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Direct Spectrophotometric Determination of Proteins Immobilized on Bead Cellulose and Dissolved in Cadoxene

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Abstract

A direct spectrophotometric method has been developed for the determination of the amount of proteins immobilized on bead cellulose. The method is based on dissolution of conjugates in cadmium tris(ethylenediamine) hydroxide (trivial name, cadoxene) that is optically transparent in the region of the UV spectrum in which proteins absorb. The method developed for the determination of immobilized proteins is simple, rapid, and readily reproducible. The sensitivity of the method is dependent on the $A^{1\%}_{1cm}$ value of the free protein and on the amount of bound protein m (mg protein/g carrier). In the terms of $A^{1\%}_{1cm} \geq 25$ and $m \geq 120$ mg/g, the sensitivity of the mentioned method was comparable with those obtained by amino acids analyses by the ninhydrin reaction, by the modified Lowry reaction, or by radiometric determination. The possibility of substituting the direct spectrophotometric method for amino acid analysis was statistically evaluated; it does not seem to be rejected at the highest level of significance, $P \leq 1\%$. The differences in paired observations were significant, but nonetheless proportional over the whole range of protein concentrations, making both methods correlable. The absolute accuracy of the direct spectrophotometric method could be influenced by the change of $A^{1\%}_{1cm}$ value during solvolysis of the conjugate in cadoxene.

Index Entries: Immobilized proteins, direct spectrophotometric determination of; immobilized proteins, solutions in cadoxene; protein-bead cellulose conjugate; immobilization of proteins, reductive alkylation; immobilized proteins, methods of determination; spectrometric determination, of proteins immobilized on cellulose; cellulose, spectrometric determination of proteins immobilized on.

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Introduction

Direct spectrophotometric determination of immobilized proteins has many advantages. It is rapid and sensitive, the sample in most cases is not destroyed, and the method provides further qualitative data. However, measurements in the UV region are connected with difficulties concerning the preparation of samples. The most convenient mode of preparation seems to be the procedure using intact conjugate (*1*), provided that a transparent matrix employing special geometric arrangements to diminish the light scattering of matrix beads is used. The other possibility is to dissolve the conjugate under conditions leading to partial degradation of the sample (*2, 3*). None of the aforementioned methods can be used with cellulose as the matrix. The cellulose in spherical, porous form (bead cellulose) represents a matrix with multiple uses in solid-phase biochemistry and biotechnology (*4, 5*) and fulfils even the most rigorous crtieria required for the enzyme/protein carrier (*6,7*).

Cadmium tris(ethylenediamine) hydroxide (cadoxene), as a solvent for cellulose and proteins, is suitable for spectrophotometric measurements in the UV region (*8*). The present communication deals with cadoxene as a solvent for direct spectrophotometric determination of the amount of proteins immobilized on bead cellulose. The results obtained by this method have been compared with those obtained by other stanedard methods (*9*): by amino acids analysis, by reaction with ninhydrin, by the modified Lowry method, and by radiometric determination.

The conjugates were prepared by binding the protein on a periodate-oxidized bead cellulose (OC),† followed by reduction with borohydride. Reductive alkylation is a simple, cheap, and rapid procedure not requiring the use of highly toxic chemicals. It can be applied for many enzymes since it is known that ϵ-aminolysine residues utilized for binding (*10*) are not essential for the catalytic activity of numerous enzymes. This was also proved in experiments with immobilization of trypsin on different aldehyde derivatives of bead cellulose where highly active preparations were obtained (*7*).

Materials and Methods

Chemicals

Macroporous bead cellulose (dry weight, 13.7–15.0% w/v) was obtained from the Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague. Cadoxene was prepared by the general procedure (*11*) without repeated aftersaturation with CdO. It contained 5.6–5.8% Cd, 26.4–28.2% ethylenediamine, and 1.6% NaOH; d^{25}, 1.085 g/cm^3. If needed, anhydrous ethylenediamine was redistilled from zinc powder.

