ARGININE-RICH POLYPEPTIDES IN SALINE EXTRACT OF CHLORELLA PYRENOIDOSA-^{14}C.

Zdenek Nejedly and Karel Hybs.

Institute for Research, Production and Uses of Radioisotopes, Pfistavni 24, Prague 7, Czechoslovakia .

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SUMMARY

The radiochromatographic analysis of the hot 10% sodium chloride extract of the alga Chlorella pyrenoidosa-^{14}C indicated the presence of compounds rich in L-arginine, besides of nucleic acids and glucose-type polysaccharide estimated previously. L-Arginine is present in a bound form, most likely as a protamine-type polypeptide or a histone-type protein containing L-arginine as a dominant component, besides of smaller amounts of L-glutamic acid, L-aspartic acid and L-serine. A simple procedure of the isolation of L-arginine-^{14}C/U/ from the saline extract of radioactive Chlorella cells, on a preparative scale, is described.

INTRODUCTION

Radioactive algae are a highly estimated source of a wide range of essential metabolites for biochemical and biological studies. Our attention was therefore focussed towards making this procedure more attractive, in particular for simultaneous preparation of aminoacids, sugars and nucleic acid components, nonspecifically labelled with ^{14}C. A new simple apparatus was designed for cultivation of Chlorella pyrenoidosa in an atmosphere of $^{14}CO_2$, enabling the production of ^{14}C labelled compounds of high specific activity[1]. The relatively selective manner of isolation of biologically important groups of compounds from cell material[2,3] and the determination of the effect of some environmental factors in relation to the production of nucleic acids and proteins by the growing alga[4] ensure a high effectiveness of the procedure.

One of the important requirements of effective production of labelled compounds by the technique mentioned above consists in an appropriate fractionation of the cell material. In our case, a part of such procedure is the extraction of nucleic acids from cells with hot 10 % sodium chloride. This type of extraction of nucleic acids belongs to the conventional procedures of biochemical analysis; the principal possibilities along this direction as well as their modifications have been published in review articles[5]. It is generally assumed that nucleic acids isolated in this way are not undesirably degraded. The views on this point vary somewhat as to the quantitative character of isolation of nucleic acids, especially in the case of deoxyribonucleic acid[5]. The method finds application most of all during analysis of bacterial and animal tissues, although some data were also obtained by applying the method to the extraction of nucleic acids from cells of higher plants[6,7] and unicellular algae[1-4, 8-10].

Nevertheless, certain aspects of the method, important especially from the viewpoint of optimal production of a wide range of biologically active substances, have not yet been analysed. First of all, the complete analysis of the 10 % NaCl extract composition has not yet been undertaken. We have found earlier that another significant component of the saline extract is a glucose-type polysaccharide[3]. In the present paper we report on other findings made with the 10 % NaCl extract, indicative of the presence of remarkable amounts of L-arginine and of smaller amounts of some other L-amino acids.

MATERIALS

Chemicals

The substances used as standards were obtained from Calbiochem, USA /aminoacids/ and from Fluka, Switzerland /sugars/ in chromatographically homogenous form. The other nonactive organic and inorganic chemicals were supplied by Lachema, Czechoslovakia, in reagent-grade purity. The source of ^{14}C was $Ba^{14}CO_3$ obtained from USSR of specific activity 170 mCi/mmole.

Microorganism

A pure culture of the unicellular alga Chlorella pyrenoidosa, Chick, Coll. Pringsheim, from the Collection of cultures of autotrophic organism in Prague, was applied. The alga was propagated in the above laboratory on Benson's medium containing 0,1 % glucose in the presence of yeast extract, in the course of several days, at 20-23° C and illumination of 2,000 lux. A seven-day culture was used for radioactive photosynthesis.

METHODS

Technique and Apparatus for Cultivation of the Alga

Cultivation of Chlorella pyrenoidosa in an atmosphere of $^{14}CO_2$ was carried out in a closed system constructed especially for purposes of producing radioactive algae of high specific activity[11]. The cultivation executed under such conditions yielded a total of 600.0

mg of radioactive alga of total radioactivity of 625 mCi.

