STUDIES ON DEOXYNUCLEOSIDIC COMPOUNDS

II. DEOXYCYTIDINE DIPHOSPHATE CHOLINE IN SEA URCHIN EGGS

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SUMMARY

1. A new deoxynucleotide derivative was found in sea urchin eggs and was identified as deoxy-CDP-choline.

2. Deoxy-CDP-choline was synthesized chemically.

3. In addition to deoxy-CDP-choline, a large amount of CDP-choline was found in sea urchin eggs.

4. The structural characteristics and biological significance of the "masked" deoxynucleosidic compounds including deoxy-CDP-choline have been discussed.

INTRODUCTION

In the first paper of this series, a modified microbiological assay method for deoxynucleosidic compounds was reported[1,2]. The characteristic feature of the modified method is the digestion of the samples with snake venom enzyme prior to the microbioassay. By applying this method to the acid-soluble extracts of eggs and embryos of the sea urchin and tissues of the rat, a group of deoxynucleosidic compounds was found which would not have been detected if the usual microbiological method had been used. They were tentatively designated as "masked" deoxynucleosidic compounds[1,2] so as to distinguish them from simple deoxynucleosides and deoxynucleotides, which are microbiologically active without any pretreatment[3].

Soon afterwards, one of the "masked" deoxynucleosidic compounds of sea urchin eggs was isolated and identified as deoxy-CDP-choline[4].

This paper is devoted to giving the full details of its isolation, identification, and chemical synthesis. Some discussion of the chemical nature and biological significance of the "masked" deoxynucleosidic compounds is also presented.

METHODS AND MATERIALS

Determination of deoxynucleosidic compounds. Simple and "masked" deoxynucleosidic compounds were assayed microbiologically as previously described[1].

Abbreviations: deoxy-CDP-choline, deoxycytidine diphosphate choline; CDP-choline, cytidine diphosphate choline; CMP, cytidylic acid; deoxy-CMP, deoxycytidylic acid; P-choline, phosphoryl choline; DNA, deoxyribonucleic acid; Dd-CPP, dideoxycytidine pyrophosphate; DCC, dicyclohexyl carbodiimide; AF, ammonium formate; PCA, perchloric acid.

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Determination of choline. Choline was assayed microbiologically using cholineless *Neurospora* mutant[5]. Prior to the microbioassay, samples were digested with snake venom. Conditions for the venom digestion were the same as those used for the assay of "masked" deoxynucleosidic compounds[1].

Ultraviolet-absorption measurement was carried out with a Beckman DU spectrophotometer, using the quartz cell of 1 cm light path.

Phosphorus was determined by the FISKE–SUBBAROW method[6]. Absorption at 660 mμ was read in a Coleman junior spectrophotometer.

Paper chromatography was carried out on Toyo No. 7 or Azumi No. 7 paper using the following solvent systems: (1) 0.02 *N* acetic acid in 60% ethanol[7]; (2) *n*-propanol–ammonia (d 0.88)–water (7:3:1); (3) saturated $(NH_4)_2SO_4$–water–isopropanol (79:19:2)[8]; (4) ethanol (95%)–ammonium acetate (pH 7.5, 1 *M*) (7.5:3)[9].

Ultraviolet-absorbing spots were located by Matsuda germicidal lamp (GL-15) fitted with Corning No. 9863 filter. Choline was detected on paper by spraying with Dragendorff reagent[10].

Paper electrophoresis. The following buffers were used: (1) 0.05 *M* ammonium formate, pH 3.5; (2) 0.05 *M* sodium phosphate, pH 7.8; (3) 0.05 *M* sodium borate, pH 9.3.

Ion-exchange chromatography. Dowex-1-formate resins were used. Details of the column operation are described in RESULTS.

Acid-soluble extracts were prepared as described previously[1] from the lyophilized powder of unfertilized eggs of the sea urchin, *Hemicentrotus pulcherrimus*.

The lyophilized egg powder was kindly provided by Dr. R. OKAZAKI of Nagoya University.

Chemicals

Deoxycytidine*, cytidine, deoxy-CMP*, CMP, and choline chloride were commercial preparations. CDP-choline[7] was the gift of Dr. E. P. KENNEDY of University of Chicago. Calcium salt of P-choline was given by Mr. S. IWANAGA of Kyoto University. The calcium salt was converted to the free acid by treatment with Dowex-50-H^+. DCC was supplied by Dr. K. OKAWA. of Osaka University.

