FRACTIONATION OF THE INSOLUBLE MATERIAL OF *CHLORELLA* CELLS

LUISE STANGE*, V. MOSES** AND M. CALVIN

Lawrence Radiation Laboratory and Department of Chemistry, University of California, Berkeley, Calif. (U.S.A.)

(Received December 1st, 1959)

SUMMARY

A procedure is described for the fractionation of *Chlorella* cell material, labeled with ^{14}C, based on solvent extraction followed by serial digestion with several enzymes, and extraction of the breakdown products following each enzymic treatment. The cell material was divided into fractions containing the following substances or their breakdown products: water- and ethanol-soluble metabolic intermediates; lipids and pigments; ribonucleic acid; deoxyribonucleic acid; proteins and polysaccharides; some radioactivity remained in the residue after extraction.

There was some variation in the purity of the various fractions. Of the ethanol- and water-insoluble materials, the fractions prepared by the use of ribonuclease and proteolytic enzymes contained only those substances to be expected following treatment with these enzymes. The deoxyribonucleic acid fraction was contaminated with glucose and some unidentified substances. However, clear evidence was obtained for the incorporation of ^{14}C into thymine, a substance characteristic of deoxyribonucleic acid.

The polysaccharide fraction, after hydrolysis, contained some amino acids from residual protein, but these could easily be separated from the sugars by paper or column chromatography.

INTRODUCTION

Most of the past work to investigate the incorporation of $^{14}CO_2$ during photosynthesis has been restricted to short-term studies of the ethanol- and water-soluble materials[1]. However, there are many problems in which the patterns of incorporation of the labelled carbon into the ethanol- and water-insoluble materials would be relevant. Although many techniques are available for the quantitative analysis of the insoluble materials of plant cells (proteins[2,3], carbohydrates[2], fats[2], pigments[2], nucleic acids[2,4], etc.), there are no methods available, as far as we are aware, for the actual physical separation of the various constituents in order that they may be separately assayed for incorporated radioacitivity. Chemical analysis of the fractions may be

* Present address: Institut für Entwicklungsphysiologie, Universität Köln, Köln, Germany.
** Present address: Department of Chemical Pathology, King's College Hospital Medical School, London, England.

performed in the presence of other fractions provided that a specific method exists for the determination of the particular fraction under investigation. This type of methodology is not applicable to radioactivity measurements. In the latter case the criterion by which a fraction is assayed is the same for all the fractions studied, viz. the radioactivity. It is therefore obvious that unless the fractions can actually be separated one from the other, no estimate can be made of the radioactivity incorporated into each one.

While purely chemical methods exist for the fractionation and separation of bacterial cell materials, such as the scheme devised by ROBERTS *et al.*[5] for the analysis of *Escherichia coli*, these methods tend not to be satisfactory for plant cells owing to the large amounts of polysaccharide present in the latter, which often interfere with the various acidic extraction procedures for such substances as nucleic acids. No suitable methods along these lines have yet been worked out for plant cells (see ref. 2, p. 17).

It was therefore decided to attempt to develop a fractionation scheme based on enzymic analysis, since a greater degree of specificity for each fraction could be expected than when using purely chemical extraction procedures. The present communication describes such a scheme which has proved to be reasonably suitable for *Chlorella* cells, though further investigation has shown that it cannot be applied without modification to all plant tissues. It consists essentially of the following steps: (a) the removal of the ethanol- and ether-soluble materials; this is followed by a successive treatment by the following enzymes: (b) ribonuclease (c) deoxyribonuclease, (d) trypsin and chymotrypsin alternately and (e) diastase.

METHODS

Growth and photosynthesis

Cells were grown in the continuous culture apparatus described by HOLM-HANSEN *et al.*[6]. The cells were harvested, washed with fresh nutrient solution, and 1–2 ml of wet-packed cells were resuspended in 60 ml of nutrient solution MYER'S medium[7] and placed in the "steady state apparatus" described by BASSHAM *et al.*[8] and WILSON AND CALVIN[9]. 1 ml of $NaH^{14}CO_3$ (0.026 *M*; 400 μC) was added to the suspension and the cells were allowed to carry on photosynthesis for 4 h at a light intensity of 3000 foot candles. At the end of this period the cells were centrifuged, resuspended in 2 ml of distilled water, and lyophilized.