Fractionation of Cell Material

The radioactive alga was fractionated as described before[2,3] schematically shown in Fig. 1/. The grown cells were freed of lipids and coloured matter by treatment with hot 96 % ethanol and further with a mixture of ethanol and ether /3:1/ at room temperature. The algal dry weight thus obtained was then extracted with 10 % sodium chloride at 100 ° C for 9 hours. The extract /F 8/ acidified to pH 5 with acetic acid was overlayered with three volumes of 96 % ethanol. By standing overnight at 3-4 ° C a precipitate separated, containing mostly cell nucleic acids and polysaccharides. By centrifugation, this precipitate /F 11/ was separated from the 70 % aqueous-ethanolic supernatant fraction /F 10/. The analysis of this fraction F 10 represents the object of the present communication. The degree of incorporation of radioactivity of ^{14}C into the individual fractions may be seen in Table 1.

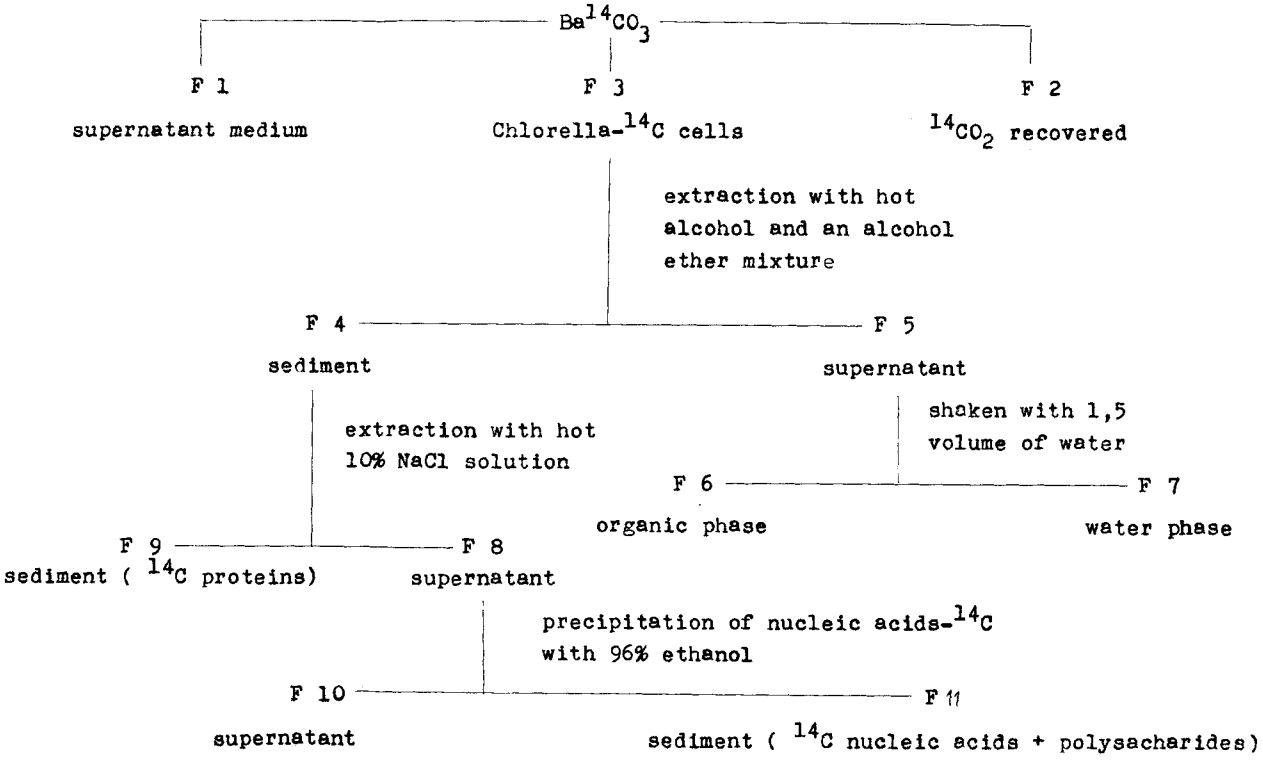
TABLE 1

Incorporation of radioactivity of ^{14}C into fractions of Chlorella pyrenoidosa
/Total $Ba^{14}CO_3$ used /625 mCi / = 100 % /

Fraction	Radioactivity ^{14}C %
F 1 - Nutrient solution	15.8
F 2 - $^{14}CO_2$ absorbed in NaOH	-
F 5 - Ethanol-ether extract	11.8
F 8 - 10% sodium chloride extract	32.2
F 9 - cell debris containing ^{14}C proteins	40.2
F 10 - 70% ethanol supernatant	17.9

FIG.1

Scheme of fractionation of Chlorella pyrenoidosa-^{14}C cells



Analytical procedures

/a/ Paper chromatography of aminoacids and sugars

The paper chromatography of both aminoacids and suggars was carried out on Whatman No. 3 paper sheets in n-butanol:acetic acid:water /4:1:5/ in descending direction at room temperature for 14 hours. In some cases which shall be mentioned in the experimental section below, the unidirectional chromatography was supplemented with chromatography in another direction using phenol:water:ethanol /2:1:1/ solvent system.