RESULTS

Ion exchange fractionation of acid soluble deoxynucleosidic compounds of sea urchin eggs

Cold PCA extracts of 800 mg egg powder was neutralized with KOH to pH 8.5. After removal of K-PCA the neutralized extract was passed through a column of Dowex-1-formate. The column was washed with water until the optical density at 260 mμ of the effluent disappeared, and then eluted with various concentrations of AF. The progress of elution was followed by measuring the optical density at 260 mμ (Fig. 1). 14 bulked fractions were obtained by combining tubes as indicated on the abscissa of Fig. 1. Each bulked fraction was concentrated, lyophilized to remove AF, dissolved in a given amount of water, and assayed for the content of deoxynucleosidic compounds. The results are summarized in Table I, which shows that three fractions, Nos. 5, 11 and 13 contained large amounts of "masked" deoxynucleosidic compounds. Of these, Fraction No. 5 aroused interest because its ultraviolet absorption spectra

* Products of California Corporation for Biochemical Research.

in acid and alkali were very similar to those of CMP. However, in order to decide whether or not the deoxynucleosidic compound contained in Fraction No. 5 is a derivative of deoxy-CMP, further purification using more starting material was needed, since the fraction was undoubtedly contaminated with a large amount of a ribosidic compound(s).

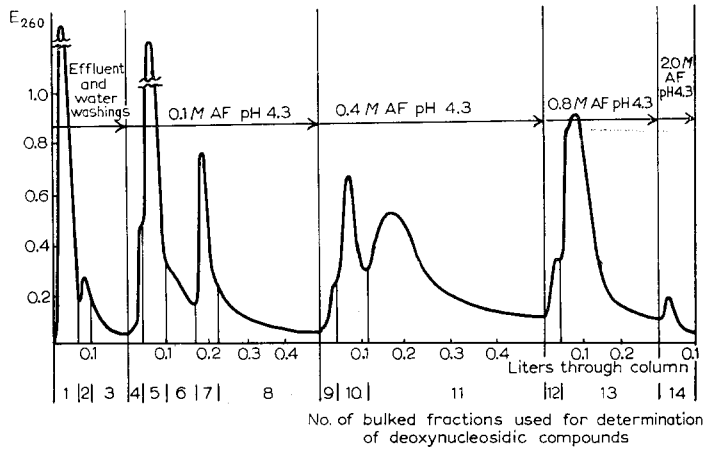

Fig. 1. Ion-exchange fractionation of acid-soluble deoxynucleosidic compounds of sea urchin eggs Sample: neutralized acid-soluble extracts of 800 mg of the egg powder. Exchanger: Dowex-1-formate, X-2, 200–400 mesh, 1 × 24 cm. Elution: as indicated. Amounts of deoxynucleosidic compounds in each bulked fraction are shown in Table I.

TABLE I

AMOUNT OF DEOXYNUCLEOSIDIC COMPOUNDS IN EACH BULKED FRACTION OBTAINED BY ION-EXCHANGE CHROMATOGRAPHY IN STEP I

Fraction No.	*Concn. of AF (M)*	*Deoxynucleosidic compounds*		
		u (before enzyme treatment)*	*t* (after enzyme treatment)*	*t — u* "masked"*
1		5	5	0
2	0	0	5	5
3		0	10	10
4		0	0	0
5		0	200	200
6	0.1	0	28.5	28.5
7		5	21.0	16.0
8		27.5	55.0	27.5
9		0	25.5	25.5
10	0.4	0	10	10
11		22.5	260	237.5
12		0	0	0
13	0.8	0	110	110
14	2.0	0	0	0

* Expressed as mμmole thymidine equivalent.

Purification and isolation of the "masked" deoxynucleosidic compound found in Fraction No. 5

Step I. Acid-soluble extracts of 8.6 g of the lyophilized egg powder, which contained 0.58 μmole and 8.7 μmoles of simple and "masked" deoxynucleosidic compounds respectively, were chromatographed in a similar manner to the above but different in that the column size was enlarged. Tubes corresponding to Fraction No. 5 were combined, concentrated, and lyophilized to remove AF. The residue was dissolved in 50 ml of water to make a fraction, F-I, which showed a spectrum similar to CMP and contained 3.35 μmoles of deoxynucleosidic compound as a "masked" form.

