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On the Mechanism of the Stable Red Colour Expression of Cellulose-bound Carthamin

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ABSTRACT

Four Carthamus *pigments were mixed separately with cellulose powder or cellulose ion-exchangers in a neutral solution and their bonding capacities were compared. Only carthamin was adsorbed by cellulose powder at a specifically marked high level. Test pigments tended to bind with anion exchange celluloses rather than cationic ones, though some discrepancies were found in the tendency. Carthamin showed less selective affinity for ion-exchange cellulose. It was efficiently taken up by ECTEOLA-cellulose (3·02), PEI-cellulose (2·34), CM-cellulose (2·43), PAB-cellulose (2·82) and SE-cellulose (1·67%) per milligram of them. Precarthamin, safflor yellow A and safflor yellow B were preferentially bound to ECTEOLA-, PEI- and PAB-cellulose. The above-listed pigments showed little or no affinity for CM-Sephadex C-25. A conspicuous augmentation in the adsorption rate was also emphasized, particularly in carthamin, by increasing the amount of cellulose powder and ion-exchange celluloses. Carthamin was separable from the reddish cellulose–carthamin complex with aqueous methanol, ethanol, ethylacetate, acetone, N-vinylpyrrolidone and urea, while the pigment bound to ECTEOLA-cellulose was not. The ion-exchange cellulose-trapped carthamin could only be recovered by using diluted formic acid, acetic acid or ammonia. The experimental findings are discussed in relation to the Saito Effect proposed for the stable colour expression of cellulose-bound carthamin. The possibility of using bound carthamin as a red food colour is also disputed.*

INTRODUCTION

Carthamin is efficiently adsorbed by cellulose and greatly stabilized through a specific interaction between carthamin and cellulose named the 'Saito

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Effect' (Saito & Fukushima, 1986). The effect is so strong that the carthamin may be retained for more than a thousand years without appreciable change in the red coloration. Although initial formation of intramolecular hydrogen bonding may contribute to the pigment stabilization, what process is operative in the attributive tinctorial property, or which site furnishes carthamin with its stable colour expression, still remains largely unknown.

Besides red carthamin, other synonymous yellow or orange–yellow quinoid-chalcone pigments, precarthamin (Takahashi *et al.*, 1984*a*), safflor yellow A (Takahashi *et al.*, 1982) and safflor yellow B (Takahashi *et al.*, 1984*b*), are contained in *Carthamus* flowers. All of these are, of course, soluble in water and are relatively stable in aqueous solutions or in cellular media, except for precarthamin, which is easily turned to red carthamin through enzymic (Saito *et al.*, 1983) or non-enzymic (Saito & Takahashi, 1985) processes. In a preliminary study we observed that the yellow pigments had little affinity for cellulose (Saito & Fukushima, 1986). This clearly indicates that they commonly lack a specific active group(s) to interact with cellulose, by which they are tightly arrested. Therefore, studies on the affinity of carthamin, as well as its related pigments, for cellulose and ion-exchange celluloses must furnish some contributive new suggestions about the regulatory mechanism of the Saito Effect, which makes carthamin form a stable bond with cellulose and subsequently stabilizes the natural red colour.

As an introduction to the study of the mechanism of carthamin red colour stabilization, leading to general application of the pigment, affinitive properties of carthamin, as well as other synonymous colouring matters, were examined under restricted conditions using cellulose and cellulose ion-exchangers. The evidence will be seen below, suggesting that many unknown factors are involved in the chemical interaction between quinoid-chalcone glycosides and cellulose or cellulose derivatives.

ABBREVIATIONS

ECTEOLA = epichlorohydrin triethanolamine, PAB = *p*-aminobenzyl, PEI = polyethyleneimine, CM = carboxymethyl, P = phosphoric ester, SE = sulphoethyl.

MATERIALS AND METHODS

Chemicals

Pure samples of carthamin, precarthamin, safflor yellow A and safflor yellow B were from our laboratory collection obtained by the published methods

from fresh *Carthamus* florets as reported previously (Fukushima *et al.*, 1987). Cellulose powder (MN 100) was purchased from Macherey Nagel (Doren, FRG). CM-Sephadex C-25 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). End group substituted cellulose derivatives, ECTEOLA-, PAB-, PEI-, CM-, P- and SE-cellulose, were provided by Seikagaku Kogyo Co., Ltd. (Tokyo, Japan). *N*-Vinylpyrrolidone and guanidine hydrochloride were supplied from Wako Pure Chemicals Ind., Ltd. (Osaka, Japan). Glassfibre filter (1·0 cm diameter and 0·7 mm thick, Toyo GA 100) was obtained from Toyo Roshi Co., Ltd. (Tokyo, Japan). Other chemicals and solvents were of reagent grade and obtained commercially. Water used throughout this study was deionized and glass-distilled just before initiation of the experiments.

