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Biosynthesis and Function of The Pigment of Blepharisma Intermedium (*Indian species*)

CHANDER KANTA KHURANA

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C. K. Khurana

C . K . KHURANA

Sivatosh Mookerjee

SIVATOSH MOOKERJEE
Supervisor

Sivatosh Mookerjee

SIVATOSH MOOKERJEE
Dean

School of
Life Sciences
Jawaharlal Nehru
University
New Delhi

A C K N O W L E D G E M E N T S

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C. K. Khurana

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PREFACE

The colouration in an organism has always evoked curiosity of a zoologist and a chemist. The interest of a zoologist is in the biological function of the zoochrome while that of a chemist is in its structure, origin; and the corelation of these two with the function. From both directions much light has been shed on the many mysteries regarding the nature, origin and function of the zoochromes (Needham, 1974).

Blepharisma, a holotrich ciliate is characterised by the presence of a photo-sensitizing pink to red pigment called 'zoopurpurin' by Arcichovskij (1905) and 'Blepharismin' by Sevanant (1965). This pigment is unique and the only animal pigment reported so far which not only kills its host cell but also the other colourless ciliates, flagellates, marine eggs etc. in the presence of strong light (above 2,000 foot candle) and oxygen (Giese, 1953, 1946, 1957; Giese and Zeuthen, 1949). Stentor nigor, another ciliate also contains photosensitising pigment but unlike Blephasimin it does not kill its own cell (Tartar, 1961). With the exception of few wild type bacteria,

the photosensitising pigments resembling Blepharismin in behaviour have been reported only in the mutant forms of bacteria, plants, animals including man (for summary, Giese, 1971 b). It seems then that the Blepharisma is the animal which has incorporated in wild type, in its genome a liability in the form of Blepharismin. Yet surprisingly, no albino has been collected in nature or reported, except for one short lived albino mutant of pink strain of B. stolci V. narai collected in Japan (Inaba et al, 1958). On the other hand, it has been observed that the laboratory generated albino mutant of pink Pedersoo strain and another from a cyst of a pink strain of B. americanum remained colourless for three months and later became pink again (Giese, unpublished).

Why has Nature bestowed upon Blepharisma a liability, and yet invariably almost all the species are pigmented and above all the deeply pigmented ones always appear to be more vigorous and are bigger in size compared with the phenotypic albinos and the less pigmented varieties (Giese, 1973)?

Giese (1965) has observed that the Blepharismin acts as a screen and prevent the animal from the damaging far-uv radiations; if that is the only function of the pigment then how are the colourless protozoa surviving in nature. Is it due to the photorevoral enzyme system which is about 95% developed in protozoan as compared to only 30% in Blepharisma (Giese, 1967), that the nature is to compensate the defect,

if so then why in a lethal form? To get some answer to these anomalous queries the present study has been undertaken.

Three inter-related aspects were taken using B. intermedium (indian species) as the experimental material. This species was chosen for its large size (200-350 µm) and well organised deep pink pigment granules:

First aspect concerns the structure of pigment: This is an essential prelude to biosynthesis of any pigment. Møller (1962), and Sevenant (1965) have shown that pigment of B. undulans (american species) is some what like hypericin; a photosensitising plant pigment of the family Guttiferae. They have based their observation on the absorption, infrared and fluorescence spectra and have proposed only a tentative structure. In this laboratory the molecular weight, molecular formula and structural formula have been elucidated. The present findings are based on the spectro-scopic techniques like nuclear magnetic resonance, mass spectroscopy besides the ultraviolet absorption, infrared and fluorescence spectroscopy.

Second aspect concerns with the biosynthesis of the pigment. No tangible work has been so far done in this area. The present study can be summarised in two stages, one where attempts have been made to find out the route of the pigment formation, viz. it's formation during protein synthesis from the gene or during the metabolic processes and secondly the

nature of precursors involved in pigment synthesis. In the former the studies have been done using DNA, RNA, protein synthesis and metabolic inhibitors while the later studies have been done using radio isotope feeding of the precursors and the enzymatic studies.

The third aspect is the function of the pigment. The biological function of the pigment as a photo protector in dim light vis a vis its role in giving size, vigorousity has been studied. The albinos have been generated in the laboratory (Giese and Grainger, 1970) and the effect of those wave lengths which are absorbed by the pigment and the protein synthesis in the pigmented and albinos has been measured. From the finding an attempt has been made to corelate the function with the structure of the pigment.

CHAPTER I

STRUCTURE OF THE PIGMENT

INTRODUCTION

Blopharisma intermedium has deep pink pigment granules, concentrated mainly below the pellicle. Some insight into the structure was essential before the experiments on biosynthesis could be undertaken. The only work reported in the literature is on the pigment of B. undulans (american species) (Møller, 1962; Sovenant, 1965) and a tentative structure based on indirect evidences has been proposed. The detailed chemical study probably could not be undertaken because of the fact that the animal contains the pigment only in micro quantity. Fortunately, with the recent advances in the better resolution of the spectro-scopic methods, it is now possible to elucidate the structure more cogently with quantities available in micro-grams and milligrams.

In the present investigations a structural formula is proposed based on the direct evidences and compilation of the data obtained from ultra-violet visible, infra-red spectroscopy, nuclear magnetic resonance and also mass spectroscopy. The fluorescence spectrum was taken mainly to compare the pigment with the pigment of B. undulans. The qualitative tests which preceeded the spectroscopic studies were done to get some idea specially about the presence of phenolic groups in the molecule.

MATERIALS AND METHODS

MATERIALS

Wild type Blepharisma intermedium constituted the material. The Blepharismas were cultured in 0.5% hay infusion, which after boiling, cooling and filtering was fortified with Horlick milk. About 500 mg horlicks was added to one litre of the hay medium. The culture was maintained in the B.O.D. chamber in the dark at 25±3°C.

Chemicals - The chemicals and their grade, used in this study are:

Acetone A.R., Sodium Chloride, carbontetra-chloride(A.R.), diethylether, distilled absolute alcohol, potassium bromide, Folin-ciocalteu reagent, ferric chloride, sodium carbonate, sodium hydroxide, aceticanhydride

All these reagents were from the British Drug house. Pure nitrogen gas was obtained from Indian Oxygen Company.

METHODS

(1) Collection of the animals:

On the fifth day after inoculation of the medium, the hay medium having thick suspension of animals were centrifuged at 1,000 to 1,500 rpm for 1 minute. The animals settle at the bottom of the centrifuge tube.

(2) Purification of the pigment:

The pigment from the centrifuged animals was extracted in acetone and the extract dried at room temperature. The dry

film of the pigment was washed three times with carbon-tetrachloride to remove the lipids; and the contents dried in nitrogen. The pigment was redissolved in acetone and transferred to a separating funnel (Giese and Grainger, 1970). Diethyl ether and dilute solution of sodium chloride were added and the contents shaken. By this step the pigment from acetone goes into the ether. Acetone and other water soluble impurities were removed by several washings (5-10) with dilute sodium chloride solution. The pigment extract was filtered and dried in nitrogen atmosphere at 60°C.

(3) Qualitative tests :

A few qualitative tests were done for ascertaining the presence of phenolic hydroxyl group in the pigment molecule.

(a) 1 ml of concentrated solution of the pigment was treated with 1 ml of Folin-ciocalteu reagent and 2 ml of 20% (v/v) solution of sodium carbonate. The reaction mixture was warmed to about 60°C. (Folin and Ciocalteu, 1927).

(b) The concentrated solution of the pigment in 50% ethyl alcohol was treated with few drops of 0.5 N freshly prepared neutral ferric chloride solution (Bray and Thorpe, 1967).

(c) The pigment solution in acetone was made alkaline with sodium hydroxide and then acidic with dilute sulphuric acid. (Thomson, 1971).

(4) Spectroscopic methods :

(i) Ultra-voilet visible absorption spectrum :

The purified pigment was dissolved in distilled ethyl alcohol and the spectrum was taken on the Beckmann spectrophotometer.

(ii) Infra-red spectrum :

The IR spectrum of the solid was taken as KBR pellet. The measurement was done in Grub Parson (London) double beam infra-red spectrophotometer at Indian Institute of Technology, New Delhi.

(iii) Nuclear magnetic resonance spectrum :

The nuclear magnetic resonance of the purified pigment was done in deutero-acetone in 60 MH Varian spectrometer at Reading University, Reading (U.K.). The readings were taken at 250 and 500 sweep widths.

(iv) Mass Spectrum :

Mass spectrum was taken in mass spectrometer at 200°C at Reading University, Reading (U.K.)

(v) Flouresconce spectrum :

The spectrum of pure pigment using acetone solvent was taken in flourimeter fabricated in the Life Sciences department of Jawaharlal Nehru University, New Delhi.

EXPERIMENTAL RESULTS

Qualitative tests

(a) Folin-ciocalteu tests :

The pigment solution when treated with Folin-ciocalteu reagent and sodium carbonate solution on warming to 60°C developed blue colour. The formation of blue colour is characteristic of phenols, that is the benzene hydroxyl groups.

(b) Ferric chloride test :

The pigment solution when treated with neutral ferric chloride solution developed greenish colour. This again indicates the presence of phenolic group in the pigment molecule.

(c) Colour change with pH :

The pigment solution which was red changed to blue in the alkaline pH when treated with sodium hydroxide solution and again turned pink on acidification (acidic pH). The change in colour in the alkaline solution indicates that the molecule is a hydroxyquinone.

Thus the presence of phenolic hydroxyl group and hydroxylquinone group in the pigment were indicated from the above tests.

Spectroscopic results

(i) Ultra-violet visible spectrum :

The ultra-voilet visible spectrum of the pigment in alcohol shows absorption in the far uv, near uv and the

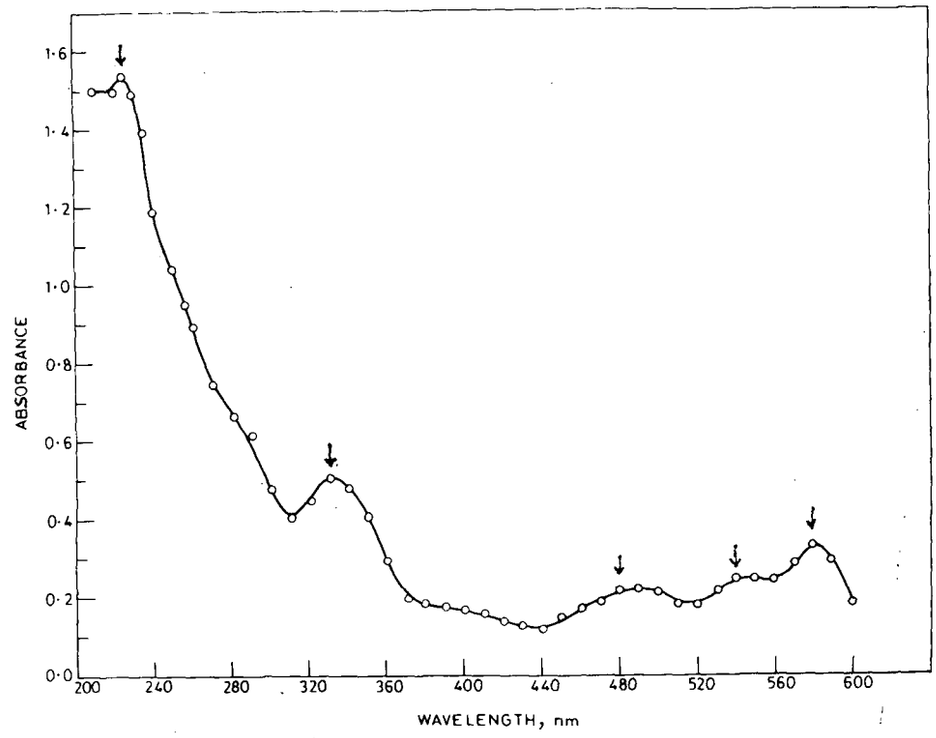

FIG. 1.1. UV ABSORPTION SPECTRUM IN ALCOHOL

Fig. 1.1 **The ultra-violet visible absorption spectrum of pure pigment in absolute alcohol. The arrows indicate absorption peaks.**

visible regions. The absorption peak in the far uv is at 225 nm; in the near uv it is at 330 nm, and in the visible region the peaks are at 480, 540 and 580 nm (Fig. 1.1 and Table 1.1). The maximum absorption (λmax) is in the far uv. The 225 nm band represents the quinone absorption overlaping the benzenoid absorption, 330 nm is the benzenoid absorption while the bands at 480, 540 and 580 nm are due to α-hydroxy groups. The number of α-hydroxyl groups may be four or more and the general spectrum seems to be belonging to α-hydroxy higher quinones (Scott, 1964: Brockmann, 1957).

Table 1.1

The ultra-voilet visible absorption spectrum of purified pigment in alcohol. The absorption peaks are marked (-)

Wave length in nm	Absorbance	Wave length in nm	Absorbance	Wave length in nm	Absorbance
210	1.5	310	0.41	450	0.14
215	1.5	315	0.42	460	0.17
220	1.5	320	0.45	470	0.19
225	1.55	325	0.49	480	0.22
230	1.5	330	0.50	490	0.22
240	1.2	335	0.50	500	0.21
245	1.2	340	0.49	510	0.18
250	1.0	350	0.41	520	0.18
255	0.95	360	0.30	530	0.22
260	0.90	370	0.20	540	0.25
265	0.85	380	0.19	550	0.25
270	0.75	390	0.18	560	0.24
275	0.70	400	0.17	570	0.29
280	0.67	410	0.15	580	0.33
290	0.62	420	0.14	590	0.30
295	0.54	430	0.13	600	0.18
300	0.48	440	0.13		

(ii) Infra-red spectrum :

The IR spectrum is shown in Fig. 1.2. The position, nature and significance of peaks are given in Table 1.2.

Table 1.2

The location, nature and significance of the peaks of infra-red spectrum of the purified pigment

S.No.	Peak in Wave length (μ)	Wave number (cm^{-1})	Nature of peak	Remarks
1.	2.9 to 2.96	3448-3378	Weak broad	O-H stretching Vibration
2.	3.42	2924	Sharp Strong	A-symmetrical C-H stretching of alkane
3.	3.5	2857	Sharp Strong	symmetrical C-H stretching of alkane
4.	4.27	2342	Sharp Strong	Impurity
5.	5.88	1701	Sharp Strong	Carbonyl stretching vibration
6.	6.17	1621	Weak	Carbonyl vibration
7.	6.79 to 6.9	1473-1449	Sharp Strong broad	A-symmetrical bonding C-H vibration of $-CH_3$($\delta as\ CH_3$) or skeltal vibration due to C-C stretching within the ring
8.	7.21	1387	Weak	-C-O stretch of phenol ring or symmetrical bending vibration of $-CH_3$($\delta_s\ CH_3$)
9.	7.95	1285	Sharp Strong	C-O stretching vibration of phenol
10.	8.21	1218	Weak	C-O stretching vibration of phenol and also due to aromatics
11.	8.66	1155	Medium	
12.	9.31	1074	Weak	Inplane bending vibration of C-H of aromatics
13.	13.9	719	Medium	Out of plane bending of the ring C-H bonds of polynuclear aromatics

The above spectral data reveal the presence of following four groups in the pigment molecule:

(i) Alkyl group :

The alkyl groups (R) belonging to the normal paraffins predominate the spectrum. Their presence is indicated from the strong intense peaks at 2924, 2857, 1473-1449 and weak peak at 1387 cm^{-1}.

(ii) Carbonyl group :

The carbonyl group (c=o) is indicated by the strong peak at 1701 and a weak peak at 1621 cm^{-1}.

(iii) Phenolic hydroxyl group :

The hydroxyl (-OH) group is indicated by the peaks 3448-3378, 1387, 1285 and 1218 cm^{-1}.

(iv) Aromatic rings :

The aromatic rings are indicated by the peaks at 1473-1449, 1285, 1155, 1074 and 719 cm^{-1}. The peak at 719 cm^{-1} shows the presence of polynuclear aromatic system in the molecule.

(iii) Nuclear magnetic resonance spectrum :

The spectrum taken in deutero-acetone is shown in Fig.1.3. The graph also shows the integration of protons in the molecule. The position of the peaks and their significance is given in table 1.3.

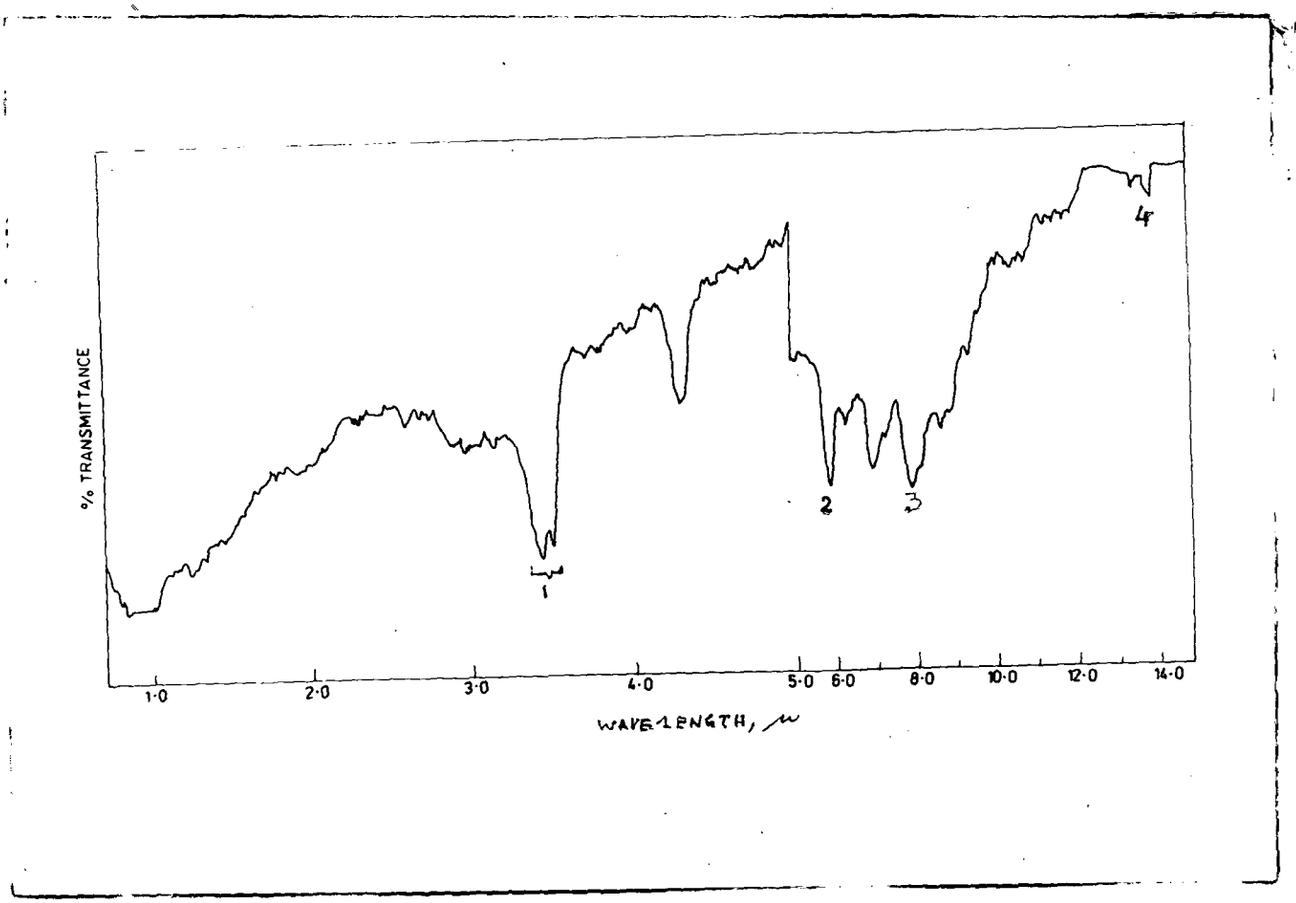

Fig. 1.2 Infra-red spectrum of pure pigment taken as KBr pellet. Marked 1,2 and 3 main peaks represent the alkyl, carbonyl and phenolic group whereas peak 4 is due to polynuclear structure of the pigment molecule.

Table 1.3

The position of peaks in the NMR spectrum; the groups and the protons associated with each of the peak

S. No.	Signal position in δ	Signal position in τ	Nature of the peak and the group associated with it	Number of protons
1.	1	9	Probably due to grease used in the instrument	-
2.	1.35	8.65		
3.	2.87	7.13	Singlet most prominent peak. It is due to anthracene or anthraquinone methyl	The integration shows the maximum number of protons, and these are present in the same chemical environment
4.	3.44	6.56	Doublet due to phenolic OH groups	The protons associated with phenols in the molecule are much less compared to those of benzylic protons
5.	4.27	5.73		
6.	7.74	2.26	Aromatic ring protons	The protons are least showing that most of the positions in the molecules are substituted

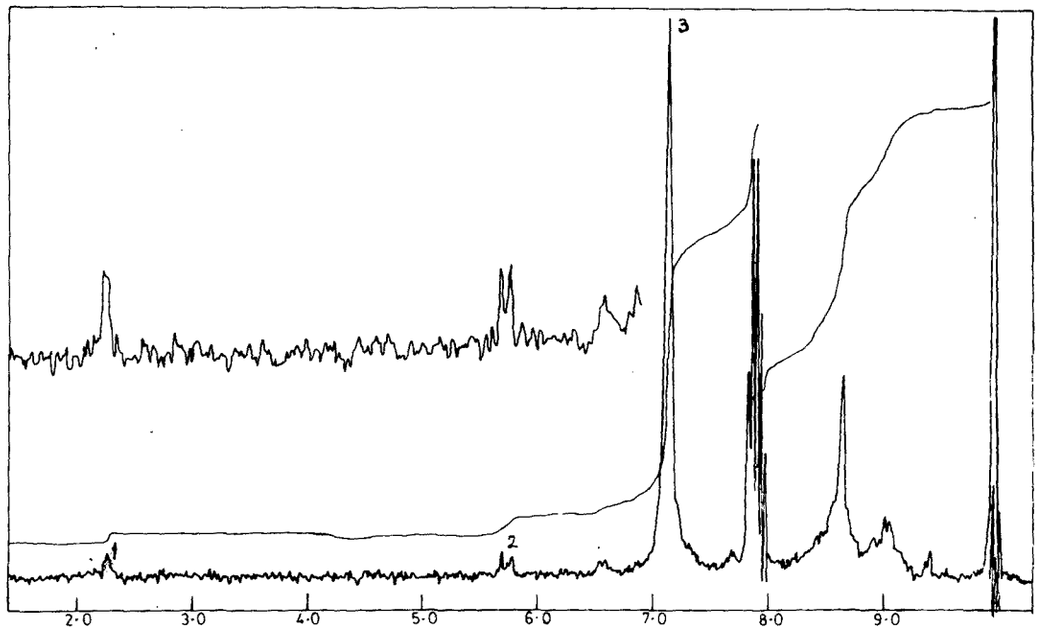

FIG 1.3.NMR SPECTRUM OF PURIFIED PIGMENT IN DEUTERO ACETONE

Fig. 1.3 Nuclear magnetic resonance spectrum of the pure pigment. The peaks 1,2 and 3 are due to aromatic, phenolic and benzylic groups respectively.

The NMR spectrum is simple indicating that the molecule is symmetrical. There are three major peaks. These are atτ 2.26, 5.73-6.56 and 7.13. These peaks represent the aromatic rings, phenolic hydroxyl groups and the methyl groups joined to the benzene rings (Silverstein and Bassler, 1967, Dyer, 1969) respectively. The most prominent peak in whole of the spectrum is due to aromatic methyls. (Fig. 1.3). The integration that is number of protons associated with these three groups (Fig. 1.3) show that the aromatic protons (τ 2.26) are the least and are two in number only, the protons associated with the hydroxyl group (τ5.73-6.56) are four while the maximum number of protons are associated with the aromatic methyl groups (τ7.13). The peak at τ7.13 is a singlet indicating that the chemical environment of the methyl groups is the same, while low number of aromatic protons indicate that the molecule is substituted at all places except the two.