Step II. Fraction F-I of Step I was adjusted to pH 8.5 with aqueous ammonia and applied to a column of Dowex-1-formate. This time, the column was eluted by gradient elution with formic acid (Fig. 2). The deoxynucleosidic compound under con-

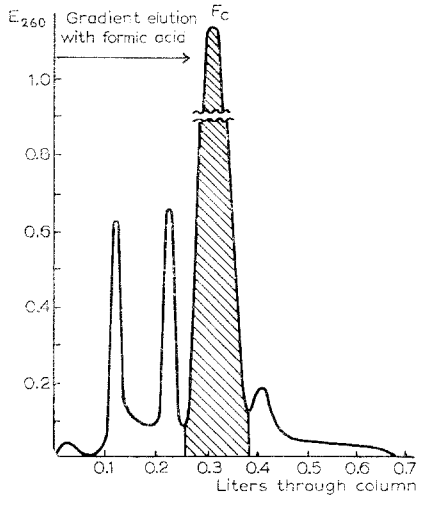

Fig. 2. Ion-exchange chromatography in Step II. Sample: F-I of Step I. Exchanger: Dowex-1-formate, X-2, 200–400 mesh, 1 × 28 cm. Gradient elution: mixer contained 500 ml of water and reservoir 0.02 *N* formic acid.

sideration was eluted together with the main ultraviolet-absorbing peak F_C (indicated as a shaded area in Fig. 2). F_C showed a CMP-like spectrum and contained 2.49 μmoles of "masked" deoxynucleosidic compound. At this purification stage, however, the amount of the deoxynucleosidic compound estimated microbiologically as deoxycytidine was not more than one-tenth of that calculated from the optical density measurement. This apparent discrepancy indicated that F_C still contained a considerable amount of a ribosidic compound which seemed to be a CMP-derivative. Therefore, to separate effectively the deoxynucleosidic compound from the contaminating ribosidic one, the next purification procedure was designed to utilize a borate system in ion-exchange chromatography, and it was found to be successful.

Step III. Fraction F_C was added to two volumes of water, adjusted to pH 8.5 with aqueous ammonia, and then applied on a column of Dowex-1-formate. All ultraviolet-absorbing materials were adsorbed on the resin. After washing with water, it was eluted by gradient elution with AF, pH 7.8, containing sodium borate, the

concentration of the former being gradually increased and that of the latter gradually decreased (Fig. 3). By this procedure, two separate fractions, F_1 and F_2, were obtained, both having spectra similar to CMP. As seen in Fig. 3, microbioassay of each chromatographically separated fraction made it clear that Fraction F_1 was the "masked" deoxynucleosidic compound in question. This result together with the result of spectral measurement suggested that F_1 would be a derivative of deoxy-CMP and F_2 a ribosidic analogue of F_1. It should be mentioned here that, in this ion-exchange chromatography, F_1 was eluted slightly faster than F_2 and both fractions emerged from the column much more rapidly than authentic deoxy-CMP and CMP, indicating the less anionicity of the former (F_1 and F_2) than the latter (Fig. 3).

F_1 and F_2 were further purified by rechromatography with the same borate-containing system, and finally by rechromatography with the formic acid system described in *Step II*. Formic acid was removed by lyophilization. F_1 thus obtained

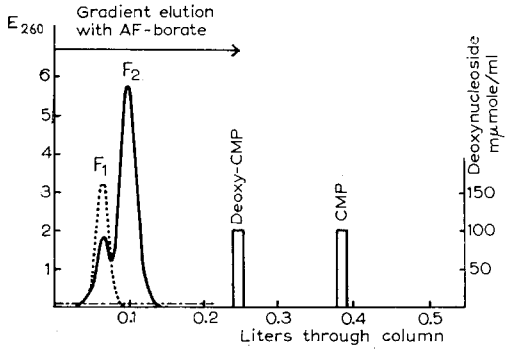

Fig. 3. Ion-exchange chromatography in Step III. Sample: Fc of Step II. Exchanger: Dowex-1-formate, X-8, 200–400 mesh, 1 × 10 cm. Gradient elution: mixer contained 500 ml of 0.0005 *M* sodium borate, pH 7.8 and reservoir 0.5 *M* AF, pH 7.8. ——, chromatogram followed by optical density measurement. - - - - -, chromatogram followed by microbiological measurement of deoxynucleosidic compound after venom digestion. – · – · – · –, the same as above but before venom digestion. The two columns indicate the positions where authentic samples of deoxy-CMP and CMP emerge.

was shown by paper chromatography to be contaminated with a trace amount of F_2, whereas the preparation of F_2 was free of F_1. The yield of the "masked" nucleosidic growth activity in this step was about 80 %.