Conditioning of cellulose and cellulose derivatives

A weighed amount of cellulose and its derivatives (30 g dry weight each) was suspended in about three times per gram of 0·2M NaOH and left overnight at 22–24°C. The suspension was washed with water many times by the batch-method and it was immersed again in 0·2M HCl, which was allowed to stand overnight. Washing of the suspension was repeated with distilled water. The washed materials were activated with 0·2M HCl (P-, SE-, CM-cellulose and CM-Sephadex C-25) and 0·2M NaOH (PAB-, PEI- and ECTEOLA-cellulose), respectively. The cellulose and the activated ion-exchange celluloses were stocked in deionized and distilled water at 2°C after washing them several times with 70% aqueous methanol.

Test of the affinity of *Carthamus* pigments for cellulose and cellulose derivatives

Wet cellulose and the derivatives were loaded on a Büchner funnel and the water removed with suction. The resulting semi-dried materials were divided into six groups with different weights, 1, 10, 20, 30, 40 and 50 mg. Each material was mixed with 2·0 ml of solution containing 50 μM test phenolic pigments. After stirring for 10 s, the mixture was passed through a glassfibre filter and the filtrate immediately used for estimating the binding rate of the test pigments with cellulose or cellulose derivatives. The rate of the adsorption was determined by examining the amount of the remaining pigments in the filtrate.

Test of exogenous substances for release of carthamin bound to cellulose powder and ECTEOLA-cellulose

Two millilitre of 50·0 mM citrate-phosphate buffer, pH 7·0, containing carthamin (50 μM) was blended to cellulose powder or ECTEOLA-cellulose

(50 mg wet weight each) for 10 s. Each mixture was filtered and the residue washed with 5·0 ml of deionized and distilled water. The washed material on the filter was then treated with each 5·0 ml of aqueous methanol, ethanol, ethylacetate, acetone, urea, *N*-vinylpyrrolidone, guanidine hydrochloride, formic acid, acetic acid or ammonia. The eluates with water and other solvents used were directly analyzed spectrophotometrically. The solutions eluted with formic acid and acetic acid were adjusted to pH 3·0 with liquid ammonia and used for the spectrophotometric estimation. The eluate of ammonia was acidified with acetic acid to pH 3·0 prior to spectral analysis. The amount of carthamin released from cellulose or ECTEOLA-cellulose was determined by examining change in absorbance at 510 nm with a Shimadzu type 150-02 spectrophotometer.

RESULTS

The amount of pigments bound to glucosyl polymers in a defined experimental period

Carthamin, precarthamin, safflor yellow A and safflor yellow B in a neutral buffer solution were separately mixed with ionically active and inactive celluloses. The contents of the remaining pigments in test filtrates were monitored within 60 s after initiation of the mixing. The data are summarized in Table 1. Carthamin is adsorbed most efficiently by ECTEOLA-cellulose, PAB-cellulose, CM-cellulose, PEI-cellulose or cellulose powder. Precarthamin is fixed with ECTEOLA-cellulose or PEI-cellulose, while it shows no appreciable affinity for cellulose powder. Safflor yellow A is preferentially adsorbed by anion-exchangers such as ECTEOLA-cellulose and PEI-cellulose. Safflor yellow B has an affinity for ECTEOLA-cellulose or PEI-cellulose, while it binds with PAB-, P-, SE-, CM-cellulose and cellulose powder at only a negligible level. None of the test pigments binds appreciably with CM-Sephadex C-25 (Table 1).

Effect of varied amount of cellulose and cellulose derivatives on the adsorption of *Carthamus* pigments

Four *Carthamus* pigments were stirred quickly in a solution with varied weights of cellulose powder, six cellulose ion-exchangers and one Sephadex cation-exchanger. After 10 s, the spectral change in the filtrates was measured spectrophotometrically and the contents of the test pigments were determined by consulting with the standard curves. Figures 1–7 show the adsorption pattern of each pigment to seven different types of celluloses. In

TABLE 1
Comparison of the Specific Bonding of *Carthamus* Pigments to Cellulose Powder, CM-Sephadex C-25 and Cellulose Ion-Exchangers

Glucosyl polymer	*Pigment (pmol mg^{-1} glucosyl polymer min^{-1} × 10^2)*			
	Carthamin	*Precarthamin*	*Safflor yellow A*	*Safflor yellow B*
Cellulose powder	20·4	0·56	1·02	0·66
ECTEOLA-cellulose	22·0	6·96	2·18	3·18
PAB-cellulose	17·4	0·20	1·52	0·18
PEI-cellulose	18·9	5·32	1·86	2·12
P-cellulose	12·7	0·38	0·04	0·30
SE-cellulose	12·7	0·18	1·42	0·14
CM-cellulose	18·0	0·86	0·56	0·38
CM-Sephadex C-25	0·82	0·10	0·06	0·22

