DYNAMIC ASPECTS OF THE NUCLEOTIDE POOL OF BREWER'S YEAST DURING GROWTH

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SUMMARY

Chromatography of ethanolic and aqueous trichloracetic acid extracts prepared successively from a particular strain of yeast at various stages of growth revealed, in addition to nucleotides already recognised in other yeasts, a number of new compounds of this nature including derivatives of uridine diphosphate, various nucleotide-peptide anhydrides which may be intermediates in protein synthesis, and polynucleotides. During the onset of growth of the yeast the pool of alcohol-soluble nucleotides decreased, while the reservoir of nucleotides soluble in trichloroacetic acid increased; but later a steady state was attained. The consequent hypothesis that the alcohol-soluble compounds are precursors of the nucleotides soluble in trichloracetic acid, which in turn are converted into insoluble compounds, was supported by the results of experiments in which the uptake and subsequent metabolism of [^{14}C]adenine and uracil were examined. Entry of [^{14}C]uracil into a nucleotide-peptide anhydride was observed.

INTRODUCTION

Recent investigations[1,2] have shown that yeasts contain nucleotide pools analogous to the amino-acid pools detected earlier[3,4]. Furthermore[5], study of the incorporation of [^{14}C]purines into the nucleic acids of *Candida utilis* have shown that the adenine of the soluble nucleotides was labelled far more extensively than that of the nucleic acids, implying that the nucleotides have a greater turnover rate than the nucleic acids. Both added purines and nucleotides are ultimately incorporated into the nucleic acid of *C. utilis*, the acid-soluble nucleotides forming direct precursors of nucleic acid[6].

In order to gain further insight into the synthesis of nucleic acids and proteins in yeasts, in particular those used in brewing, an investigation of the nucleotide pool of a selected strain during aerobic growth has been made.

Abbreviations: DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide; AMP, adenosine-5′-monophosphate; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate; GMP, GDP and GTP, guanosine-5′-mono-, di-, and triphosphate; UMP, UDP, and UTP, uridine-5′-mono-, di-, and triphosphate; CMP, CDP, and CTP, cytidine-5′-mono-, di-, and triphosphate; UDPAG, uridine diphosphate acetylglucosamine; UDPG, uridine diphosphate glucose; TCA, trichloroacetic acid.

MATERIAL AND METHODS

Culture of yeast

Saccharomyces cerevisiae (No. 240, British National Collection of Yeast Cultures) was grown in shake culture in sterile hopped wort (sp. gr., 1.040) for 48 h and was then centrifuged off and washed with water as quickly as possible (20 min). An aliquot portion was removed for extraction and analysis as described below and further samples (6 g fresh weight) were weighed into separate batches (250 ml) of the same brewers' wort in Roux bottles and growth was allowed to proceed with shaking at 24°. The individual crops of yeast were rapidly removed after various time intervals by filtration on kieselguhr pads[7]. It is relevant to note that in earlier investigations[8] the yeast was centrifuged off as rapidly as possible, but comparison of ion-exchange chromatograms of the extracted nucleotides (see below) with those prepared from extracts of filtered yeast showed that some decomposition of nucleotides, particularly adenosine triphosphate, was incurred in the earlier procedure.

Extraction of nucleotides from yeast

Each yeast crop was killed immediately by admixture with powdered carbon dioxide and treated with ethanol (1 ml/g yeast); the mixture was then allowed to come to room temperature and was then warmed for 3 min at about 50° to complete extraction of nucleotides, after which the yeast residue was filtered off. This residue was warmed twice more with 50 % ethanol (2 ml/g original yeast) and the ethanolic extracts were combined and stored at 0° prior to analysis.

