CARBOHYDRATE METABOLISM IN MARINE RED ALGAE *PORPHYRA*

II. Photoassimilation of $C^{14}O_2$ into Carbohydrate Fractions*

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In the previous paper (Su *et al.*, 1965), we have reported the results of fractionation and analyses of *Porphyra* polysaccharides which are in accord with the hypothesis that etherification of galactosyl residues in the *Porphyra* galactan (porphyran) to form 3,6-anhydro-L-galactose and 6-0-methyl-D-galactose units takes place at the polysaccharide level and galactan sulfate serves as the intermediate (Su and Hassid, 1962b).

Rees (1961 a, 1961 b) prepared an enzyme from *Porphyra umbilicalis* which was capable of liberating sulfate from porphyran to form 3,6-anhydro-L-galactose units within the polymer. From the available data, we believe that methylation of D-galactose to form 6-0-methyl-D-galactose also takes place at the polymer level, however, evidence pertaining to the reaction is still lacking.

If the synthesis of 6-0-methyl-D-galactose takes place as postulated, then the porphyran isolated from the alga exposed to $C^{14}O_2$ for a longer period of time will show a lower ratio of radioactivities residing in galactose and 6-0-methylgalactose units. In the following are reported the results of an experiment designed to prove this point, and also the pattern of $C^{14}O_2$ assimilation into various metabolite and saccharide fractions of the red alga *P. crispata.*

Materials and Methods

The red alga *P. crispata* used in this experiment was collected by hand-picking at the beach near Keelung. Immediately after collection, sands and shells were removed by washing in sea water. The alga was kept in ice chilled sea water and brought to the laboratory as soon as possible. In the case that the sample was not used immediately, it was kept in a cold room at 4° and aerated to keep the alga alive.

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An apparatus similar to that described by Bean and Hassid (1955) was used for carrying out the photosynthetic experiment. Externally generated $C^{14}O_2$ (the $BaCO_3$ used in this experiment had a specific activity of 9 μc/mg) was introduced by vacuum transfer into the photosynthesis chamber, which contained weighed amount of algal leaves and a couple of drops of water. After photosynthesizing for a definite period, the residual $C^{14}O_2$ was swept off with CO_2-free air and the algal specimen decanted into boiling 95% ethanol. After adjusting the concentration of ethanol to 70%, the heating was continued for 5 minutes to extract low molecular weight substances. Extraction with boiling 70% ethanol was repeated until the extract showed no chlorophyll color. The 70% ethanol extracts were combined, the volume measured and an aliquot was drawn for radioactivity assay. The combined solution was evaporated first in a vacuum evaporator at 40° and then in a vacuum desiccator over sulfuric acid and KOH pellets to obtain an immobile sirup.

The residue from the ethanol extraction was heated with 2% acetic acid to extract porphyran. The extract was concentrated, if necessary, and 5 volume of ethanol was added to precipitate the polysaccharide. Radioactivity assays were made on the extract and the supernatant liquid after separation of the polysaccharide, and the radioactivity of the polysaccharide was obtained by the difference.

The residues from 2% acetic acid extraction were extracted successively with 4% and 10% NaOH, and the alkali soluble polysaccharides were precipitated with ethanol as above. The polysaccharide precipitates were washed with 95% ethanol several times and dried. The supernatant liquid was deionized by passing through a column of Dowex-50 resin in hydrogen form and evaporated under reduced pressure to obtain a hard mass. For the estimation of the total radioactivity in the alkali extract, an aliquot was drawn before precipitation of the polysaccharide, deionized with the cation exchange resin, and plated for counting.

Amino acids in the ethanolic extracts were separated by adsorbing on a column of Dowex-50(H^+) resin and desorbing from the column with dilute ammonium hydroxide. The amino acid mixture was first fractionated by paper electrophoresis in 0.1*M* ammonium acetate pH 5.7, and the separated acid and neutral fractions were then paper chromatographed in the solvent systems butanol-acetic acid-water, 4:1:1, and *m*-cresol-phenol-0.064*M* Sodium borate buffer pH 9.3, 25:25:7 (w/w/v), respectively (Levy and Chung, 1953). No basic amino acid was found. Polysaccharide hydrolysates were also separated in the butanol-acetic acid-water system. For the separation of galactosylglycerol from hexoses, the solvent system ethyl acetate-pyridine-water, 8:2:1, was used. Electrophoresis was conducted with an apparatus

similar to that described by Crestfield and Allen (1955) at a field strength of 28 v/cm. The buffer used for fractionating ethanol solubles was 0.1 *M* potassium phosphate, pH 7.5, and that for analyzing neutral saccharides and hexitols was 0.05 *M* sodium tetraborate, pH 9.2.