The values are mean specific binding activities of quinochalcone pigments to test polymers in a neutral buffer solution. The numerical data were obtained from three replications of the adsorption experiment.

this study carthamin is shown to be adsorbed most prominently by increasing amounts of cellulose powder, ECTEOLA-, PAB-, PEI-, CM-, P- and SE-cellulose. Although precarthamin is changed by increasing concentrations of ECTEOLA- and PEI-cellulose, its adsorption rate is not proportionate to the varied weight of cellulose powder. Safflor yellow A is entrapped by increasing ECTEOLA-cellulose and PEI-cellulose; however, the amount is almost negligible. Safflor yellow B adsorption is increased by additional ECTEOLA- and PEI-cellulose only weakly (see Figs 2 and 4). All *Carthamus* pigments tested are inert to CM-Sephadex C-25.

Recovery of carthamin adsorbed by cellulose powder and ECTEOLA-cellulose

Carthamin was bound to cellulose powder or to ECTEOLA-cellulose which, after washing with water on a funnel, was treated by aqueous alcohols, ester, ketone, nitrogenous compounds, acids and base. Tables 2 and 3 list the effects of organic solvents and nitrogenous compounds on release of carthamin which was arrested by cellulose powder or ECTEOLA-cellulose. Aqueous methanol, ethanol, ethylacetate and acetone are effective to elute bound carthamin from cellulose powder. More carthamin is released at the 30% than at the lower 10% level. None of these solvents can separate ECTEOLA-cellulose-arrested carthamin. Solutions of urea, *N*-vinyl-pyrrolidone and guanidine hydrochloride are also found to be isolation effectors for powdered cellulose-bound carthamin. At higher molar