Each yeast residue was dried by successively washing with ethanol, ethanol–ether mixture (1:1, v/v) and ether and stored at 0° prior to extraction with 5 % TCA. The TCA was cooled to 0°, mixed with the yeast residue (1 ml/g original yeast) and the suspension frozen and kept overnight at —15°. After thawing, the residue was immediately filtered off and the bulk of the TCA rapidly extracted from the filtrate by shaking (3 × 0.5 vol), the remaining TCA being removed by continuous extraction with ether for 16 h. The aqueous solution was stored at 0° in the presence of a few drops of chloroform until required for analysis.

Chromatographic separation of nucleotides

Aliquot portions of the ethanolic extract corresponding to 4 g of original yeast at each growth stage were loaded individually on to Dowex 1-X4 (formate) columns (20 cm × 1.77 cm^2) and the nucleotides eluted successively in a formic acid–ammonium formate gradient as described by GILBERT AND YEMM[7], the eluate being collected in 5 ml fractions and the u.v. light absorption at 260 mμ of the fractions taken as a measure of their nucleotide content.

Aliquot portions of the TCA extracts corresponding to 18 g of original yeast were similarly chromatographed on Dowex 1-X4 (chloride) columns as described by PONTIS AND BLUMSON[9].

Identification of nucleotides

The nucleotides were provisionally identified by comparison of their positions on the ion-exchange chromatograms and their u.v. light absorption spectra at various pH values with those of authentic nucleotides. In this connection, however, it was frequently impossible to measure complete absorption spectra directly on the eluates

from the ion-exchange columns, particularly the solutions containing formate ions, which themselves absorb u.v. light strongly at wavelengths less than 245 mμ. The eluates obtained from the Dowex chloride columns did not suffer from this disadvantage but, it was on the other hand often not possible to measure their u.v. absorption in alkali owing to their becoming opaque.

Further characterization of the nucleotides was effected by (a) electrophoresis on paper using acetate buffer solution at pH 4.0 and a voltage gradient of 10–20 V/cm, (b) chromatography on paper in the solvent system II of KIRBY[10] and the isopropanol–hydrochloric acid solvent of WYATT[11] and (c) hydrolysis to the constituent purine base or pyrimidine nucleotide by means of 6 *N* hydrochloric acid at 100°. The purine bases and pyrimidine nucleotides thus formed were themselves identified by two-dimensional chromatography on paper using solvent systems composed of *n*-butanol–acetic acid–water (4:1:1, v/v) and *n*-butanol–ethanol–2 *N* ammonia (20:2:5, v/v) respectively[12]. The solvent composed of isopropanol and hydrochloric acid[11] was also employed.

The nucleotides and derived bases were detected on paper chromatograms and electropherograms by means of their absorption of u.v. light at 254 mμ.

Incorporation of labelled purines and pyrimidines

[8-^{14}C]adenine and [2-^{14}C]uracil were employed separately at the rate of 0.4 μmole/ml of wort. Yeast (0.5 g) was inoculated into wort (50 ml) containing the labelled base and grown up aerobically for 30 h in shake-culture at 24°. Aliquot portions (3 ml) of the suspension were removed from time to time; the yeast was removed from each by filtration through a thin kieselguhr pad and washed twice with distilled water and the mixture of yeast and kieselguhr was placed immediately in a mixture of 60 % ethanol and solid carbon dioxide. The whole procedure of collection, washing and inactivation of the yeast took about 1 min. Further extraction of the yeast with aqueous ethanol was effected as before and the whole extract was evaporated in the cold to a small volume, which was then made up to 10 ml. The yeast debris was extracted with aqueous TCA as before and the extract brought to 10 ml.

In further experiments labelled adenine or uracil was added to the yeast at different stages of growth using 20 ml of wort, 0.25 g of yeast and 4 μmole of labelled uracil or 10 μmole of labelled adenine.

Measurement of radioactivity

For measurement of radioactivity the growth medium and corresponding yeast extracts (0.5–1.0 ml) were individually mixed with cellulose powder, which was dried, combusted and the disintegrations of the carbon dioxide formed counted in a gas counter. Chromatograms and electropherograms of medium and extracts were scanned for radioactive components, using an end-window counter and autoradiographs prepared using Kodak X-ray no-screen film and a contact time between chromatogram and film of 10–20 days.

