INVESTIGATIONS OF THE EFFECTS OF SURFACE ACTIVE AGENTS OF THE PROPERTIES OF CHLOROPLAST LAMELLAR FRAGMENTS

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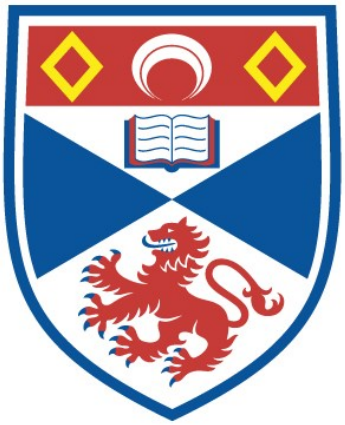
A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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being a thesis presented by

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to the University of St. Andrews in application
for the degree of Doctor of Philosophy

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DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was conducted in the Department of Biochemistry in the United College of St. Salvator and St. Leonard, the University of St. Andrews, under the direction of Professor G.R. Tristram.

CERTIFICATE

I hereby certify that David Williams has spent nine terms engaged in research work under my direction, and that he has fulfilled the conditions of Ordinance No. 16, (St. Andrews) and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosopy.

ACADEMIC RECORD

I matriculated at the University of London (Chelsea College of Science and Technology) in October 1963, and graduated with the degree of Bachelor of Science, First Class Honours in Chemistry and Botany in June 1966. My ancilliary subject was Geology at inter-B. Sc. level. In October 1966, I matriculated as a research student in the Department of Biochemistry, University of St. Andrews.

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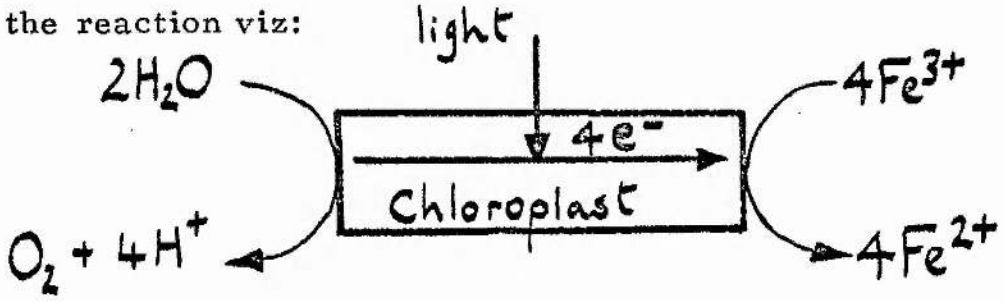
I. 1. Historical Introduction

It is now accepted that the chloroplast of green plants is a self-contained photosynthesising unit capable of all the reactions whereby carbon dioxide is reduced to form sugars at the expense of water, which is oxidised to molecular oxygen.

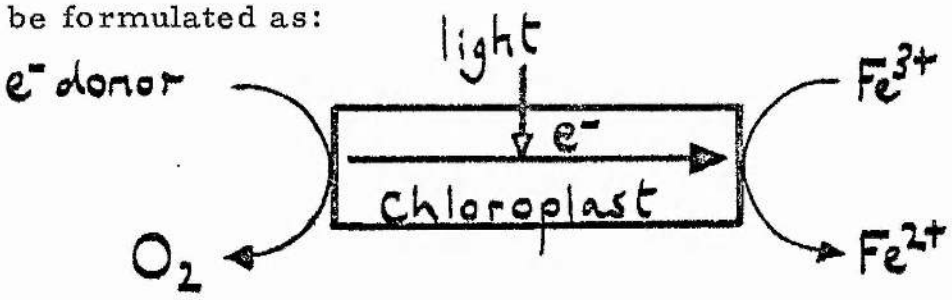
The first recognition that photosynthesis is the property of the green parts of the plant was by the Dutch chemist Jan Ingen-Housz (1779). The pigment conferring the green colour was termed chlorophyll by Pelletier and Caventou, (1818), and its importance stressed by Dutrochet (1837) who recognised chlorophyll as being essential to photosynthesis. Sachs, (1862) postulated starch as a direct product of photosynthesis and described experiments to show that starch production by illuminated green plant tissues takes place in the chloroplast. In 1873, Pfeffer showed that starch formation only occurs in illuminated leaves if the atmosphere contains carbon dioxide. That the chloroplasts are also

the centres of oxygen release in photosynthesis was demonstrated by Engelmann (1884), who showed that oxygen-sensitive motile bacteria migrated to the position of the chloroplasts upon illumination of the tissue. Further support for the localisation of the light processes within the chloroplast came from the experiments of Hill (1937) (1939) (1940), who showed that isolated chloroplasts when illuminated would evolve oxygen if a suitable acceptor of electrons was present. The electron acceptor used was a ferric salt which was reduced to the ferrous form during the reaction viz:



Some electron donor was oxidised to yield oxygen gas with the concomitant reduction of Fe^{+++}. The identity of the electron donor was established by Ruben (1941) and Vinogradov (1941), who demonstrated with 0^{18} labelling experiments that oxygen evolved in photosynthesis —

has the same O^{18}/O^{16} ratio as the oxygen of the water in which the cells are suspended. Hence the Hill Reaction can be formulated as:



Thus the Hill Reaction differs from photosynthesis in that iron rather than carbon dioxide is the terminal electron acceptor. Attempts to employ carbon dioxide as the Hill Oxidant did not meet with success until 1954 when Arnon ultimately demonstrated the chloroplast as being capable of carrying out full photosynthesis without any requirement for cytoplasmic materials.

Earlier work by Blackman (1905, 1911) had indicated that the photosynthetic process may be resolved into (1) a temperature insensitive step initiated by light energy and (2) an enzymic, temperature dependent light insensitive step. These are termed the light and dark reactions respectively and are now known to be not only

physiologically separable in time, but also spatially separable from one another in the chloroplast: the stroma being the site of operation of the dark reactions and the green lamellae the functional site of the light processes. Trebst (1958) Park (1961).

The major pigment of photosynthesising plants is the porphyrin chlorophyll a. A considerable body of evidence developed from the original proposal of Van Niel (1935;1941), has accumulated in recent years that this chlorophyll functions in the light reactions by producing and maintaining a charge separation in the highly ordered lamellar structure. A photo-excited chlorophyll molecule may donate an electron to an acceptor of lower reduction potential, the energy for the process being provided by absorbed solar radiation. The absorption spectrum of chlorophyll a extracted from leaves by organic solvents was noted by Hagenbach (1870) to display a shift of the red adsorption band, 10-20 nm to shorter wavelengths compared with the band position in vivo. (Fig 2). Hagenbach later (1874) noted that the maximum of the

strong chlorophyll fluorescence in solution was displaced in the same way with respect to the weak fluorescence of the leaf. Among suggestions that offer explanations for these observations are that the leaf pigment is dispersed in (Tschirch, 1883) or combined (Palladin, 1910) with lipoid; that the pigment is colloidally dispersed (Herlitzka, 1912), or adsorbed as a monomolecular layer on protein. (Willstatter, 1913 and Noack, 1927). The latter author found that chlorophyll precipitated with protein on adding protein precipitants. More recently it has been found (Rodrigo 1953, 1955; Sapozhnikov 1956 Vishniac 1957). that small amounts of photosynthetic activity can be detected in artifically prepared chlorophyll-protein complexes. It is currently believed that the principal association in vivo of chlorophyll is with protein. Treatment with organic solvents, destroys the weak forces binding chlorophyll to its molecular environment, thus altering its spectral properties. The use of spectrophotometric techniques capable of greater resolution, (e.g. low

temperature absorption spectroscopy, Butler 1960, 1961; derivative spectroscopy, French 1957, 1958, Brown 1959; and difference spectroscopy, Kok 1956a) has resulted in the characterisation of several forms of chlorophyll a (and possibly two forms of chlorophyll b, Shlyk, (1963)) each having different absorption maxima in the red band. Metzner (1963) employing a reflectance spectrophotometer noted nine different shoulders in the chlorophyll absorption band from Chlorella cells between 665 and 685 nm. The majority of the spectral measurements on intact plant material however, have indicated that the bulk of the chlorophyll a exists in two forms, with absorption maxima near 672 nm and 683nm, (Brown 1963) whilst about 0.25% of the chlorophyll a exists in a form absorbing maximally at about 700nm (Kok, 1960). In addition, certain lower plants possess a form of chlorophyll a which exhibits an absorption peak at 695nm. One group, Michel-Wolwertz (1965) and Sironval (1965) has reported however that the different forms of chlorophyll a observed in the intact chloroplast are due to isomeric species which may

be chromatographically separated in the pure state. The possibility that isomerisation occurred during the purification procedures has not been eliminated however, and the currently accepted view is that the different chlorophyll a molecules vary only in their molecular environment.

The present scheme for the functioning of chlorophyll in photosynthesis envisages it as photoionising - ejecting an electron when in the excited state. The investigations by Emerson (1943, 1957) on the 'red drop' in the quantum efficiency of photosynthesis, and the enhancement (Emerson 1957) by light of wavelengths of below 680nm of the quantum efficiency induced by light of above 680nm, led to the theory that photoionisation of chlorophyll a occurs in two different environments or systems with diverse accessory pigments and different electron transfer components. These two systems have different loci for their absorption maximum in the red, and are termed system 1 (long wavelength) and system 11 (short wavelength).

(Duysens 1961, 1963). Thus contemporary theory suggests that diverse partial photochemistry takes place according to the wavelength of the light absorbed. (see Fig. 1). The product in each of the photochemical steps is an oxidised chlorophyll a molecule (Emerson 1960) and an electron. The positively charged chlorophyll ions of the two systems are of appreciably different redox potential. In neither case is the nature of the immediate electron acceptor known, though in one case (system 1) the ultimate acceptor appears to be ferredoxin, while in the other (system 11) it may be a quinone and a cytochrome electron transport complex. Oxidised chlorophyll is reduced to its neutral form by oxidising an aqua-dismutase (system 11) with the evolution of oxygen, while in system 1 it becomes reduced by itself oxidising cytochrome f (Fork, 1965) or the copper protein plastocyanin.

Thus the two photochemical systems are envisaged as accumulations of pigment molecules which transfer excitation energy either by resonance energy transfer (Duysens, 1957) or by semiconduction (Nelson, 1957;

light induced electron flow in chloroplasts

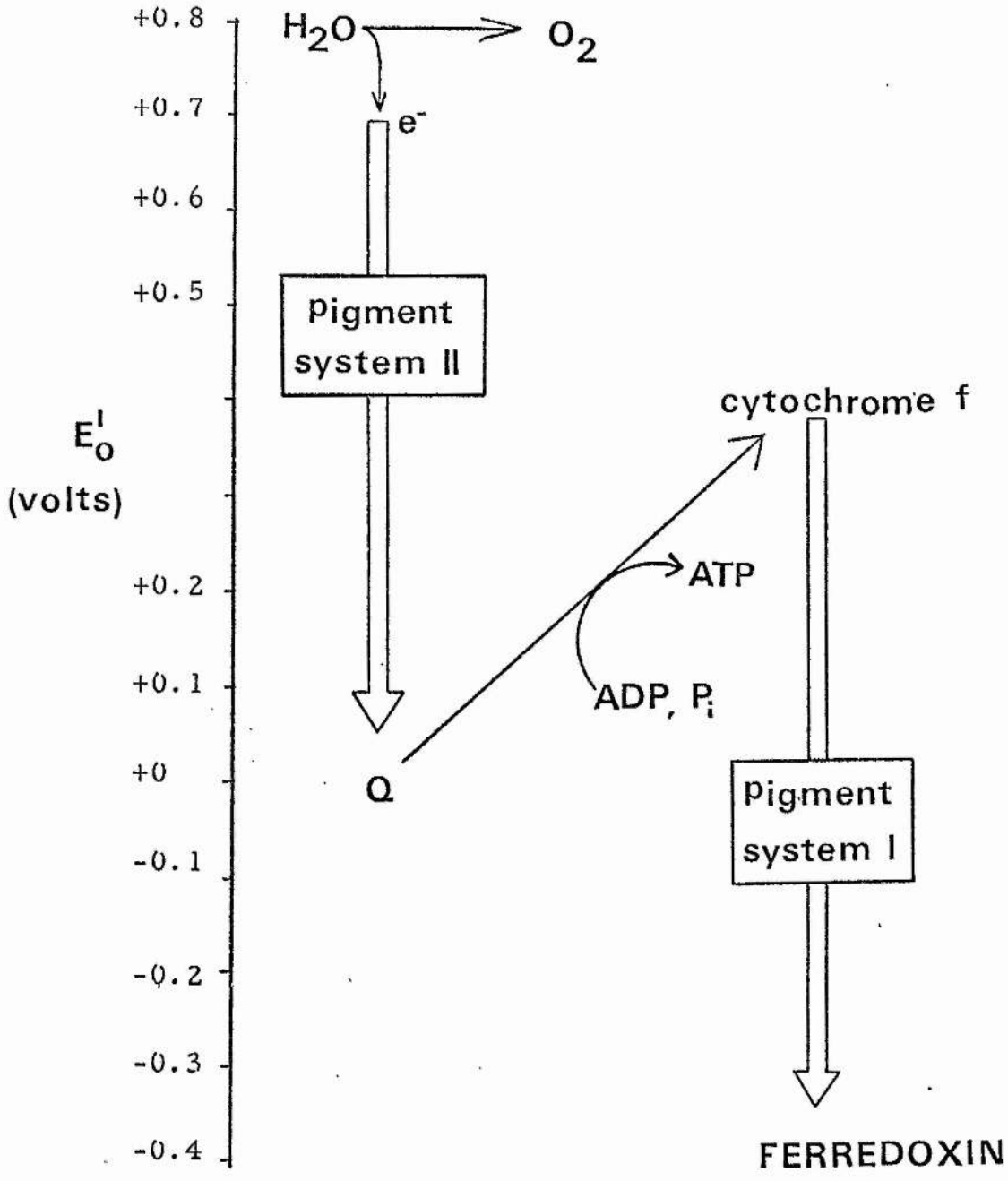


Fig. 1

(After Vernon, 1965_b)

Arnold, 1957, 1958) to a collecting point or reaction centre (Gaffron, 1936). This reaction centre is thought to be the form of chlorophyll a absorbing maximally at about 700nm, termed P700 (Kok,1956a, 1960, 1961, 1963), for the long wavelength system, but the nature of the photocatalyst for system 11 has as yet been unidentified.

The nature of the non-photocatalytic pigment molecules, differs in the two systems. The first evidence (Engelmann, 1894) that carotenoids and phycobilins can promote photosynthesis has been confirmed in recent years (Dutton, 1941; Emerson, 1942; Arnold, 1950; Duysens, 1952), and in all cases studied, the excitation spectra for photosynthesis featuring peaks due to accessory pigments as well as to chlorophyll a have the same form as corresponding excitation spectra for fluorescence of the chlorophyll a (Dutton, 1943; French, 1952; Duysens, 1952). In photosynthetic plants, system 11 is sensitised by phycobilins, or chlorophyll b (Losada 1961) and short wavelength non-photocatalytic chlorophyll a, whilst

the accessory pigments involved in the system 1 response are carotenoids and long wavelength non-photocatalytic chlorophyll a.

As stated, not all chlorophyll a molecules possess a photocatalytic role. The early flashing light experiments of Emerson (1932 a, 1932 b) and later experiments of Gaffon (1936) and Kok (1956 b) have shown that the primary photochemical event of photosynthesis in plants is initiated by one chlorophyll a molecule which is structurally linked to approximately 300 other chlorophyll a molecules plus accessory pigments. A single reaction centre, plus the amount of light-harvesting pigment molecules associated with it is termed a photosynthetic unit. This concept of several hundred pigment molecules acting in concert suggested that the photosynthetic unit may be a discernible morphological entity. Thomas (1953) measured the Hill reaction activity of small chloroplast fragments produced by sonication, as a function of particle size, and concluded that Hill reaction activity was maintained at normal rates

down to a particle size of approximately 10^6 $\mathring{A}^3$ but below this critical volume, activity was rapidly lost. Such particles would contain about 100 chlorophyll molecules. Following the original report of Steinmann (1952) of a repeating structure on the surface of the lamellar membrane, Park (1961, 1964) and collaborators noted in electron micrographs of chloroplast lamellae that the lamellar surface had a cobblestone appearance with units of about 200 $\mathring{A}$ diameter. These units, termed quantasomes have been proposed as the morphological expression of the photosynthetic units, as chemical analysis indicated there to be 240 chlorophyll molecules per quantosome (Park, 64). Theoretical photochemical mechanisms involving two separate pigment systems suggest the possibility of two different photosynthetic units of different pigment composition and function, and much effort has been expended in recent years in attempts to isolate lamellar particles to which could be ascribed some functional integrity with respect to the photosynthetic light reactions.

There have been two main approaches to the problem:

1. Physical disruption without addition of any solubilising agent

Initial disruption of the lamellae may employ ageing (Tachiki, 1969) or osmotic shock in a hypotonic medium (Becker, 1962) followed by ultrasonication (Thomas, 1952), or passage through a small aperture the size of which is controlled by a needle value (Milner, 1950). Comparisons made by French (1951) between sonic and pressure disintegration showed that the latter gave a much more complete dispersion of the sample. A limited degree of success has been achieved by this approach, particularly by Allen (1963a, 1963b, 1963c) who isolated a characterisable, photochemically active chlorophyll-protein complex from Chlorella cells. Further fractionation of the fragments when carried out, has involved differential centrifugation (Brown 1959a Park,1961, Butler 1963).

It should be noted that isolation of chlorophyll proteins from Chenopodium species by ultrasonication and

ammonium sulphate precipitation probably involves participation of natural detergents present in the cell sap of these plants.

2. Disruption of lamellae in the presence of solubilising agents

(a) Small organic molecules

Takashima (1952) employed 55% aqueous a-picoline in isolation of a lamellar chlorophyll protein in a method modified later by Thirkell (1964) who used 55% aqueous pyridine as solvent. 40% acetone has also been successfully employed in isolation of chlorophyll proteins (Thomas 1964, 1966), but none of these organic solvents yielded chlorophyll protein species capable of any photochemical activity characterisable with in vivo activity or absorption spectra not displaying a marked shift of the main peaks towards the blue. This shift for the acetone solubilised chlorophyll proteins is reversible, but not so for the others and evidence from other workers indicates that the association of the chlorophyll with protein is only loosely physical (Krasnovsky, 1954; Anderson, 1954).

(b) Large organic polymers

A major development in studies on lamellar fragmentation occurred when Smith (1938, 1940a, 1940b, 1941a, 1941b, 1941c, 1942) discovered the ability of natural and synthetic detergents to clarify turbid chloroplast suspensions, producing clear non-fluorescent solutions. Detergents, by virtue of their combined lipophilic and hydrophilic molecular construction, are capable of solubilising both the lipophilic chlorophyll, and its partially hydrophilic lamellar environment still combined together. The solubilising effect of organic solvents such as picoline is much less gentle, displaying a preference fot the lipophilic moiety and thus the association between the chlorophyll and its lipoprotein is either destroyed or weakened. In detergent-solubilised lamellae however, the chlorophyll may or may not be separated from its lipoprotein according to the conditions (Ke, 1956) and the nature of the detergent employed (Smith 1941c).

Despite the vast number of synthetic surface active agents available, their employment in this field has not been very extensive in terms of different detergents. Indeed, only four have been studied in detail. These are the steroidal detergent digitonin, the anionic detergents sodium dodecyl sulphate (S.D.S.) and sodium dodecyl benzene sulphonate (S.D.B.S.) and the non-ionic surfactant Triton X-100. Using these, lamellar particles have been isolated with molecular weights in the range 10,000-60,000 (Wolken, 1953, 1956; Bailey, 1966) - allowance having been made for the contribution of the detergent micelle. The tendency of detergent molecules to aggregate into large macromolecules or micelles has severely hampered estimation of molecular weights of solubilised fragments, since the contribution of the detergent micelle to the apparent molecular weight of the complex (detergent-chlorophyll-lipoprotein), is liable to vary (See Appendix, p. 210 - 213). A similar problem arises on fractionation of detergent solubilised lamellae (see Discussion Section p. 199). The most obvious solution in each case is to employ detergents in concentrations

below their critical micellar concentrations (c.m.c.) - the concentration of a given detergent at which micelle formation begins. Such concentrations however tend to be too low to bring about solubilisation of the lamellae to any appreciable extent (e.g. c.m.c. of SDS is 0.008 moles/litre. Mysels et. al. 1959).

No reports have appeared in the literature to date of investigations on general effects of a variety of synthetic detergents upon the physical, chemical and photochemical properties of chlorophyll protein in the chloroplast.

The aim of the present investigation has been to screen several detergents in this way, with particular interest in producing lamellar fragments characterisable with either of the photosynthetic photosystems.

A review of similar work conducted by other investigators up to mid-1969 is given below:

I. 2. Solubilisation of chloroplast lamellae

An investigation of the relative abilities of different detergents to solubilise chloroplast lamellae does not appear to have been carried out to date though Anson (1940) compared the effectiveness of Duponol PC and bile salts in extracting chlorophyll, and Smith (1941a) noted that for equivalent concentrations, sodium deoxycholate is slightly more effective in solubilisation than either digitonin or bile salts.

This work has attempted a survey of the ability of several detergents to promote lamellar fragmentation under different conditions of pH and concentration of detergent.

1.3. Spectra of detergent-solubilised lamellae

(a) Absorption spectra

The absorption spectra of detergent-solubilised lamellae have been studied by other authors, whose results are summarised in table 1. It will be noted that the range over which the major red absorption peak extends (663-679mμ) is quite broad.

Differentially centrifuged lamellar preparations treated with digitonin by Anderson et. al. (1966) showed differences in the spectra according to the magnitude of the r.c.f. employed in sedimentation.

The spectrum of aqueous quantasome preparations (fig 2) shows a maximum absorption in the red at about 680mμ. The effect of most detergents and of organic solvents is to produce hypsochromic shifts in the position of the red maximum (fig 3).

In this thesis, the spectra of lamellae solubilised by several detergents under varying conditions of concentration, pH and temperature were studied, in an attempt to determine the factors responsible for such spectral shifts.

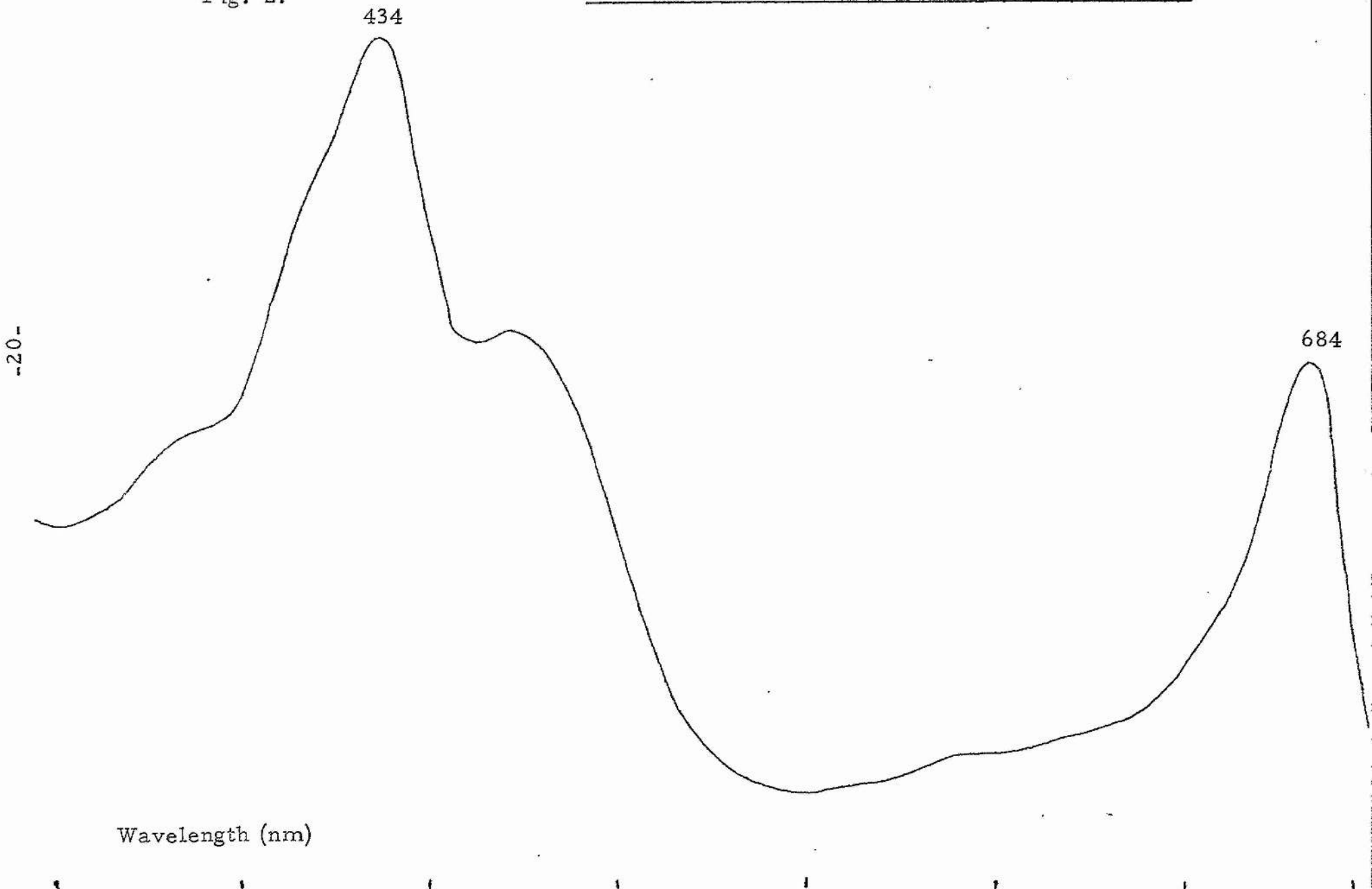
TABLE 1

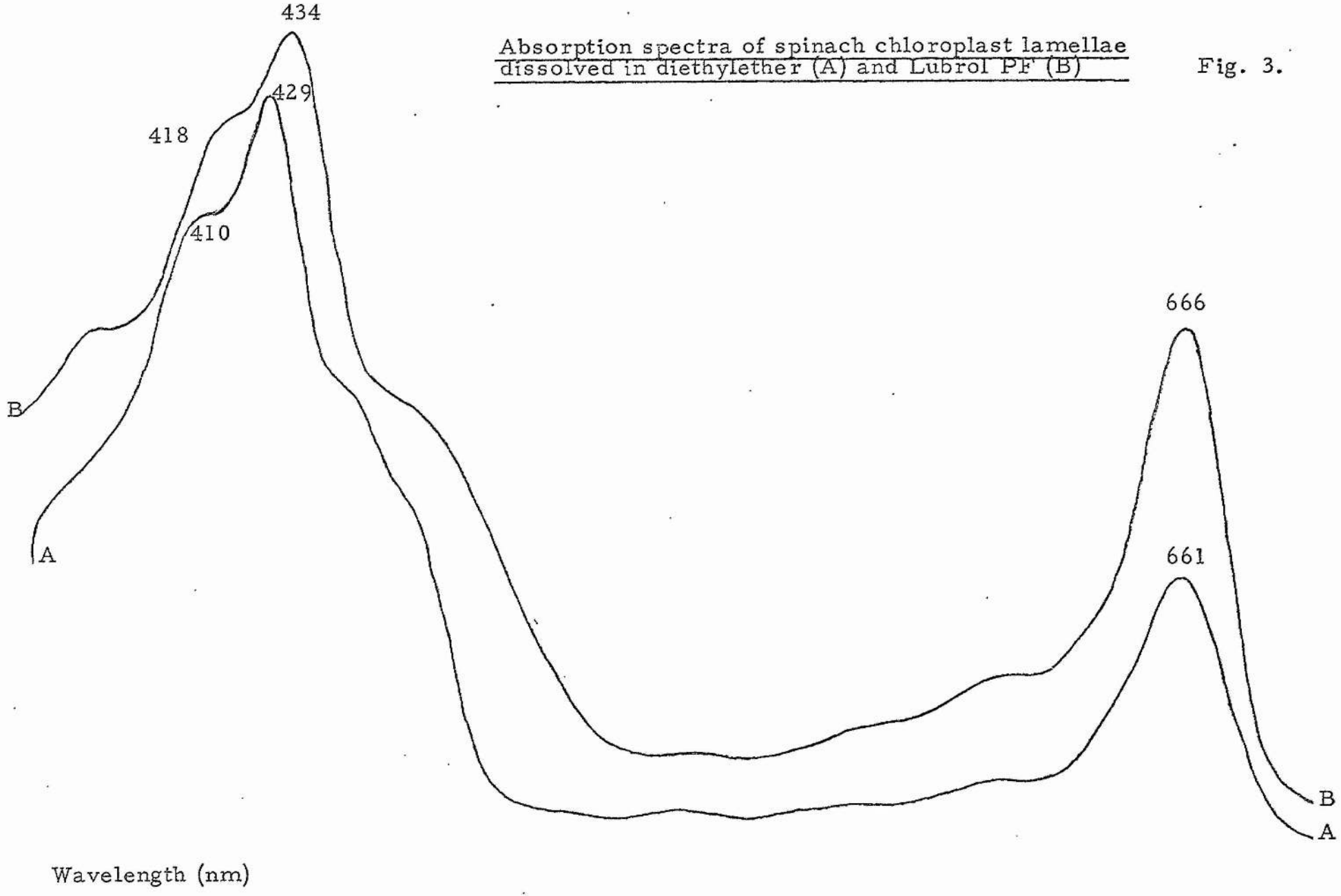
Reference	Detergent	Red λ max	Blue λmax	other bands	Blue/Red Peak heights	Notes
Smith E.L. (1938) (1941a)	Digitonin	675	437	470	1.6	Single extract
Brown J.S. (1964)	S.D.S	680-672	437*	470	1.5*	Two successive extracts, separated by high speed spin. Showed small but consistent differences in absorption in the ranges 670-680 and 460-480 independent of increased detergent concentration. λmax fell to 672 for both extracts on (i) increasing detergent concentration (ii) ageing 48 hours.
Anderson N.K. et al. (1966)	Triton X-100 Nonidet P40 Digitonin	671-672 677.5-679.5	437*	470*	1.6*	Single extract fractionated into several components by differential centrifugation
Chiba Y. (1960)	Duponol C and Span 80	675	437	421, 470, 544, 624	1.7*	Single extract.
Kahn et al. (1965a, 1965b)	TritonX-100	671	436		1.7*	A chlorophyll protein complex extracted with 1% Triton and purified on DEAE cellulose column. Addition of Triton to pure complex lowers λ max from 671 to 668 nm.
Ogawa et al. (1966)	S.D.S.	675, 672 and 671	438		1.8* 2.2* and 1.8*	Red λmax and Blue/Red peak heights quoted for respectively components I, II, and III. See same ref. in Table 2. Study carried out at pH 10.3.
Vernon et al. (1966a)	Triton X-100	678 675	438 438	473 -	1.6* 1.6*	P8 fraction PD10 fraction - produced by centrifuging for 8 and 10 hours respectively, lamellae solubilised in 4% Triton.
Smith (1941a)	Bile Salts, Sodium deoxycholate	671-672				Single extract.
Ke et al. (1956)	S.D.S. Tween 20 - picoline	670				Single extract.

* Approximate values. Not stated by authors but adduced from published graphs.
All spectral values at room temperature.

Fig. 2.

Aqueous quantasome suspension:visible spectrum





Absorption spectra of spinach chloroplast lamellae dissolved in diethylether (A) and Lubrol PF (B)

Fig. 3.

(b) Fluorescence Spectra

It was shown by Smith (1941b) that SDS-clarified leaf extracts showed a small but significant red fluorescence which is stronger than that of the untreated preparation but appreciably less than that of an equivalent concentration of chlorophyll in organic solvents.

Kahn et. al. (1965) prepared a chlorophyll-protein from spinach using dilute Triton X-100 which on excitation with light of 406 and 436nm fluoresced at 680nm with a quantum yield of 0.01. (Yield of chlorophyll a in acetone=0.30).

The fluorescence spectra of subchloroplast particles produced by the action of 4% Triton X-100 were studied by Ke et. al. (1967) and the results are summarised below:

	Heavy Particles		Light Particles	
	20°C	-196°C	20°C	-196°C
Peak (nm)	682.5	696 685	680	678 726
Band (nm) Centre	-	730	-	-

Briantais (1967) confirmed Ke's finding that fluorescence of the heavy fraction was stronger than that of the light fraction, and also recorded a low temperature far red peak at 720nm.

Ogawa et. al. (1966) separated on polyacrylamide gels two chlorophyll proteins produced by the action of SDS on spinach lamellae which fluoresced red when illuminated at 365nm.

No other reports of fluorescence of detergent-produced subchloroplast particles are known.

A brief study has been made in this work of the fluorescence of lamellar fragments produced by some detergents not reported on to date.

1.4 Chlorophyll a : b ratios of fragmented lamellae

Evidences implicating the participation of chlorophyll b specifically in the system II response, are numerous (Losada et. al. 1961; Arnon et. al. 1961). This has resulted in several attempts to fragment chloroplast lamellae into particles enriched in chlorophyll a (and hence possibly representative of system 1) and particles enriched in chlorophyll b (system II). See table 2.

An attempt has been made in the present work to estimate such fractionation based on chlorophyll a : b ratios using several detergents not covered in the literature to date.

TABLE 2

Reference	Detergent	High Ca/Cb Fraction	Low Ca/Cb Fraction	Detergent (D) Chlorophyll (C) Ratio (W/W)	Notes
Thornber et al. (1967a)	SDBS (0.5%)	12.0:1	1.2:1	2.5(D):1 (C)	First extract discarded. Second extract 2 fractions separated by disc-gel electrophoresis. Slow moving = High Ca/Cb fraction. Fast moving = Low Ca/Cb fraction.
Anderson et. al. (1966)	Digitonin (0.5%) Digitonin (2.0%)	5.34:1 4.65:1	2.27:1 2.25:1	50(C):1 (D) 8 (C):1 (D)	Single extract differentially centrifuged.
Brown and Duranton (1964)	SDS (conc. not stated)	3.15:1	- 2.42:1	3(D):1 (C) 10(D):1 (C)	Two successive extracts. No further fractionation. Extract 1 : High fraction. Extract 2 : Low fraction.
Vernon et. al. (1966a, 1966b)	Triton X-100 (4%)	5.7:1	2.0:1	33.3(D):1 (C)	Single extract. 10,000 xg sediment discarded, supernatant spun at 144,000 xg for one (low fraction) and ten (high fraction) hours.
Ogawa et. al. (1966)	SDS (conc. not stated)	7.1:1	1.9:1	*185(D):1(C)	As Thornber above, but a 3rd , faster fraction obtained which unlike the other two, did not contain protein.
Wessels J.S.C. (1966)	Digitonin	6.0:1	3.0:1	5(D):1 (C)	High Ca/Cb fraction produced by centrifugation at 80,000 xg
Briantais J. M. (1967)	Triton X-100 (conc. not stated)	4.3:1 -	- 2.0:1	3(D):1 (C) 12(D):1 (C)	Preparations produced by two separate procedures. High fraction produced from 80,000xg spin in Treatment 1. Low fraction produced from 10,000 xg spin in Treatment 2.

* Molar Ratio

I, 5. Trace Element Analysis

It is believed that manganese is required for the oxygen evolving sequence of photosynthesis (Pirson, 1937); Kessler, 1957; Spencer et. al. 1961) and also that the iron-containing protein ferredoxin and the cytochromes f, b_6, and the copper containing protein plastocyanin, are situated close to the site of NADP reduction on the photosynthetic electron pathway. (Vernon et. al. 1965a).

Fragmentation of chloroplasts by Anderson et. al. (1964) using digitonin produced a light fraction enriched in iron and copper, and a heavy fraction containing a high proportion of manganese relative to the magnesium content of intact chloroplasts, which indicated that some physical separation of systems I and II had occurred during lamellar breakdown by the digitonin.

Thornber et. al. (1967a) also studied the manganese distribution in SDBS treated lamellae but could find no difference between their two chlorophyll-protein complexes

in terms of manganese content. (Inference was made of a separation of systems I and II on other evidences however).

In the present work, the ability of a number of detergents to dissociate lamellae into fragments of differing trace element content was investigated.

I. 6. Detergent-pigment complex affinities of solubilised lamellae

Benzene extractability of the pigment, in detergent-solubilised lamellae was employed by Ke et. al. (1956) to estimate the affinity of a detergent for pigment components. Digitonin, SDS and purified plant saponins have sufficient affinity for the pigments at concentrations above 10^{-2}M to prevent their removal from the aqueous phase by benzene, but have insufficient affinity at concentrations below 10^{-2}M to prevent colouration of the organic phase. Cationic detergents were found to have a very low affinity - the pigments were found to be completely extractable by benzene from lamellae solubilised by zephiranchloride at as high a concentration as 1% $^{w}/_{v}$.

I. 7. Effect of detergents on the photosynthetic light reactions

Apart from the work of Ke et. al. (1956) no authors have investigated the inhibition of photochemical activity of chloroplasts by a range of detergents.

The results of Ke et. al. (1956) are summarised in Table 3.

It will be noted that at a concentration of 0. 2%, digitonin abolishes system II activity.

Wolken et. el. (1953), employing the same detergent at 2. 0% concentration in a suspension of chloroplasts however demonstrated that photoevolution of oxygen was not abolished. The same result was found by Eversole et. al. (1958) though Wessels (1962) states that digitonin has a selective inhibitory effect on system II, resembling that of DCMU, (which abolishes system II activity, but permits system I activity if an external electron donor is supplied).

Thornber et. al. (1967a)noted that a final concentration of 0. 012% SDBS caused photoevolution of oxygen by a chloroplast suspension to cease within a few seconds, and NADP reduction to be inhibited immediately.

Vernon et. al. (1965b) made a detailed survey of the effects of Triton X-100 on the photochemical activities of chloroplasts, noting the following points:-

(i) Low concentrations of detergent <0.007% stimulated Hill activity with ferricyanide or DCPIP as electron donors.

(ii) Concentrations anove 0.01% abolish all oxygen photoevolution.

(iii) Low concentrations of Triton (0.008 to 0.009%) stimulated NADP photoreduction with water as electron donor (systems I and II operative).

(iv) Slightly higher concentrations (0.014%) decreased the same form of NADP reduction greatly.

(v) Still higher concentrations (> 0.02%) abolished all activity of system II but permitted high rates of NADP photoreduction with ascorbate/DCPIP as electron donors (system I only, operative).

These and other observations indicated that as the concentration of Triton X-100 is increased, chloroplast photochemical activities follow three phases:

(a) Stimulation of electron transfer reactions at low Triton X-100 concentrations.

(b) Inhibition of electron transfer reactions at intermediate concentrations.

(c) Reappearance at high concentrations of Triton X-100 of some simple electron transfer reactions.

Detergent	Concentration (%)	% inhibition of O_2 photoevolution after 20 mins	% Inhibition of photo-synthesis (steady state)
S.D.S.	0.0095	15	0
	0.0190	30	0
	0.0285	60	15 (average)
	0.0380	85	15 (average)
	0.0240	100	37
Tween 20	0.120	65	0
	0.410	100	0
Zephiran chloride	0.050	100	100
Digitonin	0.070	55	0
	0.20	100	0
Saponin (Soapbark)	0.830	20	38
	1.670	100	60

TABLE 3

Inhibition of photochemical activity in isolated chloroplasts and Chlorella cells, by detergents. (Ke et.al. 1965).

I.8 Photochemical activities of particles fractionated from detergent solubilised lamellae

(1) From Digitonin

Several reports have employed digitonin to disrupt lamellae into fractions separable by differential centrifugation (Thornber et. al. 1967a; Boardman et. al. 1964; Anderson et.al. 1966; Wessels 1962, 1963).

Their results are all essentially similar and are summarised in Table 4.

(II) From Triton X-100

Vernon et.al. (1966a) and Ke et. al. (1967) using 4% Triton X-100 have produced fractions very similar to those resulting from 0.5% digitonin treatment (See Table 5).

Thus, Triton and digitonin have both been shown to yield subchloroplast particles of two main types:

(a) heavy particles which can carry out photochemical reactions typical of the short wavelength (system II response),

(b) light particles which have observed photochemical properties expected for a particle containing photosystem I.

The light particle has the capacity to photoreduce NADP using ascorbate/DCPIP as electron donor at a rate of 1980 μ mole/mg chlorophyll/hour in the presence of almost saturating plastocyanin at pH 6.0. (Vernon et. al. 1966b). This reaction coupled with its enzymatic requirements indicates that a high degree of structural integrity is maintained in this particle.

It should be noted that Anderson et. al. (1966) employed Triton X-100 (and another nonionic detergent, Nonidet P-40) at a concentration of 0.1% to produce particles in the same way as for digitonin fragmentation, and obtained fractions devoid of photochemical activity. This however may find an explanation in the observation of Vernon et. al. (1965b) that such concentrations inhibit electron transfer reactions, which subsequently reappear at higher concentrations of detergent.

(III) From anionic detergents

SDS-solubilised lamellae were fractionated by Ogawa et. al. (1966) electrophoretically but none of the fractions produced exhibited any detectable photochemical activity. The same was found of similar fractions produced by the related detergent SDBS studied by Thornber et. al. .(1967).

(IV) From mixed detergents

Chiba (1962) treated osmotically ruptured chloroplasts (4 vols.) with a 3:1 mixture of 5% Duponol C and 5% Span 80 (1 vol.) and centrifuged at 20,000xg.

The clear supernatant of solubilised chloroplasts:

(a) Had no detectable activity in Hill Reaction

(b) Showed 58.5% greater activity in ascorbate/DCPIP photo-oxidation than was carried out by osmotically ruptured chloroplasts.

(c) Showed a rapid photo-oxidation of ferro-cytochrome c.

Photochemical Activities of chloroplast fragments

Fractions	Rates of reduction (μ moles/ mg chlorophyll/hour)				
	TCPIP	$Fe(CN)_6^{-3}$	NADP	NADP + asc/DCPIP	Methyl Red + asc/DCPIP
C	152	255	96	64	-
Cd	81	138	33	18	-
1	139	209	24	14	-
144	0	0	0	123	-

Table 4. (After Anderson et. al. 1966)

P20	-	260	55	193	221
S175	-	155	45	700	790
P-1	-	0	0	7	10
P-D10	-	0	0	320	92
P-D10[1]	-	-	-	1980	-

Table 5 (After Vernon et. al. 1966a and Katoh et. al. 1966)

KEY:

C: Untreated chloroplasts.
Cd: Chloroplasts in 0.5% digitonin.
1: Material sedimented at up to 1000xg.
144: Material sedimented at 50 to 144000xg.
P20: Heavy fraction from sonicated, detergentless chloroplasts.
S175: Light fraction from sonicated, detergentless chloroplasts.
P-1: Heavy fraction from chloroplasts treated with 4% Triton X-100.
P-D10: Light fraction from chloroplasts treated with 4% Triton X-100.
P-D10[1]: As P-D10 but experimental conditions according to Katoh et. al. 1966
- : No measurement.

I.9 Fractionation of detergent-solubilised lamellae

Fractionation of lamellar fragments dispersed by detergents has been attempted by numerous workers in efforts to isolate characterisable chlorophyll proteins. (See Discussion Section p.199).

(a) Polyacrylamide disc gel electrophoresis

This method employed by Thornber et.al. (1967a) using SDBS as dispersing agent and by Ogawa et.al. (1966) using SDS to fragment the lamellae has been successfully applied only to anionic detergents.

Both reports indicated separation of the solubilised lamellae into photoinactive fractions, each having some compositional properties of one of the two photosynthetic photosystems. Ogawa (1966) also subjected chloroplast extracts of Tween and digitonin to electrophoresis. No migration occurred.

(b) Ion Exchange Column Chromatography

Kahn (1963, 1964) purified a chlorophyll protein complex from chloroplast lamellae disrupted with Triton X-100,

on a DEAE cellulose column. The complex was subsequently characterised (Kahn, 1965) but no reports have appeared since of isolation of chlorophyll proteins in this way from lamellae solubilised by other detergents.

(c) Adsorption chromatography

Adsorption chromatography was employed by Ke. et.al. (1956) using celite, starch, paper alumina, tricalcium phosphate and silica gel as adsorbents. All but the first three, gave a sharply defined chlorophyll-protein band from SDS-solubilised lamellae, but no evidence of separation of components within the band, using several different aqueous developing solutions was obtained.