Lysozyme (EC 3.2.1.17) from chicken egg white, twice crystallized, salt-free, and lyophilized, was purchased form ICN Pharmaceuticals (Cleveland) and

†Abbreviations used: BSA-cel, bovine serum albumin–cellulose conjugate; LYS-cel, lysozyme–cellulose conjugate; OC, oxidized cellulose; OVA-cel, ovalbumin–cellulose conjugate; TRY-cel, trypsin–cellulose conjugate.

chicken ovalbumin flakes from BDH (Poole). Trypsin (EC 3.4.21.4) crystallized from bovine pancreas and bovine serum albumin, lyophilized and additionally made free of fatty acids (*12*), were supplied by SPOFA and SEVAC Prague, respectively.

^{125}I-labeled human serum albumin (37.0 MBq/mL) was delivered by INR Swierk TN (Otwock), ^{125}I-labeled trypsin (1.0×10^5 cpm/mg) was prepared by iodination of trypsin with K^{125}I and chloramine T (*13*).

Preparation of Protein–Cellulose Conjugates

Albumins were dissolved in 50 m*M* NaCl, lysozyme, and trypsin in water. After acidifying with 1*M* HCl the solutions were neutralized with 1*M* NaOH. If needed, the solutions were centrifuged (10,000*g*, 10 min) and ^{125}I-labeled protein (37–74 kBq) was added. Proteins were immobilized by reductive alkylation on a periodate-oxidized cellulose (OC) (*7*). The reaction of OC with 0.5% solutions of proteins and the subsequent reduction with sodium borohydride were accomplished at pH 9. The conjugates were washed successively with water, 0.1*M* acetic acid, water, 0.2*M* borate buffer containing 1.075*M* NaCl and 0.1% (v/v) Tween 20, water, ethanol, and acetone and were air-dried. The conjugates of lysozyme (LYS-cel), trypsin (TRY-cel), ovalbumin (OVA-cel), and serum albumin (BSA-cel) with bead cellulose were also prepared in this way.

Determination of $A^{1\%}_{1cm}$ for Proteins in Cadoxene

All UV spectra were recorded on a Specord UV VIS (Zeiss, Jena) double-beam recording spectrophotometer equipped with a temperature cuvet holder. The solution of protein (0.4–3.0% w/v) was added to cadoxene, maintained at 25 ± 0.2°C in a stoppered quartz cuvet, so that its volume did not exceed 5% v/v. UV spectra were recorded during 2 h at 25 ± 0.2°C and absorbances read at one wavelength were extrapolated to $t = 0$. These values, corrected for absorbance of cadoxene, were used to calculate $A^{1\%}_{1cm}$ (ex). The values of absorbances obtained from the first measurements after the addition of a protein solution into cadoxene were used for calculation of $A^{1\%}_{1cm}$. Concentrations of proteins in stock solutions were determined from $A^{1\%}_{1cm}$ values for aqueous solutions (Table 1).

TABLE 1
$A^{1\%}_{1cm}$ Values for Proteins in Aqueous Solutions and Cadoxene

	Aqueous solutions[a]			Cadoxene		
Protein	$A^{1\%}_{1cm}$	λ, nm	Conditions	$A^{1\%}_{1cm}$[b]	$A^{1\%}_{1cm}$[c] (ex)	λ, nm
Lysozyme	26.7	280	0.1*M* HCl	25.8	25.6	291.5
Trypsin	16.0	280	0.1*M* HCl	17.2	17.0	294
Ovalbumin	7.5	280	0.1*M* NaOH	8.5_5	8.4	294
Serum albumin	6.7	279	0.1*M* KCl	9.0	8.6	294

[a]According to refs. (*14–16*).
[b]Calculated from the Eq. (1).
[c]Calculated from the Eq. (2).

