Compounds (III) and (IV) were synthesized analogously. The yield of (III) was 5.75 g (66%), R_f 0.53. Hydrochloride with mp 66-68°C; methiodide with mp 106-108°C. IR spectrum (ν, cm^{-1}): 2830 (trans-quinolizidine), 1735 (—OCO—), 1210 (—COC—). PMR spectrum (δ, ppm): 3.8-4.2 (2H, m, OCH_2), 2.84 (2H, d, $H_{2\bar{e}}$ and $H_{10\bar{e}}$, J = 10.6 Hz), 1.2-2.3 (46H, m, CH_2), 0.84 (3H, t, CH_3).

The yield of (IV) was 4.68 g (54%); R_f 0.68, n_D^{20} 1.3544; methiodide with mp 239-242°C.

IR spectrum (ν, cm^{-1}): 2810 (trans-quinolizidine), 1750 (—OCO—), 1220 (—COC—). PMR spectrum (δ, ppm): 5.22 (2H, t, CH=CH), 3.95-4.3 (2H, m, OCH_2), 2.76 (2H, d, $H_{2\bar{e}}$ and $H_{10\bar{e}}$, J = 10.6 Hz), 1.0-2.4 (42H, m, CH_2), 0.83 (3H, t, CH_3).

The samples for determining the activities of the enzymes contained 0.4 ml of a 0.001 M solution of Ellman's reagent, 0.7 ml of 0.1 M phosphate buffer with pH 8.0, 0.3 ml of an aqueous solution of ACE or BuCE (prepared to give 0.2 activity unit per 1 ml), 0.2 ml of a solution of the substrate acetylcholine (ATC) (the concentration in the sample varied from $1.25 \cdot 10^{-4}$ M to $7.5 \cdot 10^{-5}$ M), and 0.2 ml of a 0.2 M solution of the substance under investigation.

As control we used a sample in which the 0.2 ml of the solution of the substance under investigation had been replaced by an equivalent amount of water. To exclude the possibility of irreversible inhibition of the substance under investigation and to show the reversible nature of the inhibition of the enzyme, before the inhibition of the substrate the sample was incubated with the inhibitors under investigation at 30°C for ~20 min in the reaction mixture, and then the ATC was added and hydrolysis was carried out. The constant of reversible inhibition was found from a graph of double reciprocal values of $\bar{K}_i$ according to [3].

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PREPARATION OF CONJUGATES OF PEPTIDES IMITATING PART OF THE ANTIGEN-DETERMINANT SECTION OF PROTEIN VP1 OF FOOT-AND-MOUTH DISEASE VIRUS A_{12} WITH VARIOUS SUPPORTS

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The preparation of conjugates of peptides 143-148, 153-159, 149-159, 146-159, and 143-159, imitating a section of a protein of the foot-and-mouth disease (FMD) virus of type A_{12}, with bovine serum albumin, with a copolymer of N-vinylpyrrolidone with acrylic acid, and with a copolymer of N-vinylpyrrolidone with maleic anhydride is described. The dependence of the degree of conjugation on various factors is discussed.

At the present time there are two approaches to investigations of the creation of synthetic vaccines. The first, proposed by Sela [1] consists in obtaining the determinant groups of the antigens synthetically and their subsequent conjugation with synthetic poly(amino acid)s or with native proteins. This approach has been used for those viruses in

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TABLE 1. Initial Information and Results Obtained on Conjugation

Peptide	Initial molar ratio of support to peptide			Degree of conjugation					
				% w.r.t. peptide			moles of peptide/mole of support		
	BSA	NA	NM	BSA	NA	NM	BSA	NA	NM
1. 154—160	1:30	1:30	1:30	23,3	88,3		7	26,5	
2. 156—160	1:30	1:30	1:20	13,3	53,3	20	4	16	4
3. 147—160	1:30	1:20	1:20	30	46,6	45	9	13—14	9
4. 144—160			1:20			15			3
5. 144—149	1:30	1:30	1:20	16,6	26,6	25	5	8	5
6. Nle—154—160	1:40			55			22		
7. Nle—150—160	1:40			20			8		
8. Nle—147—160									
9. Nle—144—160	1:40			7,5			3		

which the antigen-determinant sections have been determined. The second approach, developed by Petrov et al. [2], consists in the complexing or covalent binding of haptens or the determinant groups of antigens, obtained synthetically or isolated from the causative agent, with synthetic polyionic molecules (polyelectrolytes) fulfilling the role of immunoadjuvant. It possesses all the advantages of the first approach, and, in addition, by its aid it is possible to increase the immunogenic activity even of intrinsically weak immunogens.