A number of analytical procedures were investigated and the following has given the most satisfactory results. A summary of this procedure appears in Table I.

Soluble materials

A portion of the dried cells (40 mg) was homogenized in a Potter homogenizer with 6 ml of 80 % (v/v) ethanol, and the suspension subsequently heated in a bath of boiling water until the ethanol began to boil. The cell residue was removed by centrifugation, and was re-extracted with 2 ml of boiling 80 % ethanol. This extraction was repeated twice more. After determination of the radioactivity in each extract, they were pooled. The residue was next extracted with 5 ml of 20 % (v/v) ethanol for 2 min at 70°. This extraction was repeated twice more with 2-ml amounts of 20 % ethanol.

TABLE I

DISTRIBUTION OF ACTIVITY IN VARIOUS CHEMICAL FRACTIONS OF *Chlorella* CELLS AFTER PHOTOSYNTHESIS FOR 4 H WITH RADIO-BICARBONATE

After photosynthesis the cells were lyophilized and extracted with various solvents and enzymes in the sequence shown below. The values given are the percentages of the total radioactivity fixed by the *Chlorella* cells; this was $128.5 \cdot 10^6$ counts/min/40 mg of lyophilized cells.

Sample No.	*Extracted with*	*Temp.*	*Extraction period*	*Activity (% of total)*	*Total activity in whole fraction (% of total)*
Ethanol–water and ethanol–ether soluble fractions					
1	80% ethanol	Boiling	—	27.48	
2	80% ethanol	Boiling	—	2.29	
3	80% ethanol	Boiling	—	0.98	31.21
4	80% ethanol	Boiling	—	0.46	
5	20% ethanol	70	2 min	4.70	
6	20% ethanol	70	2 min	0.68	5.81
7	20% ethanol	70	2 min	0.43	
8	Distilled water	70	5 min	0.56	0.72
9	Distilled water	70	5 min	0.16	
10	Ethanol–ether (3:1)	70	5 min	0.12	0.12
	A total ethanol soluble fraction				37.86
	A 1, ether-extract of A			15.64	—
	A 2, residue from A 1			22.22	—
Ethanol–water and ethanol–ether insoluble fractions					
11	Ribonuclease	37	6 h*	2.56	
12	Distilled water rinse	—	—	0.29	
13	Ribonuclease	37	3 h	0.22	3.22
14	Distilled water rinse	—	—	0.15	
15	Deoxyribonuclease	37	3 h**	0.47	
16	$MgSO_4$ rinse	—	—	0.14	
17	Deoxyribonuclease	37	1 h	0.13	0.79
18	$MgSO_4$ rinse	—	—	0.05	
19	Trypsin	37	39 h	27.17	
20	Distilled water rinse	—	—	2.59	
21	Trypsin	37	24 h	2.67	
22	Distilled water rinse	—	—	0.02	
23	Chymotrypsin	37	24 h	8.19	
24	Distilled water rinse	—	—	1.41	
25	Chymotrypsin	37	24 h	3.32	
26	Distilled water rinse	—	—	0.92	49.53
27	Trypsin	37	24 h	0.81	
28	Distilled water rinse	—	—	0.38	
29	Trypsin	37	24 h	0.31	
30	Distilled water rinse	—	—	0.18	
31	Chymotrypsin	37	60 h	0.69	
32	Distilled water rinse	—	—	0.27	
33	Diastase	37	20 h	3.57	
34	Distilled water rinse	—	—	0.44	
35	Diastase	37	20 h	0.86	6.04
36	Distilled water rinse	—	—	0.33	
37	Diastase	37	20 h	0.70	
38	Distilled water rinse	—	—	0.14	
39	Residue	—	—	8.34	8.34
Total recovered activity				105.98	

* 3 h is the optimum period for the first ribonuclease extraction (see Table II).
** 1 h is the optimum period for the first deoxyribonuclease extraction (see Table II).