Methods for Determination of the Protein Content in Conjugates

Method A Direct spectrophotometry (cadoxene method). Suspensions of conjugates in cadoxene (0.2–2.0% w/v) were stirred at 25 ± 0.2°C until the total dissolution and transferred into the tempered cuvet. The UV spectra were recorded during 2 h, maintaining the temperature at 25 ± 0.2°C. The solutions of bead cellulose in cadoxene were prepared and measured in the same way. The values of absorbances were used to calculate the protein content m (mg/g) in the conjugate according to:

$$m = (A_k - A_c)c_1/10^{-3}A_p c_2 \tag{1}$$

where A_k and A_c are the absorbance values for the solutions of the conjugate and the cellulose, respectively, at the same concentration c_2 (g/mL), A_p is the absorbance of a 1% solution of protein in cadoxene always at the same wavelength, and c_1 (mg/mL) denotes the concentration of this solution. The A_k and A_c values were obtained from the first measurements after dissolution of the conjugate and cellulose, respectively. The protein content m_{ex} was calculated from:

$$m_{ex} = (A_{k,ex} - A_{c,ex})c_1/10^{-3}A_{p,ex}c_2 \tag{2}$$

where $A_{k,ex}$ and $A_{c,ex}$ are the extrapolated ($t = 0$) absorbance values of solutions of the conjugate and the cellulose, respectively, and $A_{p,ex}$ is the absorbance of a 1% solution of protein in cadoxene at $t = 0$. Both methods were also accomplished in a simplified manner when, instead of water, the respective cadoxene solutions of cellulose were used as the references. In this case the quantities A_c and $A_{c,ex}$ have been omitted from the Eqs. (1) and (2).

Method B Analysis of amino acids. The conjugate (25–100 mg) containing a protein (2–3 mg) was hydrolyzed with 6N HCl (2.5–10 mL) for 24 h at 110°C *in vacuo*. The mixture of protein (2–3 mg) and bead cellulose (25–100 mg), respective, was hydrolyzed simultaneously. Byproducts of hydrolysis were removed on the strong cation exchangers (Dowex 50 W × 4, Amberlite IR-120 × 8) by elution with water. The hydrolyzates eluted with 10% pyridine/water (v/v) or 1*M* ammonia after Koelsch et al. (*1*) were subjected to amino acid analysis (Amino Acid Analyzator, type AAA Hd 1200 E, Czechoslovakia). The protein content was calculated on the basis of at least four amino acids in the conjugates and in the mixtures of proteins and celluloses.

Method C Reaction with ninhydrin. The hydrolyzates prepared for the amino acid analysis were used. These were suitably diluted to 1 mL with water and after the reaction with ninhydrin reagent (1 mL), freshly prepared and free of hydrindantine, were worked up and measured (*17*). The protein content in the conjugate was read from the calibration curve constructed after measuring the hydrolyzates of the mixture of protein and cellulose.

Method D Modified Lowry reaction. Prior to determination (*18*), the conjugates (2–20 mg) were processed for activation in 1*M* NaOH (0.2 mL) at room tem-

perature for 16 h. The protein solutions (5–150 μL) for calibration curves were worked up in a similar way.

Method E Radiometric determination. Conjugates labeled with ^{125}I were measured (*6*, *7*) using a scintillation probe (typ NaI/Tl, Well) and a scintillation counter (NZQ 717-T, Tesla, Czechoslovakia).

Statistical Evaluations

The statistical evaluations of results achieved by A and B methods were done with a *t*-test applied to paired observations as well as with correlation analysis (*19*). Parameters of the line equation were computed using a least-squares fitting program.

Results

Preliminary spectrophotometric measurements showed that proteins in cadoxene solutions retained the typical absorption properties observed with their aqueous solutions. The spectra of lysozyme in 0.1*M* HCl and of cadoxene illustrated by Fig. 1 may serve as examples. The protein–cellulose conjugates were dissolved in cadoxene up to the concentration of ca. 2% w/v. The UV spectra of these solutions did not differ in their shapes from those of free proteins in cadoxene solutions as evidenced by the spectrum of the conjugate LYS-cel in the cadoxene solution (Fig. 1). Changes in absorbances at 280–295 nm of the conjugates in cadoxene, e.g., TRY-cel (Fig. 2), were ascribed to changes of the protein content in the conjugate calculated from the Eq. (1). When both the conjugate and cellulose dissolved in cadoxene equally well, the cadoxene solution of cellulose was used as the reference solution. This was the case with TRY-cel (Fig. 2) and LYS-cel, i.e., when the concentrations of cellulose used were below 0.5%.

