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Dye-Binding Stoichiometry of AO 12, AB 10B and OG with Etalon Proteins, Feed and Feedingstuffs and its Application for Reactive Lysine Determination

Ibolya Molnár-Perl, Margit Pintér-Szakács

Institute of Inorganic and Analytical Chemistry, L. Eötvös University, VIII., Múzeum krt. 4B, Budapest, Hungary

Ágnes Kővágó, István Petróczy

Hajduság Agrarian Industrial Association, Hajduság, 4181 Nádudvar, Hungary

U. Pál Kralovánszky & Jakab Mátyás

Managing Department for Protein Researches, 1056 Váci u 81, Budapest, Hungary

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ABSTRACT

Our recently developed method for the determination of the reactive lysine content of soya bean proteins with the dye, Orange G, has been extended. The interactions of several etalon proteins and high protein-containing food and feedstuffs with Orange G, Acid Orange 12 and Amido Black 10B have been studied. The stoichiometric investigations showed that the reactive lysine content can be quantitatively determined by dye-binding although total nitrogen content measurements are no more advantageous than by Kjeldahl. A probable mechanism for the interaction has been given.

INTRODUCTION

Our earlier work (Perl *et al.*, 1985*a*), aimed at the study of the stoichiometry of soy bean proteins showed that, contrary to literature

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data, the dye Orange G (OG) reacts with only one molecule of basic amino acid (BAA) per molecule despite its two sulphonic acid groups. In the present work, we summarize research on the interaction of model proteins and protein-rich matrices (their detailed description is given in the 'Experimental' section) of economic importance with AO 12 (1-phenylazo-2-naphthol-6-sulphonic acid), AB 10B (1-amino-2*p*-nitrophenylazo-7-phenylazo-8-naphthol-3,6-disulphonic acid) and OG (1-phenylazo-2-naphthol-6,8-disulphonic acid) dyes (Fig. 1) along the

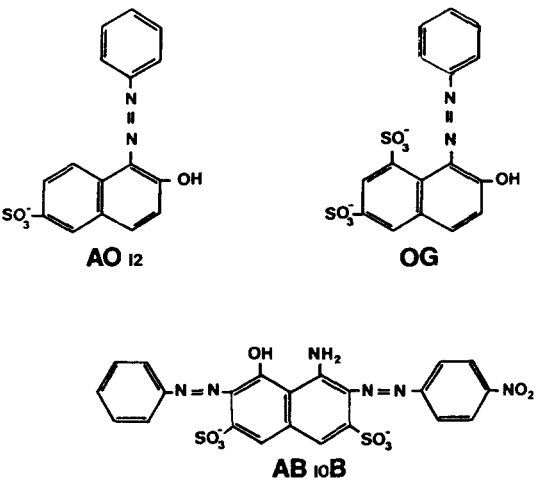

Fig. 1. Structure of dyes used.

following lines. Interaction of all three dyes with all the protein matrices, applying the same procedures:

(a) Attention to the stoichiometric observation that AO 12 interacts with protein in a molecular ratio differing from 1:1.

(b) An attempted proof that OG combines with every model and natural protein matrix in a molar ratio of dye: BAA $\simeq$ 1.

(c) Observation that sulphonic acid groups of AB 10B are involved in the interaction with every protein.

(d) The efficiency and usefulness of the suggested procedure as illustrated by the stoichiometric study of the total dye-binding capacity (TDBC) and the dye-binding capacity after propionylation (DBCAP) of numerous samples.

MATERIALS AND METHODS

Model materials

Standard proteins: Bovine albumin (produced by Phylaxia; abbr., BA). Albumin lyoph. (from human serum, produced by Reanal; abbr., HA). Human gamma globulin (produced by Human; abbr., HGG). κ'-Casein (produced by Human). Protein matrices: Soy samples, numbered 1 to 5, of the same type (Varietas: NKS 1346, *Glycine max.*), from different commercial sources; sample 1 is made fat free; samples 2–5 contain fat. Subscripts show the treatment (samples 1–3) and the origin (samples 4 and 5) of the samples. Samples 1_1 and 1_3 were prepared by microwave heat treatment (Benedek *et al.*, 1984) at 100 °C for 30 and 5 min, respectively. Samples 1_2 and 2_1 were denatured by acid treatment (Dévényi *et al.*, 1981), sample 1_2 until pH $\simeq$ 2 was reached without neutralization, with hydrochloric acid and sample 2_1 with a 2:1 mixture of phosphoric and hydrochloric acids to pH $\simeq$ 2 by acidification and subsequent neutralization with a mixture of sodium and calcium carbonate. Milk powder samples were of Hungarian production. Skim milk powder, prepared by spray drying, and milk proteins (treated first enzymatically then acid treated) were prepared in 1981 and 1984, respectively. Meat and bone meal samples 1 and 2 were also of Hungarian origin, prepared by pressing and making fat free by extraction, respectively. Fish meal samples 1 and 2 were commercial products originating from the FRG and Sweden, respectively.