RESULTS AND DISCUSSION

The nucleotide content of the yeast at various times during aerobic growth is shown diagrammatically in Fig. 1, (a) and (b), (a) referring to the ethanolic extract and

(b) to the TCA extract. Incidentally, the advantage of employing the dual extraction procedure rather than direct extraction of the total nucleotides in aqueous TCA or perchloric acid as commonly carried out, is manifest, because the ethanolic solvent is more selective and the interpretation of the ion-exchange chromatograms is therefore simplified.

The ethanolic extracts of the yeast (Fig. 1 (a)) contained AMP, ADP, ATP, GMP, GDP, GTP, UMP, UDP and UTP as previously observed by SCHMITZ[1] and by AYENGAR *et al.*[13], together with CMP, DPN, TPN and various other substances among which were provisionally identified UDPAG and UDPG. It is apparent that, as in *C. utilis*[7], cytidine diphosphate and cytidine triphosphate must be present in relatively small concentration in the ethanol extracts, as no discrete peaks due to them were discerned. The previous observation[8] that the bulk of the pyridine nucleotide in the initial yeast was TPN, was not borne out in the present investigation and the requisite conditions for obtaining only TPN in the culture were not found again.

Only small amounts of nucleotide derivatives reactive towards hydroxylamine[14] as detected by HARRIS *et al.*[8] were recovered from Dowex 1 (formate) columns, because they are not adsorbed as they are when Dowex 1 (chloride) is used (*cf.* DAVIES AND HARRIS[12]).

The TCA extracts (Fig. 1 (b)) contained a number of nucleotides, none of which was identified with any known compound. Several of the earlier peaks however had

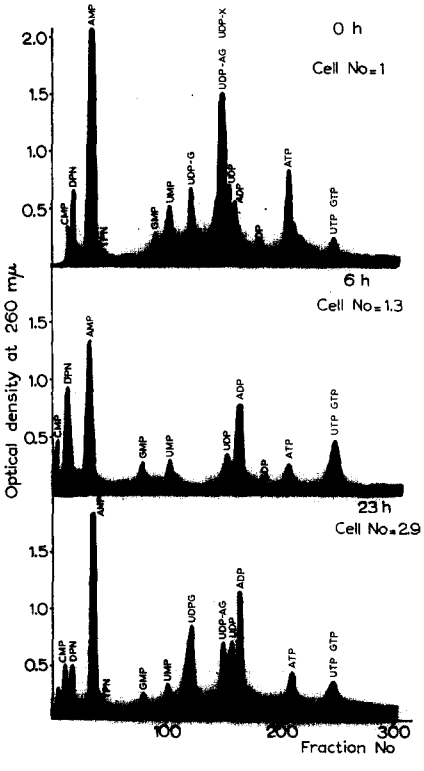

Fig. 1 (a). Ethanol-soluble nucleotide content of yeast at various stages of growth (for details see text).

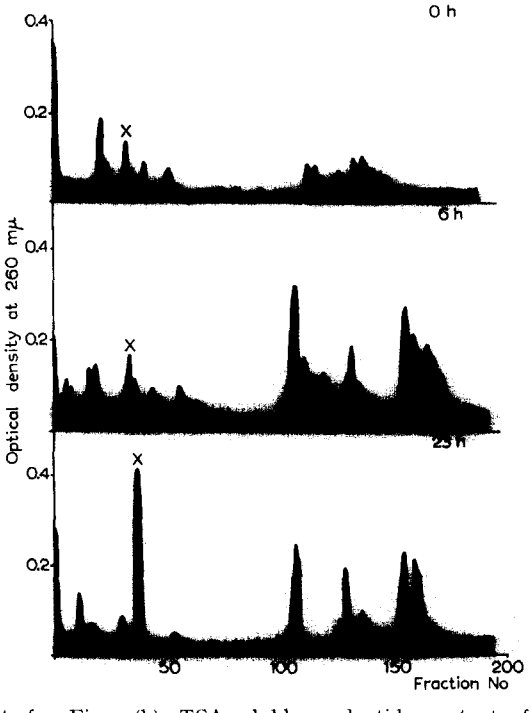

Fig. 1 (b). TCA-soluble nucleotide content of yeast at various stages of growth (For details see text).