The results of determinations of the protein content in conjugates by the cadoxene and other standard methods are summarized in Table 2. The reproducibility achieved by the cadoxene method (SE below, 3.8%) was in most cases comparable to that of other standard methods. However, the sensitivity of this method was lower with proteins having $A^{1\%}_{1cm} < 25$ and $m < 120$ mg/g (albumins, trypsin) when compared with the sensitivity of the other standard methods. The advantage of the cadoxene method lies in the rapidity of determination as well as in the simple chemicals and measuring instruments applied.

The cadoxene solutions of proteins were unstable as indicated by absorption changes. This led to extrapolation in determination of $A^{1\%}_{1cm}$ (ex) for proteins in cadoxene solutions (Fig. 3). For comparison we present also the $A^{1\%}_{1cm}$ values both for aqueous proteins solutions taken from literature data and cadoxene solutions of proteins determined without extrapolation (Table 1). Changes of absorbances in the conjugates were minimal within 2–3 h of measurements (Fig. 4). Extrapolation was chosen with regard to difficulties in establishing the moment of dissolution of the conjugate as well as in order to eliminate the changes of absorbances of proteins during the process of dissolution. This procedure led to the

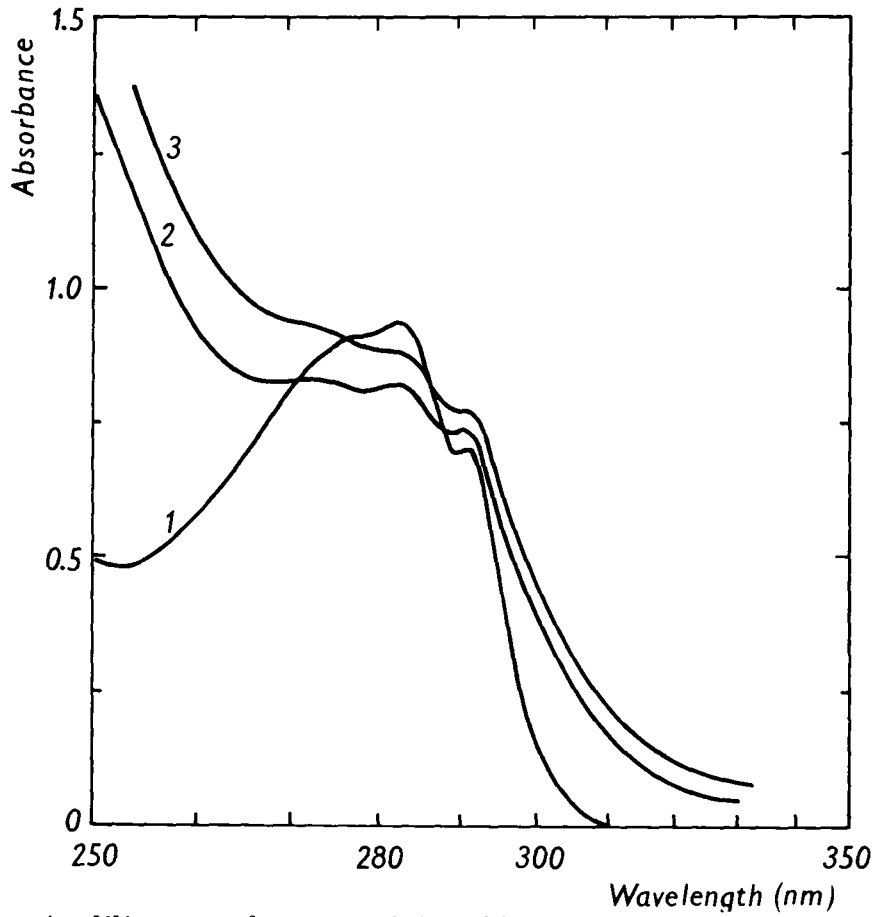

Fig. 1. UV spectra of aqueous solution of lysozyme and cadoxene solutions of lysozyme and the conjugate LYS-cel. (1) lysozyme in 0.1*M* HCl, 0.04% w/v; reference: 0.1*M* HCl. (2) lysozyme in cadoxene, 0.03% w/v; reference, water. (3) LYS-cel in cadoxene, 0.2% w/v; reference, water. 1.0 cm cuvet. The UV spectra were recorded immediately after the addition of the protein solution (within 1 min) and dissolution of the conjugate (within ca. 30 min), respectively.

determination of the protein content according to the Eq. (2); the results are presented in Table 3.