After the extractions with ethanol, the residue was extracted with 15 ml of distilled water for 5 min at 70°; this extraction of the residue was repeated once more with 6 ml of water. The next extraction of the residue was with 6 ml of ethanol–ether (3:1, v/v) for 5 min at 70°. The above extractions removed those substances from the cells which are usually considered as being in the ethanol- and water-soluble fractions: the soluble metabolites, pigments, and lipids.

The total ethanol–, water– and ethanol–ether extracts were combined and extracted 3 times with 5 ml of ether. The total ether extracts were combined and the activities of the ethanol–water-soluble and ether-soluble fractions were measured.

Nucleic acids

The residue from the previous extracts was treated for 6 h with 3 mg of ribonuclease dissolved in 3 ml of water at 37°. After 3 h an aliquot was removed for determination of radioactivity in the supernatant after centrifugation. After the 6 h extraction period, the suspension was centrifuged and the residue was washed with 3 ml of distilled water. The residue was treated with a further 3 mg of ribonuclease in 3 ml of distilled water for 3 h at 37°, and the residue was again washed with 3 ml of water.

Following treatment with ribonuclease, the residue was digested with 3 mg of deoxyribonuclease in 3 ml of 0.003 *M* $MgSO_4$ for 3 h at 37°. After 1 h of this digestion, an aliquot was removed for assay of the radioactivity in the supernatant after centrifugation. At the end of the 3-h digestion period, the suspension was centrifuged and the residue washed with 3 ml of 0.003 *M* $MgSO_4$. The digestion with deoxyribonuclease was repeated (2 mg of enzyme in 2 ml of $MgSO_4$ solution) for 1 h and the residue was again washed with 2 ml of $MgSO_4$ solution.

Protein

The residue from the deoxyribonuclease extractions was incubated at 37° for 39 h with 20 mg of trypsin dissolved in 2 ml of 0.01 *M* potassium phosphate buffer, pH 7.6. An aliquot was removed after 15 h for radioactivity determinations on the supernatant. The residue was washed with 2 ml of distilled water. The extraction with trypsin was repeated for a further 24 h at 37°, and the residue was again washed with 2 ml of water. The residue was next extracted with chymotrypsin: 20 mg of enzyme was dissolved in 2 ml of 0.01 *M* phosphate buffer, pH 7.6, and the extraction was allowed to proceed at 37° for 24 h. After washing with 2 ml of distilled water, the extraction with chymotrypsin (24 h) and the washing with water was repeated.

The residue was again extracted with 2 ml of trypsin solution in phosphate buffer for 24 h, followed by washing with 2 ml of water. The trypsin digestion was repeated once more (24 h), followed by another washing with 2 ml of water. A final extraction was made with 2 ml of chymotrypsin solution in phosphate buffer (60 h) and the residue was again washed with water.

Polysaccharides

After removal of the proteins, the residue was treated for 20 h at 37° with 90 mg of malt diastase dissolved in 3 ml of distilled water. 8 h after the beginning of the incubation period, an aliquot was assayed for radioactivity in the supernatant following centrifugation. The residue was washed with 2 ml of water. The extractions with

diastase (for 20 h periods) were repeated twice more. A residue remained which was not further extracted.

Analysis of the fractions

The successive extractions for each fraction were pooled and an aliquot was analyzed by paper chromatography to check the nature of the substances removed from the cells by each enzymic treatment. All chromatograms were made on oxalic acid-washed Whatman No. 4 filter paper, and were developed in phenol–water in the first dimension and in *n*-butanol–propionic acid–water in the second dimension[10]. Radioactive substances were located by exposure of the chromatograms to Dupont X-ray film No. 507E. Substances were identified by their chromatographic positions, by cochromatography with known markers, and by other properties such as absorption of u.v. light and reaction with appropriate sprays.