Reagents

Propionic anhydride, anhydrous sodium acetate and the components of dye solutions were, all reagents of analytical grade; AO (CI 15970), AB 10B (CI 20470) and OG (CI 16230) dyes were products of Sigma (USA), Reanal (Hungary) and Reachim (USSR), respectively. Glass filter paper was Grade GF/A Whatman (Great Britain). Standard dye solutions (approximately 0·04M) were prepared by measuring (with analytical accuracy) 7 g and 9 g of AO 12 and OG dyes, respectively, and to each were added 300 cm^3 of glacial acetic acid, 100 g of oxalic acid dihydrate, 17 g of potassium dihydrogen phosphate and making up to 5 litres. The pH of these solutions, independently of the dye dissolved, was 1·3 (see below, buffer$_1$). The 5 litres of AB 10B and AO 12 dye solutions

contained 12·5 g and 7 g of dye, respectively, as well as 10 g each of disodium hydrogen phosphate and 80 g of citric acid. The pH of these solutions, independently of the dye dissolved, was 2·4 (see below, buffer$_2$). It should be noted that: (i) The dyestuffs used in preparing the stock solutions were recrystallized twice and dried according to the method of Udy (1971). Thus, the water content of the dyestuffs, maintained in a glass-stoppered vessel, was constant, below 1 %, as controlled by repeated measurements. (ii) The reaction of OG was measured in buffer$_1$, and that of AB 10B in buffer$_2$, respectively, in all cases. The dye-binding capacity of AO 12, soluble and stable in both buffers, was investigated primarily in buffer$_1$, as advised in the literature (Tables 1–7) to obtain stoichiometric data; checking the dependence of pH, i.e. the buffer composition effect, on the dye-binding capacity, AO 12 was also used in buffer$_2$ for two characteristic proteins (Table 8).

Apparatus

Electric shaker (Labor MIM), accommodating 24 flasks. Spectrophotometer (Spectromom 361, MOM).

Procedures

All measurements were carried out at ambient temperature (20–22 °C) with the exception of investigations summarised in Table 9 which were made at 50 °C (±0·2 °C).

Determination of the total dye-binding capacity (TDBC)

The protein, or protein-containing matrix (weighed with analytical precision* and powdered below the particle size of 60 mesh) was placed into a 100 cm^3 glass-stoppered flask; glass beads and 2 cm^3 2M sodium acetate were also added and the flask was shaken for 10 min. After that time, 40 cm^3 of AO 12 or OG, or 20 cm^3 of AB 10B dye is introduced, followed by 30–120 min† of shaking. After quantitative dye-binding, the

* Optimum dye binding is achieved when weighing (in mg), based on the results summarised in Tables 2–6; soy bean, 150; milk powder, 200; milk protein, 100; meat and fish meals, 100; model proteins, 50.

† Optimum shaking times needed for dye-binding are, in minutes: soy bean, 60; milk powder and milk protein, 30; meat and fish meals, 120; model proteins, 30.

precipitated solution was filtered through the glass filter paper. The filtrate was diluted (in the case of AO 12 and OG dyes in a ratio of 1:100; for AB 10B dye in a ratio of 1:200). The optical densities of the diluted filtrates were measured in a 1 cm cuvette at wavelengths of 475 nm for AO 12 and OG, and at 615 nm for AB 10B against distilled water.

Determination of the dye-binding capacity after propionylation (DBCAP)

The procedure described for the determination of the total dye-binding capacity is used, with the following difference: before the reaction of the reagents with the dye solution, 0·4 cm^3 of propionic anhydride is added to the sample homogenized previously with sodium acetate for 10 min. In all cases, acylation is quantitative in 30 min. Thereafter, reaction with the dye solution is carried out.

RESULTS AND DISCUSSION

(1) Conclusions about the uniform procedure. Our model investigations were started for every protein and dye by establishing the optimum time of interaction. In the course of these studies, we measured the reaction of dye to protein BAA in a molecular ratio of 2:1, based on our experiences with soy bean proteins. In all cases studied it could be proved that the time needed for maximum binding is independent of the nature of the dye; it is a characteristic feature of the protein (Table 1).

(2) Results of experiments with different molar ratios of dye to protein BAA have shown that:

(i) Interactions in broad molar ratios are non-existent.

(ii) On applying the proposed molar ratios, the quantitative change in the reactive lysine content is nearly stoichiometric.

(iii) The optimum range for all three dyes and all protein matrices is the molar ratio of dye:protein BAA = 2:1, in accordance with our earlier experiences.

(iv) On increasing the molar ratio of reactants (M/M > 2), binding capacities larger than the theoretical value are obtained; on applying smaller molar ratios (M/M < 2), smaller binding capacities are obtained (M/M data in parentheses in Tables 2–6).