The radioactive spots on paper were located by radioautographic technique using Kodak no-screen X-ray film. Silver nitrate -0.05*N*-NaOH dipping reagent and ninhydrin in ethanol were used to reveal sugar and amino acid spots, respectively. For the radioactivity assay, samples were plated on aluminium planchets and counted with an ultrathin-window gas-flow counter at the proportional region.

Seventy % ethanol extract was used directly for paper chromatographic and electrophoretic analyses. The 2% acetic acid and alkali soluble fractions were hydrolyzed in a sealed tube with 50 volume of 1 *N* sulfuric acid at 110° for 4 hours. The hydrolysate was neutralized with barium carbonate and the precipitate removed by centrifugation. After concentrating in a vacuum desiccator over KOH pellets, the thin sirup was analyzed by paper chromatography.

The final residue was hydrolyzed with 50 volume of 2 *N* sulfuric acid at 110° for 5 hours and then treated as above.

Results and Discussion

The distributions of the tracer carbon in various solvent soluble fractions of the alga exposed to $C^{14}O_2$ for various length of time are presented in Table 1, and some of the data are represented in a graphical form in Figure 1. From these data, it is seen that in the ethanol soluble fraction is accumulated the largest portion of the radioactivity no matter how long is the period of photosynthesis. Since the 70% ethanol soluble fraction is consisted of low molecular weight compounds such as monosaccharides, oligosaccharides, sugar phosphates, sugar nucleotides, amino acids, organic acids, etc., the rapid decrease in the relative amount of the tracer carbon in this fraction during the first hour of photosynthesis is considered to indicate that, some active metabolites, such as phosphate esters, amino acids, etc., are rapidly turned over and some stable compounds, such as galactosylglycerols, are accumulated, and finally a steady state is established.

It is interesting to note that the kinetic curves of the two alkali soluble fractions follow the pattern of initial rise and a subsequent decline, while that of the dilute acetic acid soluble fraction is in the reversed fashion. This is considered to indicate that porphyran, the main constituent of the dilute acetic acid soluble fraction, bears the metabolic behavior of starches of higher plants, since it is one of the early photosynthetic products as evidenced by the initial

Table 1. *Distribution of radioactivity in various fractions of Porphyra crispata after photosynthesis in $C^{14}O_2$.*

Time \ Extracting Solvent		70% Alcohol	2% Acetic acid		4% NaOH		10% NaOH	
			Super-natant	Precipitate	Super-natant	Precipitate	Super-natant	Precipitate
15 minutes	c.p.m.	7.52×10^6	1.11×10^5	3.5×10^5	1.56×10^5	7.88×10^5	5.56×10^4	4.43×10^5
	%	79.8%	1.18%	3.72%	1.66%	8.35%	0.59%	4.70%
			4.90%		10.01%		5.29%	
30 minutes	c.p.m.	9.77×10^6	2.79×10^5	3.77×10^5	8.6×10^4	2.25×10^6	3.52×10^4	1.12×10^6
	%	70.3%	2.02%	2.78%	0.62%	17.2%	0.25%	8.08%
			4.8%		17.8%		8.33%	
1 hour	c.p.m.	7.75×10^6	1.89×10^5	1.40×10^5	1.97×10^5	2.86×10^6	6.24×10^4	1.24×10^6
	%	62.3%	1.52%	1.12%	1.58%	23%	0.52%	10%
			2.64%		24.58%		10.52%	
2 hours	c.p.m.	7.49×10^6	4.74×10^5	2.49×10^5	1.06×10^5	3.11×10^6	3.52×10^4	1.12×10^6
	%	58.58%	3.77%	1.97%	0.84%	24.7%	0.28%	8.86%
			5.74%		25.54%		9.18%	
5 hours	c.p.m.	4.56×10^7	5.82×10^6	5.47×10^6	6.44×10^6	9.78×10^6	2.51×10^5	3.9×10^6
	%	59%	7.53%	7.08%	8.35%	12.68%	0.32%	5.05%
			14.61%		21.03%		5.37%	

decline of the progress curve and since it is accumulated on prolonged photosynthesis as indicated by the increase of the slope of the curve following the initial decline.