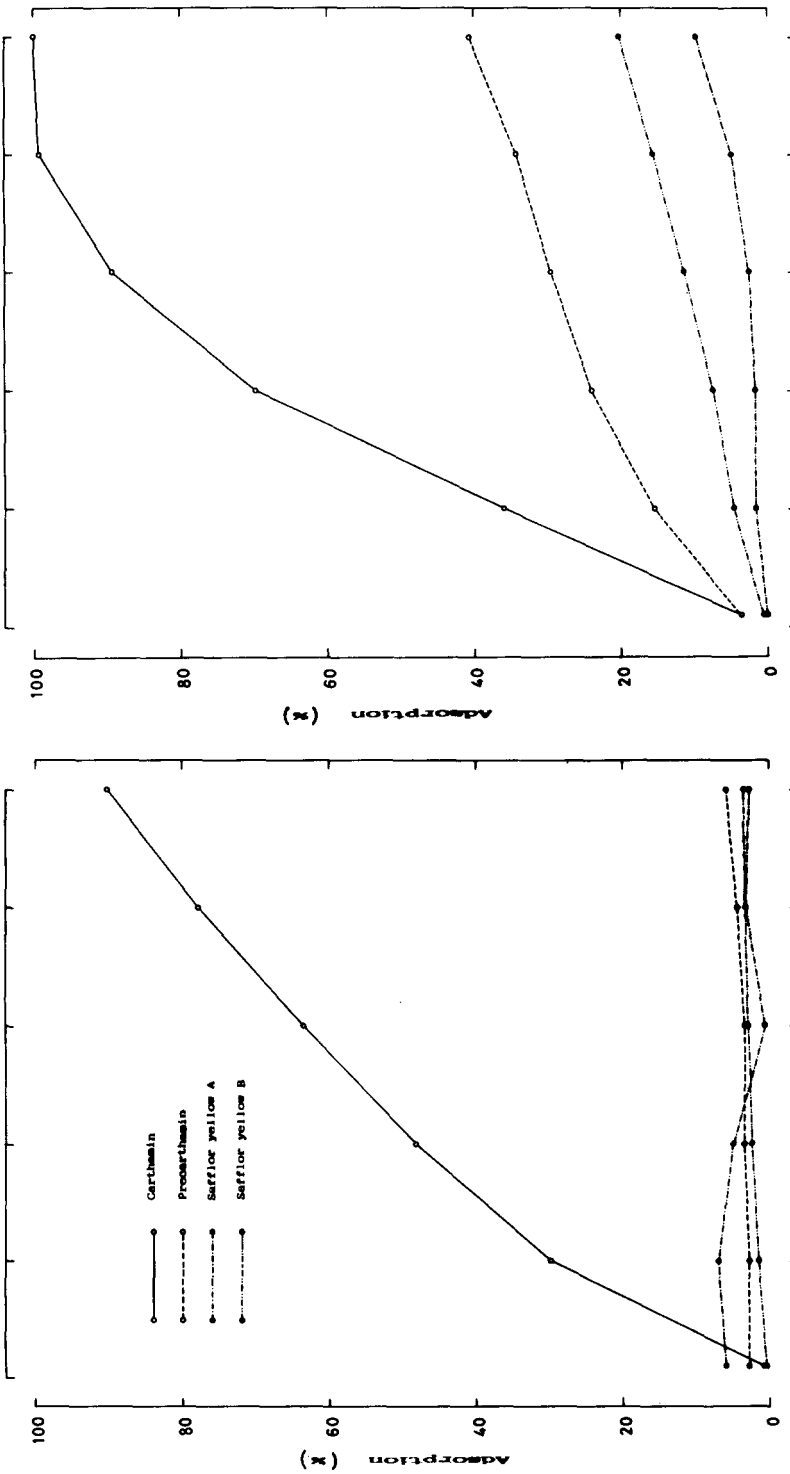


Fig. 1. Effect of increasing amount of cellulose powder on the adsorption of *Carthamus* pigments. Moisture content of cellulose powder was 21·5%. For details of assaying pigment contents see 'Materials and Methods'.

Fig. 2. Effect of increasing amount of ECTEOLA-cellulose on the adsorption of *Carthamus* pigments. Moisture content of ECTEOLA-cellulose was 14·8%. For details of other explanations see 'Materials and Methods', and Fig. 1.

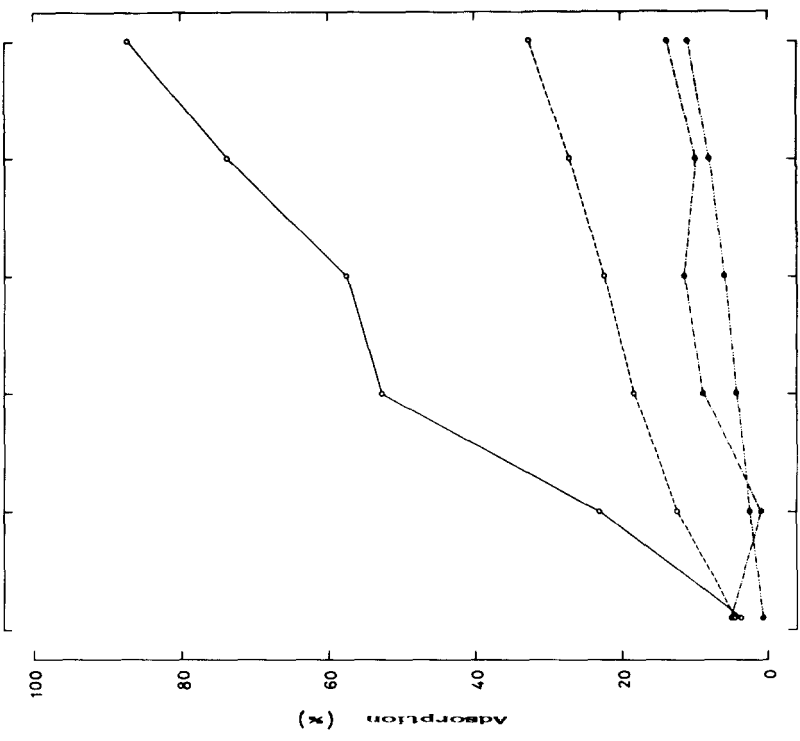


Fig. 4. Effect of increasing amount of PEI-cellulose on the adsorption of *Carthamus* pigments. Moisture content of PEI-cellulose of 8·0%. For details of other explanations see 'Materials and Methods', and Fig. 1.

