponding CMP-compound. Because of the small amount present it has not been possible to carry out a rigorous identification. (The small UV-absorbing peak between CDPEA and CMP presented in the histogram of Fig. 2 is caused by tyrosine.)

CMP and DPN have been identified by co-chromatography and UV-absorption spectra. Towards the end of the DPN-peak some unidentified cytidine compound CX, the concentration of which was very low, emerged from the column. The compound was so labile that it decomposed before it could be investigated further.

AMP has been identified by its absorption spectrum and co-chromatography.

Succinyl adenine (Succ. A). After the AMP peak a compound is eluted from the column which contains neither ribose nor phosphorus. Its UV-

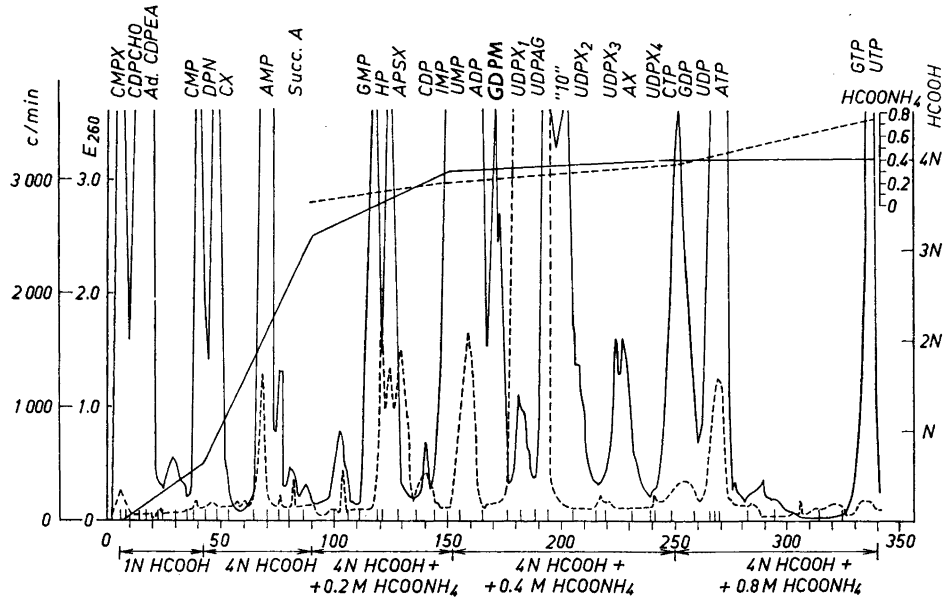

Fig. 2. Dowex-1 ion exchange chromatogram of an acid extract of *Candida utilis* yeast. Solid curve UV-absorption; dashed curve radioactivity. As eluents 1—4 N formic acid until fraction No. 90, then 4 N formic acid + ammonium formate in increasing concentrations. X means unknown derivative of the nucleotide indicated. Abbreviations explained in the text.

absorption spectrum is exactly the same as the one which Carter and Cohen [18] have presented for succinyladenine. It is a free base.

The peak corresponding to the fractions 95—105 (Fig. 2) also contained a compound which had a deep green colour (E_{max} 735 mμ) but not a UV-absorption maximum. The compound was labile and because of the small quantity present could not be identified.

GMP has been identified by its absorption spectrum, its location on paper chromatograms and on the basis of ribose and phosphorus determinations.

Hexose phosphates, HP. The radioactivity of fractions 120—135 is due to hexose monophosphates, all of which are eluted in this region. They have been identified by non-absorbability on Norite and 2-dimensional paper chromatography before and after hydrolysis.

APSX. This compound, which in paper chromatography moves very slowly in the first direction and fast in the second direction, has an adenosine-type spectrum but does not give a cyanide complex spectrum and hence is not TPN. When stored unlyophilized at —20°C it is decomposed into two adenosine derivatives, which move on paper identically with ADP and AMP. The original compound gives a positive reaction with Feigl's sulphate test.[16] The compound may be 3-phosphoadenosine-5′-phosphosulphate (PAPS).

CDP, IMP, UMP, and ADP have been identified by co-chromatography, UV-absorption spectra and ribose and phosphorus determinations.

GDPM. The main compound of this peak is *guanosine diphosphomannose.* It has been identified by co-chromatography, the UV-absorption spectrum of guanosine, by ribose and phosphorus determinations, and by co-chromatographic identification of the mannose released when the compound is hydrolysed with 0.01 N hydrochloric acid for 15 min at 100°C. This peak contained in addition another compound, *adenosine diphosphoribose* ADPR, which has been identified by its spectrum and by ribose and phosphorus determinations. This compound is probably an artefact, a decomposition product of DPNH created during the Dowex run.[19] In addition to the two compounds mentioned the fraction contained several decomposition products: GDP, GMP, a slight amount of UDP, and very small amounts of 3 unknown compounds.