In the creation of synthetic vaccines against the foot-and-mouth disease (FMD) virus, it is possible to use both these approaches. The positions and limits of the antigen-determinant sections of this virus have been determined and peptides have been synthesized the immunization of animals with protein conjugates of which has protected them from a control infection [4, 6, 10]. Ivanov et al. [3] have reported that a peptide from the VP1 proton of the FMD virus of type O1 protected animals from infection without being conjugated with supports. We have previously synthesized various peptides from the suggested antigen-determinant section of the VP1 protein of the FMD virus A_{12} [6]. In the present paper we report the conjugation of these peptides with bovine serum albumin and with polymeric immunostimulators.

All the initial information and the results obtained are given in Table 1. When peptides 153-159, 149-159, 146-159, and 143-148 were conjugated with BSA by the carbodiimide method using p-nitrophenol, the conjugates obtained had low degrees of conjugation – 7, 4, 9, and 5 moles of peptide per 1 mole of BSA, respectively. As is known, condensation by the carbodiimide method in the presence of p-nitrophenol proceeds through the formation of activated (p-nitrophenyl) esters. We therefore assumed that the use of N-hydroxysuccinimide, giving more reactive esters, in place of p-nitrophenol, might lead to an increase in the degree of conjugation.

An increase in the molar ratio of protein to peptide may also lead to an increase in the degree of conjugation. To check this hypothesis, in the conjugation of the peptides Nle—153—159, Nle—149—159, and Nle—143—159 with BSA, in place of p-nitrophenol we used N-hydroxysuccinimide and increased the molar ratio of protein to peptide to 1:40. And in actual fact, such changes did lead to a considerable increase in the degree of conjugation: 7 and 22 moles of peptide per 1 mole of protein in the case of peptides 153—159 and Nle—153—159; 4 and 8 in the case of peptides 149—159 and Nle—149—159; and 9 and 41 in the case of peptides 146—159 and Nle—146—159. The low degree of conjugation of peptide Nle—143—159 with BSA (3 moles of peptide per 1 mole of protein) can be explained by the great length of the peptide and, possibly, by the steric hindrance arising as a result of this, and also by the poor solubility of the peptide in the solvent.

The calculation of the number of moles of peptide attached to the BSA from the results of amino acid analysis was made by two methods. By the first method the epitopic density was determined from the ratio of such amino acids as glycine, phenylalanine, leucine, and alanine in the initial BSA and in the conjugate. The second method used was that proposed in [11]. In this case, all the amino acids present both in the initial BSA and in the peptide were taken into account. Here the difference in the amounts of amino acids present in the peptide, in the BSA, and in the conjugate were determined. The discrepancy in the values of the epitopic densities obtained by these methods amounted to 1-2 moles. Table 1 gives the average values. The epitopic densities of the conjugates of peptides Nle—153—159,

Nle–149–159, Nle–146–159, and Nle–143–159 calculated from the amount of norleucine (Nle) in the conjugate scarcely differed from those calculated by the method of [11].

For the conjugation of the peptides with polymeric immunostimulators we used polymers differing by the structure of their chains and containing different functional groups. On conjugation with a copolymer of N-vinylpyrrolidone and maleic anhydride, conjugation takes place through the opening of the anhydride ring, while on conjugation with the copolymer of N-vinylpyrrolidone and acrylic acid the formation of an amide bond takes place through the active N-hydroxysuccinimide groups of the polymer. Nevertheless, a comparison of peptides 149–159, 146–159, and 138–148 showed that the nature of the functional group affected the degree of conjugation only for peptide 149–159.

We also found that on the conjugation of different peptides with one and the same polymer, the degrees of conjugation were different. This is apparently connected not only with the different solubilities of the peptides but also with the nature of the amino acids present at the N-ends of the peptides. Thus, for example, for peptides with the sterically hindered amino group of valine the degree of conjugation on all the supports was lower than for peptides containing aspartic acid residues at their N-ends.