(d) Differential Centrifugation

This simple method of particle separation according to size has been used predominantly with nonionic detergent preparations, being employed for Triton X-100 (Vernon et.al. 1965_b, 1966_a, 1966_b, and Ke et.al. 1967) and digitonin (Boardman et.al. 1964, Anderson et.al. 1966; Wessels 1962, 1963,) to produce distinct chlorophyll-protein moieties.

Similar fractionations of solubilised lamellae apart from adsorption chromatography have been employed in the present work.

I.10. Chlorophyll - protein Relationships

It was suggested by Smith (1941a) that if a true combination exists between chlorophyll and protein in lamellae, there should be a definite quantitative relationship between them. The subsequent discovery of the double photosystem in photosynthesis has led to a concept of there being two such chlorophyll-protein complexes.

The effect of detergents on the relationship between chlorophyll and protein was investigated by Smith (1940b) and Ke et. al. (1956), who showed that some detergents (e.g. digitonin, bile salts, sodium deoxycholate and Tween 20) cause dissociation of protein from chlorophyll, whilst others (e.g. SDS) do not.

More recent work suggests that this view is untenable without reference to the concentration of detergent, relative to that of chlorophyll. The action of detergents is to split lamellae into progressively smaller units which retain a multicomponent identity down to a certain detergent-chlorophyll ratio, beyond which the lipid and protein moieties become separated. Shibuya et. al. (1968) propose a four-step fragmentation of chloroplast lamellae by Nonidet P-40 (See fig.4) in which

the final stage occurring at Nonidet : chlorophyll molar ratios of above 60 involves separation of protein from lipid.

Similarly it was reported by Kahn (1965a) that addition of Triton X-100 to a protein-chlorophyll complex isolated in 1% Triton X-100 produced a colloidal suspension of chlorophyll in Triton which could also be prepared by adding pure chlorophyll a in a small volume of acetone to Triton X-100.

Estimations of the ratio of chlorophyll to protein in detergent treated lamellar preparations show considerable variation:

(a) in similar extracts produced by different detergents (Wolken et. al. 1956)

(b) in successive extracts of the same lamellar sample by a single detergent. (Brown et. al. 1964). (See Table 6).

Thornber et. al. (1967b) and Ogawa et. al. (1966) separated chlorophyll-protein complexes which were considered to represent separated photosystems, though none of which were photochemically active. Thornber et. al. (1967b) showed a different ratio of chlorophyll to protein in the separated complexes.

Four Step Solubilsation of chloroplast lamellae by Nonidet P-40

Nonidet/Chlorophyll (molar)

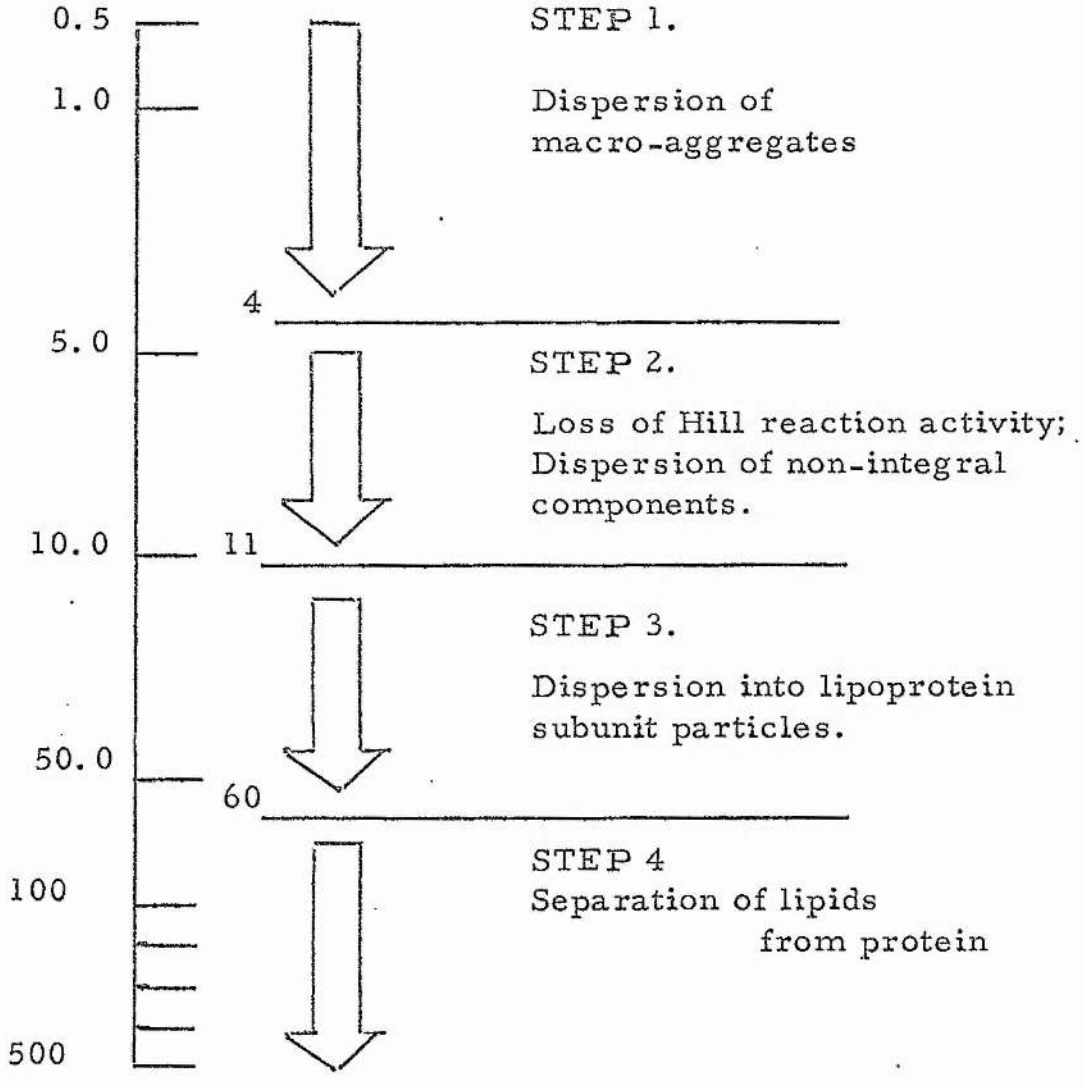


Fig. 4 (after Shibuya et. al. 1968)

Chlorophyll:protein relationships in detergent-extracted chloroplasts

Reference	Mass Ratio (Chlorophyll:Protein)	Protein per chlorophyll molecule (In mol. wt. units*)	Detergent	Notes
Thornber et. al. (1967b)	0.114 0.075	7830 11900	SDBS	Complex I Complex II
Ogawa et. al. (1966)	0.447	2000 ∔	SDS	Components 1 and 2
Brown et. al. (1964)	0.128 0.094 0.210	6977 9500 4252	 SDS	Whole chloroplast 1st extract 2nd extract
Wolken et. al. (1956)	0.0362 0.0350 0.0390 0.0028 0.0022 0.0072	24700 25500 22900 322900 415300 124100	))Digitonin)))Nacconal)NRSF ∅	

Table 6

* Kupke et. al. (1961) - based on a 1:1 chlorophyll:protein ration

∔ approximate value

∅ anionic alkyl sulphonate detergent.

METHODS

M. 1. Isolation of chloroplast lamellae

The isolation procedure was based on the method of Anderson et. al. (1966) with slight modifications.

Three types of leaf material were used: Garden Spinach (Spinacea oleracea) Swiss Chard (Beta vulgaris); and Broad Bean (Vicia faba).

Vicia leaves were homogenised intact; the others were de-midribbed prior to homogenisation.

Leaf material (x gm) was blended in 10 x ml of 0. 04M phosphate buffer pH 7. 1 containing 0. 35M sodium chloride; 0. 01M potassium chloride and o. 002M EDTA (Jensen et. al. 1966) using a Potter-Elvehjem homogeniser. The resultant brei was filtered through nylon cloth of 50μ mesh size (Type T 11 material, Henry Simon Ltd., Stockport, Cheshire.) to remove unbroken cells, cell debris, and nuclei. The crude chloroplast fraction was isolated from this filtrate by sedimentation at 1000xg for 10 minutes. The chloroplast precipitate was blended by means of a cottonwool pad in a fresh aliquot of homogenising medium and recentrifuged at 1000xg for 10 minutes. The resultant

precipitate of intact chloroplasts and chloroplast fragments was suspended in 0.04M phosphate pH 7.1 containing 0.01M potassium chloride and 0.002M EDTA (hypotonic buffer), by means of a cottonwool pad and a Potter-Elvehjem homogeniser, to give a homogeneous suspension of chloroplast fragments. The volume of hypotonic buffer employed was kept minimal to ensure a chlorophyll concentration of >1mg/ml. After estimation of the chlorophyll concentration (see p.46) the volume of the suspension was adjusted by addition of hypotonic buffer to yield a lamellar suspension containing 1.00 mg chlorophyll/ml.

M. 2. Chlorophyll Estimation

Analysis of chlorophyll was carried out according to the method of Arnon (1949) by measuring optical absorbancies of an 80% aqueous acetone solution of the test material at pH 7.0 using 1cm silica cells.

Absorbancies were measured at 645 and 663nm.

Particular use was made of equations (iv), (v), (vii) and (ix) of Arnon's work:

$$Ca = 0.0127\, E_{663} - 0.00269\, E_{645} \qquad \text{(eq. v)}$$

$$Cb = 0.0229\, E_{645} - 0.00468\, E_{663} \qquad \text{(eq. iv)}$$

$$Ct = 20.20\, E_{645} + 8.02\, E_{663} \qquad \text{(eq. vii)}$$

Where Ca, Cb and Ct are concentrations of chlorophylls a and b, and total chlorophyll respectively, in mg per ml of 80% acetone solution. E_{645} and E_{663} are the optical densities of the acetone solution at 645 and 663nm respectively.

Using 1cm spectrophotometer cells, the method is accurate on the S.P.500 for 80% acetone solutions containing approximately 0.0025 to 0.05mg chlorophyll per ml. It was normally found necessary to shake the chloroplast extract vigorously on transference to acetone and stand in darkness in a stoppered flask for five minutes or until precipitation of protein was complete. Spectrophotometric readings were taken

following removal of the precipitated proteins by filtration or centrifugation.

Additional checks on the total chlorophyll concentration were made by measuring the optical densities of the 80% acetone solutions at 652nm (pH 7.0) and substitution in Arnon's equation (ix):

$$Ct = \frac{E_{652}^{1cm}}{34.50}$$

where Ct is the concentration of total chlorophyll in mg per ml of 80% acetone solution, and the denominator is the calculated specific absorption coefficient of chlorophyll at the wavelength (652nm) at which the light absorption curves of chlorophyll a and b intersect. (MacKinney, 1941).

M. 3 Solubilisation of chloroplast fragments and estimation of degree of solubilisation

In all cases the method employed was as follows:-

The cold detergent solution was added to the chilled chloroplast lamellar suspension. During addition, the latter was stirred by means of a magnetic stirrer and the mixture then dispersed in a Potter-Elvehjem homogeniser (with an ice core) for a standard time in the range 30 seconds to 2 minutes.

Estimation of the relative effectiveness of detergents in solubilising lamellae was carried out by solubilising x ml chloroplast fragments containing 1.0mg chlorophyll/ml, in 6.25x ml 4% detergent buffered in 0.05M acetate, phosphate and carbonate/bicarbonate buffers at various pH's. (Final detergent:chlorophyll ratio of 250:1). The period of Potter homogenisation was fixed at 55-60 seconds.

Following homogenisation, the solubilised suspension was centrifuged at 100,000xg for 30 minutes at $4^{\circ}C$ and the O.D. of the supernatant or carefully measured dilutions of the supernatant, read at 530 nm (607 filter) on the EEL Colourimeter. The readings of the diluted supernatants

were multiplied by the appropriate dilution factor to make the results comparable (e.g. 1 ml supernatant plus 9 ml 0.05M buffer optical density multiplied by 10.).

M. 4. Spectral Analyses

Spectral measurements were carried out in this work with the aid of the following instruments:

Absorption Spectrophotometry

1. Unicam S. P. 500 ultraviolet absorption spectrophotometer, for accurate fixed wavelength estimations in the visible and ultraviolet regions.
2. Unicam S. P. 800 automatic spectrophotometer for plotting absorption over the visible and ultraviolet range.

Fluorescence Spectrophotometry

Equipment was not available, suitable for accurate quantitative fluorescence measurements. The fluorescence spectrophotometer employed, (Baird-Atomic, Inc. 'Fluorispec' fluorescence spectrophotometer, Model S. F. 1) was of use only as a fluorescence detector and was used as such.

M. 5 Trace Element Analysis

These analyses were conducted by two methods:

1. Colorimetric analysis according to the method of Bradfield (1956) for determination of manganese.

2. Analysis by polarography and colorimetry for determination of manganese, iron and copper. (Readings using these methods were carried out by British Titan Products Co. Ltd., using a cathode ray polarograph and spectrophotometer.)

In all estimations, the materials employed were rigorously purified. Detergents were freed of contaminating cations by passing down a column of Whatman carboxy methyl cellulose CM-22.

The buffer used in all these analyses was 0.05M phosphate pH 7.2, as employed by Anderson et. al. (1964) which was purified according to the method of Gentry and Sherrington (1950): trace elements were removed from 50ml aliquots of aqueous buffer solution by extraction in a separating funnel with 10ml of a 1% solution of 8-hydroxyquinoline in chloroform. The combined aqueous layers were then extracted with pure chloroform and finally, residual chloroform removed by evaporation under reduced pressure.

Tris buffer could not be employed as a buffering medium, as it has been shown (Anderson et. al. 1969) that this buffer leaches manganese from chloroplast lamellae.

The fractions analysed were derived from detergent-solubilised lamellae and were produced by differing fractionation techniques (see Methods Section pp. 48, 67).

In all cases the trace element content was related to the magnesium content assuming that all the magnesium present originated in chlorophyll.

Colorimetric Estimation of Manganese (Bradfield, 1956)

Assays were conducted on dried material, which was digested with concentrated nitric acid (25ml per 1g dried material) until completely dissolved. Then, 2.5ml 60% perchloric acid was added and digestion continued until fumes of perchloric acid were evolved. Following complete removal of perchloric acid fumes, the solution was cooled, diluted with 25ml of water, boiled and filtered through a Whatman No. 540 filter paper into a 100ml volumetric flask which when cool was diluted to volume.

By pipette, an aliquot containing 10 to 50 ug of manganese was transferred to a 50ml volumetric flask, diluted to about 30ml, and treated first with 5.0ml of H.E.E.D.T.A. solution (see below) and then sodium hydroxide solution (see below) dropwise until the pH of the solution was 7. This was followed by addition of 1.0ml of diluted formaldoxime reagent (see below) and 2.0ml of sodium hydroxide solution. The flask was then heated to 65^{o}C for two hours, cooled and made up to volume. The optical density of the flask contents was then estimated at 450 nm using 4cm glass cells.

In each series of estimations, a blank determination was performed by carrying out the same procedure on the reagents only.

Reagent solutions:

H.E.E.D.T.A. solution : 10% aqueous solution of N-hydroxyethylethylenediamine triacetate

Formaldoxime solution : 20g. paraformaldehyde and 55g. hydroxylamine sulphate in total volume 100ml.
(For use, this solution is diluted x 10)

Sodium hydroxide solution : 10% aqueous solution of A.R. sodium hydroxide.

M. 6 Benezene extractability of chlorophyll in aqueous, chloroplast dispersions containing detergents

The effects of detergents upon the benzene-solubility of chlorophyll in aqueous chloroplast suspensions were determined according to the method of Ke. et. al. (1956).

The chlorophyll content of all chloroplast preparations tested was adjusted to 0. 05mg/ml prior to cold liquid-liquid extraction with benzene. Each chloroplast preparation was buffered, in 0. 04M phosphate at pH 7. 0, and in 0. 1M carbonate/bicarbonate at pH 10. 0.

Buffered detergent solutions were added to aliquots of the chloroplast preparation to give different final concentrations of detergent in the mixture.

The liquid was then homogenised for 30 seconds in a Potter-Elvehjem homogeniser (see p.48) before transference to a separatory funnel containing an equal volume of cold benzene.

The degree of extraction of chlorophyll by the benzene layer was estimated on the S. P. 500 by measuring the absorbancy at 665 nm.

Blank chloroplast preparations, differing only in having no detergent present were also tested for benzene extractability.

A certain amount of difficulty was experienced in obtaining reproducible results in the experiments carried out at pH 10.

Stability of recordings was obtained by leaving the shaken mixture to stand in darkness for 15 minutes before separation of the layers.

M. 7 Estimation of the effect of detergents on System 1 and System II activity of lamellar fragments

System 1 activity:

The estimation of reduced pyridine nucleotides is normally carried out by optical density measurements at 340 nm.

Many synthetic detergents are found to absorb strongly at this wavelength however.

Estimations recorded in the literature (Vernon 1965$_b$, 1966$_a$) of NADP photoreduction by lamellar fragments produced by detergent action have employed the standard method, from which it must be concluded that in such cases the detergent does not absorb sufficiently at 340 nm to affect the assay.

This work required an unambiguous alternative method of estimating $NADPH^+$ suitable for use in the presence of all detergents.

The method developed was to estimate the amount of DCPIP required to re-oxidise reduced NADP produced by illumination of the test sample. The test chloroplast material (0.1 ml) containing 0.05 to 0.20 mg chlorophyll was suspended in 7.0 ml of reaction medium (see below) in darkness, mixed, and split equally between two 1 cm spectrophotometer cuvettes. One (unilluminated) was used for

a blank and the other was illuminated for periods of three minutes by an Atlas A1/215 100w 12v projector lamp focussed to a 3 x 6 cm rectangle through a 1.46 steradian condenser. Undue heating of the illuminated sample was avoided by passing the light beam through a heat filter of cupric sulphate solution (4.10^{-2}M) thickness 2 cm. The cuvette was 80 cm from the condenser and received approximately 5×10^4 lux, which is saturating light intensity.

After illumination of the sample cuvette, 0.01ml aliquots of DCPIP containing 1.0 umoles DCPIP/ml were added and the absorbancy change at 607 nm recorded.

Two variations of the method were employed:

1. Addition of DCPIP aliquots to both control and illuminated sample.

This method is preferable as it eliminates the possibility of dilution effects and also gives quantitative indication of any endogenous reducing material present in the chloroplast preparation. A typical graph obtained is as shown in fig. 5.

2. Addition of DCPIP aliquots to the illuminated sample only.

This procedure, followed in the early assays has neither advantage of the previous method. A typical graph is as shown in fig. 6.

Reaction medium:

2.0 mg NADP

1.05mg Ferredoxin

0.25mg Ferredoxin-NADP reductase

Made up to 23ml with diluting medium.

Diluting medium:

20ml 0.5M phosphate pH 7.1 (500 μmole/ml)

20ml 0.01M ammonium sulphate A.R. (10 μmole/ml)

20ml 0.005M potassium chloride A.R. (5 μmole/ml)

Made up to 200ml with distilled water.

(Each cuvette contained 175 μmole phosphate; 3.5 μmole ammonium sulphate; 1.5 μmole potassium chloride; 0.3mg NADP; 0.15mg ferredoxin and 0.035mg ferredoxin - NADP reductase).

Fig. 5

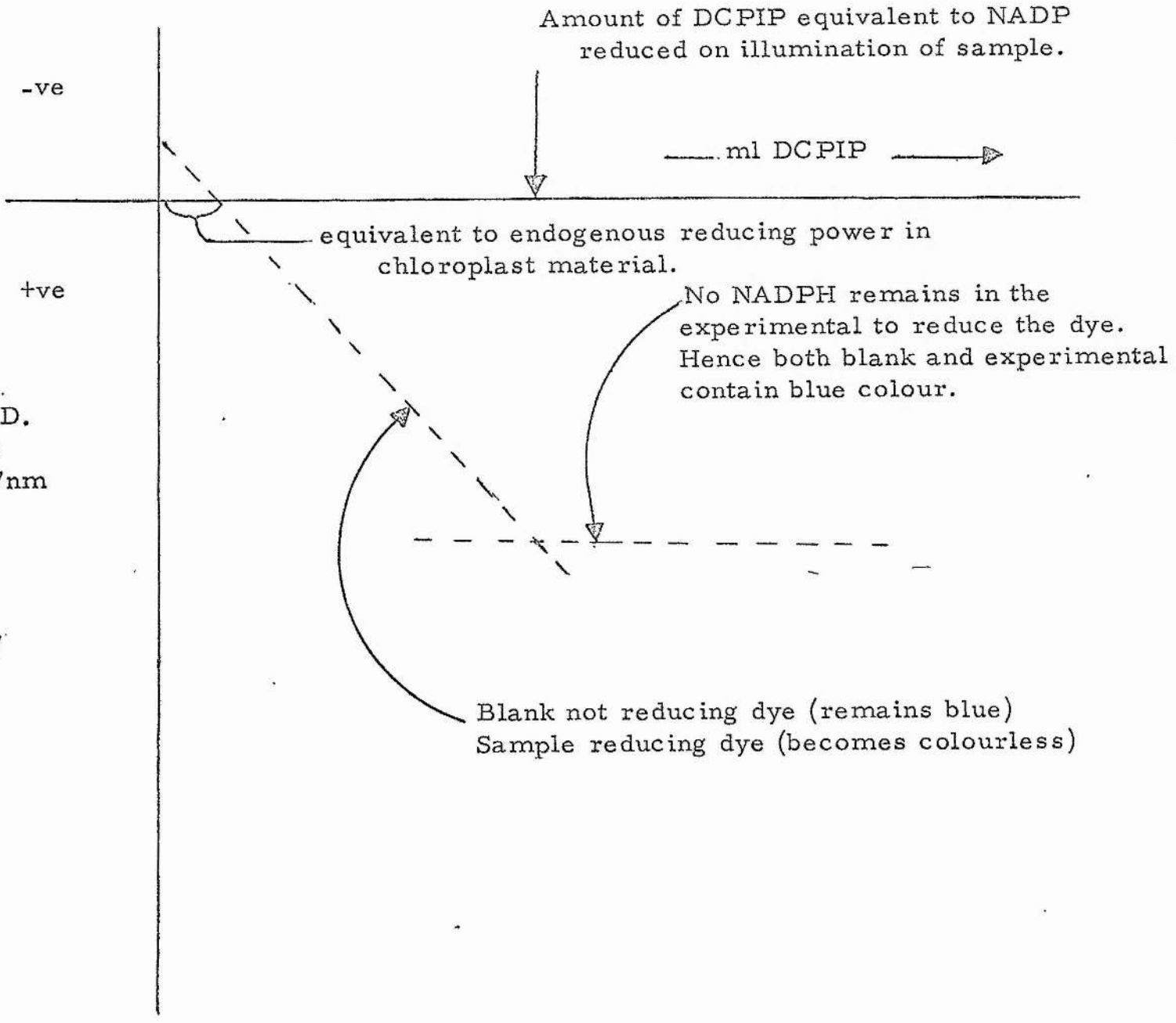
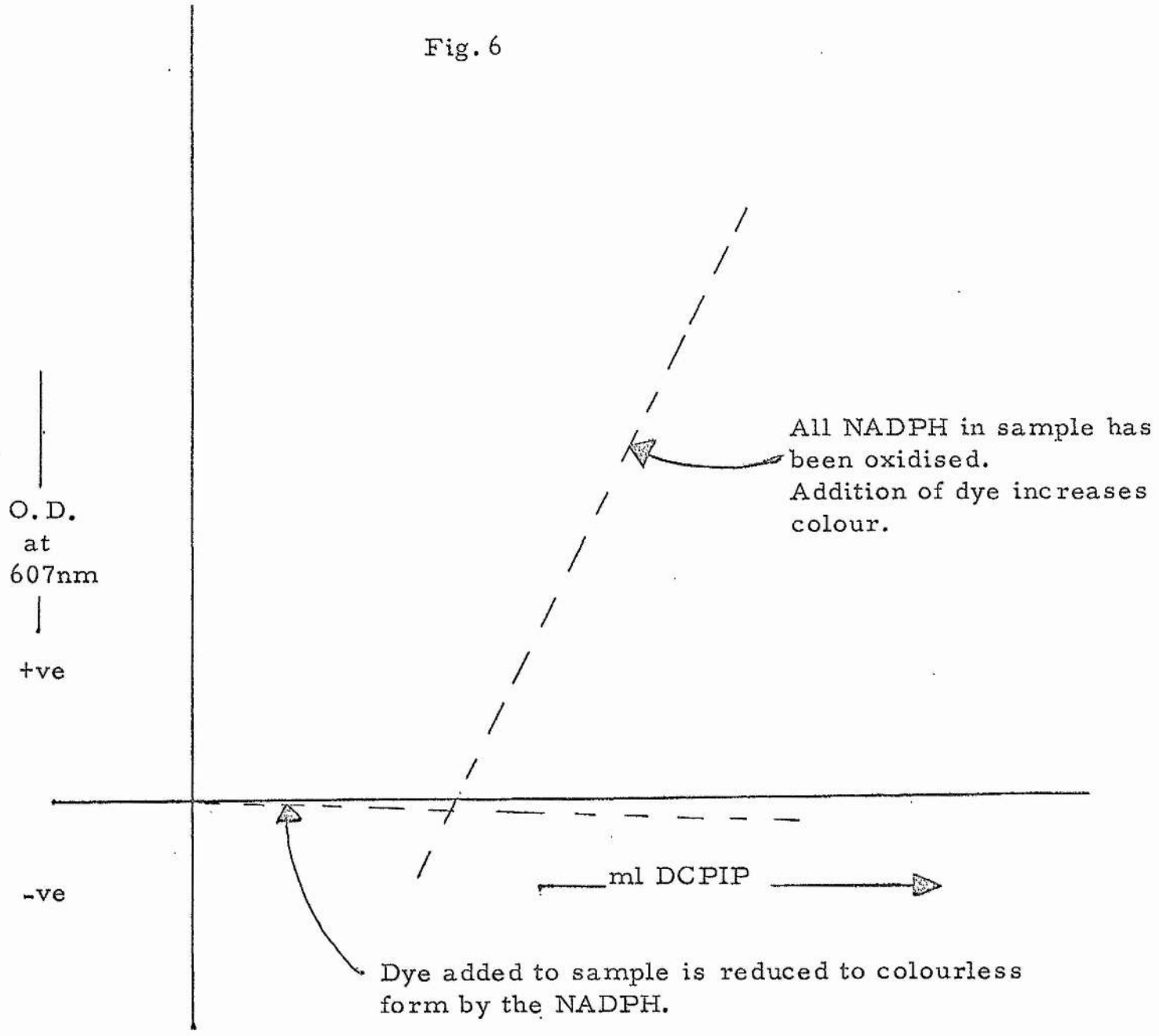

Amount of DCPIP equivalent to NADP reduced on illumination of sample.
-ve
ml DCPIP
equivalent to endogenous reducing power in chloroplast material.
+ve
No NADPH remains in the experimental to reduce the dye. Hence both blank and experimental contain blue colour.
Blank not reducing dye (remains blue)
Sample reducing dye (becomes colourless)

Fig. 6


O. D.
at
607nm
+ve
-ve
All NADPH in sample has been oxidised.
Addition of dye increases colour.
ml DCPIP
Dye added to sample is reduced to colourless form by the NADPH.

System 11 activity: (photoevolution of oxygen)

Evolution of oxygen by a suspension of chloroplast material in a Rank oxygen electrode (Rank Bros. Bottisham, Cambs.) was recorded electronically. For each assay, a 5 ml aliquot of reaction medium (previously freed of oxygen by saturating with nitrogen gas) was pipetted into the electrode cell followed by a sample (up to 0.05ml) of chloroplast material containing 0.03 to 0.2mg chlorophyll. The cell cover was then replaced, care being taken to eliminate air bubbles from above the medium, and the magnetic stirrer in the cell switched on.

The effect of detergents on the photoevolution of oxygen was followed by adding 0.01 to 0.05ml aliquots of detergent solution buffered at pH 7.1 to the electrode cell from a syringe through a small hole in the cell cover. Additions were made as soon as the normal oxygen photoevolution had reached a steady rate, and the effect on oxygen production noted.

(The cell, jacketed with water at 20°C was illuminated from the side by a Vickers Intense microscope lamp (40w) focussed from 15cm through a heat filter of $4.\times 10^{-2}$ M cupric sulphate thickness 2cm. The beam was also passed through an Ilford 205 red filter (cut-off below 680nm) to avoid bleaching of the chlorophyll. Under

these conditions the chloroplast material is light saturated).

In all experiments, the behaviour of the unilluminated chloroplast material was also observed as a control.

Reaction medium:

1ml 0.25M phosphate pH 7.1 (250 umoles)

1ml 0.005M potassium chloride (5 umoles)

1ml 0.005M ferricyanide (5 umoles)

1ml 0.0025M ammonium sulphate (2.5 umoles)

1ml distilled water.

M. 8 Photo-oxidation of ascorbate by detergent treated lamellae

The method employed was a modification of that of Chiba et. al. (1962). All procedures were carried out under a dark green safety lamp emitting light of about 530nm. All solutions were saturated with nitrogen gas.

Each reaction mixture consisted of chloroplast preparation equivalent to 1.0-1.2 mg chlorophyll, 2.0ml 0.1M phosphate buffer pH 6.5, 0.4ml 0.1M EDTA, 0.2ml 0.01M DCPIP and 0.6ml 0.01M sodium ascorbate made up to 18.0ml with distilled water.

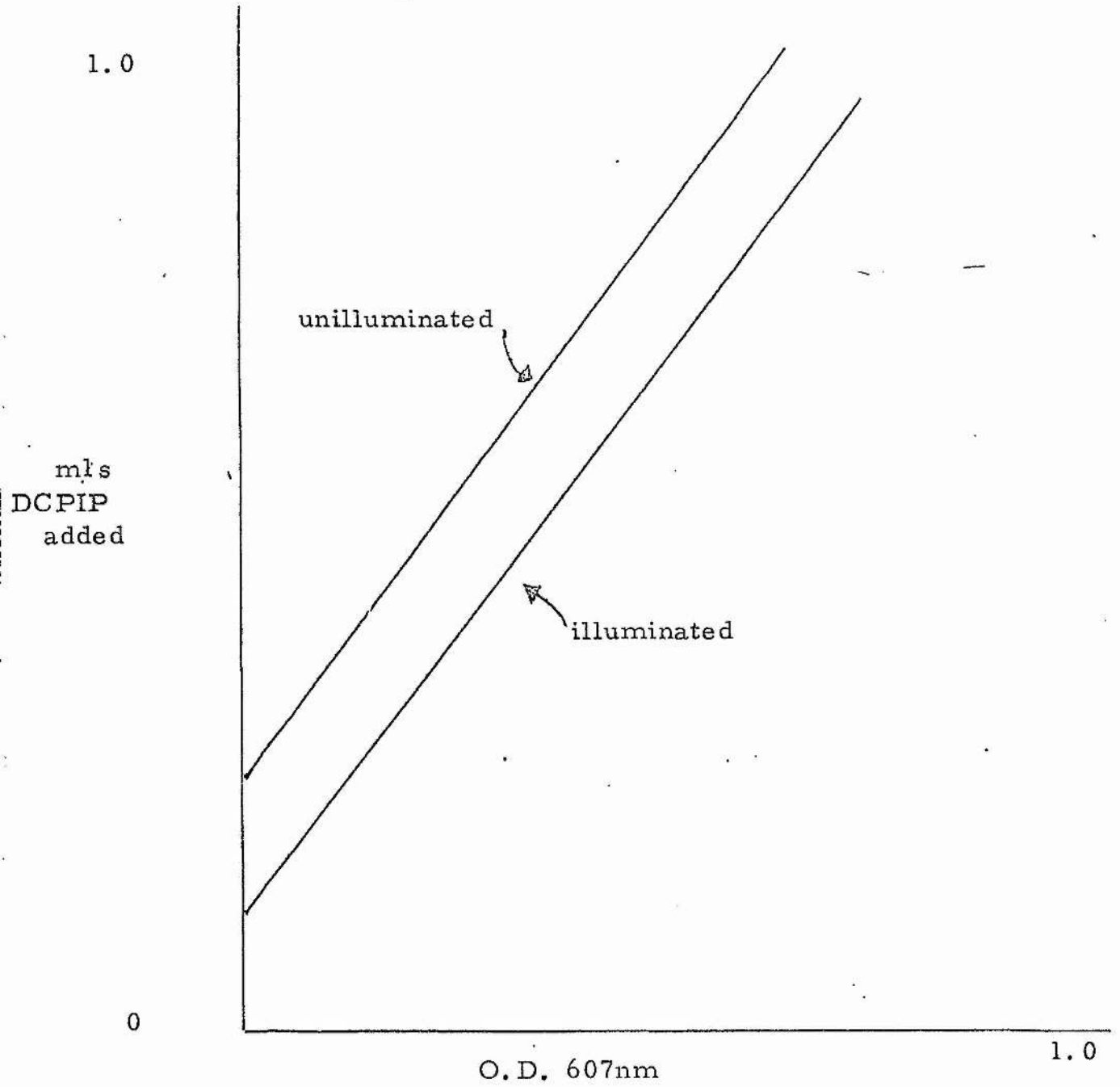
For each assay, two 3.0ml samples of the reaction mixture were pipetted into each of two 1cm spectrophotometer cuvettes and one of these placed in the beam from an Atlas A1/180 140 v 500w lamp, focussed to an area of 12" x 12" through a 2" x 2" Voigtlander Perkeo slide projector. Under these conditions, the illuminated cuvette was light saturated. The other cuvette was kept in darkness.

The illuminated sample was kept in the light beam for a fixed time of between 1 and 10 minutes, following which, the optical densities of both the unilluminated and illuminated samples at 607nm were read on a S.P.500 spectrophotometer, using distilled water as reference. To each of the experimental cuyetttes, prior to each reading, an aliquot of 0.01ml of 0.01M DCPIP buffered in 0.04 phosphate at pH 6.5 was added.

It is recommended (Roe; 1954) that oxidation of ascorbate by DCPIP should be carried out at pH 3.5. At this pH, the dye colour is pink however, and of too weak an intensity to be of practical use in spectrophotometric assays where addition of small aliquots is desirable. For this reason, the dye was buffered at pH 6.5, despite the fact that at higher pH's, the action of reducing substances other than ascorbate becomes more prominent, since allowance for the presence of such substances is made in adding the dye to both illuminated and unilluminated cuvettes.

A typical graph is shown in fig. 7.

Fig. 7



M. 9 Preparative ion exchange Chromatography

Column Chromatography (preparative)

Columns of DEAE cellulose (Whatman DE-11 and DE-22) were employed both in the fractionation of detergent-solubilised lamellae and in the preparation of ferredoxin, ferredoxin-NADP-reductase and plastocyanin. Further details on the latter procedure are given on p. 73.

Fractionation of chloroplast solutions was carried out basically as reported by Kahn (1964), but the concentrations of detergents were varied in order to give equivalent lamellar solubilisations.

Preparation of DEAE cellulose column:

Whatman DE-11 fibres were activated by washing successively in distilled water (2 hours); 0.5N sodium hydroxide (2 hours; solution changed each hour) and finally, distilled water (several extractions, as above). The cellulose was then equilibrated in 0.05M tris buffer pH 8.0 for 3 hours and bedded in a column of diameter 3.2cm to a height of 30cm.

The column was washed with 5 x bed volume of 0.05M tris buffer pH 8.0 containing detergent of concentration x%. (See below).

Preparation of soluble chloroplast extract

A suspension of chloroplast fragments buffered in 0.05M tris pH 8.0 and saturated with nitrogen gas at 4°C, containing 0.5mg chlorophyll/ml, was suspended in an equal volume of a 2x% solution of detergent in 0.05M tris pH 8.0 and homogenised in a Potter Elvehjem homogeniser for 45 seconds. (See p.48). The material was then centrifuged at 50,000xg for 30 minutes. The supernatant was discarded and the precipitate resuspended in the same volume as above, of a 20x% solution of detergent in 0.05M tris pH 8.0. The suspension was then homogenised for 45 seconds in a Potter Elvehjem homogeniser and centrifuged at 144,000xg for 60 minutes. The supernatant was dialysed for 12 hours at 4°C against distilled deionised water through which nitrogen gas was passed.

Chromatography of the chloroplast extract:

25ml of the dialysed extract was layered onto the column below 5ml of x% detergent in 0.05M tris pH 8.0. The column was then prepared for linear gradient elution and eluted to a final concentration

of 0.5M sodium chloride plus x% detergent in 0.05M tris pH 8.0. The eluate was collected in 10.0 ml aliquots and every third fraction was assayed for protein, (after dialysis against distilled deionised water for 24 hours at 4°C) chlorophyll and ascorbate photooxidation. (See pp. 82, 46, 64 respectively).

The chromatography of the extract was conducted at 4°C and under the illumination of a dark green safety lamp (emitting light of about 530nm) in order to minimise bleaching of the chlorophyll.

The detergents employed in these experiments and their respective values of x, were:

Detergent	x(%)
Triton X-100	0.05
Renex 698	0.05
Lubrol E	0.05
Lissapol NXP	0.05
G 711	0.05
Brij 96	0.35
Calsolene Oil	0.50

M. 10 Disc-Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out on a Shandon Analytical Polyacrylamide Electrophoresis Apparatus with a Shandon Vokam power supply, initially using non-ionic and cationic detergent solutions buffered in phosphate buffers of differing molarities and pH's.

The gels varied in concentration between 4% and 10% and were equilibrated by electrophoresis (5ma/tube) for fifteen minutes before adding the detergent-chloroplast extract. Each extract was the supernatant from a 30 minute, 50,000 x g centrifugation of detergent-solubilised lamellae and was mixed with propylene glycol (10% v/v) to increase density and avoid pre-electrophoresis diffusion prior to layering under buffer onto the top of the gel column. The supernatants produced by Polychol 15, Crillet 4, Tween 80, Crillet 1, Tween 20, Dispersol VLX, Brij 35, Brij 58, Lubrol AL18 and Brij 98, were of too light a colour to be applied directly to the gel columns and were dialysed against a 35% solution of polyethylene glycol 4000 overnight in darkness at 4^{o}C to concentrate.

After further electrophoresis for 10 to 45 minutes, the gels were stained by immersion in a 1% solution of Amido Black 10B for 20 minutes and destained by washing in methanol-acetic acid-water (5:1:5 v/v) for 24 hours, or by use of an electrophoretic destaining apparatus.

Gels prepared using a high ($> 0.2\%$) detergent concentration and/or low gel concentration ($< 7.5\%$) were formed in tubes having slightly constricted lower ends to prevent the gels from falling through the tubes into the lower buffer compartment.

In the case of anionic detergents, lamellar extracts were prepared according to a modification of the method of Thornber et. al. (1967b).

Isolated lamellae (chlorophyll concentration 1mg/1ml) were dispersed in an equal volume of 1% detergent in 0.10M sodium borate pH 8.0.

The suspension was centrifuged at 144,000 x g for 30 minutes and the precipitate re-extracted with the same volume of buffered detergent as used in the first extraction. The suspension was centrifuged at 144,000 x g for 30 minutes and the supernatant mixed with propylene glycol (10% v/v) prior to

layering under 0.05M sodium borate pH 8.0 onto the top of the column.

As in the other experiments the gels were equilibrated by electrophoresis (5ma/tube) for 15 minutes before adding the detergent-chloroplast extract.

Following electrophoresis, the loaded gels were stained in 1% Amido Black 10B as described above.

M.11 Preparation of Ferredoxin, Flavoprotein and Plastocyanin

The principal electron transfer proteins were isolated from Spinacea leaves by a modification of the methods of San Pietro et. al. 1958 (ferredoxin); Shin et. al. 1963 (flavoprotein) and Katoh, 1960 (plastocyanin). 1 kg of leaves were homogenised with 1.08 litres of acetone chilled to -15°C, 400 ml of water and 100 ml M tris buffer pH 7.7 in a Waring blender. The homogenate was filtered through nylon cloth of 50μ mesh size (see p.44) and centrifuged at 2000 x g for 20 minutes. To the supernatant, 1.16 volumes of cold (-15°C) acetone was added and the mixture beaten and left to stand for one hour. The relatively clear green supernatant was discarded and the sticky brown precipitate centrifuged at 1500 x g and collected. The precipitate was transferred to minimum volume (50ml) of a solution of 0.01 M tris pH 7.7 in 0.2M sodium chloride and dialysed overnight against two changes each of the same solution at 4°C, with stirring. The insoluble material was centrifuged off and the supernatant passed through a column, 3cm diameter and 15 cm packed length of diethyl aminoethyl (DEAE) cellulose (Whatman DE22) equilibrated with the tris-sodium chloride solution referred to above. Ferredoxin was retained on the column

whilst the other proteins were not. The latter were eluted out of the column and dialysed against a large volume (eluate volume x 50) of saturated ammonium sulphate solution for 12 hours at 4°C. The proteins precipitated within the dialysis sac were collected by filtration and redissolved in 0.05 M tris buffer pH 7.7. Insoluble residue having been discarded, the solution contained a mixture of flavoprotein and plastocyanin in its reduced (yellow) form.

Ferredoxin was purified from residual flavoprotein and plastocyanin by elution from the DEAE cellulose column with a solution of 0.01 M tris buffer pH 7.7 in 0.2 M sodium chloride, followed by passage down a column of Sephadex G.25 (3 cm diameter and 20 cm packed length) equilibrated with distilled water, to remove ions, and then further chromatographed as above on a DEAE cellulose column.

DEAE cellulose column chromatography was employed to separate plastocyanin from flavoprotein using a linear concentration gradient in 0.01 M tris buffer pH 7.7 from 0.05 M sodium chloride to 0.20 M sodium chloride. Plastocyanin was eluted (in the reduced, yellow form) at higher ionic strengths and was oxidised to the blue form by addition of a crystal of potassium ferricyanide.

The separated proteins were lyophilised and stored at -5°C.

M.12 Preparation of Chlorophylls

Chlorophylls were prepared by a method based on that proposed by Strain et.al. (1966): 200g demidribbed leaves were dropped into 2 litres of boiling water. After two minutes, the water was cooled with an excess of cold water. The water was decanted, the leaves washed once with cold water and dried between paper towels. The leaves were then separated from one another and placed in methanol (500 ml) plus petrol ether (125 ml, boiling range 20°-40°C). The leaves were then homogenised in a Waring blender. The extract was then filtered through nylon bolting cloth (mesh size 50μ) and centrifuged to remove all solid material. The supernatant was then transferred to a 2 litre separating funnel and diluted with the same volume of saturated salt solution. This transferred most of the pigments to the petrol ether layer. The petrol ether extracts were reduced in volume on a rotary evaporator in darkness at room temperature and purified by reversed phase chromatography on thin layer plates of kieselguhr impregnated with paraffin oil.

The plates were first impregnated by running in 8% paraffin oil in petrol ether (boiling range 20° - 40°) and dried at 40°C for 12 hours to remove all traces of petrol ether. The crude chlorophyll solution was then applied to the plates by means of a strip applicator and the plates developed in methanol/ water/ acetone (20:4:5 v/ v).

Chlorophyll a occurs as a slow flowing blue-green band and chlorophyll b as a fast flowing yellow-green band (Rf values 0.33 and 0.63 respectively).