Uridine diphosphoacetylglucosamine, UDPAG, and the unknown $UDPX_1$. In the region of this peak the inorganic orthophosphate begins to be eluted from the column, being present in the next 20 to 30 fractions. At the beginning of the peak some unknown UDP compound is eluted first, and after this UDPAG, which has been identified not only by its spectrum but also by co-chromatography of itself and of the glucosamine split off in hydrolysis (0.01 N HCl 15 min 100°C) with the respective authentic compounds.

$UDPX_2$. This peak contains an unknown UDP compound, which is decomposed when lyophilized, leaving only UDP and UMP detectable.

Unknown No. 10. The spectrum of the compound present in these fractions is the same as that presented by Carter and Cohen [18] for succinyl adenosine monophosphate. For the molar ratio base:phosphorus we obtained 1:1.2. A strongly acidic hydrolysis (6 N HCl 1 h, 100°C) split AMP and adenine from the compound. After this hydrolysis the sulphate test (of Feigl) gave a positive reaction. On paper chromatography the compound was divided into two spots "10" and "10B"; the latter had an adenosine spectrum and the

ratio base: phosphorus was 1:1. "10B" also contained sulphate. On the basis of this information the compounds have been identified as succinyladenosine-5′-phosphosulphate (SAPS) and adenosine-5′-phosphosulphate (APS). Succinyl-adenosine-5′-monophosphosulphate has been found by Tsuyuki *et al.*[20] in the sperm of salmon. Carter and Cohen[18] isolated succinyl-adenosine-5′-monophosphate from yeast.

$UDPX_3$ and $UDPX_4$ are UDP-sugar compounds, which were decomposed during the treatment to such an extent that it was not possible to identify the sugar components.

AX is an unknown compound which has the spectrum of adenine and is decomposed during treatment. It might be ADPRP, which is produced as a decomposition product of TPNH in the Dowex column[19] or a cyclic A3,5DP.[25]

CTP, GDP, UDP, ATP, GTP, and UTP have all been identified through their UV-absorption spectra and ribose and phosphorus determinations. Along with GTP and UTP several phosphorus compounds are eluted from the column, compounds, which do not move at all with the basic solvent in paper chromatography. These compounds are probably various polyphosphates (Fig. 1, P_1 and P_2).

DISCUSSION

The methodology used here has several weak points. Ion exchange chromatography carried out on a preparative scale has not sufficient resolution for the separation of individual compounds, and so rechromatography is necessary. Removal of ammonium formate is cumbersome, but can be achieved by cation exchange, using a resin in H^+ form. Even so, formic acid causes hydrolysis and the most labile compounds are lost. Several labelled compounds, in addition to those reported, were observed in our original extracts. These compounds were decomposed during chromatography and therefore could not be studied in detail. In this paper we have only described compounds whose identity we believe to have been proved with reasonable reliability. Identification is mostly based on co-chromatography with an authentic preparation, identification of the decomposition products and UV spectrum at different pH's. Once the compounds have been isolated from an organism and their structure has been elucidated, a chromatographic map can be compiled, which is very useful in metabolic studies. Location on the chromatogram, fortified by co-chromatography and other specific tests, is then often sufficient for identification. This has been the aim of this study.

Since this study was completed, papers have appeared on thin-layer chromatography of nucleotides[21] and sugar phosphates.[22] The resolution seems to be of about the same order as in the paper chromatographic technique used in this study, or perhaps even slightly better. In addition, thin-layer chromatography has the great advantage of being more rapid and sensitive.

Ion exchange column chromatography has been automatized in recent years and separations superior to ours can be obtained at least with pure nucleotides[23] at pH 4 to 4.5. Work with rather small amounts of extract is necessary for good resolution, however. On the other hand, ion exchange chromatography has been used for fractionation of yeast nucleotides on a

much larger, semi-industrial scale.[7] In addition, Ingle [24] has studied the soluble nucleosides in yeast and Su and Hassid [25] in the red alga *Porphyra perforata*, by ion exchange chromatography. Ingle only identified the main components, but Su and Hassid separated from the alga, in addition to the common nucleotides, the cyclic adenosine-3′,5′-pyrophosphate, GDP-mannose, GDP-galactose, UDP-glucose, UDP-galactose, and UDP-glucuronic acid, of which we were able to identify only GDP-mannose and UDP-glucose in yeast. It is quite possible, however, that our AX is the above cyclic ADP and that the other three phosphates were among the compounds decomposed before or during lyophilization. These three nucleotide-sugars are partially overlapped by other nucleotide-sugars in our paper chromatographic system and would not be easy to identify in the original extract. It is quite possible that if all the operations could be carried out at nearly neutral pH and at low temperature, some ten more nucleotide derivatives could be demonstrated in the food yeast used in this study.

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