Anti-Inflammatory and Antishock Water-Soluble Polyesters of Glucocorticoids with Low Level Systemic Toxicity

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Purpose. The objective of this study was to evaluate the pharmacological activity of glucocorticoid hormones incorporated into the structure of water-soluble carbochain polymers via the esterified 21-CH_2OH group.

Methods. Polymer analogs of glucocorticoids were prepared by radical copolymerization of 1-vinyl-2-pyrrolidone with cortisol or dexamethasone 21-crotonates and crotonic acid or 2-(diethylamino) ethylcrotonate which served as ionogenic comonomers. Anti-inflammatory, immunosuppressive and catabolic activities for ionogenic tertiary copolymers and previously prepared non-ionogenic binary copolymers were evaluated in standard animal models. The antishock activity of some of the copolymers was evaluated using the "declamping shock" model.

Results. Water-soluble tertiary copolymers with a steroid content up to 14 mol% and an intrinsic viscosity up to 0.30 dl/g in ethanol were synthesized. It was shown that the copolymers were stable in aqueous solutions at pH 5.2–7.3. All of the polymers studied suppressed inflammatory reactions at the level of free hormones when administered interperitoneally. The antishock activity was considerably higher compared to free steroids. The copolymers, unlike unmodified glucocorticoids, did not influence the physical development of young animals. They also caused much lower thymus hypotrophy than free hormones. No clear effect of the presence and nature of ionogenic units in copolymers on the pharmacological performance of the copolymers was detected.

Conclusions. The water-soluble polymers bearing glucocorticoid 21-residues in the side chains retain the anti-inflammatory activity of free steroids and exhibit a higher antishock, a lower immunosuppressive and no catabolic effect.

KEY WORDS: glucocorticoids; steroids anti-inflammatory; esters crotonic; copolymers water-soluble.

INTRODUCTION

Glucocorticosteroids are widely used in the therapy of many chronic inflammatory diseases and in emergency situations (collapse, shock). Despite recommendations for their rational use, systemic toxicity of these hormones limits the use of glucocorticoid preparations on a regular basis and in administration to children (1). Side effects are largely due to cellular uptake of the exogenous hormones which disturbs the inner glucocorticoid balance in the body (1).

Therefore, it seems urgent to search for new methods of structural modification in order to influence the biotransportation of these substances and prevent adverse pharmacological side effects. The conjugation of glucocorticoids with polymers is particularly relevant because macromolecules are transported into the cell by endocytosis rather than by diffusion (2). This diffusion limitation accounted for the inability of polymer analogs of steroid hormones to penetrate target cells freely (3,4).

Polymer conjugates bearing glucocorticoid residues covalently linked to a polymer-carrier by a hydrolytically labile bond (carbonate, ester etc.) and capable of a sustained release of the steroid under physiological conditions have recently received much attention (5–7) in attempts to reduce negative side effects of topical corticosteroids. On the other hand, a weakened systemic toxicity reported for water-soluble copolymers of glucocorticoid 21-maleates with VP was, at least in part, due to the inability of the bound steroid to enter the cell (8).

In a previous paper (9), we communicated the synthesis of binary copolymers of VP with glucocorticoid 21-crotonates which exhibited a much higher hydrolytic resistance in comparison with their maleic congeners (8). The water solubility of these more stable conjugates was limited to the steroid content of 3–5 mol% (9). The copolymers were shown to have a high degree of alternation of VP and steroid units with no adjacent steroid units (10) which might be favorable for binding of the modified hormones to target cells.

In this work, we attempted to obtain ionogenic tertiary copolymers of glucocorticoid 21-crotonates exhibiting a higher solubility in aqueous solutions (thus capable of a higher specific activity) than corresponding non-ionogenic copolymers. In animal models, we also assessed how the incorporation of glucocorticoids into polymer structure affected their anti-inflammatory, antishock, catabolic and immunosuppressive properties.

MATERIALS AND METHODS

Reagents and Solvents. Cortisol and dexamethasone (analytical grade) were purchased from Sigma Chemical Co. (St. Louis, MO). CC (pure) was obtained from Merck and Co. (West Point, PA). HPLC grade VP, AIBN and organic solvents were obtained by additional purification of the appropriate commercial substances (pure or analytical grade) by standard procedures (9,10). DEAEC was prepared as described elsewhere (11). Other chemicals (all analytical grade) were used as received from suppliers. Distilled water was used without further purification.

Apparatus. Preparative and analytical size-exclusion chromatography of the copolymers was performed with equipment supplied by Pharmacia (Uppsula, Sweden) and Waters Div. Millipore Corp. (Milford, MA). UV-spectra were recorded on a Specord M-40 spectrophotometer. ^{1}H-NMR spectra were recorded at 200 MHz on an AC-200 Bruker instrument in $CDCl_3$ solutions. The intrinsic viscosity of polymers was measured in an Ubellode viscosimeter in methanol at 25.0 ± 0.5 °C unless otherwise specified.

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ABBREVIATIONS: AIBN, 2,2′-azobis(isobutyronitrile); CA, crotonic acid; CC, cortisol 21-crotonate; DC, dexamethasone 21-crotonate; DEAEC, 2-(diethylamino)ethylcrotonate; VP, 1-vinyl-2-pyrrolidone.