Preparation of chlorophylls by reversed-phase thin layer chromatography

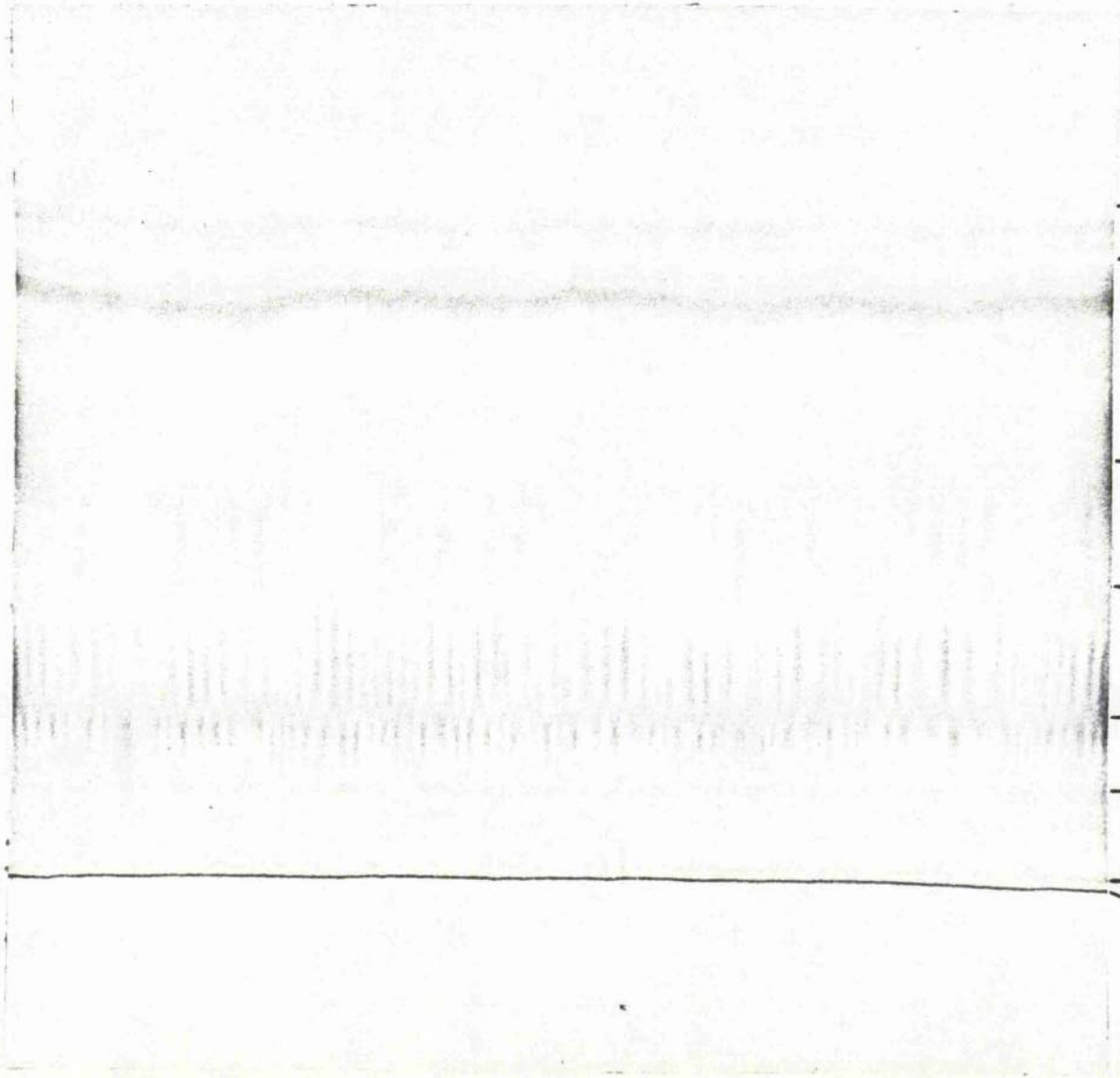


Fig. 8.

M. 13 Estimation of degree of bleaching of chlorophylls and alteration of pigment composition in detergent-solubilised lamellae when exposed to visible light

It was noted by Kahn (1964) that a photochemically active chlorophyll-protein complex isolated from lamellae solubilised in Triton X-100 was bleached if exposed to strong light, with resultant loss in photochemical activity. Detergent solutions of lamellae have been tested in this thesis for stability when subjected to strong visible illumination under varying conditions of detergent and chlorophyll concentrations, pH and oxygenation of the suspension, temperature and time of illumination.

The light source employed was a Mazda 400 $\bar{w}$ MBFR/u lamp. which was suspended from a height of one metre above the test material. The test material was contained in a shallow circular dish of radius 15cm (surface area 700 sq. cm.) which afforded a substantial surface area:volume ratio. Lamellar solubilsation was carried out essentially as described before (see p. 48). Following solubilisation, each suspension was centrifuged at 20,000xg for the minimal period of time required to produce a clear supernatant and the supernatant transferred to the circular dish.

Experimental Conditions:

Six different detergents were employed; Lissapol NXP, G711, Cetavlon, Brij 35, Tergitol 7, and Renex 698.

The concentrations of these detergents in the buffer media employed for lamellar solubilisation were as follows:

Lissapol NXP	:	Chlorophyll	200 : 1
G711	:	Chlorophyll	150 : 1
Cetavlon	:	Chlorophyll	200 : 1
Brij 35	:	Chlorophyll	350 : 1
Tergitol 7	:	Chlorophyll	300 : 1
Renex 698	:	Chlorophyll	200 : 1

a) Effect of variation of the chlorophyll concentration

In these experiments, the supernatants were diluted with 0.04M phosphate buffer of pH 7.0 to decrease the chlorophyll concentration within the range 3.0 to 0.05mg chlorophyll/ml. Each supernatant was illuminated at 20°C whilst gently gassed with a stream of nitrogen for a total period of 5 days.

b) Effect of pH variation

Here, the lamellar solubilisation was carried out at three different pH's (4.0, 7.0, and 10.0) and the resultant supernatants

after dilution to a chlorophyll content of 1.0 mg/ml, illuminated whilst being gently gassed with nitrogen at 20°C for a total period of 5 days. The buffers employed for the different pH's were 0.04M acetate (pH 4.0), 0.04M phosphate (pH 7.0), and 0.05M carbonate/bicarbonate (pH 10.0).

c) Effect of Oxygenation

In these experiments carried out at 20°C and at pH 7.0, the chlorophyll content having been adjusted to 1.0mg/ml, the supernatant was illuminated for a five day period whilst being stirred with an overhead stirrer and gassed with a stream of air.

Each oxygenation experiment was compared with two similar experiments which were conducted under the same conditions, using:

1) a solution of chlorophylls extracted from Spinacea leaves, in acetone (see p. 75) and of the same chlorophyll concentration.

2) a fresh lamellar suspension in 0.04M phosphate buffer pH 7.0 of the same chlorophyll concentration, containing no detergent.

These controls were performed in order to demonstrate the differences in stability of chlorophyll to bleaching in differing environments and to ascertain whether the detergent exerts any stabilising influence on the bleaching of chlorophyll in the presence of leaf prôtein.

d) Effect of Temperature

These experiments were performed on the solubilised lamellae (chlorophyll content 1.0 mg/ml) at pH 7.0 without agitation and with slow passage of nitrogen through the supernatant, whilst under illumination for a total period of 5 days. Temperatures used were $4^{\circ}C$ and $20^{\circ}C$.

M. 14 Nitrogen Estimation

The method used to determine nitrogen was based on the development of the Kjeldahl technique (1883), by Chibnall et. al. (1943) and differed mainly by employing sodium sulphate-copper sulphate rather than selenium, digestion catalyst. Each aliquot of test material was treated with 2. 0 ml of nitrogen-free sulphuric acid in a small Kjeldahl flask, and a tablet of catalyst added. The flask contents were then digested for twelve hours on an electric heating rack. Care was taken to ensure that no carbon or other undigested material remained. The flask contents were then made alkaline by addition of 15ml of 40% potassium hydroxide solution and steam distilled using the distillation apparatus designed by Tristram (1966) (fig 9). The distillate was collected in a small flask containing 3ml of 2% boric acid and titrated with N/70 sulphuric acid to a grey end-point using the mixed indicator (methyl red/bromo cresol green) employed by Ma et. al. (1942). Each 5. 00 ml of 1. 00 x N/70 acid is equivalent to 1. 00mg nitrogen in a sample. All estimations

were performed in triplicate.

Protein estimations were conducted according to the method of Takashima (1952) in which assumption is made that the nitrogenous content of protein amounts to 16% and that negligible quantities of non-protein nitrogen are present.

Preliminary work in which attempts were made to estimate protein by the microbiuret method (Itzhaki et. al. 1964) and by the Folin-Lowry method (Lowry et. al. 1951) in the presence of detergents were unsuccessful.

Apparatus for Kjeldahl determination of nitrogen (Tristram 1966)

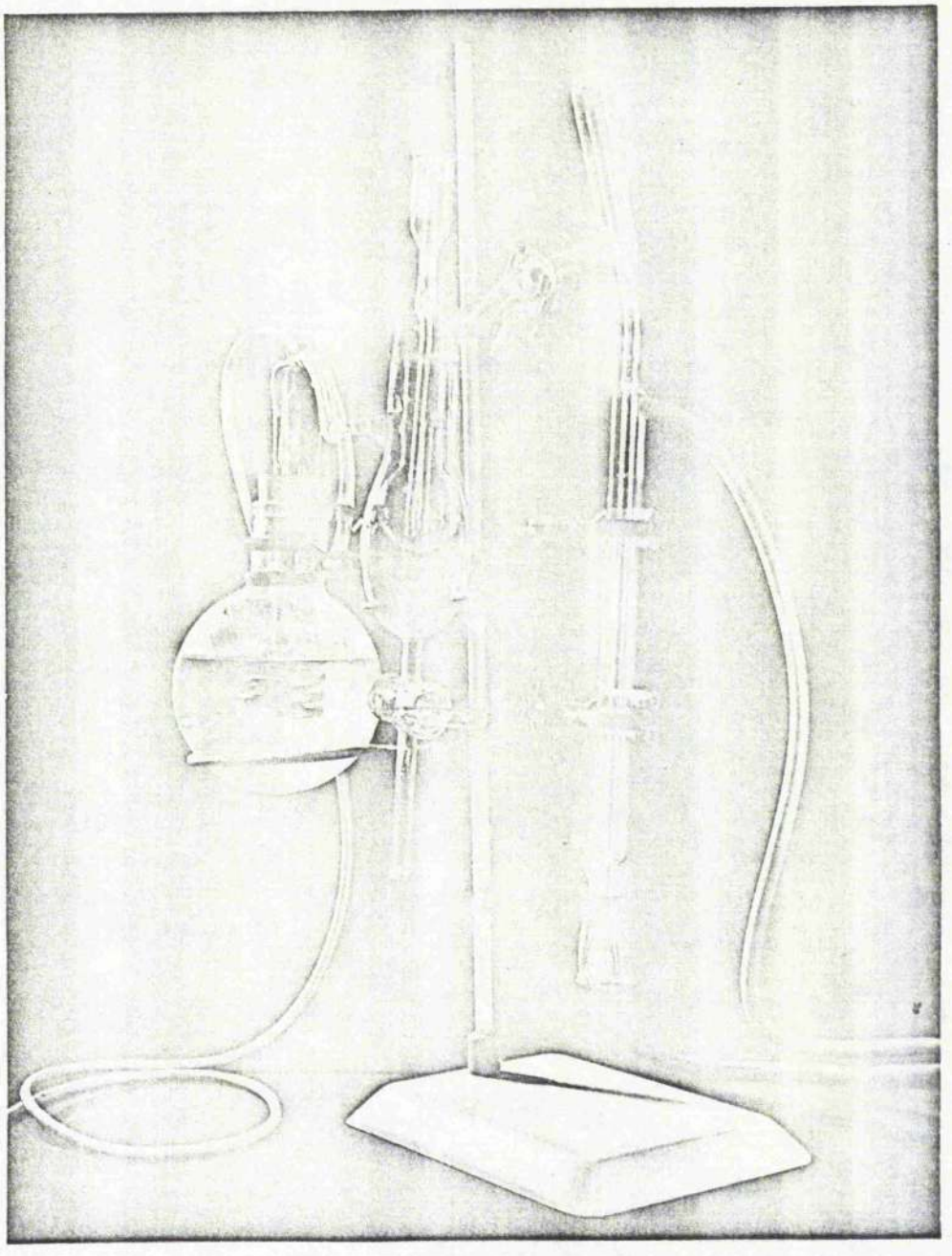


Fig. 9

RESULTS

R.1 Chlorophyll a/b ratios in detergent treated fractionated lamellae

Estimation of the chlorophyll a/b ratios of lamellar particles fragmented by the detergents under survey, has shown there to be three basic types of lamellar dispersion with respect to chloropyll content:

1. The detergent G711 (see figs. 10, 11) was found to split lamellae in such a way as to retain almost all the chlorophyll b in the more readily sedimentable fractions, irrespective of the detergent/chlorophyll ratio, resulting in the supernatant from high speed centrifugations possessing very high chlorophyll a/b ratios.

2. Other detergents (e.g. Renex 698; see Table 7) were found to yield fragments rich in chlorophyll a which are more readily sedimentable, but produced no fractions as rich as in chlorophyll a as the high speed centrifugation supernatant from the G 711 treated lamellae.

3. The effect of the third group of detergents (remainder) was found to resemble the action of sonication (Vernon, 1966b) since none of the fractions produced by differential centrifugation showed any large deviation from the lamellar norm.

Chlorophyll a : Chlorophyll b ratios of fractions produced from lamellae by detergent solubilisation

	Ratio Detergent: Chlorophyll	Chlorophyll a/b ratio 10,000 x g fraction	Chlorophyll a/b ratio 144,00 x g fraction
Calsolene Oil	50:1 300:1	2.8:1 2.9:1	4.9:1 5.3:1
Digitonin	5:1 50:1	2.3:1 2.6:1	5.0:1 5.2:1
Lissapol NXP	5:1 50:1	2.3:1 2.8:1	5.7:1 5.5:1
Lubrol E	10:1 100:1	2.2:1 2.9:1	6.0:1 5.4:1
Lubrol L	25:1 250:1	2.6:1 3.2:1	4.8:1 4.3:1
Lubrol PF	50:1 300:1	3.2:1 3.2:1	4.4:1 4.6:1
Nonidet P42	5:1 50:1	2.7:1 2.9:1	5.0:1 4.6:1
Renex 698	5:1 50:1	3.0:1 2.6:1	5.7:1 6.2:1
SDBS	5:1 50:1	2.3:1 2.8:1	5.3:1 5.1:1
SDS	5:1 50:1	2.5:1 2.4:1	4.5:1 4.0:1
Tergitol 7	50:1 300:1	2.6:1 3.5:1	6.6:1 5.9:1
Triton X-100	5:1 50:1	2.2:1 2.7:1	5.6:1 5.8:1

Table 7

Except at high detergent concentrations, all the detergents studied showed a consistently lower chlorophyll a/b ratio than intact lamellae (though very slight in the case of group 3 detergents) in the lowest g sedimented fraction, and a higher ratio than intact lamellae in its supernatant.

Of the detergents studied only G 711 displayed the properties referred to above, in group 1.

Group 2 is represented by Calsolene Oil, Digitonin, Lissapol NXP, Lubrol E, Lubrol L, Lubrol PF, Nonidet P42, Renex 698, SDBS, SDS, Tergitol 7 and Triton X-100.

The remainder of the detergents under survey, were found to belong to the third group.

See also Preparative anion exchange column chromatography, p. 139 .

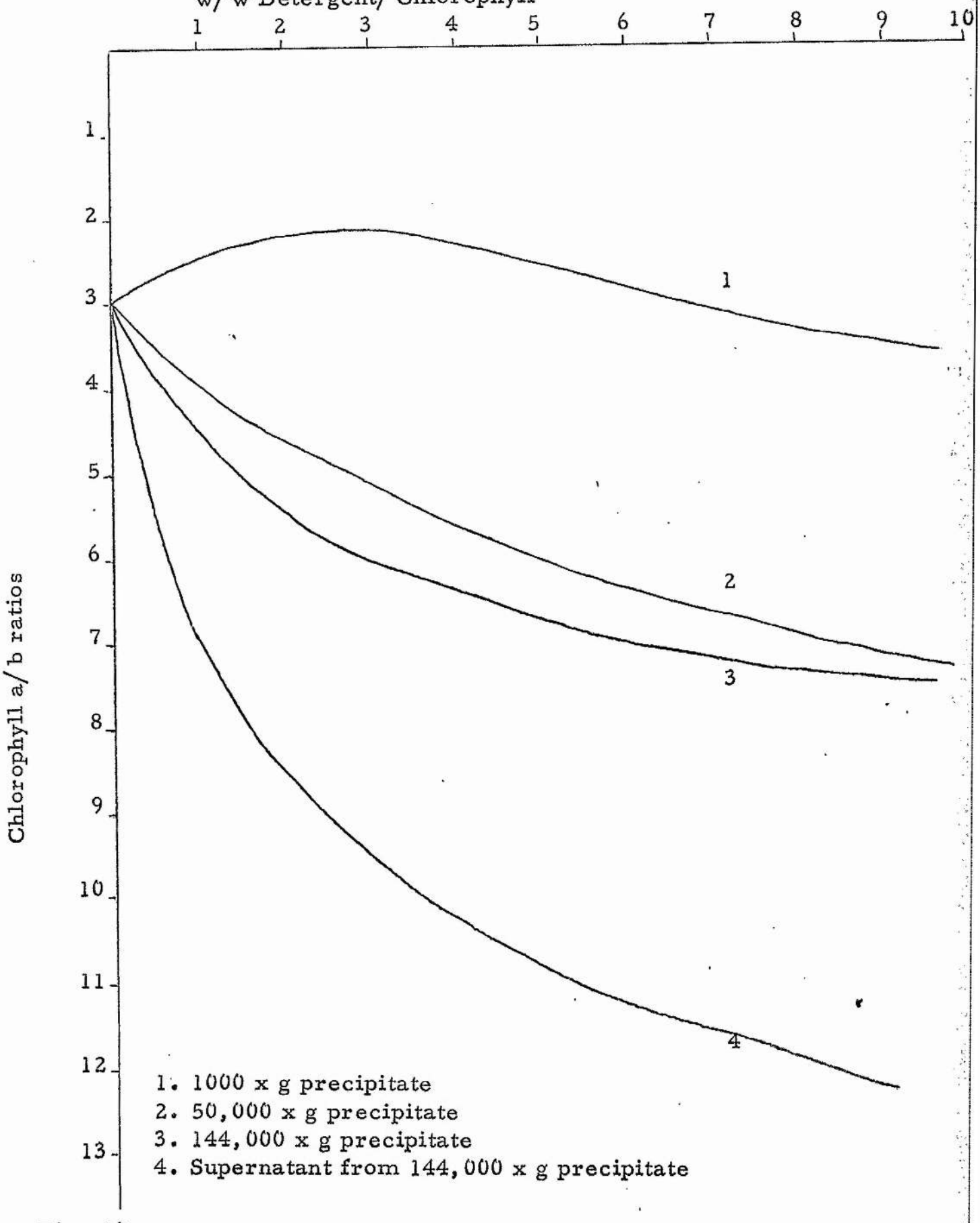

Chlorophyll a/ b ratios of lamellar fractions produced by action of G711
w/ w Detergent/ Chlorophyll
1
2
3
4
5
6
7
8
9
10
Chlorophyll a/ b ratios
1
2
3
4
5
6
7
8
9
10
11
12
13
1
2
3
4
1. 1000 x g precipitate
2. 50,000 x g precipitate
3. 144,000 x g precipitate
4. Supernatant from 144,000 x g precipitate

Fig. 10

Effect of the concentration of G 711 on the chlorophyll ratios of sedimented fractions

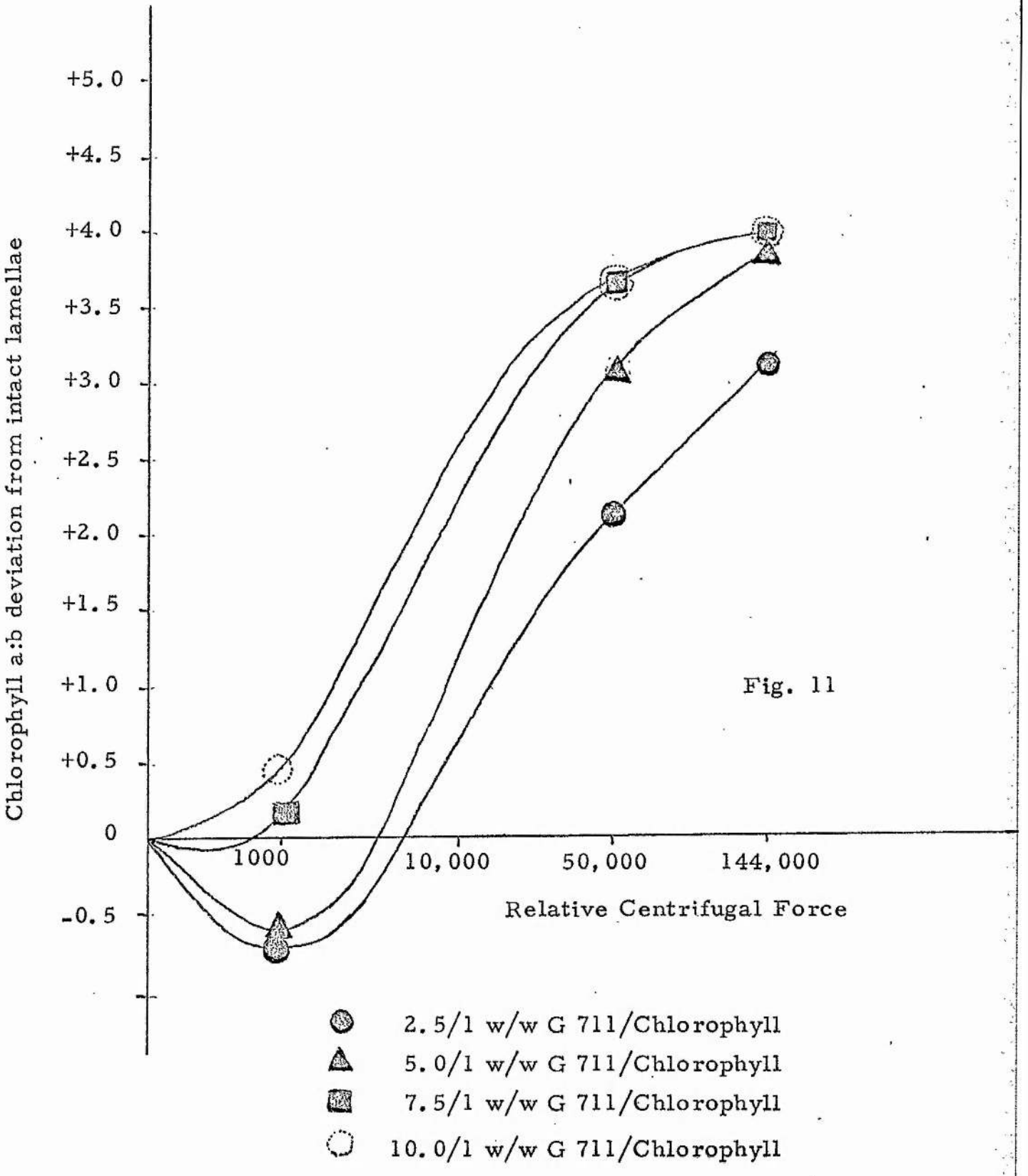


Fig. 11

R. 2 Lamellar Solubilisation

The variation of the solubilising effect of the detergents studied is quite considerable as may be seen from table 8 .

Experiments conducted according to the method detailed on p.48 but employing varying ratios of detergent : chlorophyll, indicate that two types of lamellar solubilisation profile are obtainable:

1. As shown in fig. 12, a profile which rapidly forms a plateau, beyond which an increase in the detergent : chlorophyll ratio has no effect on the degree of solubilisation of chloropyll.

This curve is shown by aliphatic polyoxyethylene detergents.

2. As shown in fig. 13 , a profile which shows a gradual and increasing degree of solubilisation of chlorophyll up to high detergent : chlorophyll ratios.

(Detergent : chlorophyll $\geqslant$ 250 : 1)

This curve is shown by polyoxyethylene detergents possessing aromatic moieties. Ionic detergents studied include examples of both solubilisation profiles.

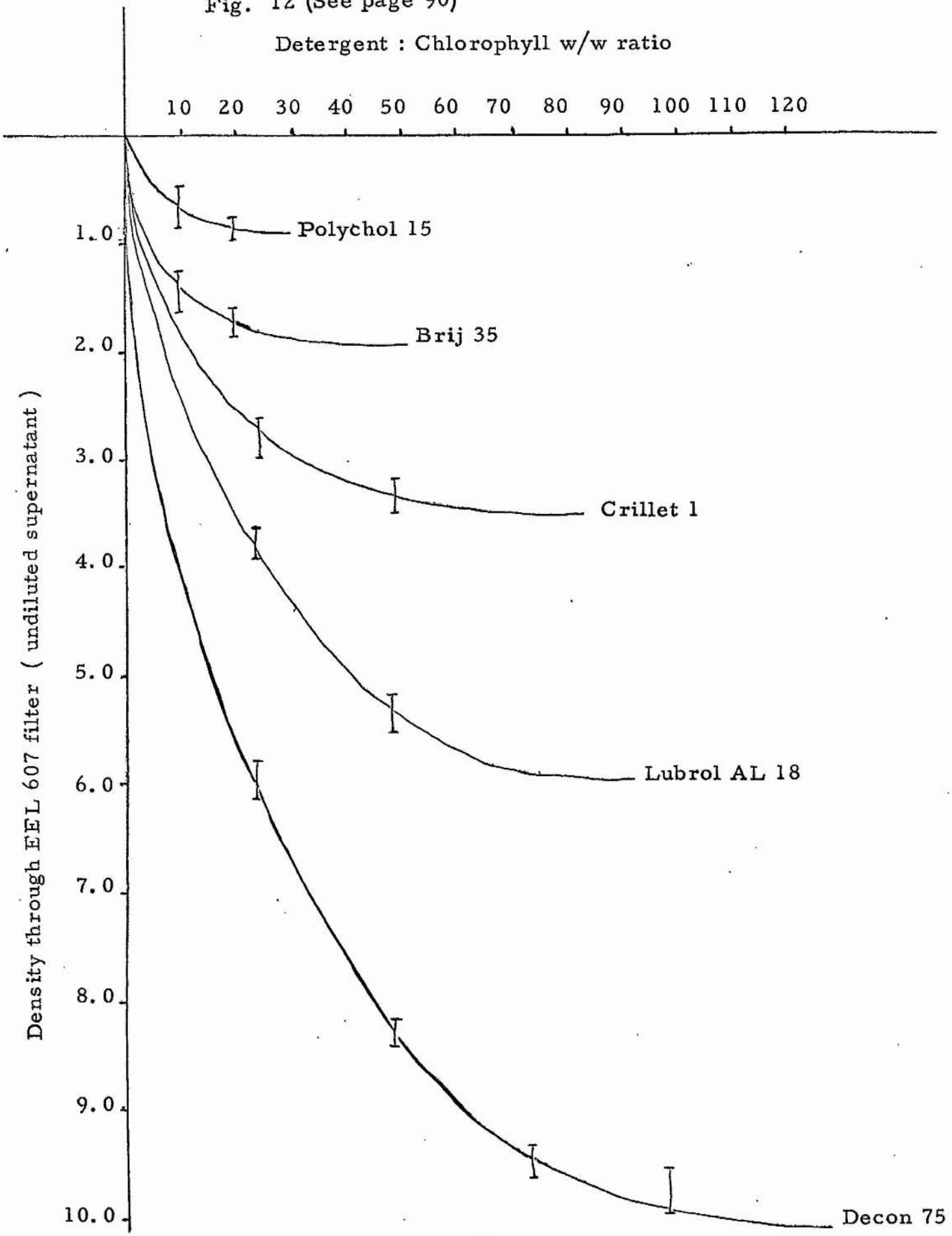
Detergent Effectiveness in Lamellar Solubilisation

(Average of three estimations)

Polychol 15	0.92	Tergitol 7	14.71
Crillet 4	1.53	Volpo N-10	22.34
Tween 80	1.77	Cithrol A	27.52
Brij 35	1.96	Lubrol L	28.55
Dispersol VLX	2.10	Brij 96	32.32
Brij 58	2.20	Lubrol E	42.35
Manoxol OT	2.26	Vantoc AL	43.20
Brij 98	2.84	Nonidet P42	45.28
Tween 20	3.00	Lissapol LS powder	46.21
Crillet 1	3.50	Triton X.100	46.77
Lubrol W	5.35	Renex 698	46.93
Tergitol Anionic 08	5.70	Lissapol NXP	47.12
Lubrol AL18	5.87	Digitonin	48.20
Lubrol PF	6.13	SDBS	48.64
Decon 75	10.93	SDS	48.80
Calsolene Oil	11.73	G711	56.00

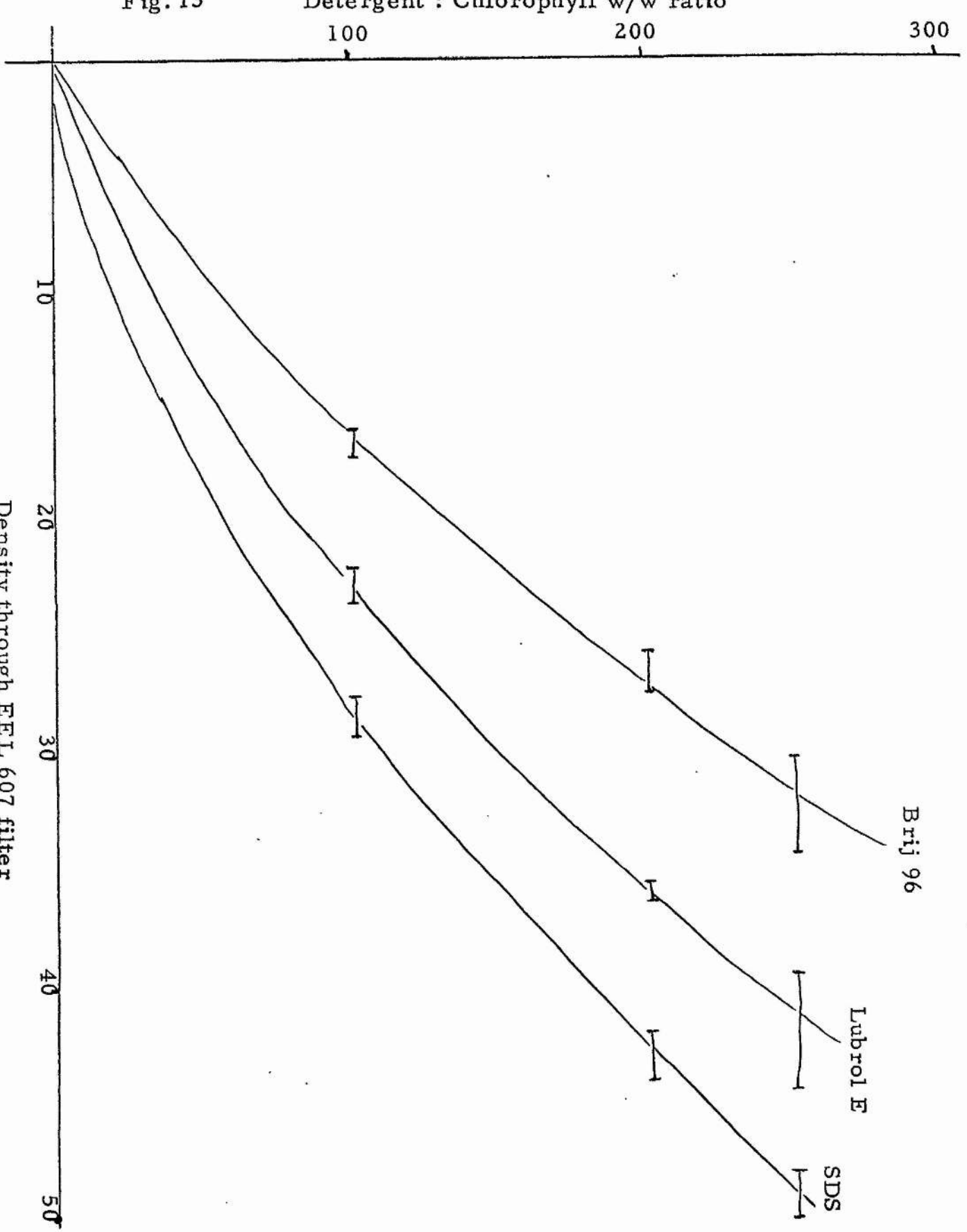
Table 8.

Fig. 12 (See page 90)



(See page 90)

Fig. 13



The effect of pH variation on the solubilisation of lamellae is described in table 9 , which summarises the results of solubilisation experiments conducted at pH's 7.1, 10.0, and 4.0. (For method, see p. 48).

Detergents which show only slight solubilisation at neutral pH, show little variation at other pH's. With increasing solubilisation at pH 7.1 however, the trend is toward a greater degree of solubilisation at pH 10 and a markedly lower solubilisation of lamellae at acid pH.

Effect of pH variation on lamellar solubilisation

Detergent	Optical Density (EEL Colourimeter) pH 4.0*	pH 7.1ø	pH 10.0‡
Lubrol PF	5.74	6.13	6.84
Calsolene Oil	10.69	11.73	12.90
Volpo N-10	16.13	22.34	24.02
Lubrol L	22.78	28.55	31.72
Lubrol E	33.47	42.35	47.41
Vantoc AL	31.71	43.20	41.57
Renex 698	35.20	46.93	53.58
Digitonin	30.83	48.20	57.75
SDBS	28.47	48.64	50.13
G 711	31.65	56.00	52.27

* 0.04 M acetate buffer pH 4.0 employed to make up the detergent solution

ø 0.05 M phosphate buffer pH's 7.1 and 10.0 employed to make up the detergent solution.

‡ 0.05M carbonate/ bicarbonate buffer pH 10.0 employed to make up the detergent solution.

Table 9.

R. 3 Visible absorption spectra of detergent solubilised lamellae

As may be seen from figs. 14-19 , the visible absorption spectra of lamellae solubilised by the detergents studied fall into six recognisable categories as outlined below:

Category 1

This most closely resembles the absorption spectrum of chloroplast lamellae in detergent-free buffer pH 7. 1 (Fig. 2) and shows the following characteristics:

Rounded shoulder at near 384 nm.

Indistinct shoulder at near 416 nm.

Sharp peak at near 435 nm.

Sharp shoulder at near 460 nm.

Sharp peak at near 670 nm.

Category 2

Here, the main Soret band maximum is much more rounded, and the absorption at near 460 nm is accentuated in the form of a sharp peak. viz:

Indistinct shoulder at near 382 nm.

Broad peak at near 430 nm.

Sharp peak at near 479 nm.

Sharp peak at near 670 nm.

Category 3

This resembles category 1, but the shoulder at near 460 nm is greatly reduced. The following characteristics are observed:

Rounded shoulder at near 380 nm.

Indistinct shoulder at near 416 nm.

Sharp peak at near 435 nm.

Indistinct shoulder at near 460 nm.

Sharp peak at near 670 nm.

Category 4

Here, both shoulders near 416 and 460 are very indistinct viz:

No shoulder at near 380 nm.

Indistinct shoulder at near 416 nm.

Fairly sharp peak at near 435 nm.

Indistinct shoulder at near 460 nm.

Sharp peak at near 670 nm.

Category 5

In this spectrum, there is considerable loss of structure of the Soret absorption band viz:

Rounded shoulder at near 380 nm.

Rounded peak at near 418 nm.

Indistinct shoulder at near 437 nm.

Indistinct shoulder at near 469 nm.

Sharp peak at near 670 nm.

Category 6

This spectrum displays complete loss of Soret band definition with strongly rising absorption towards the near ultra violet region of the spectrum. viz:

No characterisable absorption in blue region of the spectrum.

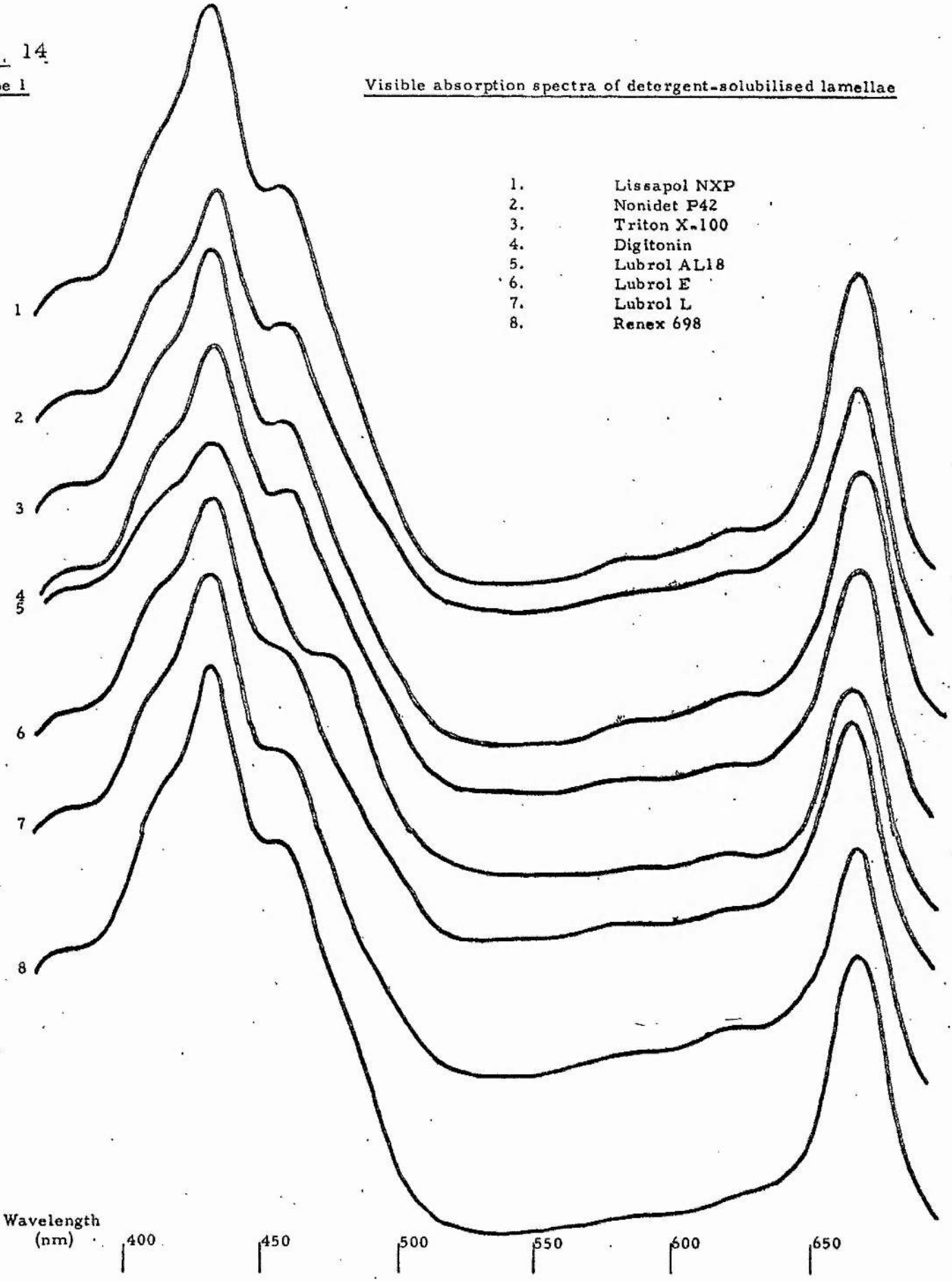
Rounded peak at near 670 nm.

Category	Examples
1.	Triton X-100, Renex 698, Lissapol NXP, Nonidet P42, Digitonin, Lubrol E, Lubrol L, Lubrol AL18.
2.	Dispersol VLX, Brif 58, Tween 20, Tween 80, Crillet 1, Crillet 4.
3.	Vantoc AL, G 711, Volpo N-10, SDBS.
4.	Tergitol 7, Cithrol A, Brij 96.
5.	Decon 75, SDS.
6.	Manoxol OT, Polychol 15.