Step IV. The lyophilized preparation of F_1 of Step III was dissolved in a small amount of water, applied on a sheet of Toyo No. 7 paper as a band, and developed with Solvent 1. The main faster band of F_1, located by ultraviolet lamp, was cut out, eluted with water, and lyophilized. This final preparation was completely free of F_2 and migrated as a single spot in paper chromatography and paper electrophoresis carried out under various conditions (see the next section). Its deoxynucleosidic growth activity, determined microbiologically after venom digestion and expressed as mμmoles of deoxynucleoside, showed good agreement with the deoxycytidine content estimated from the optical density measurement (Table III). This means that F_1 is a single compound containing only deoxycytidine as its nucleosidic constituent.

TABLE II

$R_{deoxycytidine}$ VALUES OF F_1, F_2, THEIR DEGRADATION PRODUCTS, AND THE RELATED SUBSTANCES

	Solvent 1		*Solvent 2*	*Solvent 3*	*Solvent 4*
	*u.v.**	*Dragendorff**	*u.v.**	*u.v.**	*u.v.**
Deoxy-CMP	0.50		0.37	1.08	0.04
F_1 after acid hydrolysis	0.49	trailed	—	—	0.03
Deoxycytidine	1.0		1.0	1.0	1.0
F_1 after venom digestion	1.02	1.08	—	—	0.98
F_1	0.35	0.35	0.44	1.12	0.11
Deoxy-CDP-choline	0.34	0.34	0.45	1.13	0.10
CDP-choline	0.25	0.25	0.35	1.22	0.06
F_2	0.25	0.25	0.33	1.21	0.06
F_2 after venom digestion	0.79	1.08	—	—	0.73
Cytidine	0.81		0.78	1.11	0.70
F_2 after acid hydrolysis	0.43	trailed	—	—	0.04
CMP	0.44		0.32	1.19	0.04
Choline		1.07	—	—	—

Solvents: see MATERIALS AND METHODS.

* U.v.: detected under ultraviolet lamp; Dragendorff: detected by spraying Dragendorff reagent (see MATERIALS AND METHODS).

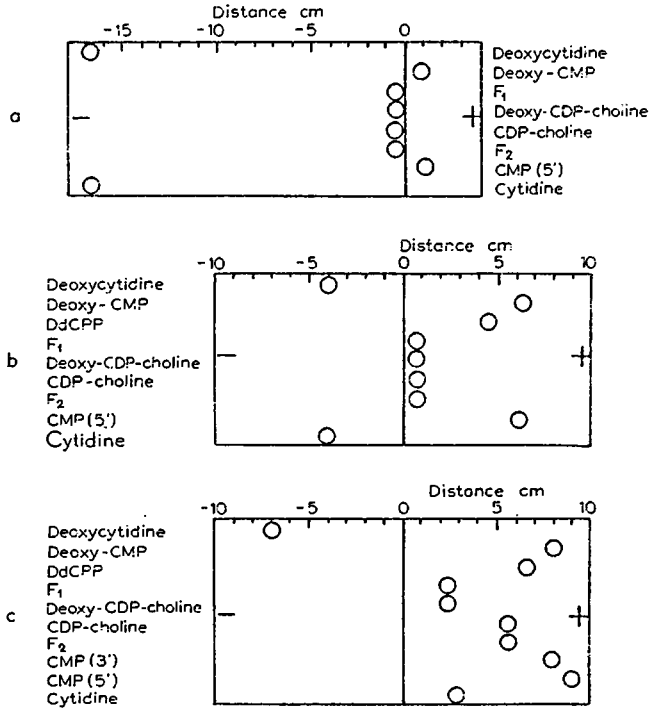

Fig. 4. Paper electrophoresis of F_1, F_2, and the related substances. Conditions: (a) Buffer 1, pH 3.5, 10 V/cm, 7 h. (b) Buffer 2, pH 7.8, 10 V/cm, 3.5 h. (c) Buffer 3, pH 9.3, 10 V/cm, 5.5 h.