/b/ High-voltage electrophoresis of aminoacids

For the electrophoretic separation of aminoacids we used a borate buffer of pH 9.0. Separation was carried out at 2.5 kV, 0° C, for 1 hour, using a 3 cm wide and 50 cm long strip of Whatman No. 3.

/c/ Spectrophotometric estimation of L-arginine content

The content of L-arginine was determined after reaction with the ninhydrin reagent spectrophotometrically at 570 nm on a double-beam spectrophotometer /Optica, Milano/, using a calibration curve.

/d/ Detection methods

Sugars were detected on the chromatograms by using a method of Buchan and Savage[11]. Aminoacids on the chromatograms and electrophoretograms were detected by the ninhydrin test. The L-arginine was also identified by a modified Sakaguchi reaction[12,13]. Autoradiography of chromatograms and electrophoretograms with radioactive ^{14}C-compounds was carried out by application of X-ray ORWO films. The quantitative evaluation of radioactive spots on chromatograms and electrophoretograms was proved by counting them under a β-tube of the Friese-ke-Hoepfner chromatogram scanner and by planimetry of the graph obtained.

/e/ Determination of ^{14}C radioactivity

The radioactivity of the aqueous solutions of compounds labelled with ^{14}C was determined with the aid of an end-window GM counter of defined geometric angle. The liquid tested was in a saturated layer with respect to the radionuclide ^{14}C and calibration was done in the same geometry with the aid of the ER-2 ^{14}C standard/product of this Institute/.

RESULTS

The heretofore unidentified fraction F 10 was analyzed, mostly with regard to the possible presence of compounds containing aminoacids and sugars as the basic building blocks. The presence of the radioactive substances mentioned was tested in acid hydrolyzates of equivalent portions of fraction F 10. For this purpose, we used portions of this fraction freed of salts and hydrolyzed then with 6N hydrochloric acid /105° C, 20 hours/ for aminoacid analyses and with 2N hydrochloric acid /100° C, 3 hours/ when detecting traces of sugars.

Removal of salts from the fraction

Ethanol was evaporated from an aliquot of fraction F 10 /about 1.0 mCi/ in vacuo and the residue was dissolved in 0.5 ml 12N hydrochloric acid precooled to -10° C. After 2-3 min. of standing at this temperature sodium chloride precipitated from the solution. The salt was removed by centrifugation at -10° C and 6,000 g for 15 min. After separation of the liquid phase the salt sediment was washed in the centrifuge tube with 1.0 ml hydrochloric acid precooled to -10° C. By centrifuging the suspension under the above conditions and by combining the liquid phases we obtained a solution containing organic compounds of fraction F 10, freed practically of all inorganic salts and fully satisfactory for chromatographic analysis. As no requirements were made to the prevention of partial destruction of the organic compounds present, the desalting procedure was found quite adequate for the purposes of the analysis. The radioactive balance is also favourable. The described procedure of desalting was repeated several times and it was found that the liquid phase contains usually 90 - 95 % radioactivity of ^{14}C of the F 10 fraction.

For subsequent analytical estimations an equivalent part of the 12N hydrochloric acid extract was made with distilled water to 6N or 2N hydrochloric acid and the solutions obtained were hydrolyzed as described above.

Chromatographic and electrophoretic analyses

Qualitative analysis of the fraction studied yielded the following findings.

The radiochromatographic record of the 12N hydrochloric acid extract of the F 10 fraction indicated that all the radioactive compounds present either remain at the start or move a negligible distance /R_F = 0.0 - 0.01/. The presence of free sugars or of aminoacids in the extract may be thus excluded.

It follows from a radiochromatographic record of the 2N and of the 6N HCl hydrolyzates /Fig. 2/ that with 2N acid four radioactive compounds are clearly separated. Nevertheless, in this case a greater part of the radioactivity /69 %/ remains at the start. The other radioactive compounds /a total of 31 %/ correspond to L-arginine, L-serine, L-glutamic acid and L-alanine. Only the compound corresponding to L-arginine by its R_F /some 10 % of total radioactivity/ gave a positive reaction for ninhydrin. It should be noted that in the alternative tests where the R_F values of radioactive compounds were compared with those of sugars used as internal standards, in no single case was it possible to observe the identity of the autoradiographic and the chemical detection.