Fig. 14

Type 1

Visible absorption spectra of detergent-solubilised lamellae



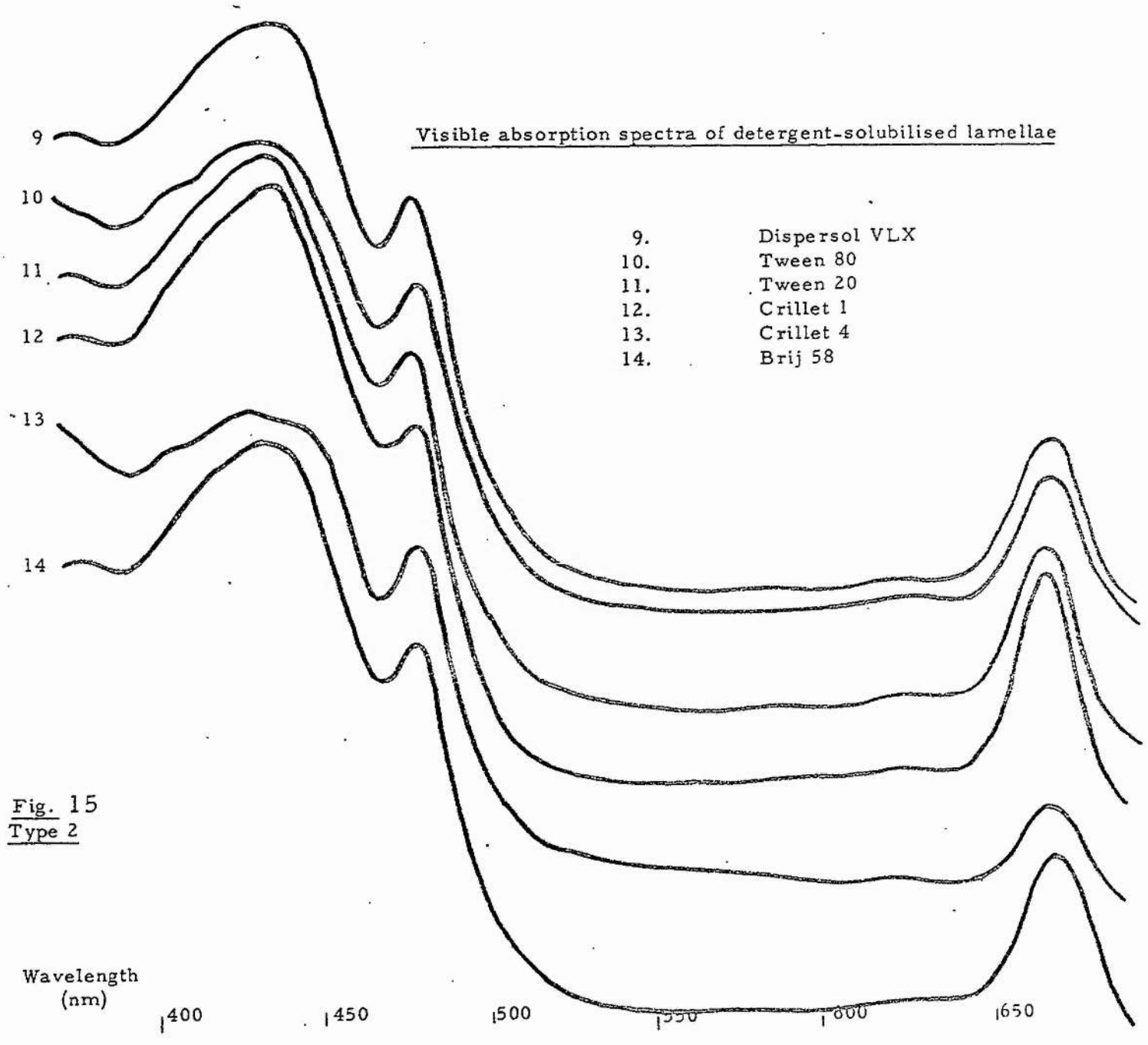

Visible absorption spectra of detergent-solubilised lamellae
9. Dispersol VLX
10. Tween 80
11. Tween 20
12. Crillet 1
13. Crillet 4
14. Brij 58
9
10
11
12
13
14
Wavelength (nm)
400
450
500
550
600
650

Fig. 15
Type 2

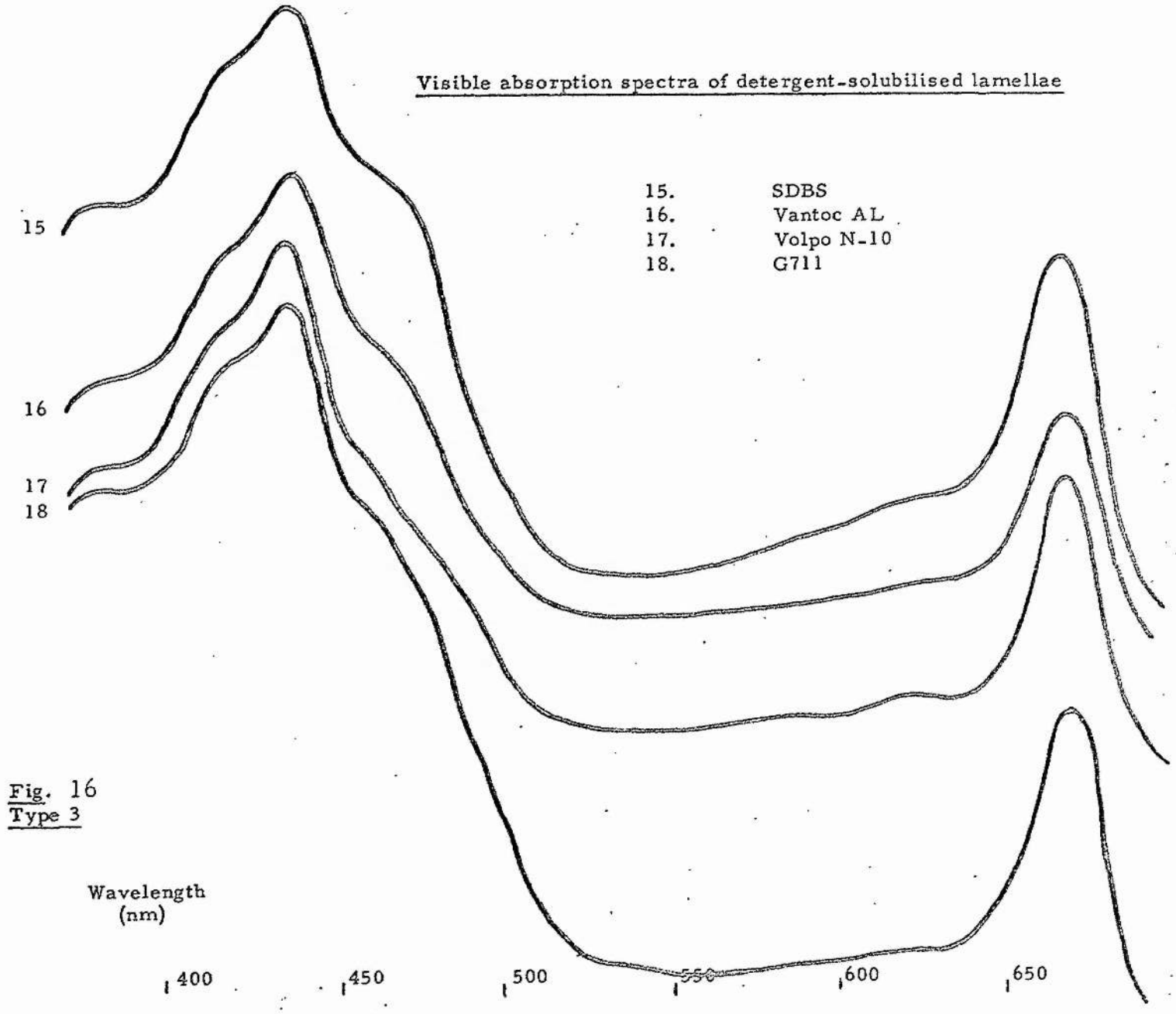

Visible absorption spectra of detergent-solubilised lamellae
15. SDBS
16. Vantoc AL
17. Volpo N-10
18. G711
15
16
17
18
Wavelength (nm)
400
450
500
550
600
650

Fig. 16
Type 3

Visible absorption spectra of detergent-solubilised lamellae

19. Tergitol 7
20. Cithrol A

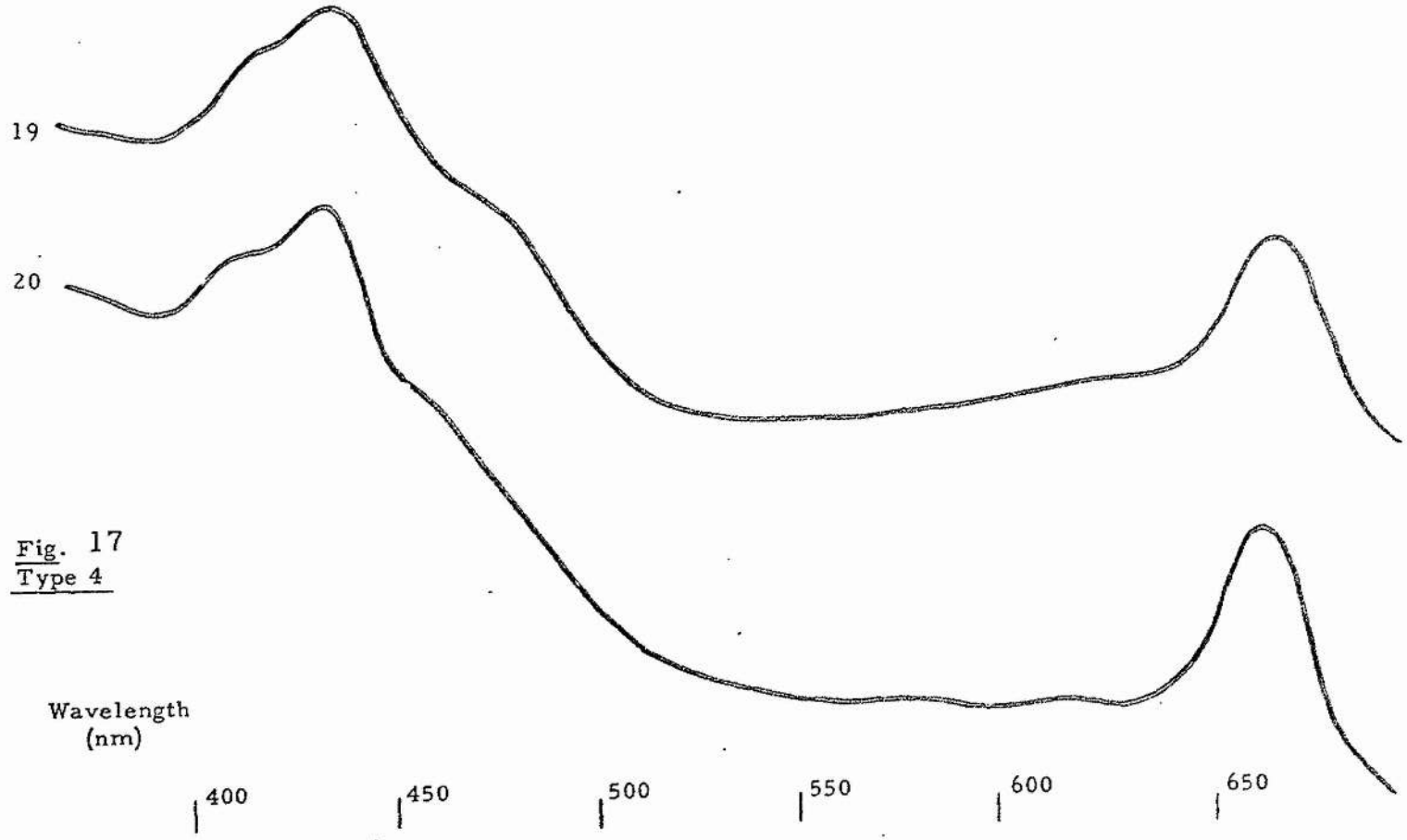


Fig. 17
Type 4

Visible absorption spectra of detergent-solubilised lamellae

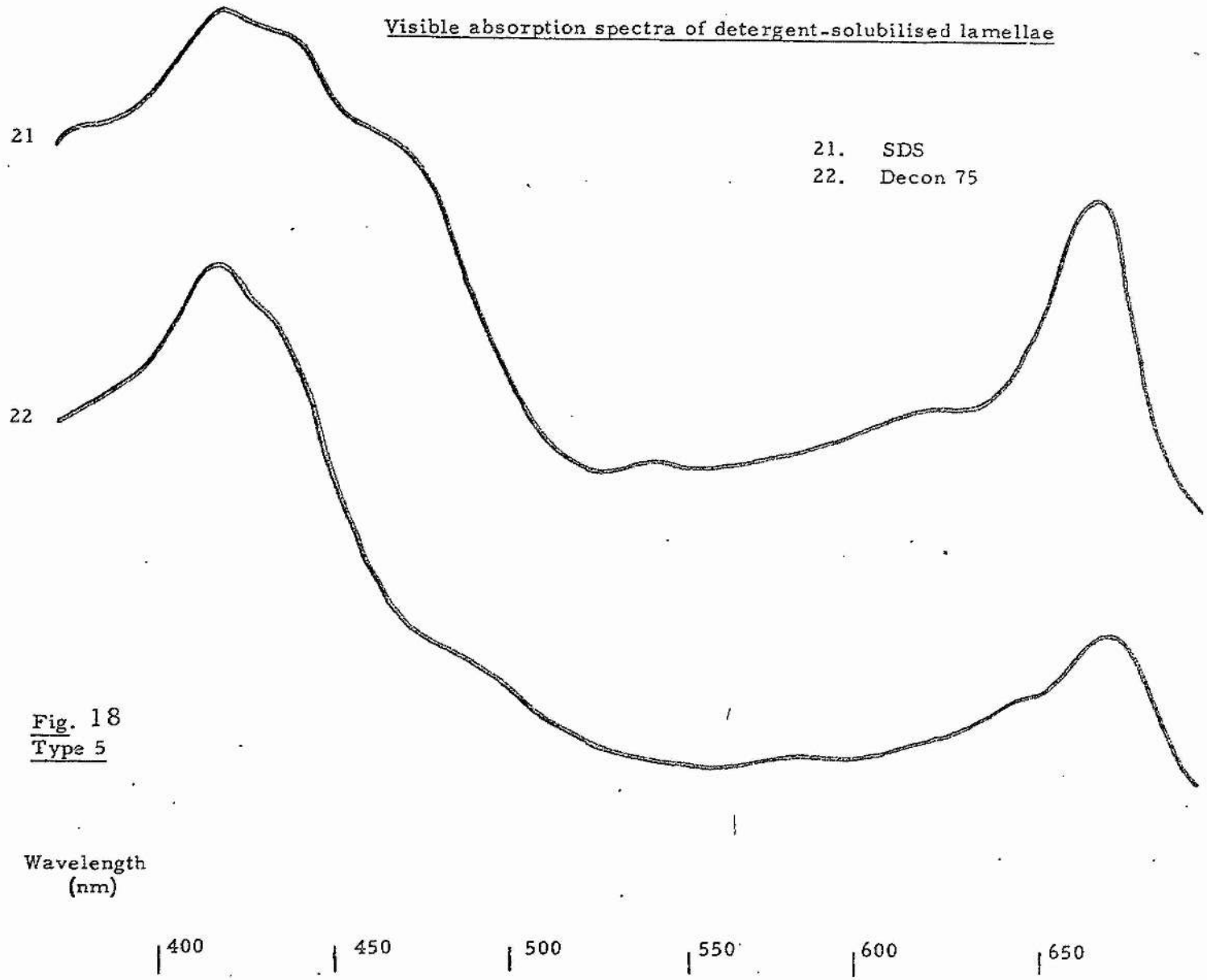


Fig. 18
Type 5

Visible absorption spectra of detergent-solubilised lamellae

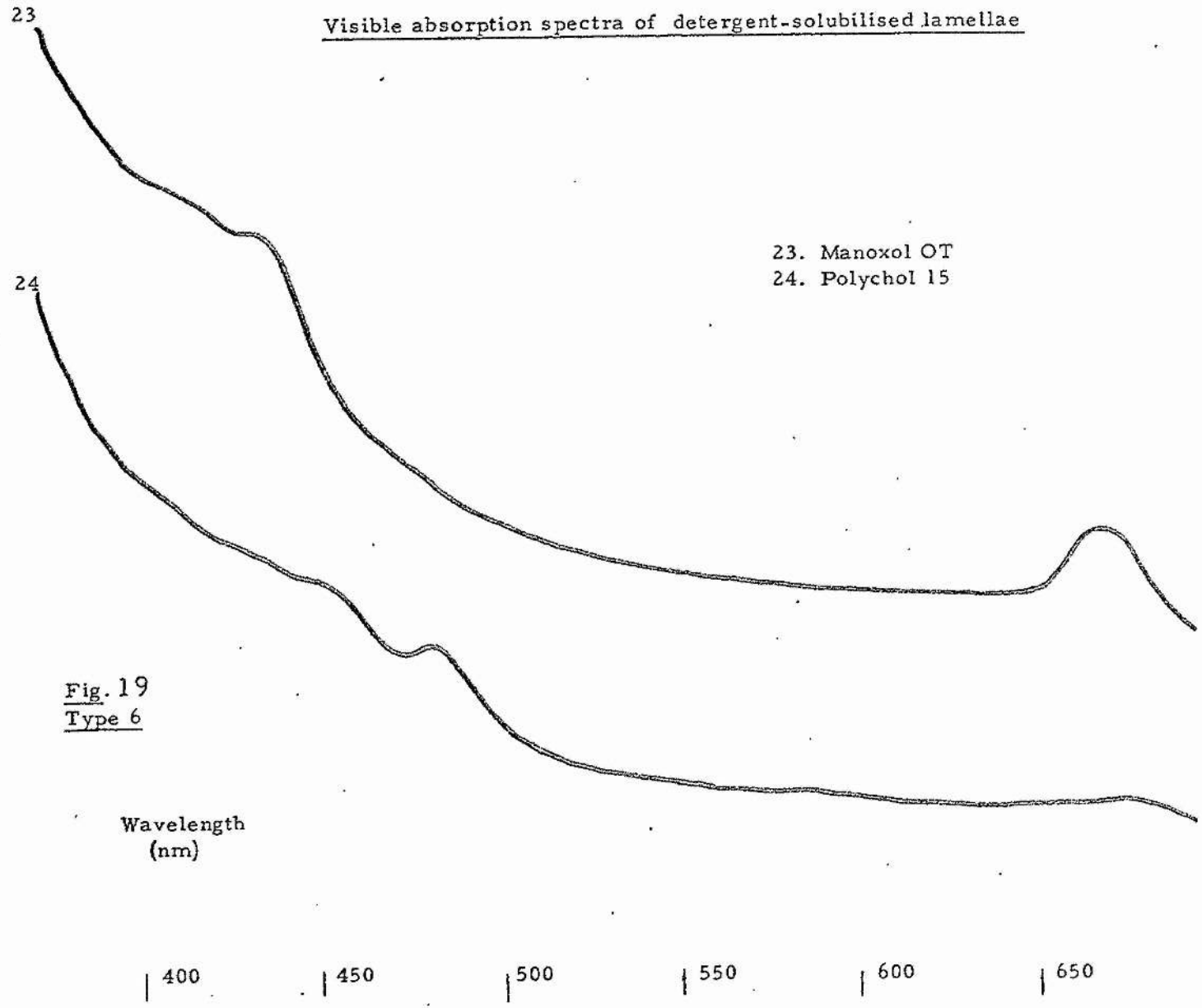


Fig. 19
Type 6

Absorption spectra of illuminated lamellar extracts from G711

KEY:
1. Unilluminated precipitated material
2. Unilluminated supernatant from 144,000 x g centrifugation
3. As 2, after 3 days illumination)
4. As 2, after 5 days illumination)

see p. 161

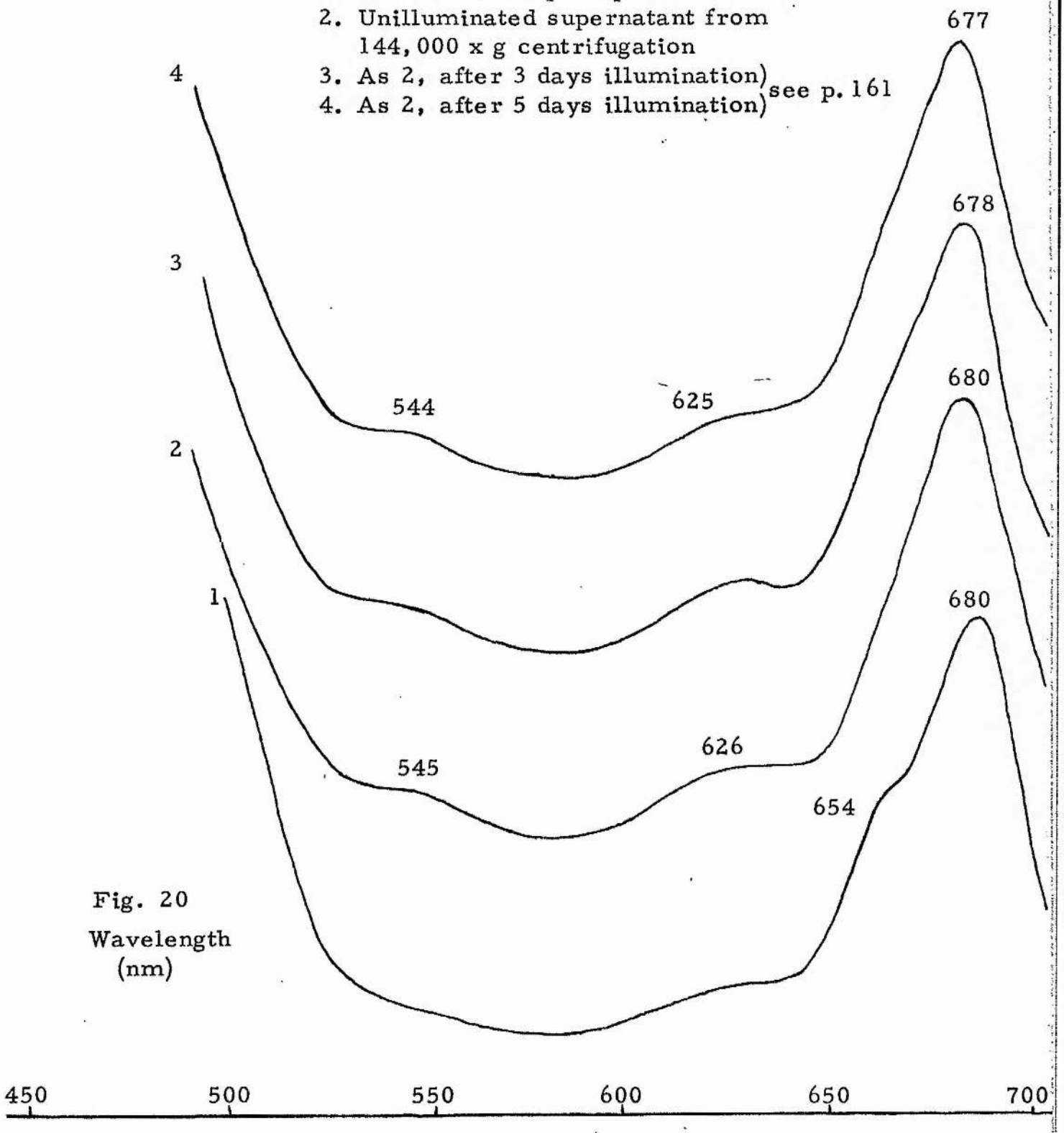


Fig. 20

Wavelength (nm)

Fluorescence Spectra of detergent solubilised lamellae

Fluorescence measurements were made on all lamellar extracts prepared according to the method detailed in Methods Section p. 48 .

The 144,000 x g supernatant of lamellae extracted using a chlorophyll:detergent ratio of 1:250 were observed on the Fluorispec SF 1 employing an excitation wavelength of 345 nm.

Table 10 shows which detergent extracts exhibited fluorescence and the λ maximum of fluorescent emission.

Detergent blanks were also checked for fluorescence employing an excitation wavelength of 345nm. All the detergents listed in table 10 were tested, using 1% aqueous solutions buffered in 0.05M phosphate at pH 7.0. None displayed any fluorescence.

Fluorescence measurements of detergent extracts

Solubilising Detergent	Red fluorescence measured	Red λ maximum	Blue fluorescence measured	Blue λ maximum
Brij 35	-	-	-	
Brij 58	+	683	-	
Brij 96	+	684	-	
Brij 98	-	-	-	
Calsolene Oil	+	686	-	
Cithrol A	+	685	+	465
Crillet 1	-	-	-	
Crillet 4	-	-	-	
Decon 75	-	-	-	
Digitonin	++	686	-	
Dispersol VLX	+	684	-	
G 711	+	686	+	457
Lissapol LS powder	+	684	-	
Lissapol NXP	++	681	+	392
Lubrol AL 18	+	684	-	
Lubrol E	++	686	+	470
Lubrol L	++	686	+	462
Lubrol PF	+	685	-	
Manoxol OT	-	-	-	
Nonidet P42	++	687	+	400, 460

cont'd...

Fluorescence measurements of detergent extracts (cont'd..)

Solubilising Detergent	Red fluorescence measured	Red λ maximum	Blue fluorescence measured	Blue λ maximum
Polychol 15	-	-	-	
Renex 698	+	682	-	
SDBS	-	-	-	
SDS	-	-	-	
Tergitol 7	+	685	+	486
Tergitol Anionic 08	+	684	-	
Triton X-100	+	686	-	
Tween 20	-	-	-	
Tween 80	-	-	-	
Vantoc AL	+	685	+	441
Volpo N-10	+	684	-	

KEY:

-	No fluorescence
+	Very slight fluorescence
++	Slight fluorescence

Table 10

R.4 Trace element analysis of detergent treated lamellae

The trace element analyses carried out by British Titan Products Co. Ltd., using polarographic and colorimetric techniques on differentially centrifuged detergent-treated lamellae are detailed in table 11.

For comparison, the results of similarly centrifuged digitonin lamellar extracts produced by Anderson et. al. (1964) and analysed by atomic absorptiometry are given in table 12. It will be noted that the results of Anderson et. al. bear no resemblance to those of the present work, though it should be observed that a review of the literature indicates that atomic absorptiometry is a less sensitive analytical technique than colorimetry or polarography; the working limits (in parts per million) for the determination of iron, copper and manganese being as follows:

	Fe	Cu	Mn
Atomic absorptiometry	2.0	2.0	2.0
Colorimetry	0.05	0.05	0.50
Polarography	0.001	0.02	0.10

(Whitehead, 1969)

Estimation of manganese only, was conducted by the method of Bradfield (1956) (see Methods Section p.53) on differentially centrifuged lamellar extracts produced by treatment of lamellae with nineteen different detergents. All extracts except three (those produced from Cithrol A, Lissapol NXP and Tergitol Anionic 08) displayed a higher manganese content in the heavier fraction though in the majority of cases the difference is only slight. Four detergents however, (Digitonin, G711, Renex 698 and Tween 20) produced lamellar extracts which on differential centrifugation exhibited a distinct fractionation in terms of relative manganese content. In these cases, the manganese content of the heavy (10,000 x g) fraction was at least twice that of the material sedimented at 144,00 x g. (Table 13).

The smallest particles sedimented from G711 treated lamallae contained only 15.75% as much manganese as was found in the heavy fraction (relative to the chlorophyll content).

Further experiments on differentially centrifuged G711 lamellar extracts were conducted employing different detergent concentrations, and the results are given in table 14 , from

which it may be seen that detergent concentrations in excess of a 50:1 G711:chlorophyll ratio are required to give an appreciable difference in manganese distribution between the heavy and light precipitated fractions (Fig. 21).

Manganese estimations were also conducted on the chlorophyll-protein fractions eluted from DEAE cellulose columns using lamellar preparations from Brij 96, Triton X-100 and Renex 698 (see pp. 67 - 69, and figs. 37, 32, 33). The distribution of manganese (in ug/mg magnesium) in these fractions is given in Table 15. No distinct differences between the fractions is observable.

Trace element content (in ug/mg magnesium) of the 144,000 x g and 10,000 x g precipitated fractions produced from broad bean chloroplast lamellae fragmented by various detergents

Results are average of two experiments conducted polorographically and colorimetrically by British Titan Products Co. Ltd.

(See Methods section p. 51)

Detergent	144,000 x g			10,000 x g		
	Cu	Fe	Mn	Cu	Fe	Mn
Triton X-100	9.16	16.36	5.50	3.47	14.85	3.09
G 711	5.96	16.60	3.94	1.12	2.86	1.78
Renex 698	2.13	6.01	1.94	2.68	4.08	2.44
SDBS	6.17	10.30	3.78	11.07	24.47	6.49
Brij 96	5.15	4.12	1.03	6.78	14.45	1.02
Digitonin	10.14	2.45	0.35	1.004	0.262	0.212

Table 11

Manganese content (in μg/mg magnesium) of the 144,000 x g and 10,000 x g precipitated fractions produced from broad bean chloroplast lamellae fragmented by various detergents

(Results are average of three experiments conducted according to the method of Bradfield (1956); see Methods Section, p. 53)

Detergent	Manganese Content	
	144,000 x g	10,000 x g
Brij 35	0.74	1.08
Brij 96	1.68	1.79
Cetavlon	1.15	1.92
Cithrol A	1.61	1.34
Crillet 4	1.93	2.78
Digitonin	2.21	4.66
G 711	0.40	2.54
Lissapol LS Powder	2.76	3.10
Lissapol NXP	1.67	0.98
Lubrol E	1.85	2.72
Lubrol L	1.70	2.37
Nonidet P42	1.23	1.28
Renex 698	1.55	3.43
SDBS	2.03	2.61
Tergitol 7	1.89	2.58
Tergitol Anionic 08	1.42	1.05
Triton X-100	1.68	1.95
Tween 20	1.12	2.46
Vantoc AL	2.38	2.41

(In all the above experiments, the detergent:chlorophyll ratio employed in lamellar extraction was 250:1)

Table 13

Trace element content (in μg/mg magnesium) of the 144,000 x g and 10,000 x g precipitated fractions produced from spinach chloroplast lamellae fragmented in 0.5% digitonin by Anderson et. al. (1964).

Estimations carried out by atomic absorptiometry.

Untreated Chloroplasts			144,000 x g			10,000 x g		
Cu	Fe	Mn	Cu	Fe	Mn	Cu	Fe	Mn
18.8	41.6	13.7	16.2	34.1	4.1	10.0	21.1	19.3

Table 12

Manganese content (in μg/mg magnesium) of differentially centrifuged lamellar extracts produced by G 711

Detergent: Chlorophyll ratio employed in lamellar extraction	Fraction				
	Whole lamellae	10,000 x g	50,000 x g	144,000 x g	super-natant
250:1	2.15	2.54	1.42	0.40	0.74
100:1	1.93	2.73	1.03	0.49	0.51
50:1	1.86	2.38	1.21	0.53	0.91
10:1	2.27	2.06	1.46	1.12	0.58

(Results are average of three estimations conducted according to the method of Bradfield; 1956).

Table 14

Effect of G711 concentration upon distribution of manganese between light and heavy precipitated fractions from chloroplast lamellae

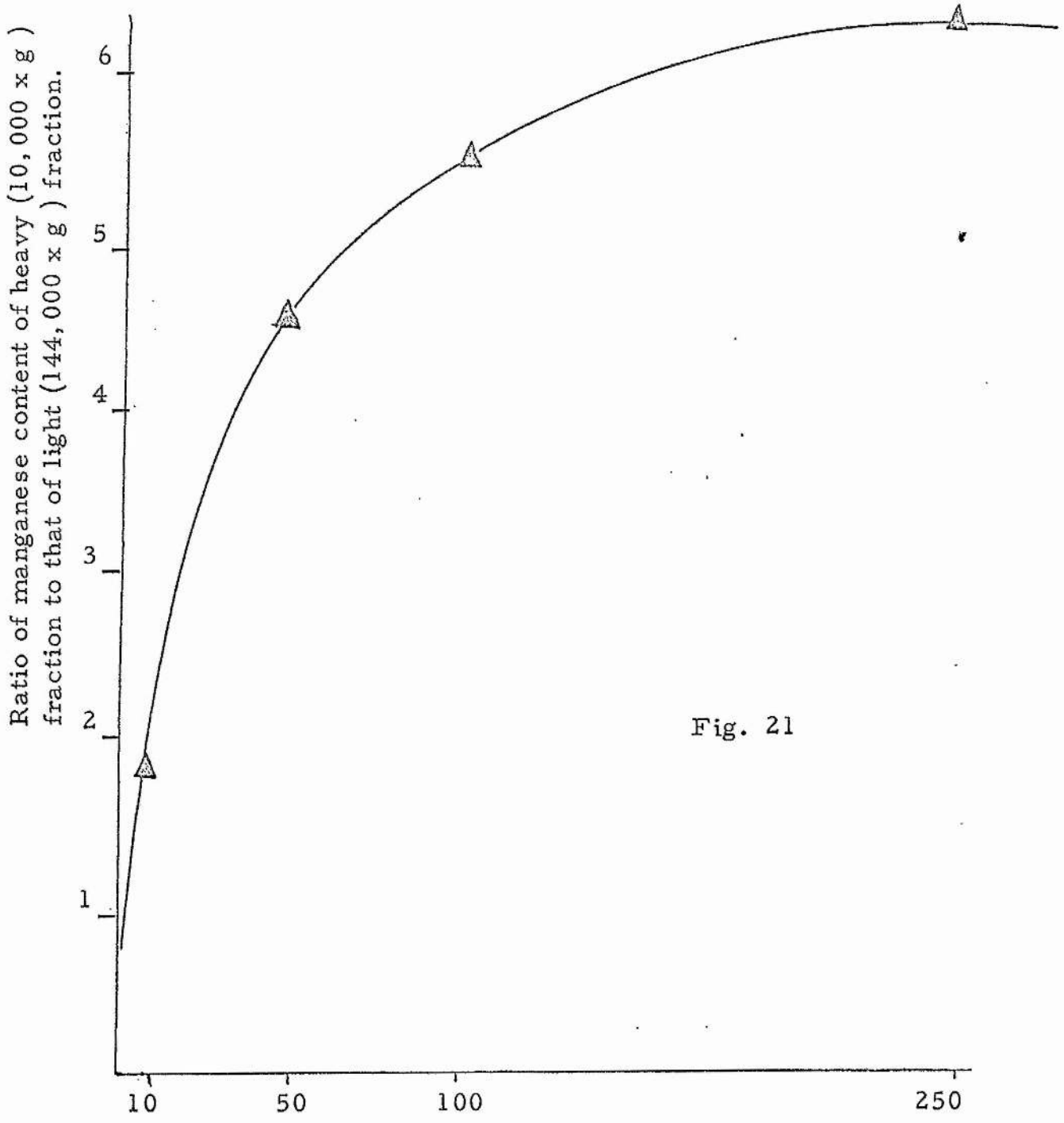


Fig. 21

Manganese distribution in chlorophyll-protein fractions separated by DEAE cellulose column chromatography

Detergent	Eluted fraction numbers*	Manganese content, in μg/mg chlorophyll
Brij 96	18 - 30	0.26
	35 - 50	0.38
Triton X-100	15 - 25	0.47
	30 - 45	0.42
Renex 698	18 - 25	0.34
	35 - 45	0.52

* See figs. 32, 33, 37.

Table 15

Standard Graph for manganese analysis
(Method of Bradfield 1956; see Methods Section, p.53)

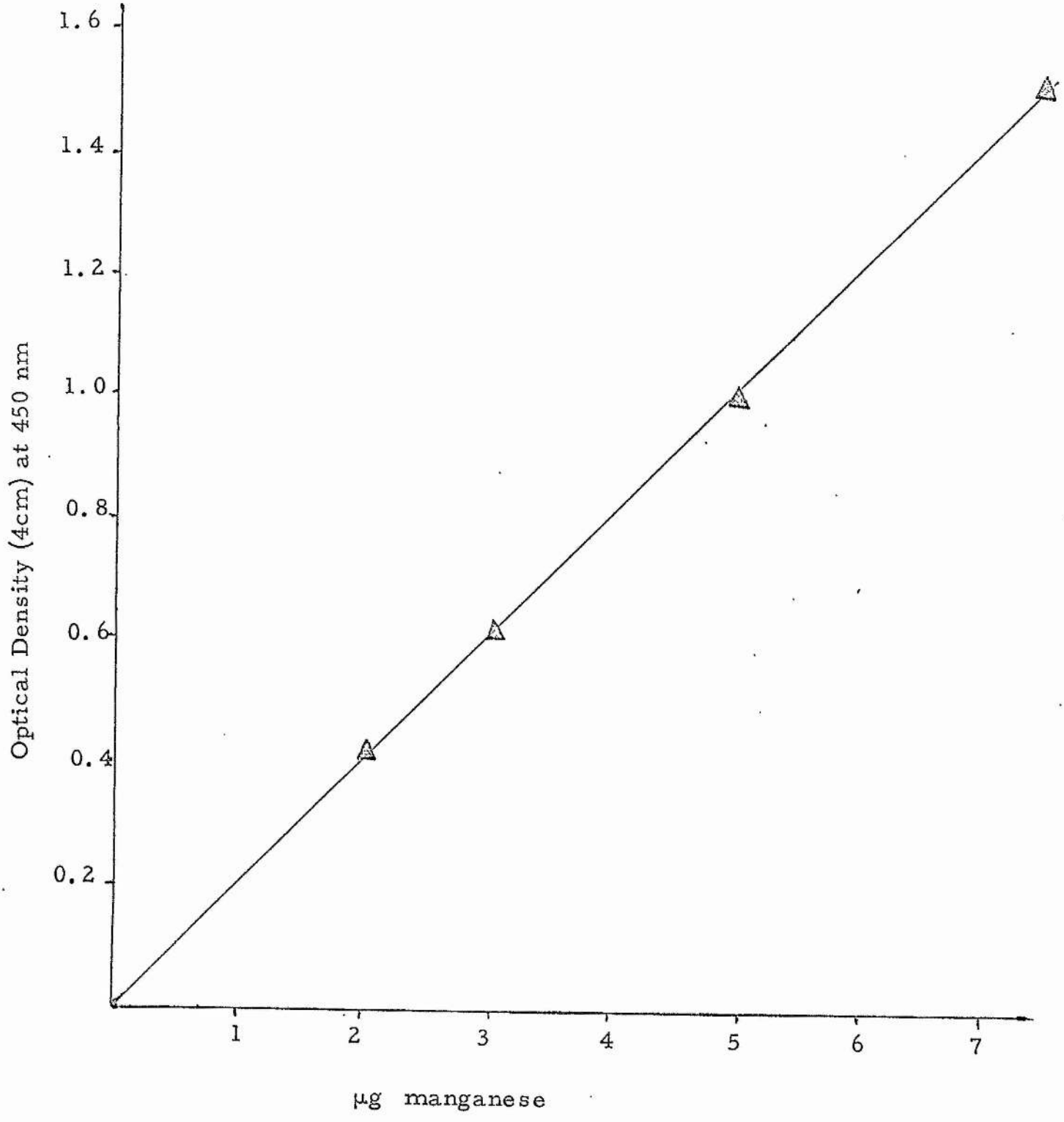


Fig. 22

R.5 Benzene extractability of chlorophyll in aqueous chloroplast dispersions containing detergents

The benzene extractability of chlorophyll in detergent-containing aqueous lamellar dispersions, is detailed in figs. 23, 24.

The higher pH was found to induce more facile extraction of the chlorophyll by the benzene, particularly in the cases of Lissapol NXP (fig. 24), SDBS and Crillet 4 (fig. 23).

The steroidal detergent digitonin and the cationic detergents Cetavlon and Vantoc AL were found to have a sufficiently high affinity for the freed chloroplast pigments to largely prevent pigment removal from the aqueous phase by the benzene (fig. 24), which is in contradiction with the generalisation put forward by Ke et. al. (1956) that cationic detergents do not have sufficient affinity for the chloroplast pigments to prevent their removal from the aqueous phase by benzene.

The results obtained by Ke. et.al. (1956) for digitonin are confirmed and the results obtained in this work for the detergent Crillet 4, which resembles Tween 20 are similar to those obtained by Ke for Tween 20.

Effect of pH upon the benzene extractability of chlorophyll in aqueous chloroplast dispersions containing SDBS and Crillet 4

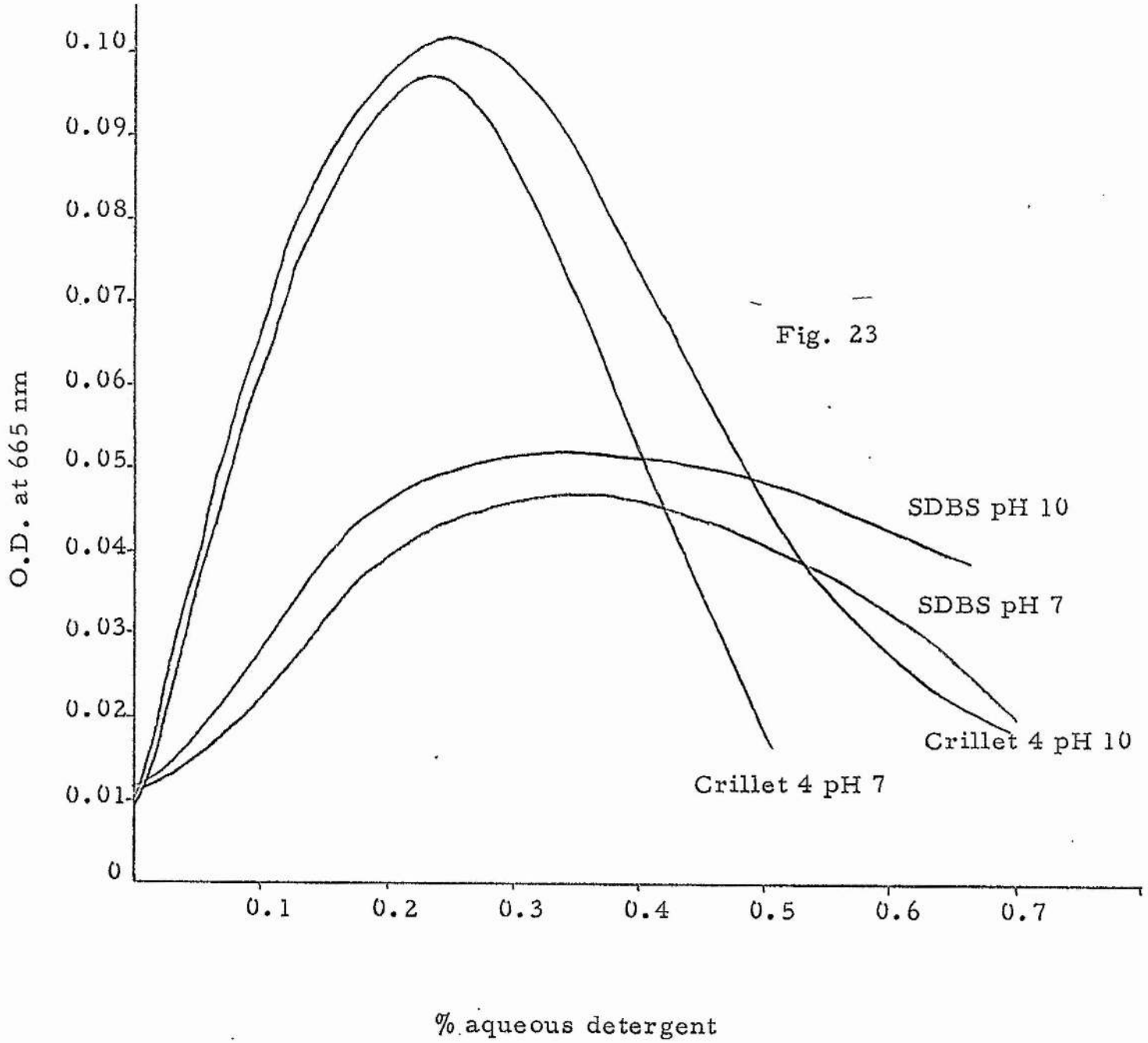


Fig. 23

Benzene extractability of chlorophyll in aqueous chloroplast dispersions containing Triton X-100, Lissapol NXP, Vantoc AL, Cetavlon and Digitonin

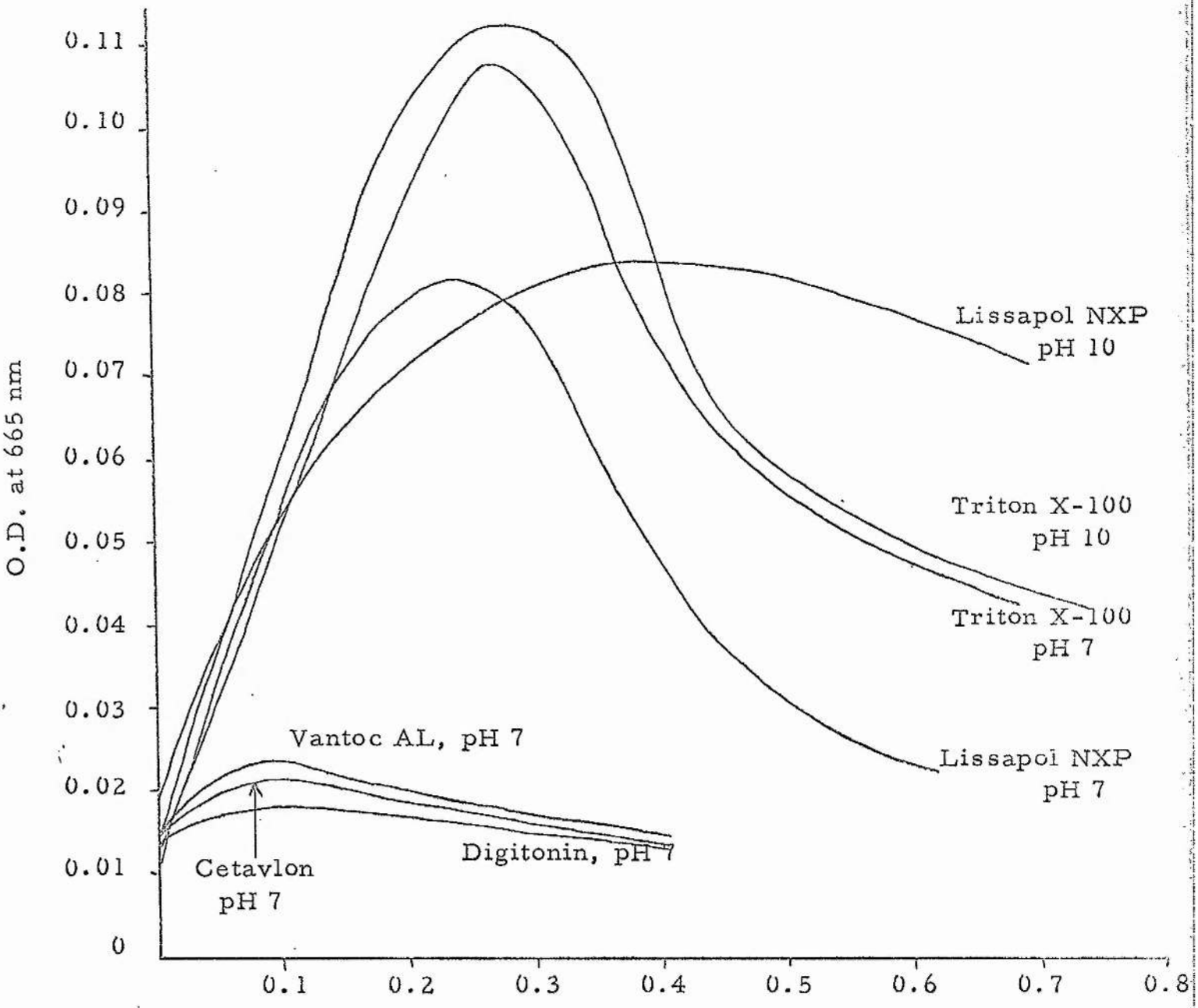


Fig. 24

R.6 Estimation of the effect of detergents on System 11 and System 1 activity of lamellar fragments

Following the method detailed in Methods section p.62 , the effect of detergent addition on the photoevolution of oxygen by isolated lamellae was investigated.

With the exception of G711, Tergitol Anionic 08 and Polychol 15 which will be discussed separately, all the detergents studied were found to abolish oxygen photoevolution at a final surfactant concentration of 0.05% or less.

Excluding the exceptions noted above, it is possible (fig.25.) to distinguish four groups of detergent in terms of their effect on oxygen photoevolution:

Group 1

Detergents belonging to this group had an immediate and rapid inhibitory effect on the photoevolution of oxygen, total inhibition being manifested at a final detergent concentration of 0.006 or less. (Table 16).

Group 2

Though requiring a greater final detergent concentration (up to 0.022%) to abolish oxygen photoevolution, the surfactants

belonging to this group displayed a similar action profile to the detergents of group 1 (Table 16).