In order to look more closely into the changes of the properties of ethanolic extract during the course of photosynthesis, they were electrophoresed in 0.1 *M* phosphate, pH 7.5, and the electrophoretograms cut into strips of 1 cm width, eluted with water and the eluates plated for radioactivity assay. The distributions of the tracer compounds on the electrophoretograms are shown in Table 2. In the diagrams, the peaks with corresponding positions are disignated as A, B, C, D, E and F. Peak A consisted of neutral compounds, and paper chromatographic and electrophoretic analyses revealed that, in addition to very small amounts of glucose and fructose, a galactosylglycerol constituted the bulk of radioactivity. The galactosylglycerol was further characterized by chromatographing the acid hydrolysis products and reducing the isolated

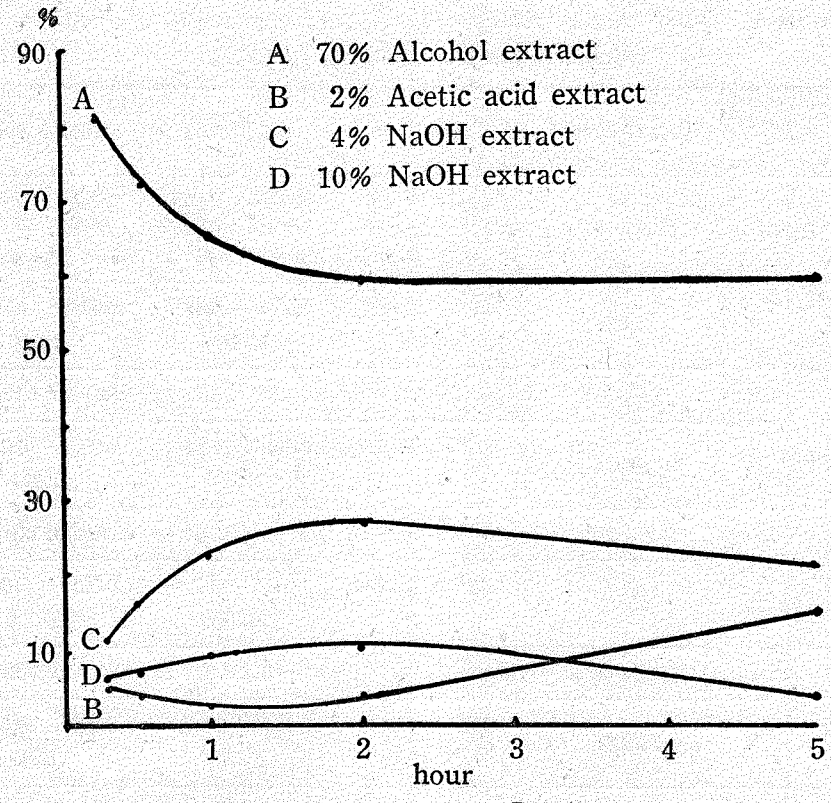

Fig. 1. Kinetic analysis of relative amounts of C^{14} in various extracted fractions from *P. crispapa.*

Table 2. *Distribution of radioactivity in the electrophoretograms.*

Time	Peak	A	B	C	D	E	F	Total c. p. m.
15 minutes	c. p. m.	9,389	586	14,337	7,581	—	2,382	37,384
	%	25.11%	1.54%	38.35%	21.00%	—	6.37%	
30 minutes	c. p. m.	14,113	423	7,452	266	—	2,181	25,358
	%	55.65%	1.66%	29.38%	1.04%	—	8.60%	
1 hour	c. p. m.	12,120	835	5,440	548	5,463	2,369	28,688
	%	42.24%	2.56%	18.96%	1.91%	19.04%	8.25%	
5 hours	c. p. m.	98,440	5,751	12,033	—	6,081	5,700	131,127
	%	75.07%	4.38%	9.17%	—	4.63%	4.34%	

galactose to dulcitol, which could be identified by electrophoresis in tetraborate, pH 9.2.