TABLE 1
TDBC of Etalon Proteins and Protein Matrices as a Function of the Reaction Time[a]

Sample	*Expressed as a percentage of the maximum dye binding*																	
	Reaction time (min)																	
	10			*30*			*45*			*60*			*90*			*120*		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
BA	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
HA	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
HGG	100	54	100	100	78	100	100	80	100	100	88	100	100	100	100	100	100	100
κ-Casein	65	56	55	78	65	71	85	79	76	90	79	83	97	90	91	100	100	100
Soya bean	74	75	88	85	88	97	100	100	100	100	100	100	100	100	100	100	100	100
Skim milk powder	97	97	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Milk protein	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Fish meal	77	73	71	85	83	80	90	87	86	94	91	91	98	96	94	100	100	100
Meat and bone meal	86	84	82	90	91	86	94	93	94	96	97	95	97	94	97	100	100	100

[a] On the basis of the reactions carried out with optimal amounts—detailed in the 'Materials and Methods' section—of proteins: a = OG; b = AO 12; c = AB 10B.

Stoichiometric Comparison of the TDBC and DBCAP for the Reactive Lysine Content Determination of Etalon Proteins with the Dyes OG, AO 12 and AB 10B

Sample	*Protein* (%) ($N \times 6{\cdot}25$)	*Dye*		*Bound dye (mg/g protein)*											
				A	*B*	*A–B*	*A*	*B*	*A–B*	*A*	*B*	*A–B*	*A*	*B*	*A–B*
				'20'			'40'			'50'			'100'		
BA	88·7	OG	a	620 (0·88)	238	377 (0·94)	633 (0·90)	273	359 (0·89)	618 (0·87)	278	335 (0·84)	595 (0·84)	273	325 (0·81)
			b	614	241		633	274		603	271		593	265	
		AO 12	a	617 (1·14)	290	331 (1·06)	617 (1·14)	285	337 (1·08)	617 (1·13)	281	341 (1·10)	533 (0·99)	275	267 (0·86)
			b	626	291		626	285		616	270		547	271	
		AB 10B	a	466 (0·48)	160	297 (0·54)	482 (0·50)	198	290 (0·53)	458 (0·48)	188	279 (0·51)			
			b	450	162		488	193		469	188				
HA	90·1	OG	a	655 (0·94)	276	379 (0·94)	654 (0·93)	276	377 (0·94)	658 (0·93)	272	381 (0·95)			
			b	671	291		649	274		651	275				
		AO 12	a	622 (1·14)	239	383 (1·23)	625 (1·15)	273	355 (1·14)	636 (1·14)	273	350 (1·13)			
			b	624	242		632	274		609	273				
		AB 10B	a	514 (0·54)	228	293 (0·54)	493 (0·53)	193	290 (0·53)	481 (0·49)	208	272 (0·50)	458 (0·48)	185	275 (0·50)
			b	514	213		486	208		457	187		461	185	
				'30'			'60'			'90'			'120'		
HGG	85·0	OG	a	467 (1·04)	229	241 (0·96)	468 (1·04)	230	236 (0·94)	476 (1·06)	236	240 (0·96)	437 (0·98)	224	215 (0·86)
			b	463	219		464	230		476	236		444	228	
		AO 12	a	478 (1·36)	256	225 (1·15)	460 (1·32)	231	228 (1·17)	445 (1·28)	226	218 (1·12)	422 (1·20)	216	202 (1·04)
			b	471	244		464	237		449	231		417	220	
		AB 10B	a	358 (0·59)	201	161 (0·47)	332 (0·55)	197	137 (0·40)	330 (0·54)	193	134 (0·39)	303 (0·50)	194	113 (0·33)
			b	359	194		335	196		329	198		304	187	
				'50'			'100'			'150'			'200'		
κ-Casein	76·0	OG	a	313 (1·16)	105	201 (1·30)	296 (1·11)	106	194 (1·29)	295 (1·09)	97	186 (1·20)	278 (1·03)	93	184 (1·19)
			b	313	119		297	101		287	115		276	92	
		AO 12	a	313 (1·45)	76	220 (1·83)	293 (1·47)	119	231 (1·92)	278 (1·37)	95	185 (1·94)	261 (1·26)	86	177 (1·47)
			b	288	85		317	87		294	106		265	86	
		AB 10B	a	168 (0·46)	62	106 (0·50)	168 (0·45)	65	101 (0·48)	158 (0·43)	61	97 (0·46)	157 (0·43)	65	93 (0·44)
			b	170	64		164	64		157	61		159	65	

In quotation marks: amount of weighed sample (mg).
In parentheses: Bound dye/BAA = M/M, in the case of A values. Bound dye/L = M/M, in the case of A–B values.
BAA = Basic amino acids in total: i.e. the sum of lysine, arginine and histidine. L = Lysine.
Theoretical TDBC: 1 Mole of dye/1 Mole of lysine + arginine + histidine (using OG or AO 12). 0·5 Mole of dye/1 Mole of lysine + arginine + histidine (using AB 10B).
Theoretical DBCAP: 1 Mole of dye/1 Mole of lysine (using OG or AO 12). 0·5 Mole of dye/1 Mole of lysine (using AB 10B).
The basis of calculations *BAS:* $1{\cdot}55 \times 10^{-3}$ M/1 g protein; $9{\cdot}91 \times 10^{-4}$ M/1 g protein; $5{\cdot}94 \times 10^{-4}$ M/1 g protein. *Lysine:* $8{\cdot}88 \times 10^{-4}$ M/1 g protein (in the cases of BA and HA: Gehrke & Leimer, 1971); $5{\cdot}54 \times 10^{-4}$ M/1 g protein (in the case of HGG: Bruckner, 1974); $3{\cdot}43 \times 10^{-4}$ M/1 g protein (in the case of casein: Gehrke & Leimer, 1971).
'a' and 'b' correspond to two parallel measurements carried out with the same dye.

