A COLORED WATER-SOLUBLE CARBODIIMIDE AS A REAGENT FOR THE MODIFICATION OF PROTEINS

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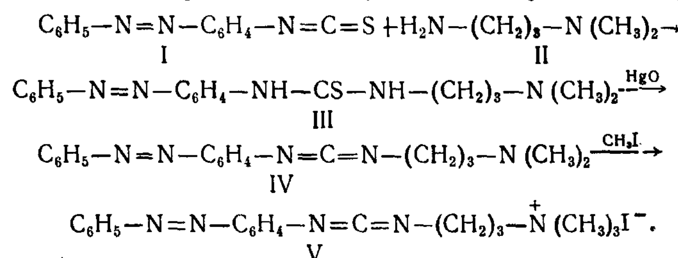
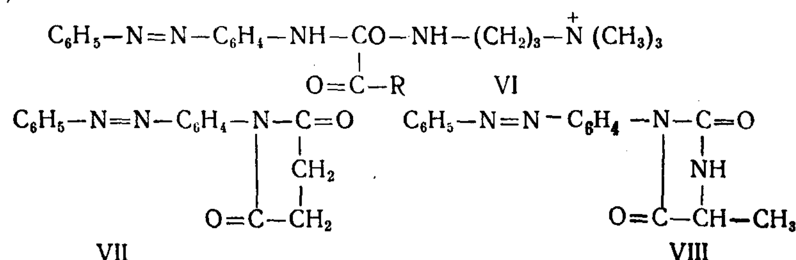
Water-soluble carbodiimides (CDIs) are widely used for the chemical modification of proteins. In this process, the CDIs are not only activating agents for the condensation of the protein with an amine but may also take part directly in interaction with the protein. For the quantitative evaluation of this reaction, in addition to labelled CDIs it is desirable to use colored CDIs. In view of this, we have synthesized a water-soluble CDI including a phenylazophenyl chromophoric group which provides the possibility of monitoring the course of its reaction with a protein. The synthesis was performed by the following route



Aqueous solutions of the CDI (V), and the urea and thiourea (III) corresponding to it have absorption maxima in the UV spectra at 355 nm. The reaction of the CDI (V) with pepsin, pepsinogen, and the pepsin-like acid proteinase of _Aspergillus awamori_ leads to derivatives with their absorption maxima at 280 and 325 nm (Fig. 1), the proteolytic activity of which is considerably reduced (Table 1). When the modified pepsin was chromatographed on DEAE-cellulose, it was eluted earlier than the native pepsin, which shows a decrease in the total negative charge of the protein as a result of the reaction of its carboxy group with the CDI.

Since the most probable product of the reaction with CDI may be the urea corresponding to the CDI acylated at the least basic nitrogen atom (VI), we synthesized model compounds having the same environment of the acylated nitrogen as in (VI) – p-phenylazophenylsuccinimide (VII) [1] and 5-methyl-3-p-phenylazophenylhydantoin (VIII):



Compounds (VII) and (VIII), like the proteins modified with the CDI, have an absorption maximum at 325 nm in their UV spectrum. This confirms the hypothetical structure (VI). To determine the number of CDI residues incorporated in the protein molecule, we used the values of the molar extinction coefficient determined for model compounds: 22,000 for (VII) in 10% DMFA and 20,000 for (VIII) in ethanol.

The presence of an aromatic substituent in the molecule of the CDI (V) is responsible for its higher reactivity than that of a CDI with an aliphatic structure CME–CDI. Reaction with the CDI (V) takes place

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TABLE 1. Results of the Modification of Proteins

Excess of CDI with respect to the protein	Pepsin		Pepsinogen		Aspergillus awamori	
	No. of CDI residues per molecule of protein	activity with respect to hemoglobin after 60 min, %	No. of CDI residues per molecule of protein	pepsinogenic activity	No. of CDI residues	activity with respect to hemoglobin after 60 min, %
1	0,79	80	0,66	86	—	—
2	1,57	68	1,45	91	—	—
4	2,42	50	2,3	52	—	—
5	2—2,4	38	—	—	1,2	7,5
8	4,8	30	—	—	—	—
10	4—5	23—24	3,5	20	2,04	0
15	5,6	10—20	—	—	—	—
20	5,6—6	11—17	5,5	—	—	—
30	6,5	17	—	—	2,4	0