From the electrophoretic mobilities, Peak B was considered to contain organic acids and Peaks C and D phosphate esters, such as hexose monophos-

phates, sugar nucleotides, phosphoglyceric acid, glycerol phosphate, etc. Peak E has the mobility identical with that of fructose 1,6-diphosphate, but so far we have not been able to match peak F with a known compound.

In Figure 2 are presented the progress curves showing the changes of percent radioactivity of electrophoretically separated peaks as the function of time. From these curves, it is seen that components in Peaks C and D are the early products of algal photosynthesis, while those in A are the primary stable products, such as the ones represented by sucrose in higher plants. From the facts that Peak A consists almost exclusively of galactosylglycerol, and that in Peaks C and D are present phosphoric esters, we may be able to say that the present experiment confirmed the view of Bean and Hassid (1955) that in red algae galactosylglycerols take part of sucrose in higher plants but otherwise the pathway of photosynthetic carbon assimilation in the algal is in accord with that of green plants. This view is further substantiated by the results of amino acid analyses. We obtained serine, glycine, alanine, glutamic acid and aspartic acid as the early products of photosynthesis and these results are in conformity with those obtained for green plants.

The porphyran and alkali soluble fractions were hydrolyzed, the hydrolysis products chromatographed on paper and the radioactive spots corresponding to galactose, 6-0-methylgalactose, mannose and xylose were cut, eluted, and assayed for radioactivity. The radioactivity ratios of the separated saccharides were calculated by taking the activity of methylgalactose as unity. The results are summarized in Table 3. It is interesting to note that, although no mannose and xylose could be detected in porphyran by chemical means (Su *et al.*, 1965), radioactive spots migrating as these two saccharides were found when the porphyran fraction obtained in the present experiment was hydrolyzed and chromatographed.

When the activity ratios galactose/methylgalactose are plotted against time, Figure 3 is obtained. As we have pointed out, porphyran acquires tracer carbon earlier than the alkali soluble cell wall polysaccharides. The very steep slope of curve A of Figure 3 indicates that, at the early stage of $C^{14}O_2$ assimilation, the tracer gets into the polysaccharide in the form of galactose units. This may imply either: a) as we have stated in introduction, methylation of galactose takes place at the polymer level, or, b) the methylation reaction takes place at the precursor level, but the pool size of the methylgalactose donor is so large that, even after five hours of photosynthesis (at which time a considerable amount of radioactivity is accumulated in the polysaccharide), the galactose/methylgalactose ratio still remains at a high value of 11.5. The latter view seems to be very unlikely, however, from the facts that, in known porphyran preparations, galactose (almost one half of which is in L-configuration) and 6-0-methyl-D-galactose units are present in