u.v. absorption spectra like that of cytosine but one of the major constituents (X in Fig. 1(b)) had a spectrum like that of uridine. This component is probably identical with the substance 17 noted by HARRIS *et al.*[8]. The large peaks eluted later and particularly prominent in the 6 and 23 h samples were, on the other hand, complex, because on electrophoresis the bulk of u.v. absorbing material migrated towards the anode only as a diffuse band, and on hydrolysis with hydrochloric acid it yielded a mixture of adenine, guanine, uridylic acid and cytidylic acid. Moreover, in many cases the original peak substances contained not only negatively-charged nucleotides but also nucleotide-peptide anhydrides migrating towards the cathode[8,12], these being present because the TCA extracts were chromatographed on the chloride form of Dowex-1.

The nucleotide pattern of the yeast extracts after 6 h development of the cells was of particular interest, because it represents an early stage in the onset of growth. The amounts per cell of the majority of the alcohol-soluble nucleotides, with the exception of DPN, ADP and the UTP-GTP peak, had undergone a considerable reduction as observed earlier[8]. In view of the well-established role of ATP in energy transfers associated with synthetic processes in the cells, its depletion and the accompanying increase in ADP concentration may well reflect the demands of the onset of these synthetic processes. The decrease observed in other nucleotides, *e.g.*, UDPAG, also probably indicates the draining off of raw materials for the synthesis of proteins, nucleic acids and cell-wall components[15–17] at this stage of the life cycle of the yeast. Correspondingly, a large increase both in amount and diversification of the nucleotides extracted by TCA was observed. The later samples of yeast contained nucleotide pools which resembled more closely the original material.

From the above data it appeared possible that the nucleotides soluble in TCA were formed wholly or in part from the alcohol-soluble compounds. An attempt to gain information on this point was made using [^{14}C]adenine, uracil and thymine. It was shown in preliminary trials that the two former bases were taken up by the strain of yeast used. In the present experiments nearly 80 % of the added radioactivity in adenine was taken up by the yeast in 30 h and the only radioactive compound apart from adenine detected in the medium was a trace of adenosine. The progress of uptake of radioactivity is shown in Fig. 2 and agrees well with the results previously obtained by HARRIS AND PARSONS[18] using non-labelled adenine. The distribution of the absorbed radioactivity between ethanol-soluble, TCA-soluble, and insoluble material within the cell is given also in Fig. 2 on the basis of percentage of absorbed radioactivity, while the distribution of activity in these fractions in the total culture and in unit weight of cells is shown in Fig. 3. It is apparent that the

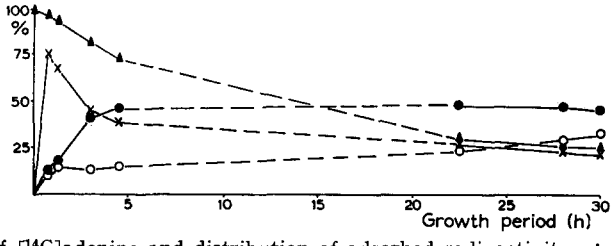

Fig. 2. Uptake of [^{14}C]adenine and distribution of adsorbed radioactivity. ▲▲▲, % of added radioactivity remaining in medium; × × ×, % distribution of adsorbed radioactivity between ethanol extract; ● ● ●, TCA extract; O O O, insoluble residue.

radioactivity of all fractions increased from the time of addition, a large proportion of the absorbed activity initially entering ethanol-soluble material. After 1 h the relative activity of this fraction (Fig. 2) then declined, and that of the TCA extract increased, during a period (1–4 h) in which little relative increase of activity in insoluble material took place, although the absolute values (Fig. 3) were of course continuing to increase throughout. During the logarithmic phase of growth (4–24 h) the relative activity of the insoluble material continued to increase, while that of the TCA-soluble fraction was steady and that of the alcohol-soluble fraction fell, presumably because a steady rate of turnover of the acid-soluble material occurred. In the later phases, synthesis of insoluble material continued at the expense of both alcohol-soluble and acid-soluble fractions.