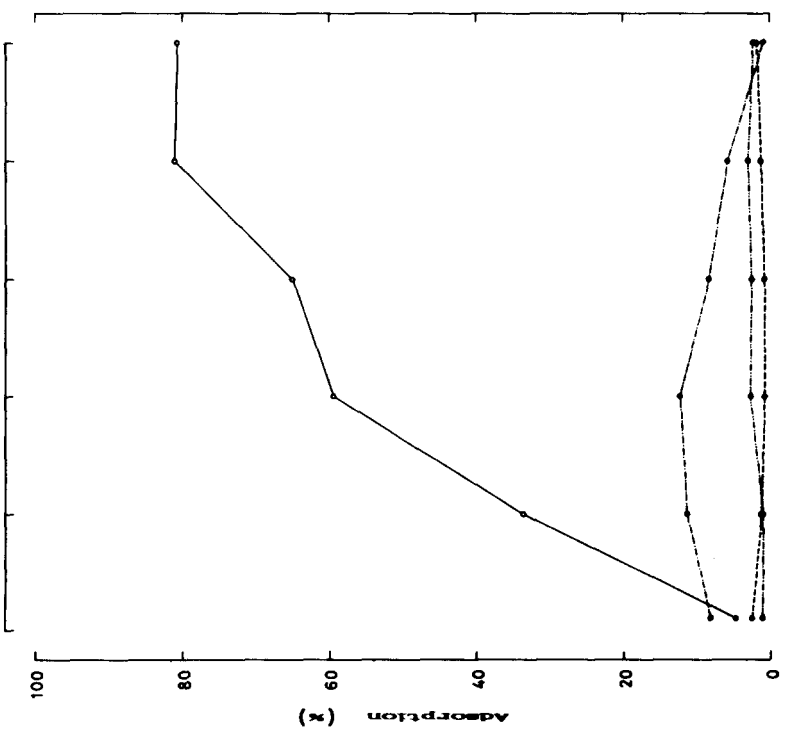


Fig. 3. Effect of increasing amount of PAB-cellulose on the adsorption of *Carthamus* pigments. Moisture content of PAB-cellulose was 9·9%. For details of other explanations see 'Materials and Methods', and Fig. 1.

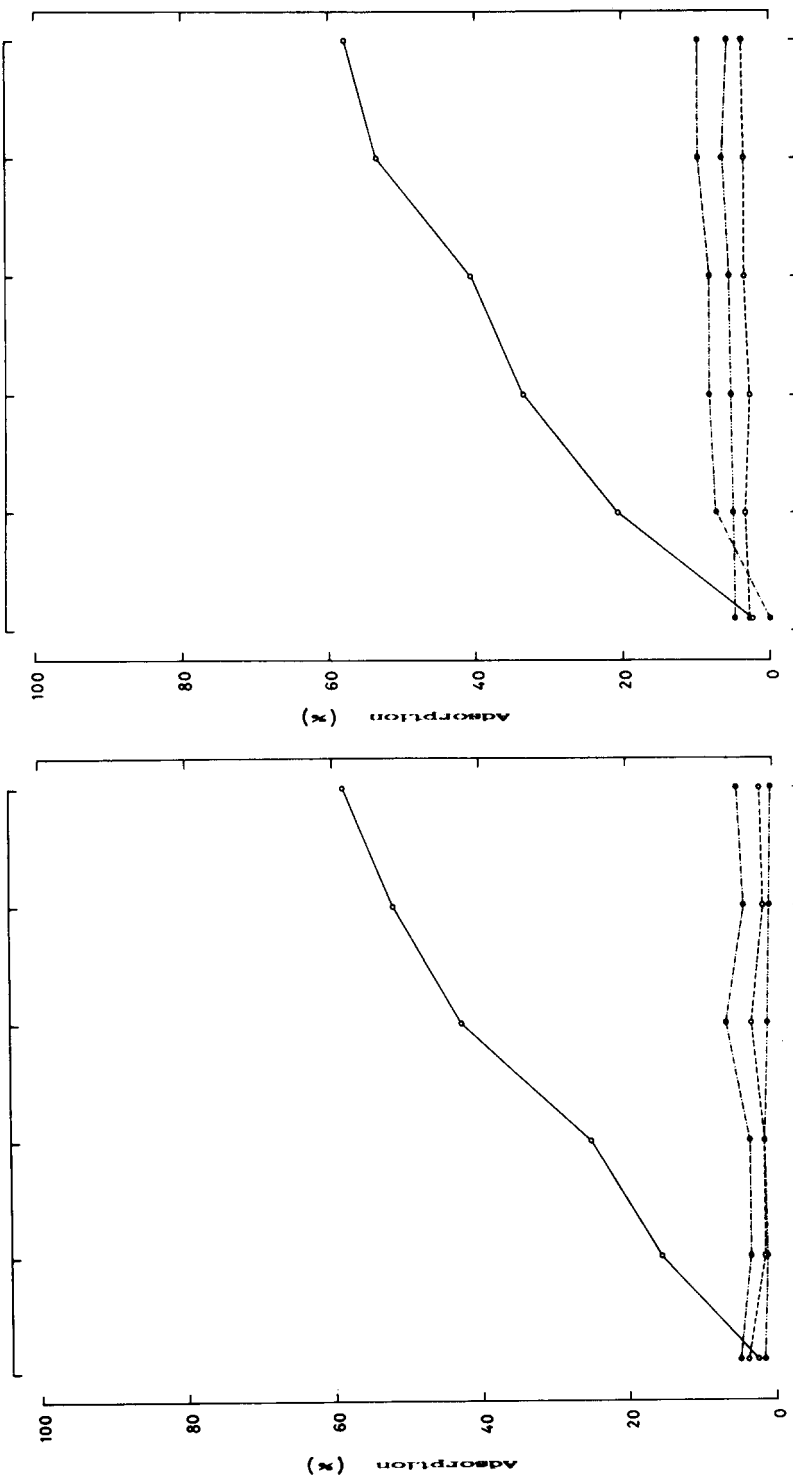


Fig. 5. Effect of increasing amount of P-cellulose on the adsorption of *Carthamus* pigments. Moisture content of P-cellulose was 16·1%. For details of other explanations see 'Materials and Methods', and Fig. 1.