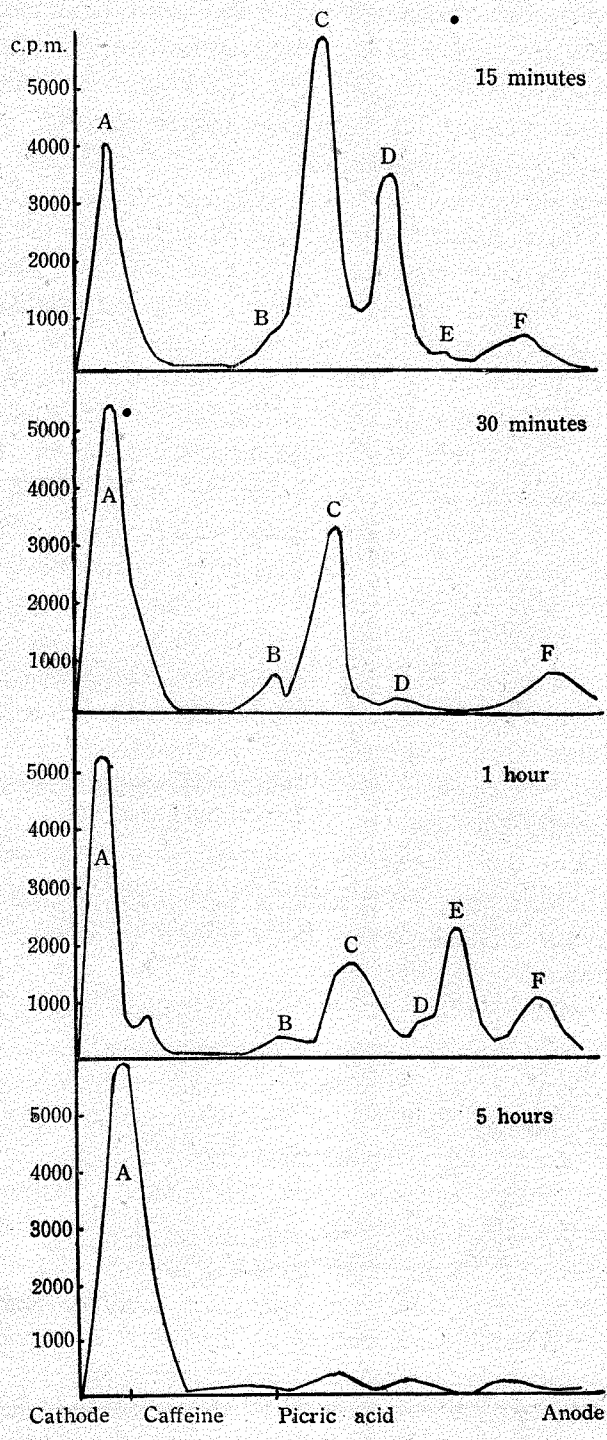

Fig. 2. Difference of distribution of radioactivity in the electrophoretograms of 70% alcoholic extracts with respect to time.

Table 3. *Radioactivity ratio of constituent monosaccharides of Polysaccharide fractions.*

Extracted fraction / Time	I	II	III
	Gal-act-ose : Man-nose : Xyl-ose : Methyl galac-tose	Gal-act-ose : Man-nose : Xyl-ose : Methyl galac-tose	Gal-act-ose : Man-nose : Xyl-ose : Methyl galac-tose
15 minutes	42 : 5 : 2.5 : 1	34.5 : 3.88 : 2 : 1	18 : 5.1 : 1 : 1
30 minutes	21.2 : 6 : 2 : 1	38 : 5.1 : 2.85 : 1	18 : 4.7 : 1 : 1
1 hour	17.25 : 0.75 : 1.5 : 1	15.7 : 1.4 : 1.08 : 1	14 : 6 : 1 : 1
2 hours	11.3 : 0.86 : 0.85 : 1	5.9 : 0.86 : 1.05 : 1	10.6 : 9.6 : 1 : 1
5 hours	11.5 : 1.15 : 0.63 : 1	8.2 : 0.81 : 0.95 : 1	6 : 3 : 1 : 1

I. Alcoholic precipitate from 2% acetic acid extract
II. Alcoholic precipitate from 4% NaOH extract
III. Alcoholic precipitate from 10% NaOH extract