All fractions extracted from the insoluble material gave unsatisfactory chromatograms* unless they were first subjected to acid hydrolysis. After several trial attempts, the following hydrolysis conditions were found to be the most satisfactory: (a) for the ribonucleic acid fractions: heated at 100° for 2 h with 1 *N* HCl; (b) for the deoxyribonucleic acid fraction: heated at 120° for 2 h with 6 *N* HCl; (c) for the protein fractions: 1 volume of solution heated for 16 h at 110° with 8 volumes of a mixture of equal parts of 10 *N* HCl and glacial acetic acid; (d) for the polysaccharide fractions: heated with *N* HCl for 3 h at 100°. After hydrolysis, each deoxyribonucleic acid and each protein sample was evaporated to dryness, redissolved in water, taken to dryness twice more, and finally dissolved in water and applied to the chromatogram.

Radioactivity determinations

Samples for assay of radioactivity were dried onto thin aluminium planchettes and counted with a thin-window Scott-type Geiger-Muller tube connected to a scaler. Substances counted directly from paper chromatograms were counted with a similar tube. The tubes were flushed continuously with a gas mixture consisting of 99.05 % (v/v) helium and 0.95 % (v/v) isobutane.

Enzymes

The enzyme preparations used were obtained from the following sources: ribonuclease (crystallized) and chymotrypsin (crystallized, salt-free) from Armour Laboratories, Chicago, Ill.; deoxyribonuclease (once crystallized) from Worthington Biochemical Corp., Freehold, N.J.; trypsin (twice crystallized; contains approximately 50 % $MgSO_4$) from Mann Research Laboratories, New York, N.Y.; malt diastase from Nutritional Biochemicals Corp., Cleveland, Ohio.

RESULTS

Quantitative results

In almost every case a single extraction with a solvent or an enzyme is not sufficient to achieve a quantitative extraction of the substances involved. However, even though repeated extractions remove lesser and lesser amounts with each successive treatment, it is extremely difficult to reach a stage where no material at all is

* The chromatograms were made unsatisfactory by immovable origins and streaking spots.

removed with a further extraction[8]. For this reason it is necessary to select some limit beyond which a particular extraction would not be pursued. In practice each extraction was stopped when no more than about 0.5 % of the total radioactivity originally present in the sample of lyophilized cells was removed by a further treatment; for the nucleic acid fractions this limit was reduced to about 0.2 %.

Table I shows the sequence of extractions and washings and gives the radioactivity found in each fraction expressed as a percentage of the total radioactivity present in the whole sample of lyophilized cells before extraction.

Extraction times

During the first extraction with ribonuclease, deoxyribonuclease, trypsin, and diastase, aliquots were removed from the reaction vessel at various times in order to determine the optimum extraction period for each of these enzymes (see METHODS section). Table II shows the activity extracted after various incubation periods with these enzymes. The results demonstrated that, while there was no added advantage in extending the extraction periods with ribonuclease and deoxyribonuclease for longer than 3 h and 1 h, respectively, extraction was not complete with trypsin after 15 h, and 24 h was adopted as the standard extraction period. Extraction with diastase was incomplete after 8 h, and 20 h was made the standard reaction period.

TABLE II

EFFECT OF THE LENGTH OF THE INCUBATION PERIOD ON THE EXTRACTION OF THE INSOLUBLE CELL MATERIAL BY VARIOUS ENZYMES

The values given are the percentages of the total radioactivity fixed by the *Chlorella* cells. See Table I for identity of the sample nos.

Sample No.	*Extracted with*	*Extraction period*	*Activity % of total*
11	Ribonuclease	3 h	2.43
11	Ribonuclease	6 h	2.56
15	Deoxyribonuclease	1 h	0.41
15	Deoxyribonuclease	3 h	0.47
19	Trypsin	15 h	19.61
19	Trypsin	39 h	27.17
33	Diastase	8 h	2.63
33	Diastase	20 h	3.57

Composition of the extracted fractions

Ethanol–water-soluble fraction: The nature of the compounds extracted by each procedure was investigated chromatographically. The ethanol–water-soluble fractions contained sugar phosphates, amino acids, organic acids, free sugars, pigments, and lipids. After extraction of this fraction with ether, the ether contained the pigments, lipids, and phospholipids. These fractions were not investigated further.