Fig. 6. Effect of increasing amount of SE-cellulose on the adsorption of *Carthamus* pigments. Moisture content of SE-cellulose was 17·7%. For details of other explanations see 'Materials and Methods', and Fig. 1.

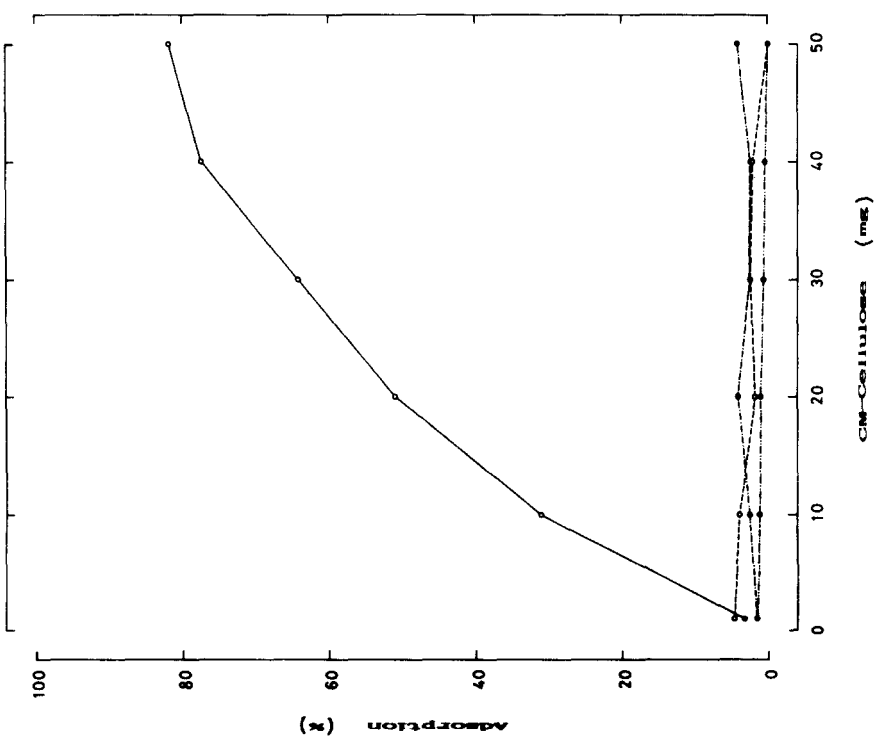


Fig. 7. Effect of increasing amount of CM-cellulose on the adsorption of *Carthamus* pigments. Moisture content of CM-cellulose was 15·4%. For details of other explanations see 'Materials and Methods', and Fig. 1.

TABLE 2
Effect of Organic Solvents on the Release of Carthamin Bound to Cellulose Powder and ECTEOLA-cellulose

Compound	*Concentration* (%)	*Carthamin released (nmol)*	
		Cellulose powder	*ECTEOLA-cellulose*
Methanol	10	23·8	0
	20	32·1	0
	30	37·1	0
Ethanol	10	24·8	0
	20	34·4	0
	30	52·3	1·68
Ethylacetate	3	26·7	0
	6	25·8	0
	10	32·0	0
Acetone	10	40·7	1·00
	20	47·4	0
	30	51·0	0

69·8 and 5·00 nmol bound carthamin were released by pre-washing with 5·0 ml deionized–distilled water from cellulose powder and ECTEOLA-cellulose, respectively, in an average of twelve different determinations.

TABLE 3
Effect of Nitrogenous Compounds on the Release of Carthamin Bound to Cellulose Powder and ECTEOLA-cellulose

Compound	*Concentration* (M)	*Carthamin released (nmol)*	
		Cellulose powder	*ECTEOLA-cellulose*
Urea	2·0	12·1	0
	4·0	18·9	0
	8·0	28·3	2·8
N-Vinylpyrrolidone	0·01	20·9	0
	0·05	19·3	0
	0·1	26·6	0
Guanidine hydrochloride	1·0	0	0
	2·5	0	24·1
	5·0	12·3	44·1

58·4 nmol and a negligible amount of bound carthamin were released by pre-washing with 5·0 ml deionized and distilled water from cellulose powder and ECTEOLA-cellulose, respectively, in an average of nine different determinations.