It was possible to observe a similar relationship in the conjugation of the peptides with BSA. In the case of peptides 153–159 and 146–159 the N-terminal amino acids were arginine and aspartic acid, while for peptides 149–159 and 143–148 they were serine and valine. The degrees of conjugation of peptides 153–159 and 146–159 (7 and 9 moles/mole, respectively) were higher than for peptides 149–159 and 143–148 (4 and 5, respectively). Since peptide 146–159 was four amino acids longer than peptide 149–159 and peptide 143–148 was five amino acids longer and the degree of conjugation for it was greater, it is possible that the influence of the structure of the N-terminal acids on the degree of conjugation is more considerable than the influence of the length of the peptide.

Thus, the results obtained show that the replacement of p-nitrophenol by N-hydroxysuccinimide in the conjugation of peptides with a protein leads to an increase in the degree of conjugation for the given peptides. Apart from the activating reagents, the degree of conjugation is also affected by the structure of the amino acid present at the N-end of the peptide, and this influence is probably the stronger the greater the length of the peptide.

The peptides obtained and their conjugates were studied for their capacity for inducing the primary and secondary humoral immune responses in mice $(CBA \times C57BL/6)F_1$. The animals were immunized with the free peptides and their conjugates and the amount of antipeptide IgGs in the blood sera of the mice were determined by the method of solid-phase immunoenzyme assay. Considerable levels of antipeptide antibodies were observed in both the primary and the secondary immune responses. The results of the immunological investigations will be published separately.

EXPERIMENTAL

For conjugation we used peptides imitating sections of the VP1 protein of FMD virus A_{12} with the following sequences:

1. Arg-Val-Ala-Arg-Gln-Leu-Pro- (154–160);
2. Ser-Leu-Ala-Pro-Arg-Val-Ala-Arg-Gln-Leu-Pro (150–160);
3. Asp-Phe-Gly-Ser-Leu-Ala-Pro-Arg-Val-Ala-Arg-Gln-Leu-Pro (147–160);
4. Val-Arg-Gly-Asp-Phe-Gly-Ser-Leu-Ala-Pro-Arg-Val-Ala-Arg-Gln-Leu-Pro (144–160);
5. Val-Arg-Gly-Asp-Phe-Gly (144–149).

In addition, norleucine was added as a label to the N-ends of peptides 1, 2, 3, and 4, and the resulting peptides were numbered 6, 7, 8, and 9, respectively.

As high-molecular-mass supports we used bovine serum albumin (BSA) (Fluka, Switzerland), a copolymer of N-vinylpyrrolidone with acrylic acid (NA) with a molecular mass of 100,000 [7], and a copolymer of N-vinylpyrrolidone with maleic anhydride (NM) having a molecular mass of 40,000 [8]. The NA contained 3.15 μmole/mg of N-hydroxysuccinimide groups.

The amounts of peptides in the conjugates and the degrees of conjugation were determined from the results of amino acid analysis. Amino acid analysis was carried out after hydrolysis in 6 N HCl at 110°C for 24 h on Biotronic-5001 and Hitachi-635 amino acid analyzers.

The succinylation of the BSA was carried out by a standard procedure [9]. The BSA (1.0402 g) was added to a solution of 24 g of guanidine hydrochloride in 50 ml of distilled water. The mixture was stirred until the BSA had dissolved completely. The pH of the mixture was brought to 9.3 with a 1 N solution of NaOH, and 3.75 g of succinic anhydride was added in several portions, the pH of the reaction mixture being kept at ~9.3 by the addition of NaOH. After the addition of the last portion of succinic anhydride, the mixture was stirred at room temperature for 1 h, and then 9 g of hydroxylamine hydrochloride was added and stirring was continued for another 1 h. The reaction mixture was dialyzed against distilled water and was freeze-dried. This gave 800.7 mg of succinylated BSA.

Conjugation of the Peptides with BSA. Peptides 153–159, 149–159, 146–159, and 143–148 were conjugated with the succinylated BSA by the use of dicyclohexylcarbodiimide (Merck) and p-nitrophenol [10]. The initial molar ratio of protein to peptide was 1:30. The peptides Nle–153–159, Nle–149–159, Nle–146–159, and Nle–143–159 were conjugated with the succinylated BSA by the procedure of [10], except that N-hydroxysuccinimide was used instead of p-nitrophenol. The molar ratio of protein to peptide was 1:40.