Ribonucleic acid fraction: Chromatography of this fraction after hydrolysis with *N* HCl showed the presence of ^{14}C, in (a) ribose (14.6 % of the total radioactivity in this fraction), (b) in adenine (10.9 %), (c) in guanine (16.4 %), (d) in uridine (7.7 %), (e) in uridylic acid (23.7 %), (f) in an unknown u.v.-absorbing spot (19.2 %), (g) a small amount in glucose (2.4 %) and (h) in two unknown spots and the origin (5.1 %) (Fig. 1). It was found that the proportion of labeled glucose appearing in this fraction

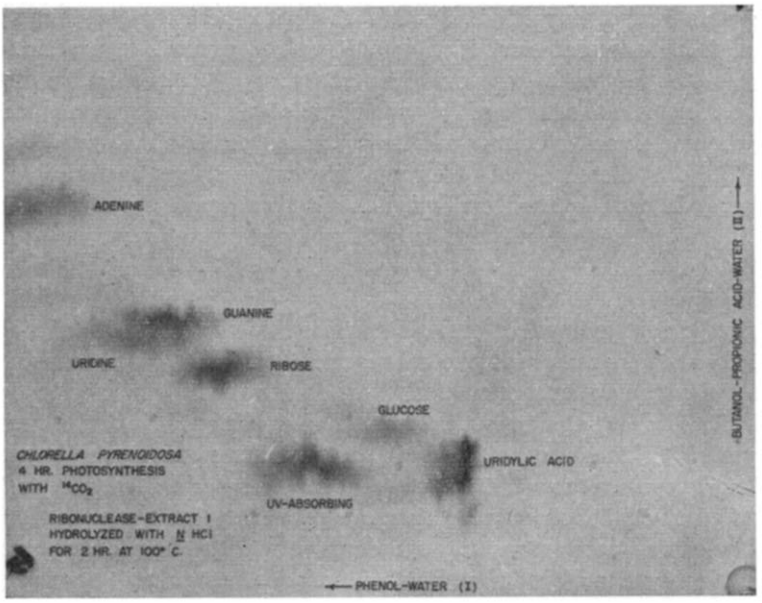

Fig. 1. Radioautogram of a chromatogram of the ribonuclease extract of *Chlorella pyrenoidosa* after hydrolysis with 1 *N* HCl for 2 h at 100° (4 h photosynthesis with $^{14}CO_2$).

increased considerably if the total extraction period with ribonuclease was prolonged for more than 6 h.

In another analytical procedure, cell residues, which had previously been extracted with ethanol–water and ethanol–ether, were extracted with 10 % (w/v) perchloric acid for 18 h at 4° (see ref. 4); this extract had the same qualitative and quantitative composition as extracts prepared with ribonuclease. Perchloric acid extracted 3.40 % of the total ^{14}C of the lyophilized cells compared with a total of 3.22 % in all the ribonuclease extracts and washings (Table I).

Deoxyribonucleic acid fraction: Very little ^{14}C was found in this fraction (Table I). While some of the constituents in this fraction, following hydrolysis, could be identified as nucleic acid components, this fraction was also contaminated with glucose and some unidentified compounds. Some of the radioactivity in this fraction coincided chromatographically with u.v.-absorbing substances which moved chromatographically in the positions of nucleosides, nucleotides and bases. As it was very difficult to decide whether the u.v.-absorbing materials originated from deoxyribonucleic acid, or residual ribonucleic acid, a specific search was made for the presence of thymine, the only purine or pyrimidine base characteristic of deoxyribonucleic acid as distinct from ribonucleic acid.

The isolation of deoxyribose is particularly difficult since chemical procedures to remove the phosphate from the deoxyribose nucleotides invariably destroy the sugar[12]; thymine was therefore used as the criterion of deoxyribonucleic acid extraction. Labeled thymine was indeed detected, indicating that this fraction did contain deoxyribonucleic acid although it was seriously contaminated with other substances. The following distribution of activity was found: thymine (4.7 %); adenine (2.8 %),