TABLE 4
Release of Carthamin Ionically Fixed with ECTEOLA-cellulose

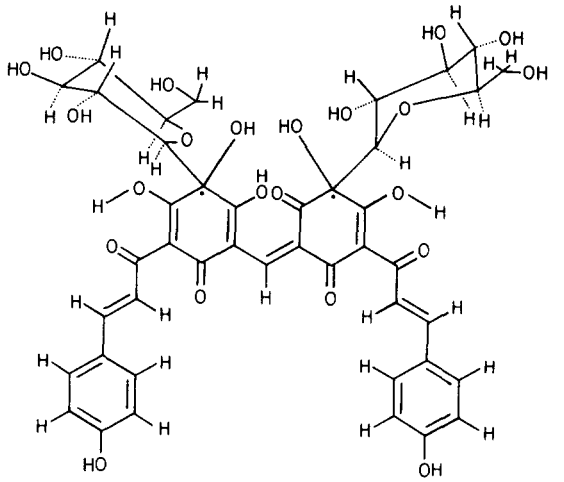
Compound	*Concentration (%)*	*Carthamin released (nmol)*
Formic acid	10	0
	20	19·9
	30	34·0
	50	39·8
	70	64·6
	90	75·8
Acetic acid	10	0
	20	0
	30	0·98
	50	10·8
	70	43·7
	90	51·1
Ammonia	0·04	3·0
	0·4	12·2
	4·0	21·1

No carthamin was detected in the eluates from pre-washing with 5·0 ml deionized–distilled water.

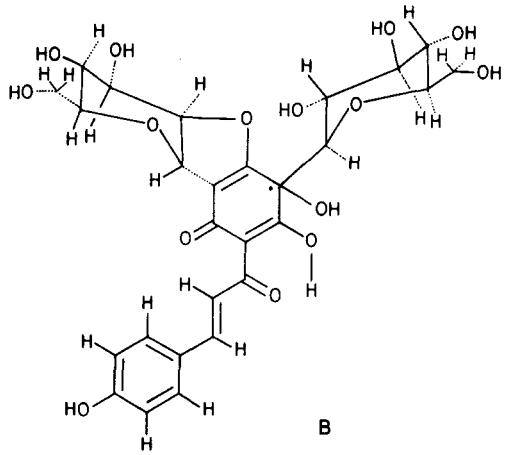
concentrations, carthamin is solubilized from the red powders more readily. Urea and guanidine hydrochloride separate carthamin from ECTEOLA-cellulose by 1·4, 12·1 and 22·1 μM in two millilitre eluates at solvent concentrations of 8, 2·5 or 5M, respectively. Ionically fixed carthamin could be recovered from ECTEOLA-cellulose by using diluted formic acid, acetic acid and ammonia (Table 4). The recovery rate of the bound pigment rises in proportion to increasing concentration of the base and acids examined.

DISCUSSION

The present studies provide new evidence that *Carthamus* pigments are entrapped by various cellulose derivatives with or without ionically charged end-groups. The rates of the trapping were markedly different according to whether cellulose or its derivatives were tested. These findings in part, support our previous postulation, that the specific affinity or carthamin for cellulose might be closely related to structural configuration (Saito & Fukushima, 1986). *Carthamus* pigments used for the present investigation were composed of the following chemical structures, though precarthamin



A



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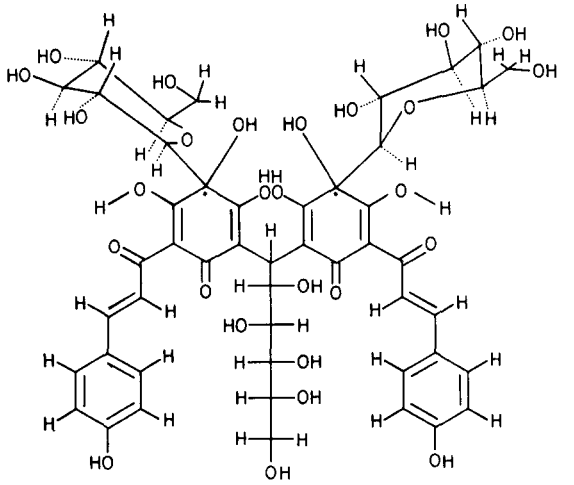