The record of distribution of ^{14}C radioactivity after hydrolysis with 6N hydrochloric acid is quite different. First of all, there is no radioactivity of ^{14}C at the start. Autoradiography revealed a total of 11 radioactive compounds; chemical detection with a ninhydrin reagent was positive in seven cases. The identity of the autoradiographic and the ninhydrin detection is quite pronounced, corresponding by its R_F to L-arginine, L-serine, L-glutamic acid and L-alanine, the amount of radioactivity associated with L-arginine representing some 60 % of the total.

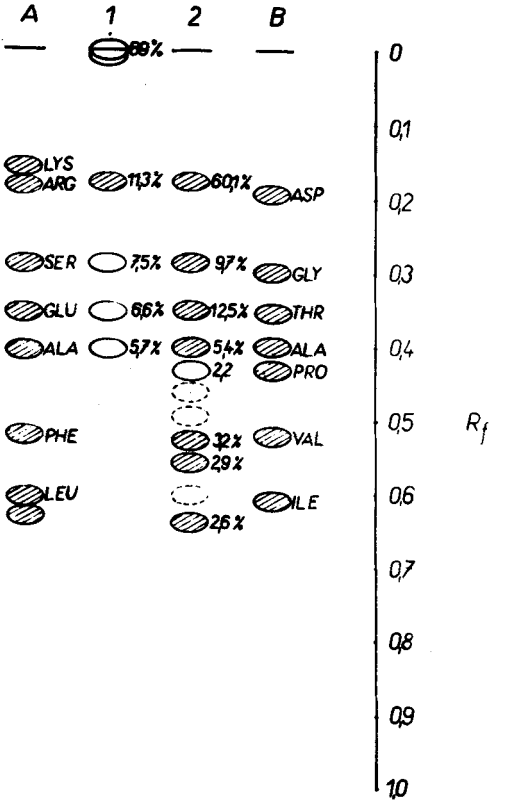


Fig. 2 - Chromatographic analysis of acid hydrolyzates of F 10 fraction (Radioactivity of compounds separated is expressed in % of radioactivity of acid hydrolyzates).

A, B - Aminoacids standards.

1 - 2N hydrochloric acid hydrolyzate of F 10 fraction.

2 - 6N hydrochloric acid hydrolyzate of F 10 fraction.

- positive detection with ninhydrin.

The isolation of L-arginine- C /U/ on a preparative scale

On the basis of results obtained by the qualitative analysis, L-arginine- C/U/ was produced by the above procedure on a preparatory scale. An aliquot of the F 10 fraction /9.96 mCi/ was freed of salts, hydrolysed with 6N hydrochloric acid and after evaporation of the acid, chromatographed. The radioactive compounds corresponding to four standards of amino acids as well as 6 other unidentifield radioactive compounds were eluted with distilled water and the radioactivity of the eluates was determined. The radioactivity balance sheet is shown in Table 2.

TABLE 2

Balance sheet of ^{14}C radioactivity of compounds present in 6N hydrochloric acid hydrolyzate of fraction F 10 / Radioactivity of compounds is expressed in % of F 10 fraction/

Compound / Fraction /	Radioactivity %
F 10 fraction	100.0
12N HCl extract	91.3
L-Arginine - ^{14}C/U/	32.9
L-Serine - ^{14}C/U/	5.0
L-Glutamic acid - ^{14}C/U/	7.7
L-Alanine - ^{14}C/U/	3.9
L-Aminoacids - ^{14}C/U/, total	49.5
Unidentified compounds - ^{14}C/U/	30.4
Losses of ^{14}C radioactive compounds during chromatography	11.4

The identity of the aminoacids was checked by the method of internal standards, using two-dimensional paper chromatography and high-voltage electrophoresis with subsequent autoradiography and chemical detection with ninhydrin. In the case of L-arginine, this was supplemented with detection with Sakaguchi 's reagent. With the exception of L-serine-^{14}C where two-dimensional chromatography and electrophoresis showed the presence of two other ninhydrin-negative compounds representing almost 50 % of the eluted radioactivity, the other aminoacids were found to be chromatographically homogeneous.