(iv)Mass Spectrum :

The mass spectrum of the pigment was run at 200°C. Mass upon charge values of the different ions along with their relative percentage abundance values are given in table 1.4. The diagramatic representation of these values are given in Fig. 1.4.

The most characteristic feature of the spectrum is the appearance of ions even after the molecular ion peak which

is at 279. The significant peaks which reveal the structure of the molecule are at m/e 167, 149 and 121.

The mass spectrum shows that the pigment molecule is a dimer, molecular ion peak appears at 279, the molecular weight is 279 x 2 that is 558. That molecule is a quinone is established mass/charge peaks at 149 and 121. The valve represent the fragment formed after the loss of carbon-mono oxide molecule (molecular weight 28) from the fragment having m/e valve 149. The positive ion at m/e 149 is formed from 167 after the loss of a molecule of water (molecular (weight 18). (Budzikiewicz et al, 1967, Beynon and Williams, 1960).

Table 1.4

The mass upon chargo (m/o) and the relative percentage abundance of the ions obtained from the mass spectrum of purified pigment

m/o	Length of peak in cm	% abundance	m/o	Length of peak in cm	% abundance
17	2.2	6.6	82	2.3	6.9
18	6.5	19.5	83	7.5	22.5
27	3.5	10.5	84	2.7	8.1
28	1.5	4.5	85	1.7	5.1
29	16.5	49.5	91	1.2	3.8
39	2.8	8.4	93	2.2	6.6
41	23.5	70.5	94	0.7	2.1
42	6.0	18.0	95	1.7	5.1
43	27.5	82.5	96	0.8	2.4
44	1.3	3.9	97	2.4	7.0
50	0.8	2.4	98	0.7	2.1
53	1.6	4.8	99	0.7	2.1
54	1.5	4.5	104	5.3	16.9
55	18.0	5.4	105	3.0	9.0
56	8.0	24.0	107	0.7	2.1
57	33.5	100	109	1.2	3.8
58	1.9	5.7	111	1.0	8.1
60	1.0	3.0	112	3.7	8.1
65	3.5	10.5	113	5.5	16.5
67	2.4	7.2	121	2.6	7.8
68	1.4	4.2	122	1.3	3.9
69	9.0	27.0	123	0.7	2.1
70	15.0	45.0	128	0.8	2.4
71	16.0	48.0	129	1.0	3.0
72	1.2	3.8	132	1.8	5.4
73	1.2	3.8	149	27.0	81.0
76	3.5	10.5	150	6.0	18.0
77	2.0	6.0	167	11.5	34.5
79	1.5	4.5	168	1.2	3.8
81	2.4	7.2	279	4.5	13.5
			280	0.8	2.4
			281	0.2	0.6
			285	0.4	1.2

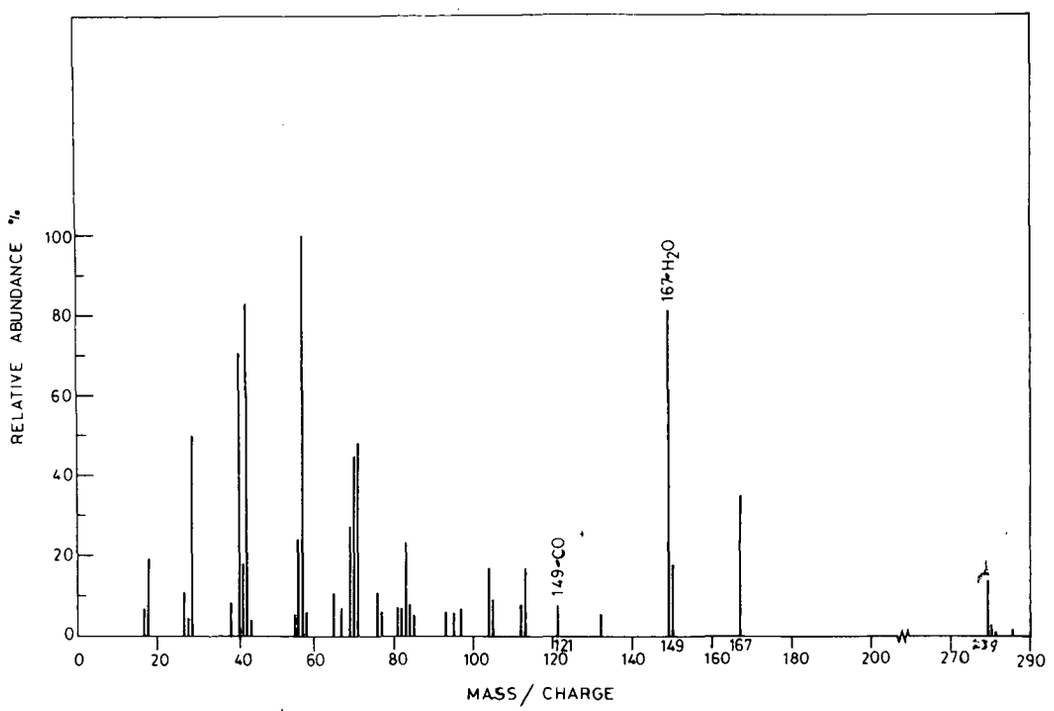

FIG.1.4. MASS SPECTRUM OF PURIFIED PIGMENT

Fig. 1.4 Diagramtic representation of the mass spectrum of the pure pigment. The peak marked as 1 at M/e 279 is the molecular ion peak giving half the molecular weight of the pigment molecule.

(v) Fluorescence spectrum :

The data of fluorescence spectrum of the purified pigment in acetone is given in Table 1.5

Table 1.5

Fluorescence emission of the pigment in acetone at different wave lengths

Wave length nm	Fluores-cence emission	Wave length nm	Fluores-cence emission	Wave length nm	Fluores-cence emission
492	0.04	580	0.04	642	0.12
496	0.04	592	0.06	647	0.12
502	0.04	596	0.08	653	0.12
507	0.04	602	0.12	658	0.12
513	0.04	608	0.14	669	0.10
518	0.04	614	0.15	680	0.08
524	0.04	618	0.16	692	0.08
534	0.04	624	0.16	692	0.08
546	0.04	630	0.14	714	0.06
555	0.04	636	0.12	724	0.04
568	0.04				

The fluorescence emission data in the table 1.5 shows that the pigment fluorescence in the red part (λ 618) of the spectrum. This suggests molecule contains a polynuclear aromatic system (Becker, 1969).

DISCUSSION

Ultra-voilet visible spectrum :

The absorption of the uv-visible light in the readily accessible portion 200-750 nm of a spectrophotometer depends on the electronic structure of a molecule. Only those conjugated systems which can have electronic transitions from n to π^* and or n to σ^* and or π to π^* will absorb in this region of the spectrum. The electronic transitions involving the non-banding n electrons to the antibonding π orbital are associated with least energy and are absorbed at the lower wave length viz. in the ultra-voilet region. The energy required by the n to sigma star orbital is the highest and the absorption occurs in the longer wave length or visible region of the spectrum. The pi to pi star transitions require intermediate energy and thus have absorption in between the ultra-voilet and the visible region (Dyer, 1969; Silverstein and Bassler, 1967).

A lone benzene ring shows the maximum absorption at 184 nm and as the number of rings increase the absorption band shifts to the longer wave length; viz. anthracene with three rings absorb at 256 nm. The pure aromatic hydrocarbons involve pi to pi star transitions, and if a carbonyl group (c=o) is present in the molecule; an intense absorption band is observed between 200-250 nm. This is due to

electronic transitions n to pi star. These transitions are due to the non-bonding electrons of oxygen atom. The substitution of alkyl group in the benzene ring shifts the absorption to the longer wave length. The absorption occurs in the visible region of the spectrum when a compound contains hydroxyl groups. This is due to fact that the non bonding electrons of oxygen facilitate the pi to pi star transitions of the benzene rings. The shift in the visible region increases with the increase in the hydroxyl groups; and the α-hydroxyl have more pronounced effect than the β-hydroxyls.

It must, however, be mentioned that in the case of complex molecules the overlapping of the absorption bands is bound to occur. This overlapping of bands makes the interpretation of the spectrum difficult, thus it is customary to compare the spectrum of the unknown compound with that of the known.

The uv-visible spectrum of the pigment of B.intermedium shows absorption bands at 225, 330, 480-490, 540 and 580 nm (Fig. 1.1, table 1.1). The maximum absorption is at 225 nm. In the light of the above discussion, the band at 225 is due to presence of carbonyl group, while 330 band is the benzenoid band and the absorption in the visible regions are due to hydroxyl groups.

Møller's (1962) and later Sevanant (1965) showed that the pigment of B. undulans resembles hypericin. Hypericin is a plant pigment belonging to the family Guttiferae. Both the pigments were shown to have broadly the same uv-visible absorption spectra and gave nearly the same spectral shifts when treated with different reagents. From analogy with hypericin, which has the mesonaphthodianthrone structure, a similar structure was given to the Blepharisma undulan's pigment.

The uv-visible absorption spectrum of B. undulans in alcohols showed the band at 330, 490, 540 and 580 nm (Sevanant, 1965). These peak positions are similar to those of pigment of B. intermedium (Fig. 1.1). This means then that the pigments of two Blepharisma species are similar, in their basic structure*.

Infra-red spectrum :

A molecule is constantly vibrating and its bonds are always stretching or contracting and bending with respect to each other. These normal vibrations are affected when the molecule is subjected to infra-red radiations, and the infra-red bands appear at definite frequency or wave number. The position of these bands is characteristic of the nature of groups present in the molecule. Thus the alkanes show

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*The spectral shift studies with the pigment of B. intermedium could not be done in this laboratory, due to the non-availability of hypericin.

C-H stretching absorption bands in the region of 3,000-2,840 cm^{-1} (3.3 - 3.5 μ) and C-H bending vibrations at 1350-1470 cm^{-1}. The saturated hydrocarbons containing the methyl groups show two distinct bands in 3.3 - 3.5 region, and these two bands are intensified with the increase in the number of methyl groups. The polynuclear aromatics show the characteristic absorption in three regions; out of which the out of plane bending vibrations at 900-675 cm^{-1}(11.11 - 14.81 μ) are the most important. The other two vibrations are in the regions 1300-1100 cm^{-1} (7.7 to 10 μ); and 1600-1585 cm^{-1} (6.25-6.31 μ); 1500-1400 cm^{-1} (6.67-7.14 μ). 1300-1100 cm^{-1} are the in plane bending of the ring C-H bonds; whereas 1600-1585 cm^{-1} and 1500-1400 cm^{-1} are the C-H stretching vibrations within the ring . The C-O stretching vibration of a carbonyl group appear as a strong band at 1870-1540 cm^{-1} (5.35 - 6.50 μ). The characteristic bands for phenols are due to the O-H stretching, C-O stretching and O-H bending vibrations. Free -O-H stretching vibrations appear at 3650-3590 cm^{-1}(2.74-2.79μ); the C-O stretching vibration produce a sharp strong band in the 1260-1000 cm^{-1} (7.93-10.0 μ). The O-H bending vibrations are of little diagnostic value.

In the light of above discussion the pigment of B.intermedium has three functional groups present on a polynuclear structure. The aromatic vibrations are at 1074 cm^{-1} 1155 cm^{-1} and 719cm^{-1}. The vibrations at 719cm^{-1} indicate the polynuclear nature of

the structure. The three functional groups are normal alkyl, carbonyl and phenolic groups. The alkyl group predominate the spectrum; and this is evident from the sharp strong bands at 2924 cm^{-1} , 2857 cm^{-1}, 1473-1449 cm^{-1}. The carbonyl group is represented by the sharp strong absorption band at 1701 cm^{-1} and a weak band at 1621 cm^{-1}, The phenolic groups are represented by the vibrations 3448-3378 cm^{-1}, 1387 cm^{-1}, 1285 cm^{-1} and 1218 cm^{-1} the vibrations 1285 cm^{-1} are the most conclusive. As the pigment molecule is complex, the overlapping of bands of different groups is bound to be there.

Although the infra-red spectroscopy is used in finding the different groups present in a molecule, its chief application is in finding the carbonyl group. The carbonyl absorption of p-benzoquinone falls at 1669 cm^{-1} (in solution) and as the number of linear fused rings increase the absorption frequency increases, thus 9, 10 anthraquinone absorbs at 1678 cm^{-1} (Thomson, 1971). The carbonyl absorption for B. undulans is at 1740 cm^{-1} and for hypericin 1710 cm^{-1} (Sovanant , 1965), and that of B.intermedium is at 1701 cm^{-1} , this once again reflects some similarity in these three pigments. Although the carbonyl frequency of hypericin and B.undulans pigments do not differ much, Sevanant, 1965 found significant differences at all positions of carbon to hydrogen response.

Nuclear magnetic resonance spectrum :

The nuclei of certain atoms spin and develop charge as a result they behave like tiny bar magnets. The nucleus which

is referred to in the NMR spectroscopy is the hydrogen proton, that is the hydrogen atom, atomic weight one. For taking the NMR spectrum the compound having protons is subjected to an external magnetic field at constant radiation frequency. The magnetic field strength is varied, the protons depending upon their nature absorb radiation at definite magnetic field strength and give signals. This results in NMR spectrum in which number of signals of different intensity and different field strength are recorded.

The number of signals in the NMR spectrum reveal the different 'kind' or 'type' of protons present in the molecule. The protons with the same environment absorb at the same field strength while the protons with different environment absorb at different field strength. This means that one can tell how many sets of equivalent protons are there in the molecule.

The position at which the signal appears or the chemical shift value of a proton reflects its nature viz. whether it is aliphatic, aromatic, benzylic, phenolic etc. The chemical shift values are expressed either in delta (δ) or in tau (τ) scale. In the delta value the signal for tetra-methylsilane, the reference compound is taken as zero, where as it is taken as 10 on the tau scale, i.e. $\tau = 10-\delta$. The difference in the chemical shifts of different protons is due to the shielding and deshielding effects of the electrons of the groups to

which they belong. The alkane protons are shielded maximum and appear in the high magnetic field strength, whereas aromatic protons are less shielded and appear at the low magnetic field strength. The equivalent protons have the same chemical shift values. The aromatic protons (Ar-H) absorb at τ 1.5-4 ; the benzylic protons (Ar-C-H) absorbs between τ 7-7.8 and the phenolic protons (Ar-O-H) absorb between τ-2 to 6 . (Dyer 1969, Silverston, 1967).

The relative intensities of signals as indicated from the size of the absorption peaks, is directly proportional to the number of protons giving rise to that signal. The number of protons under each peak is measured from the peak area. The peak area is measured by an electronic integrator, and is always shown on the graph along with the nmr signals. The NMR spectrum of the pigment of B.intermedium is extremely simple, indicating that the molecule is symmetrical. The three signals at values τ2.26, 5.73-6.56 and 7.13 representing aromatic, phenolic and benzylic protons respectively. The signals at values 9 and 8.65 are impurities probably due to the grease. This assumption is based upon the fact that the aliphatic alkyl groups have neither been confirmed by the infrared spectrum nor do they fit in the nmr and the final structure derived after the compilation of all the spectral data. The integration of the protons show the presence of least number of aromatic protons, which are two only. This is

followed by the phenolic protons which are four in number. Rest all the protons are contributed by the benzylic groups. As there is singlet at τ7.13, this means all the benzylic protons have the same environment. The value 7.23 is characteristic of alpha methyl groups belonging to anthracene or anthraquinone compounds (Mehandale et al, 1968), this means then that pigment molecule also has number of methyl groups, but which may not be at the α position.

Mass spectrum :

The mass spectrum tells the mass or molecular weight of a molecule. In the mass spectrophotometer compound is bombarded with energetic electrons as a result the molecules breaks into many fragments which are highly characteristic of the original molecule. These fragments are mostly positively charged ions but a few are neutral fragments. Each fragment has a definite mass/charge (m/e) value. The ions are univalent and m/e value gives the mass of the ion. The fragments have different intensity depending upon their relative abundance. The peak or fragment with the highest intensity is called the base peak and its value is taken as 100, and the intensities of other peaks are expressed relative to the base peak. The peak at the highest mass upon charge value is the molecular ion (M^+) peak and gives the molecular weight of the molecule. A dimer molecule when put

in the mass spectrometer breaks and gives the molecular ion for the monomer only. The molecular weight is calculated by multiplying this molecular ion value by two.

The mass spectrum of the pigment of B-intermedium shows the molecule to be dimer; the molecular ion peak of monomer is at 279, therefore, the molecular weight of the molecule is 279x2 = 558. The insight into the structure of the molecule is provided by the three peaks at m/e values of 167, 149 and 121. The peak 149 represents the loss of one molecule of water (molecular weight 18) from 167; whereas 121 represents the loss of one molecule of carbon-mono-oxide (molecular weight 28) from 149. The loss of one or two molecules of carbon-mono-oxide is characteristic of a quinone structure (Budzikiewics et al, 1967), therefore, it confirms that the pigment molecule is quinone.

Fluorescence spectrum :

Fluorescence is the emission of radiations by an excited molecules. The molecule becomes excited by absorbing the energy given from the external source. The emission of fluorescence reflects the structure of the molecule, thus benzene emits fluorescence in the ultra-voilet (270 nm) region and as the number of rings increase the emission shifts to the longer wave length; thus anthracene with three rings emits in the blue and pentacene with five rings emits in the red region of the spectrum (Becker, 1969). The presence of alkyl and hydroxyl

groups also shift the emission to the longer wave length.

The pigment of B-intermedium gives fluorescence emission at 618 nm, the red region of the spectrum (Table 1.5). The emission in the red region shows that the molecule is a poly-nuclear aromatic compound. The pigment of B-undulans and hypericin also emit fluorescence in the red region (Sevanant, 1965), this means that there is similarity in the structures of pigments of two Blepharisma species, and that both have structural similarity with hypericin.

Summing up all the above spectral data, the structure possible for the pigment of Blepharisma intermedium is:[o]

This structure fits in all the above spectral data that is,

(i) It is dimer having molecular weight 558.

(ii) It has two aromatic, four phenolic and 24 benzylic protons. The benzylic protons have the same environment and thus give a singlet at 7.13 in the NMR spectrum.

(iii) The infra-red spectrum data of three groups alkyl, hydroxyl and the carbonyl fit in it and finally

(iv) The basic structure as given by the ultra-violet absorption spectrum resembling hypericin fits in the above structure.

The molecular formula from above data is $(C_{18}H_{15}O_3)_2$ i.e. the structure proposed $C_{36}H_{30}O_6$.

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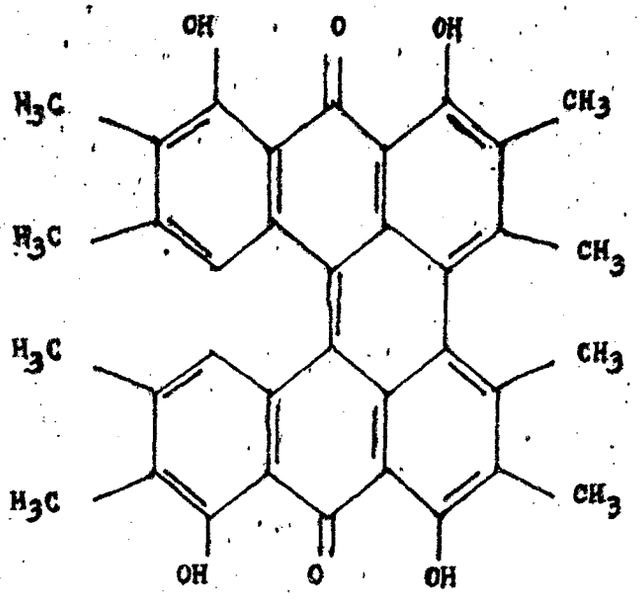
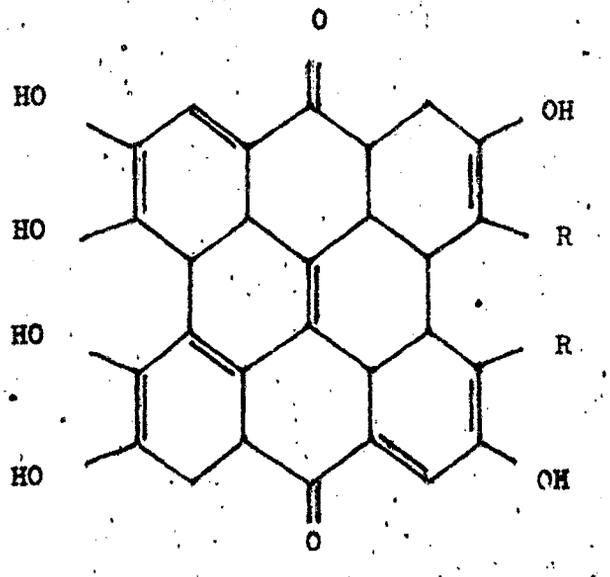
[o]given on next page



Proposed Structure of B.intermedium pigment



Proposed Structure of B.undulans pigment(Sevanant,1965)

The structure for B-undulans as given by Sevanant (1965)is:

This structure is different from the structure of B-intermedium pigment found in this laboratory in the following respects :

(1) It has mesonaphthodianthrone structure, whereas the our proposed structure belongs to helianthrone and has a protohypericin nucleus rather than the hypericin nucleus.

(2) The number of aromatic and hydroxyl protons in the B-undulans pigments are four and six respectively whereas they are two and four respectively in the pigment of B-intermedium.

(3) The number of alkyl groups are two in the B-undulans whereas they are four in the pigment of B-intermedium. The nature of R has not been given in the B-undulans pigment whereas it has been established as methyl in B-intermedium.

Future experiments :

It will really be worth-while to confirm the structure of pigment of B-intermedium by its synthesis from its two basic 2,3,6,7 tetra methyl anthranol units.

con r - ^ page 34

SUMMARY

The structure elucidation of the pigment of B-intermedium using spectroscopic techniques reveal the following salient features :

1. The pigment molecule is a dimer and has protohypericin as its basic structure.
2. The molecular weight is 554 and the molecular formula is $C_{36}H_{30}O_6$.
3. The groups present are the methyl, the hydroxyl and the carbonyl.

CHAPTER II

BIOSYNTHESIS OF THE PIGMENT

INTRODUCTION

As established from the spectroscopic studies the colour of the pigment of B.intermedium is due to a dimeric polycyclic quinone, formed from two anthraquinone derivative units.

In the animal kingdom, the quinones, excepting ubiquinones and vitamin K group quinones, are present mainly in the phyla Arthropoda, Echinodermata and to a much lesser extent in Ciliata and Annelida (Thomos, 1971). Structurally the quinone pigments in these phyla vary from the simplest benzoquinone derivatives to highly condensed polycyclic extended quinones. All the pigments are para quinones except annelids which are ortho quinones (Prota et al, 1971). The simplest benzoquinone derivative pigments are present in phylum Arthropoda, order Insecta, Diplopoda and Arachinida (Weatherston, 1967; Estable et al, 1955). The napthaquinones and the Anthraquinones in the phyla Arthropoda and Ehinodermata form well defined, structurally similar pigments which are known by the definite names viz the Echinoderm naphthaquinone and anthraquinone pigments are called the 'Sphinochromes'(Gough and Sutherland, 1964; Moore et al, 1966; Mathieson and Thomos, 1971) and 'Rhodo-Comatulins' respectively (Dimlow, 1958, Powell and Sutherland, 1967). The napthaquinone pigments of phylum Arthropoda are known as 'Aphins'(Cameron & Lord Todd, 1967) and the anthraquinone pigments all have the basic structure unit

belonging to deoxyerythrolaccin (Gadgil et al, 1968; Pandhare et al, 1966, 1967, 1969; Mehandale et al, 1968; Yates et al, 1964; Bhide et al, 1965) The polycyclic quinones resembling hypericin are present in ciliates, Stentor nigor, Stentor coeruleus (Barbier et al, 1956; Møller, 1962), in jurassic crinoidea Apiocrinus (Blumer, 1968) and in two Australian pseudo coccids (Banks et al, 1976).