Paper chromatography and paper electrophoresis of F_1, F_2, their degradation products, and the related substances

F_1 and F_2 were compared with deoxycytidine, cytidine, CMP, deoxy-CMP, and CDP-choline by paper chromatography and paper electrophoresis. Results are summarized in Table II and Fig. 4. F_2 seemed to be identical with CDP-choline. F_1 appeared to be the deoxyanalogue of F_2, *i.e.* deoxy-CDP-choline, since the positional relationship of F_1 to F_2 (or CDP-choline) was very similar to that of deoxycytidine to cytidine and of deoxy-CMP to CMP.

F_1 as well as F_2 showed a strongly positive spot test on paper with Dragendorff reagent, providing additional evidence for the presence of such a quaternary ammonium as choline in their structure. In fact, the venom digestion of F_1 gave deoxycytidine and choline, and F_2 cytidine and choline, identified by paper chromatography (Table II). The presence of choline residues in F_1 and F_2 fits in well with the fact that, in anion-exchange chromatography at pH 7.8 (Fig. 3) or paper electrophoresis at pH 7.8 (Fig. 4b) and pH 9.3 (Fig. 4c), F_1 and F_2 appeared less anionic than deoxy-CMP and CMP in spite of their high phosphorus content (analytical results are shown in a later section, Table III).

Hydrolysis of F_1 and F_2 with 1 *N* HCl for 15 min at 100° yielded deoxy-CMP and CMP, identified by paper chromatography (Table II).

TABLE III

ANALYTICAL DATA OF F_1, F_2

Material	*μmole/μmole of deoxycytidine or cytidine**				
	*Deoxyriboside***		*Phosphorus***	*Choline***	
	Before venom treatment	*After venom treatment*		*Before venom treatment*	*After venom treatment*
F_1	0.15	0.93	1.93	0	0.83
F_2	0	0	2.03	0	0.87
Deoxy-CDP-C (synthetic)	0.1	0.98	1.96	—	—

* Estimated from optical density measurement at 260 mμ at pH 2 using $\varepsilon = 6{,}200$.
** See the text.

Microbiological determination of choline

The presence of choline residues in F_1 and F_2 was further confirmed microbiologically using choline-less *Neurospora* as a test organism.

Also in this case, it was noticed that neither F_1 or F_2 supported the growth of the test organism, choline-less *Neurospora*, unless they were hydrolysed with snake venom (Table III). This situation is very similar to that observed in the microbiological determination of deoxynucleoside of F_1, and served as an additional proof that choline is bound to the nucleoside moiety through a linkage sensitive to the venom enzyme.

Analytical data of F_1 and F_2

The absorption spectra of F_1 and F_2, completely coincided with those of deoxy-CMP or CMP. Analytical data of F_1 and F_2 are summarized in Table III. From these

data and several lines of evidence already mentioned, it was concluded that F_1 is deoxy-CDP-choline, having a structure shown in Fig. 5, and that F_2 is CDP-choline, which was previously found by KENNEDY AND WEISS[7,11].

That the structure of this newly found compound, F_1, is that of deoxy-CDP-choline was finally confirmed by its chemical synthesis.

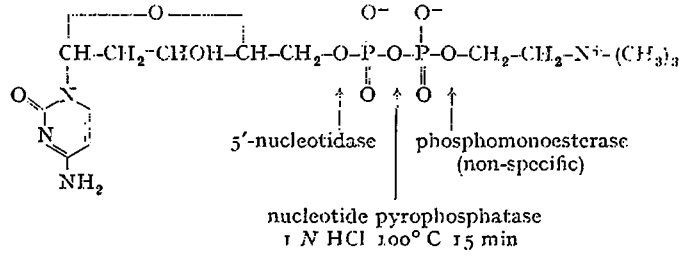

Fig. 5. Structure of F_1, deoxy-CDP-choline. Arrows indicate the positions of cleavage by hydrolysis with the snake venom or acid.

Chemical synthesis of deoxy-CDP-choline

The synthetic method was the same as that described by KENNEDY[7] for the synthesis of CDP-choline except that deoxy-CMP was used in place of CMP.

The reaction products obtained from 130 μmoles of deoxy-CMP and 110 μmoles of phosphoryl choline were fractionated by ion-exchange chromatography according to KENNEDY. Ultraviolet-absorbing materials were eluted from the column as three peaks, I, II and III, in this order. Each fraction was adsorbed on charcoal, eluted with ammonical ethanol, concentrated under reduced pressure, and lyophilized.