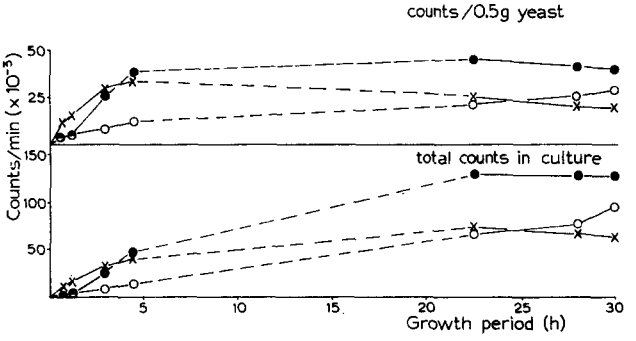

Fig. 3. × × ×, radioactivity present in ethanol extract; ● ● ●, TCA extract; ○ ○ ○, insoluble residue resulting from uptake of [^{14}C]adenine.

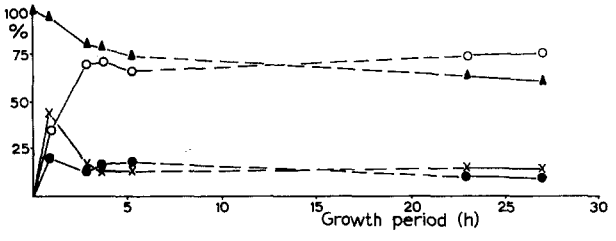

Fig. 4. Uptake of [^{14}C]uracil and distribution of adsorbed radioactivity. ▲ ▲ ▲, % of added radioactivity remaining in medium; × × ×, % distribution of adsorbed radioactivity between ethanol extract; ● ● ●, TCA extract; ○ ○ ○, insoluble residue.

The results for uracil were different in that a much smaller proportion of the added radioactivity was ingested by the yeast (Fig. 4) and that of the ingested base, a much higher proportion, promptly appeared in the insoluble and TCA-soluble fractions than was the case with adenine. Only in the first hour was the proportion soluble in ethanol greater than that in the other fractions, and thereafter the bulk of the radioactivity ingested was found in insoluble material (*cf.* Fig. 5).

Using labelled thymine of the same level of radioactivity as the other bases, only 0.5 % of the added thymine entered the yeast after 24 h and it therefore appears that the yeast has an impediment to the entry of this pyrimidine.

It is apparent that the above results for adenine and uracil support the earlier suggestion that in the initial phases of uptake of adenine and uracil the alcohol-soluble pool functions as a precursor of the TCA-soluble pool of intermediates.

Confirmation and extension of this result was obtained by adding labelled adenine and uracil to the yeast at different times early in development and comparing the uptake of these bases into the various fractions at these different times. The results are shown in Fig. 6 (a) and (b) from which it may be seen that the relative rate of uptake of labelled adenine into the TCA-soluble fraction was much slower than that into the alcohol-soluble materials during the first 2–4 h, but that later (4–8 h) it increased and subsequently predominated as growth proceeded. The results for uracil were similar, except that, as before, the relative incorporation into the acid-soluble fraction was much greater.

