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INVESTIGATION OF A CHLOROPHYLL–LIPOPROTEIN COMPLEX

D. THIRKELL

Department of Physiology and Biochemistry, St. Salvator's College, University of St. Andrews

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Abstract—The nature of the chlorophyll–lipoprotein complex, first isolated by Takashima[4] was surveyed using several analytical techniques. Initial findings were all consistent with the view that such a complex could be isolated by Takashima's method. Paper chromatography showed that the crystalline material was not pure, but a mixture of three pigments and two protein-staining bands. The amino acids of the protein(s) were identified by paper chromatography after previous acid hydrolysis. After separation of the material on Sephadex G.50, one of the protein-staining bands was no longer present and there was no overlapping of the remaining protein band with any of the three pigment bands. It was concluded from the available evidence that if a complex exists, the association of chlorophyll and protein is by physical rather than chemical bonding.

BY 1940 there was a growing volume of evidence which suggested that in the chloroplast, the protein and chlorophyll were in combination (French,[1,2] Smith[3]). Takashima[4] published details of a crystalline complex from chloroplasts and Chiba,[5] using a modified method, found that the crystals of the complex had at least two components, each of which could be crystallized separately. Osipova[6] also suggested such a complex between protein and chlorophyll, while indicating that pigment is also present in the free state. Anderson, Spikes and Lumry[7] isolated the crystals using the same type of procedure, but they maintained that the chlorophyll was not chemically bound to protein. A new investigation of Takashima's chlorophyll–lipoprotein complex was therefore undertaken.

METHODS AND RESULTS

Isolation of the Complex

The method of Takashima[4] was followed closely except that pyridine was substituted for α-picoline. Sugar beet leaves were obtained fresh and stripped of their midribs and main veins. They were suspended in 5 vol. (w/v) M/50 phosphate buffer at pH 7·0 (Cole[8]) and disintegrated in a homogenizer. The slurry was centrifuged at 2500 rev/min for 20 min and the supernatant filtered through a large pad of cotton wool giving a green opalescent solution. With constant stirring, the solution was made 55% with respect to pyridine and dialysed against 55% pyridine for 20 hr. This was repeated with three changes of solvent. The contents of the dialysis sack were filtered and dioxan added to the filtrate to make it 20% with respect to dioxan. After 5 days at 0° the crystals present were spun down and dissolved in M/1200

[1] C. S. FRENCH, *J. Gen. Physiol.* **21**, 71 (1938).
[2] C. S. FRENCH, *J. Gen. Physiol.* **23**, 469 (1940).
[3] E. L. SMITH, *J. Gen. Physiol.* **24**, 583 (1941).
[4] S. TAKASHIMA, *Nature, Lond.* **169**, 182 (1952).
[5] Y. CHIBA, *Arch. Biochem. Biophys.* **54**, 83 (1955).
[6] O. P. OSIPOVA, *Fiziol. Rastenii, Akad. Nank SSSR.* **4**, 28 (1957).
[7] D. R. ANDERSON, J. D. SPIKES and R. LUMRY, *Biochim. et Biophys. Acta* **15**, 298 (1954).
[8] S. W. COLE, *Practical Physiological Chemistry* (10th Edn.), Heffer and Sons Ltd., Cambridge (1955).

phosphate buffer, pH 7·0 (Cole[8]). The solution was filtered and the filtrate again made 20% with respect to dioxan and placed in the refrigerator. During the next 3 weeks, four crops of crystals were collected, lyophilized and stored in a dark bottle.

Examination of the Complex

The following tests were carried out—microscopic examination (before lyophilization), tests for protein (Biuret, Xanthoproteic and Ninhydrin), ash content, nitrogen content (microkjeldahl[9]), chlorophyll content,[10] phosphorus content[11,12] and determination of minimum molecular weight. The latter was calculated on the basis that one molecule of the complex would contain a minimum of one gram molecule of chlorophyll per molecule of protein, the contribution of any other lipid which may be present being ignored. The results of these general tests carried out on the complex are shown in Table 1.

TABLE 1. GENERAL DATA ON THE COMPLEX

Investigation	Result	Comment
Crystalline nature	Star-shaped crystals	Crystals fragmented with the least disturbance of the solvent
Protein nature:		
(*a*) Biuret test	Positive?	After boiling with 10% NaOH a green solution remained which made final colour determination difficult
(*b*) Xanthoproteic test	Positive	Aromatic amino acid(s) present
(*c*) Ninhydrin test	Positive?	Ninhydrin over the crystals slowly turned purple
Minimum molecular weight	8642	See discussion
Ash content	3·41%	—
Nitrogen content	8·92%	—
Chlorophyll content	9·20%	—
Phosphorus content:		
(*a*) King method[11]	2·10%	—
(*b*) Allen method[12]	2·10%	—

Paper Chromatography of the Complex

Chromatograms of the complex were run before and after acid hydrolysis.