TABLE 3

Stoichiometric Comparison of the TDBC and DBCAP of Untreated (Sample 1), Heat-Treated (Sample 1/1) and Acid-Treated (Sample 1/2) Soya Beans as a Function of the Dye Applied and of the Dye–Protein Interaction Ratio

Sample	*Protein* (%) ($N \times 6{\cdot}25$)	*Dye*		*Bound dye (mg/g protein)*											
				A	*B*	*A–B*	*A*	*B*	*A–B*	*A*	*B*	*A–B*	*A*	*B*	*A–B*
				'50'			'100'			'150'			'200'		
1	44·0	OG	a	564 (1·17)	342	205 (1·06)	493 (1·05)	301	197 (1·02)	482 (1·02)	283	193 (1·00)	426 (0·91)	276	155 (0·80)
			b	540	351		501	298		480	292		434	274	
		AO	a	505 (1·38)	294	206 (1·38)	499 (1·35)	323	177 (1·18)	467 (1·28)	293	174 (1·16)	441 (1·26)	300	141 (0·94)
		12	b	504	314		490	314		470	298		446	304	
		AB	a	392 (0·61)	266	129 (0·49)	388 (0·61)	262	129 (0·49)	377 (0·59)	262	115 (0·44)	361 (0·56)	252	112 (0·43)
		10B	b	398	265		393	262		379	264		363	249	
1/1	40·4	OG	a	422 (0·88)	311	108 (0·56)	388 (0·82)	237	165 (0·85)	381 (0·81)	241	141 (0·73)	366 (0·80)	243	125 (0·65)
			b	417	312		390	230		388	247		386	259	
		AO	a	389 (1·08)	313	98 (0·66)	372 (1·03)	282	93 (0·62)	381 (1·03)	257	125 (0·84)	356 (0·97)	256	100 (0·67)
		12	b	406	288		380	283		372	254		356	254	
		AB	a	298 (0·46)	219	73 (0·28)	285 (0·44)	219	67 (0·25)	290 (0·45)	220	71 (0·27)	270 (0·42)	206	63 (0·24)
		10B	b	299	231		286	219		292	220		267	206	
1/2	43·1	OG	a	500 (1·06)	326	176 (0·91)	479 (1·02)	303	170 (0·88)	443 (0·92)	270	170 (0·88)	414 (0·89)	265	198 (0·82)
			b				484	320		441	274		425	259	
		AO	a	495 (1·35)	325	164 (1·10)	452 (1·25)	292	169 (1·13)	432 (1·18)	278	156 (1·04)	415 (1·14)	263	152 (1·02)
		12	b	497	341		457	282		431	274		418	266	
		AB	a	320 (0·50)	214	109 (0·41)	319 (0·49)	205	114 (0·43)	303 (0·47)	200	103 (0·39)	299 (0·47)	193	104 (0·40)
		10B	b	325	214		319	206		303	200		301	199	

Meanings of abbreviations, etc., as in Table 2. Plus: BAS = $1{\cdot}046 \times 10^{-3}$ M/1 g protein; L = $4{\cdot}27 \times 10^{-4}$ M/1 g protein (Kellor, 1974).

TABLE 4

Stoichiometric Comparison of the TDBC and DBCAP of Different Soya Beans for the Determination of Their Reactive Lysine Content