The transfer of activity from radioactive base to ethanol-soluble nucleotide, and in turn to TCA-soluble nucleotide, even during the very early period of growth, shows,

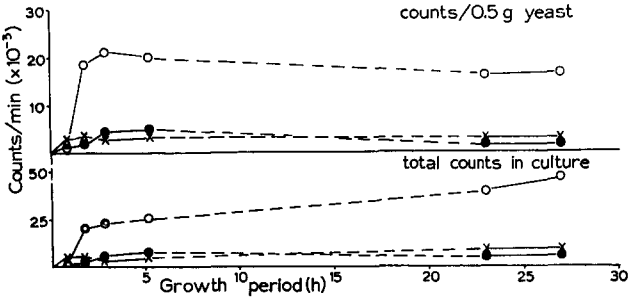

Fig. 5. × × ×, radioactivity present in ethanol extract; ● ● ●, TCA extract; O O O, insoluble residue resulting from uptake of [^{14}C]uracil.

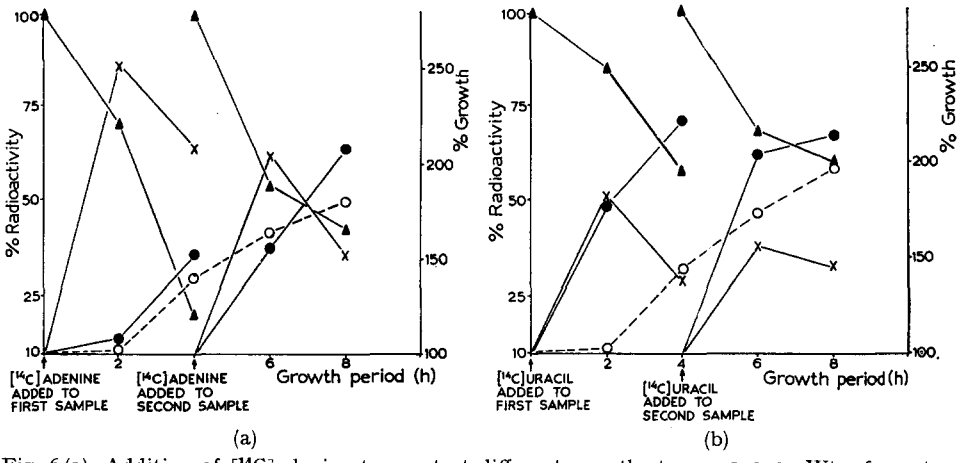

Fig. 6(a). Addition of [^{14}C]adenine to yeast at different growth stages. O O O, Wt. of yeast as a % of wt. of original inoculum; ▲ ▲ ▲, % of added radioactivity remaining in medium; × × ×, % distribution of total radioactivity extracted between ethanol; ● ● ●, TCA extracts. Fig. 6(b). Addition of [^{14}C]uracil to yeast at different growth stages. Details as for Fig. 6(a).

not only that the ethanol-soluble materials act as precursors of the acid-soluble compounds, but also that a substantial synthesis of nucleotides (see below) takes place during the lag phase of growth.

In the case of adenine particularly, the results observed might have been due simply to the accumulation of large amounts of free adenine in the alcohol-soluble

fraction in view of the rapid incorporation of this purine into the cell. However, it was remarkable that a large proportion of the absorbed adenine was converted into nucleotide very rapidly. This fact was established by two-dimensional chromatography of the alcohol-soluble fractions and estimation of the radioactivity present in the various zones. The results are shown in Fig. 7(a) from which it is seen that after only 1¼ h 46 % of the adenine was in the form of nucleotide, the remainder being in the form of the free base (8 %) and corresponding nucleoside (16 %) together with guanine[5] (7 %), guanosine (10 %) and, interestingly enough, the degradation product hypoxanthine (7 %). The compounds A and B (Fig. 7(a)) are presumably also degradation products, because they are more mobile than adenine in the chromatographic solvents used and are probably, therefore, of relatively low molecular weight. Despite