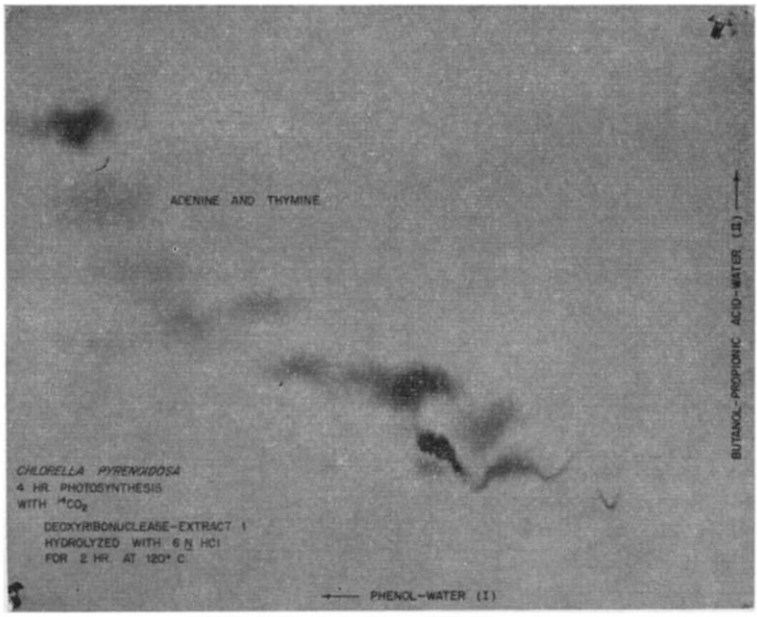

Fig. 2. Radioautogram of a chromatogram of the deoxyribonuclease extract of *Chlorella pyrenoidosa* after hydrolysis with 6 *N* HCl for 2 h at 120° (4 h photosynthesis with $^{14}CO_2$).

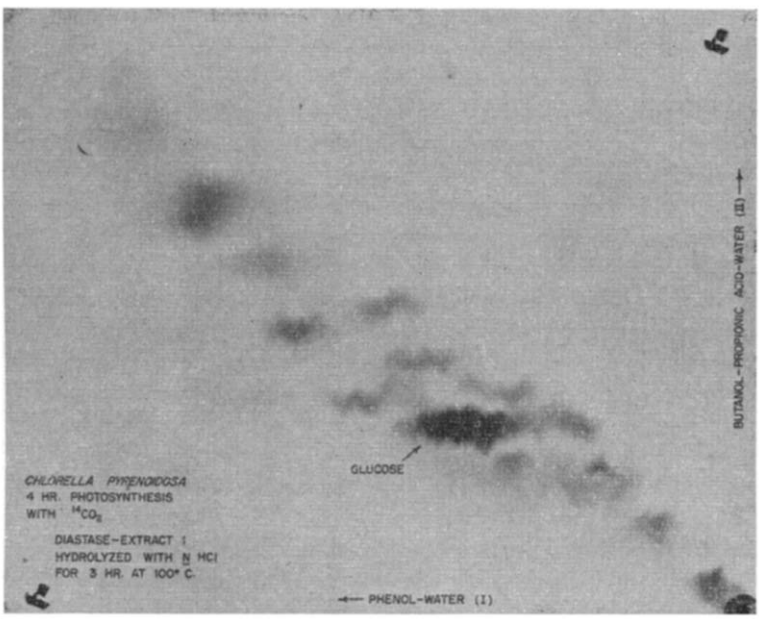

Fig. 3. Radioautogram of a chromatogram of the diastase extract of *Chlorella pyrenoidosa* after hydrolysis with *N* HCl for 3 h at 100° (4 h photosynthesis with $^{14}CO_2$).

other u.v.-absorbing substances (11.4 %); glucose plus levulinic acid (derived from glucose by acid treatment) (30.6 %); unidentified substances (51.5 %) (Fig. 2). Thymine and adenine are not well separated chromatographically with the solvents mentioned in the METHODS section. The mixed spot of thymine and adenine was therefore resolved by chromatography using water adjusted to pH 10 with ammonium hydroxide (see ref. 4, p. 252).

In order to detect free thymine vigorous hydrolysis conditions for deoxyribonucleic acid were necessary[13]. Under these conditions deoxyribose is destroyed[12].

Protein fraction: After hydrolysis this fraction consisted solely of amino acids: each radioactive area corresponded exactly with a ninhydrin-reacting area, and each ninhydrin-reacting area was radioactive. No differences were seen in extracts made with trypsin or with chymotrypsin, and the relative amounts of each of the amino acids remained constant through successive treatments with the proteolytic enzymes.