Sample	*Protein* (%) ($N \times 6{\cdot}25$)	*Dye*		*Bound dye (mg/g protein)*											
				A	*B*	*A–B*	*A*	*B*	*A–B*	*A*	*B*	*A–B*	*A*	*B*	*A–B*
					'50'			'100'			'150'			'200'	
2	48·7	OG	a	472 (1·00)	295	175 (0·91)	475 (1·01)	289	186 (0·96)	468 (0·99)	282	186 (0·96)	441 (0·93)	289	159 (0·79)
			b	478	307		477	292		470	283		440	289	
		AO	a							454 (1·24)	287	168 (1·12)			
		12	b							458	289				
		AB	a	380 (0·59)	280	108 (0·44)	387 (0·60)	264	123 (0·47)	380 (0·59)	261	120 (0·46)	372 (0·58)	261	109 (0·41)
		10B	b	379	264		387	264		379	259		369	263	
2/1	39·3	OG	a	482 (1·02)	295	186 (0·96)	494 (1·06)	296	199 (1·03)	488 (1·04)	297	192 (0·99)	471 (1·00)	293	178 (0·92)
			b	496	305		507	306		492	298		472	294	
		AO	a							486 (1·31)	303	173 (1·16)			
		12	b							475	310				
		AB	a	385 (0·60)	246	140 (0·53)	371 (0·58)	256	115 (0·44)	376 (0·58)	245	126 (0·48)	355 (0·55)	243	112 (0·43)
		10B	b	382	241		376	261		373	250		356	244	
3	31·8	OG	a	480 (1·04)	333	166 (0·86)	486 (1·03)	286	202 (1·05)	467 (0·99)	273	195 (1·01)	460 (0·99)	273	192 (0·99)
			b	501	317		488	279		472	276		472	275	
		AO	a							487 (1·32)	293	192 (1·28)			
		12	b							478	288				
		AB	a	379 (0·59)	265	115 (0·44)	393 (0·61)	265	127 (0·48)	390 (0·61)	263	129 (0·49)	374 (0·58)	251	120 (0·46)
		10B	b	386	270		397	271		393	263		373	257	
3/1	32·6	OG	a	477 (1·00)	283	194 (1·00)	457 (1·00)	292	171 (0·89)	466 (0·99)	294	174 (0·90)	459 (0·97)	285	172 (0·89)
			b	477	273		489	292		469	294		457	286	
		AO	a							462 (1·27)	299	171 (1·14)			
		12	b							470	291				
		AB	a	372 (0·59)	254	121 (0·46)	369 (0·58)	257	114 (0·43)	374 (0·58)	258	116 (0·44)	370 (0·57)	250	121 (0·46)
		10B	b	385	257		373	257		375	259		369	248	
4	47·9	OG	a	482 (1·00)	310	165 (0·85)	489 (1·03)	298	191 (0·99)	462 (0·98)	292	172 (0·89)	439 (0·92)	290	147 (0·76)
			b	471	311		484	292		465	291		435	289	
		AO	a							467 (1·27)	289	173 (1·16)			
		12	b							463	294				
		AB	a	353 (0·55)	256	100 (0·38)	359 (0·56)	244	119 (0·45)	362 (0·56)	240	125 (0·47)	352 (0·55)	239	113 (0·43)
		10B	b	354	252		369	246		363	236		351	238	
5	45·4	OG	a	471 (1·02)	281	199 (1·03)	470 (0·99)	276	184 (0·95)	458 (0·97)	282	179 (0·93)	436 (0·92)	281	152 (0·79)
			b	490	282		464	290		459	279		450	284	
		AO	a							471 (1·29)	300	174 (1·16)			
		12	b							476	299				
		AB	a	347 (0·53)	224	121 (0·46)	354 (0·55)	241	114 (0·43)	358 (0·55)	243	118 (0·45)	349 (0·54)	239	107 (0·41)
		10B	b	341	222		357	243		358	236		348	244	

Meanings of abbreviations, etc., as in Table 3.

TABLE 5

Comparative Stoichiometric Measurements for the Determination of the Reactive Lysine Content of Meat and Fish Meals

Sample	*Protein* (%) ($N \times 6{\cdot}25$)	*Dye*		*Bound dye (mg/g protein)*														
				A	*B*	*A B*	*A*	*B*	*A B*	*A*	*B*	*A–B*	*A*	*B*	*A B*	*A*	*B*	*A B*
					'25'			'50'			'100'			'150'			'200'	
Meat meal$_1$ (Hungary)	61·0	OG	a	289 (0·85)	156	125 (0·87)	296 (0·88)	147	148 (1·02)	309 (0·93)	159	152 (1·05)	311 (0·91)	166	140 (0·97)	287 (0·96)	169	120 (0·83)
			b	281	163		295	149		314	161		301	165		290	169	
		AO	a	316 (1·22)	236	88 (0·79)	319 (1·23)	194	125 (1·12)	320 (1·24)	185	137 (1·23)	304			291 (1·12)	180	113 (1·01)
		12	b	319	223		319			325	186		310			291	175	
		AB	a	208 (0·46)	138	71 (0·36)	207 (0·46)	138	70 (0·36)	199 (0·44)	138	61 (0·31)	205 (0·45)	141	64 (0·33)	202 (0·44)	158	41 (0·21)
		10B	b	210	138		210	138		199	138		205	141		196	158	
Meat meal$_2$ (Hungary)	59·5	OG	a	271 (0·83)	174	102 (0·71)	272 (0·81)	169	100 (0·69)	285 (0·86)	170	116 (0·80)	283 (0·83)	167	113 (0·78)	264 (0·79)	170	95 (0·66)
			b	277	170		273	177		292	176		274	164		264	167	
		AO	a	292 (1·19)	193	101 (0·90)	315 (1·24)	202	118 (1·06)	298 (1·16)	183	114 (1·02)	292 (1·13)			276 (1·05)	184	88 (0·79)
		12	b	328	224		329	206		302	190		293			270	186	
		AB	a	226 (0·49)	182	41 (0·21)	226 (0·49)	181	41 (0·21)	232 (0·51)	181	50 (0·25)	232 (0·51)	182	50 (0·25)	221 (0·48)	180	40 (0·20)
		10B	b	223	186		223	187		232	182		232	182		219	180	
Fish meal (FRG)	66·4	OG	a	339 (0·88)	191	146 (0·82)	352 (0·92)	189	162 (0·91)	368 (0·96)	186	183 (1·03)	363 (0·94)	186	173 (0·97)	334 (0·87)	183	150 (1·03)
			b	336	192		352	192		372	188		357	187		334	185	
		AO	a	405 (1·40)	211	199 (1·57)	405 (1·36)	215	197 (1·43)	387 (1·32)			389 (1·30)	201	185 (1·34)	383 (1·26)	203	170 (1·23)
		12	b	426	223		402	199		398			387	204		365	204	
		AB	a	197 (0·38)	131	66 (0·27)	208 (0·40)	130	76 (0·31)	220 (0·42)	134	88 (0·36)	218 (0·42)	131	85 (0·35)	210 (0·40)	131	79 (0·33)
		10B	b	197	131		207	133		221	131		220	136		209	131	
Fish meal (Sweden)	63·7	OG	a	313 (0·83)	159	160 (0·90)	350 (0·91)	176	179 (1·01)	360 (0·93)	175	181 (1·02)	345 (0·90)	180	164 (0·92)	321 (0·84)	178	143 (0·80)
			b	327	163		350	186		354	176		345	181		323	179	
		AO	a	309 (1·08)	123	194 (1·40)	330 (1·11)	174	150 (1·09)	334 (1·12)			320 (1·08)	169	152 (1·10)	299 (1·01)	160	136 (0·99)
		12	b	334	132		330	185		329			324	170		302	170	
		AB	a	210 (0·40)	132	75 (0·31)	247 (0·47)	148	100 (0·41)	242 (0·46)	148	91 (0·37)	239 (0·46)	147	91 (0·37)	228 (0·44)	147	83 (0·34)
		10B	b	204	132		248	147		235	148		239	151		229	145	