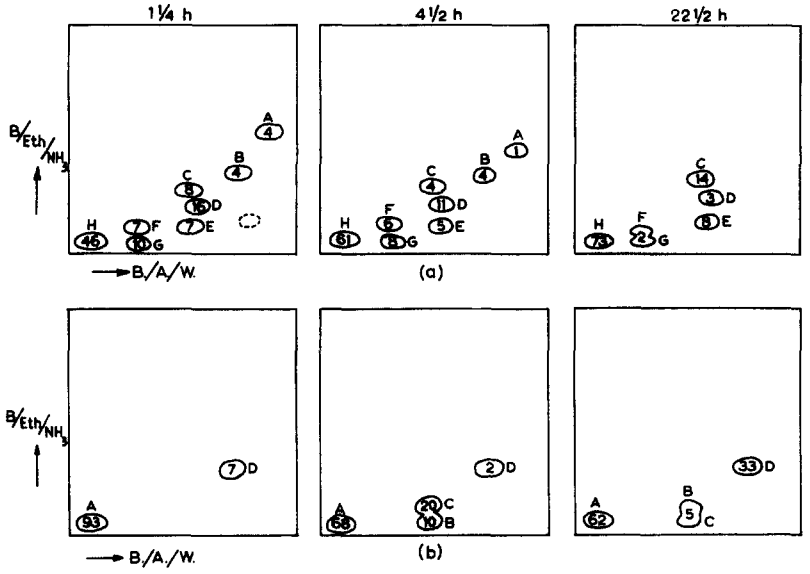

Fig. 7(a). % distribution of radioactivity in ethanol extracts of yeast, [^{14}C]adenine added at 0 h. A and B = ?; C, adenine; D, adenosine; E, hypoxanthine; F, guanine; G, guanosine; H, nucleotides.
Fig. 7(b). % distribution of radioactivity in ethanol extracts of yeast, [^{14}C]uracil added at 0 h. A, nucleotides; B, nucleotide-peptide; C, uridine; D, uracil.

the continuing ingestion of adenine during development of the yeast the proportion of nucleotide continued to increase, reaching 61 % after 4.5 h and 73 % after 22.5 h in this series. The radioactivity of the TCA-soluble fraction was contained almost entirely in nucleotides, the bulk of which, as already indicated for the fractions separated by ion-exchange chromatography, migrated towards the anode on electrophoresis at pH 4. This material gave on hydrolysis both labelled adenine and labelled guanine, as was anticipated from the earlier-observed conversion of the former to the latter purine[5].

As was expected from the more rapid rate of incorporation of uracil into the insoluble and TCA-soluble fractions, an even greater proportion (93 %) of uracil was located in nucleotides (Fig. 7(b)) and only 7 % remained free after 1 h. However, as uracil uptake proceeded throughout this trial, the proportion of chromatographically-immobile nucleotide decreased and after 4 h 20 % of the nucleoside uridine was

observed, while after 23 h 33 % of the radioactivity in the ethanol-soluble fraction was present as uracil itself, presumably because the cell's requirements for this base for synthetic processes had by then been satisfied. Particularly noteworthy was the observation, made independently by measuring the combined radioactivity of the one zone containing uridine and nucleotide-peptides on electropherograms of the alcohol-soluble fractions, and substracting the activity of separated uridine as measured on the two-dimensional chromatograms (Fig. 8b), that as much as 10 % of the labelling was in the form of the nucleotide-peptides mentioned earlier. These nucleotide-peptides on alkaline degradation[12] yielded labelled uridine-5'-phosphate only. It appears that nucleotide-peptides derived from uridine may have a special significance in yeast, presumably as forerunners of proteins, and in the extracts examined they were found to exceed in quantity similar materials derived from adenine. Nevertheless, at the 4 h stage of growth of the cells 90 % of the total labelling derived from uracil in the ethanol extracts is in the form of other nucleotides, of which a major proportion must consist of the uridine compounds shown in Fig. 1. In this connection the specific incorporation of uridine derivatives into ribonucleic acid comes to mind, but it remains to be seen what part is played by the alcohol-soluble uridine compounds in the biosynthesis of this nucleic acid in yeast.

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