Results obtained with the cadoxene method (method A) were compared with those of amino acid analysis (method B). This was done both with the pairs of values (A/B ratio, Tables 2 and 3) and with all the group (statistical evaluations, Table 4). The *t*-test pointed out that the original hypothesis concerning the suitability of the spectrophotometric method with respect to the amino acid analysis cannot be rejected at the highest level of significance $P \leq 1\%$ (*19*). The correlation analysis offered linear relations with the correlation coefficients $r \geq 0.95$.

The differences in paired observations were significant (*t*-test), nonetheless proportional (correlation analysis) over the whole range. With respect to the parameters of corresponding equations of the correlation analysis, the amino acid analysis afforded lower values than the cadoxene method. The generally accepted view is

that in the amino acid analysis under conditions of conventional hydrolysis, the recoveries are often poor, especially for hydrophobic proteins (*20*), and that hydrolysis of protein-loaded polysaccharide gels gives rise to amino acid losses because of side reactions (*1*).

The hydrolyzates for amino acid analysis and for the reaction with ninhydrin were processed using two combinations of cation exchanger–eluent (Table 2). The differences in the results (TRY-cel 1, LYS-cel) exclude a systematic error. To determine the amount of lysine and the number of its linkages in the conjugate, the use of Amberlite and 1*M* ammonia as the eluent was found to be advantageous.

The number of linkages via lysine increased with the decreasing molecular weight of the protein and with the increasing amount of the bound protein. Simul-