None of the detergents of either groups 1 or 2 displayed any stimulatory effect on System II activity at any concentration.

Group 3

Detergents of this type (fig.26) displayed an initial and very slight stimulation in the photoevolution of oxygen up to a final detergent concentration of 0.009% followed by a gradual inhibitory effect which was complete at a final concentration not exceeding 0.028%.

Group 4

The most characteristic feature of the effects of this group of detergents on the rate of oxygen photoevolution was found to be a marked initial stimulation of up to 43%, followed (at final detergent concentrations of above 0.01%) by a strong inhibitory effect which became more gradual with increase in detergent concentration but was complete at final concentrations above 0.05%. (Table 17).

Other Detergents

G 711

This detergent was found to be of particular note, in stimulating oxygen photoevolution at low concentrations (0.015%) but exerting only a very gradual inhibitory effect with a further increase in detergent concentrations.

At a final G 711 concentration of as much as 0.06%, the initial rate of photoevolution of oxygen was reduced by only 50%. (fig. 27).

Tergitol Anionic 08

Although this detergent exhibited no stimulatory effect on System II activity of isolated lamellae, its degree of inhibition of oxygen photoevolution was found to be notably slight with increase in concentration of detergent. At a final Tergitol Anionic 08 concentration of 0.2%, the rate of oxygen photoevolution was depressed by only 10.5% (fig. 28).

Polychol 15

An apparently massive stimulation of oxygen evolution at all detergent concentrations was observed for lamellae treated with Polychol 15. It has since been learned that this

detergent contains peroxides which account for oxygen evolution and mask any effect of the detergent on System II activity of lamellae.

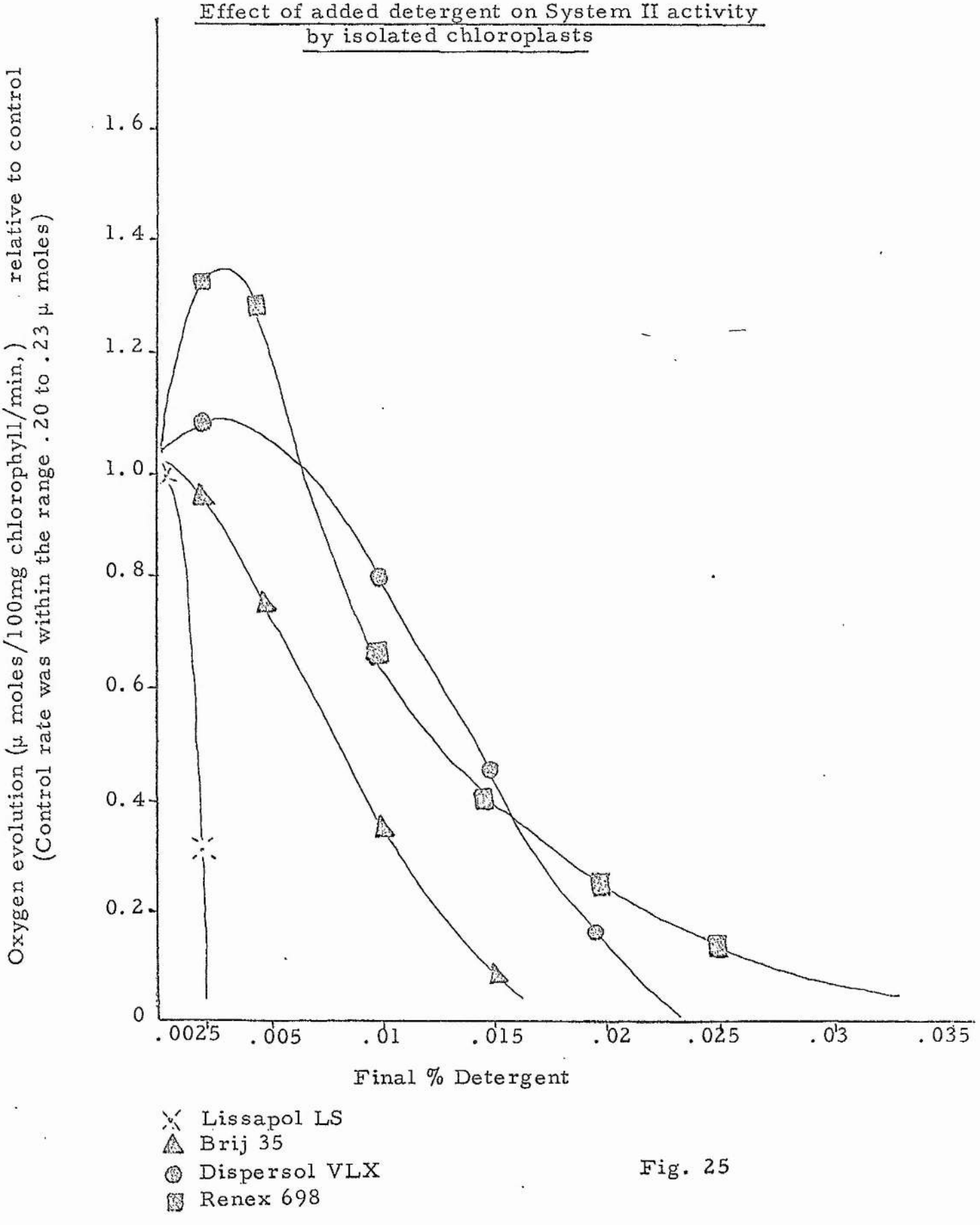

Effect of added detergent on System II activity
by isolated chloroplasts
Oxygen evolution (μ moles/100mg chlorophyll/min,) relative to control
(Control rate was within the range .20 to .23 μ moles)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
.0025
.005
.01
.015
.02
.025
.03
.035
Final % Detergent
Lissapol LS
Brij 35
Dispersol VLX
Renex 698

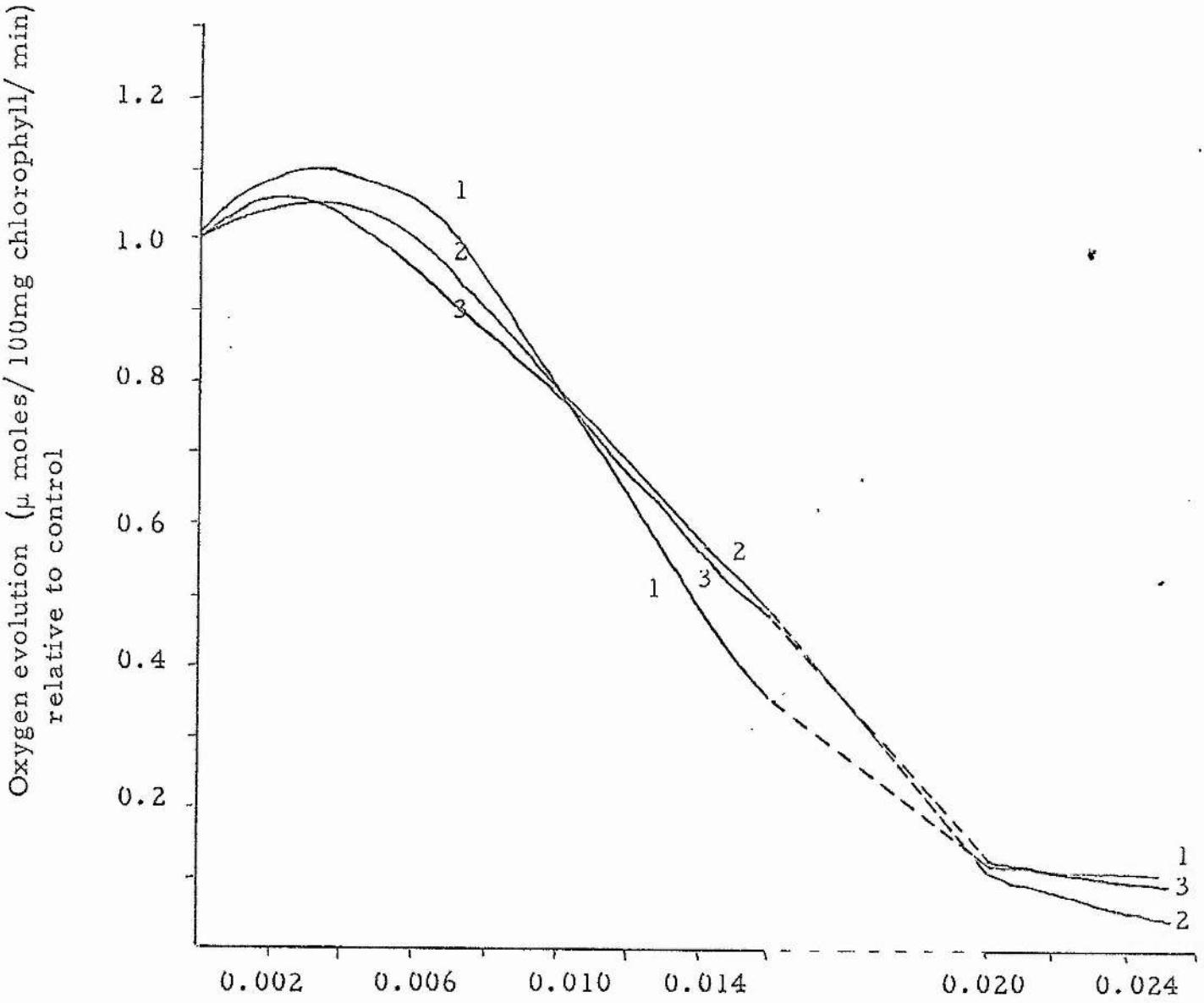
Fig. 25

Detergent concentrations (final) required to inhibit System II activity of isolated chloroplasts by 50%

Detergent	Group	Concentration (%)
Brij 96	1	0.0050
Brij 98	1	0.0056
Calsolene Oil	1	0.0039
Cetavlon	1	0.0027
Cithrol A	1	0.0047
Decon 75	1	0.0028
Lissapol LS Powder	1	0.0021
SDS	1	0.0036
SDBS	1	0.0030
Vantoc AL	1	0.0025
Volpo N-10	1	0.0036
Brij 35	2	0.0086
Brij 58	2	0.0082
Crillet 1	2	0.0103
Crillet 4	2	0.0095
Lubrol AL18	2	0.0080
Lubrol PF	2	0.0071
Tergitol 7	2	0.0076
Tween 20	2	0.0097
Tween 80	2	0.0090

Table 16

Effect of addition of Digitonin Lubrol L, and Dispersol VLX upon System II activity by isolated chloroplasts (Group 3 detergents)



1 : Digitonin

2 : Lubrol L

3 : Dispersol VLX

Fig. 26

Effect of detergent addition (Group 4 detergents) on System II activity by isolated chloroplasts

	Oxygen evolution relative to control (see fig.25)	Final concentration (%) of detergent
Renex 698	1.34	0.0025
	1.29	0.005
	0.65	0.01
	0.41	0.015
	0.28	0.02
	0.18	0.025
Lubrol E	1.26	0.002
	1.20	0.004
	1.05	0.006
	0.71	0.01
	0.45	0.016
	0.27	0.022
Nonidet P42	1.15	0.001
	1.38	0.002
	1.25	0.004
	1.00	0.006
	0.47	0.01
	0.32	0.015
	0.24	0.02
	0.09	0.025
Triton X-100	1.11	0.001
	1.40	0.0025
	1.18	0.005
	0.76	0.01
	0.48	0.015
	0.28	0.02
	0.17	0.025
Lissapol NXP	1.24	0.001
	1.29	0.002
	1.06	0.004
	0.85	0.006
	0.43	0.01
	0.30	0.015
	0.11	0.02

Table 17

Effect of Tergitol Anionic 08 addition upon System II activity of isolated chloroplasts

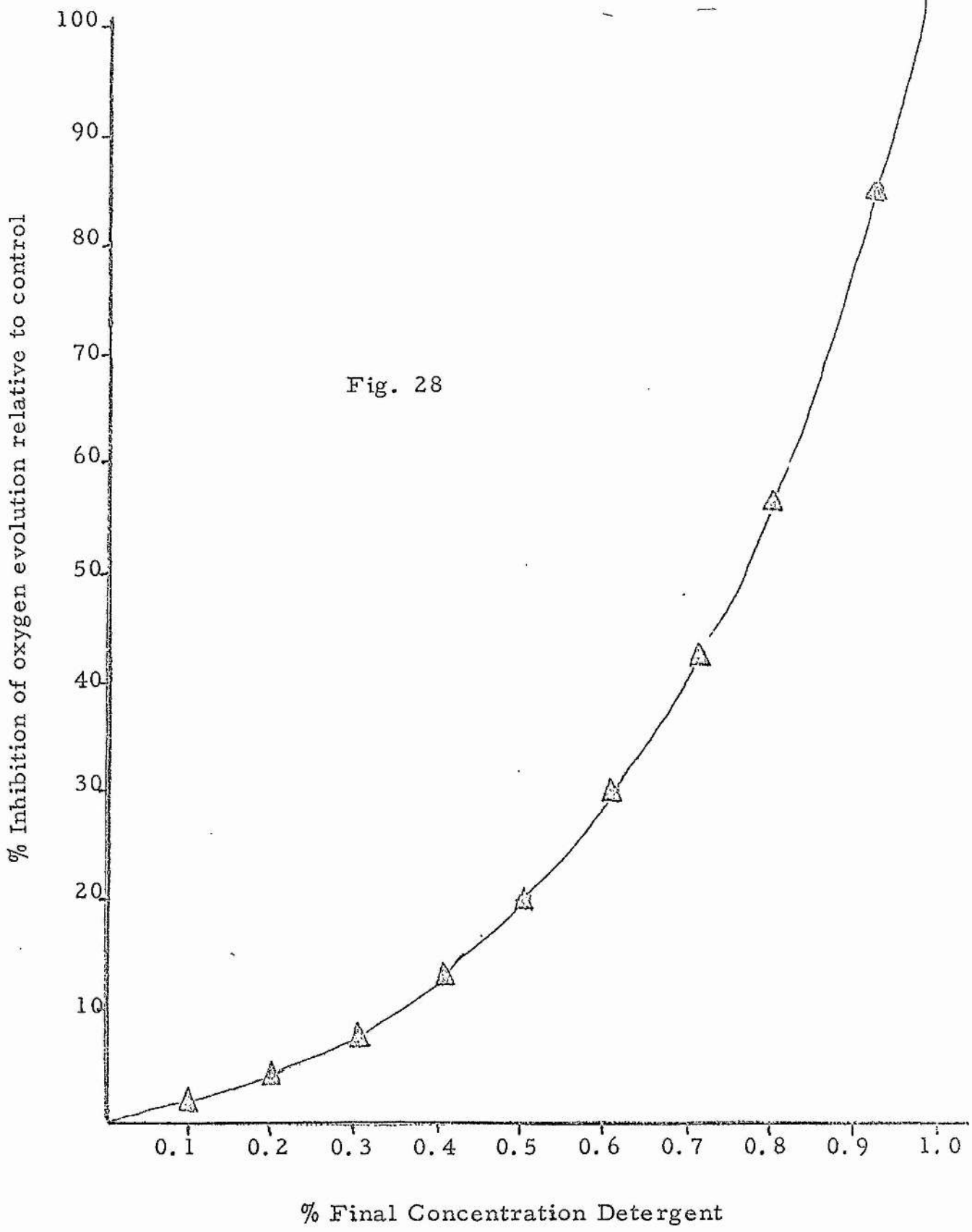


Fig. 28

Estimation of the effect of detergents on System I activity of lamellar fragments

Following the methods detailed in Methods section, pp. 57-61 (figs. 5,6) the effect of detergent addition upon the photoreduction of NADP by isolated lamellae was investigated. The detergents studied were found to be less variable in their effect on System I activity (with DCPIP/ascorbate as electron donor) than in their effect on System II activity. Thus whereas four general groups of detergent, plus some additional exceptional cases were recognisable in terms of system II activity, the detergents studied were of two main groups as regards their effect on NADP photoreduction.

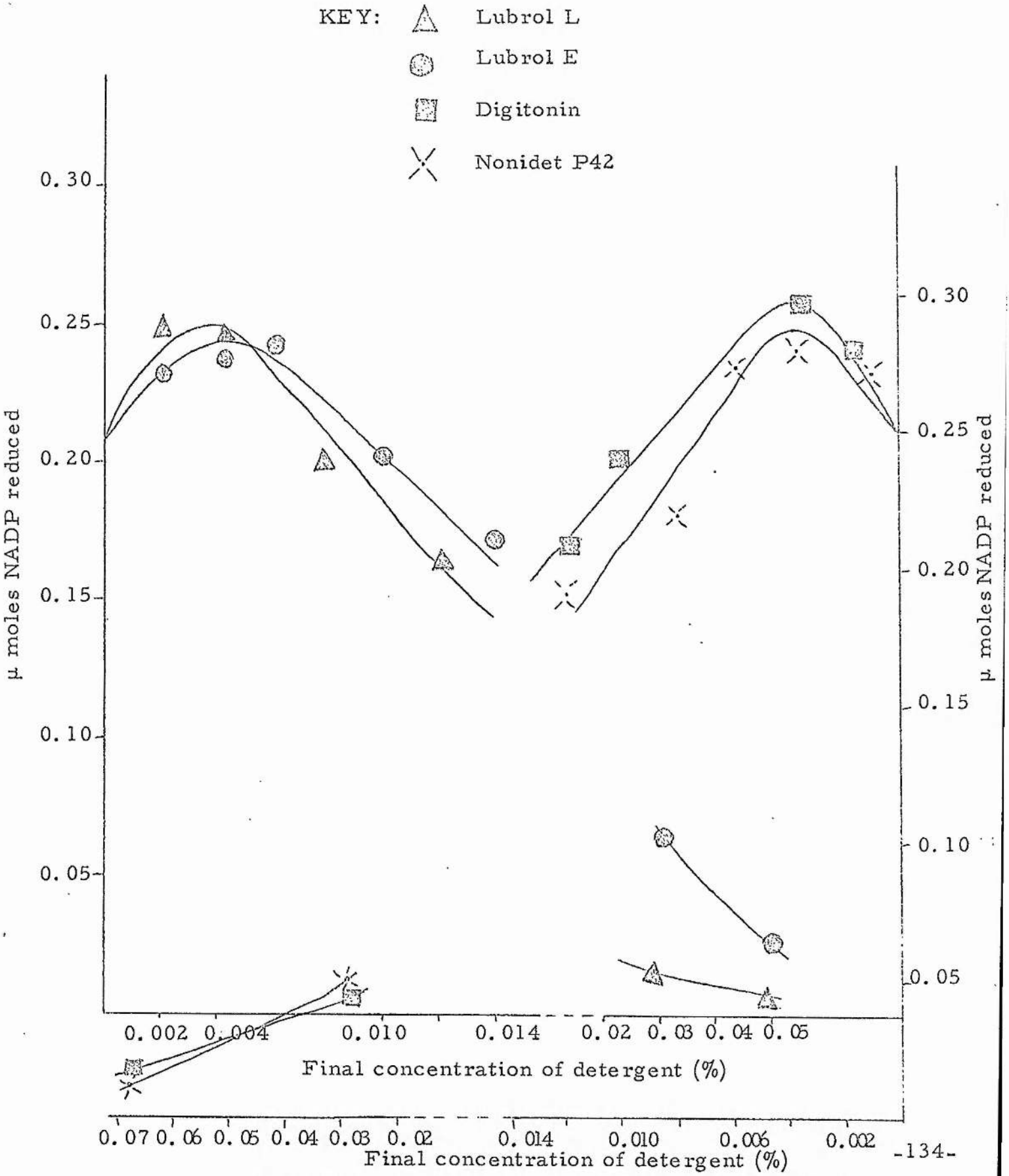
1. As shown in figs. 29,30, the detergents digitonin, Lubrol E, Lubrol, Lissapol NXP, Nonidet P42, Renex 698 and Triton X-100, all exhibited an initial stimulation of NADP photoreduction at final detergent concentrations of 0.005 to 0.010%, followed by a gradual inhibition of NADP photoreduction as the final concentration of detergent is increased.

It will be noted that most of these detergents are polyoxyethylene alkyl phenol condensates.

2. As shown in fig. 31 , the other detergents observed (e.g. Brij 58, Brij 96, Cithrol A, Calsolene Oil, G 711, SDBS, SDS, Tergitol-7, Tergitol Anionic 08, Tween 20, Tween 80, and Vantoc AL) induced an immediate depression of the rate of NADP photoreduction and completely abolished photoreductive activity at concentrations appreciably less than required in the first group.

Fig. 29

Effect of addition of the detergents digitonin, Lubrol E, Lubrol PF and Nonidet P42 upon System I activity of isolated chloroplasts



Effect of addition of the detergents Triton X-100, Lissapol NXP and Renex 698 upon System I activity of isolated chloroplasts

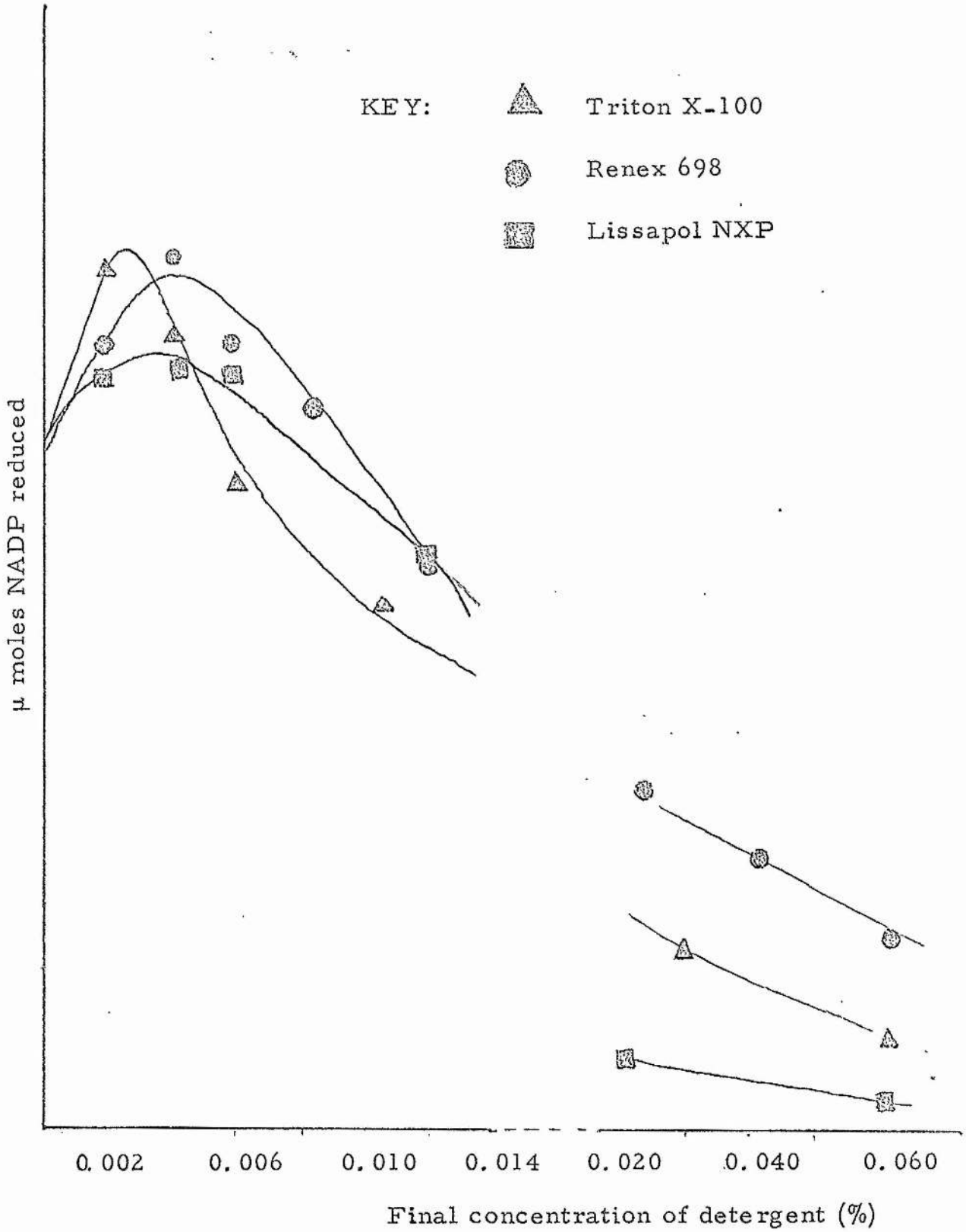


Fig. 30

Effect of addition of the detergents Vantoc AL, G711 and Tween 80 upon System I activity of isolated chloroplasts

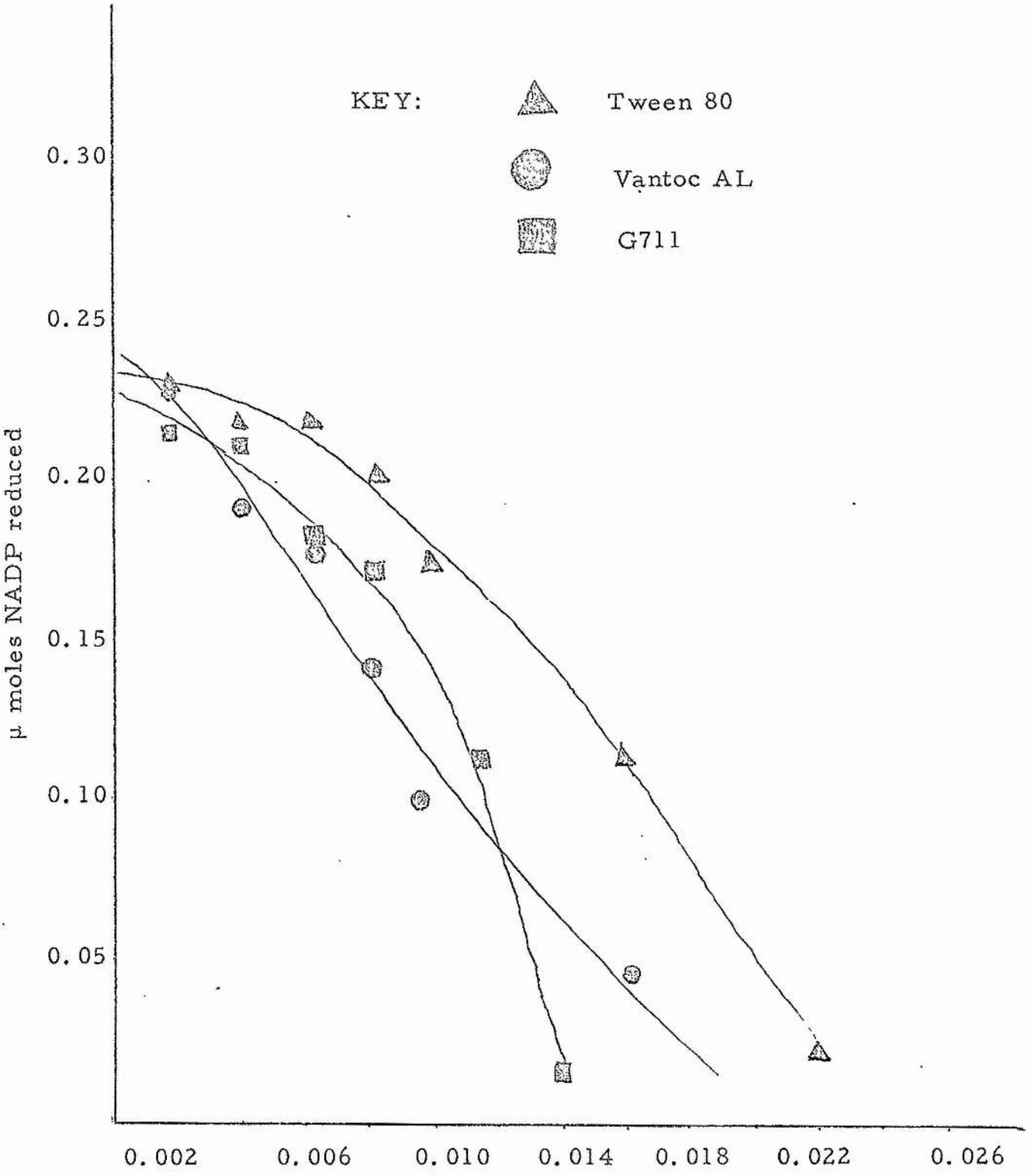


Fig.31

R.7 Preparative anion exchange column chromatography

Following the method based on the work of Kahn (1964) detailed in Methods section, p. 67 , the seven detergents studied showed considerable variation in the elution pattern of solubilised lamellae from the DEAE cellulose column, as is shown in figs.32, 38.

Protein and Chlorophyll profiles:

1. Triton X-100 (Fig. 32)

Chromatographic separation of two complexes was observed but the results of this investigation differ from those of Kahn in showing no elution of a protein rich sample or definable chlorophyll-protein in the later fractions.

2. Renex 698 (Fig. 33)

This detergent also gave an elution pattern showing separation of two approximately coincidental chlorophyll-protein moieties. Again, no prominent elution of chlorophyll or protein appeared in the later fractions. In this case, the relative amount of protein to that of chlorophyll, in the first complex is appreciably higher than in complex 1 from Triton X-100 preparation.

3. Lubrol E (Fig. 34)

4. Lissapol NXP (Fig. 35)

Similar elution patterns were produced by both these detergents, with an initially high concentration of chlorophyll relative to protein. Only a single sharply defined green band was observed, which at no stage became divided. The later fractions contained very little chlorophyll but appreciable quantities of protein.

5. G 711 (Fig. 36)

The elution diagram for this detergent showed a broad chlorophyll peak containing only small amounts of protein. The protein concentration of fractions was found to increase steadily as the column was eluted, the curve displaying no minimum at any stage.

6. Brij 96 (Fig. 37)

Lamellar preparations from this detergent displayed a double green band on the column, both parts of which were distinctly separate though the slower moving one appeared to be considerably more diffuse than the first eluted band. The leading edge of the faster moving band contained no protein, but the subsequent fractions contained greater amounts of protein than chlorophyll.

7. Calsolene Oil (Fig. 38)

The profile of protein content in the eluted fractions for this detergent resembles that of Lissapol NXP and Lubrol E, whereas the profile of chlorophyll content is similar to that of Triton X-100. The faster moving band appears to represent a distinct chlorophyll-protein complex since the chlorophyll and protein curves are of similar shape and position.

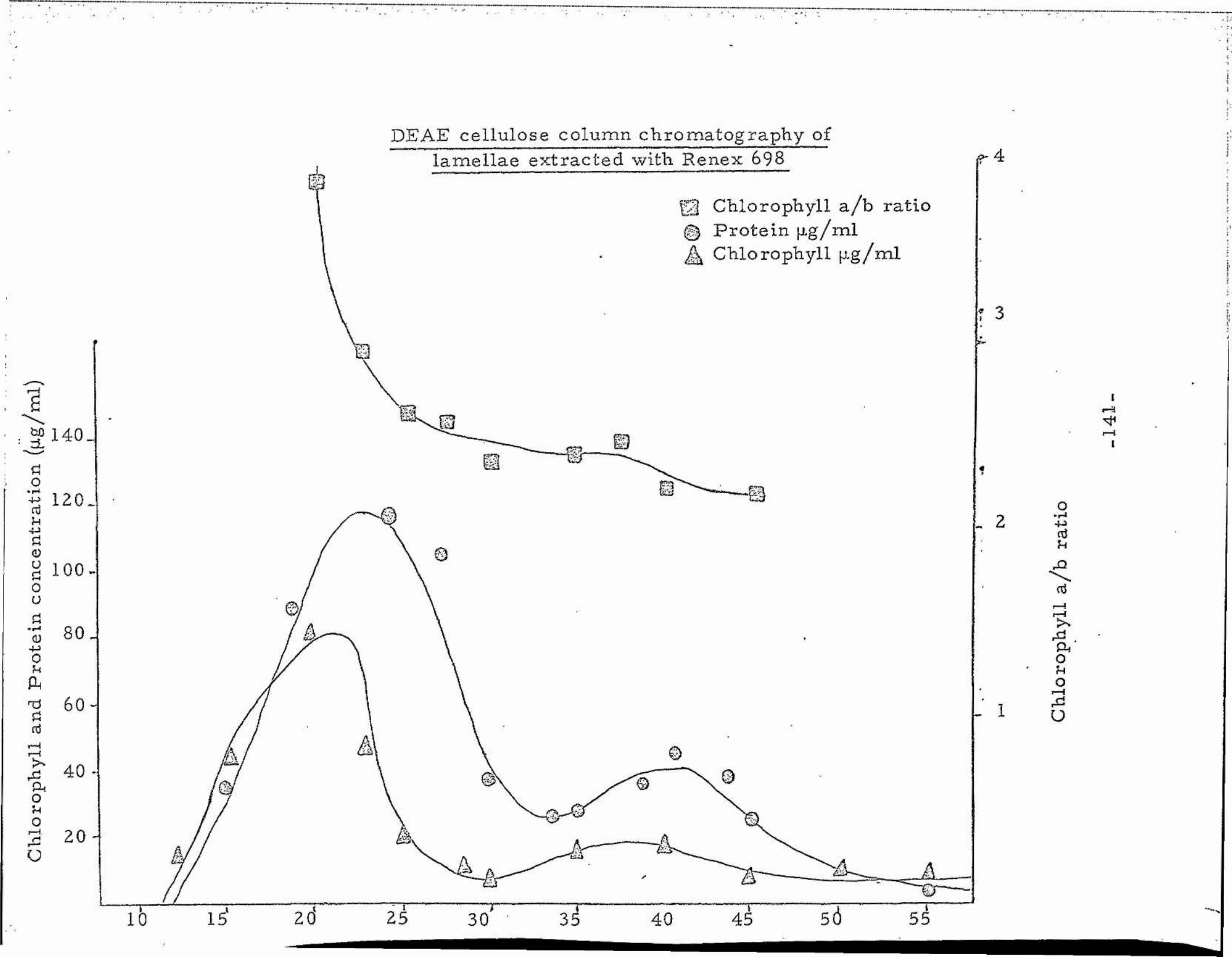
Chlorophyll a/b profiles

A feature of the chlorophyll a/b profiles shown by the samples eluted with all the detergents, is that the maximum ratio occurs in the first eluted green fraction.

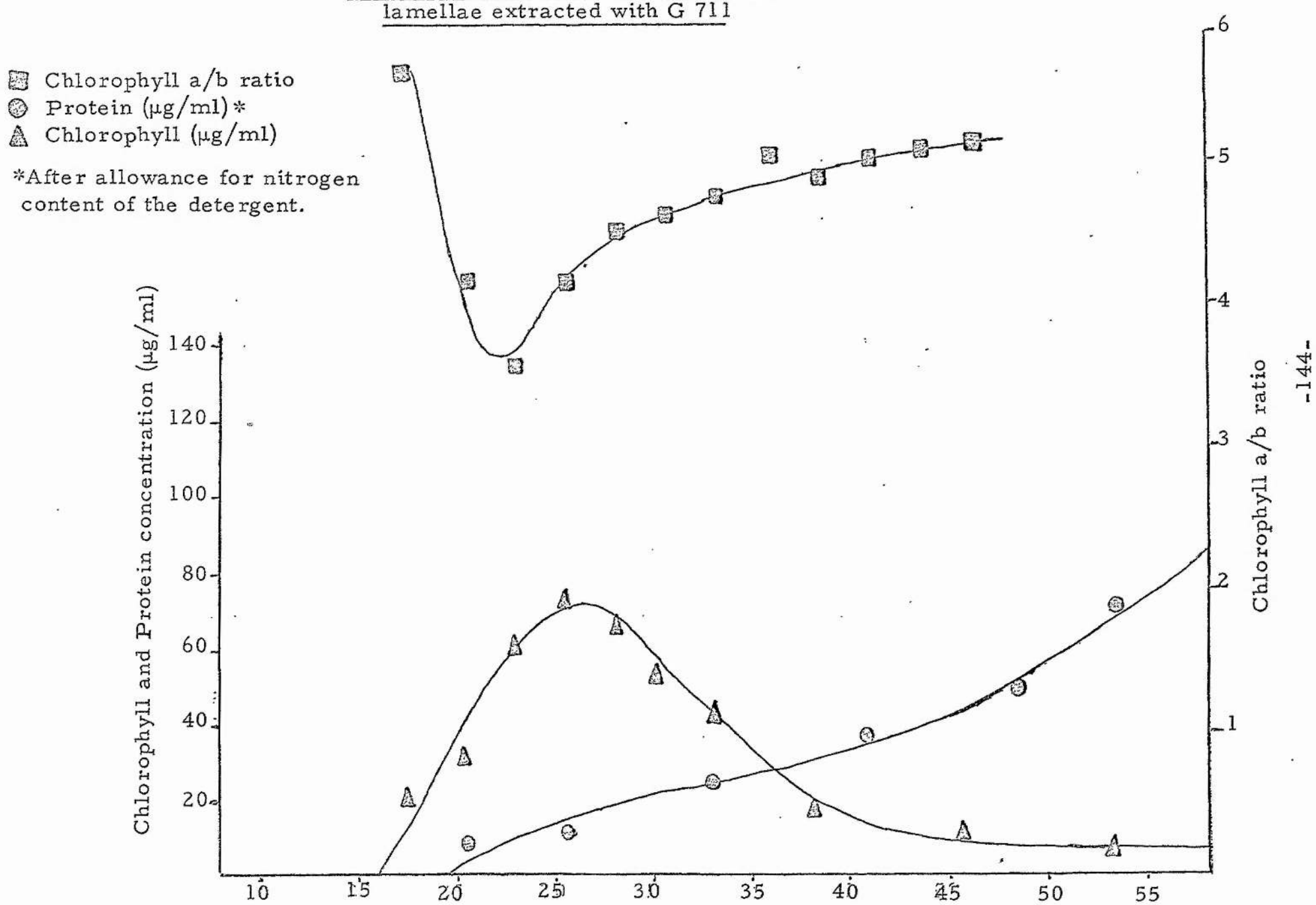
With the exception of G 711, all the detergents display a decrease in the chlorophyll a/b ratio with increasing fraction number.

The diagram for Triton X-100 (Fig. 32) shows a slight rise in the chlorophyll a/b ratio in the light green fraction eluted between the two complexes. The fractions eluted from G 711 treated lamellae (Fig. 36) show an inverse proportionality of the chlorophyll a/b ratio to the paucity of chlorophyll in the sample. Thus the fraction containing the highest concentration of chlorophyll, displays a minimum in the ratio of chlorophyll a/b.

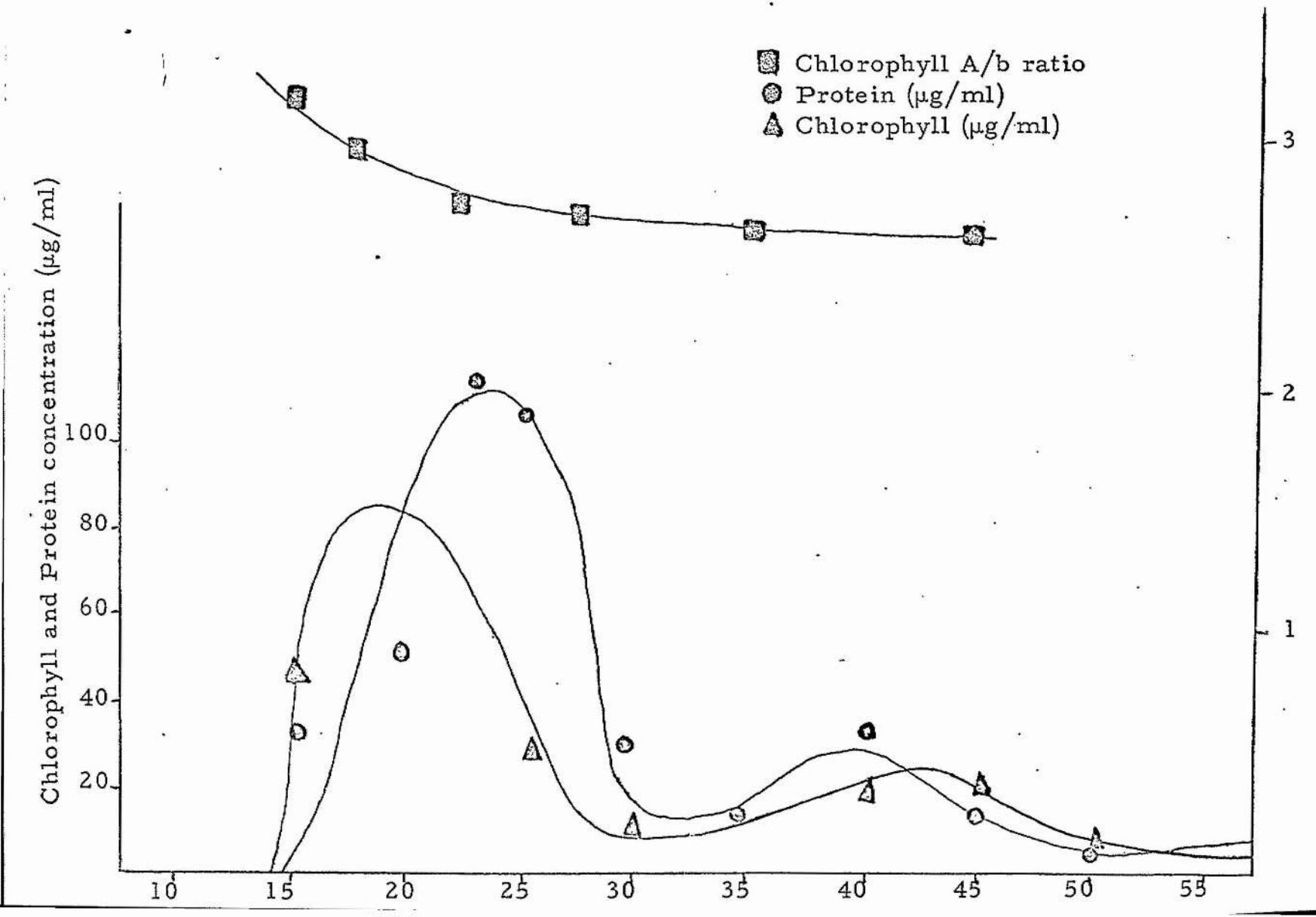
DEAE cellulose column chromatography of lamellae extracted with Renex 698



DEAE cellulose column chromatography of lamellae extracted with G 711



DEAE cellulose column chromatography of lamellae extracted with Brij 96



DEAE cellulose column chromatography of lamellae extracted with Calsolene Oil

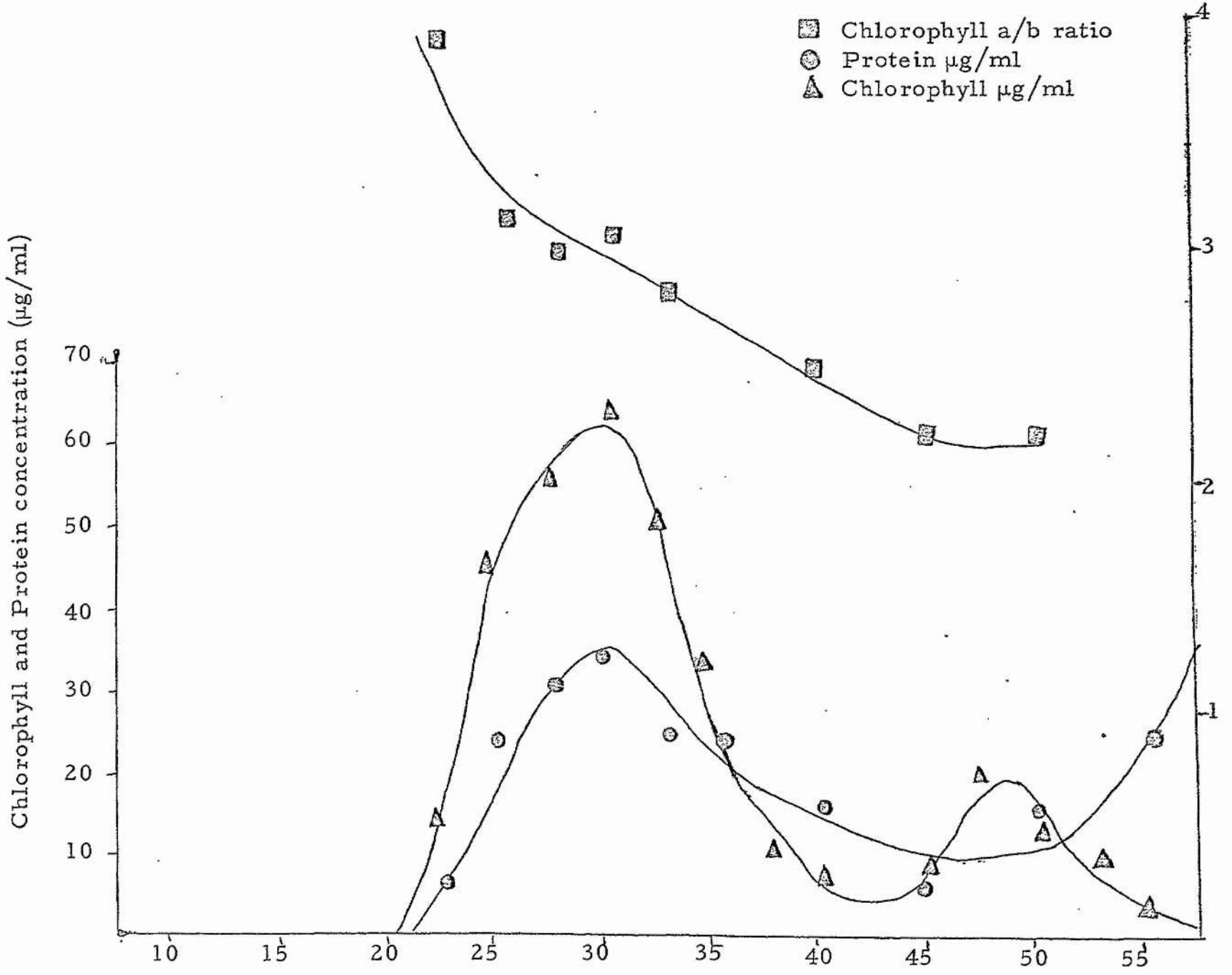


Fig. 38

R. 8 Polyacrylamide Disc Gel Electrophoresis

Attempts to separate characterisable chlorophyll-protein complexes from lamellar detergent extracts were made (see Methods Section p.70), using the entire range of detergents under survey.