Meanings of abbreviations, etc., as in Tables 2–4, plus *BAS*: $7{\cdot}41 \times 10^{-4}$ M/1 g protein; $8{\cdot}49 \times 10^{-4}$ M/1 g protein. *Lysine*: $3{\cdot}19 \times 10^{-4}$ M/1 g protein (in the case of meat and bone meal); $3{\cdot}94 \times 10^{-4}$ M/1 g protein (in the case of fish meal). Basis of calculations in both cases: Hubell (1983).

TABLE 6

Comparative Stoichiometric Measurements for the Determination of the Reactive Lysine Content of Skim Milk Powder and Milk Proteins

Sample	*Protein* (%) ($N \times 6{\cdot}25$)	*Dye*		*Bound dye (mg/g protein)*											
				A	*B*	*A–B*	*A*	*B*	*A–B*	*A*	*B*	*A–B*	*A*	*B*	*A–B*
					'100'			'150'			'250'			'300'	
Skim milk powder (Hungary)	34·6	OG	a	395 (1·10)	148	243 (0·97)	380 (1·08)	153	227 (0·91)	367 (1·06)	138	234 (0·94)	354 (1·00)	146	212 (0·85)
			b	385	147		381	153		378	138		355	139	
		AO	a	407 (1·16)	154	251 (1·30)	398 (1·45)	145	247 (1·28)	375 (1·39)	143	226 (1·17)	361 (1·33)	150	214 (1·10)
		12	b	410	162		396	154		386	146		364	148	
		AB	a	306 (0·64)	115	185 (0·54)	300 (0·62)	132	169 (0·50)	301 (0·63)	132	174 (0·51)	294 (0·62)	132	169 (0·50)
		10B	b	308	130		301	129		306	128		300	124	
					'25'			'50'			'100'			'150'	
Milk protein (Hungary, 1981)	81·1	OG	a	334 (0·96)	108	232 (0·93)	354 (1·00)	126	231 (0·92)	355 (1·01)	122	234 (0·94)	326 (0·93)	130	195 (0·78)
			b	347	108		354	121		161	127		327	134	
		AO	a	416 (1·50)	116	295 (1·52)	400 (1·46)	145	253 (1·30)	366 (1·34)	149	222 (1·15)	278 (1·41)	136	137 (0·69)
		12	b	408	118		400	148		369	143		268	135	
		AB	a	302 (0·64)	178	132 (0·39)	271 (0·55)	128	136 (0·40)	248 (0·51)	123	122 (0·36)	230 (0·48)	126	106 (0·31)
		10B	b	305	166		263			246	126		229	122	
		OG	a	356 (1·02)	142	220 (0·88)	349 (0·99)	129	215 (0·86)	359 (1·03)	143	220 (0·88)	324 (0·92)	129	195 (0·78)
			b	363	136		350	142		367	143		326	132	
Milk protein (Hungary, 1984)	83·7	AO	a	363 (1·30)	149	207 (1·07)	365 (1·34)	—		354 (1·31)	137	219 (1·13)	327 (1·19)	135	191 (0·99)
		12	b	349			370			362	140		325	135	
		AB	a	351 (0·53)	124	131 (0·38)	272 (0·56)	127	144 (0·42)	254 (0·52)	127	128 (0·38)	248 (0·51)	130	118 (0·35)
		10B	b	257	121		272	128		251	123		247	130	

Abbreviations as in Tables 2–5. Plus: BAS = $7{\cdot}82 \times 10^{-4}$ M/l g protein. Lysine = $5{\cdot}53 \times 10^{-4}$ M/l g protein. Basis of calculations: De Lange *et al.* (1979) and Hubell (1983).