30 μmoles of deoxy-CDP-choline, which gave a strongly positive spot test on paper with Dragendorff reagent, were obtained from peak I. Peak II and III were deoxy-CMP and Dd-CPP, respectively.

Comparison of F_1 with the synthetic deoxy-CDP-choline by paper chromatography (Table II) and paper electrophoresis (Fig. 4), and by chemical and microbiological analyses (Table III) proved that F_1 was deoxy-CDP-choline.

DISCUSSION

Experimental results reported in this paper clearly show that one of the "masked" deoxynucleosidic compounds of sea urchin eggs was deoxy-CDP-choline, which was the first conjugated deoxynucleotide found in nature at the time when the preliminary report appeared[1].

Independently, POTTER AND BUETTNER-JANUSCH discovered deoxy-CDP-ethanolamine in calf-thymus[12]. Soon afterwards, SCHNEIDER AND ROTHERHAM reported the occurrence of deoxy-CDP-choline and deoxy-CDP-ethanolamine in Novikoff Hepatoma[13]. OKAZAKI *et al.* found a conjugated thymidylic acid, thymidine diphosphate-X*, in microbial cells[14].

It is of great interest that all of these deoxynucleosidic compounds, which must be digested with snake venom or other enzymes[13] to reveal their full microbiological

* Recently X has been identified as rhamnose[26].

activity towards *L. acidophilus* R-26, have such a common structural feature that the deoxynucleoside moiety is linked through a pyrophosphate bond to the second component (choline, ethanolamine, etc.). The attachment of pyrophosphate to the deoxynucleoside "masks" the deoxynucleosidic growth activity, and such enzymes as cleave the pyrophosphate bond to give a deoxynucleoside or deoxynucleoside monophosphate can be used to activate the "masked" deoxynucleotides. Thus, the action of snake venom, which liberated deoxycytidine and choline from deoxy-CDP-choline, can be explained by its nucleoside pyrophosphatase[15–18] and phosphomonoesterase[19–22] (including 5'-nucleotidase) activities.

This view coincides well with the fact that di- or tri-phosphates of pyrimidine deoxynucleosides do not support the growth of *L. acidophilus* R-26 unless they are deophosphorylated to monophosphates[23], and that chemically synthesized Dd-CPP, which was microbiologically inactive, could support the growth of *L. acidophilus* when it was hydrolysed with snake venom[24]. Similar results were reported by ROTHERHAM AND SCHNEIDER[25], who used intestinal phosphatase instead of the snake venom*.

The biochemical function(s) of deoxy-CDP-choline and other "masked" deoxynucleotides is as yet unknown. However, their coenzyme-like structure and distribution strongly suggest that they must actively participate in some biosynthetic reactions of rapidly dividing cells or cell nuclei, where rapid DNA synthesis proceeds.

Further investivation is needed in order to decide what specific functions the conjugated deoxynucleotides, including deoxy-CDP-choline, may carry out.

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* Our venom preparations, vacuum-dried powder of venoms of *Trimeresurus flaroviridus* and *Agkistrodon blomhoffi*, did not contain appreciable nucleosidase activity, while cobra venom was found to contain the nucleosidase activity[25].

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INFLUENCE DE DIVERS AGENTS CHIMIQUES SUR LA CAROTÉNOGÉNÈSE DE *RHODOTORULA MUCILAGINOSA*

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SUMMARY

The influence of various chemical agents on the carotenogenesis of Rhodotorula mucilaginosa

The action of phenol, phenylethylacetamide, acenaphthene, naphthalene, phenantrene, streptomycin, and diphenylamine on the carotenogenesis of *Rh. mucilaginosa* was studied; where the action was inhibitory, it was accompanied by a decline in the growth of the cultures: a fact that indicates the complex nature of the phenomenon.

Biphenyl incorporated in the culture medium at a concentration of 50 μg/ml has practically no effect on the growth of *Rh. mucilaginosa*, and permits the obtention of a white yeast. Among the diphenyl derivatives studied, only the asymmetrically substituted ones inhibited the carotenogenesis.

INTRODUCTION

Une des méthodes permettant d'aborder le mécanisme de la biosynthèse des caroténoïdes, réside dans l'utilisation d'inhibiteurs spécifiques. Ce procédé largement employé par de nombreux auteurs et chez divers microorganismes[1] ne semble pas,

* Directeur: Professeur F. KAYSER.