To 3 ml of DMFA was added 0.75 μmole of succinylated BSA, and the mixture was stirred with a magnetic stirrer until a homogeneous suspension had been obtained (2.5 h). Then 21.25 mg (187.5 μmoles) of N-hydroxysuccinimide was added. After 15 min, 19.31 mg (93.75 μmoles) of DCHC was added and the mixture was stirred for 3 h. Then 30 μmoles of the peptides and 375 μl of TEAS (2.866 μmoles) was added and the reaction mixture was stirred in the refrigerator at 4°C for two days. When the reaction was complete the mixture was dialyzed against distilled water. After dialysis, if a precipitate had deposited, it was centrifuged off, and the soluble fraction was lyophilyzed.

Conjugation of the Peptides with NA. Peptides 153–159, 149–159, 146–159, and 143–159 were conjugated with NA at a molar ratio of polymer to peptide of 1:30. The NA contained 3.15 μmoles/mg of N-hydroxysuccinimide groups.

The polymer (0.5 μmole) was dissolved in 5 ml of DMFA, and 15 μmoles of triethylamine and 15 μmoles of the peptide were added. The reaction was carried out at room temperature for three days. Then the reaction mixture was dialyzed against distilled water. Before dialysis, imidazole was added to the reaction mixture. After dialysis the precipitate was filtered off, and the soluble fraction was freeze-dried.

Conjugation of the Peptides with NM. Peptides 149–159, 146–159, 143–159, and 143–148 were conjugated with NM at a molar ratio of polymer to peptide of 1:20.

The peptide (7.9 μmoles) was dissolved in 1 ml of DMFA, and a solution of 0.305 μmoles of the polymer in 1.5 ml of DMFA was added to the solution. Then 7.9 μmoles of pyridine was added. The reaction was continued at 45-50°C for three days. After the end of the reaction, the reaction mixture was dialyzed against distilled water. If a precipitate had formed, it was filtered off, and the soluble fraction was freeze-dried.

The final purification of all the conjugates obtained to remove unchanged peptide and other low-molecular-mass substances was effected by gel filtration on a column (1.6 × 36 cm) of Sephadex 6-50 in 0.1 M NaCl solution at a rate of elution of 0.4 ml/min. The fractions containing the conjugates were combined and freeze-dried.

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SOME FUNCTIONAL PROPERTIES OF COTTON PROTEIN HYDROLYSATES

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A comparative study of the solubility and emulsifying properties of cotton protein hydrolysates produced by the enzyme preparation Pektofoetidin has shown that the optimum functional properties are possessed by hydrolysates with a degree of proteolysis of 8-13%.

At the present time, the question of the search for new sources of food proteins and the expansion of the range of their application is becoming acute [1]. In addition to the biological value of protein substances, an important factor limiting the sphere of their use is undoubtedly their functional properties [2]. Among the considerable arsenal of known methods of regulating the functional properties of proteins, a definite place is occupied by proteolysis. The hydrolysis of proteins at the peptide bonds under the action of enzymes generally improves their solubility, particularly at the isoelectric point (IEP). At the same time, some other functional properties of the hydrolysates obtained (emulsifying, gel-forming, etc.) also change [3-7].

Protein isolates obtained by acid extraction from cottonseed meal possess a low solubility in the neutral pH range at low ionic strength of the solution [8]. In the present paper we give results of a study of the change in the solubility and emulsifying properties of cotton protein hydrolysates obtained on proteolysis of an isolate by the enzyme preparation Pektofoetidin P10x, which is used in industry [9].

Analysis of the products of the proteolysis of the cotton protein isolate by Pektofoetidin (Fig. 1) show that the enzyme exhibits considerable activity, and the rate of proteolysis depends on the enzyme:substrate ratio. As can be seen from Fig. 1, the maximum degree of proteolysis of the protein (α) amounted to 20.1% at a time of hydrolysis of 24 h.

Since proteins exist in aqueous solutions in the form of bipolar ions and the solubility of cotton protein at the IEP is low, it is possible to study their solubility with the aid of turbidimetric titration. The region of the pH for maximum precipitation of all the

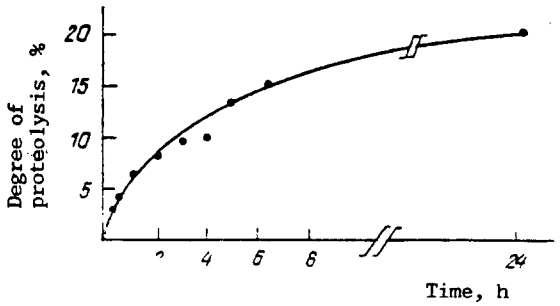

Fig. 1. Degree of proteolysis of hydrolysates of cotton protein by Pektofoetidin as a function of the time of the reaction.

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