(3) Experiences summarized in point (2) indicate that interactions based on dye-binding are unfavourable for the determination of the total nitrogen content of proteins; the results achievable with this method are not equivalent to these obtained by the Kjeldahl method.

(4) The determination of the reactive lysine content of protein matrices, based on the dye-binding capacity measured directly (TDBC) and after propionylation (DBCAP), may be useful information. As may be seen from the footnotes to Tables 2 to 6, our calculations are based on the determination of the basic amino acid (BAA) and lysine (L) contents measured by selective methods and taking into account a large amount of literature data. It should be noted that the amino acid content of the majority of samples studied and determined gas chromatographically as *N*-TFA-*n*-butyl ester, agrees with the literature data within the limits of experimental error.

The reactive lysine contents measured have shown that the theoretical mole number was best approximated in the order OG, AB 10B and AO 12. It is a general experience that dyes of OG and AB 10B are bonded approximately as expected, while the dye AO 12 agrees less with respect to the calculated value.

From the above it is clear that, in order to determine the change in the reactive lysine content during treatments, the untreated and treated samples should be studied simultaneously under strictly identical conditions.

(5) The differences in the reactive lysine contents of treated and untreated soy bean samples were in strict accordance with the treatment ('denaturation') and the saccharide contents measured (Perl *et al.*, 1984, 1985*b*) in the samples. (Tables 3 and 4, samples 1–3.)

The greatest change in the reactive lysine content* was measured in sample 1/1, heat-treated at 100 °C for 30 min; the decreases were, for OG, AO 12 and AB 10B, 27 %, 28 % and 39 %, respectively. The changes were also significant for sample 1/2, treated with hydrochloric acid and not neutralized; in the reactions with OG, AO 12 and AB 10B they were 12 %, 10 % and 11 %, respectively.

Smaller decreases could be measured for sample 3/1 heat-treated at 100 °C for 5 min; they were 11 %, 11 %, and 10 % for OG, AO 12 and AB 10B, respectively. In soy bean sample 3/1, denatured by mild

* Based on dye-binding capacities obtained with weighed amounts of 150 mg corresponding to the optimum molar ratio, dye:BAA $\simeq$ 2.

acidification and subsequent neutralization, the measured reactive lysine content was identical to that of the untreated sample 3 within the limits of experimental error (Table 7).

(6) In milk powder, the amount of basic amino acids (BAA) was smaller, whereas that of lysine (L) was higher, with respect to the calculated values (sample 1 in Table 6). The composition of milk proteins (sample 2/3, Table 6), according to our measurements, did not significantly change in the three years of storage.

(7) Results concerning Hungarian meat and bone meals, as well as fish meals from abroad, have shown that:

(a) The reactivity of these samples in interaction with AB 10B is uniformly lower than calculated.

(b) The reactive lysine contents of foreign fish meals and the meat $meal_1$ (Hungary) are appropriate, as shown by their reactions with OG and AO 12 dyes, whereas that of meat $meal_2$ (Hungary) is smaller (by 20%) than the calculated value; consequently, it does not conform to requirements.

(8) The reproducibility of our procedure is illustrated in Table 7. The relative standard deviation for the reactions of different matrices with different dyes is less than 2·7%.

(9) The different buffer systems applied are based on the following considerations: (i) the buffer systems used for the three different dye–protein interactions were identical with those in the cited literature, (ii) the solubility of AB 10B in buffer containing oxalic acid, acetic acid and KH_2PO_4 ($buffer_1$) is not sufficient ($\sim$0·01 M) and is also (in this low concentration), unstable. OG is more soluble in the buffer system containing citric acid and Na_2HPO_4 ($buffer_2$) but at the standard concentration of 0·04 M, by shaking, 2–5% of it became insoluble. Based on the above detailed considerations, the only possibility was to carry out the stoichiometric interaction studies with the dye AO 12, which was soluble and stable in both buffers. Table 8 shows that the dye-binding capacities of the characteristic proteins with AO 12 in both buffers are the same within the experimental error of our method.

(10) Temperature studies reported earlier (Perl *et al.*, 1985*a*) for soya bean–OG interactions proved that reaction temperature had no effect on the dye-binding process. Temperature interactions in the present experiments were carried out at ambient temperature (at 20–22 °C).

To investigate the temperature (in)dependence of the interactions in a

TABLE 7
Reproducibility of the TDBC Measurements of Different Proteins[a]

Sample	*Bound dye (mg/g protein)*									*Standard deviation*	*Relative standard deviation (%)*
	Single								$\bar{x}$		
Soya bean											
OG	451	443	449	445	444	446	436	446	445	4·47	1·0
AO 12	474	464	474	488	468	465	467	468	471	7·70	1·6
AB 10B	335	345	347	345	348	399	339	338	342	4·80	1·4
Skim milk powder											
OG	367	367	367	354	365	358	360	359	362	5·03	1·4
AO 12	399	384	385	389	381	374	377	375	383	8·29	2·2
AB 10B	291	294	309	312	303	297	296	311	302	8·25	8·7
Fish meal											
OG	390	388	389	391	390	390	390	390	390	0·93	0·24
AO 12	392	395	399	407	404	404	408	416	403	7·68	1·9
AB 10B	215	213	217	218	217	215	214	213	215	1·93	0·9

[a] Carried out with optimal conditions of the samples proposed in the 'Materials and Methods' section.