C

Fig. 8. Structures of (A) carthamin; (B) safflor yellow A; (C) safflor yellow B

has not yet been chemically characterized. Carthamin: 6-β-D-glucopyranosyl-2-[[3-β-D-glucopyranosyl-2,3,4-trihydroxy-5-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]-6-oxo-1,4-cyclohexadien-1-yl]-methylene]-5,6-dihydroxy-4-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]-4-cyclohexen-1,3-dione (Takahashi *et al.*, 1982; Obara & Onodera, 1979) (Fig. 8(A)), safflor yellow A: 6-β-D-glucopyranosyl-2,3,4a,6,9b-hexahydro-3,4,6,7-tetrahydroxy-2-(hydroxymethyl)-8-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl-9H-pyrano[3,2-b]benzofuran-9-one (Takahashi *et al.*, 1982) (Fig. 8(B)), safflor yellow B: 1-deoxy-1,1-bis[3-β-D-glucopyranosyl-2,3,4-trihydroxy-5-[3-(4-hydroxyphenyl)-1-oxo-2-propenyl]-6-oxo-1,4-cyclohexadien-1-yl]-D-glucitol (Takahashi *et al.*, 1984) (Fig. 8(C)). The relative binding rate of the pigments with ionically uncharged cellulose or ionically charged cellulose ranged in the following order (%, in average of four and six different determinations): carthamin (90·1:78·4), precarthamin (2.5:10·7), safflor yellow A (4·5:5·8) and safflor yellow B (2·9:5·0). The differences in these affinity rates seem to be closely related with the charge forms of the test adsorbents, though carthamin showed less selective affinity for cellulose and cellulose derivatives. For example, precarthamin and safflor yellow B were attracted by ECTEOLA- and PEI-cellulose far more strongly than ionically uncharged cellulose powder. They bound more tightly with anion-exchangers (ECTEOLA-, PAB- and PEI-cellulose) than cationic (P-, SE, and CM-cellulose). In general, phenolic substances are recognized to be negatively charged in polar media (Lam & Shaw, 1970). Therefore, it is reasonable that *Carthamus* pigments, which are new types of quinonoidal chalcone glycosides, are readily bound with anionic-exchangers. However, it still remains obscure why only carthamin showed a high affinity for cellulose powder and how carthamin was attracted strongly by both cationic and anionic-exchangers (see Table 1 and Figs 1–7). Possibly this resulted from two different affinity mechanisms of carthamin through hydrogen bonding with the hydrophilic hydroxyl group(s) on cellulose or cellulose derivatives and ionic bonding with the ionically charged substituent(s) on cellulose ion-exchangers. Based on the present studies, the above supposition can be clearly justified from the experiments with hydrogen bond dissociation reagents such as aqueous methanol, ethanol, ethylacetate and acetone which resulted in a marked loss of the cellulose-bonding capacity of carthamin with increased reagent concentrations. The release of bound carthamin by urea, pyrrolidone and guanidine hydrochloride also strongly indicates that the pigment was fixed with cellulose by hydrogen bonding. On the other hand, carthamin, from the ECTEOLOA-cellulose–carthamin insoluble complex, could only be recovered by washing with diluted formic acid, acetic acid, ammonia, and/or with urea and guanidine hydrochloride at higher concentrations. The differences in the binding mechanisms of carthamin to cellulose and

ECTEOLA-cellulose are also indicated by the fine colour expression. Cellulose powder-bound carthamin kept its natural red colour for a long time, while ECTEOLA-cellulose-bound carthamin rapidly lost its characteristic red colour and turned to reddish purple or purple within a few minutes. This colour change on ECTEOLA-cellulose is indicative of a hypsochromic chemical shift of the red colour resulting from contact of the chromospecific action site(s) of carthamin with the epichlorohydrin triethanolamine substituent(s) on the cellulose anion-exchanger, though the bonding mechanism remains to be studied precisely. Other yellow or orange–yellow pigments used may also be bound to anion-exchangers through almost the same process.

The above results provide the first direct evidence that carthamin is bound to cellulose ion-exchangers *via* a different binding mechanism from that of cellulose powder. Therefore, carthamin may have two specific binding sites in its structure and the hydrogen bonding site must play an important role in keeping the stable colour expression on cellulose, which leads to the control mechanism of the Saito Effect. It is interesting to note that an inert substance, precarthamin, is changed readily (batho-shift) to the chemically active product, carthamin, through a naturally occurring oxidation reaction. Upon searching for the catalytic action site(s) between carthamin and cellulose, the mechanism of the stable red colour pronunciation may be realized chemically and/or theoretically. The bound carthamin can preserve its specific red colour for a long time on both wet and dried pigment-cellulose conjugates. These facts strongly indicate the possibility that carthamin is applicable more universally to manufactured foods or medical tablets as a natural harmless colour additive. In planning the practical use of the bound carthamin, with an economic aim, the above evidence will surely provide a contribution.

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