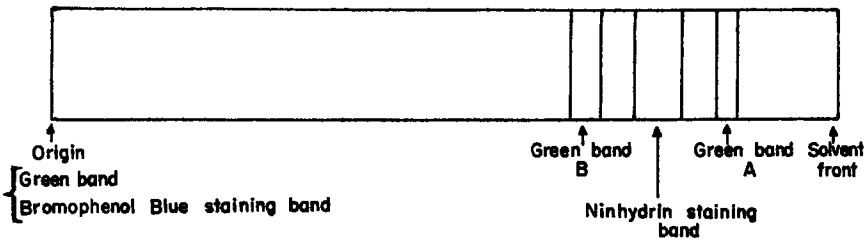
(*a*) Before hydrolysis: The complex was solubilized in 55% pyridine and run in an ascending direction on paper 31 with a pyridine/water (55/45:v/v) solvent for 7 hr. After drying, the papers were examined in daylight, under ultra-violet light and after staining with either ninhydrin or bromophenol blue[13] (Fig. 1). Three green bands were clearly visible, one of which remained at the origin while the other two moved with R_f's of 0·68 and 0·84 respectively. The band at the origin exhibited a red fluorescence in ultra-violet light and stained with bromophenol blue. The bands with R_f's of 0·68 and 0·84 did not fluoresce, or

[9] A. C. CHIBNALL, M. W. REES and E. F. WILLIAMS, *Biochem. J.* 37, 354 (1943).
[10] *Methods of Analysis A.O.A.C.* (8th Edn.) Washington, D.C., U.S.A. (1955).
[11] E. J. KING, *Biochem. J.* 2, 292 (1932).
[12] R. J. L. ALLEN, *Biochem. J.* 34, 858 (1940).
[13] I. SMITH, *Chromatographic and Electrophoretic Techniques*, Vol. 2, p. 11, W. Heineman Press, London and New York (1960).

react with ninhydrin or bromophenol blue. There was however, evidence of a ninhydrin-reactive band (R_f 0·78) midway between these bands. On this evidence it would appear therefore, that only the band with zero mobility is directly associated with protein and that the mobile green bands contain only free pigment.

(*b*) After hydrolysis: The complex was hydrolysed in a sealed tube for 18 hr at 105°. All traces of the acid were removed by re-distillation of the hydrolysate. Descending chromatograms were developed for varying periods of time with butanol/acetic acid/water (70/12/25: v/v). After drying, they were stained with ninhydrin. Twelve amino acids were found which, from the positions of the markers, appeared to be leucine, valine, alanine, threonine, glutamic acid, aspartic acid, histidine, lysine, glycine, serine, proline and tyrosine. These amino acids

FIG. 1. ASCENDING PAPER CHROMATOGRAM OF A 55% PYRIDINE SOLUTION OF THE COMPLEX.



Paper: Whatman No. 31; Solvent: Pyridine/Water (55:45, v/v).

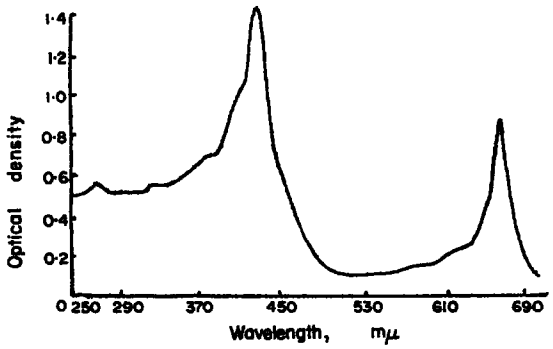


FIG. 2. ABSORPTION SPECTRUM OF A LIGHT PETROLEUM EXTRACT (b.p. 60–80°) OF THE CRYSTALS.

may have been liberated from a chlorophyll–protein complex or from the free moving, ninhydrin reactive band.

Absorption Spectroscopy

This was carried out on light petroleum extracts (b.p. 60–80°) of (*a*) the crystals (between 250–700 mμ) and (*b*) the green bands separated by paper chromatography (between 360–700 mμ).