The quinone pigments are secondary metaboic products and are formed either from acetate malonate pathway (Richardson and Hendrickson, 1964) or from shikimic acid pathway (Springson, 1960) or from both. But in spite of the advancement in chemical knowledge of the anthrones since 1960, very little work has been done about the biosynthesis. Many a time the predictions have been made about the pathway without any experimental evidences. The only experimental work for the biosynthesis of simple quinones is that of Meinwald et al (1966). He found the existence of both the pathways in tenebrionid beetle, Eleodes longicollis by feeding ^{14}C isotopes precursors of both the pathways. In the lac insect (coccidae) pigments which have amino acid residue, the existence of both the pathways has been suggested; the amino acid residue coming from the shikimic acid route while the anthraquinone structure from acetate-malonate pathway. The spino-chromes seem to be formed from

acetate pathway. The experimental evidence for this is the incorporation of labelled acetate in 6 ethyl - 2,3,7 trihydronaphthazarin by Arabacia pustulosa (Salaque et al, 1967). There are also indications (Sutherland, 1969) that rhodocomatulin pigments are formed from acetate pathway.

In contrast to the zoochromes the pigments in plants have been studied extensively from the biosynthetic angle. As the Blepharismin is an anthraquinone derivative the plants which have anthraquinone pigments have been considered for the present study. The anthraquinones are the largest group present in the plants. They are present in fungi, lichen, and in higher plants. In moulds they are present in Aspergillus and Penicillium species (Shibata, 1967; Bu'Lock and Smith,1968). In higher plants they are present maximum in family Rubiaceae (Burnett and Thomson, 1968) and to a lesser extent in Rhamnaceae, Leguminosae,Polygonaceae, Bignoniaceae, Verbenaceae, Scrophulariaceae and Liliaceae. In plants biosynthetically, it has been found out through labelling experiments that the quinones having emodin like structure originate via acetate malonate pathway (Birch and Donovan, 1953; 1955; Birch et al 1958; Gatenbeck, 1958; 1960; 1962; Shibata and Ikekawa, 1963) while the anthraquinone of the alizarin and purpurin type are formed involving both the shikimic acid and acetate pathways. Burnett and Thomson, (1968; 1967) Leistner and Zenk, (1968; 1967) have found out further that the shikimic acid contribute

one benzene ring and half of the carbonyl group of the quinone while the rest of the molecule is formed from the acetate units.

In the present study of biosynthesis of Blepharisimin four different aspects were taken:

(i) Gene-pigment relationship :

This aspect was undertaken to see if the pigment is formed during the protein synthesis. The inhibitors of DNA, mRNA and protein synthesis were used and the pigment formation in the laboratory generated albino Blepharismas was studied.

(ii) Carbohydrate metabolism and the pigment formation :

The study on this aspect was undertaken to see if the pigment is a metabolic product, formed by the secondary reactions. Metabolic inhibitors blocking specific reaction steps of the metabolism were used and the pigments formation in the albino Blepharismas was measured. The role of carbohydrate in the pigment formation was confirmed by ^{14}C glucose feeding experiments.

(iii) Acetate pathway and the pigment formation :

The quinone pigments have been known to be formed by acetate malonate pathway, involving acetate units. The study was carried out to see if the acetate has any role in the pigments biosynthesis. For this the animals were fed with radioactive sodium acetate and the incorporation of the label was measured in the

pigment, after it was purified chromatographically.

(iv) Shikmic acid pathway and the pigment formation :

In certain plants and bacteria and the quinones are formed involving shikmic acid as a precursor. This aspect was considered to see the role of the precursor in forming the Blepharisma pigment. The enzyme shikmate reductase which forms the shikmic acid was assayed; and the pigment formation was studied after blocking specifically the above enzyme.

MATERIALS AND METHODS

MATERIALS

Pigmented and albino Blepharisima intermedium (Indian species) constituted the material for the present studies. The animals were cultured at $25 \pm 3^{\circ}C$ in dark in hay medium fortified with 'Horlicks' milk as described earlier in Chapter I.

The chemicals used and their sources were:

Acetone, nickel chloride, ethyl ether, sodiumhydroxide, sodium fluoride, toluene and trichloroacetic from the British Drug House.

Streptomycin, hydroxyurea, Actinomycin D, cycloheximide, refampicin, iodoacetic acid, iodo-acetamide, 2-4 Dinitrophenol, NADP, glycine, shikimic acid, sodium azide, PPO, POPOP were from Sigma Chemicals (USA).

^{3}H Thymidine, ^{14}C uridine, ^{14}C isoleucine, ^{14}C leucine, $^{14}C(U)$ glucose and $^{14}C(U)$ sodium acetate were from the Bhabha Atomic Research Centre, Trombay.

Parachloro mercuribenzoate (PCMB)was from the Patel Chest, New Delhi.

METHODS

(1) General

(i) De-pigmentation of the rod Blopharisima :

The rod Blopharisima were de-pigmented by immersing the centrifuged animals for 30 seconds in a beaker maintained at -2° to -3°C as suggested by Gieso and Grainger, 1970. The albinos were quickly brought to the room temperature by immersing the tube with the animals in another beaker maintained at 35° to 40°C for a few minutes (5-6 minutes). The pigment extract was removed from animals by three successive washings with distillated water or with freshly prepared hay medium, and centrifuging everytime to remove the supernatant.

(ii) Measurement of pigment concentration :

The pigment from the animals was dissolved in acetone. For 5,000 to 7,000 animals 2-3 ml of solvent was used. The debris was removed by centrifugation. The optical density was taken at 340 nm in Beckmann Spectrophotometer.

(iii) Regeneration of the pigment :

The time taken by the albinos to get nearly the same optical density at 340 nm as that in the same number of pigmented animals before depigmentation, was used as the index for pigment regeneration. For this equal number of animals were taken in four centrifuge tubes.

The animals in the three tubes were made albinos. The pigment from the red pigmented animals and pigment from one of the tube having albino animals was extracted separately in equal volumes of acetone and optical density of the extract was measured. The optical density from the other two tubes was determined after 24 and 48 hours.

(iv) Use of 50 ug/ml streptomycin in all experiments :

The transcriptional and translational studies carried out showed that to get consistent results (Table 2:3) one has to add streptomycin. Therefore, all the experiments conducted were in the nutrient medium containing 50 ug/ml of streptomycin.

(2) Gene pigment relationship

The general procedure followed for all these studies was as follows:-

(i) Finding of optimum dose of inhibitor :

About 1,500 to 2,000 Blepharisima/ml were treated with different concentrations of the inhibitor, in a freshly prepared hay medium containing 50 ug/ml of streptomycin. The Blepharisimas were labelled after 22 hrs with the respective ^{14}C radio-isotope. After two hours i.e. twenty four hours after the treatment with the inhibitor the reaction was stopped by keeping the tubes at $0^{\circ}C$. The animals were centrifuged at 9,000 to 10,000 rpm for ten minutes.

The inhibitor was removed by washing two to three times with the fresh hay medium. The animals were homogenised in the cold and 10 percent cold TCA added, to give the final concentration to 5 percent. The tubes were kept at 0°C for overnight for the complete precipitation of nucleic acid and proteins. The nucleic acids and protoins were filtered using millipore filters. The precipitate was washed 3 to 4 times with cold 5 percent TCA. The final washing was given with alcohol ether mixture (3:1). The dried filter papers were taken in the scintillation vials and the counts taken in 7 ml of toulene based scintillation fluid, containing 4 gm of PPO and 50 mg of POPOP per litre of toluene, on the liquid scintillation counter.

In the earlier experiments carried out with Rifampicin and Cyclohoximide no antibiotic was added. It was found that such experiments did not yield repeatable results, and the concentration of the inhibitor was also much higher to produce the desired results. The inhibitor of DNA synthesis was Hydroxy urea. The concentration tried was 2 to 8 mM, the labelling was done with 5 μci/ml ^{3}H Thymidine (specific activity 6,100 mci/m mole). The inhibitor used for RNA synthesis was Actinomycin D. The concentration tried were 50 and 75 ug/ml. The

labelling was done with 5 µci/ml of Uridine TG ^{14}C. The protein synthesis inhibitor used was cycloheximide in the concentration 100 µg/ml labelling was done with ^{14}C Isoleucine (specific activity 122 mci/m mole). All the radio-isotopes were from Bhabha Atomic Research Centre.

(ii) Pigment formation with the optimum dose of inhibitor :

Equal number (5,000 to 8,000) of albino Blepharisimas were taken in two different tubes. The experimental animals were treated with optimum dose of inhibitor. The optical density of the pigment formed in blank* and that in treated animals was taken at 340 nm after 24 hours of the treatment. The optimum concentration of Hydroxy urea used was 8 mM, Actinomycin D 75 ug/ml and cycloheximide 100 µg/ml. The treatment with inhibitor was given only once in all cases except in cycloheximide experiments. In this case, the animals were treated again after 12 hours. This was done because the percentage inhibition decreased in this case after 12 hours.

(3) Carbohydrate metabolism and the pigment formation

The general procedure for all the experiments was the same, that is equal number of (5,000 to 8,000) albino Blepharisma in hay medium were taken in different tubes. All experiments had 50 µg/ml of streptomycin. Except in the blank; different concentrations of inhibitor were given

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*Blank wherever : : mentioned stands for the Control.

to the albinos. The optical density of the pigment of the blank as well as those of experimental one was taken after 24 hours at 340 nm.

The inhibitors used were iodo-acetic acid 0.1 to 0.6 mM; Iodo-acetamide 0.02 to 0.04 mM; sodium fluoride 2 to 10 mM; sodium azide 0.1 to 0.8 mM; 2,4, Dinitrophenol 0.01 to 0.19 mM and finally nickel chloride in the concentration range of 0.1 to 4 mM.

(4) Acetate pathway and the pigment formation

The acetate pathway was studied through feeding experiments with ^{14}C labelled glucose and ^{14}C labelled sodium acetate. The general procedure followed was as follows :

The animals were fed with the labelled compound, which was added in the culture medium containing 50 ug/ml of streptomycin. After specified time the animals were killed and the pigment extracted in acetone.

The pigment was purified chromatographically, on Kieselguhr paper using 88 percent acetone, 10 percent ethylether and 2 percent water as the developing solvent system (Sevenant 1965). The paper with the red band of pigment was cut and the incorporation of label measured in 7 ml of toulenescintillation fluid on liquid scintillation counter.

The experiments done under this were:

(i) Glucose feeding experiments :

(a) Incorporation of label in the pigment :

About 400/ml of albino Blepharisimas were fed with 0.05 uci/ml of uniformly labelled ^{14}C glucose (specific activity 175 mci/m mole) in nutrient medium containing 50 ug/ml of streptomycin. The animals were killed, pigment extracted and purified as per method given above and the counts taken after 48 hours of feeding.

(b) 500 /ml of albinos were fed with 0.05 uci/ml of glucose and the incorporation in the pigment was measured after 12,24,36 and 48 hours.

(c) Equal number (about 1,000) of albinos and pigmented animals were fed with 0.125 uci/ml of glucose. The incorporation was measured in pigment after 2, 4 and 6 hours of feeding.

(ii) Sodium acetate feeding experiments :

The experiments done were:

(i) Effect of sodium acetate on pigment formation :

Equal number of albinos (about 10,000) were taken and treated with different concentration of sodium acetate ($1x10^{-5}$M to $1x10^{-3}$M). The blank was run under similar conditions. The optical density at 340 nm of treated animals and blank were taken after 24 hours.

(ii) Feeding experiments with ^{14}C(U) sodium acetate Incorporation of label in the pigment :

(a) 5,000 albinos were fed in culture medium containing 50 ug/ml of streptomycin with 0.3 uci/ml of uniformly ^{14}C labelled sodium acetate having specific activity 37.27 mci/m mole. Pigment was extracted, purified and counts taken after 48 hours.

(b) 500/ml albinos were fed in four different tubes with 0.1 uci/ml of radio-isotope and the ^{14}C incorporation in the purified pigment was studied after 12, 24, 36 and 48 hours of feeding.

(c) Albino and red Blepharisima taken in equal numbers about 500/ml were fed with 0.25 uci/ml of isotope. The counts measuring the ^{14}C incorporation in the purified pigment were taken after 2, 4 and 6 hours.

(5) Shikmic acid pathway and the pigment

The experiments conducted under this head were :

(a) Assay of the enzyme shikmate reductase or 5-Dehydroshikmic acid reductase or Shikmate Dehydrogenase (Balinsky and Davis 1961)-E.C. 1.1.1.25. About 7,000 per ml of pigmented animals were taken and homogenised in 1 ml distilled water in the cold room for 10-15 minutes. The solution centrifuged to remove the debris at 10,000 rpm in the cold for 10 minutes.

To 2 ml of 0.1 M glycine sodium hydroxide buffer (pH.9.0) was added, 0.2 ml of 0.005 M NADP, 0.2 ml Blopharisma extract and 0.2 ml of shikmic acid. All these solutions were taken in quartz cuvette. The blank was with 2 ml of glycine buffer, 0.2 ml of NADP and 0.2 ml of Blopharisma extract minus the shikmic acid. The increase in the optical density at 340 was recorded in the Beckmann spectrophotometer for 4 minutes at intervals of 30 seconds.

The enzyme is present in pea seedlings, and a similar experiment was run using pea seedlings, 3-4 days old, grown in the dark.

(b) Inhibition of the enzyme and pigment formation

(i) Para-chloromercuribenzoate (PCMB) and pigment formation :

(A) Chloromercuribenzoate from Patel-chest, in concentrations $2x10^{-5}$M to $2x10^{-6}$M was used and the pigment formed in the albino Blepharisimas was measured at 340 nm after 24 hours.

(B) Treatment of $1x10^{-5}$M PCMB and the pigment formation - $1x10^{-5}$M the optimum dose of PCMB found at b(i) was used and the pigment formed in the treated and untreated albino animals (6,000/ml) after 8,20,24 and 44 hours was

measured by taking the optical density at 340 nm.

(ii) Absorption spectra of PCMB treated albinos :

Equal number of albinos (about 7,000/ml) were taken in two tubes. One was used as blank. The animals in the other tube were treated with $1x10^{-5}$M PCMB solution. The absorption spectra of both samples was taken after 24 hours in A.R. acetone.

EXPERIMENTAL RESULTS

1. (i) Regeneration of pigment in the albino animals :

The pigment is regenerated in laboratory made albino animals, when they were placed in the nutrient medium. The results are given in Table 1.1.

Table 2.1

The regeneration of the pigment

No.of red animals /ml	Optical density of pigments from red Blepharisma at 340 nm	No.of albinos /ml taken	Optical density of pigments from the albinos at 340 nm	Optical density at 340 nm after		% pigment formed in	
				24 hrs	48 hrs	24 hrs	48 hrs
6,500	0.35	6,500	0.11	0.21	0.31	60	88.5

(ii) Absorption spectrum of the pigment extracted from about 10,000 animals in acetone is shown in Fig.2.1. The optical density at different wave lengths is tabulated in Table 2.2.

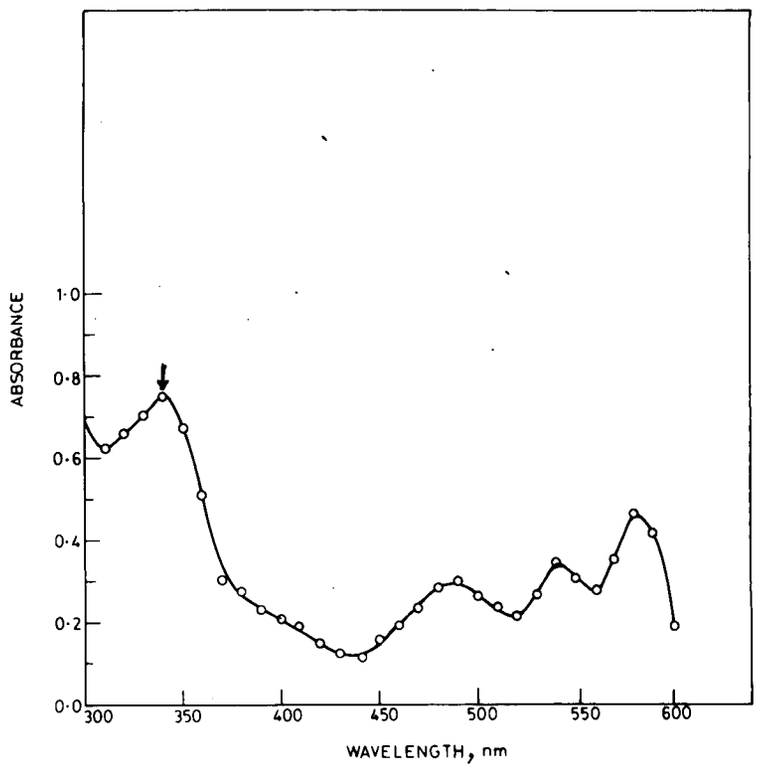


FIG 2.1. ABSORPTION SPECTRUM OF PIGMENT IN ACETONE

Fig. 2.1 **Absorption spectrum peaks of the pigment between 300-600 nm. The maximum absorption at 340 nm is shown by the arrow.**

Table 2.2

The absorption spectrum of the pigment in acetone

Wave length (nm)	Optical density	Wave length (nm)	Optical density	Wave length (nm)	Optical density
300	0.69	400	0.21	510	0.23
310	0.62	410	0.10	520	0.22
320	0.66	420	0.15	530	0.27
330	0.69	430	0.13	540	0.35
335	0.72	440	0.12	550	0.31
340	0.75	450	0.15	560	0.28
350	0.67	460	0.20	570	0.35
360	0.57	470	0.24	580	0.47
370	0.31	480	0.29	590	0.42
380	0.27	490	0.30	600	0.19
390	0.23	500	0.23		

The result indicates that the pigment has maximum absorption at 340 nm.

2. Gene Pigment relationship :

(i) Experiments without streptomycin :

The transcriptional and translational studies done without adding streptomycin are given in tables 2.3 and 2.4 DNA dependent RNA synthesis was blocked

with Refampicin while the protein synthesis was blocked with cycloheximide. In the former experiments the labelling was with 5 uci/ml ^{14}C uridine TG in the later in labelling was with 10 uci/ml of ^{14}C isoleucine (specific activity 122 mci/m mole).

The data from both the inhibitors indicate the inconsistent results.

Table 2.3

The effect of different concentration of Rifampicin on inhibition of RNA synthesis

No.of animals/ml			Concentration of Rifampicin in ug/ml													
Blank	Experimental	Blank Counts	50		100		150		200		250		300		350	
			*C	%I **	C	%I	C	%I	C	%I	C	%I	C	%I	C	%I
150	150	410	281	38.4	147	64.2	319	22.2	282	31.1	252	37.7	224	45.4	186	54.7

(*) = 50 seconds counts of experimental animals

(**) = Percentage inhibition

Table 2.4

The effect of different concentrations of cycloheximide on inhibition of protein synthesis

No.of animals/ml		Blank Counts	Concentration of cycloheximide in mμ/ml							
			50		100		150		200	
Blank	Experimental		C *	%I **	C	%I	C	%I	C	%I
1,200	1,200	16,385	15,468	6.4	9,579	41	14,791	9	13,944	14.7

(*) = 50 seconds counts of experimental animals

(**) = Percentage inhibition

(ii) Experiments using 50 ug/ml streptomycin :

(a) Effect of streptomycin on animals :

Before doing any experiment with streptomycin, its effect on the animal was seen. The animals containing 50 ug/ml of antibiotic did not have any ill-effect and the animals were normal healthy even after 48 hours.

(b) Transcriptional studies :

(A) Finding optimum dose of Actinomycin D :

The animals were treated with 50 and 75 ug/ml of Actinomycin D and labelled with 5 uci/ml of uridine TG in the last two hours. The results are given in Table 2.5.

Table 2.5

The percentage inhibition of RNA synthesis with different concentration of Actinimycin D in presence of 50 ug/ml of streptomycin

No. of albino animals taken/ml		ug/ml of Actinomycin D		50 sec counts after 24 hours		% inhibition
Blank	Experimental	Blank	Experimental	Blank	Experimental	
1,000	1,000	Nil	50	334	75	78
1,000	1,000	Nil	75	334	32	90.5

(B) 75 ug/ml Actinimycin D gave 90.5% inhibition of RNA synthesis. The effect of this concentration on the pigment formation was studied. This is shown in Table 2.6.

Table 2.6

The effect of 75 ug/ml Actinomycin D on pigment formation

No.of albinos animals/ml		ug/ml of streptomycin		ug/ml Actinomycin D		Optical density after 24 hours at 340 nm	
Blank	Experimental	Blank	Experimental	Blank	Experimental	Blank	Experimental
5,000	5,000	50	50	Nil	75	0.228 ≈0.23	0.225 ≈0.23

It is quite clear from the results the pigment formation is independent of the RNA synthesis.

(c) Translational studies :

(A) Finding Optimum dose of cycohexamide : 100 ug/ml of the inhibitor was tried. The results of the inhibition obtained with this concentration after 12 and 24 hours are given in Table 2.7.

Table 2.7

The effect on inhibition of protein synthesis by 100 ug/ml of cycloheximide in presence of streptomycin

No. of albino/ml taken		ug/ml of streptomycin		ug/ml of cycloheximide		Counts after 12 hours for 50 seconds		Counts after 24 hours for 50 seconds		% inhibition	
Blank	Experimental	Blank	Experimental	Blank	Experimental	Blank	Experimental	Blank	Experimental	Blank 12 hrs	Experimental 24 hrs
1,000	1,000	50	50	Nil	100	145	10	519	453	93	12.8

(D) Effect of 100 ug/ml of cycloheximide on pigment formation :

Equal number of albinos were treated with 100 ug/ml of inhibitor the results are given in Table 2.8. Since the results of Table 2.7 showed that after 12 hours the effect of the inhibitor decreases to a great extent, it was considered proper to remove the inhibitor and the nutrients medium. The animals were placed in fresh medium containing the streptomycin and again treated with 100 ug/ml of inhibitor. The optical density was taken after 24 hours.

Table 2.8

The effect of 100 ug/ml cycloheximide on pigment formation

No. of albinos taken		ug/ml of streptomycin		ug/ml of cycloheximide		Optical density at 340 nm	
Blank	Experimental	Blank	Experimental	Blank	Experimental	Blank	Experimental
10,000	10,000	50	50	Nil	200	0.8	0.65

(d) Inhibition of DNA synthesis and the pigment formation :

A. Finding optimum dose of Hydroxy urea to give about 90·/. inhibition of DNA synthesis :

The concentration tested were 3.5 mM and 8 mM. The results are given in Table 2.9.

Table 2.9

The effect of Hydroxy urea on inhibition of DNA synthesis

S.No.	No.of albinos/ml taken		ug/ml of streptomycin		mM of -Hydroxy urea added		50 secs. counts after 24 hours		% inhibition
	Blank	Experimental	Blank	Experimental	Blank	Experimental	Blank	Experimental	
1.	1,000	1,000	50	50	Nil	8	1,620	286	82
2.	1,000	1,000	50	50	Nil	8	1,620	425	74
3.	1,000	1,000	50	50	Nil	8	1,620	199	88

(D) The effect of 8 ml, Hydroxyurea on the pigment Formation :

The result as given in Table 2.9 showed that 8 ml hydroxy urea gives about 90 % inhibition, therefore, the effect of this concentration on pigment formation was studied. The results are given in Table 2.10.

Table 2.10

The effect of 8 mlkurea on pigment formation

No. of albinos taken/ml		ug/ml of streptomycin		Hydroxy urea/ml		Optical density after 24 hours	
Blank	Experimental	Blank	Experimental	Blank	Experimental	Blank	Experimental
5,000	5,000	50	50	Nil	8 ml	0.21	0.20

3. Role of Carbohydrate metabolic inhibitors on the pigment formation :

The metabolic inhibitors were used to see the relation between the pigment formation and the carbohydrate metabolism. The effect of different concentrations of inhibitors was studied till the stage beyond which the animals die. The inhibitors used were :-

(i) Iodoacetic acid and Iodoacetamide :

The concentration used were 0.1 to 0.6 ml and 0.02 to 0.04 ml respectively. The results are given in Tables 2.11(a) and 2.11(b), and Figs. 2.2(a) and 2.2(b).