1. Non ionic detergents

Initial investigations were conducted on the 50,000 x g supernatant of lamellar extracts prepared from lamellae containing a final chlorophyll concentration of 0.5mg/ml in a final detergent concentration of 0.2%, and buffered at pH 7.1 with 0.04M phosphate buffer. The gel concentration used was 9%.

No migration of a coloured band occurred in any instance. A decrease in the pH of the buffer had no effect; the coloured material remained at the origin. An increase in the buffer pH to 9.5 and an increase in the molarity to 0.10M (carbonate/bicarbonate buffer) caused migration of the coloured material into the gel towards the anode in all cases with the exception of the following detergents: Tween 20, Tween 80,

b) Anionic detergents

All the anionic detergents studied, with the exception of Tergitol Anionic 08, when employed according to the modified method of Thornber et. al. (1967a) (see Methods section p. 70) exhibited some form of migratory green band which travelled towards the anode on electrophoresis.

The results of Thornber et. al. (1967a) were confirmed for SDBS: Three green bands were observed, all of which stained blue with Amido Black 10B solution.

In the cases of Lissapol LS powder and Tergitol 7, three green bands were separable, the central band of each being weakly stainable with Amido Black 10B solution. (Fig. 40).

Two green bands only were visible from Calsolene Oil preparations, both of which stained blue/brown with Amido Black 10B solution. (Fig. 40).

Crillet 1, Crillet 4, Polychol 15, Brij 35, Digitonin and Lubrol W. The above conditions, whilst causing band migration in most instances did not induce any band separation.

In the case of the non ionic detergent Volpo N-10 however, a decrease in the gel concentration to 6% caused a separation of two green bands, the lower of which was slightly stainable with Amido Black 10B solution. (Fig. 39).

No separation of coloured bands was observed for any of the other non ionic detergents studied, despite variation of the conditions as detailed in table 18.

2. Ionic detergents

a) Cationic detergents

The cationic detergents Cetavlon and Vantoc AL, whilst giving brilliantly clear green lamellar solutions which migrated through polyacrylamide gels and stained blue with Amido Black 10B solution, did not yield preparations which could be fractionated into different components.

Table 19 summarises the conditions employed, all of which caused migration of a single band towards the cathode on electrophoresis.

Conditions employed in disc-gel electrophoresis of non-ionic detergent-lamellar extracts which caused migration of a single green band

Detergent	Experimental Conditions	
	Expt. 1.	Expt. 2.
Brij	ADH	AEH
Brij 98	AEF	AEG
Dispersol VLX	AEH	-
Lissapol NXP	BEF	-
Lubrol AL18	BEH	-
Lubrol E	BEF	CEG
Lubrol L	CEF	-
Lubrol PF	CEH	-
Nonidet P42	BDF	BEF
Renex 698	CDF	CEG
Triton X100	AEG	-

KEY:

Gel concentrations
A = 6%
B = 7.5%
C = 9.0%

Buffers (0.1M)
D = pH 7.0 (phosphate)
E = pH 10.0 (carbonate/bicarbonate)

Detergent concentrations (final)
F = 0.2%
G = 0.5%
H = 4.0%

Table 18

Conditions employed in disc-gel electrophoresis of cationic detergent-lamellar extracts which caused migration of a single green band

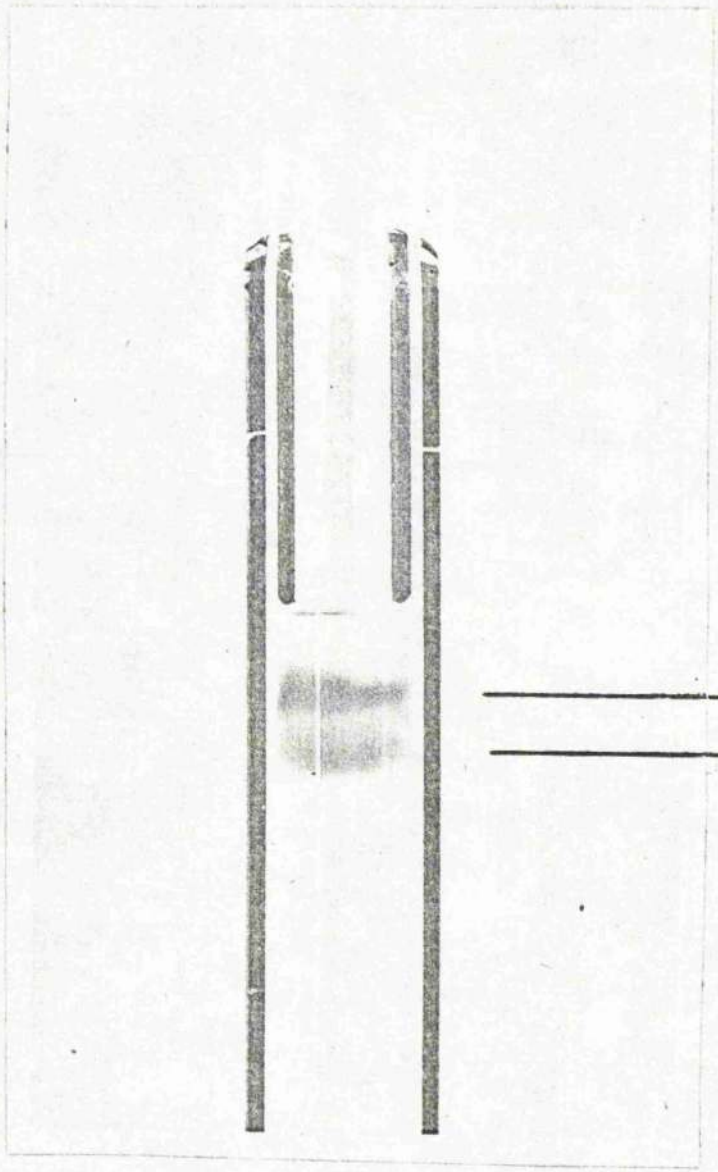
Detergent	Experimental Conditions		
	Expt. 1.	Expt. 2.	Expt. 3.
Cetavlon	BEH	DEH	CFJ
"	BEG	BEJ	AFH
Vantoc AL	BEH	DEH	CFJ
"	BEG	BEJ	

KEY:

Gel concentrations	A = 4%
	B = 6.0%
	C = 7.5%
	D = 9.0%
Buffer molarities (phosphate pH 6.0)	E = 0.04M
	F = 0.10M
Detergent concentrations (final)	G = 0.2%
	H = 0.5%
	J = 4.0%

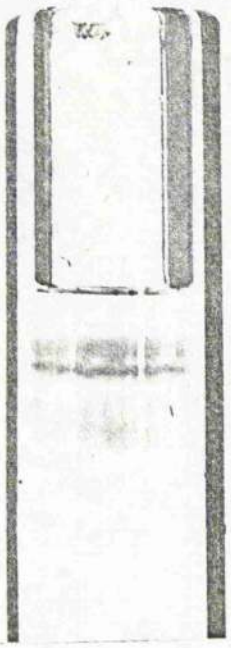
Table 19

Fig. 39



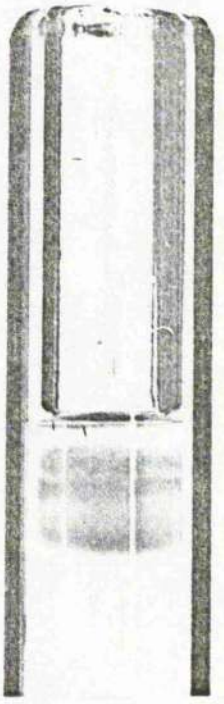
Volpo N-10

Fig. 40



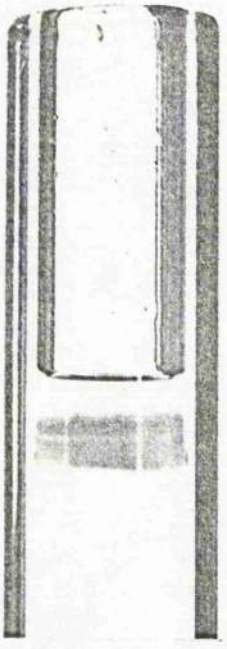
Band 1 (non staining)
Band 2 (stainable)
Band 3 (non staining)

Lissapol LS powder



Band 1 (non staining)
Band 2 (stainable)
Band 3 (non staining)

Tergitol 7



Band 1) Blue/brown stain with
Band 2) Amido Black 10 B

Calsolene Oil

R. 9 Photo-oxidation of ascorbate by detergent treated lamellae

Photo-oxidation of ascorbate was estimated on lamellar particles fractionated from differentially centrifuged detergent extracts and on chlorophyll proteins isolated from DEAE cellulose columns (see p. 137 -146).

1. Fractions produced by differential centrifugation

Seven detergents were studied : Digitonin, G 711, Lissapol NXP, Lubrol L, Renex 698, Tergitol 7 and Vantoc AL.

In all cases, the method of solubilisation was as detailed in Methods section p.48. The fractions sedimenting at 10,000 x g, 50,000 x g and 144,000 x g were assayed for ascorbate photo-oxidation as described previously. (Methods section p.64).

None of the fractions produced from Tergitol 7 or Vantoc AL preparations showed any activity in ascorbate photo-oxidation at all.

Only the 144,000 x g fraction of the G 711 treated lamellae would photo-oxidise ascorbate (fig. 41) and only the 144,000 x g and 50,000 x g fractions of Lissapol NXP, Lubrol L and Renex 698 were found to be active in ascorbate photo-oxidation (fig.41).

The digitonin fractions however were all found to possess ascorbate photo-oxidation activity (fig. 42) with a distinct increase in activity accompanying decrease in particle size. The unfractionated material was also capable of photo-oxidising ascorbate by approximately the same amount as the 10,000 x g fraction.

Photo-oxidation of ascorbate by lamellar extracts in Lubrol L, Lissapol NXP, Renex 698 and G 711 isolated by differential centrifugation

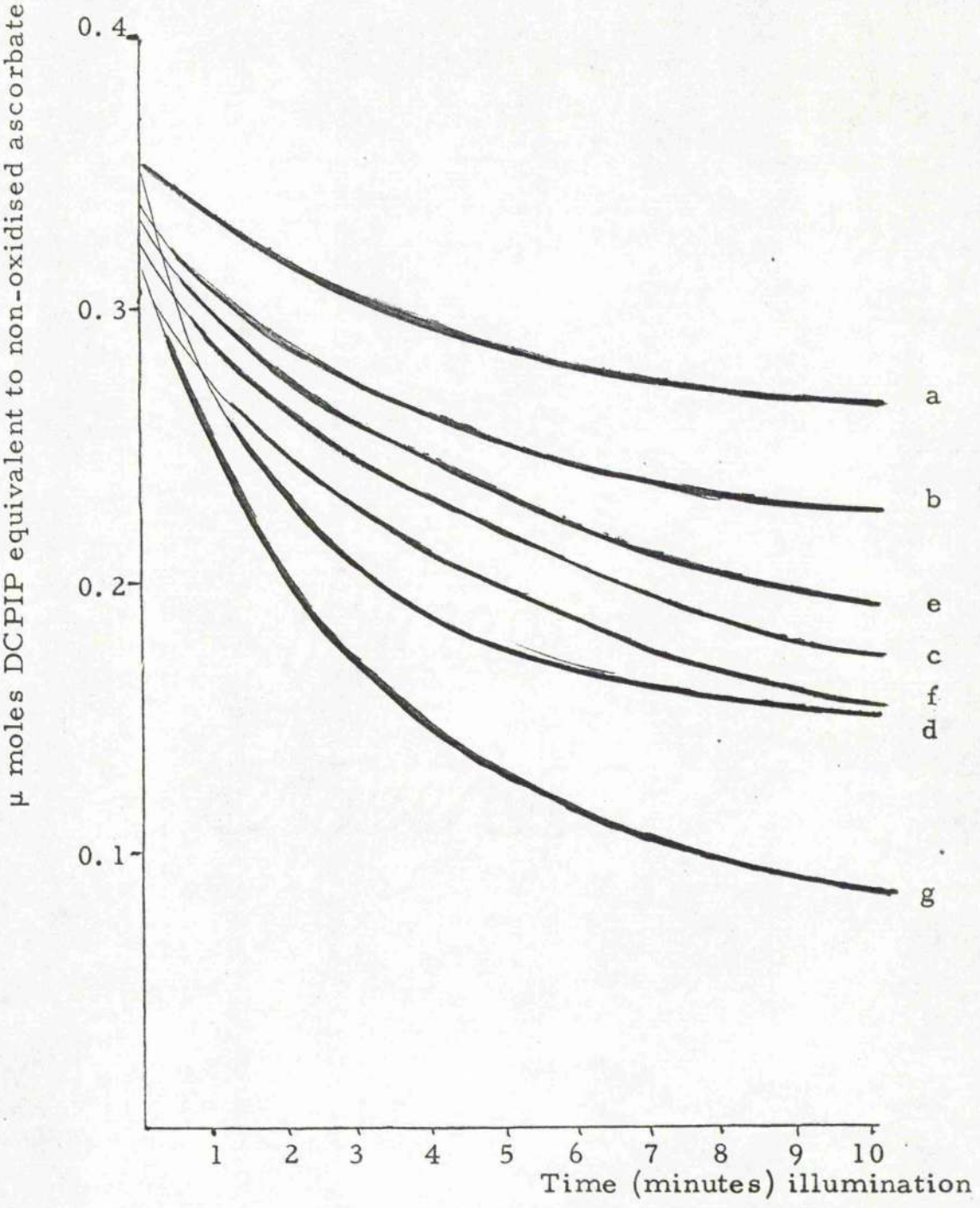


Fig. 41

a Lubrol L 50,000 x g fraction
b Lubrol L 144,000 x g fraction
c Lissapol NXP 50,000 x g fraction
d Lissapol NXP 144,000 x g fraction
e G 711 144,000 x g fraction
f Renex 698 50,000 x g fraction
g Renex 698 144,000 x g fraction

Photo-oxidation of ascorbate by lamellar extracts in digitonin, produced by differential centrifugation

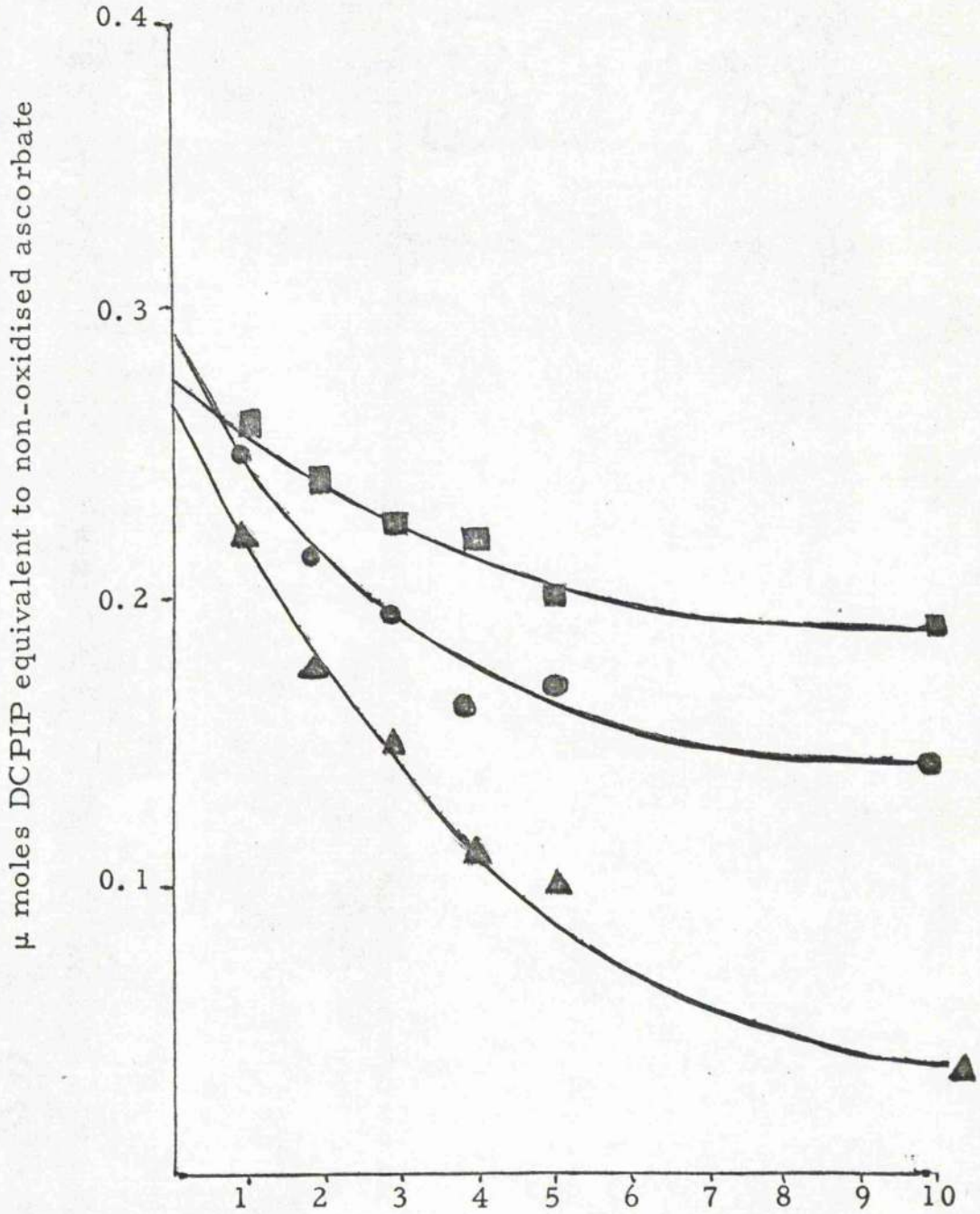


Fig. 42

■ 10,000 x g fraction and unfractionated material.
● 50,000 x g fraction
▲ 144,000 x g fraction

2. Fractions produced by DEAE cellulose column chromatography of detergent extracts

The chlorophyll protein fractions eluted from DEAE cellulose columns using lamellar preparations from Calsolene Oil, Brij 96, Triton-X and Renex 698 (see pp. 137-146 and figs. 38, 37, 32, 33) were assayed for ascorbate photo-oxidation. The results are summarised below:

Table 20

Detergent	Eluted fraction numbers*	Ascorbate photo oxidative activity
Brij 96	18 - 30	Nil
	35 - 50	Nil
Triton X-100	15 - 25	Nil
	30 - 45	Nil
Renex 698	18 - 25	Slight (see fig. 43)
	35 - 45	Nil
Calsolene Oil	23 - 35	Slight (see fig. 43)

* See figs.

Photo-oxidation of ascorbate by lamellar extracts in Calsolene Oil and Renex 698 isolated by DEAE cellulose column chromatography

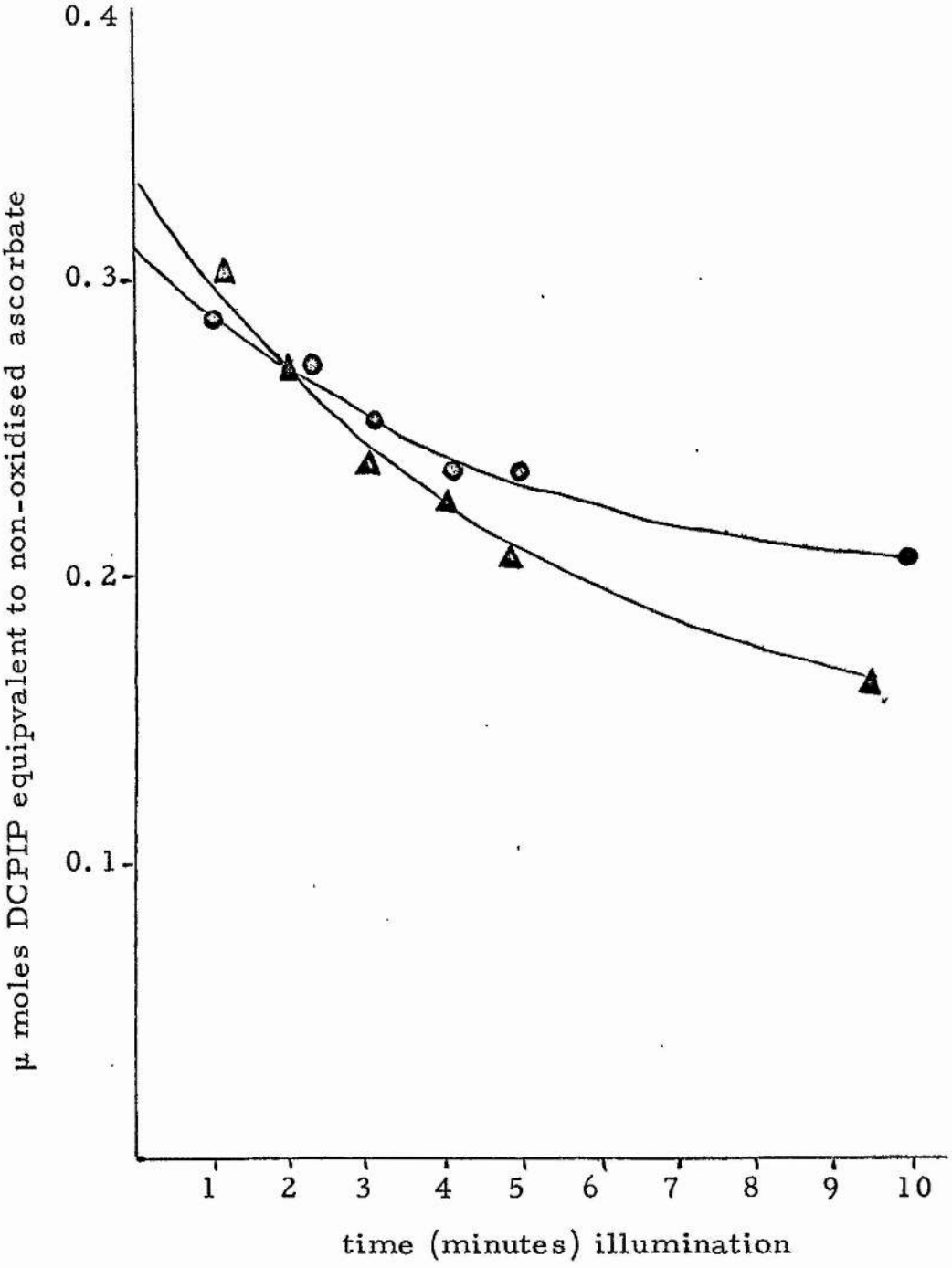


Fig. 43

*See pp. 137-146 and figs. 33, 38.

R.10 Stability of lamellar detergent extracts under differing conditions of continuous illumination

Experiments were conducted according to the methods detailed in Methods section pp. 78 - 81.

a) Effect of chlorophyll concentration of extracted lamellae upon pigment stability in continuous light

The results of these assays, given in tables 21 - 26 indicate appreciable variation in the effects of detergents on pigment stability:

Brij 35 solutions show equal bleaching of both chlorophylls a and b at an approximately constant rate over the five day period, independent of the diluted chlorophyll concentration.

Cetavlon solutions are relatively stable at chlorophyll concentrations in excess of 0.1mg/ml for periods of three days or less illumination, but show rapid chlorophyll breakdown with preferential bleaching of chlorophyll a if lower concentrations, or longer periods of illumination are employed.

Lissapol NXP solutions resemble lamellar solutions in Cetavlon, but differ in the more concentrated solutions (1.0mg chlorophyll/ml) being less resistant to degradation after long periods of illumination (4-5 days), whereas the less concentrated

solutions (0.10 mg chlorophyll/ml) are more resistant to degradation after long periods of illumination.

Tergitol 7 solutions showed very rapid chlorophyll breakdown at all concentrations once the bleaching process had commenced, but exhibited a distinct stability in the early period of illumination. Renex 698 and G 711 solutions both possessed considerable stability to breakdown of pigments at all concentrations measured.

The visible absorption spectra of the G 711 extracts were examined throughout the period of investigation, as detailed in fig. 20 and support the results of table 26 .

b) Effect of pH variation on bleaching of chlorophyll in detergent treated lamellae (Figs. 44 - 49)

With the exception of Tergitol 7 lamellar extracts, the pH 4.0 assays showed rapid chlorophyll decomposition which in all cases except that of G 711 preparations occurred almost to completion within the first 24 hours.

The pH 10.0 assays indicated a distinct stability of the extracts to loss of chlorophyll with the exception of preparations from Cetavlon.

c) Effect of oxygenation on bleaching of chlorophyll in detergent treated lamellae (Figs. 50 - 55)

Of the six detergents studied, three, (Brij 35, G 711 and Renex 698) conferred a degree of resistance upon lamellar extracts to chlorophyll breakdown in the presence of light and oxygen, in comparison with the stability of untreated lamellae and of chlorophyll a in acetone. Lissapol NXP, Cetavlon and Tergitol 7 extracts however, underwent rapid photo-oxidative bleaching.

d) Effect of temperature on bleaching of chlorophyll in detergent treated lamellae (Figs. 56 - 61)

Lower temperature was found in all cases studied to delay breakdown of chlorophyll in illuminated lamellar extracts, though not to the same extent for each detergent. The temperature effect in the case of Brij 35 extracts was found to be only marginal, whereas the effect for Tergitol 7 extracts was sufficient to result in the illuminated preparation after five days at 4^{o} possessing more than double the chlorophyll concentration of the equivalent sample maintained at $20^{o}C$.

Effect of chlorophyll concentration of Brij 35 lamellar extracts upon pigment stability in continuous light

Initial chlorophyll concentration (mg/ml)	1.68	1.68	1.68	1.68	1.68
Diluted chlorophyll conc. and chlorophyll a/b ratio: before illumination.	1.00 2.54	0.50 2.57	0.100 2.53	0.050 2.57	0.0100 2.57
Total chlorophyll conc. and chlorophyll a/b ratio: after 24 hours concentration.	0.97 2.56	0.44 2.55	0.094 2.50	0.048 2.57	0.0101 2.53
Total chlorophyll conc. and chlorophyll a/b ratio: after 2 days illumination.	0.90 2.52	0.35 2.42	0.092 2.48	0.040 2.50	0.0090 2.49
Total chlorophyll conc. and chlorophyll a/b ratio: after 3 days illumination.	0.81 2.46	0.23 2.37	0.079 2.40	0.029 2.41	0.0076 2.38
Total chlorophyll conc. and chlorophyll a/b ratio: after 4 days illumination.	0.73 2.43	0.19 2.30	0.064 2.37	0.017 2.33	0.0058 2.35
Total chlorophyll conc. and chlorophyll a/b ratio: after 5 days illumination.	0.65 2.40	0.16 2.28	0.041 2.31	0.011 2.26	0.0039 2.29

Table. 21

Effect of chlorophyll concentration of Cetavlon lamellar extracts upon pigment stability in continuous light

Initial chlorophyll concentration (mg/ml)	2.90	2.90	2.90	2.90	2.90
Diluted chlorophyll conc. and chlorophyll a/b ratio: before illumination.	1.00 3.03	0.50 2.99	0.100 2.92	0.050 2.96	0.010 2.92
Total chlorophyll conc. and chlorophyll a/b ratio: after 24 hours illumination.	1.01 3.00	0.49 3.01	0.099 2.94	0.043 2.70	0.0081 2.69
Total chlorophyll conc. and chlorophyll a/b ratio: after 2 days illumination.	0.97 2.97	0.45 2.90	0.090 2.86	0.031 2.21	0.0053 2.02
Total chlorophyll conc. and chlorophyll a/b ratio: after 3 days illumination.	0.95 2.91	0.41 2.74	0.085 2.73	0.025 1.96	0.0044 1.93
Total chlorophyll conc. and chlorophyll a/b ratio: after 4 days illumination.	0.93 2.90	0.38 2.53	0.061 2.19	0.021 1.92	0.0040 1.89
Total chlorophyll conc. and chlorophyll a/b ratio: after 5 days illumination.	0.91 2.89	0.33 2.26	0.047 1.92	0.020 1.88	0.0037 1.87

Table. 22

Effect of chlorophyll concentration of Lissapol NXP lamellar extracts upon pigment stability in continuous light

Initial chlorophyll concentration (mg/ml)	2.79	2.79	2.79	2.79	2.79
Diluted chlorophyll conc. and chlorophyll a/b ratio: before illumination.	1.00 2.46	0.50 2.49	0.100 2.52	0.050 2.47	0.010 2.47
Total chlorophyll conc. and chlorophyll a/b ratio: after 24 hours illumination.	0.97 2.31	0.46 2.46	0.091 2.44	0.048 2.41	0.0094 2.34
Total chlorophyll conc. and chlorophyll a/b ratio: after 2 days illumination.	0.97 2.29	0.46 2.41	0.084 2.39	0.040 2.07	0.0086 2.26
Total chlorophyll conc. and chlorophyll a/b ratio: after 3 days illumination.	0.94 2.17	0.40 2.12	0.080 2.22	0.032 2.00	0.0077 2.11
Total chlorophyll conc. and chlorophyll a/b ratio: after 4 days illumination.	0.71 2.09	0.34 2.03	0.072 2.01	0.028 1.96	0.0063 1.99
Total chlorophyll conc. and chlorophyll a/b ratio: after 5 days illumination.	0.65 2.12	0.32 2.06	0.067 1.97	0.026 1.96	0.0059 1.95

Table. 23

Effect of chlorophyll concentration of Tergitol - 7 lamellar extracts upon pigment stability in continuous light

Initial chlorophyll concentration (mg/ml)	1.84	1.84	1.84	1.84	1.84
Diluted chlorophyll conc. and chlorophyll a/b ratio: before illumination.	1.00 4.16	0.50 4.10	0.100 4.24	0.050 4.17	0.0100 4.13
Total chlorophyll conc. and chlorophyll a/b ratio: after 24 hours illumination.	1.02 4.16	0.48 4.11	0.098 4.13	0.049 4.14	0.0074 3.71
Total chlorophyll conc. and chlorophyll a/b ratio: after 2 days illumination.	0.91 3.98	0.40 3.85	0.061 3.18	0.030 3.02	0.0036 2.07
Total chlorophyll conc. and chlorophyll a/b ratio: after 3 days illumination.	0.84 3.80	0.29 3.14	0.042 2.14	0.018 2.00	0.0014 1.50
Total chlorophyll conc. and chlorophyll a/b ratio: after 4 days illumination.	0.47 2.21	0.17 2.13	0.020 1.86	0.005 1.42	-
Total chlorophyll conc. and chlorophyll a/b ratio: after 5 days illumination.	0.33 2.05	0.09 1.71	0.013 1.45	- -	- -

Table. 24

Effect of chlorophyll concentration of Renex 698 lamellar extracts upon pigment stability in continuous light

Initial chlorophyll concentration (mg/ml)	3.17	3.17	3.17	3.17	3.17
Diluted chlorophyll conc. and chlorophyll a/b ratio: before illumination.	1.00 3.14	0.50 3.13	0.100 3.18	0.050 3.14	0.0100 3.19
Total chlorophyll conc. and chlorophyll a/b ratio: after 24 hours illumination.	1.00 3.12	0.46 3.13	0.098 3.14	0.51 3.15	0.0092 3.10
Total chlorophyll conc. and chlorophyll a/b ratio: after 2 days illumination.	0.98 3.14	0.48 3.16	0.099 3.12	0.046 3.08	0.0090 3.02
Total chlorophyll conc. and chlorophyll a/b ratio: after 3 days illumination.	0.97 3.11	0.45 3.10	0.096 3.14	0.043 2.99	0.0081 2.93
Total chlorophyll conc. and chlorophyll a/b ratio: after 4 days illumination.	0.96 3.09	0.47 3.12	0.097 3.16	0.044 2.96	0.0076 2.84
Total chlorophyll conc. and chlorophyll a/b ratio: after 5 days illumination.	0.94 3.05	0.47 3.06	0.086 3.00	0.041 2.90	0.0070 2.75

Table.25

Effect of chlorophyll concentration of G 711 lamellar extracts upon pigment stability in continuous light

Initial chlorophyll concentration (mg/ml)	3.34	3.34	3.34	3.34	3.34
Diluted chlorophyll conc. and chlorophyll a/b ratio: before illumination.	1.00 7.32	0.50 7.61	0.100 7.50	0.050 7.53	0.010 7.41
Total chlorophyll conc. and chlorophyll a/b ratio: after 24 hours illumination.	0.98 7.34	0.48 7.57	0.101 7.49	0.049 7.50	0.0096 7.38
Total chlorophyll conc. and chlorophyll a/b ratio: after 2 days illumination.	0.98 7.27	0.49 7.60	0.098 7.49	0.051 7.51	0.0102 7.40
Total chlorophyll conc. and chlorophyll a/b ratio: after 3 days illumination.	0.94 7.28	0.49 7.59	0.098 7.47	0.047 7.48	0.0090 7.16
Total chlorophyll conc. and chlorophyll a/b ratio: after 4 days illumination.	0.99 7.21	0.47 7.58	0.098 7.42	0.048 7.47	0.0084 6.80
Total chlorophyll con. and chlorophyll a/b ratio: after 5 days illumination.	0.96 7.25	0.47 7.58	0.097 7.46	0.047 7.49	0.0071 5.98

Table. 26

Effect of pH on bleaching of chlorophyll in lamellae solubilised with Brij 35

BRIJ 35

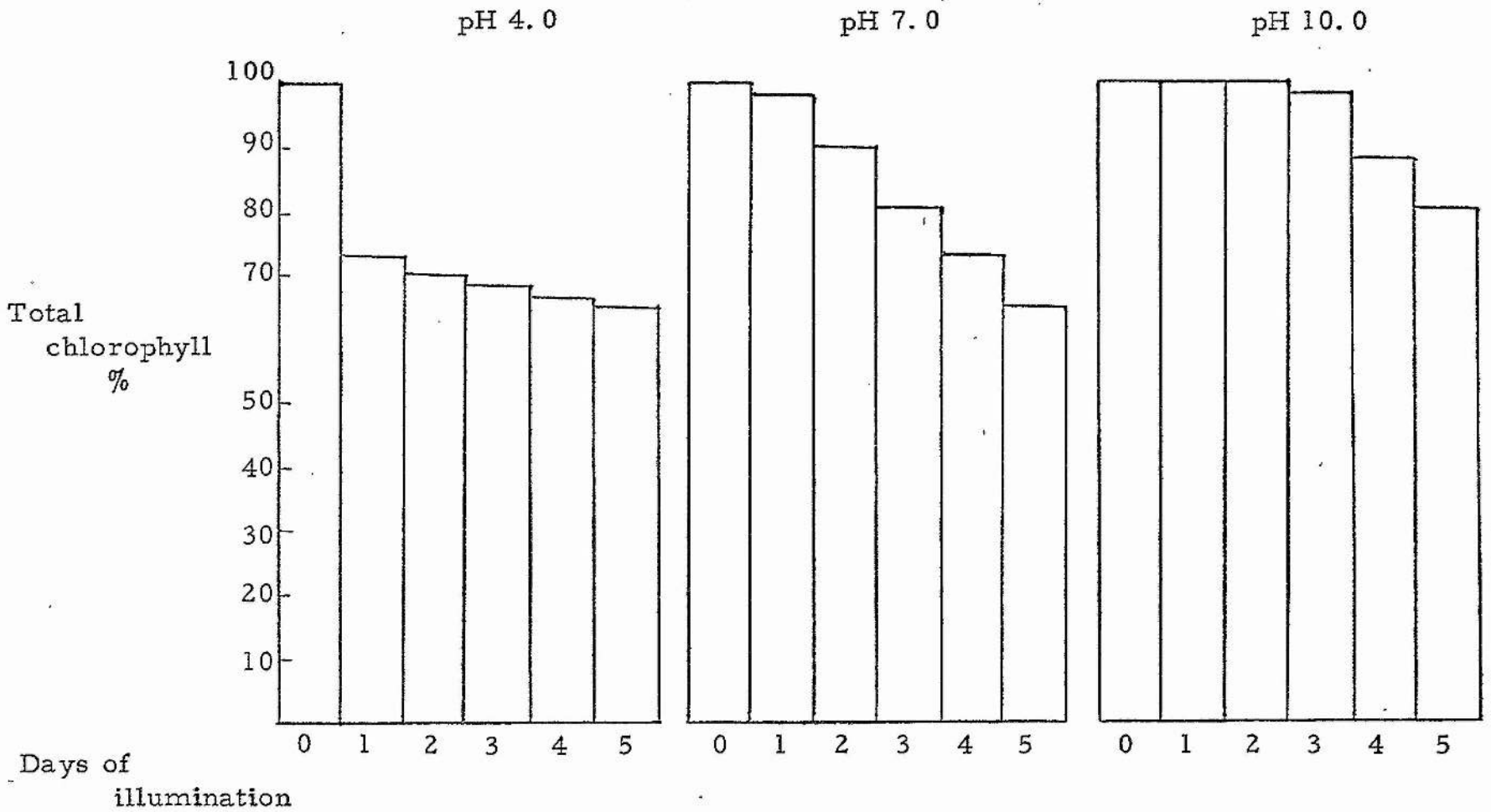


Fig. 44

Effect of pH on bleaching of chlorophyll in lamellae solubilised with Cetavlon

CETAVLON

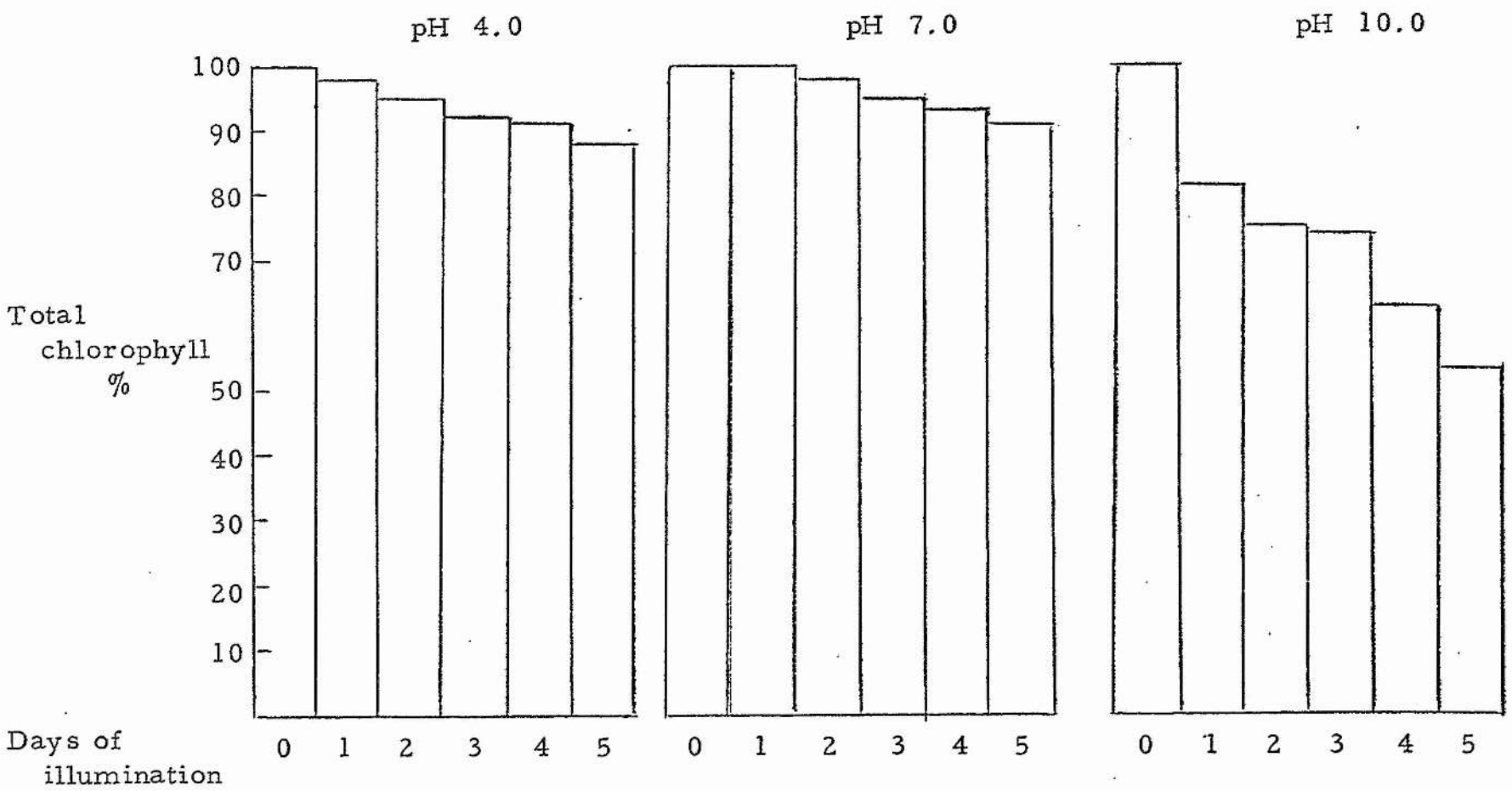


Fig. 45

Effect of pH on bleaching of chlorophyll in lamellae solubilised with Lissapol NXP