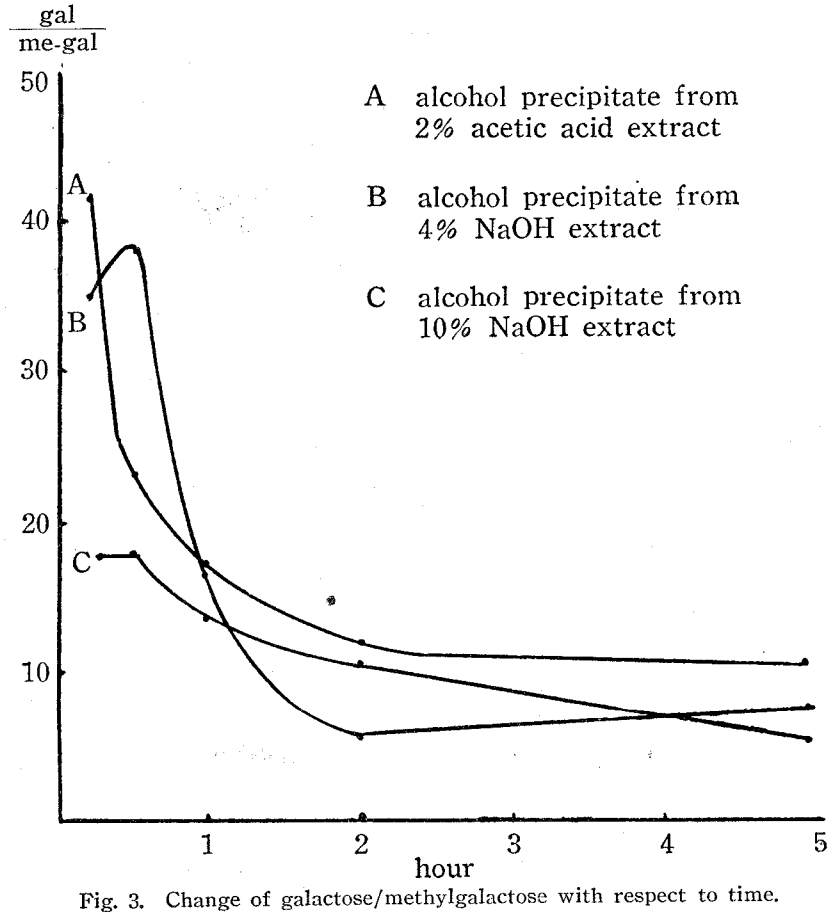

Fig. 3. Change of galactose/methylgalactose with respect to time.

nearly equimolar proportions (Nunn and von Holdt, 1958; Su and Hassid, 1962), and that, in the alga, the D-galactose donor pool is backed up by a very large pool of active D-galactosyl compound, *viz.* D-galactosylglycerol, which may occupy as high as 25% of the algal body on dry matter basis (Su, 1956).

It is concluded therefore that methylation of D-galactose to form 6-0-methyl-D-galactose takes place most probably at the polymer level as we have postulated previously.

紅海藻 *Porphyra* 之醣類代謝

II. 放射性二氧化碳光同化作用之探討

林耀輝　蘇仲卿

本實驗乃研究紅海藻 *Porphyra crispata* 光同化放射性二氧化碳產生醣類的概況。

自動力學的觀點研究可溶於不同溶劑的紫菜各成份裏放射性物質之分佈，得知甘油半乳糖（galactosyl glycerol）之存在於紅海藻是相當於蔗糖（Sucrose）之存在於高等植物體的主要貯藏性醣類，而半乳聚醣（galactan）則相當於高等植物體的澱粉（starch）。

大多數光合作用產生的磷酯可認爲和綠色植物大致相同。初期光合作用產生之氨基酸也以甘氨酸（glycine），絲氨酸（serine），丙氨酸（alanine），天門冬酸（aspartic acid）及麩氨酸（glutamic acid）爲主。

從各種多醣之構成單糖比率推證 *Porphyra* 所含有之複雜半乳聚醣其產生過程是先合成單純 DL-galactan 後發生分化作用，將 galactosyl 基轉變成衍生物。

Literature Cited

BEAN, R. C. and W. Z. HASSID. Assimilation of $C^{14}O_2$ by a photosynthesizing alga, *Iridophycus*. J. Biol. Chem., **212**: 411-425, 1955.

CRESTFIELD, A. M. and F. W. ALLEN. Improved apparatus for zone electrophoresis. Anal. Chem., **27**: 422-3, 1955.

LEVY, A. L. and D. CHUNG. Two-dimensional chromatography of amino acids on buffered papers. Anal. Chem., **25**: 396-399, 1953.

NUNN, J. R. and M. M. v. HOLDT. Red-seaweed polysaccharides. Part II. *Porphyra capensis* and the separation of D- and L-galactose by crystallization. J. Chem. Soc., 1094-7, 1957.

REES, D. A. Enzymic desulfation of Porphyran. Biochem. J. **80**: 449-53. 1961.

REES, D. A. Enzymic synthesis of 3,6-anhydro-L-galactose within porphyran from L-galactose-6-sulfate units. Biochem. J. **81**: 347-52, 1961.

SU, J. C. Studies on saccharides of marine algae. I. Identification of floridoside and a DL-galactan in a red alga, *Porphyra crispata*, Kjellm. J. Chinese Chem. Soc., Ser. II. **3**: 65-71, 1956.

SU, J. C. and W. Z. HASSID. Carbohydrates and nucleotides in the red alga *Porphyra perforata*. I. Isolation and identification of carbohydrates. Biochemistry, **1**: 468-474, 1962.

SU, J. C. and W. Z. HASSID. Carbohydrates and nucleotides in the red alga *Porphyra perforata*. II. Separation and identification of nucleotides. Biochemistry, **1**: 474-480, 1962.

SU, J. C., T. M. CHANG and C. C. WANG. Carbohydrate metabolism in marine red algae *Porphyra*. I. General survey of polysaccharides. J. Chinese Agr. Chem. Soc., Special issue, 1965.