Polysaccharide fraction: This fraction contained, after hydrolysis, mainly glucose (37.4 % of the total ^{14}C in this fraction), together with some fructose (3.5 %), amino acids, (39.3 %), and some unidentified substances (19.8 %), one of which may have been a pentose (Fig. 3). The amino acids may be separated from the sugars either by treatment with Dowex 50, or by paper chromatography, and the relative amounts of ^{14}C in each determined separately.

Residue after extraction: Hydrolysis of the final residue either with *N* HCl or with a mixture of equal parts of 10 *N* HCl and glacial acetic acid demonstrated that most (65–85 %) of the residual ^{14}C was present in amino acids.

DISCUSSION

The fractionation method described in this paper had been shown to be applicable to the separation of the various insoluble constituents of *Chlorella* cells. There exist in algal cells, as in all others, a large number of different compounds, and different groups of compounds, the physical separation of all of which in a pure state would represent an enormous undertaking. In the present study an attempt has been made only to separate the cells into a small number of fractions, all of which must be mixtures of many different individual substances. Nevertheless, it has been possible to separate in a more or less pure state five fractions from ^{14}C labeled *Chlorella* cells: ethanol- and water-soluble metabolic intermediates; fats, pigments and other ether-soluble components; nucleic acids; proteins; and polysaccharides. There remained a small residue which was shown to contain ^{14}C mainly in amino acids.

The ethanol–water-soluble, ether-insoluble material, containing metabolic intermediates involved in the carbon reduction cycle, tricarboxylic acid cycle, etc., have been studied elsewhere[1] and have not been further considered in the present work; the ether-soluble materials (lipids and pigments) have also not been further fractionated[14]. The ribonucleic acid fraction, as extracted from the cell residues by ribonuclease, contained only ribonucleic acid as far as the ^{14}C content was concerned, provided that the extraction conditions as described in the METHODS section were properly observed. If the incubation period with ribonuclease was prolonged beyond a total of 6 h, labeled glucose began to appear in significant amounts in the extract. On the other hand, too short an extraction period resulted in the presence of labeled ribose in the deoxyribonucleic acid fraction.

The deoxyribonucleic acid fraction was the least satisfactory as regards purity. Some radioactivity was associated with thymine, indicating that the fraction indeed contained deoxyribonucleic acid. However, many contaminating substances were also present, and the hydrolysis conditions necessary for the isolation of thymine precluded the detection of deoxyribose. That the separation of RNA and DNA was not complete is demonstrated by the presence of a trace of thymine associated with the adenine from the RNA'se extract; the adenine-thymine area obtained from the DNA'se extract was largely thymine with some (1/3) adenine.

The alternate incubation with trypsin and chymotrypsin removed substances yielding only amino acids on hydrolysis. There was a distinct advantage in the use of two proteolytic enzymes for the extraction of protein (Table I). The main disadvantage of using enzymes to extract the protein was the very long incubation periods which had to be used. Even after a total incubation period with trypsin and chymotrypsin of 219 h, proteins were still removed in the subsequent diastase extractions, and amino acids were found in the hydrolysate of the final residue. It was considered easier to separate the amino acids from the sugars by paper or column chromatography after hydrolysis of the diastase extracts rather than to continue extraction with the proteolytic enzymes until no more ^{14}C appeared in amino acids.

The substances extracted by diastase, apart from some activity in amino acids as mentioned above, contained ^{14}C mainly in glucose. The presence of activity in fructose, and in an unidentified substance (probably a pentose from its chromatographic behavior) indicated the existence of polysaccharides other than glucose polymers in this organism.

The fractionation methods described in this communication have also been applied to the liverwort *Riella helicophylla*. The procedure was not satisfactory as early as the ribonucleic acid stage, since large amounts of labeled glucose appeared in this fraction after a relatively short incubation period with ribonuclease (3 h). This organism contains large amounts of starch, as can be seen by staining with iodine, and part of this starch may be rendered soluble by incubation with ribonuclease solution at 37°. Hence it may prove necessary to develop critical extraction procedures for each new plant species depending on the particular nature of the polysaccharide content.