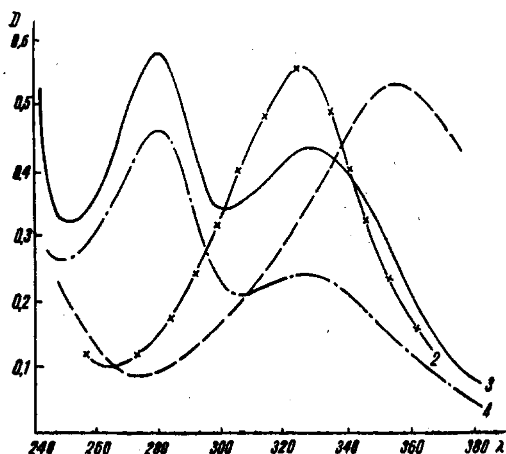

Fig. 1. Absorption spectra: 1) (V) (water); 2) (VII) (10% DMFA); 3) pepsin with two CDI residues; 4) Aspergillus awamori with 1.2 CDI residue.

even at an equimolecular ratio of CDI and pepsin (see Table 1). With a fivefold excess of CDI, three residues are incorporated, and the maximum substituent of the carboxy groups takes place with a 20-fold excess of the reagent. When CME−CDI was used under the same conditions, three CDI residues were incorporated in pepsin only with a 20-fold excess of it [2].

The difference in the structures of the CDIs also apparently affects the selectivity of their action. Thus, the inclusion of three CME−CDI residues in pepsin does not change its proteolytic activity, while the substitution of three groups of pepsin by the colored CDI (V) causes a fall in the activity to 50%. If pepsin previously modified with CME−CDI is caused to react with the CDI (V), the same number of residues of the CDI (V) are incorporated in the protein molecule as in the modification of the native pepsin. This is accompanied by a decrease in its activity.

The reaction of the CDI (V) with pepsinogen leads to the substitution of the same number of carboxy groups as in pepsin (see Table 1). After the activation of the modified pepsinogen and its subsequent purification on Sephadex, the number of incorporated residues remained unchanged. Consequently, carboxy groups present in the pepsin moiety take part in the reaction. They are not the groups of the active center but are apparently located in a region close to it.

The reaction of the CDI (V) with the pepsin-like proteinase of Aspergillus awamori, unlike that with pepsin, leads under the same conditions to the complete loss of activity of the enzyme (see Table 1). This apparently shows a difference in the structure of these proteinases. Thus, the colored CDI (V) can be used for the chemical modification of proteins.

EXPERIMENTAL METHOD

The work was carried out with a preparation of chromatographically purified porcine pepsin [3], a preparation of porcine pepsinogen (TU 10P222-68, Olaine chemical reagents factory) purified by chromatography on DEAE-cellulose at pH 5.98, a preparation of Aspergillus awamori [4], and bovine hemoglobin (Leningrad Institute of Vaccines and Sera).

The analyses of all the compounds corresponded to the calculated figures.

Modification of the Protein. A solution of 5 mg (0.143 μmole) of pepsin (or Aspergillus awamori) in 4.6 ml of water was brought to a pH of 5.6 and treated with 0.4 ml of a $1.67 \cdot 10^{-3}$ M solution of the CDI (V) in water containing 5% of DMFA (fivefold excess of CDI). The reaction mixture was stirred with the pH kept constant, and was then chromatographed on Sephadex G-25 with elution by water at pH 5.6.

Determination of Proteolytic Activity with Respect to Hemoglobin. A solution of 500 mg of bovine hemoglobin in 25 ml of 0.006 N hydrochloric acid was filtered, and 1-ml portions of filtrate was transferred to test tubes. The solutions were thermostatted at 37°C for 5 min and to each was added 0.1 ml of the solu-under investigation containing 0.1 mg/ml of protein. The mixtures were incubated at 37°C for 10 min andto

each was added 5 ml of a 5% solution of trichloroacetic acid. The precipitate was separated on a Filtrak No. 390 paper filter, and the optical density was measured at 280 nm. In a control test tube, 5 ml of 5% TCA was first added, and then 0.1 ml of protein solution. The activity of the protein before modification was taken as 100%.