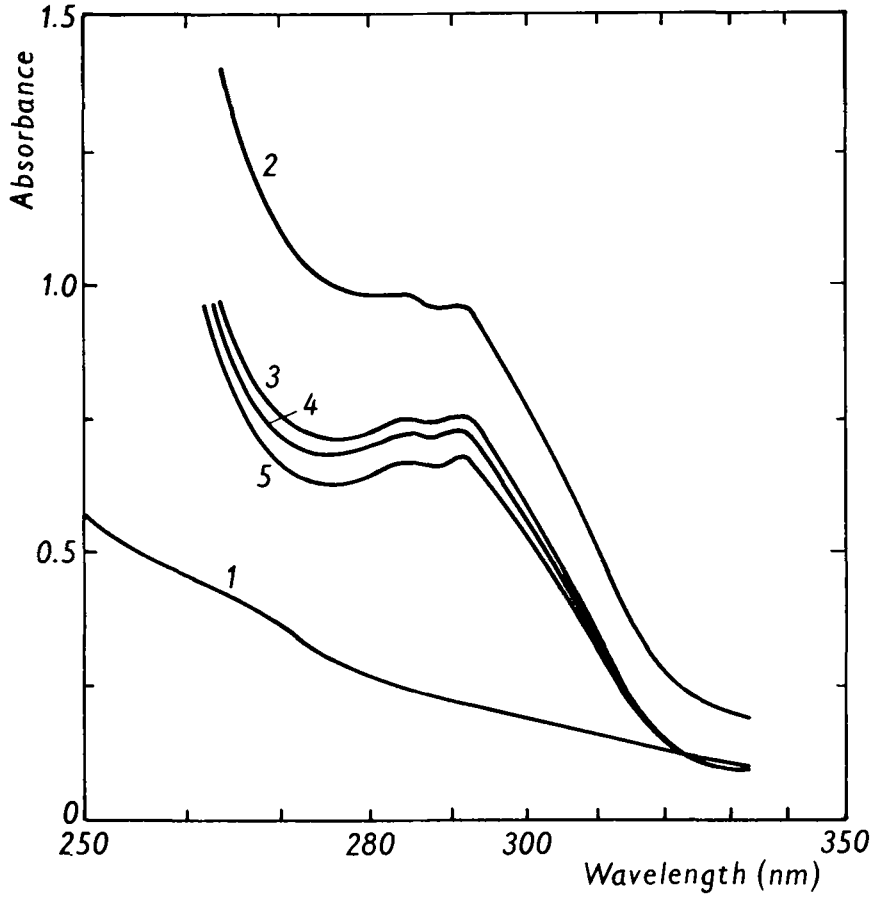

Fig. 2. UV spectra of cadoxene solutions of bead cellulose and conjugates TRY-cel. (1) bead cellulose; (2) TRY-cel 2, always 1% w/v: reference, water. (3)TRY-cel 2, 1% w/v and reference, sample 1; (4) TRY-cel 1, 0.75% w/v: reference, cadoxene solution of bead cellulose; (5) TRY-cel 3, 1% w/v: reference, cadoxene solution of bead cellulose, 32.6 ± 1.7 and 37.2 ± 0.3 mg/g determined by the methods B and A. The UV spectra were recorded immediately after the dissolution of cellulose and conjugates, respectively, in cadoxene (30 min) in a 1.0 cm cuvet at 25.0 ± 0.2°C. The cadoxene solutions of bead celluloses were prepared in concentrations corresponding to concentrations of the measured samples of TRY-cel.

TABLE 2
Protein Content in Conjugates Determined by the Cadoxene and Other Standard Methods

Conjugate	Bead cellulose batch	Method of determination mg protein/g cellulose					A/B[b]
		A, cadoxene[a]	B, amino acid analysis	C, ninhydrin	D, Lowry	E, radiometry	
BSA-cel 1	C-538/1	$29.2 \pm 0.5_5$	24.9 ± 1.6^c	23.1 ± 1.5^c	18.5 ± 0.4	21.5 ± 0.9	1.17
BSA-cel 2	op. 49	$20.9_5 \pm 0.2$	$16.6_5 \pm 0.7^d$	17.6 ± 0.6^d	14.8 ± 0.3	$19.0 \pm 0.3_5$	1.26
OVA-cel 1	C-538/1	31.3 ± 1.2	20.2 ± 1.6^c	19.9 ± 1.3^c	17.0 ± 0.4	—	1.55
OVA-cel 2	op. 49	$30.8_5 \pm 1.0$	$20.3_5 \pm 0.9^d$	18.5 ± 0.2^d	12.7 ± 0.5	—	1.52
TRY-cel 1	C-538/1	58.5 ± 0.5	66.4 ± 3.0^c	67.8 ± 0.6^c	$35.9_5 \pm 0.0_5$	—	0.88
TRY-cel 1	C-538/1	58.5 ± 0.5	72.1 ± 1.6^d	69.0 ± 2.0^d	$35.9_5 \pm 0.0_5$	—	0.81
TRY-cel 2	op. 49	41.4 ± 0.7	66.4 ± 1.2^d	53.9 ± 0.8^d	$31.8_5 \pm 0.5_5$	23.2 ± 0.6	0.62
LYS-cel	C-538/1	121.8 ± 0.7	142.7 ± 4.4^c	$115.5_5 \pm 2.5^c$	74.3 ± 2.2	—	0.85
LYS-cel	C-538/1	121.8 ± 0.7	123.6 ± 5.8^d	102.2 ± 0.6^d	74.3 ± 2.2	—	0.99

[a]Calculated from the Eq. (1).
[b]Portion of protein content in the conjugate determined by the methods A and B.
[c]Hydrolyzate purified on Dowex 50 W × 4 by elution with 10% pyridine/water (v/v.
[d]Hydrolyzate purified on Amberlite IR-120 by elution with 1*M* NH_4OH.