ACKNOWLEDGEMENTS

This work was sponsored in part by the U.S. Atomic Energy Commission and in part by the Department of Chemistry, University of California, Berkeley, Calif. One of the authors (L.S.) is a research Fellow sponsored by das Deutsche Bundesministerium für Atomkernenergie (1957–8).

REFERENCES

[1] M. CALVIN, *J. Chem. Soc.*, (1956) 1895.
[2] K. PAECH AND M. V. TRACEY, *Modern Methods of Plant Analysis*, Springer-Verlag, Berlin-Göttingen-Heidelberg, 1956.
[3] L. FOWDEN, *Biochem. J.*, 50 (1952) 355.
[4] E. CHARGAFF AND J. N. DAVIDSON, *The Nucleic Acids*, Academic Press Inc., New York, 1955.
[5] R. B. ROBERTS, P. H. ABELSON, D. B. COWIE, E. T. BOLTON AND R. J. BRITTEN, *Carnegie Institution of Washington Publication*, Washington D.C., 1955, p. 607.

[6] O. HOLM-HANSEN, P. HAYES AND P. SMITH, *University of California Radiation Laboratory Chemistry Division Quarterly Reports*, UCRL-3595, 1956, p. 56.
[7] P. W. KRAUSS, in *Algal Culture from Laboratory to Pilot Plant*, Carnegie Institution of Washington, Washington D.C., 1953, publication 600.
[8] J. A. BASSHAM, K. SHIBATA, K. STEENBERG, J. BOURDON AND M. CALVIN, *J. Am. Chem. Soc.*, 78 (1956) 4120.
[9] A. T. WILSON AND M. CALVIN, *J. Am. Chem. Soc.*, 77 (1955) 5948.
[10] A. A. BENSON, J. A. BASSHAM, M. CALVIN, T. C. GOODALE, V. A. HAAS AND W. STEPKA, *J. Am. Chem. Soc.*, 72 (1950) 1710.
[11] M. OGUR AND G. ROSEN, *Arch. Biochem.*, 25 (1949) 262.
[12] R. FEULGEN, *Chemie und Physiologie der Nukleinstoffe*, Berlin, 1923.
[13] E. CHARGAFF, R. LIBSHITZ, C. GREEN AND M. E. HODES, *J. Biol. Chem.*, 192 (1951) 223.
[14] U. BLASS, *University of California Radiation Laboratory Chemistry Division Quarterly Report*, UCRL-3836, 1957, p. 46.

Biochim. Biophys. Acta, 42 (1960) 272–282

THE INFLUENCE OF STRUCTURE ON THE ORIENTATION OF THE VERATRUM ALKALOIDS AT THE AIR/WATER INTERFACE

NORMAN L. GERSHFELD

National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. (U.S.A.)

(Received December 12th, 1959)

SUMMARY

The veratrum alkaloids—veracevine, veratridine, cevadine, and veratramine—have similar chemical structures yet are quite distinct in their pharmacological actions. Veracevine is inactive, veratridine and cevadine are "labilizers" or "unstabilizers", and veratramine is an antagonist for cevadine and veratridine. The properties of these compounds at the air/water interface also reveal marked differences. Surface tension measurements in water show surface activity decreases in the order veratramine $>$ veratridine $>$ cevadine $\gg$ veracevine. A Langmuir-type film balance was used to measure their F-A curves. Films of the alkaloids were unstable because of their slight solubility in the substrate, and a technique was devised which permits reproducible F-A measurements. Despite these precautions, veracevine did not form any films. The F-A curves for veratridine and cevadine show (a) an inflection which corresponds to their areas when oriented horizontally at the a/w interface, and (b) an area in which the molecules are tight-packed corresponding to molecular areas for the vertical orientation of the molecules. Veratramine shows no inflection in the F-A curve, but the tight-packed film occurs at an area in which this molecule is oriented almost horizontal to the a/w interface. Interpretation of these results is in terms of the distribution of hydrophobic and hydrophilic groups on the molecules; their implication for pharmacological action is discussed.