Table 2.11(a)

The effect of Iodoacetic acid on pigment formation

S.No.	No. of albinos taken/ml		ug/ml of streptomycin		Concentration of iodoacetic acid/ml given to the experimental animals in ml					
	Blank	Experimental	Blank	Experimental						
1.	6,500	6,500	50	50	0.1	0.2	0.3	0.4	0.5	0.6
Optical density after 24 hrs.	0.19	-	-	-	0.14	0.12	0.13	0.1	0.1	dead

Table 2.11 (b)

The effect of iodoacetamide on pigment formation

S.No.	No.of albinos taken/ml		ug/ml of streptomycin		Concentration of iodoacetamide/ml given to the experimental animals in ml		
	Blank	Experimental	Blank	Experimental			
1.	6,500	6,500	50	50	0.02	0.03	0.04
Optical density after 24 hrs.	0.19	-	-	-	0.14	0.11	0.11

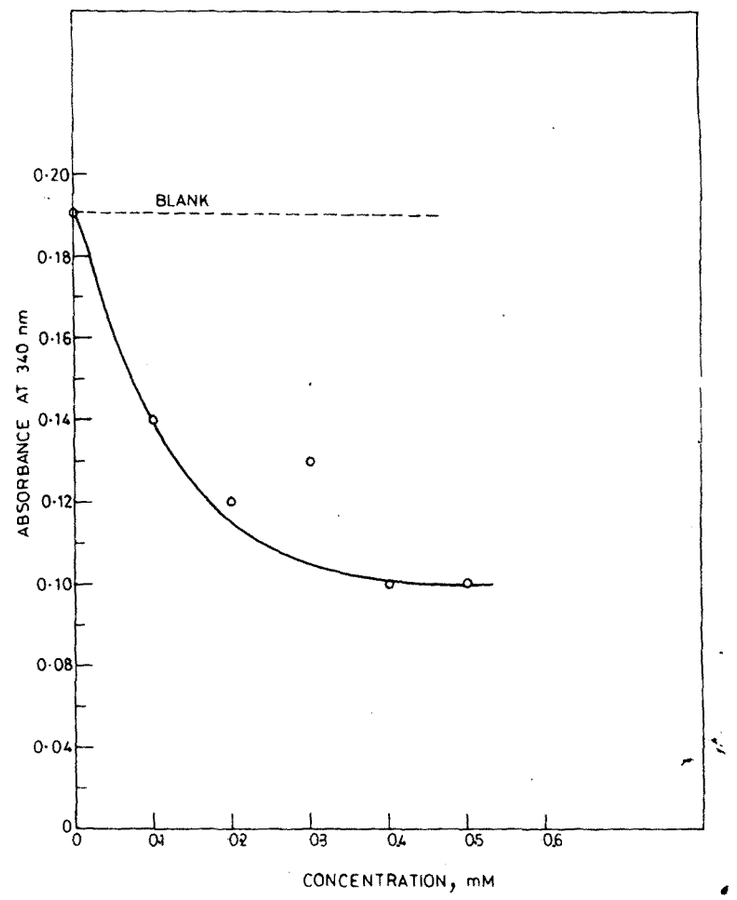


FIG. 2.2.a. EFFECT OF IODOACETIC ACID ON PIGMENT FORMATION

Fig. 2.2(a) **Effect of 0.1 to 0.5 mM iodo acetic acid on the regeneration of the pigment in the albino Blepharismas. The pigment synthesis is impaired as can be seen from the decrease in the optical density of the treated animals as compared to in the untreated(blank) animals.**

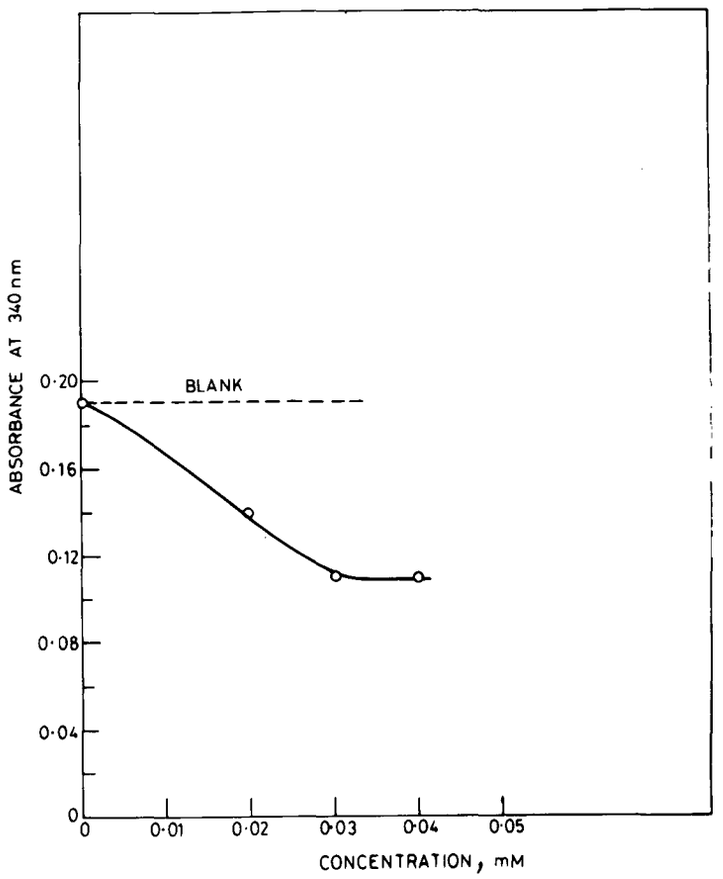


FIG 2.2b. EFFECT OF IODO ACETAMIDE ON PIGMENT FORMATION

Fig. 2.2(b) **Effect of 0.01 to 0.04 mM iodoacetamide on the regeneration of the pigment in the albinos. The regeneration of the pigment is decreased in the treated animals compared to the untreated(blank) albinos.**

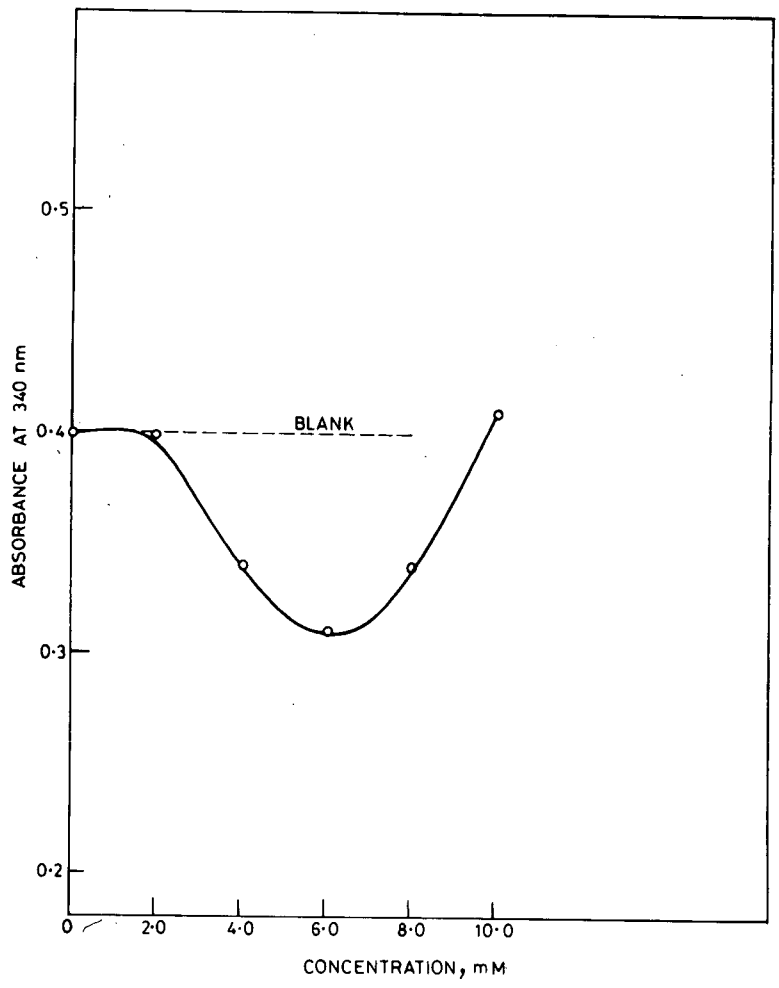


FIG.1.3. EFFECT OF SODIUM FLUORIDE ON PIGMENT FORMATION

Fig. 2.3 Effect of 2-10 mM sodium fluoride on the regeneration of pigment in the albinos. The little regeneration of the pigment is evident upto 6 mM concentration.

Table 2.13

The effect of nickelchloride on pigment formation

No.of albinos taken		ug/ml of streptomycin		Concentration of nickel chloride added to the experimental animals in mM						
Blank	Experimental	Blank	Experimental							
8,500	8,500	50	50	0.1	0.2	0.4	0.6	0.8	1	4
Optical density after 24 hrs.	0.25	-	-	0.17	0.17	0.17	0.12	0.12	0.1	.08

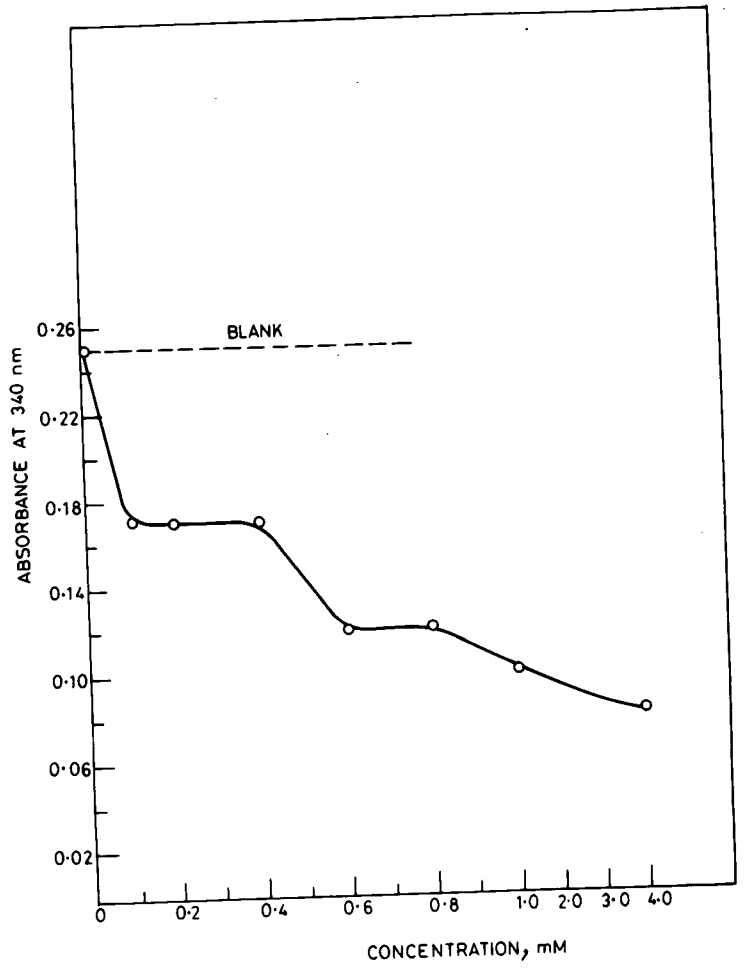


FIG 2.4. EFFECT OF NICKEL CHLORIDE ON PIGMENT FORMATION

Fig. 2.4 **Effect of 0.1 to 4 mM nickel chloride on the regeneration of the pigment in the albinos. Very little pigment is regenerated as is clear from the steep drop in the optical density of the pigment extract of experimental animals compared to pigment extract from the blank (control) animals.**

Table 2.14

The effect of 2,4 Dinitrophenol on pigment formation

S. No.	Observation	No. of albino animals/ml		ug/ml of streptomycin		Concentration of Dinitrophenol given to the albino experimental animals (ml)																
		Blank	Experimental	Blank	Experimental																	
1.		6,500	6,500	50	50	0.01	0.02	0.03	0.04	0.05	0.06	0.07	0.08	0.09	0.10	0.11	0.12	0.13	0.14	0.15	0.16	0.17	0.18	0.19
	Optical density after 24 hrs.	0.18	-	-	-	0.2	0.17	0.18	0.15	0.24	0.20	0.15	0.15	0.2	0.18	0.19	0.14	0.2	0.2	0.18	0.18	0.23		Dea

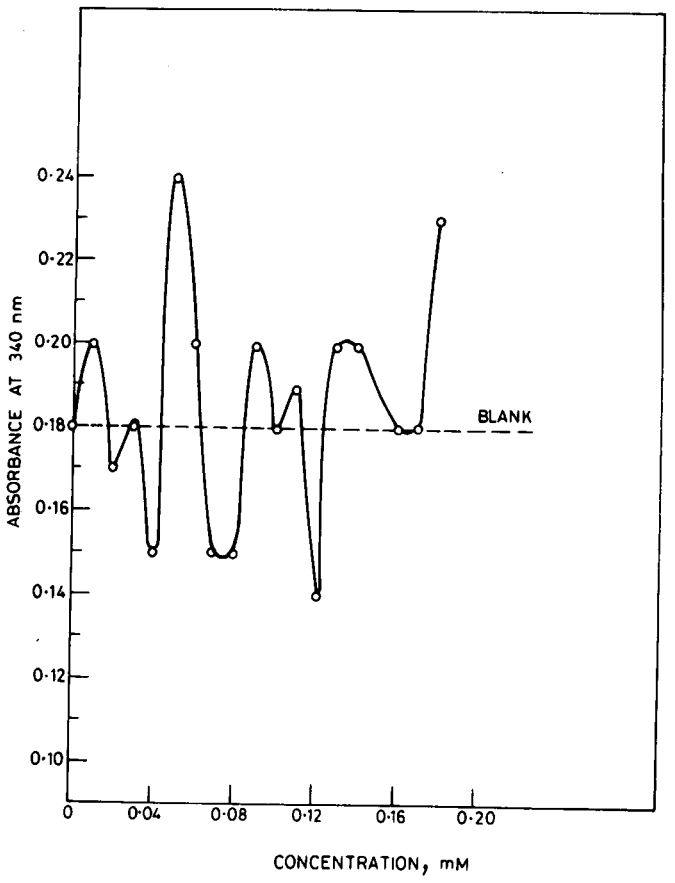


FIG.25. EFFECT OF 2,4-DINITROPHENOL ON PIGMENT FORMATION

Fig. 2.5 Effect of .01 to 0.19 mM 2,4-Dinitrophenol on the regneration of the pigment in the albinos. There is no marked decrease on the pigment synthesis in the treated and untreated (blank) albinos.

Table 2.15

The effect of sodium azide on the pigment formation

S.No.	Observation	No.of albino animals/ml		ug/ml of streptomycin		Concentration of sodium azide given to the albino experimental animals in mM						
		Blank	Experimental	Blank	Experimental							
1.		5,000	5,000	50	50	0.1	0.2	0.3	0.4	0.5	0.6	0.8
2.	Optical density after 24 hrs.	0.11	-	-	-	0.125	0.12	0.11	0.13	0.13	0.13	0.11

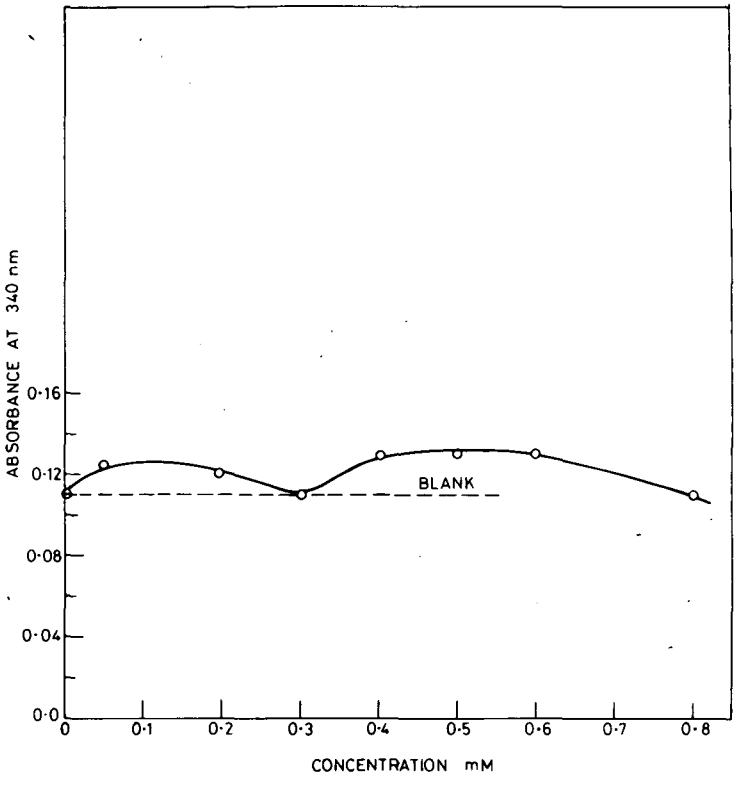


FIG.16. EFFECT OF SODIUM AZIDE ON PIGMENT FORMATION

Fig. 2.6 Effect of 0.1 to 0.8 mM sodium azide on the regeneration of the pigment in the albinos. Sodium azide increases the pigment formation as can be seen from the increase in the optical density of the pigment extracts of the albinos.

4. Acetate pathway and the pigment formation :

In order to investigate the existence of acetate pathway the experiments done were of two types. One type of experiments were those involving feeding with $^{14}C(U)$ glucose and the other type were those of feeding with $^{14}C(U)$ sodium acetate.

(i) Feeding with $^{14}C(U)$ glucose (specific activity 175 mci/mM mole) :

Three different experiments were done:

(a) This experiment was done only to find out whether the pigment takes up the label or not. In this experiment the albinos were fed with 0.05 uci/ml of glucose for 48 hours, the results obtained are given in Table 2.16.

Table 2.16

The incorporation of label in purified pigment after 48 hours of feeding

No.of albinos/ml	ug/ml of streptomycin	uci/ml of ^{14}C glucose	50 secs. counts after 48 hours
400	50	0.05	633

(b) The result of the above experiment was confirmed by feeding the albino animals with ^{14}C glucose, and purifying their pigment after 12, 24, 36 and 48 hours. The incorporation of ^{14}C was measured the results are given in Table 2.17 and Fig. 2.7.

Table 2.17

The incorporation of ^{14}C glucose during the pigment synthesis by the albino animals

No.of albinos taken/ml	µg/ml of streptomycin added	µci/ml of ^{14}C glucose	50 secs. counts after			
			12 hrs	24 hrs	36 hrs	48 hrs
500	50	0.05	251	354	856	877

(c) This experiment was done as a second confirmation of the incorporation of ^{14}C glucose in the pigment. In this case equal number of the pigmented and albino animals were fed with 0.125 µci/ml glucose and the ^{14}C incorporation measured in the purified pigment after 2, 4 and 6 hours of feeding. The results are shown in Table 2.18 and Fig. 2.8.

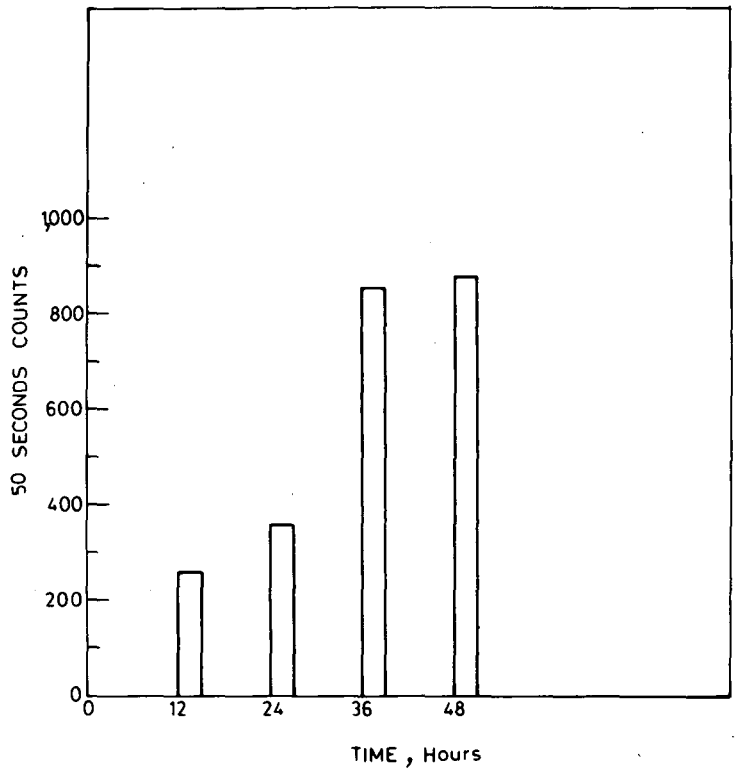


FIG. 17. ^{14}C GLUCOSE INCORPORATION DURING THE PIGMENT SYNTHESIS IN ALBINO BLEPHARISMAS

Fig. 2.7 The ^{14}C glucose incorporation in the purified pigment, synthesised by the albinos after 12, 24, 36 and 48 hours of ^{14}C glucose feeding.

Table 2.18

The ^{14}Cglucose incorporation after 2, 4 and 6 hours of feeding in pigmented and albino animals

No.of animals/ml		ug/ml of streptomycin		Glucose uci/ml		50 secs. counts after 2 hrs		4 hrs		6 hrs	
P	A	P	A	P	A	P	A	P	A	P	A
1,000	1,000	50	50	0.125	0.125	26,212	22,314	24,008	25,801	21,961	18,213

P = Pigmented animals

A = Albino animals

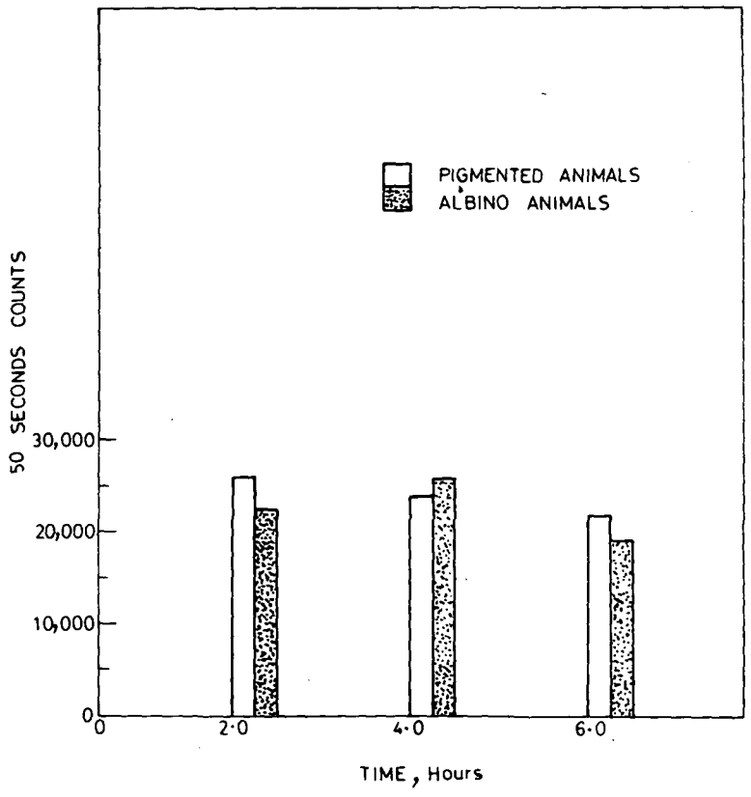


FIG.1.8. ^{14}C GLUCOSE INCORPORATION IN PIGMENTED AND ALBINO ANIMALS

Fig. 2.8 The ^{14}C glucose incorporation in the purified pigment samples extracted from equal number of pigmented and albino Blepharismas after 2, 4 and 6 hours of feeding with glucose.

All these experiment showed that glucose is incorporated in the pigment.