N-(Dimethylaminopropyl)-N'-(p-phenylazophenyl)thiourea (III). A solution of 1 g (0.004 mole) of (I) [5] in 35 ml of absolute ether was treated with 1 ml (0.01 mole) of dimethylaminopropylamine (II) [6]; an orange precipitate immediately deposited. The reaction mixture was kept at room temperature for 12 h, and then the precipitate was separated off and was washed with ether. Composition $C_{18}H_{23}N_5S$, yield 1 g (73.3%), mp 162-163°C, R_f 0.73 [paper chromatography in butanol–water–acetic acid (77 : 13 : 10) system], l 6 cm (1000 V, 40 min, pH 5.6; l being the distance migrated by the substance on electrophoresis).

N-(Dimethylaminopropyl)-N'-(p-phenylazophenyl)carbodiimide Methiodide (V). A solution of 0.5 g (0.0014 mole) of (III) in 15 ml of absolute methylene chloride was treated with 0.7 g (0.0032 mole) of freshly precipitated mercuric oxide, and the reaction mixture was stirred for two hours. The black precipitate of mercuric sulfide was filtered off and the mother solution was evaporated to dryness. The residue was extracted repeatedly with absolute petroleum ether. The petroleum ether was distilled off in vacuum. The residue of oil (IV) showed a band in the IR spectrum at 2140 cm^{-1}. A solution of (IV) in absolute ether was treated dropwise with 1 ml (0.016 mole) of methyl iodide. The reaction mixture was left at room temperature for 12 h and then the precipitate that had deposited was separated off. Composition $C_{19}H_{24}N_5I$, yield of (V) 0.228 g [50% calculated on the (III)], mp 168-170°C, R_f 0.16; l 2.5 cm (1000 V, 2 h, pH 5.6). Its IR spectrum had a band at 2140 cm^{-1}.

N-(α-Carboxyethyl)-N'-(p-phenylazophenyl)urea (IX). An alkaline solution of 0.7 g (0.008 mole) of L-alanine in aqueous pyridine (1.1), pH 9, was treated with 1 g (0.005 mole) of p-phenylazophenyl isocyanate (I). The reaction mixture was heated at 40-50°C for 30 min. The precipitate that had formed was filtered off, and the filtrate was extracted repeatedly with benzene and then with ether. When the aqueous solution was acidified to pH 2, an orange precipitate was formed, and this was separated off and washed with water. After reprecipitation with petroleum ether from acetone, 0.7 g (50%) of (IX) was obtained with the composition $C_{16}H_{16}N_4O_3$, mp 172-174°C, R_f 0.81 [Silufol; butanol–water–acetic acid (4 : 5 : 1)], l 2.8 cm (500 V, 1 h, 0.01 N Na_2CO_3).

5-Methyl-3-(p-phenylazophenyl)hydantoin (VIII). To 0.5 g (1.5 mmole) of (IX) was added 25 ml of 2 N hydrochloric acid, and the reaction mixture was boiled for 1 h. Then the precipitate was filtered off and was washed on the filter with 10% sodium carbonate solution. After reprecipitation with petroleum ether from ethanol, 0.36 g (73%) of (VIII) was obtained with the composition $C_{16}H_{14}N_4O_2$, mp 221-222°C, R_f 0.71.

SUMMARY

1. A water-soluble CDI including a chromophoric group has been synthesized.

2. The CDI obtained has been used to modify pepsin and pepsinogen.

3. A hypothesis on the nature of the bond of the CDI with the protein has been formulated.

4. The number of carboxy groups of the proteins investigated that react with CDI at various ratios of the reagents have been determined.

LITERATURE CITED

1. S. Masyuama and M. Kamada, J. Chem. Soc. Japan, 70, 138 (1949).
2. L. F. Matyash, O. G. Ogloblina, and V. M. Stepanov, Biokhimiya, 37, 1067 (1972).
3. V. M. Stepanov and T. I. Vaganova, Biokhimiya, 28, 540 (1963).
4. L. S. Lobareva, G. G. Kovaleva, and M. P. Shimanskaya, Biokhimiya, 37, 198 (1972).
5. G. B. Grippe and R. Gerraci, Gazz. Chim. Ital., 70, 381 (1940).
6. A. N. Kost and N. P. Terent'ev, Zh. Obshch. Khim., 17, 1632 (1947).