LISSAPOL NXP

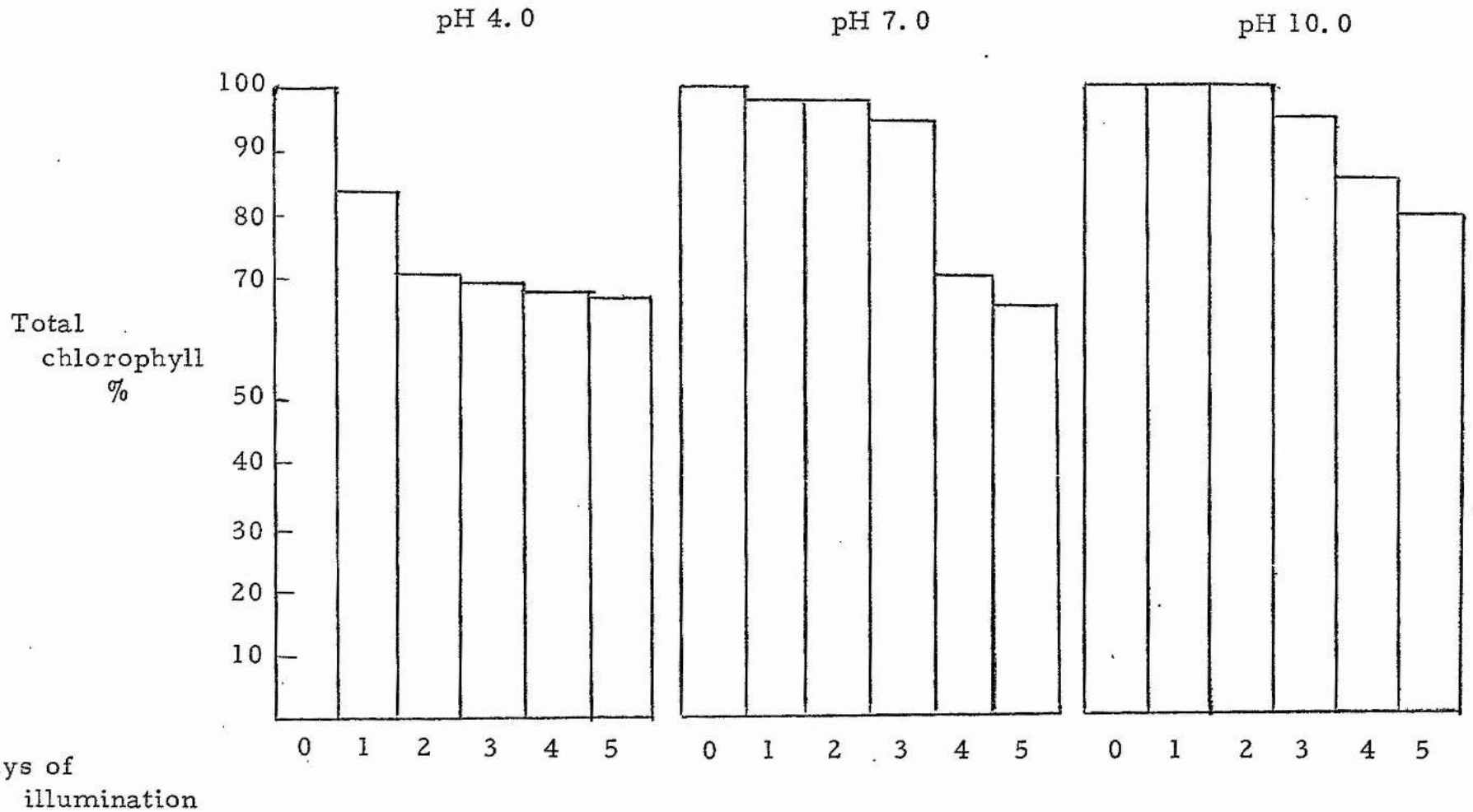


Fig. 46

Effect of pH on bleaching of chlorophyll in lamellae solubilised with Tergitol 7

TERGITOL 7

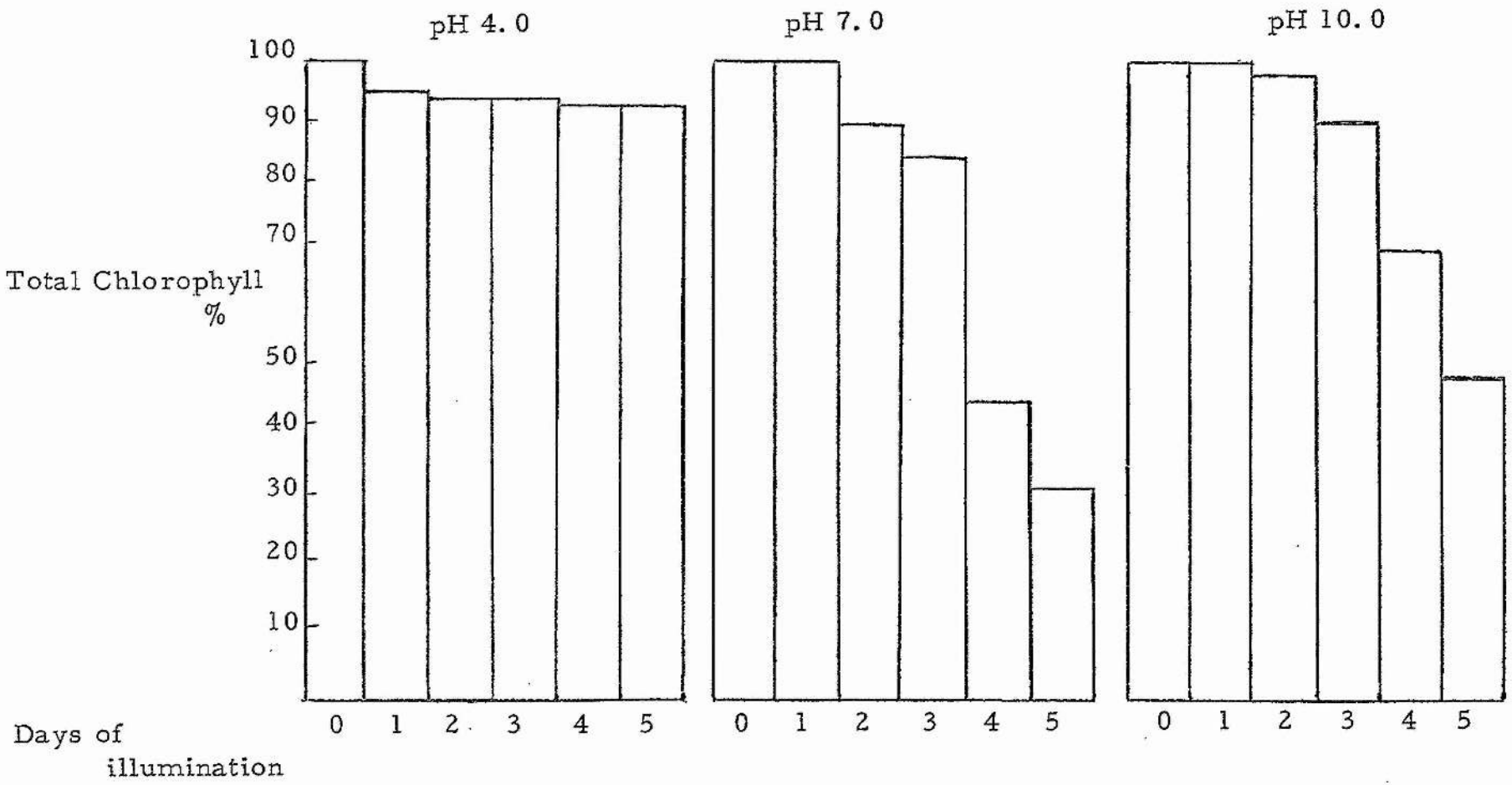


Fig. 47

Effect of pH on bleaching of chlorophyll in lamellae solubilised with Renex 698

RENEX 698

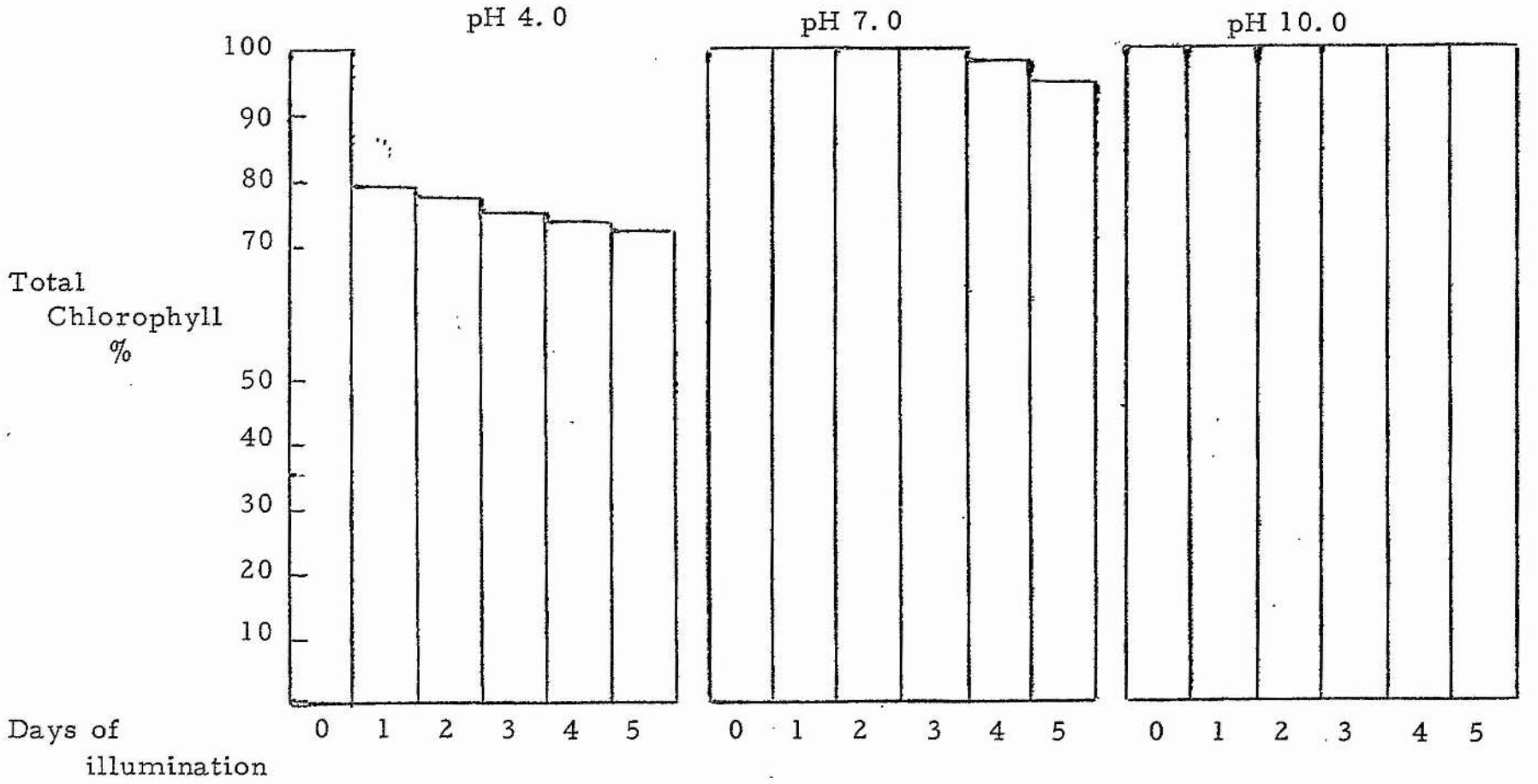
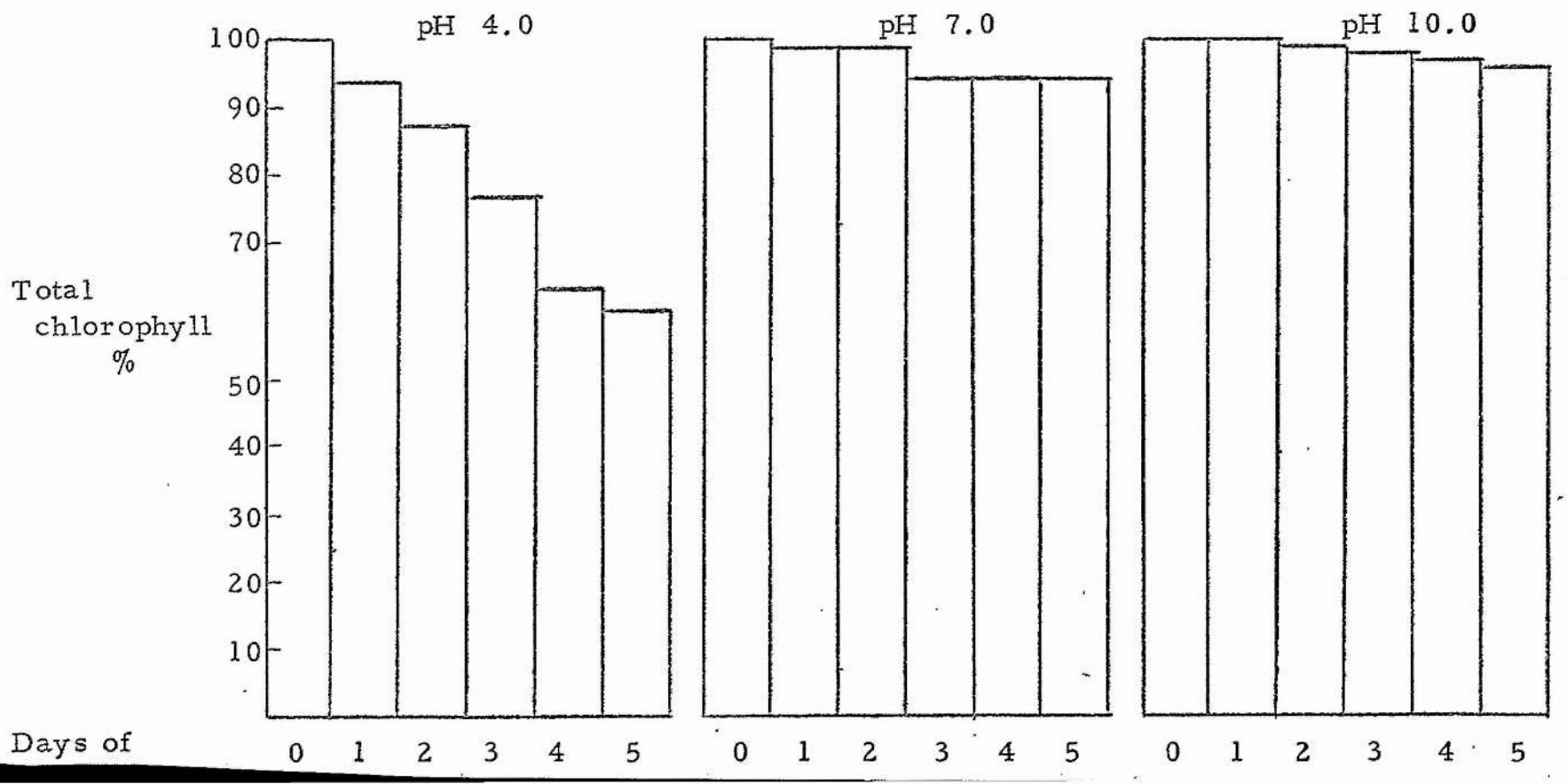


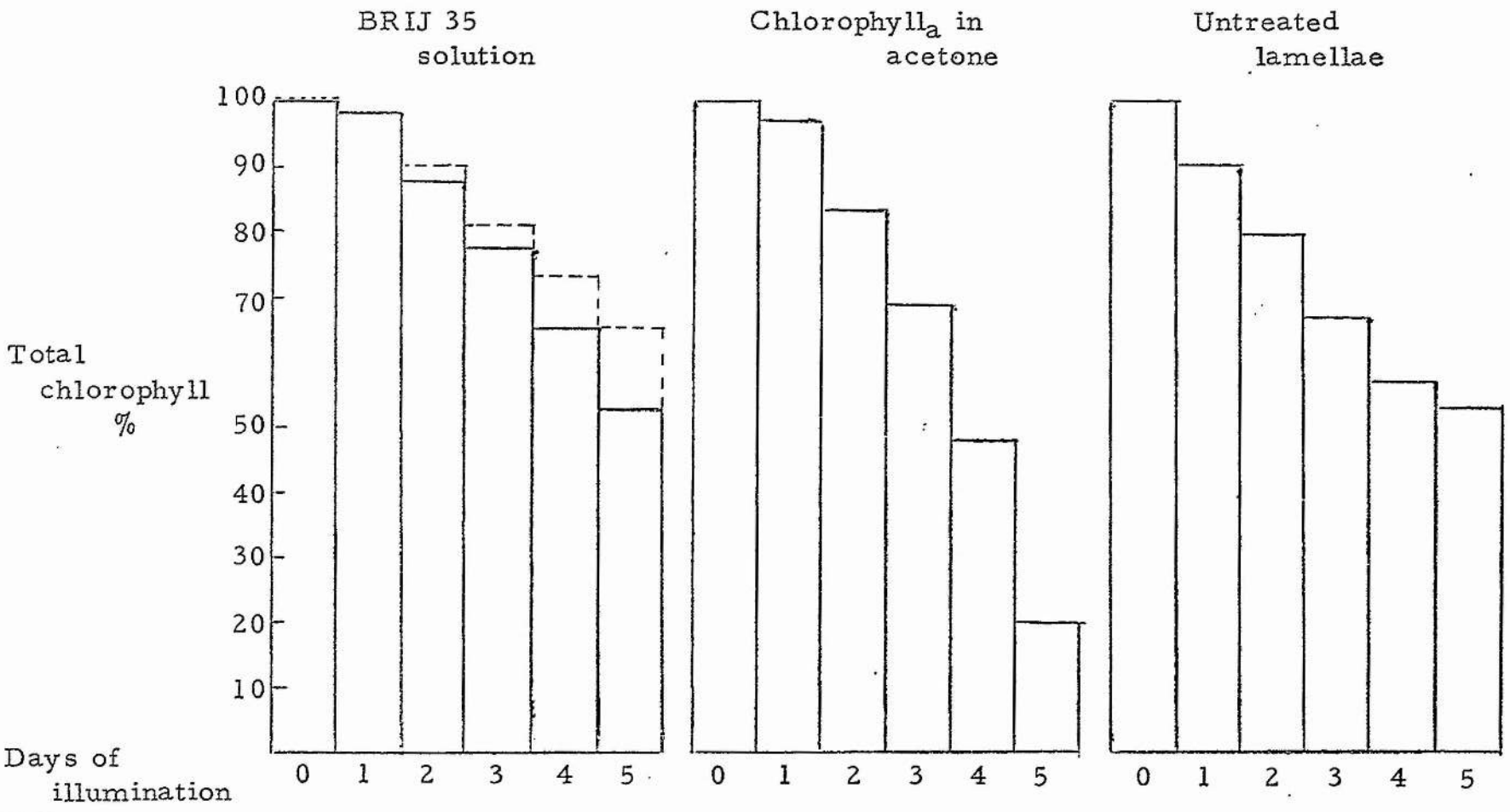
Fig. 48

Effect of pH on bleaching of chlorophyll in lamellae solubilised with G711

G711



Effect of oxygenation on the bleaching of chlorophyll in lamellar solutions prepared with the detergent Brij 35; of chlorophyll$_a$ dissolved in acetone, and of chlorophyll in untreated lamellae



Effect of oxygenation on the bleaching of chlorophyll in lamellar solutions prepared with the detergent Cetavlon; of chlorophyll$_a$ dissolved in acetone, and of chlorophyll in untreated lamellae

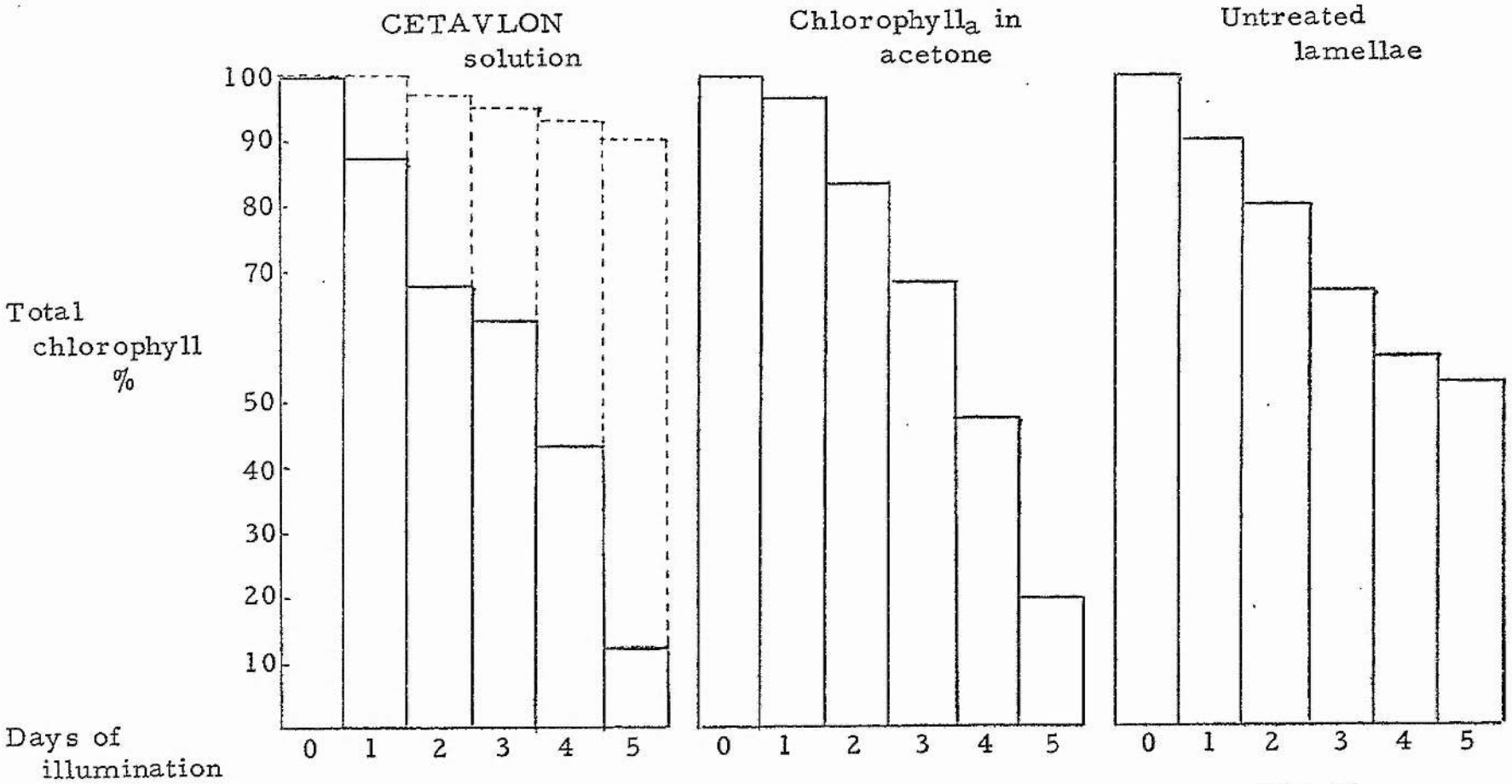
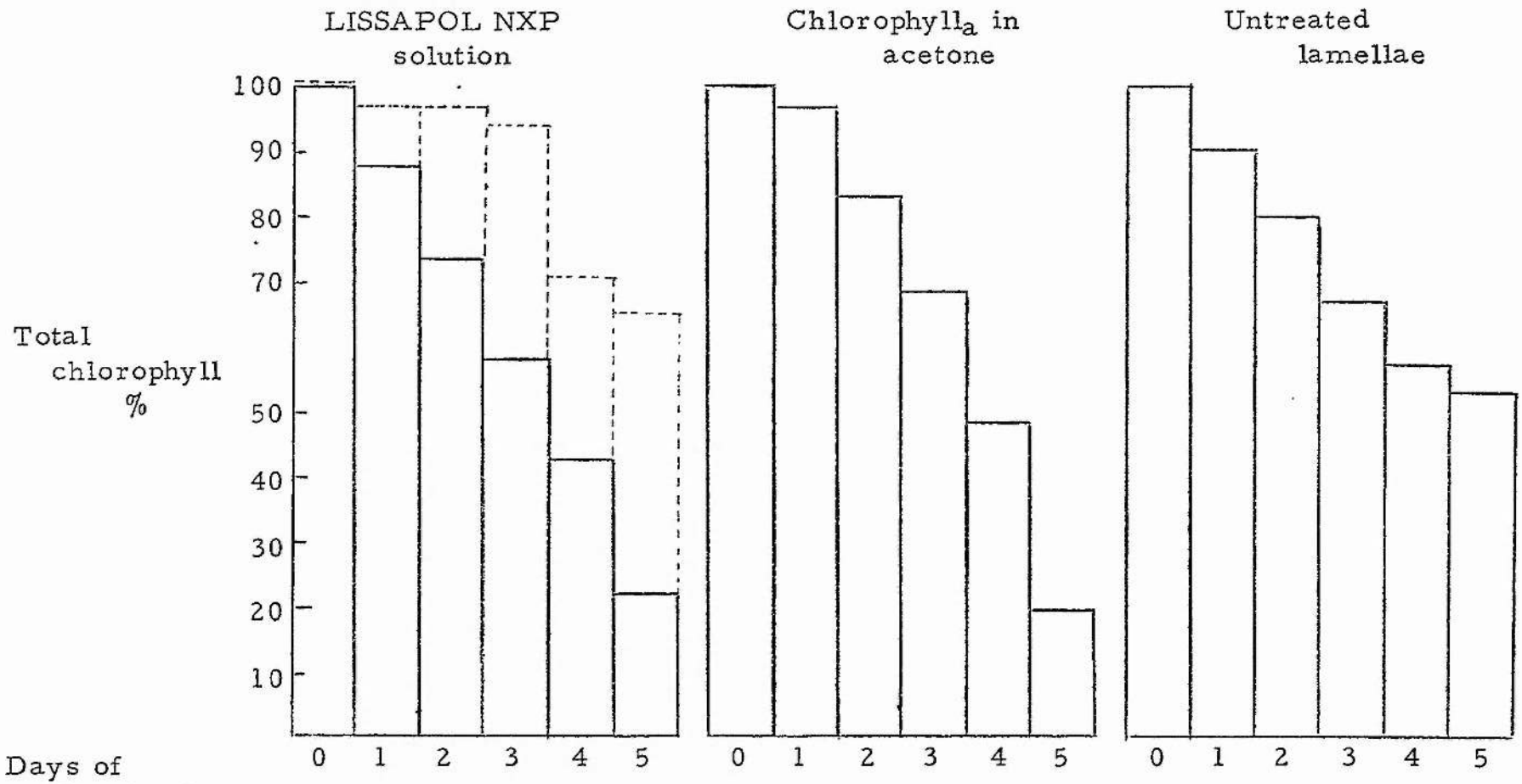


Fig. 51

Dotted lines ----- indicate chlorophyll levels for equivalent samples not oxygenated

Effect of oxygenation on the bleaching of chlorophyll in lamellar solutions prepared with the detergent Lissapol NXP; of chlorophyll$_a$ dossolved in acetone, and of chlorophyll in untreated lamellae



Dotted lines ----- indicate chlorophyll levels

Fig.52

Effect of oxygenation on the bleaching of chlorophyll in lamellar solutions prepared with the detergent Tergitol -7; of chlorophyll$_a$ dissolved in acetone, and of chlorophyll in untreated lamellae

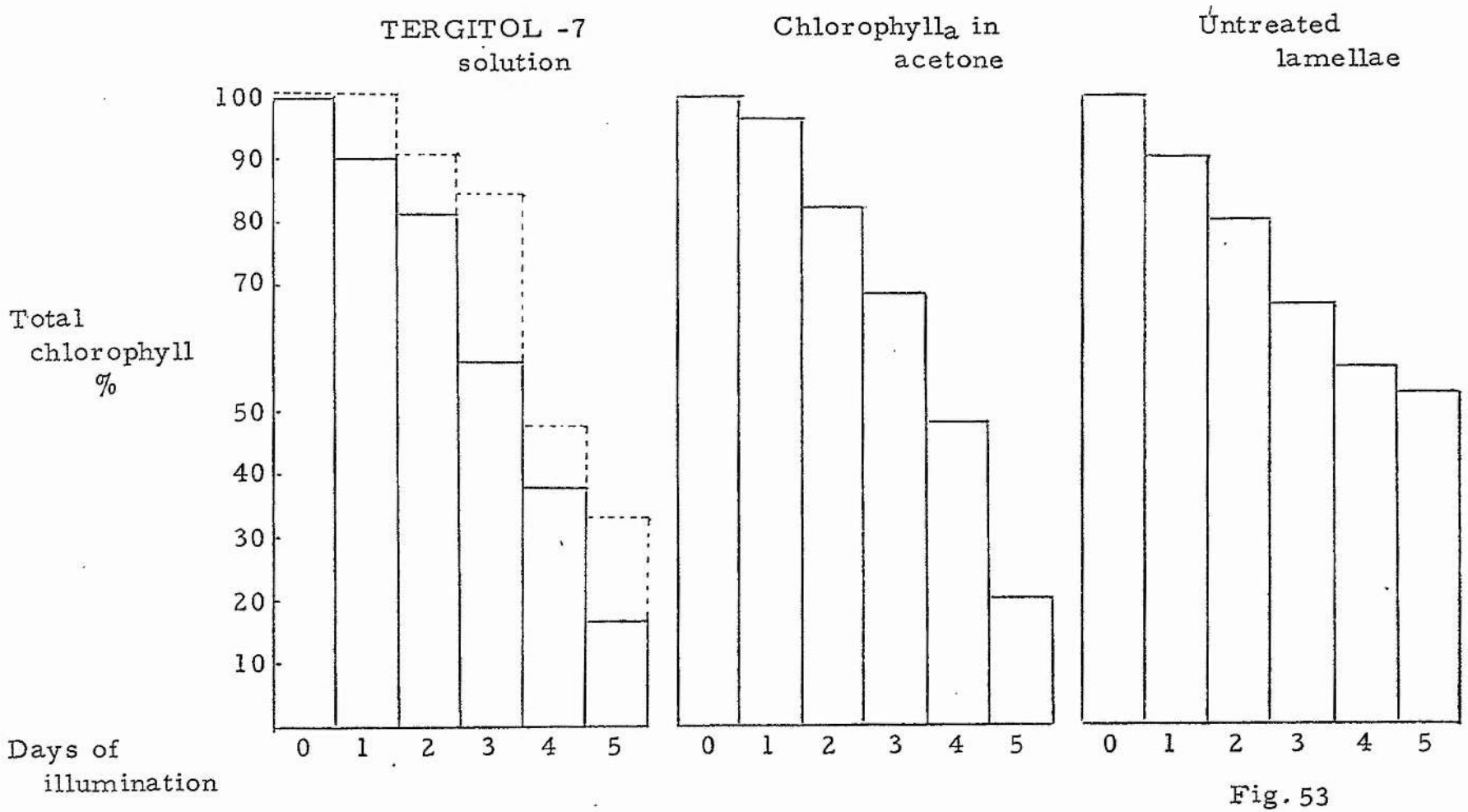
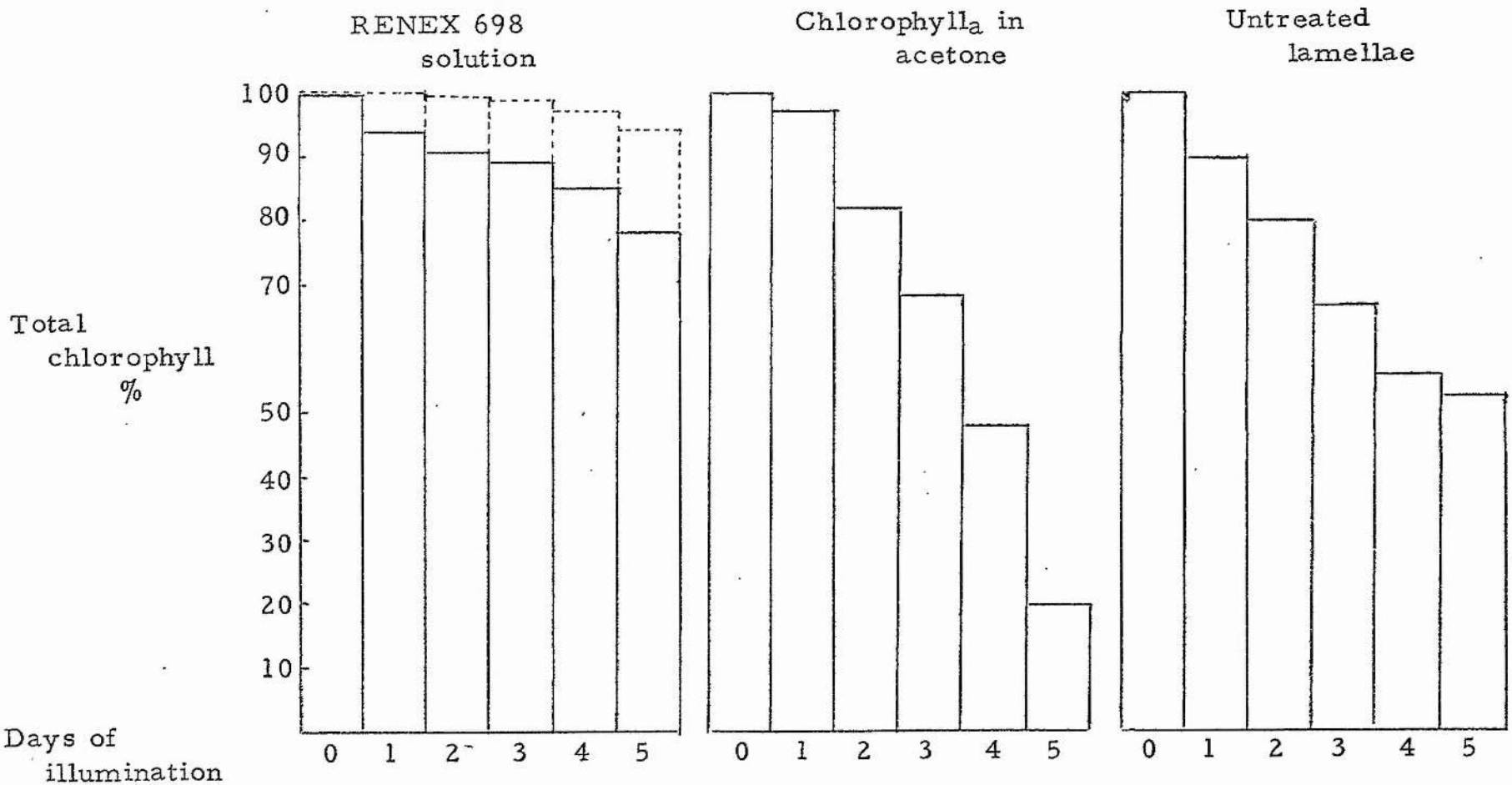


Fig. 53

Dotted lines ----- indicate chlorophyll levels for equivalent samples not oxygenated

Effect of oxygentation on the bleaching of chlorophyll in lamellar solutions prepared with the detergent Renex 698; of chlorophyll$_a$ dissolved in acetone, and of chlorophyll in untreated lamellae



Dotted lines ----- indicate chlorophyll levels

Fig. 54

Effect of oxygenation on the bleaching of chlorophyll in lamellar solutions prepared with the detergent G711; of chlorophyll$_a$ dissolved in acetone, and of chlorophyll in untreated lamellae

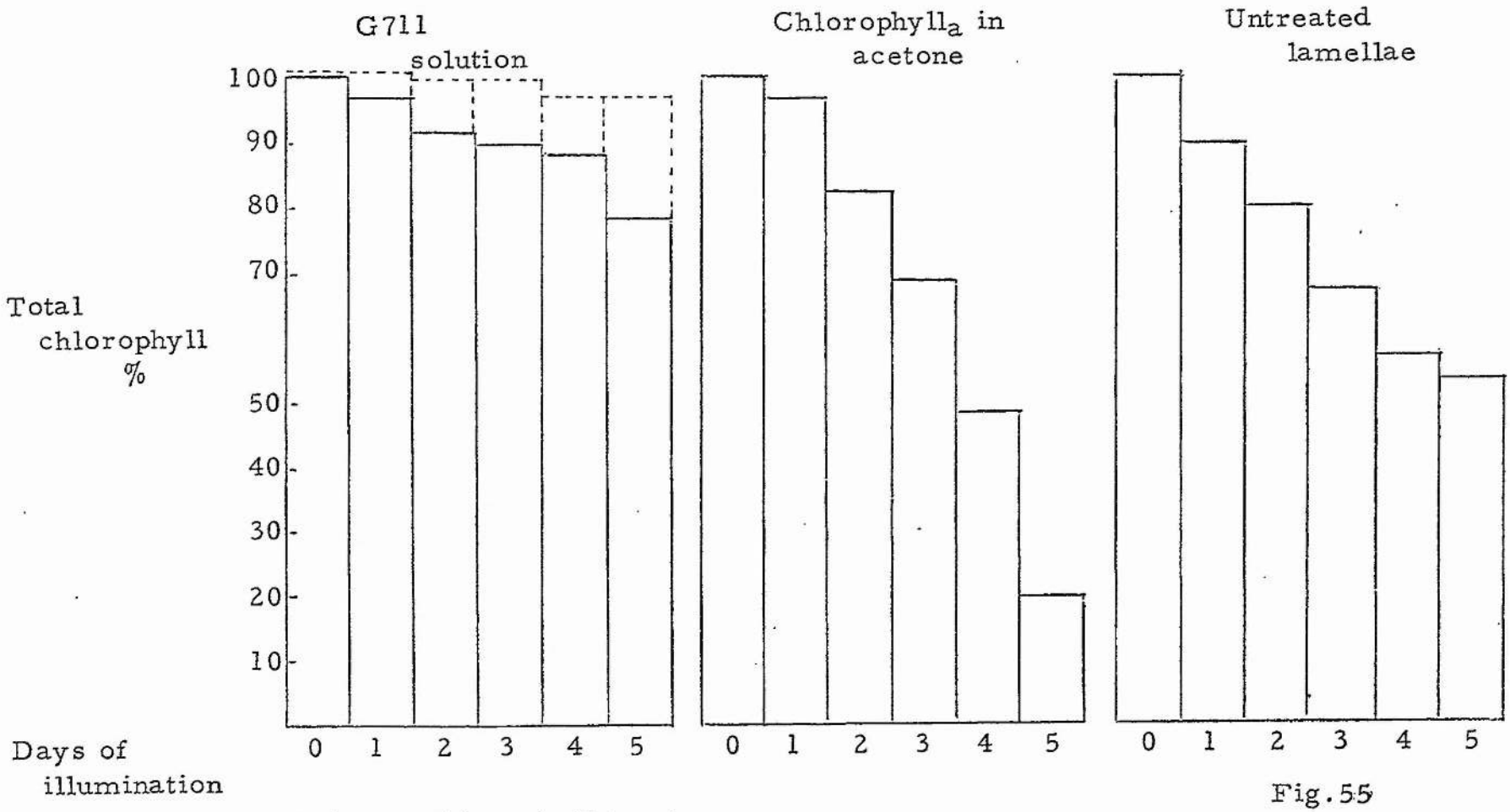


Fig.55

Dotted lines ----- indicate chlorophyll levels for equivalent samples not oxygentated

Effect of temperature on bleaching of chlorophyll in lamellae sobulised with Brij 35

BRIJ 35

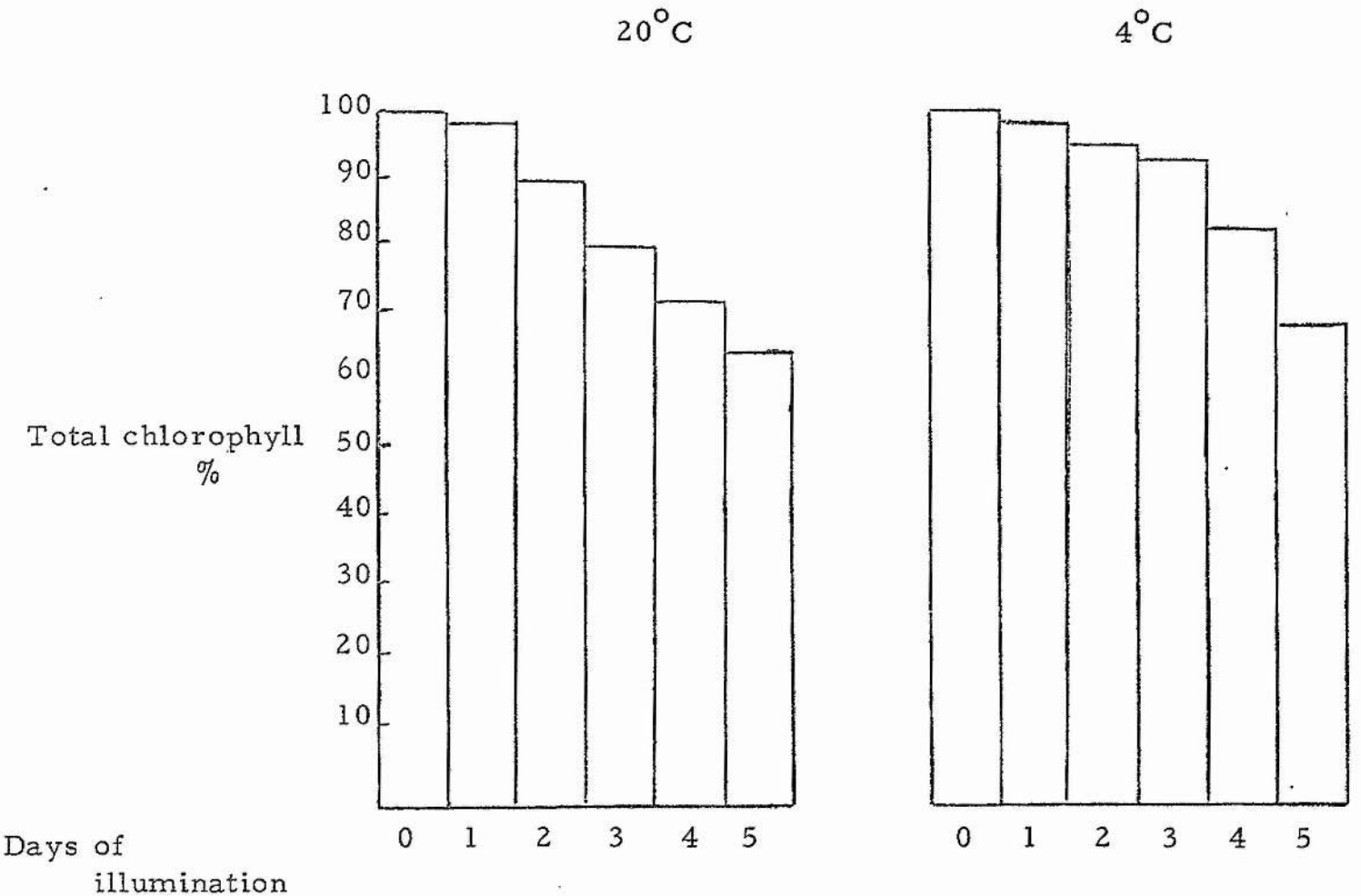


Fig. 56

Effect of temperature on bleaching of chlorophyll in lamellae solubilised with Cetavlon

CETAVLON

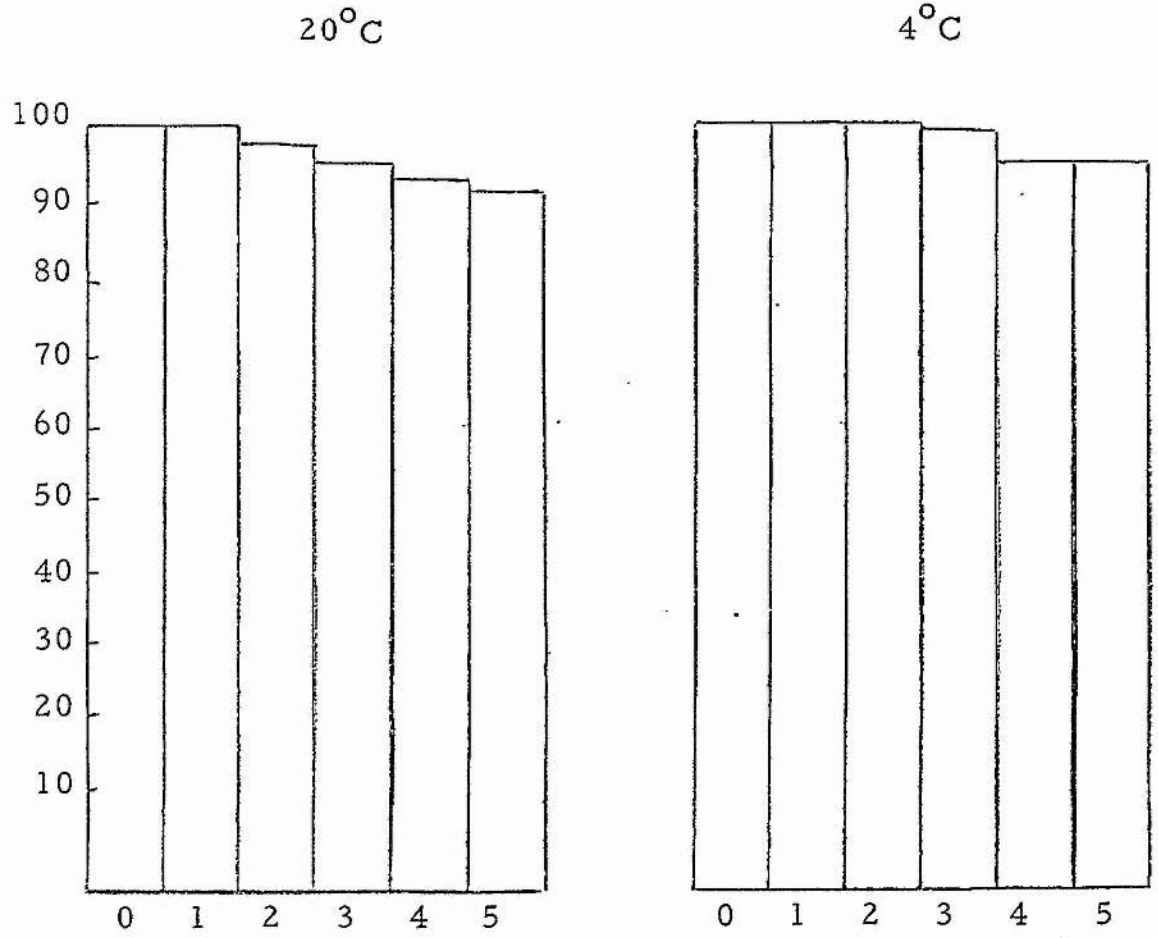


Fig. 57

Effect of temperature on bleaching of chlorophyll in lamellae solubilised with Lissapol NXP