(ii) Feeding with sodium acetate :

Four different experiments were done

Effect of sodium acetate on the pigment formation

This experiment was done to see if there is any ill-effect of sodium acetate on the pigment before feeding with ^{14}C labelled acetate. The results are given in Table 2.19.

Table 2.19

The effect of sodium acetate on pigment formation

No.of albinos taken		µg/ml of streptomycin		Concentration of sodium acetate added to the experimental animals in ml				
Blank	Experimental	Blank	Experimental					
10,000	10,000	50	50	$1x10^{-5}$	$5x10^{-5}$	$1x10^{-4}$	$5x10^{-4}$	$1x10^{-3}$
Optical density after 24 hrs. 0.56	-	-	-	0.55	0.49	0.60	0.56	0.60

It is obvious that there are no ill-effects of the sodium acetate and that the pigment is

formed as usual. The animals also looked healthy and vigrous.

Feeding with ^{14}C (U) sodium acetate

(a) The albino animals were fed with 0.3 µci/ml of ^{14}C(U) sodium acetate and incorporation, in the pigment measured after 48 hours. The result are given in Table 2.20.

Table 2.20

The incorporation of ^{14}C sodium acetate after 48 hours of feeding

No.of albinos/ml	µg/ml of streptomycin	µci/ml of ^{14}C sodium acetate	50 secs.counts after 48 hours
5,000	50	0.3	18,909

(b) The above experimental data was confirmed by feeding with 0.1 µci/ml of the radio isotope to the albinos animals and the incorporation after 12, 24, 36 and 48 hours was seen. The results are given in Table 2.21 and Fig. 2.9.

Table 2.21

/pigment The ^{14}C incorporation of sodium acetate in the purified/after 12, 24, 36 and 48 hours of feeding

No.of albinos taken/ml	µg/ml of streptomycin	µci/ml of ^{14}C acetate	50 secs counts after			
			12 hrs	24 hrs	36 hrs	48 hrs
500 in each of 4 different tube	50	0.1	441	2,234	612	1,511

(c) Further confirmation of the above experiments was obtained from this experiment, wherein both the pigmented and albinos were fed with 0.25 µci/ml of ^{14}C sodium acetate. The incorporation of ^{14}C in the purified pigment was noted after 2, 4 and 6 hours of feeding. The results are summarised in Table 2.22, Fig. 2.10.

Table 2.22

The ^{14}C incorporation in the pigment of pigmented and albino animals

No.of animals taken		µg/ml of streptomycin		µci/ml of ^{14}C sodium acetate		50 secs counts after					
						2 hrs.		4 hrs.		6 hrs.	
A	P	A	P	A	P	A	P	A	P	A	P
1,000	1,000	50	50	0.25	0.25	24,453	30,214	24,337	27,506	26,880	22,518

A = Albino animals

P = Pigment animals

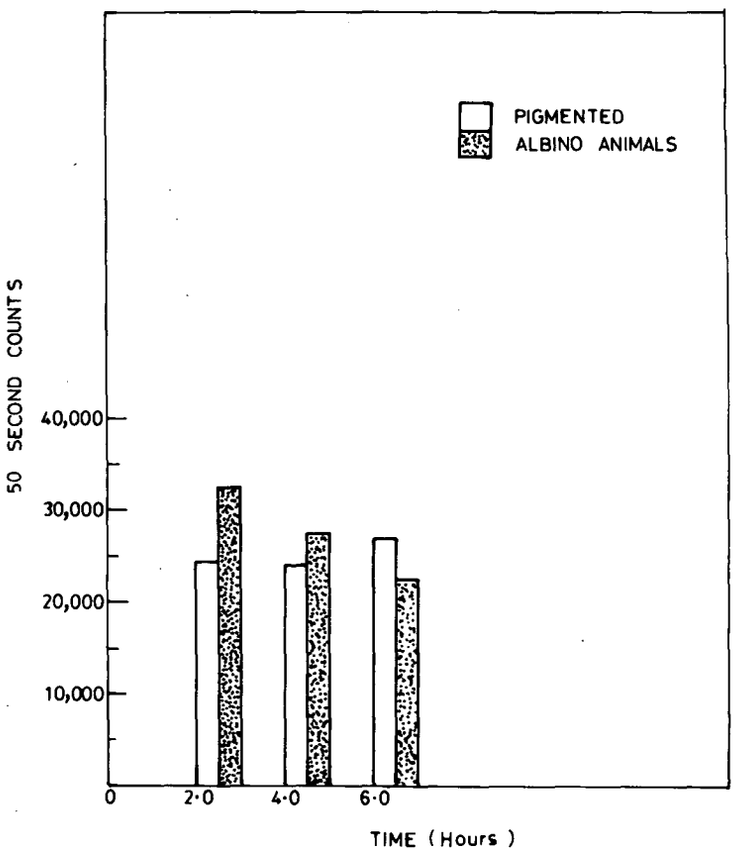


FIG.1.10. ^{14}C SODIUM ACETATE INCORPORATION IN PIGMENTED AND ALBINO ANIMALS

Fig. 2.10 The ^{14}C sodium acetate incorporation in the purified pigment samples obtained from pigmented and albino Blepharismas after 2, 4 and 6 hours feeding.

The result from (a) to (c) show that sodium acetate is incorporated in the pigment and that acetate plays a role in biosynthesis of the pigment.

5. Shikimic acid pathway and the pigment :

To study whether the shikimic acid pathway for making the quinone pigments exists in Blepharisma or not; the enzyme involved in the formation of shikimic acid was studied. The experiments done were:

(a) Assay of the enzyme shikimate reductase :

The assay of the enzyme in the pigmented animals was done as per the method of Balinsky and Davis, 1961, 3-4 days old pea seedlings grown in the dark also contain this enzyme; therefore, to confirm the presence of the enzyme in Blepharisima the enzyme was assayed simultaneously from the pea seedings as well. The results are tabulated in Table 2.23 and activity graph is shown on Fig. 2.11.

Table 2.23

Shikimate reductase in Blepharisma extract				Shikimate reductase in pea seeding extract		
Blank	Experimental	Optical density at 340nm	Time in seconds	Optical density at 340nm	Blank	Experimental
2 ml of glycine -NAOH buffer+0.2 ml NADP+ 0.2 ml animal extract	2 ml of glycine NAOH buffer+ 0.2 ml NADP+ 0.2 ml animal extract+0.2 ml shikimic acid	0.125	30	0.18	2ml of glycine -NAOH buffer + 0.2 ml NADP 0.2 ml pea seeding extract	0.2 ml of buffer+0.2 ml NADP+0.2 ml pea extract +0.2 ml of shikimic acid
		0.125	60	0.18		
		0.12	90	0.18		
		0.12	120	0.18		
		0.12	150	0.19		
		0.12	180	0.195		
		0.12	210	0.20		
		0.12	240	0.20		
		0.12	270	0.20		
		0.12	300	0.20		
		0.118	330	0.21		
		0.118	360	0.21		
		0.115	390	0.21		
		0.115	420	0.215		
		0.115	450	0.22		

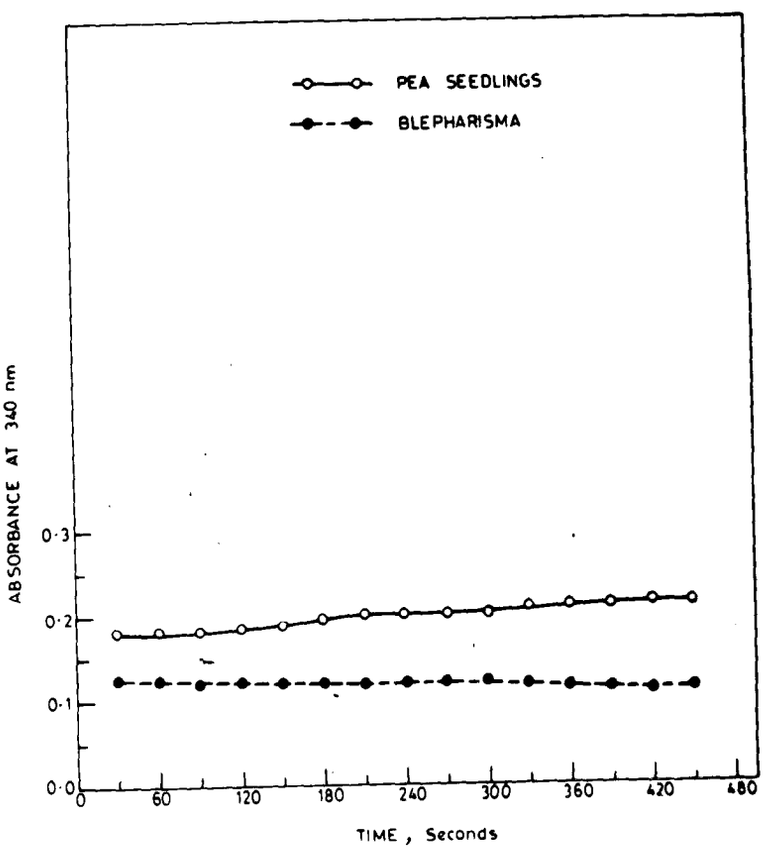


FIG. 11. SHIKIMATE REDUCTASE ACTIVITY IN BLEPHARISMA AND PEA SEEDLINGS

(b) Inhibition of the enzyme shikimate reductase with para-chloro mercuribenzoate (PCMB) :

The albinos were treated with PCMB the inhibitor of the enzyme (Balinsky and Davis, 1961) and the pigment formation was studied by taking the optical density of the pigments extract after 24 hours. The results are given below in Table 2.24.

Table 2.24

Effect of para-chloro mercuribenzoate on the pigment formation

No.of albinos/ml		µg/ml strep-tomycin added		Concentration PCMB to experimental albino animals in molar (M)					
Blank	Experimental	Blank	Experimental						
5,000	5,000	50	50	$2x10^{-6}$	$4x10^{-6}$	$6x10^{-6}$	$8x10^{-6}$	$1x10^{-9}$	$2x10^{-}$
Optical density after 24 hrs. 0.9	-	-	-	0.05	0.06	0.05	0.09	0.02	Dec

The above data indicates that with the inhibition of the enzyme shikimate reductase the pigment is practically is not formed, and that the most effective concentration is $1x10^{-9}$M.

(c) To confirm the above result the albino animals were treated with $1x10^{-5}$M PCMB, blank was run simultaneously and the optical density at 340 nm, of the pigment extracts form the treated and untreated animals was measured after 8, 20, 24 and 44 hours of the treatment. The results are summarised in Table 2.25 and shown in Fig. 2.12

Table 2.25

Pigment formation in $1x10^{-5}$ M PCMB treated albino animals

No.of albino animals taken/ml		Time in hours							
		8		20		24		44	
E	B	E	B	E	B	E	B	E	B
7,000	7,000	0.10	0.145	0.105	0.155	0.115	0.23	0.14	0.285

E - Experimental ; B - Blank

(d) The above observations were confirmed from the absorption spectra of $1x10^{-5}$ M PCMB, treated and the untreated albinos animals after 24 hours of the treatment. The data of optical density at different wave lengths is shown in below in Table 2.26 and Fig. 2.13.

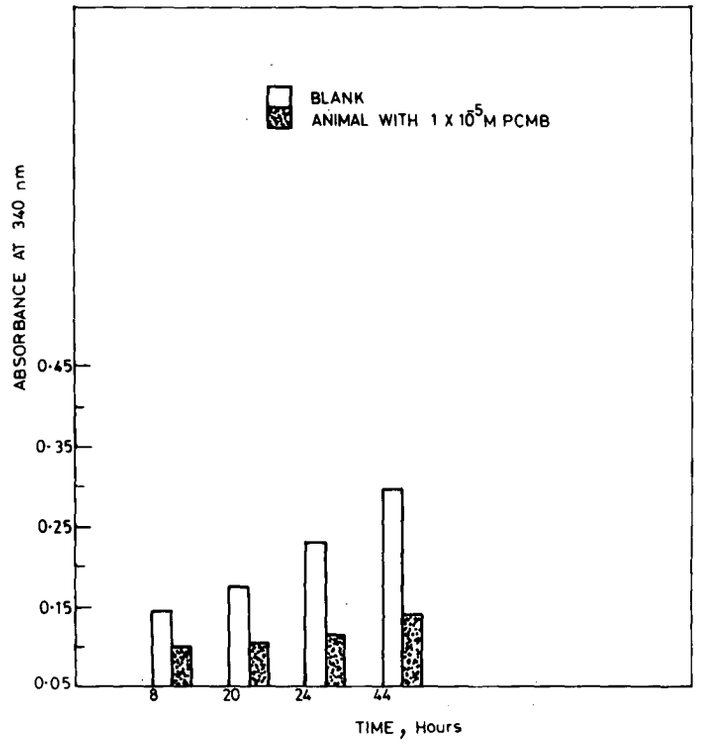


FIG 2.12. EFFECT OF 1×10^{-5}M PCMB ON THE PIGMENT FORMATION

Fig. 2.12 The effect of 1×10^{-5}M PCMB on the regeneration of pigment in the albino animals. The pigment synthesis is much less as compared to the equal number of untreated control(blank) animals.

Table 2.26

Absorption spectra of PCMB treated and untreated pigment extract

nm	Blank	PCMB
330	0.115	No OD
335	0.25	0.10
340	0.29	0.14
345	0.285	0.145
350	0.275	0.145
355	0.26	0.135
360	0.21	0.11
365	0.15	0.076
370	0.11	0.054
375	0.10	0.048
380	0.098	0.044
385	0.086	0.04
390	0.084	0.04
395	0.08	0.038
400	0.074	0.034
410	0.068	0.03
420	0.054	0.026
430	0.044	0.02
440	0.046	0.02
450	0.056	0.026
460	0.074	0.034
470	0.084	0.04
480	0.08	0.04
490	0.11	0.05
500	0.10	0.046
520	0.08	0.04
520	0.08	0.04
530	0.105	0.046
540	0.13	0.06
550	0.12	0.06
560	0.115	0.06
570	0.145	0.068
580	0.185	0.088
590	0.16	0.088
600	0.094	0.064

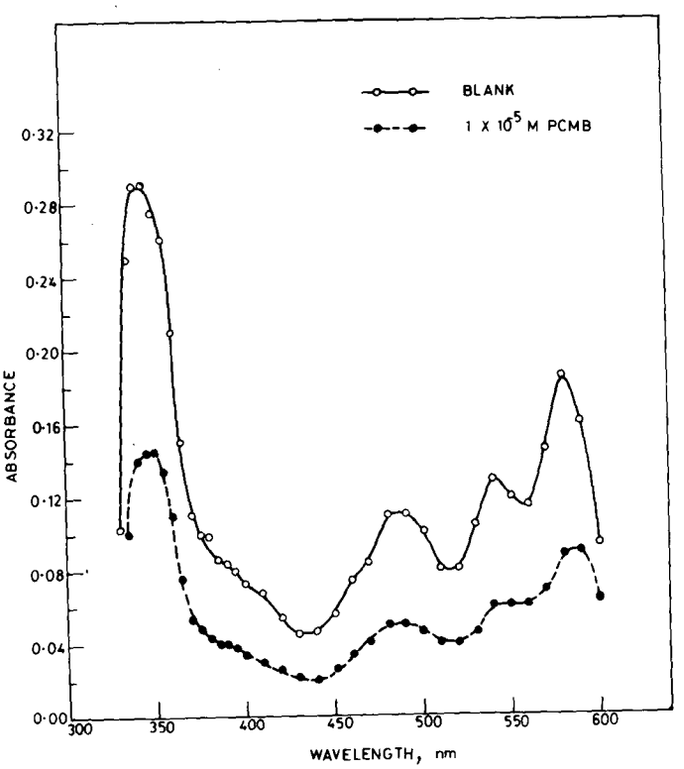


FIG. 2.13. ABSORPTION SPECTRA OF PIGMENT EXTRACTS OF 1×10^{-5} M PCMB TREATED (----) AND UNTREATED (——) BLEPHARISMAS

DISCUSSION

The laboratory made albino Blepharisma regenerate their pigment when placed in the nutrient medium and it takes about 24 hours to regenerate about 60% of the pigment, as is evident from the table 2.1. The complete regeneration of the pigment takes more than 48 hours (Giese and Grainger, 1970). The optical density at 340 nm is taken as the index for assaying the quantity of the pigment formed because the absorption is maximum at this wave length (Fig. 2.1), when the spectrum is taken from 300 to 600 nm. Since the pigment formed in the first twenty four hours is always more than that formed in the next twenty-four hours, all the studies conducted with pigment regeneration are done during the first 24 hours. The fact that the animal continuously synthesises the pigment when it is actively feeding indicates that the pigment must be having an important role, though it can not be a major one, because otherwise the animal would not have taken 48 hours to regenerate something which is vital to it.

Gene and the Pigment

The role of the gene in the pigment formation is shown in tables 2.3 to 2.10. The transcriptional studies using rifampicin inhibitor of RNA synthesis (Table 2.3)and

the translational studies using cycloheximide as the inhibitor of protein synthesis clearly reveal that the results are not consistent with the dosage applied for example with 100 ug/ml of refampicin and 100 ug/ml of cycloheximide the inhibitions is 64 and 41% respectively while with 200 ug/ml the % inhibition decreases to 31.1 and 14.71 respectively. Not only this discrepancy is observed, but the experiments also do not give repeatable data.

To over come these difficultion the gene expression studies are done in the nutrient medium containing 50 ug/ml of streptomycin. In all these experiments first the pilot experiment is run to determine the dose of inhibitor needed to give about 90% inhibition.

The transcriptional studies (Tables 2.5 and 2.6) with Actinomycin D show that 75 ug/ml of this gives 90.5% inhibition (Table 2.5); but the pigment formation is independent of the RNA synthesis, as is clear from the data of Table 2.6.

The translational studies with cycloheximide as the inhibitor of protein synthesis are shown in tables 2.7 and 2.8. Table 2.7 shows 100 ug/ml of cycloheximide gives 93% inhibition after 12 hours but after 24 hours it is only 12.8%. This may be due to the fact that the inhibitor becomes

ineffective after 12 hours. To over come this after 12 hours the fresh culture medium is added and the experimental animals are again treated with cycloheximide. The effect of cycloheximide on the pigment formation is shown in table 2.8. The result show that though there is small decrease in the optical density in the experimental animals, it is not very marked to enable one to draw any definite conclusion.

The DNA synthesis is inhibited with Hydroxy-urea and the Table 2.9 shows 8 mM given 88% inhibition. The effect of this concentration on the pigment formation (table 2.10) once again shows that pigment is formed in the experimental animals and that the DNA synthesis does not control the pigment formation.

The concluding remarks which can be made from gene expression and DNA inhibition studies are that pigment regeneration seems to be independent of the gene and its activities.

Metabolic inhibitors on the pigment formation

The different reaction steps in carbohydrate metabolism are blocked and the pigment regeneration has been studied. The inhibitor used are iodo-acetic acid, iodo-acetamide, sodium fluoride, sodium azide, nickel chloride and 2, 4; dinitrophenol.

The results are tabulated in tables 2.11 to 2.15 and drawn in Figs. 2.2 to 2.6. The steps which are blocked by these reagents are schematically shown below:-

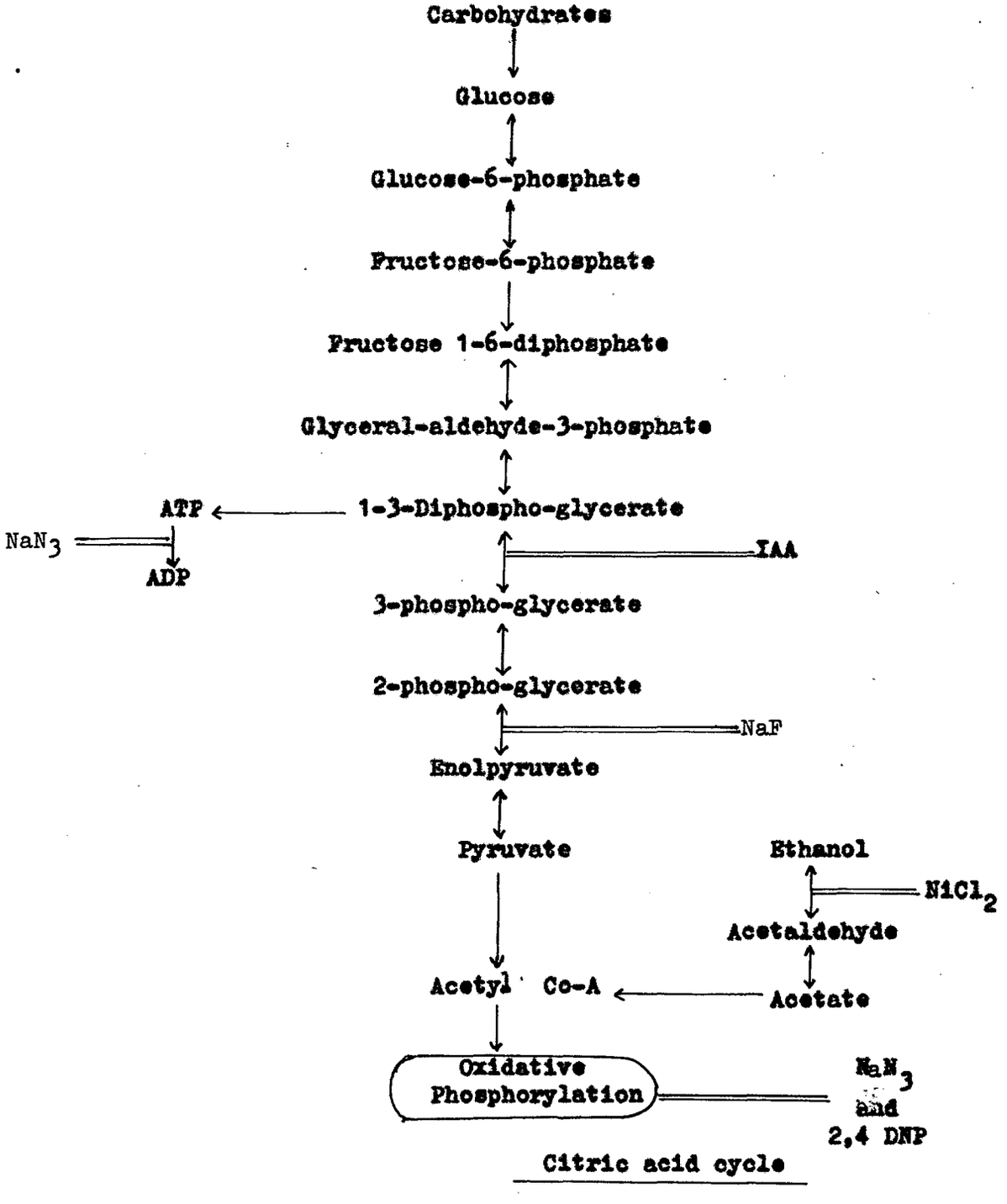


Diagram 1.

Schematic representation of Carbohydrate Metabolism showing the steps at which the inhibition occurs

(i) IAA- Iodo-acetic-acid or Iodoacetamide

(ii) NaF -Sodium fluoride

(iii) NaN_3 - Sodium azide

(iv) $NiCl_2$ - Nickelchloride

(v) 2,4 DNP - 2,4 Dinitrophenol

The treatment of animals with iodo-acetic acid and iodo-acetamide (Table 2.11a and 2.11b) (Figs. 2.2a and 2.2b) shows that the very little regeneration of the pigment takes place. Iodo-acetamide gives better result even with as low a concentration as 0.03 mM ($3x10^{-5}$M), while iodoacetic acid shows the same degree of effectiveness at 0.4 mM ($4x10^{-4}$M).

Iodoacetic acid and iodoacetamide both are alkylating agents and react with SH group of number of enzymes. The iodoacetamide is more penetrating agent than the iodoacetic acid (Goddard,1935). At low concentration the action is specific for the enzyme glycerylaldehyde-3-phosphate dehydrogenase (Racker, 1965). This enzyme is responsible for conversion of 1,3-diphospho-glycerate to 3-phospho-glycerate, or that it interfere with the first stages of the metabolic pathway of glucose utilisation and before the ATP formation takes place.