With L-arginine-^{14}C which is important for technological application of the method described here, the radioactivity estimation was supplemented with a spectrophotometric evaluation of the weight content. The specific radioactivity of L-arginine-^{14}C was 128 mCi/mmole. A total of 3.3 mCi L-arginine-^{14}C /U/ was isolated in this preparative experiment.

CONCLUSIONS

Autotrophic batch cultivation in an atmosphere of $^{14}CO_2$ at continuous illumination was used to prepare radioactive Chlorella pyrenoidosa-^{14}C. The cells of the radioactive alga, freed previously of coloured matter and lipids, were extracted for nine hours with hot 10 % sodium chloride. Radiochromatography of this extract after previous removal of nucleic acids and glucose-containing polysaccharide showed that its other important components are compounds containing aminoacids in their structure. L-Arginine, L-glutamic acid, L-serine and L-alanine were identified positively, the identity of several other ninhydrin-positive compounds remaining obscure. In all the analyses L-arginine was identified as a dominant component of the aminoacids mixture.

The radiochromatographic analysis also confirmed with certainty that none of the aminoacids is present in the extract in the free form. On the contrary, one can deduce from the relative representation of the aminoacids and from the facts established in this connection/14-19/ that native cells of the radioactive alga contain remarkable amounts of peptides or polypeptides of the protamine type or of protein of the histone type containing L-arginine as the dominant component in their primary structure, together with smaller amounts of L-glutamic acid, L-serine and L-alanine.

At any rate, it appears that L-arginine is accumulated strikingly in the cells of the growing alga not only during the phase of transition from growth in a nitrogen-limited medium to growth in the normal medium/14-17/ or in certain phases of its synchronised growth/18, 19/ but also in the course of its batch cultivation in an atmosphere of $^{14}CO_2$ during continuous illumination. Accumulation of L-arginine in cells of the growing alga was thus established in cultures obtained under different conditions of cultivation and one may hence assume a more general nature of this phenomenon. Significant deviations observed in estimating L-arginine in the algal cells after application of different conventional analytical methods can be attributed not only to the method of analysis used but also to differences in cultivation and to the external cultivation factors.

Under conditions of radioactive cultivation of the alga the arginine peptides may represent an additional potential source of L-arginine-^{14}C /U/. For this reason, a simple technological procedure for its preparation in a chromatographically pure form was suggested here.

References

1. Nejedlý, Z., Filip, J., Ekl, J., Kolina, J. and Grünberger, D. - Czechoslovak Patent 121 808 /PV 4918-65/.
2. Nejedlý, Z., Koutecký, Z. and Grünberger, D. - Coll. Czech. Chem. Commun., 30:3361 /1965/.
3. Nejedlý, Z., Filip, J. and Grünberger, D. - Proceedings of the Second International Conference on Methods of Preparing and Storing Labelled Compounds, Brussels, Nov. 28 - Dec. 3, 1966, pp. 527 - 538.
4. Nejedlý, Z., Filip, J., Hybš, K., Ekl, J. - Technical Documentary Report of the Institute for Research, Production and Uses of Radioisotopes, Prague /1966/.
5. Hutchison, W.C. and Munro, H.N. - Analyst, 86:768 /1961/.
6. Martin, E.M. and Morton, R.K. - Biochem. J., 64:221 /1956/.
7. Thomas, J.A. and Sherrat, H.S.A. - Biochem. J., 62:1 /1956/.
8. Smillie, R.M. and Krotkov, S. - Canad. J. Botan., 38:31 /1960/.
9. Ellner, P.D. - Plant Physiol., 34:638 /1959/.
10. Wacker, A., Traeger, L. and Büttner, H. - Naturwissenschaften, 49:518 /1962/.
11. Buchan, J.L. and Savage, R.I. - Analyst, 77:401 /1952/.
12. Acher, R. and Crocher, C. - Biochim.Biophys.Acta 9:704 /1952/.
13. Šorm, F., Turský, T. and Šebesta, K. - Chem. listy, 46:375 /1952/
14. Syrett, P.J. - Ann. Botan., 17:21 /1953/.
15. Syrett, P.J. and Fowden, L. - Physiol. Plant., 5:558 /1952/.
16. Thomas, W.H. and Krauss, R.W. - Plant Physiol., 30:113 /1955/.
17. Hattori, A. - J. Biochem., 45:57 /1958/.
18. Kanazawa, T. - Plant and Cell. Physiol., 5:333 /1964/.
19. Kanazawa, T., Kanazawa, K. and Morimura, Y. - Plant and Cell Physiol., 6:631 /1965/.