LISSAPOL NXP

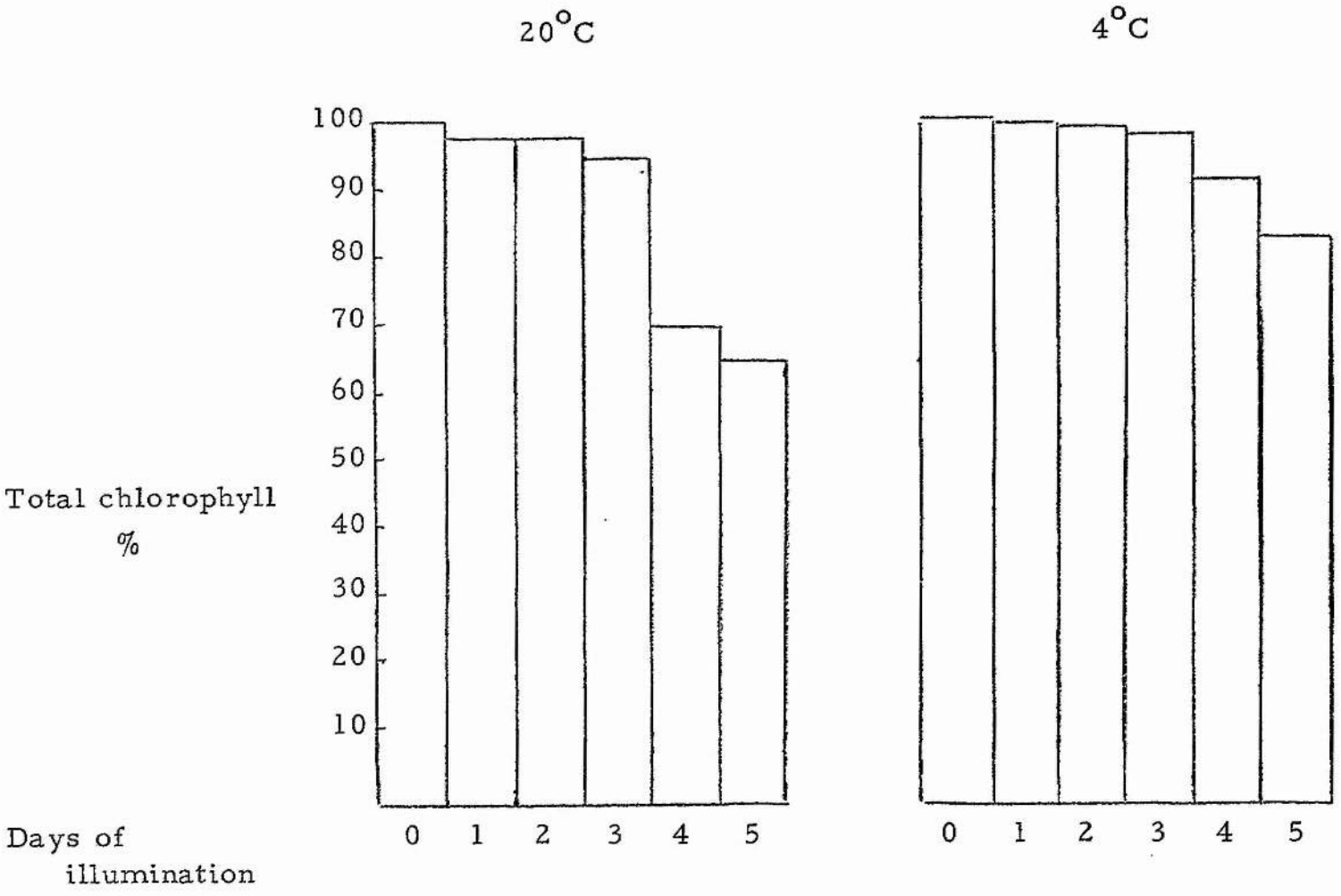


Fig. 58

Effect of temperature on bleaching of chlorophyll in lamellae solubilised with Tergitol 7

TERGITOL 7

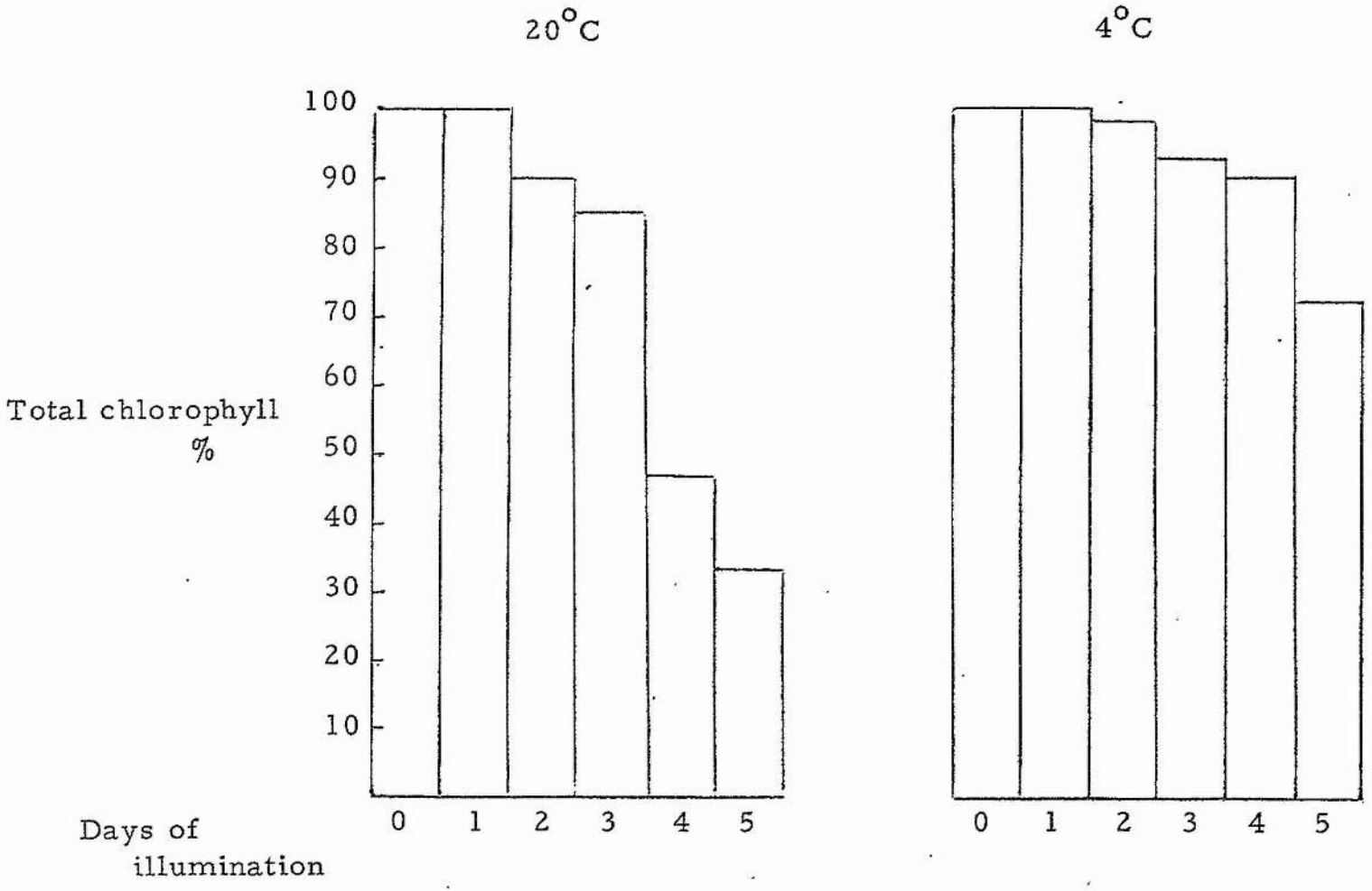


Fig. 59

Effect of temperature on bleaching of chlorophyll in lamellae solubilised with Renex 698

RENEX 698

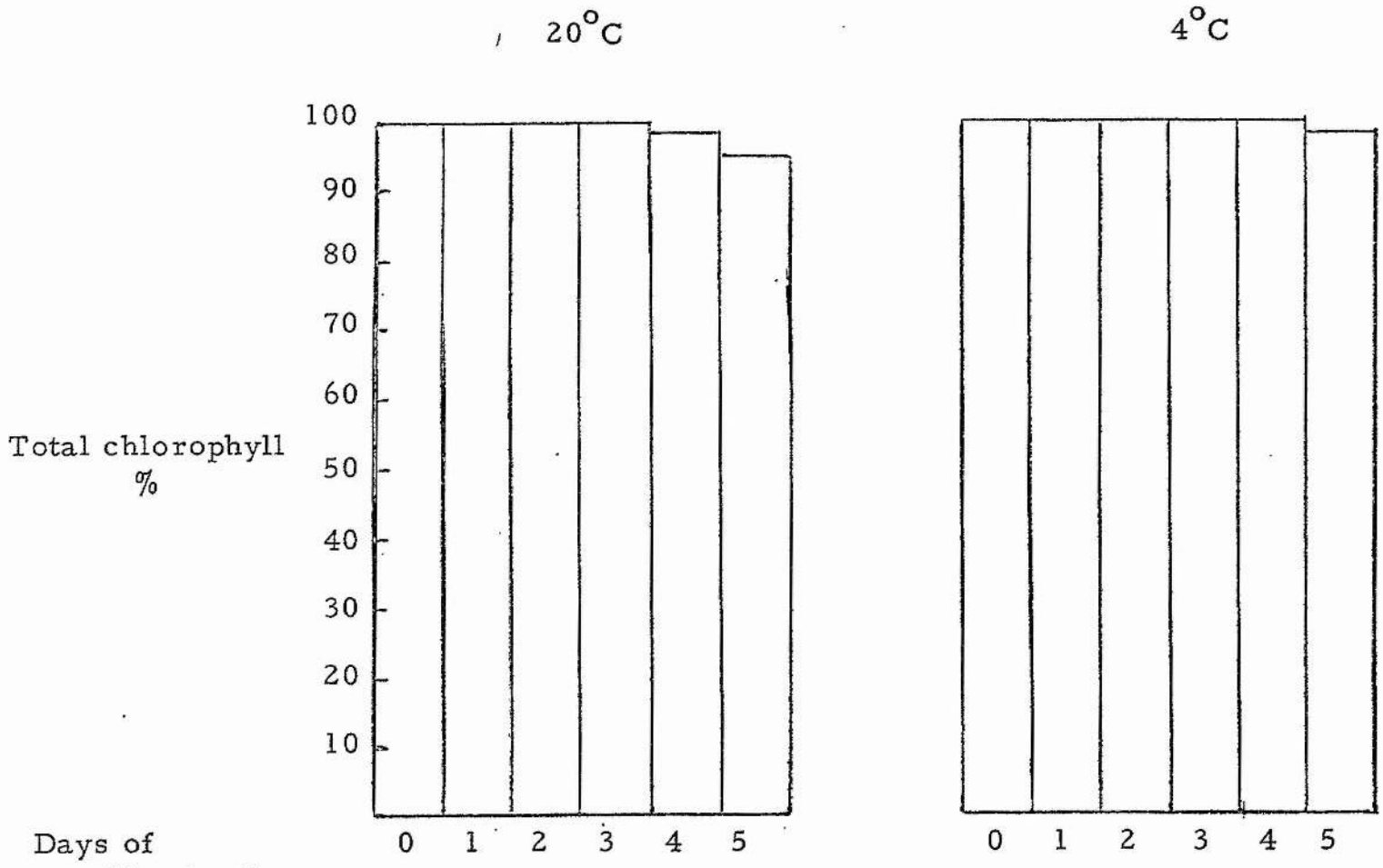


Fig. 60

Effect of temperature on bleaching of chlorophyll in lamellae solubilised with G 711

G 711

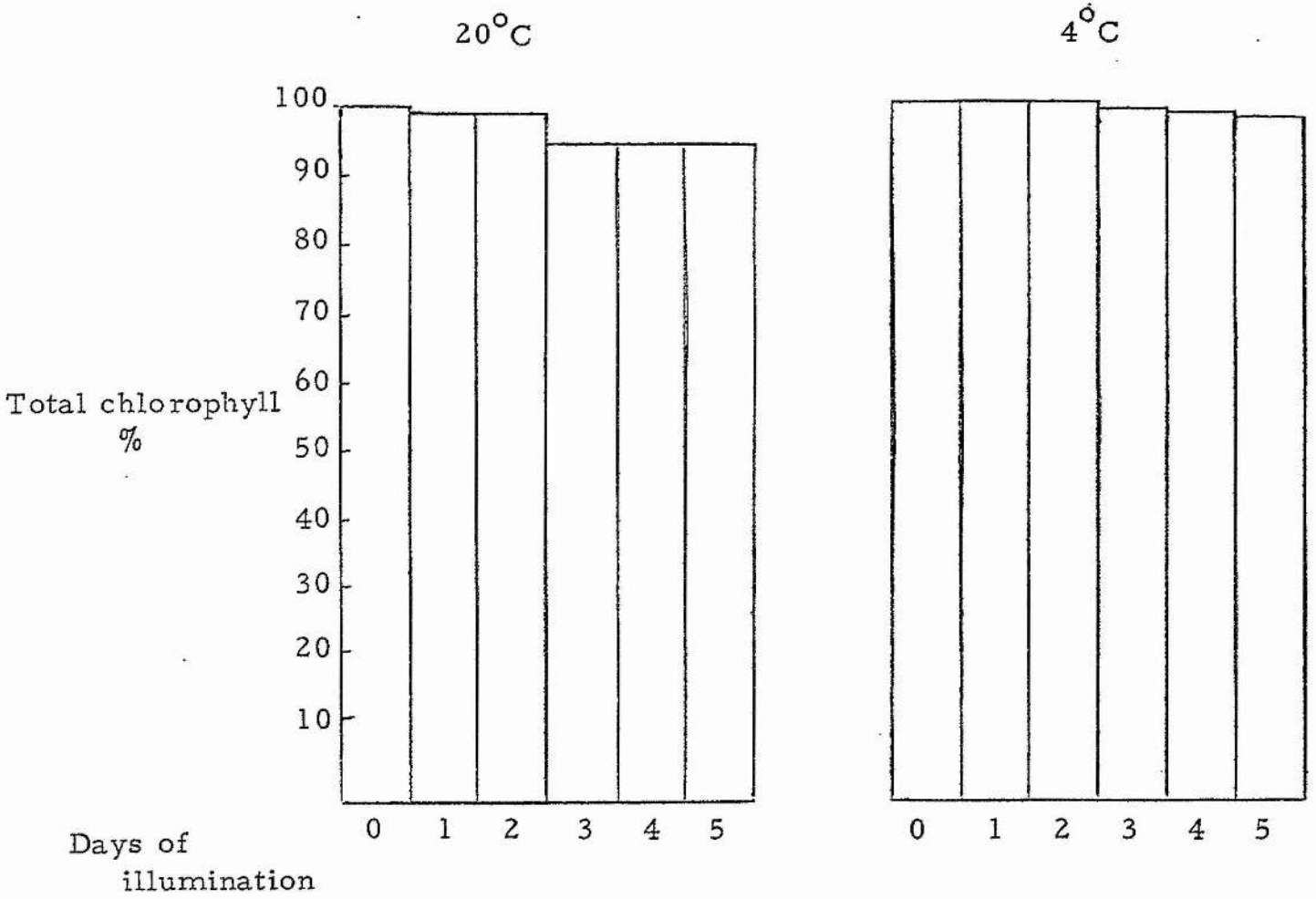


Fig. 61

R. 11 Nitrogen content of detergents

The nitrogen content of the detergents listed on pp. 214,215 was determined in accordance with the method detailed on p. 82.

Trace, or undetectable amounts of nitrogen were observed in the cases of all the detergents except five, given below:

(Results are average of three estimations)

Detergent	mg nitrogen/gram
Cetavlon	21.16
Fixanol VR	25.69
G711	24.71
Lissapol LS powder	10.35
Vantoc AL	4.43

DISCUSSION

Lamellar Solubilisation

The degree of lamellar solubilisation as estimated by extraction of pigments has been shown to vary greatly with different detergents under the same conditions. Surprisingly, there is no apparent relationship between the H.L.B. value and the ease of fragmentation. As mentioned previously, (Introduction p. 14) the bifunctional nature of detergent molecules renders them suitable solvents for both hydrophilic and lipophilic moieties. Thus it would be expected that the degree of lamellar solubilisation would bear a direct relationship to the H. L. B. value, and that there would be an optimum value for effect solubilisation. That this is obviously not so, is demonstrated by Nonidet P42 and Polychol 15, which both have an H. L. B. value of 12. 7 - 12. 8 and yet which represent extremes of the scale of solubilisation presented by the detergents under survey. It would appear from this work that the feature of over-riding importance with respect to lamellar solubilisation is the molecular structure of the detergent.

It is apparent from Table that the possession of

aromatic or other typesof ring structure, (e.g. S.D.B.S. digitonin respectively), are favourable molecular features in promoting lamellar breakdown, though there are cases (e.g. SDS, G 711) in which a strongly solubilising detergent has no ring structure. Examination of the solubilising abilities of Lissapol NXP, Lubrol L and Lubrol PF would indicate that at least in the case of polyoxyethylene alkyl aryl condensates, the length of the ethylene oxide chain is inversely related to the degree of lamellar solubilisation. (See Appendix, p. 214-215.).

This discussion makes no pretence that consideration of these features alone is exhaustive in assessing the ability of a detergent to fragment chloroplast lamellae. Other detergent properties such as micellar size would be expected to influence lamellar solubilising properties of detergents, possibly by restricting access of the solvent molecules to the lamellar matrix.

A feature of interest manifested by all the detergents studied is the increase in extractability of lamellae with increase in pH. This indicates an effect involving some charged groups within the lamellae which are required for binding of the matrix and are unstable at higher pH's due, possibly to release of counter ions. Since the effect is shown by all detergents to a relatively

similar extent, irrespective of their polarity, it is suggested that the detergent plays no part in the increased availability of lamellar material for solubilisation, but merely solubilises lamellar material fragmented by breakage of chemical bonds unstable at alkaline pH's.

Extraction of chlorophylls from lamellae by detergents

In previous reports, the maximum extent of differential extraction of chlorophylls a and b from lamellae by detergents is given by Thornber et. al. (1967a) who found that the slow-moving fraction on disc gels from lamellae solubilised with SDBS had a ratio of chlorophyll a to b of 12:1.

The lightest fractions produced from lamellae solubilised with the detergent G 711 however (which remain unprecipitated at 144,000 x g) have been found in this work to have ratios of chlorophyll a to b in excess of 18:1. This finding has been supported by spectral measurements of both heavy and light fractions produced with G 711 (fig. 2 0). It is apparent that this detergent shows selective solubilisation of chlorophyll a. It is

also of interest to note that G 711, which shows little affinity for chlorophyll b, displays remarkably little inhibition of System II reactions, which provides another evidence for the proposal of Losada et. al. (1961) that chlorophyll b is associated mainly with the oxygen evolving system, and further support for the conclusions drawn by Thornber et. al. (1967a) that System I particles are less tightly bound to the chloroplast lamellae since they are preferentially released.

A finding of this work however, could suggest an alternative theory, that the System II particles are bound to the lamellar matrix at least as strongly as the regions responsible for System I activity viz: It has been seen (table 7) that at high concentrations of detergent, the chlorophyll a/b ratio of heavy particles increases, suggesting that the extraction of System II fragments is reduced with increasing detergent concentration. Only low concentrations are required to split the System II particles from the lamellae. It is found (table 7) that higher detergent concentrations induce higher chlorophyll a/b ratios in the heavy (System II) fraction (- an observation which

agrees with the postulate that System II undergoes more ready dissociation in vitro than does System I, as discussed elsewhere, p. 191). The lower detergent concentrations which liberate particles of low chlorophyll a/b ratios, also yield light fractions with high chlorophyll a/b ratios, which suggests an approximately facile libration of the two photosystems from the lamellar matrix.

Spectral Absorption

The absorption spectral studies on lamellae solubilised by detergents performed by previous workers have done little but show that certain detergents cause a hypsochromic shift towards the blue, whilst others allow retention of the maximum of the red absorption band, at or very close to the in vivo position.

So far as is known from literature available, no report has appeared of such pronounced selective solubilisation of one of the chlorophylls by a detergent as is reported in this work for G 711, which selectively solubilises chlorophyll a in preference to chlorophyll b, evidenced by lack of absorption at around 650nm for the solubilised lamellae, and a marked shoulder in this region for the non solubilised sedimented material. (Fig. 20).

The absorption spectra which most closely resembled the spectrum of untreated chloroplast lamellae were produced by those

detergents (fig.14) which in low concentrations stimulated photochemical activities. No known previous report has cited any correlation between absorption spectral properties and photochemical activity of chloroplasts treated with detergents.

Fluorescence

Fluorescence measurements showed a distinct disparity with results reported by previous authors. Smith (1941b) noted a marked red fluorescence on illumination of SDS treated lamellae whereas no SDS lamellar extract showed any measureable fluorescence in this investigation. Further, the red fluorescence peak of Triton-solubilised lamellae was found to be higher by at least 4nm than the value of 680-682.5nm reported by Ke et. al. (1967).

Fluorescence at wavelengths in the blue region have not previously been reported. Some of the detergents covered in this survey did show blue fluorescence, but no correlation could be drawn between detergent structure and blue fluorescence. For example, extracts produced by Nonidet P-42 exhibit a double fluorescence peak at 400 and 460nm; extracts produced by the similar detergent Lissapol NXP fluoresce at 392nm when excited

with light at 345nm, but extracts from other polyoxyethylene alkyl aryl ethers (Renex 698, Triton X-100) exhibited no blue fluorescence.

Benzene extractability

According to Ke, et. al. (1956), the changes in benzene solubility of the pigments in aqueous chloroplast suspensions, induced by detergents are believed to result from the opposing effects of weakened pigment-lipoprotein bonding and affinity of the detergent for the pigments.

In all cases studied at two pH's in this survey, it was found that the higher pH induced a greater degree of extraction of the chlorophyll by the benzene. The higher pH may affect either (a) the detergent, possibly by altering the size or conformation of the micelles, so as to decrease the molecular attractive forces between the detergent and the chlorophyll lipoprotein or (b) the structure of the chlorophyll lipoprotein species arising from lamellar breakdown. It has already been noted (p. 95) that high pH's induce greater pigment extraction from the lamellae and that this is probably due to breakage of chemical bonds unstable under alkaline conditions. The greater

ease of removal of the pigments from the aqueous layer by benzene at these pH's would suggest that the bonds which undergo fracture are those joining chlorophyll to lipoprotein, thus rendering the chlorophyll more accessible to extraction by the benzene.

Trace Element Fractionation

Previously, only one case has been reported of fractionation of lamellae by detergents into particles with marked differences in trace element composition (Anderson, 1964). In this work, the action of digitonin in producing heavy lamellar fragments enriched in manganese has been confirmed and an additional three detergents (Renex 698, G 711 and Tween 20) have been shown to act on chloroplast lamellae in the same way.

It would appear that the molecular structure of the detergent is of considerable importance in this respect, since neither Triton X-100 (which closely resembles Renex 698) nor Crillet 4 (which is of similar structure to Tween 20) exhibited any discernible trace element fractionation.

No relationship may be drawn between detergent polarity and the degree of trace element fractionation of lamellae, since of the four detergents which were found to give a positive result, one is anionic, and the others are nonionic with differing polarities

(as deduced from their H. L. B. values, which lie in the range 13.0 - 16.7).

Effect of detergents on photosynthetic light reactions

The results of Vernon et. al. discussed in the Introduction (p. 28), have been confirmed for the effect of Triton X-100 on System I and System II activity. All other detergents which were found to have a similar effect were of the polyoxyethylene alkyl aryl ether type (Lissapol NXP, Lubrol E, Nonidet P-42 and Renex 698), which would indicate that this basic detergent structure promotes lamellar fragmentation in such a way as to stimulate System II activity at low detergent concentrations, possibly by sterically hindering some site normally concerned with restraint of the short wavelength response, or possibly by selective solubilisation of some regulator of this response. This effect was not found to be a manifestation of all polyoxyethylene alkyl aryl ethers however, since it was not shown by those possessing an ethylene oxide chain of more than 12 residues.

Generally, the detergent concentrations required to abolish oxygen photoevolution were found to be considerably lower than concentrations of the same detergent required for

abolition of System I activity. It has been suggested previously (Rumberg, 1964) that System I is considerably more stable than System II, since in the presence of suitable electron donors System I remains capable of carrying out its reaction after heating or prolonged storage of chloroplasts, whereas such conditions rapidly abolish oxygen photoevolution. The stability of System I in vitro is thought to reflect the strong binding of the system to its reaction centre, P700, in comparison with the affinity of System II for its reaction centre. This view is supported by the work conducted in this thesis on the relative effects of added detergents on the two photosynthetic light reactions, in which the tolerance of System I activity to added detergent was found to be consistently greater in all cases than that of System II.

It should be noted however that the easier abolition of System II activity than System I activity, by detergent action could also be a consequence of positional, steric or ionic attraction factors viz:

1. In comparison with the System I reaction centre, the System II reaction centre could occupy a more superficial position on the lamellae and hence be more susceptible to detergent penetration.

2) The shape of the System II reaction centre could be of a more closely corresponding form to the detergent micelle than that of the system I reaction centre. The former reaction centre thus undergoes more ready steric hindrance with concomitant loss of photochemical activity.

3) The ions with which System II is thought to be closely concerned (Mn^{++} and Cl^{-}) could attract charged detergent micelles towards the reaction centre, which becomes blocked and rendered photochemically inactive.

Fractionation of detergent-solubilised lamellae

During the last thirty years, as previously mentioned (Introduction, p. 37), numerous attempts have been made to isolate characterisable chlorophyll-proteins from lamellar detergent solutions. A problem which has eluded discussion in all the reports observed in the literature to date, concerns the fractionation of solutions containing micelles. Whatever method is employed to separate chlorophyll-protein species from detergent solubilised lamellae, the separation in the first instance will be a separation of micelles containing solubilised chlorophyll-protein. Thus any migration of the chlorophyll protein which is observed, may be a manifestation at least in part, of the properties of the detergent. Furthermore, it can easily be envisaged that a detergent will solubilise for example, two types of native chlorophyll-protein. Now, unless a micelle sobulilises only macromolecules of one of these types (an improbable situation, since the micelles of one detergent are unlikely to be very different in size or physical properties), it is reasonable to expect that macromolecules of both species

of chlorophyll-protein may be present dissolved in a single micelle. To fractionate further would require the micelles to be split. This is not possible without destroying the solubilising medium, and thus rendering the chlorophyll-proteins insoluble and possibly inactivated by the agents employed in the micellar breakdown.

Thus it has apparently been assumed by authors previously who have employed single detergents to fractionate more than one chlorophyll protein, that there has been selective solubilisation of different chlorophyll-protein species by different micelles. This work has attempted fractionation along similar lines to those already followed. Bearing in mind the need for caution in interpreting results of fractionation of detergent solubilised lamellae, the data indicate that some degree of fractionation is possible by DEAE cellulose chromatography or by polyacrylamide gel electrophoresis from a wide range of different types of detergent.

The tendency for higher pH's to promote electrophoretic separation of coloured bands on polyacrylamide gels indicates

that the association of these moieties involves participation of molecules or ions unstable at alkaline pH's. This is further supported by the finding that no such fractionation was found to be possible on polyacrylamide gels from lamellar solutions in cationic detergents.

Photooxidation of ascorbate

Not all the detergents studied produced photochemically active chloroplast preparations but those which did, appeared to show an increasing activity in ascorbate photooxidation with decrease in size of lamellar fragments. This may be due to the increased molecular surface area available for contact between photoreactive molecules, caused by lamellar splitting. This would be in agreement with the observation of Hinkson et. al. (1959) that lamellar fragments produced by the action of digitonin, were more than six times as active in photoxidising ascorbate in the presence of dichlorophenol indophenol as the original unbroken chloroplasts.

The general increase in photooxidative capacity with decrease in size of lamellar particles was found to be true of the material fractionated by differential centrifugation but not so for the photoreactive Renex 698 or Calsolene Oil fractions isolated on

DEAE cellulose columns from the supernatant of centrifugations at 144,000 x g, possibly owing to the removal of material necessary for efficient photooxidative activity of the chlorophyll, by the ion exchanger.

Stability of lamellar detergent extracts in light

The wide range of stability shown by lamellar detergent extracts, depending upon the nature of the detergent indicates that the modes of action by detergents upon chloroplast lamellae are of considerable variety.

Certain points emerged from this study which merit further discussion. In general, the stability of lamellar detergent extracts to bleaching has been found to be directly dependent on the concentration of the chlorophyll, in anaerobic conditions. It has been known for some time that chlorophyll bleaches rapidly in the presence of oxygen and light, (Aronoff et. al. 1943) and that this is a photooxidative bleaching requiring both light and oxygen.

In anaerobic conditions, low chlorophyll concentrations appear to promote pigment breakdown, possibly by being able to support saturating light conditions in a photodecomposition process.

The extracts of Tergitol 7, displayed a very rapid breakdown at all concentrations once the bleaching process had commenced, but exhibited a distinct stability in the early period of illumination, which suggests that one of the products of decomposition sensitises further chlorophyll breakdown. Since this does not occur with the other detergent extracts, the sensitising species may possibly require to interact in some way with the detergent molecules in order to be active.

Of the six detergents studied, the two which conferred greatest stability to bleaching on lamellar pigments, are noted to be the only two which yielded positive results in the assays on photochemical activity. Renex 698 stimulates System I activity under certain conditions, and G711, whilst not actually stimulating any photochemical activity, does show appreciably less inhibitive effect on oxygen photoevolution than the other detergents.

These observations infer a direct relationship between pigment stability to bleaching, and retention of the functional identities of the photochemical apparatus. In vivo, the pigment

molecules are stable to bleaching presumably by virtue of their association with the neighbouring protein and lipid species. It is suggested that Renex 698 confers stability upon the pigments by extracting them along with some of their normal lipoprotein environment.

The tendency for higher pH's to promote greater resistance to bleaching of the pigment molecules is of interest, as it has been stated elsewhere (p. 95). that in terms of pigment concentration, the extractability of lamellae increases with increase in pH. As noted previously it is suggested that the detergent plays no part in the increased availability of the lamellar material but merely solubilises lamellar material fragmented by breakage of chemical bonds unstable at alkaline pH's. The greater stability may result from mutual protection under non-saturating light conditions of the pigment molecules, as discussed above.

It is difficult to draw meaningful conclusions from the effects of oxygenation upon the bleaching of chlorophyll in detergent treated lamellae exposed to light. It was found that one polyoxyethylene detergent (Brij 35) did confer stability to bleaching on chlorophyll in solubilised lamellae, whereas a related polyoxyethylene derivative (Lissapol NXP) did not. Similarly, one anionic detergent (G711) rendered extracted chlorophyll stable whilst another, (Tergitol-7) had the opposite effect.

It appears that the availability of oxygen to detergent micelles containing dissolved chlorophyll lipoprotein is dependent on the detailed molecular structure of the detergent rather than its general structure or ionic nature.

Comparison of table 7 with tables 23-25 and comparison of fig. 10 with table 26, indicates from the decreased chlorophyll a : b ratio in the anaerobic experiments that oxygen has a more deleterious effect upon chlorophyll b than it does on chlorophyll a in the presence of detergent.

SUMMARY

The effects of detergent action on several photochemical and physical properties of chloroplast lamellae have been investigated.

The general observations made by other authors who have employed detergents in previous reports have been largely confirmed.

It has not however, been found possible to ascribe any of the particular effects which have been observed on the properties of lamellae, to any characteristic of molecular structure and/or polarity of the detergents studied.

One detergent of particular interest has emerged from this survey, in the form of G. 711. No other instance is known of any detergent which is an efficient solubiliser of chloroplast lamellae and yet which does not inhibit System II reactions to a high degree at low concentrations of detergent. Of the two photosynthetic photoacts, least is known of the short wavelength System II. The detergent may be of considerable use in future work on the elucidation of the mechanism of the photosynthetic photoevolution of oxygen from water.

NOTE ADDED IN PROOF

Following the completion of the majority of the work covered in this thesis, a report of considerable importance concerning lamellar fragmentation by detergents has appeared. (Huzisige et. al. 1969).

As mentioned previously, (Introduction p. 31-2) no reference had appeared in the literature with regard to the isolation of a functional photosystem II particle, uncontaminated with photosystem I activity. Huzisige et. al. however, employing the detergents digitonin and Triton X-100, separated the two photosystems in functional form by differential and density gradient centrifugation.

The most interesting feature of the particulate fractions is that they retained only one of the two photochemical activities, - completely lacking any capacity for the counterpart reaction as shown below:

Fraction	Chlorophyll a/b ratio	Photochemical Activities	
		NADP * photoreduction	O_2 ≠ photoevolution
Chloroplast suspension	3.0	72	606
System I particle	6.8	620	0
System II particle	2.0	0	813

* mu moles reduced/mg chlorophyll/minute; electron donor couple: DCPIP/ascorbate.

≠ mu moles evolved/mg chlorophyll/minite; oxidant:DCPIP.

Furthermore, the activities of the isolated particulate photosystems exceeded the corresponding activities of the chloroplast suspension which constituted the starting material.

In addition, a reconstitution of the two photosystems was attempted, and small but measurable rates of NADP photoreduction, using water as electron donor were reported.

Thus this paper describes a method whereby the two photosynthetic light dependent processes may be isolated in particulate form and then recombined, exhibiting a co-ordinated

photochemical activity, by use of more than one detergent in the fragmentation procedure.

This would indicate that a fruitful approach to this problem in future may involve use of several different detergents chosen from their known effects in lamellar fragmentation, for selective extraction of lamellar subunits.

APPENDIX

General Properties of Detergents

Detergents are a class of substances also referred to as surface active agents or surfactants, which by virtue of their molecular structure, are suitable solvents for both lipophilic and hydrophilic substances since they possess polar and apolar groupings within the same molecule.

The molecules of a detergent associate in water to form colloidal particles called micelles. These structures have an arrangement in which the apolar groups form the interior, protected from the solvent by the polar groups which are clustered around the periphery.

The apolar groups of the micelles render aqueous micelle-containing systems capable of dissolving apolar substances (solubilisates). Such apolar moieties would dissolve in the minute apolar interior of the micelles.

The single molecules which associate to form micelles may be classed into four main groups:

1. Anionic In these, the anion is the surface active species.

e.g. Sodium dodecyl sulphate (SDS)

$$CH_3 (CH_2)_{11} SO_4^- Na^+$$

2. Cationic Here, the cation is the surface active species.

e.g. Cetyl trimethyl ammonium bromide (Cetavlon)

$CH_3 (CH_2)_{15} N^+ (CH_3)_3 Br^-$

3. Ampholytic These can behave as either anionic, cationic or non ionic according to the pH of the solution.

e.g. N-dodecyl-N:N-dimethyl betaine.

Zwitterionic form is: $C_{12}H_{25}N^+(CH_3)_2 CH_2 COO^-$

4. Non ionic The polar groups of this type are usually hydroxyl groups or a polyoxyethylene chain.

e.g. Lubrol L.

$C_9H_{19}C_6H_4O(CH_2CH_2O)_{13}H$

The micellisation process of a surfactant in water occurs as a result of the large interfacial energy between the apolar groups and the water molecules. Progressive addition of the monomer to water increases the total free energy of the system. The formation of micelles is a direct result of this increase in free energy and decrease in entropy, concomitant with the dissolving of the monomers, which allows a reduction of the free energy and an increase in the entropy of the system. Micelle formation occurs

when no further free energy may be dissipated simply by adsorption of the detergent molecules at the solution/air interface or by dimerisation of the detergent molecules. The concentration at which this occurs is the critical micelle concentration (CMC). Accurate experimental work shows that this is not a single sharp concentration, but rather, a narrow range. The CMC may be determined by estimation of any physical property which varies according to whether the solute is monomeric or aggregated. (For review, see Shinoda et. al. 1963). Measurements of diffusion, viscosity, sedimentation velocity and ultrfiltration may provide information in addition to the CMC, on the size, shape and solvation of micelles. The work of Ekwall (1963) indicates that at concentrations immediately above the CMC, for ionic paraffin chain surfactants, the micelles which form are spherical, but at higher concentrations, rod-shaped or plate-like micelles appear in the system.

The degree of solubilisation of a solubilisate by a detergent is affected by temperature, by addition of

electrolytes and certain non electrolytes, as well as by the nature of the solubilisate and the surfactant. The most important factor affecting solubilisation of a solubilisate is the relative polarities of the surfactant and the solubilisate. It is convenient to assign to detergents a value known as the hydrophile-lipophile balance (HLB) which gives an indication of the hydrophilicity (see pp. ~~211, 212~~ 214, 215). Values from 0 to 20 may be obtained, the higher values representing relatively hydrophilic surfactants, and low values, those possessing a large hydrocarbon to hydrophile ratio.

Properties of detergents under review

	Detergent	Type	Nature	4%* pH 10% aqueous soln.	H.L.B. value
1	Brij 35	N	Polyoxyethylene (23) lauryl ether	2.50	16.9
2	Brij 58	N	Polyoxyethylene (20) cetyl ether	6.50	15.7
3	Brij 96	N	Polyoxyethylene (10) oleyl ether	5.40	12.4
4	Brij 98	N	Polyoxyethylene (20) oleyl ether	4.80	15.3
5	Calsolene Oil	A	Sodium salt of highly sulphated oil	7.40	30 (Z)
6	Cetavlon	C	Cetyl trimethyl ammonium bromide	4.20	X
7	Cithrol A	N	Polyoxyethylene (9) glycol oleate	8.12	11.0 - 12.0
8	Crillet 1	N	Polyoxyethylene (20) sorbitan monolaurate	3.89	16.7
9	Crillet 4	N	Polyoxyethylene (20) sorbitan mono oleate	6.27	15.0
10	Decon 75	A (M)	X	12.43	X
11	Digitonin	S	Steroidal $C_{56}H_{92}O_{29}$	7.40*	X
12	Dispersol VLX	N	Polyoxyethylene (22) condensate with cetyl/stearyl alcohols	7.10	15.8
13	Fixanol VR	C	Tetradecyl pyridinium bromide	1.70	20 (Z)
14	G 711	A	Isopropylamine dodecyl benzene sulphonate	4.45	11.7
15	Lissapol LS powder	A	Sodium oleyl p-anisidine sulphonate	8.20	20 (Z)
16	Lissapol NXP	N	Polyoxyethylene (10) nonyl phenol condensate	5.93	13.3
17	Lubrol AL18	N	Polyoxyethylene (13) condensate with fatty alcohols	4.51	13.8

Properties of detergents under review

	Detergent	Type	Nature	4% * pH 10% aqueous soln.	H. L. B. value
18	Lubrol E	N	Polyoxyethylene (7.5) octyl phenol condensate	6.25	12.3
19	Lubrol L	N	Polyoxyethylene (13) nonyl phenol condensate	7.70	14.4
20	Lubrol PG	N	Polyoxyethylene (30) nonyl phenol condensate	6.60	17.2
21	Lubrol W	N	Poloxyethylene (17) condensate with cetyl/stearyl alcohols	3.75	14.9
22	Monoxol OT	A	Di octyl ester of sodium sulphosuccinic acid	4.20	X
23	Nonidet P42	N	Polyoxyethylene (9) di octyl phenol condensate	6.30	12.8
24	Polychol 15	N	Polyoxyethylene (15) wool wax alcohol condensate	3.54*	12.7
25	Renex 698	N	Polyoxyethylene (9) alkyl aryl ether	4.25	13.0
26	SDBS	A	Sodium dodecyl benzene sulphonate	10.20	X
27	SDS	A	Sodium dodecyl sulphate	8.62	X
28	Tergitol 7	A	27% solution of sodium sulphate of 3, 9-diethyl tridecanol-6	10.09	X
29	Tergitol Anionic 08	A	40% solution of sodium sulphate of 2-ethyl-1-hexanol	9.20	X
30	Triton X-100	N	Polyoxyethylene (9) iso octyl phenoxy ethanol	5.67	X
31	Tween 20	N	Polyoxyethylene (20) sorbitan monolaurate	5.65	16.7
32	Tween 80	N	Polyoxyethylene (2) sorbitan mono oleate	5.37	15.0
33	Vantoc AL	C (M)	10% aqueous solution of higher alkyl trimethyl ammonium bromides	5.80	20 (Z)
34	Volpo N-10	N	Polyoxyethylene (10) oleyl ether	6.42	12.5

KEY: N: Non ionic; C: cationic; A: anionic; S: steroidal; (M) : Mixture X : information not available Z : approximate value

Distributors of surface active agents employed in this survey.

Detergent	Distributor	Detergent	Distributor
Brij 35	H.	Lubrol E	I.C.I.
Brij 58	H.	Lubrol L	I.C.I.
Brij 96	H.	Lubrol PF	I.C.I.
Brij 98	H.	Lubrol W	I.C.I.
Calsolene Oil	I.C.I.	Manoxol OT	B.
Cetavlon		Nonidet P42	B.
Cithrol A	C.	Polychol 15	C.
Crillet 1	C.	Renex 698	H.
Crillet 4	C.	SDBS	L.
Decon 75	M.	SDS	K.
Digitonin	S.	Tergitol 7	B.
Dispersol VLX	I.C.I.	Tergitol Anionic 08	B.
Fixanol VR	I.C.I.	Triton X-100	B.
G711	H.	Tween 20	H.
Lissapol LS powder	I.C.I.	Tween 80	H.
Lissapol NXP	I.C.I.	Vantoc AL	I.C.I.
Lubrol AL 18	I.C.I.	Volpo N-10	C.

Key

B: British Drug Houses Ltd., Macfarlane Robson Ltd., Burnfield Avenue, Thornliebank, Glasgow S.3.

C: Croda Ltd., Cowick Hall, Snaith, Goole, Yorks.

H: Honeywill Atlas Ltd., Honeywill and Stein Ltd., Mill Lane, Carshalton, Surrey.

K: Moch-Light Laboratories Ltd., Colnbrook, Bucks.

L: Lankro Chemicals Ltd., Eccles, Manchester.

S: Sigma London Chemical Co. Ltd., 12 Lettice St., London S.W.6.

ICI: Imperial Chemical Industries Ltd., Dyestuffs Division, Blackley, Manchester.

M: Medical Pharmaceutical Developments Ltd., Ellen St. Portslade. Brighton BN4 1EQ.

ABBREVIATIONS

DCPIP	:	Dichloro phenol indophenol
NADP	:	Nicotinamide adenine dinucleotide phosphate
DEAE	:	Diethyl amino ethyl (cellulose)
Chl	:	Chlorophyll
M	:	Molar
U.V.	:	Ultra violet
nm	:	nanometre
λ	:	wavelength
ml	:	millilitre
hr	:	hour

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