The effect of sodium fluoride on pigment regeneration is shown in table 2.12 and Fig. 2.3. The results show that at the concentration of 4 and 6 mM/ml the pigment reneration is blocked to a great extent. At higher concentration 8mM onward the reverse trend starts. The enzyme effected by this is the enolase (Racker, 1965) and the conversion of pyruvate from enolpyruvate is effected. The down ward trend at low concentrations once again shows that the pigment formation is in someway related to the carbohydrate metabolism.

The effect of nickel chloride on the pigment is shown in table 2.13 and Fig. 2.4). The regeneration of the pigment is seriously effected and the down ward trend which starts at 0.1 mM concentration is maintained and the maximum is achieved at 4 mM ($4x10^{-3}$M). Not only the pigment is not regenerated but the albino animals are also very sluggish.

Nickel chloride acts on the enzyme alcohol dehydrogenase (Fuhrmann and Rothstein, 1968)and inhibits the formation of acetaldehyde as shown in the above diagram 1. The regeneration of pigment in very little amount clearly shows the importance of the step in controlling the pigment formation.

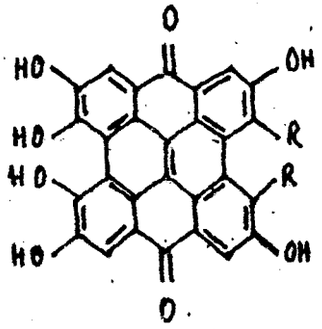
The effect of sodium azide (Table 2.15 and Fig. 2.6) shows that the pigment regeneration is not at all affected,

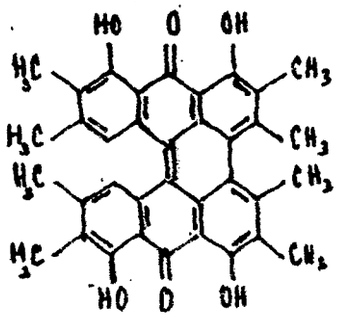
rather, on the contrary, a slight tendency to increase is seen. Similarly, from the results with 2,4 dinitrophenol (Table 2.14 and Fig. 2.5) the downward trend in the pigment regeneration is not observed. The behaviour of the animal towards this reagent is some what unusual but even then one can clearly see the increase in the pigment formation.

Both these reagents are inhibitors of oxidative phosphorylation (Racker, 1965); and thus reduce the amount of ATP produced per glucose molecule.

Now looking at the total characterisation arrived at from these five metabolic inhibitors and their relation to the pigment regeneration, the generalised conclusion is that the pigment is formed during the carbohydrate metabolism or in otherwords it is a metabolic product. It is formed during glycolysis; TCA cycle has no role to play in its generation except to provide the energy. Out of the three steps which control the pigment formation, the one which control the formation of acetate via acetaldehyde (inhibition with $NiCl_2$) plays the key role. This observation is made keeping in view the sluggish behaviour of the animals when treated with this chemical. Another aspect which emerges from this observation is that pigment is in someway related to the vigorousity in the animal.

The insight into the structure of the pigment from Blepharisma undulans has been given by Moller (1962) and Sevanant (1965) and tentative structure was given which is shown in (a). The structure of Blepharisma intermedium was elucidated in this laboratory and is given at (b). The structures shows that they are <u>dimeric polyhydroxy alkylated quinones</u>.





(a) (b)

The quinones whether in plants, bacterias or animals are formed from two pathways:-

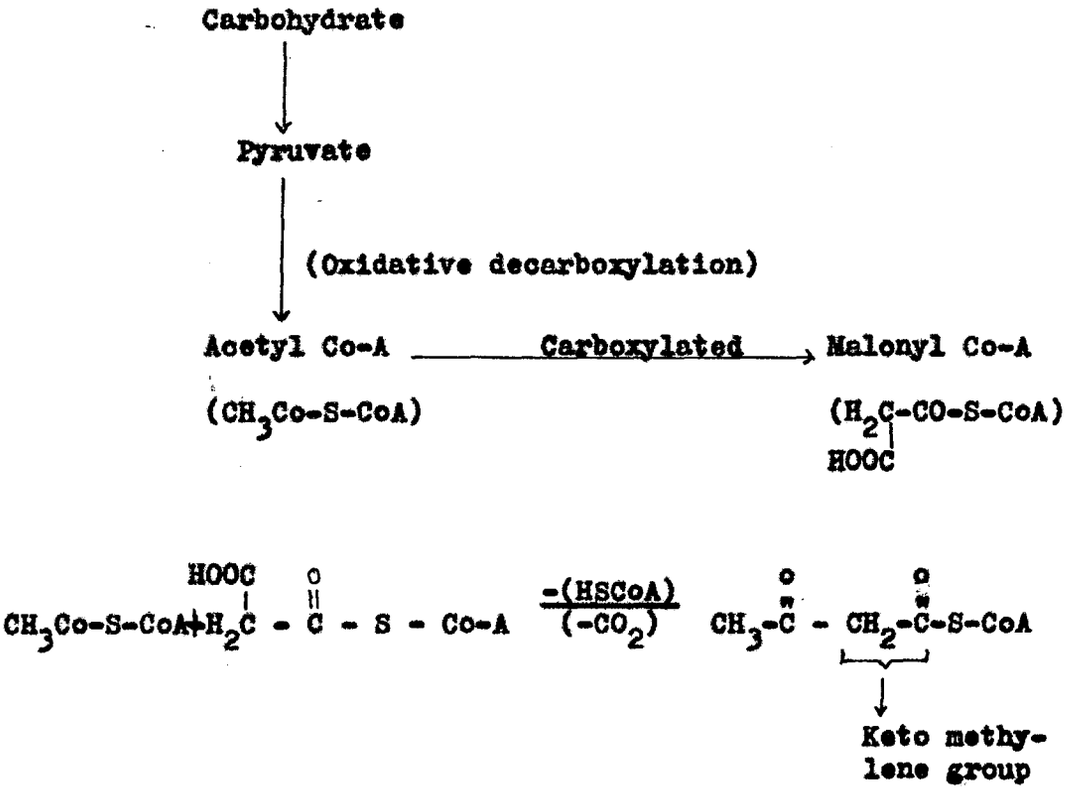
(1) Acetate-malonate pathway

(2) Shikimic acid pathway

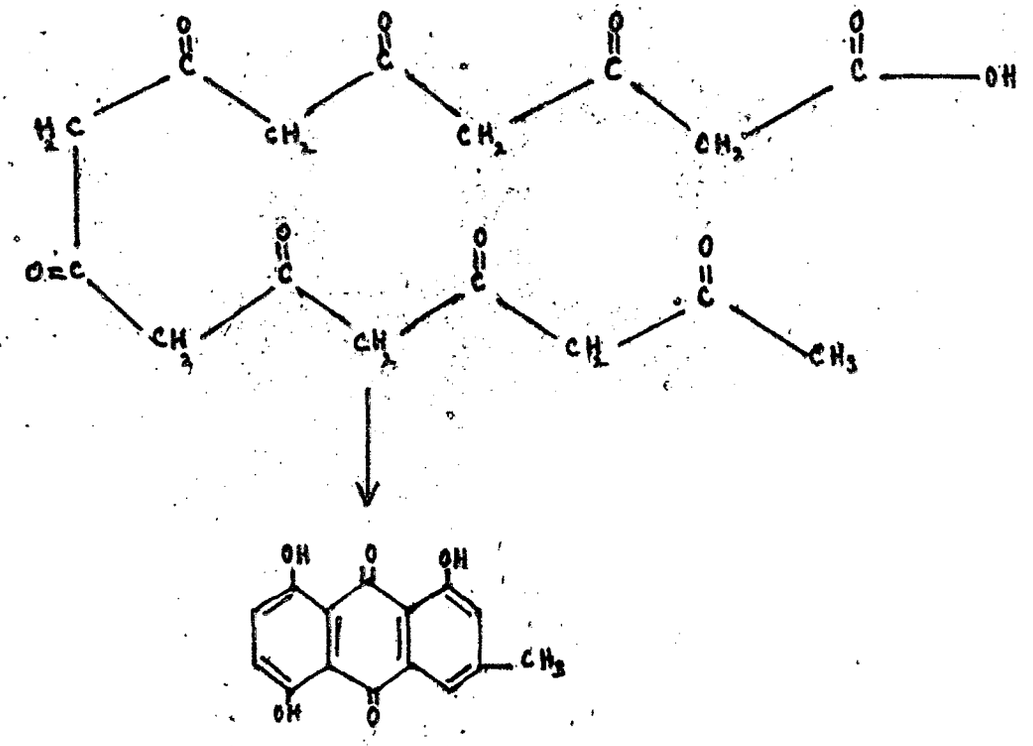
(1) <u>Acetate-malonate pathway</u> :

In this the cyclic compounds are formed by the 'head-tail' condensation of activated acetate unit (acetyl Co-A) and

malonyl Co-A units. The condensation of these two results in the formation of 'poly-keto/methylene groups', which undergo cyclisation, oxidation, reduction, alkylation etc. to form the phenols. The phenols get oxidised to form the quinones as shown :



These keto-methylene groups condense to form ring compounds viz. anthra-quinone is formed by the condensation of seven such methylene groups derived from malonic acid and one from the acetic acid.



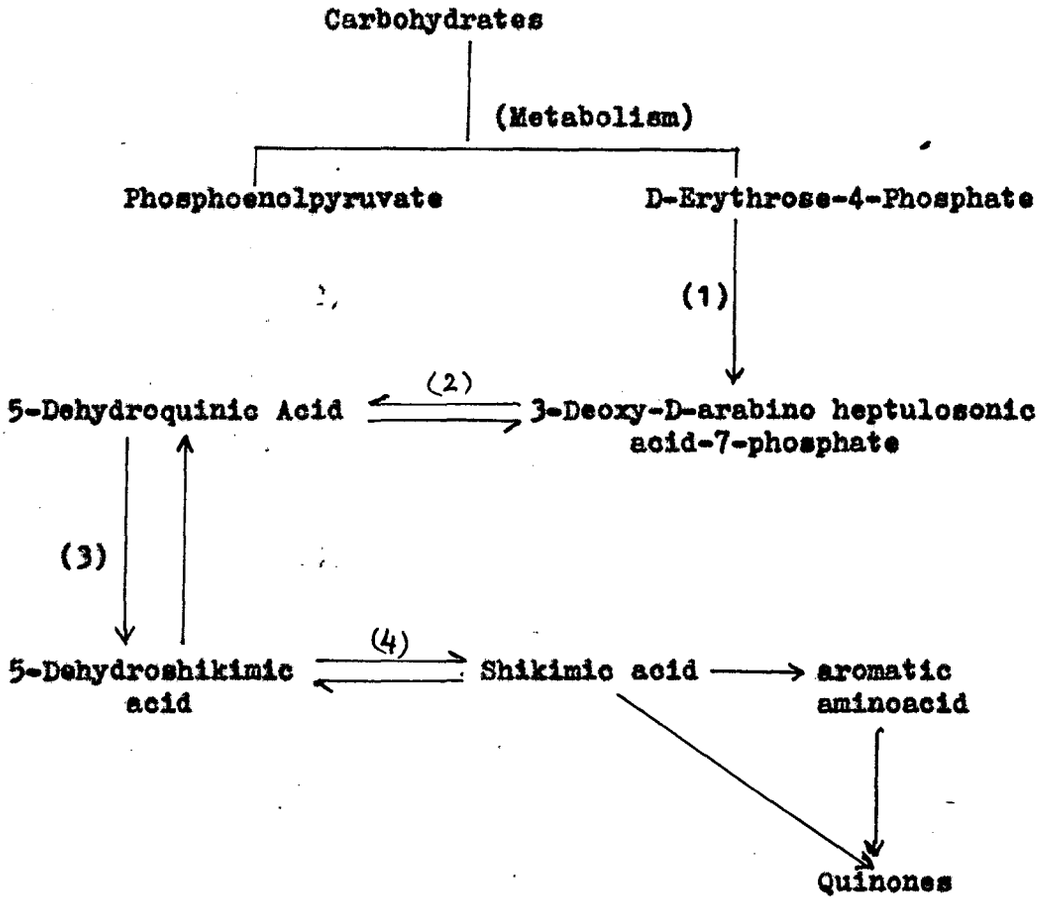
(Emodin)

The acetate unit provides one CH_3-C group only while all the other C atoms come from the malonate. These findings have been proved on different systems using ^{14}C and ^{18}O tracers (Gatenbeck, 1962; Shibata and Ikekawa, 1962,63; Birch et al, 1955; Gattenbeck & Mosbach, 1959; and Bu'lock and Smalley, 1961).

(2) Shikimic acid pathway :

This pathway is used in making aromatic amino acids, phenyl-alanine, tyrosine from aliphatic precursors derived from the carbohydrate metabolism, in plants (both higher lower) and bacteria. In animals it has been reported in Tetrahymena pyriformis (Miller, 1965) tenebrionid beetle

Meinwald et al, 1966). The quinones are formed from the shikimic acid or aromatic amino acids as shown:



Schematic representation of shikimic acid pathway for the synthesis of the quinones

The enzymes involved in steps 1 to 4 are:

(i) DAHP synthetase (Srinivasan & Sprinson, 1959)

(ii) Enzymes requiring DPN and Co (Srinivasan and Sprinson, 1957, 1959)

(iii) 5-Dehydroquinase (Mitsuhashi and Davis ,1954)

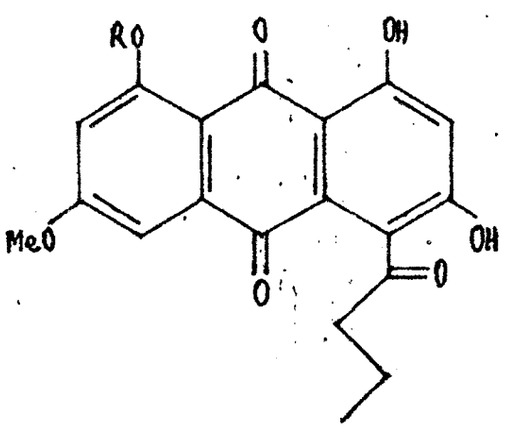
(iv) 5-Dehydro-shikimic acid reductase (Balnisky and Davis, 1961)

Therefore, obviously the main step is the formation of shikimic acid and the corresponding enzyme is 5-dehydro-shikimic acid reductase, from where the quinones are formed.

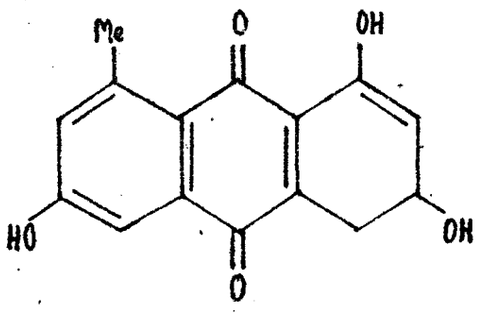
Now, discussing and analysing the results of ^{14}C glucose incorporation in the pigment in the light of above pathways (Tables 2.16 to 2.18 and Figs. 2.7 & 2.8), shows that the pigment is formed as an off-shoot during the carbohydrate metabolism, by secondary reactions. This fact is quite in line with the other secondary metabolic products like tannin, lignins, flavnoids of the plants which also originate by the similar secondary metabolic pathways. These secondary metabolites which like Blepharisma pigment are not essential for the manifestation of life but nevertheless are important for sustaining and maintaining some vital functions of the living systems in which they are present.

The incorporation of the $^{14}C(U)$ sodium acetate in the pigment shows the involvement of 'acetate' pathway in Blepharisma (Tables 2.20 to 2.22 and Figs 2.9 & 2.10). The formation of pigments of anthraquinone type are well established from this pathway viz. the fungal metabolites helminthosporin (Birch et al , 1958), emodin (Gatenbeck, 1958), islandicin (Gatenbeck, 1960) and several others are proved to have originated from acetate pathway by feeding experiments using ^{14}C radio-isotopes. The rhodocomatulin

anthraquinone pigments of crinoids (C. pectinata and C.cratera) (Sutherland and Wells, 1959; 1967) are also shown to be derived from acetate-malonate pathway (Salaque et al, 1967; and Sutherland, 1969). Similarly, the insect pigment which form a coherent group related to 'Deoxyerthrolaccin' (Venkataraman and Coworkers, 1966; 1968) also seemed to be derived from one acetate and seven malonate units though there is, no direct experimental evidence for it. The typical anthraquinone structures from crinoids and insects pigments are given below in (c) & (d) respectively:



(c) Rhodacomatulin



(d) Deoxyerthrolaccin

These are simple and smaller molecules compared to the Blepharisma's pigment molecule.

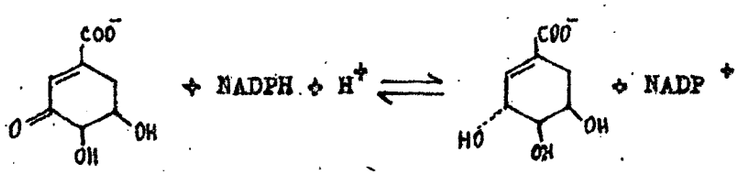
The large molecules as that of Blepharisma are the dimers formed from the simple anthraquinone derivatives.

Ref.Nos. 47,99 & 100 .

In such molecules the phenol coupling of monomers takes place and this is followed by oxidation to give the quinone structure (Imre, 1969; Sidhu and Sankaram, 1966; Sidhu et al, 1968; Fallas and Thomson 1968; and Yoshihira et al, 1970). Thus the polynuclear hypericin like quinone pigment can be the result of phenol coupling of two monomers.

Although, no experimental proof is there for the involvement of acetate pathway, for hypericin like pigments to which Blepharisma pigment belongs; the feeding experiments in echinoderm pigment elsinochrome A, which is a dimer of naphthaquinone have established the existence acetate-malonate pathway (Chen et al, 1966).

Analysis of the shikimic acid pathway results (Table 2.23 Fig. 2.11) show that the key enzyme which forms shikimic acid from dehydro-shikimic acid as per the reaction given below, is present. Since the above reaction is



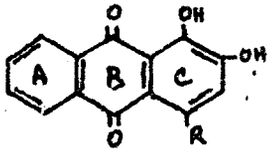
Dehydroshikimate Shikimate

reversible and it is assayed in the direction of dehydro-shikimate formation. The presence of this enzyme whose

presence was confirmed by the specific inhibition with parachloro-mercuri-benzoate (Balinsky & Davis, 1961). The non-regeneration of pigment in animals when treated with the PCMB (Tables 2.25 & 2.26 and Figs. 2.12 & 2.13) clearly shows that shikimic acid pathway plays a role in the pigment formation.

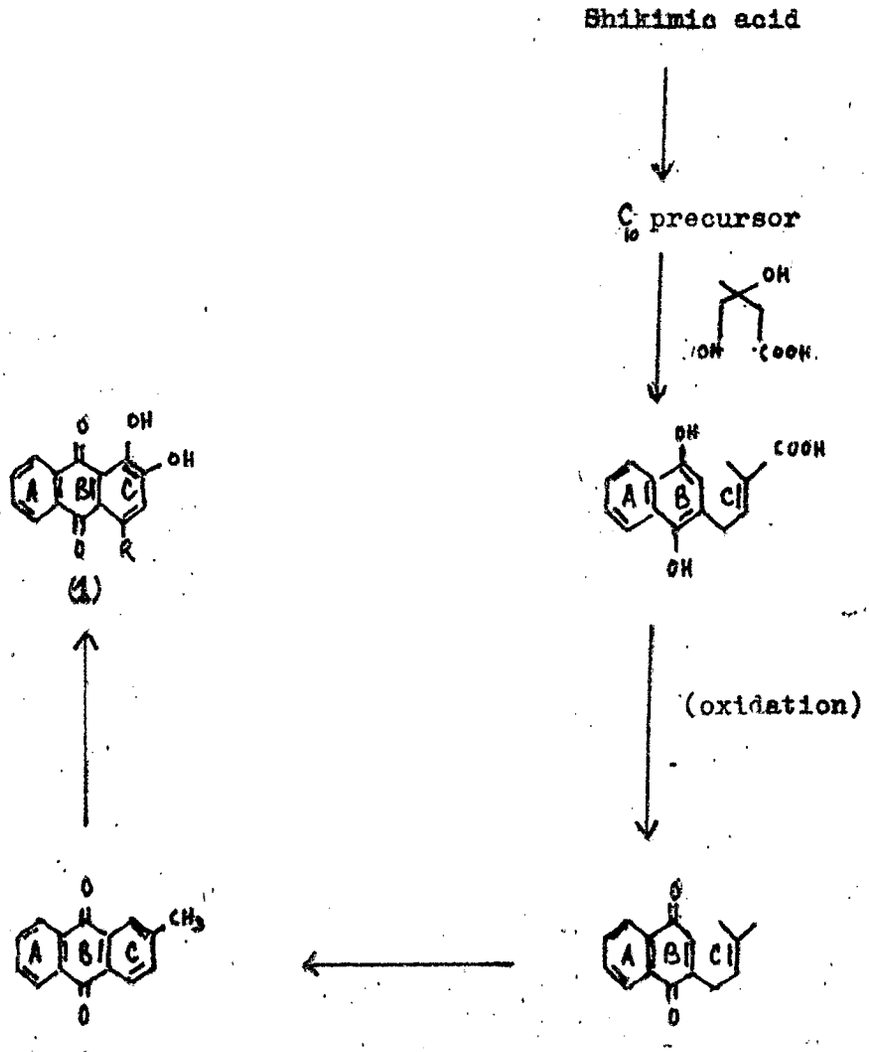
The conclusion from these studies (Tables 2.16 to 2.26) is that both the pathways are operative in Blepharisma intermedium for the formation of the pigment. To what extent each pathway plays the role is yet to be seen and established.

Though, at first glance it may appear strange and unlikely that two pathways can exist in the same organism, recent studies on plants have proved beyond doubt that two pathways can operate simultaneously; and that one benzene ring may arise from shikimic acid pathway and the other via acetate. Burnett and Thomson 1966; 1967 and Leistner and Zenk (1968) working with Rubiaceae (sub-family Rubioidae), Bignoniaceae and Verbenaceae families which all have anthraquinone pigments of the type have shown that ring 'C'

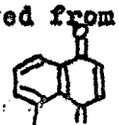
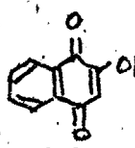


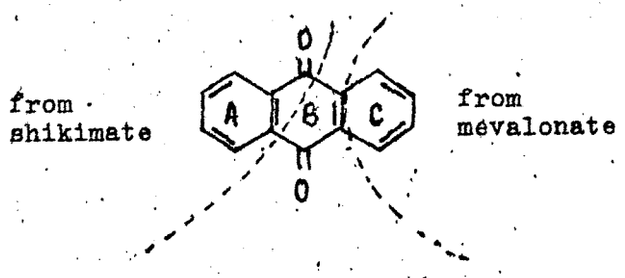
is formed from mevalonate, which in turn, is derived from 'activated' acetate (Popjak & Cornforth 1960; Wright, 1961). This fact was established by feeding the Rubiatinctorum

(madder) plant with 2-^{14}C mevalonate. The whole of ring 'A' and 50% of the carbonyl group of ring 'B' is derived from the shikimic acid as per scheme given below:



Scheme showing the formation of anthraquinone pigments from shikimic acid. If R in (1) is H then the pigment is aliqarin and if it is OH then it is purpurin.