Figure 2 shows the absorption spectrum obtained with a light petroleum extract (b.p. 60–80°) of the crystals, and Fig. 3 the spectra for the eluates of the two mobile green bands A & B (Fig. 1). Chromatography of authentic samples of chlorophylls a and b in the same solvent indicated that bands A and B were probably chlorophylls b and a respectively. A combination of these two eluates produces a spectrum which is almost identical to that

of the crystals suggesting that if the pigment is bound in the complex, the bonding has little effect on its absorption spectrum.

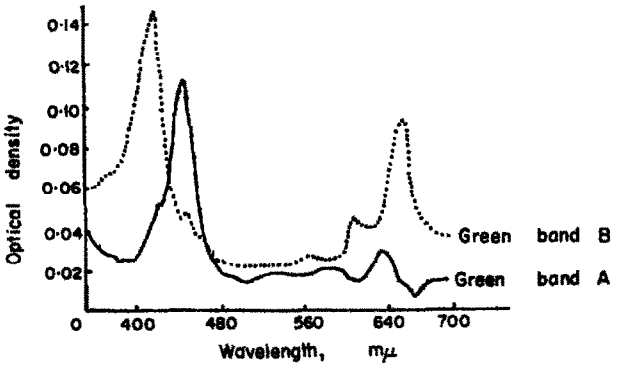


FIG. 3. ABSORPTION SPECTRUM OF A LIGHT PETROLEUM EXTRACT (b.p. 60–80°) OF THE TWO MOBILE GREEN BANDS SEPARATED BY PAPER CHROMATOGRAPHY OF THE COMPLEX.

Purification of the Complex with Sephadex G.50

A short column (15 cm × 2·5 cm) of Sephadex G.50 was made in pyridine/water (55/45; v/v). The complex was solubilized in 55% pyridine and concentrated to approximately 5 ml by blowing air onto dialysis tubing containing the solution. The concentrate was applied to the column which was developed with 55% pyridine.

The green solution eluted from the column was chromatographed on paper as before and it was seen that the three green bands still separated with similar properties under ultra-violet light as previously. With ninhydrin, staining appeared again between the two moving green bands after the chromatogram had been left to stand for some time for colour development. With bromophenol blue, no stain showed which suggested that the bromophenol blue staining material (R_f,O) had been removed by the sephadex. Thus, after sephadex treatment, no protein-staining band corresponded with any of the three green bands.

DISCUSSION

The results obtained before sephadex treatment were consistent with the view that a crystalline chlorophyll–lipoprotein complex could be isolated by this technique. They also suggested that the crystalline preparation was not a single compound, but that when the crystals were formed, free pigment and free chloroplastic protein (or peptide) were taken out of solution with them.

The analytical data, with the exception of a minimum molecular weight of approximately half the value, corresponded well with that quoted by Takashima.[4] This suggested that the substitution of pyridine for α-picoline in the extraction procedure produced a similar crystalline product. Although the crystals were very fragile and fragmented easily, there was no indication that they were colourless after removal of the crystallizing solvent as suggested by Anderson *et al.*[7] Using an ether/alcohol mixture, Takashima[4] indicated the lipoprotein nature of the complex. The presence of 2·1% phosphorus could be accounted for if the lipid was present as phospholipid. Paper chromatography of a solution of the crystals showed the presence of three green bands. Only at the origin did protein (or peptide) staining overlap a

pigment band and as there was a good separation between this and the moving bands, no other solvent was tried. After hydrolysis, twelve amino acids were detected in the material.

Sephadex treatment was tried in the hope that the ninhydrin reactive band would be removed, leaving the complex and the free pigments. As the bromophenol blue staining band was removed, this seemed to indicate that it was of fairly low molecular weight (less than 8000), but there is also the possibility that using sephadex with an organic solvent may alter its properties. As no overlap between pigment and protein-staining bands was present after sephadex treatment, it appears that the preparation is probably an artefact which may be extracted from the chloroplast, and which is a mixture of substances rather than a single crystalline complex. If the complex does exist, the association of pigment with protein must be by physical rather than chemical bonding. Spectral data of the chlorophylls in the isolated complex implies that any bonding present does not alter their absorption spectra. However, the state of chlorophylls in living cells is not fully understood and spectral differences between natural and extracted chlorophylls have been observed. In these cases the changes may be due to various types of chlorophyll-a complexes.[14]

[14] J. H. C. SMITH and C. S. FRENCH, *Ann. Rev. Plant Physiol.* **14**, 201 (1963).