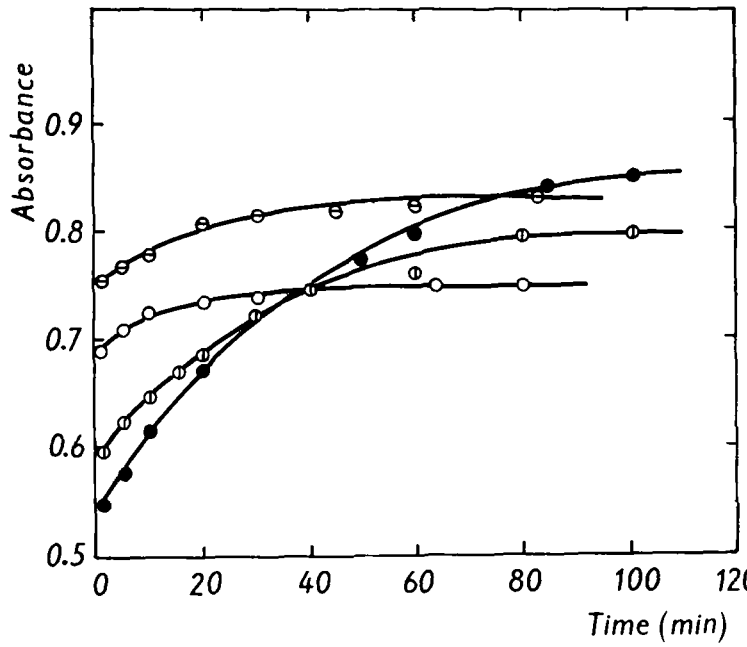

Fig. 3. Changes of absorbance of lysozyme, trypsin, ovalbumin, and serum albumin cadoxene solutions with time. (⊖) lysozyme, 0.03% w/v at 291.5 nm; (○) trypsin, 0.05% w/v at 294 nm; (⊕) ovalbumin, 0.075% w/v at 294 nm; (●) serum albumin, 0.05% w/v at 294 nm. Reference: water, 25.0 ± 0.2°C, 1.0 cm cuvet.

taneously, the ratio of A/B (Table 2) decreased in this direction. This fact excluded any contribution of absorbance by chemical bonds between protein and cellulose to the "cadoxene" value. Nevertheless, how significantly this contribution could influence the results is demonstrated by the conjugate BSA-cel 3 prepared by partial reduction at pH 5, where method A gave 92.8 mg/g and the method E (radiometry) 17.75 mg/g.

The lower values achieved by the method C (the reaction with ninhydrin) in comparison with the method B can be ascribed to the decrease of lysine residues participating in the binding of the proteins to cellulose. Also, method D (the Lowry modified reaction) afforded very low results when compared to other methods. This can be explained by the reduced absorption properties of the chromophore because of the lower ability to produce the awaited complex compound (*21*). The reliability of the results obtained by the method E (radiometry) is dependent on the manner of preparation of ^{125}I-labeled proteins. For example, radioiodination by using chloramine T (*13*) might have led to damage of the protein (*22*) that can result from the decrease in reactivity of protein in the process of reductive alkylation (TRY-cel 2, Table 2).

Discussion

There are two possible sources of inaccuracy in the method developed:

1. Dissolution of the conjugates in cadoxene lasted at least 30 min. This stage was considered to be linear in extrapolations. Because of the error caused by these