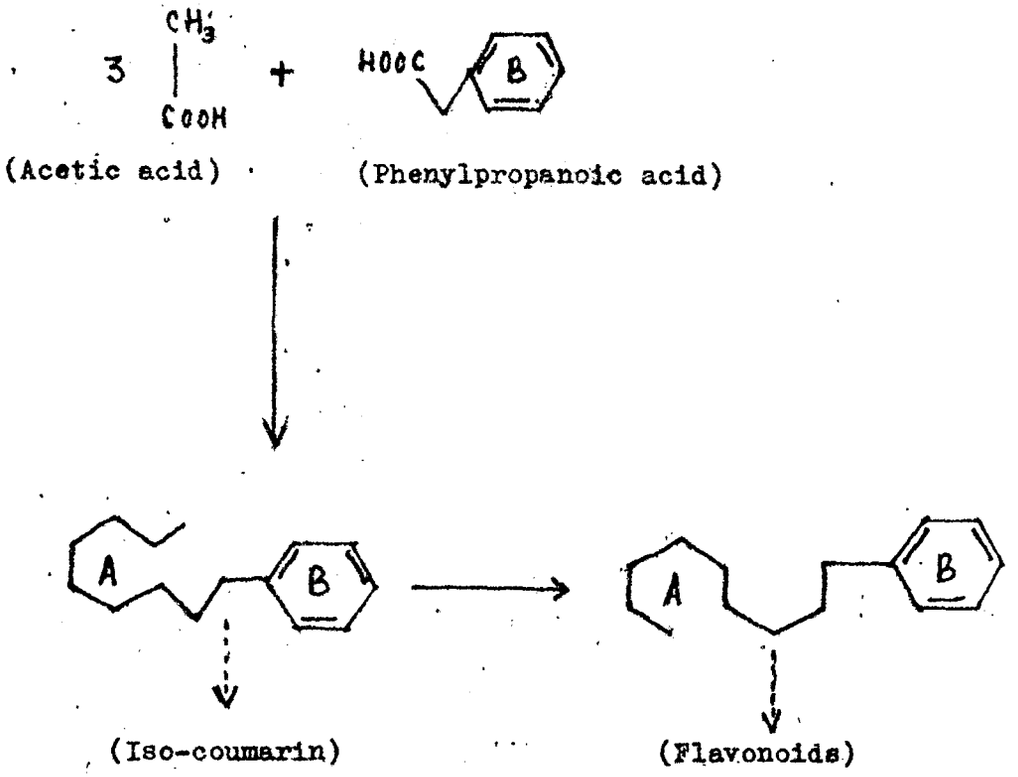
Shikimic acid forms a compound with ten carbon atoms which then condenses with the mevalonic acid (coming from the acetate units) and forms the anthraquinone pigments. Though the nature of C_{10} compound has not been identified in Rubia plant, but there is every likelyhood of its being a naphthol derivative. The C_{10} is a naphthol derivative is based on the experimental facts of Leistner and Zenk (1968; 1967) who found that 1,4 napththaquinone is incorporated in alizarin in Rubiatinctorum. They further showed that ^{14}C shikimic acid is incorporated in 'toto' into alizarin and purpurin (Leistner & Zenk 1967). The importance of C_{10} unit as forming whole of ring 'A' and 50% carbonyl group of ring 'B' has been further proved from the ^{14}C incorporation of shikimic acid in Juglone () (family Junglandaceae) and Lawsone() (family: Lythraceae) (Leistner & Zenk 1967; 68). From the above discussion it is apparent that ring 'A' and part of 'B' is formed from shikimic acid, while ring 'C' is obtained from acetate units. This leaves only three carbons of ring 'B' as shown:- These three



C atoms have been shown to be formed in Juglone and Lawsone (Campbell, 1969) from succinatesemi-aldehyde-thiamine pyrophosphate complex derived from acetate and oxaloacetate; The acetate and oxaloacetate are formed from alanine and aspartate respectively via the Krob's cycle.

Now looking to the overall picture of the three rings of anthraquinone, ring 'A' is totally from shikimic acid, ring 'C' is from acetate while ring 'B' is shared by these two.

It is worth mentioning that existence of both the pathways is not only reported in quinone pigments, but also in flavnoids, isoflavnoids and isocoumarin pigments. Here also two benzene rings are formed by different pathways. The scheme for these pigment is given below. Ring 'A' is formed from acetates while 'B' is formed from shikimic acid. The shikimic acid forms phenyl-propanoid acid (C_6-C_3 unit) (Mc-Calla and Neish, 1965) which then condenses with active acetates units to form the pigments.



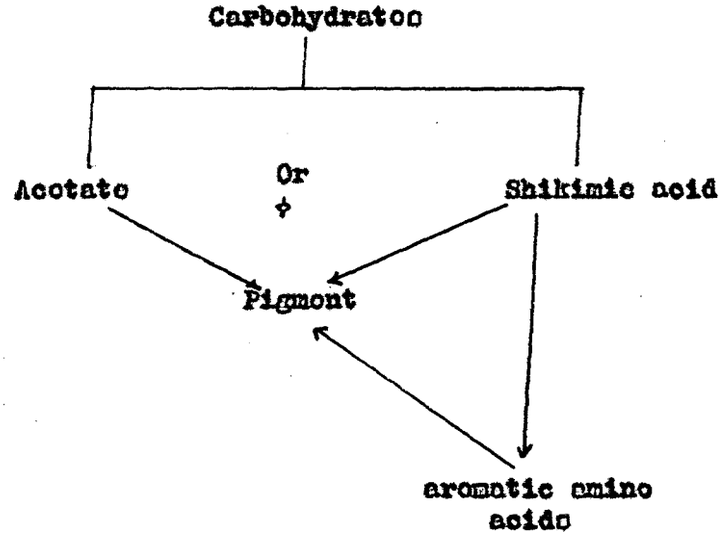
Scheme showing the synthesis of plant pigments

The isocoumarins and flavnoid are formed due to the difference in the mode of cyclisation of the intermediate formed by the condensation of acetates and shikimate acid derivatives (Robinson, 1955; Birch and Donovan, 1953; Bogorad, 1958; Neish, 1960, Grisebach, 1961, and Grisebach and Ollis, 1961).

Though all these observations have been made in higher plants, it is quite logical to extend them to animals, because the pigments of plants have similarity with the animal pigments not only structurally but biosynthetically

as well. Thus emodin is basic pigment of fungi and several higher plants while the isomer of emodin is the parent pigment found in insects (Venkatram-an et al, 1967; 1968). The Blepharisma pigment and the pigment isolated from Jurassic crinoid (Apiocrinus spp) by Blumer (1951, 1960, 1965) are all related structurally to 'Hyporicin', a plant pigment belonging to family Guttiferae (Sevanant 1965; Blumer 1968). The spinochromes of echinoderms are hydroxy naphthaquinones just like juglone, lawsone etc. of the plant kingdom.

Summing up the biosynthetic pathway in Blepharisma intermedium a simple schematic diagram may be given as:



The precursors shikimic acid and the acetate of the pigment arise from carbohydrate metabolism which alone or react together can form the pigment. The shikimic acid may

be directly involved in pigment formation or it may be through tyrosine, phenylalanine. Therefore, future studies should be done on the lines:

(i) To find out the extent of involvement of shikimic acid and acetate pathways in the pigment formation. This is to be done using ^{14}C shikimic acid and ^{14}C acetate studies, and studying the position of the incorporation of these labels in the different rings.

(ii) To find out if shikimic acid directly takes part in the pigment formation or it takes place through aromatic amino acids. This study is to be carried out by feeding ^{14}C phenyl alanine and tyrosine.

(iii) The roles of mevalonic acid and malonic acid in the formation of pigment, using ^{14}C tracer studies.

(iv) The structure of pigment shows several methyl groups in B-position. The origin of methyl groups have been through methionine in other pigments. What role does methionine play in making the pigment in Blepharisma is to be investigated. This can be done using labelled methionine.

SUMMARY

The study done on the biosynthesis of the red pigment of Blepharisma intermedium (indian species) can be summarised as:

(i) The pigment is continuously produced by the animal.

(ii) The pigment is not formed during the protein synthesis.

(iii) The pigment is a metabolic product, and it is formed as an off shoot from the carbohydrate metabolism, through secondary reactions.

(iv) The animal makes use of acetate units and the shikimic acid for synthesis of its pigment.

CHAPTER III

FUNCTION OF THE PIGMENT

INTRODUCTION

The pink to red pigment of Blepharisma is photodynamic in nature and its presence in the animal appears to be a liability rather an asset (Giese, 1972) and yet all the varieties of Blepharisma are pigmented. This present study together with other observations, such as, that the albinos have not been reported in nature except one from Japan (Inaba et al , 1958), the laboratory mutants turn pink after couple of months (Giese unpublished; Ropah, 1968) and the deeply pigmented forms are bigger and more vigorous than their albinos suggest that the pigmented animals must have some selective advantage over their albinos.

However, it must be mentioned that the pigment is lethal to the animal only in the strong light and oxygen. In its natural habitat the Blepharismas live only in the dim light and when the sun light becomes bright, they are found deep in the pond or buried in the debris. The present species of B. intermedium is found in pools throughout the country during the season between the monsoon and the drying out of the ponds (Seshachar unpublished).

The normal functions of an integumented melechromes are protection against the radiations (Davis et al 1963, Pullman, 1972), mechanical protection, chemical defence and

thermal regulation.

It was thought that since the Blepharismas discharge pigment granules under stress it might possible be a protective action against predators, since the pigment in high concentration is toxic to many kind of cells(Giese,1949). However, the Blepharismas grown in mixed cultures in dim light with other ciliates - such as Colpidum, Paramecium, Stentor, Didinium and Actinosphaerium do not discharge pigment when they collide with their neighbours. On the other hand, Actinosphaerium and small crustaceans readily eat the Blepharismas, therefore, the pigment does not seem to protect the Blepharisma against its predators.

Giese (1965) has shown that the pigmented Blepharisma intermedium are more resistant to far uv radiation damage than their albinos. He has also shown that the regeneration of pigmented B. japonicum exposed to far uv is retarded less than the albino mutant. These findings indicate that the pigment is acting as a screen and prevents the animal from the damaging effect of far uv radiations, but at the same time the interesting observation made by Giese (1967) that unlike other protozoans which possess about 95 % of photo-reversal enzyme system, the pigmented as well as the albinos have only about 30 %. This means that the animal has besides the photo-reversal enzyme system, some other agency to repair the damage of far uv radiations.

The role of the pigment in acting as a supplementary agency to the photo-reversal enzyme system to repair the far uv damage was studied in the present investigation.

Blepharisma japonicum and B. intermedium are the two deeply pigmented species of the genus Blepharisma. Those two are the largest species known having size 450-500 and 200-350 µm respectively (Giese, 1973). The role of the pigment in governing the size of B. intermedium (indian species) was undertaken.

The growth of bacteria (Buchbinder et al, 1941; Kurup and Brodie, 1966; Jagger et al, 1964; Buchard and Dworkin, 1966; Kashket and Brodie, 1962; Hollaender, 1943); fungi (Brandt, 1964; Epel and Krause, 1966); protozoa (Epel and Krause, 1966); algae (Epel and Krauss, 1966; Kovalik, 1965); higher plants (Klein and Edsall, 1967; Klein et al, 1965; Mohr, 1961) and animal tissues (Wells and Giese, 1950; Santamaria and Prino, 1964; Rounds and Olson, 1967) is delayed or inhibited under the influence of near ultra-violet and visible light. The delay in growth is due to damage of oxidative respiratory system of the mitochodria. The near uv delays growth because it damages the quinones of the respiratory chain and consequently effects the phosphorylation and production of energy (Almquist, 1937; Ewing et al, 1943; Brodie and Ballantine, 1960; Kashket and Brodie, 1962; Fujita et al, 1966; Werbin et al, 1974).

The visible light delays the growth mainly due to the damage of cytochrome oxidase system (Epel and Butler, 1969; 1970; Ninnemann et al, 1970).

Since the Blepharisma pigment is a quinone (vide Chapter I; Møller, 1962; Sevanant, 1965), it was thought that may be it is protecting the quinone system of the mitochondria by taking the rap of the radiation itself, and acting as a redox agent. So far only the ubiquinones, naphthoquinone, vitamin K group, and plasto-quinones have been shown in vivo to be the potential redox agents involved in the coupling with phorphosylation (Morton, 1965; Brodie, 1963; Mitchell and Marrian, 1965; Ito et al, 1970; Jagger, 1967; Werbin et al, 1974). Though the anthraquinones have not been studied but its nitrogen, sulphur and oxygen analogues such as acridines (Phenazines), phenthiazines (methylene blue) and phenoxazine have been shown to be potential redox agents in the laboratory (Sexton, 1963). The naphthaquinones and anthraquinones have been shown to protect the flavin system, while the phenanthraquinones catalyze the cyclic redox process in Ehrlich as-cite cells (Mitchell and Marrian, 1965; Schmidt & Butler, 1976).

The present study has been undertaken to enrich further our ideas of the photoprotection function of the pigment vis-a-vis growth. The filters were so chosen that they

transmitted only the wave lengths which were absorbed by the pigment. The specific wave length far uv filter was not available, therefore, radiations covering whole of far uv were used. Equal number of pigmented and laboratory made albino Blepharismas were simultaneously irradiated under identical conditions and the protein synthesis in both type of the animals was measured.

MATERIALS AND METHODS

MATERIALS

Blepharisma intermedium wild type and the albino Blopharisma intermedium were the materials used. The albino animals were obtained from the red animals by the method already described.

METHODS

The role played by the pigment in protein synthesis viss vis growth was studied. The albinos as well as red Blepharismas were irradiated with light of specific wave lengths simultaneously. The wave-lengths were so chosen, that they corresponded approximately to the wave-lengths absorbed by the pigment i.e. 225, 340, 480, 540, 580 nm (Tables 1.1 & 2.2). As the filter for 225 nms was not available the UV light from 30W Philips (Cat. No. 57413 P-40 K8) tube fitted in the UV chamber was used, for other wave-lengths the Russian filter numbers y Ø C 8, C 3C9, ✗3C9 and ✗C20 were used for wave-lengths 350, 490, 520 and above 560 respectively. The source of light for these experiments was high pressure mercury lamp of 125W (Philips HPL 125 catalogue No. 57236F). The bulb was fitted in a wooden box fitted both with choke and starter and having 3" diameter opening for holding the

filter.

The general procedure in all these experiments was as follows:

Separately equal number of red and albino animals in 20 ml of freshly prepared cultured medium containing 50 ug/ml of streptomycin were taken in two 50 ml corning beakers. They were irradiated simultaneously in dark with constant stirring with the magentic stirrers. After irradiation the animals were allowed to recover for half an hour at room temperature. Culture medium in which the animals were irradiated was changed with the fresh one. In different tubes equal number of red and albino animals were taken. They were labelled with ^{14}C Leucine (BARC, Bombay)specific activity 120 mci/m mole in the last one hour, that is, labelling was done after 1, 2 and 5 hours of irradiation. The reaction was stopped by keeping the tubes at $0^{o}C$ and the proteins precipated after homogenisation in cold by 10% TCA. The precipatate was filtered in milipore filter washed 3-4 times with cold 5% TCA. The final washing was done with a cold mixture of alcohol ether (3:1). The filters were dried and then counted in liquid scintillation counter in a toluene based liquid scintillation fluid, containing 4g/litre PPO and 50 mg/litre POPOP.

EXPERIMENTAL RESULTS

The same/equal number of pigmented and albino Blepharisma were irradiated simultaneously in the dark. They were exposed to the ultra-violet radiations from a 30 watt uv light for 5 and 7 minutes; and with radiation of 350, 480, 520 and above 560 nm using filters. The protein synthesis in the albinos and the pigmented was measured after 2,3 and 6 hours of irradiation. The results are shown in tables 3.1 and 3.2 and Figs. 3.1 to 3.5.

Effect of ultra-violet light :

(i) 5-minutes radiation effect :

The pigmented and the albino animals immediately after irradiation showed marked difference in their mobility. Many albinos formed clusters, and the ones which were swimming, were doing so slowly as compared to the pigmented ones, all of which were swimming and appeared normal. After twenty-fours of irradiations all the animals (pigmented and albinos) were alive but the albinos looked small, less vigorous and mobile than the pigmented ones.

As is evident from the results (Table 3.1 and Fig. 3.1a) that the protein synthesis in the pigmented animals was considerably more (more than

Table 3.1

Effect of uv light on the protein synthesis in the pigmented and albino Blepharismas

S.No.	Distance of uv tube from the sample (in cm)	No. of animals/ ml	Irradiation given in minutes	^{14}C Leucine in uci/ml	Protein synthesis					
					2 hrs		3 hrs		6 hrs	
					P	A	P	A	P	A
1.	23	1,500	5	0.5	1,749	1,332	3,709	1,489	35,885	11,327
2.	23	1,500	7	0.5	406	281	550	318	606	336

P = Pigment animals

A = Albino animals

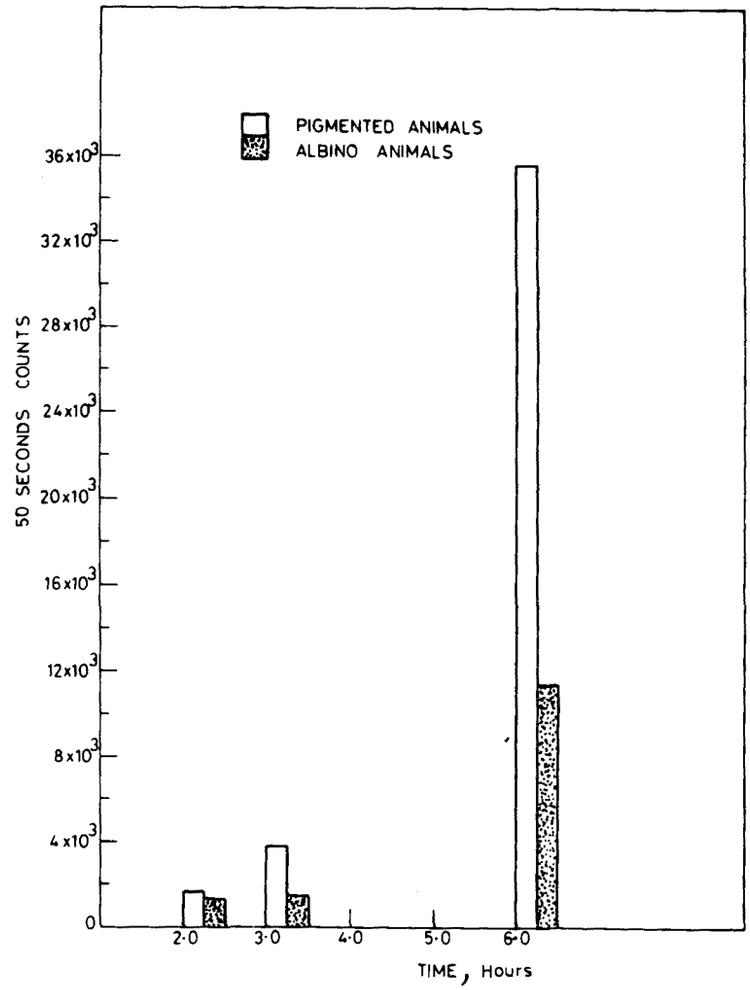


FIG. 3.1a. EFFECT OF 5 MINUTES U V LIGHT ON PROTEIN SYNTHESIS

Fig. 3.1(a) Effect of 5-minutes radiation of ultra-violet light on the protein synthesis in equal number of albino and pigmented Blepharimas. The increase in protein synthesis in the pigmented animals is evident and besides the pigmented animals seem to recover faster from the uv effect as is evident from the 50 % and above enhancement in the protein synthesis after 3 and 6 hours.

30 %) than in the albinos. Another notable feature is the quick recovery from the uv damage of the pigmented animals. This is indicated by the increased protein synthesis (60-70 %) in the animals after 3 and 6 hours (Table 3.1) over their albino counterparts.

(ii) 7-minutes radiations effect :

Like the 5-minutes the immediate effect of uv radiations was on the mobility of the pigmented and albino Blepharisma. Most of the albinos formed clustered whereas the pigmented looked sluggish and moved slowly. After twenty-four all the albinos barring a few were found dead, while the pigmented ones were alive and normal.

The protein synthesis is much more (about 50 %) in the pigmented ones than in the albinos. The results of protein synthesis after 7-minutes treatment reveal that proteins synthesis is seriously impaired in animals. This observation is based upon the observation of much less protein synthesis in the 7-minutes treated animals as compared to the 5-minutes treated ones.

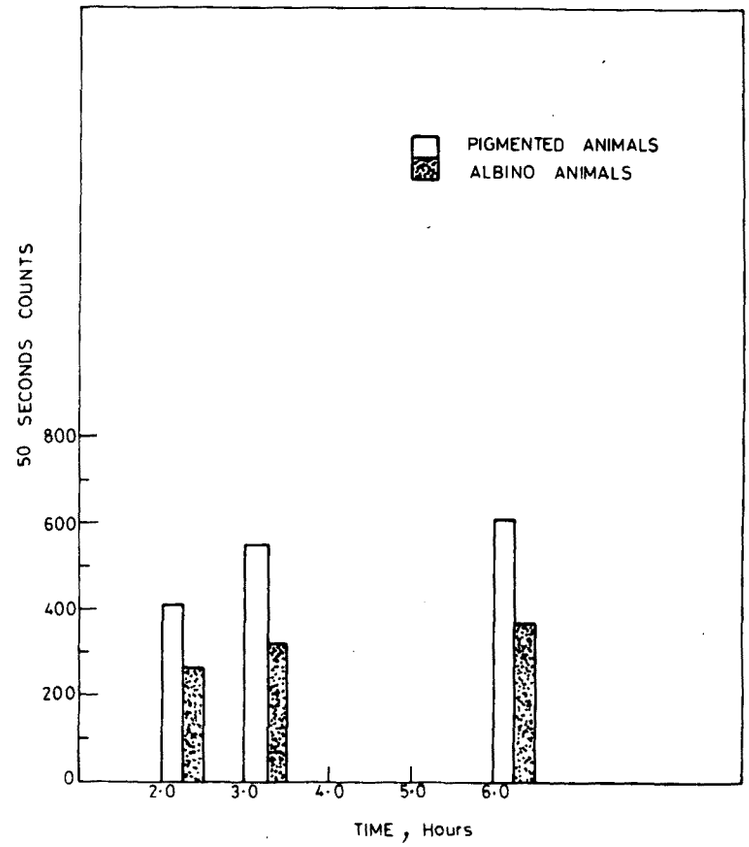


FIG.3.1b. EFFECT OF 7 MINUTES UV LIGHT ON PROTEIN SYNTHESIS

Fig. 3.1(b) Effect of 7-minutes uv radiation on the protein synthesis in equal number of albino and pigmented Blepharismas.

Effect of near uv (350 nm)and visible light (480, 520 and >560 nm) :

The absorption peaks (Table2.2 and Fig. 2.1) of the pigment are at 340, 490, 540 and 580 nm; The filters corresponding to these or nearly the same wave lengths were selected and the equal number of albinos and pigmented were irradiated for 30 minutes and the protein synthesis was measured after 2,3 and 6 hours of irradiations. The results are given in table 3.2 and Figs. 3.2 to 3.5.

(a) Effect near uv (350 nm) light :

The immediate effect after irradiation with 350 nm on the animals were on their mobility. The albinos moved slowly and a few become rounded whereas the pigmented ones looked normal and were swimming like their usual way. Next day, that is, twenty-four after irradiation the albinos though all alive looked smaller and less mobile and vigorous than the pigmented animals. The protein synthesis in the pigmented animals (Table 3.2 top line, Fig. 3.2) was about 50 % more as compared to in the albinos.

(b) Effect of visible light (480, 520 and >560 nm) :

In all these cases also the effect of radiations was felt on the mobility of the albino Blepharisma,

Table 3.2

Effect of near uv and visible light on the protein synthesis in the pigmented and albino Blepharismas

Filter No.	Filter used for wave length (nm)	No. of animals/ ml	Volume in which animal taken	Time given for irradiation	Intensity of irradiation (Lux)	^{14}C Leucine uci/ml	Protein synthesis after irradiations comes					
							2 hrs		3 hrs		6 hrs	
							P	A	P	A	P	A
1. Y ø C8	350	1,500	2 ml	30 min.	20	0.5	11,444	6725	7636	3820	4286	2197
2. C.3 C-9	480	1,500	2 ml	30 min.	410	0.5	1,710	1283	1404	572	1216	932
3. ǂ3C9	520	4,000	2 ml	30 min.	4,000	1.0	47,281	39116	45805	29196	19948	15240
4. ǂ C-20	>560	1,500	2 ml	30 min.	4,200	0.5	915	520	829	713	774	760

These animals showed decreased mobility and this observed effect decreased from 480 to 520 and 560 nm. The albinos in none of these cases formed clusters and were alive after twenty-four. The only noticeable change next day was in the size, mobility and the vigour of the albino animals. These animals except in the case of >560 nm treated albinos looked smaller, less mobile and vigorous than their counterpart red Blepharismas.