TABLE 8
Stoichiometric Comparison of the TDBC and DBCAP of Different Proteins Measured in Two Buffers with the Dye AO 12

Sample	*Buffer*	*Bound dye (mg/g protein)*[a]		
		A[b]	*B*[b]	*A–B*
BA	1	617 (1·13)	276	341 (1·10)
	2	610 (1·12)	247	363 (1·16)
HA	1	623 (1·14)	273	350 (1·13)
	2	621 (1·14)	270	351 (1·13)
Soya bean$_1$	1	469 (1·28)	295	174 (1·16)
	2	400 (1·09)	273	127 (0·85)
Fish meal (Sweden)	1	330 (1·11)	180	150 (1·09)
	2	321 (1·08)	191	130 (0·94)
Skim milk powder (Hungary)	1	381 (1·39)	145	236 (1·17)
	2	399 (1·46)	195	204 (1·05)

Abbreviations, etc. as in Tables 2–6, as well as:
[a] Investigations were carried out with the 'optimum' amount of the matrices as detailed in the 'Materials and Methods' section.
[b] All data are means of at least two measurements.

wider range we have measured the dye-binding capacity of all three dyes under the optimal reaction conditions with bovine albumin and soya beans at 50 °C and also as a function of the reaction time. Data show (Table 9) that, in the case of AO 12 and partly also in the case of AB 10B (soya beans), a lower dye-binding capacity at the elevated temperature can be observed, probably due to the fact that the so-called 'stacking' effect (Rattee & Breuer, 1974) decreases at higher temperatures. This effect was observed to a high degree in the case of AO 12 without exception. Thus, taking into consideration that a difference of approximately 28 °C in the reaction temperature resulted in about a 6 % decrease in dye-binding, slight changes (± 1 °C) of room temperature could not affect the extent of dye-binding interactions.

(11) Our hypothesis concerning the mechanism of these interactions can be summarized as follows.

Earlier literature has qualified these reactions as salt-formation (Chapman *et al.*, 1927; Rawlins & Schmidt, 1929) or complex formation (Goh *et al.*, 1979).

TABLE 9
TDBC of Bovine Albumin and Soya Bean (Sample 1) as a Function of the Reaction Time at 50 °C (±0·2 °C)

Sample	*Dye*	*Bound dye (BAA = M/M) Reaction time (min)*			
		10	*20*	*30*	*40*
BA	OG	0·84	0·88	0·91	0·88 (0·87)
	AO 12	1·05	1·06	1·06	1·05 (1·13)
	AB 10B	0·50	0·48	0·50	0·49 (0·48)
		Reaction time (min)			
		20	*40*	*60*	*90*
Soya bean$_1$	OG	0·98	1·00	0·97	0·99 (1·02)
	AO 12	1·19	1·20	1·21	1·19 (1·28)
	AB 10B	0·52	0·54	0·53	0·55 (0·59)

Data in parentheses show the TDBC at ambient temperature: 20–22 °C.
[a] On the basis of the reactions carried out with optimal amounts of proteins: detailed in the 'Experimental' section.

Since, in their free state, the basic amino acids of the protein do not react with the dyes studied, the protein (as a giant molecule containing a number of peptide bonds) should play a significant rôle in establishing the dye–protein interaction. Thus, neither salt-formation, nor simple complex formation can be responsible for these reactions. Probably the initial step of the interaction between the dye and the protein is due to the formation of hydrogen bridges by means of the NH— and —C=O groups of the protein chain. The formation of hydrogen bridges is also the precondition for stabilization of the insoluble associates of dye and protein molecules in a nearly stoichiometric ratio, being a weak interaction through the hydrogen bridges.

(12) OG and AB 10B are dye molecules, both containing two sulphonic acid groups; their different reactivities can be explained partly by the different positions of the sulphonic acid groups inside the molecule and partly by the structure of protein molecules, that is:

(a) The rotational possibilities of R groups projecting from coplanar peptide chains of the plated sheet are restricted.

(b) The reactant dye molecules are substituted sulphonic acids of the naphthaline type.

It follows from the above that the sulphonic acid groups of the planar naphthaline molecule (similarly to the R-groups of the coplanar protein chains with their free basic amino acid groups) are restricted in their movement and can only combine if their planes approach each other in a parallel fashion.

Thus, both sulphonic acid groups, in the 3 and 6 positions, being in the same plane, are reactive in the AB 10B molecules; however, with sulphonic acid groups in positions 6 and 8 in the OG molecule, only the one—in position 6—is reactive. Inactivity of the sulphonic acid group in position 8 may be caused by the space requirement of the neighbouring phenyl-azo group in position 1. We expect to prove the modifying rôle of the latter substituent on the basis of the reactivity of 2-naphthol-6,8-disulphonic acid.

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