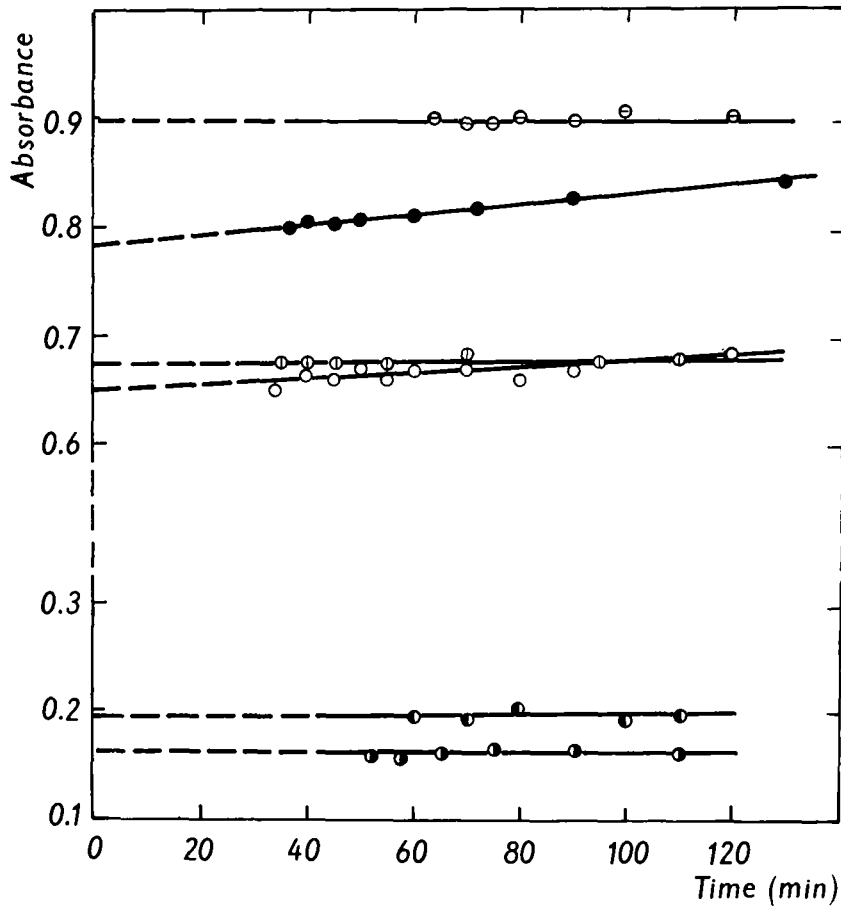

Fig. 4. Changes of absorbances of cadoxene solutions of cellulose and conjugates BSA-cel, OVA-cel, TRY-cel, and LYS-cel with time. (⦶) BSA-cel 1, 2% w/v at 294 nm; (⊖) OVA-cel 1, 2% w/v at 294 nm; (○) TRY-cel 1, 0.5% w/v at 294 nm; (●) LYS-cel, 0.2% w/v at 291.5 nm; (◐) bead cellulose, 0.5% w/v at 294 nm; (◑) bead cellulose, 0.2% w/v at 294 nm. Reference: water, 25.0 ± 0.2°C, 1.0 cm cuvet.

TABLE 3
Protein Content in Conjugates Determined by the Cadoxene Method after Extrapolation

Conjugate	m_{ex}, g/g	A/B
BSA-cel 1	30.6 ± 0.6	1.23
BSA-cel 2	21.95 ± 0.2	1.32
OVA-cel 1	31.8 ± 1.2	1.57
OVA-cel 2	31.3_5 ± 1.0	1.57
TRY-cel 1	58.1 ± 0.5	0.87_5; 0.80_5
TRY-cel 2	40.6 ± 0.7	0.61
LYS-cel	115.2 ± 0.7	0.81; 0.93

TABLE 4
Statistical Evaluations of the Results Obtained by the Cadoxene Method and Amino Acid Analysis

No.	*t*-Test (t)	Level of significance P, %	Line equation,[a] $m_2 = a + bm_1$		Correlation coefficient, r	Number of pairs, n	Calculation of m_2 according to Eq. #
			a	b			
1	4.2552	0.5	7.550	0.807	0.9678	9	(1)
2	4.7853	0.1	10.157	0.744	0.9677	9	(2)
3	3.1696	1	7.436	0.834	0.9508	7	(1)
4	4.1096	0.5	9.909	0.769	0.9499	7	(2)

[a] m_1, m_2 present the paired values obtained by methods B and A (Table 2).

simplifications, the results obtained by the method A might have been overestimated.

2. The differences in the protein contents determined by methods A and B can also be explained by changes in the $A^{1\%}_{1cm}$ values of proteins after their immobilization.

The direct spectrophotometric determination of the protein content in cellulose conjugates is based on the addivity of UV spectra connected with the increase of absorbance of the conjugates (Figs. 1, 2) and must be exclusively due to absorbance of the protein. This requirement excludes: (a) absorbance contribution caused by chemical bonds between protein and cellulose, (b) change in the UV spectrum of the protein caused by chemical modification. A method for preparation of conjugates, i.e., the reductive alkylation of proteins via dialdehydes of cellulose, should fulfill both conditions mentioned. The results have shown that the method has not introduced any additional chromophore into the conjugate and has not modified the protein in such a way that would lead to any changes in its UV spectrum. As far as these conditions are fulfilled, it is also possible to employ any other method of immobilization. The cadoxene method can be applied also in other noncrosslinked celluloses (microcrystalline, powdery, fibrous), starches and dextrans as carriers (P. Gemeiner, unpublished results).

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