The conclusion which can be drawn from all the above studies is that the pigment protects the animal from the radiations. The protection is most pronounced against the far uv radiation, followed by the near uv, 480 and 520 nm and finally >560 nm.

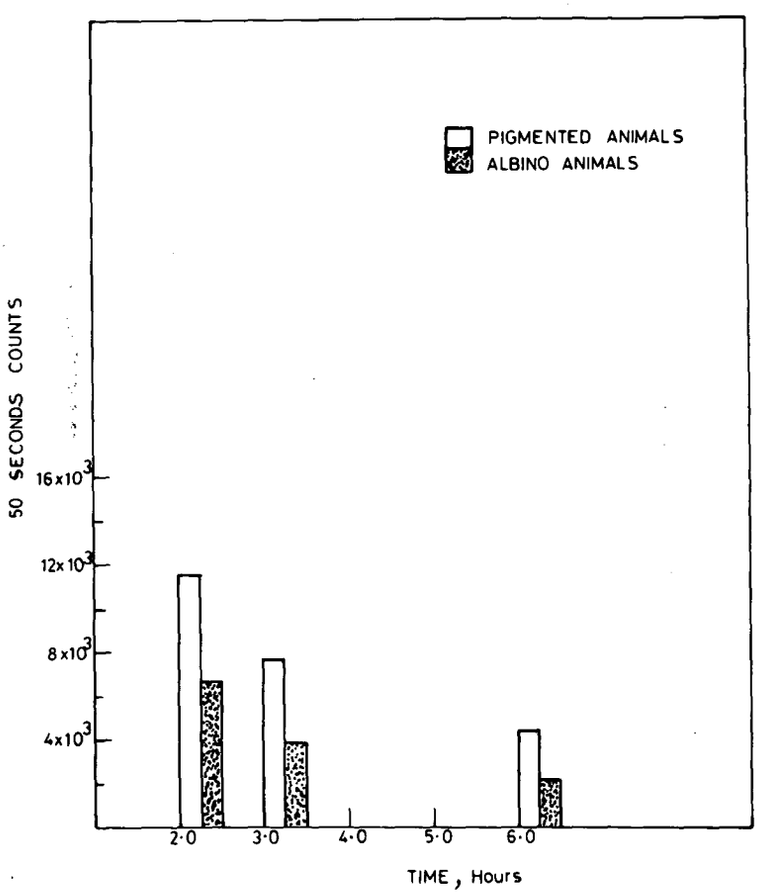


FIG.3.2. EFFECT OF 350 nm LIGHT ON PROTEIN SYNTHESIS

Fig. 3.2 Effect of 350 nm (near uv) on the protein synthesis in equal number of albino and pigmented animals. The protein synthesis is about 50 % less in the albinos than in the pigmented animals.

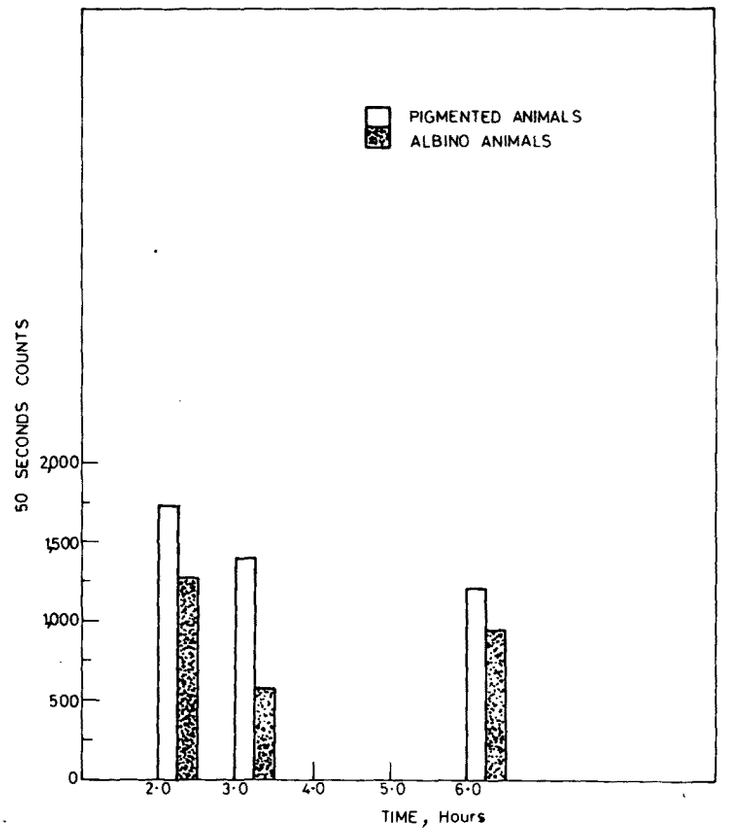


FIG. 3.3. EFFECT OF 480 nm LIGHT ON PROTEIN SYNTHESIS

Fig. 3.3 Effect of 480 nm light on the protein synthesis in equal number of albino and pigmented Blepharismas. The protein synthesis is more in the pigmented animals than in the albinos.

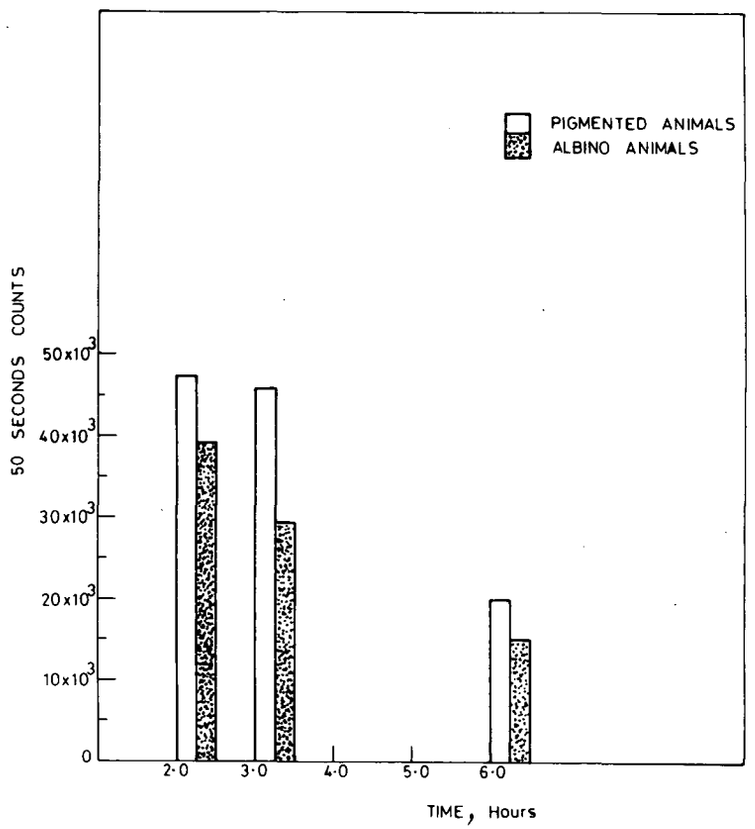


FIG. 3.4. EFFECT OF 520 nm LIGHT ON PROTEIN SYNTHESIS

Fig. 3.4 Effect of 520 nm on the protein synthesis in equal number of albino and pigmented animals.

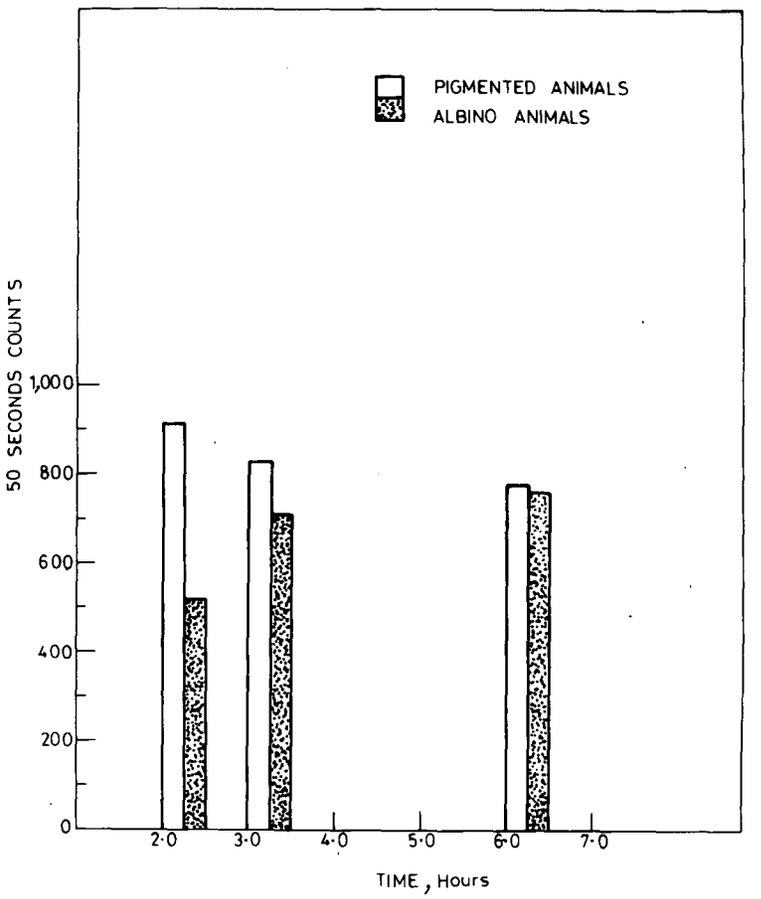


FIG.3.5. EFFECT OF < 560 nm LIGHT ON PROTEIN SYNTHESIS

Fig. 3.5 Effect of $\rangle$560 nm on the protein synthesis in equal number of albino and pigmented Blepharismas.

DISCUSSION

The following generalized observations can be made from the experiments conducted on pigmented and phenotypic albino Blepharismas using ultra-violet and visible light.

(i) The protein synthesis is always more in pigmented animals as compared to in the albino animals.

(ii) The pigmented animals always looked bigger, healthier more mobile than the albinos.

These observations indicate that the pigmented Blepharismas have a natural advantage over their albinos in terms of vigour, mobility and size.

To appreciate the role played by the pigment in Blepharisma, it is worth while to take into account the findings harvested out of similar photo-experiments in the case of other organisms.

(1) Far ultra-violet light (200-300 nm) :

The far uv radiations in 250-280 nm are mainly absorbed by the nucleus and to a much lesser extent by the cytoplasmic proteins. The nuclear absorption of these radiations denatures the DNA. The denaturation is due to the formation of pyrimidine dimers. The dimerisation prevents

or affects the DNA replication which eventually decreases the protein synthesis. The dimerization reaction is reversible. (Boukers et al, 1959; John et al, 1962; Setlow, 1961; Setlow and Carrier, 1966; Setlow et al, 1965; Wulff, 1963). The backward reaction that is monomerization of pyrimidine dimers can be done either by the photo-reactivative enzyme (present in all the cells along with DNA) or by irradiating the affected cell by far uv radiations of 239 nm (Setlow and Setlow, 1967). The reaction can be represented as:

$$2 \text{ Pyrimidine} \underset{239\ nm}{\overset{280\ nm}{\rightleftharpoons}} \text{Pyrimidine dimer}$$

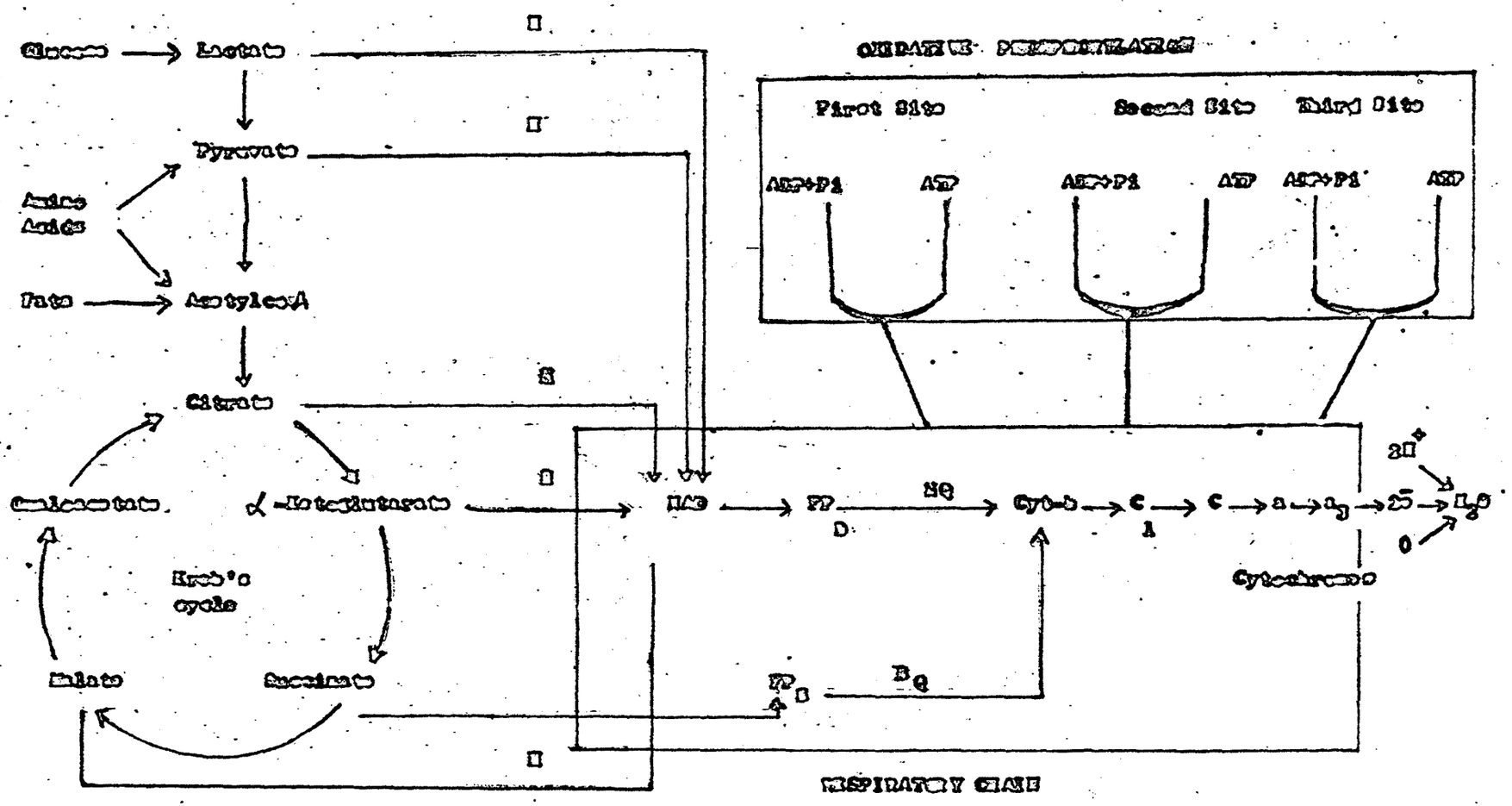
It has been found out the damage done by 280 nm of the far uv radiation is also reversed by certain dyes (Setlow and Carrier, 1967). The energy absorbed by the dyes is passed on to the DNA by energy transfer mechanisms and this energy is used in monomerising the dimers (Sutherland and Sutherland, 1969). This means that the dyes are acting in a manner similar to the 239 nm radiations.

(2) Near uv light (300-400 nm) and visible light :

The irradiation of living systems with near uv light and visible light delay the growth in bacteria, (Jagger et al, 1964), protozoa (Epel & Krause, 1966), algae (Kowallik, 1965), higher plants (Klein and Edsall, 1967; Mohr, 1961) and animal (Hela) cells (Klein and Edsall,

1967). The delay in the growth is due to the adverse effects of the near ultra-violet and visible light on the oxidative respiratory system going on in the mitochindria of a cell.

The normal pathway of the oxidative respiratory system in a cell is:-



Schematic diagram of oxidative respiratory system in mitochondria (adapted from Rackor, 1965). Sugars, proteins, and fats are partially catabolized to produce some ATP in the absence of oxygen (fermentation) and then, in mitochondria, enter the Krebs cycle, in which they are broken down to carbon dioxide, while transferring hydrogen to nicotinamide-adenine dinucleotide (NAD) to produce the highly reduced compound NADH. Electrons are then transported along the chain from NADH through flavoproteins (FP) naphthoquinone (NQ, and cytochromes b, c and a to oxygen to form water. An alternate pathway is from succinate through a flavoprotein and benzoquinone (BQ) to the cytochromes. The respiratory chain is coupled to oxidative phosphorylation, to produce large amounts of ATP, at three points.

As can be seen from the above diagram that the respiratory oxidative system is made from two intermingled and interrelated systems; one the electron respiratory chain system and the other oxidative phosphorylation system. In the electron respiratory chain system the electrons are transported from NADH (sometimes from succinate and malate) to oxygen through flavo-proteins, quinones (naphtho-quinones, vitamin K group, benzoquinones etc.), and cytochromes. In the oxidative phosphorylation the ATP is formed from ADP at three different sites as shown. Both the systems are important for the growth but Brodie and Ballantine(1960) and Lakchaura(1969) have proved from their experiments that oxidative phosphorylation is more important than the electron respiratary chain. It is quite understandable because ATP is the primary sources of chemical energy for all the aerobic organisms.

The experiments (Almquist 1937; Ewing et al, 1943; Boyer, 1959; Brodie and Ballantine; 1960; Kashket and Brodie, 1962; Fujita et al, 1966; Worbin et al, 1974; Creed et al, 1971; Jagger, 1972; Marques and Brodie,1970) with different systems have established that the quinones are the main targets of near uv radiations (300-380 nms). The experiments showed that the damage in the quinones affects the oxidative phosphorylation more than the electron respiratory chain. This results in less production of

ATP and consequently growth is delayed. The flavoproteins and cytochromes are also affected by the near uv but their sensitivity is much less as compared to the quinones. Summing up the effect of near uv on oxidative respiratory system the sensitivity of different components of respiratory chain is Naphthoquinone>Benzquinone>Flavoprotein> Cytochromes.

The delay in growth in different living systems when irradiated with visible light has been shown to be mainly due to the damage in the cytochrome oxidase system and to some extent in the flavoprotein (Epeland Krauss, 1966; Epel and Butler, 1969; Hinnemann et al, 1970). In contrast to the near ultra-violet light the respiratory chain is affected more than the production of ATP.

From the foregoing discussions it can be stated that the protein synthesis or the delay in growth is there in the living systems studied so far, and sites damaged by these radiations is different as is shown in the table 3.3.

Table 3.3

The site and damage of far, near and visible light on a cell

S. No.	Effective wave length (nm)	Site of damage	Damage of radiations	Result
1.	250-280	DNA & to a small extent cytoplasmic proteins	Formation of Primidine dimers	Decreased protein synthesis
2.	300-380	Quinones of oxidative respiratory system	Damage mainly in oxidative phosphorylation system	Decrease production of ATP
3.	400-590	Cytochrome oxidase of oxidative respiratory system	Damage mainly in electron respiratory chain and to lesser extent in oxidative phosphorylation system	Decrease in respiration and production of ATP

In the light of the above metabolic frame, the role of the pigment can now be discussed:

I. Reaction of animals to uv light :

The results of experiments of the protein synthesis of 5 and 7 minutes irradiated pigmented and phenotypic albino Blepharismas show (Table 3.1; Fig. 3.1a and b) that the protein synthesis is much more (approximately 35.5%) in the pigmented animals than in the albinos. Besides this it was observed that most of the albinos clumped together when irradiated for 7 minutes and they were found dead the next day (about 24 hours after the irradiation). The clumping of albinos was observed with 5 minutes ultra-violet treatment also but the animals did not die. The pigmented animals always remained mobile and normal, through immediately after irradiation they looked slugish.

These findings indicate that the pigment protects the DNA and the cytoplasmic proteins of animals from the damaging far uv radiations, as a result the protein synthesis and the ciliary, movements go on uninterupted.

The pigment of Blepharisma being a strong far uv absorber (Table 1.2 and Fig. 1.1) may be acting like a screen or it is also possible that the pigment is mediating as a dye, specially when the photo-reversal system in pigmented and

albino Blepharisma is only about 30 % (Giese, 1967) and the energy absorbed by the dye (pigment) is being transferred and is used for monomerising DNA dimers. It will be really interesting to experiment in vitro to find out if Blepharismin is capable of acting as energy transferring dye like proflavin and acridine dyes, which have been known to monomerise the pyrimidine dimers.

It seems then that the pigmented Blepharismas are better suited for life than the albinos. The resistant nature of the pigmented Blepharisma in far uv light has also been noticed by Giese (1965).

II. Reaction of albinos to near uv and visible light :

(a) Near uv light :

The results of protein synthesis of pigmented and their albino Blepharismas after they were irradiated for 30 minutes by 350 nm, indicate that the protein synthesis is much more (50 %) in the pigmented Blepharismas when compared to in their albinos. The ciliary movements were also more in the pigmented.

These results indicate that the pigment seems to protect the oxidative respiratory system of the animal, by absorbing the radiations (300-380) which, otherwise would have damaged the mitochondrial system. The pigment is a quinone

(Chapter I of the thesis) with maximum absorption at 340 nm and it is very likely that its behaviour is on the lines of other quinones (naphthaquinones, menaquinones, ubiquinones etc.) which as mentioned above are known to effect the oxidative phosphorylation and ATP production. Such a reaction must be taking place is evident from the fact that the red colour of the pigment becomes blue in the presence of near uv light (Giese unpublished).

(b) Visible light :

The results of protein synthesis experiments conducted on pigmented and their albinos after they were irradiate for 30 minutes with 480, 520 and above 560 nm visible light, indicate once again that the protein synthesis is more in the pigmented animals than in the albinos. The ciliary movements were also effected though much less compared to uv light. The ciliary movements were more affected at 480 nm than at 520 and 560. The protein synthesis in albinos at 560 nm after 6 hours was also practically same as in the pigmented.

These results again point out that the pigment by absorbing the radiations (Fig. 2.1; Table 2.2) prevent the damage to the oxidative respiratory system, specially to the electron respiratory chain, as a result the protein synthesis and the ciliary movements are not affected.

Summing up the above observations it can now be stated that pigment protects the animals against ultra-violet light as well from the visible light. Since the pigment can protect the animal from the radiation which are known to have adverse effect on growth, obviously then the animals become better suited and adopted to their environments. Enhanced sensitivity of phenotypic and genotypic albinos of two species of Blepharisma (name not given) as compred to pigmented animals has been observed by Giese (unpublished). Thus the nature by providing the pigment has given selective advantage to the animals.

SUMMARY

The results of experiments of protein synthesis conducted simultaneously on equal number of pigmented and laboratory made albinos Blopharismas with different filters and uv light show the following features :

1. The mobility immediately after irradiations in the albinos was always less than in the pigmented ones.

2. The pigmented animals after twenty-four of irradiations always looked normal, healthier and vigorous than the albinos.

3. The protein synthesis was invariably more in the pigmented animals than in the albinos. The protein synthesis in the albinos at lower wave lengths viz. uv 350, and to some extent at 480 nm was more seriously affected than at 520 and above 560 nm.

4. The pigment is protecting the animals from ultra-violet, and visible light and that the most effective visible region is 480 nm.

5. The recovery from the ultra-violet light damage in the pigmented animals is quicker than in the albinos.

6. The pigment in Blopharisma seems to be a nature's blessing and seems to be responsible to some extent in giving vigorousity, size and mobility over its albino counterparts.

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