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Copolymer Synthesis and Characterization. Non-ionogenic binary copolymers of VP with CC and DC were synthesized as reported previously (9). Tertiary copolymers were synthesized analogously by radical copolymerization of the monomer mixtures of VP-CC-CA, VP-CC-DEAEC, VP-DC-CA and VP-DC-DEAEC. The presence of glucocorticoid units in copolymers was confirmed by IR and UV spectroscopy (8, 12). The steroid content in binary and tertiary copolymers was determined by UV spectroscopy (12), ^{1}H-NMR and elemental analysis (11). The content of ionogenic groups in terpolymers was determined titrimetrically and by ^{1}H-NMR spectroscopy (11). The viscosity-average molecular weight of the polymers was estimated by using the parameters of the Mark-Kuhn-Houwink equation for poly(VP) (10, 13). The molecular-weight distribution was estimated by size-exclusion chromatography (14) after complete removal of steroid moieties from copolymers in alkaline conditions (11). In solubility tests, amorphous powders of the tertiary copolymers were stirred in water, sodium hydroxide or acetic acid aqueous solutions (equimolar to respective ionogenic units) at 25°C until the absorbance at 254 nm stabilized. A copolymer was considered water-soluble if the final absorbance accounted for no less than 95% of the steroid content in the copolymer. Hydrolytical stability of copolymers was determined by measuring the rate of abstraction of the steroid from the polymer in aqueous solution, at pH 5.2–9.3 and T = 23 ± 3 °C, using analytical size-exclusion chromatography with photometric detection (254 nm) (9).

Pharmacological Evaluation. Pharmacological investigation of the copolymers was carried out using white non-pedigreed male mice weighing 20–23 g and white non-pedigreed rats of both sexes at an age of 2–4 weeks. In experiments on young animals, groups of 10–12 individual rats were used. The mass and linear dimensions of animals in each group did not differ by more than 2%. Aqueous solutions of the investigated copolymers were administered to animals interperitoneally in doses of 10 and 0.2 mg/kg for cortisol and dexamethasone derivatives, respectively, based on the steroid content in the copolymer. Water was administered to animals from each litter to serve as a control. Experimental results were statistically analyzed using the Student's t-test. The significance of values with respect to a control was designated as p_c and with respect to the effect of initial steroid as p_s.

Anti-Inflammatory Activity. The anti-inflammatory activity of the polymers was evaluated by the usual procedures of the "formalin edema" and "cotton wool granuloma" (15) which served as models for exudative and proliferative phases of inflammation.

In acute experiments, 0.02 ml 3% formalin solution was injected under aponeurosis of a low extremity of mice. 0.1 ml steroid suspensions or copolymer solutions were administered before the beginning of the experiment, at the time of formalin injection and two more times at an interval of 1 hour each. After 3 hours the animals were euthanized by decapitation. Lower extremities were cut in the region of the ankle and weighed on a torsion balance. The magnitude of the edema was expressed as a mass percent of the inflammated extremity with respect to a control extremity.

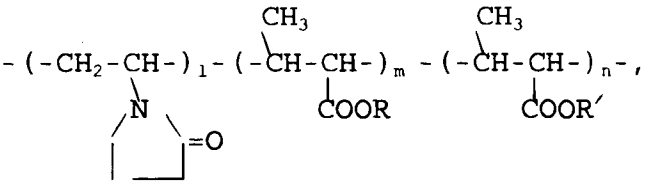
In chronic experiments, a dissection between shoulder-blades of rats at an age of 14 days was made in the sterile conditions. A ball of cotton wool weighing 20 ± 1 mg and soaked in 3% formalin solution was introduced in the cavity. Steroids and copolymers (0.2 ml) were administered once a day for 14 days in the morning hours. After 14 days the animals were decapitated and the granuloma formed was removed and weighed on a torsion balance.

Antishock Activity. The antishock activity was checked using the "declamping shock" model (16). The shock conditions in mice and rats were created by putting a tight elastic tourniquet on the femoral part of lower extremities. The tourniquets were left for 4 hours. The copolymer samples were administered 2 hours after applying the tourniquet and then immediately after its removal. The magnitude of lethality was surveyed over time. The average life-time of an animal after the tourniquet was removed was evaluated.

Systemic Toxicity. The growth rate of the animals was determined by Schmalgausen's method (17). The degree of thymus hypotrophy was also evaluated (15).

RESULTS

Copolymer Synthesis and Structure. Water-soluble polymer derivatives of glucocorticoids were synthesized by radical copolymerization of the appropriate steroid crotonic esters with VP (Table 1). The admixture of the initial low molecular weight steroid in the copolymers did not exceed 0.1 mass percent. The structures of the copolymers obtained are shown in Table 1 and in the following scheme:



where R – 21-steroid residue,
R′ – H (CC) or $CH_2CH_2N(C_2H_5)_2$ (DEAEC);
l = 56–99.8, m = 0.2–14, n = 0–30 mol %

The values of the polymer composition determined by different methods were in good agreement (Table 2).

The viscosity-average molecular weight of terpolymers depended on the conditions of copolymerization and varied in the range from 9 to 40 kDa corresponding to their intrinsic viscosity (Table 1). An analogous dependence was found earlier for binary copolymers (9). The polydispersity of both the binary and tertiary copolymers with respect to molecular weight is low (M_w/M_n = 1.5–2.1) and decreases with increasing molar fraction of each crotonic monomer in the initial reaction mixture. This also results in a decrease of the weight-average molecular weight of the copolymers (Table 3). The molecular weight and molecular weight distribution of the copolymers in the course of copolymerization remain almost unchanged until high degrees of conversion (not shown).

Water-Solubility. Tertiary copolymers containing at least 5 mol% of an ionogenic comonomer are soluble in water at a steroid content up to 5–7 mol% in anionogenic copolymers and up to 11 mol% in cationogenic copolymers. CA copolymers

Table 1. The Conditions of Synthesis and Structures of Water-Soluble Copolymers of VP with Steroid and Ionogenic Comonomers

Conditions of Synthesis								Copolymer Structure					
								Composition					
Crotonic monomers[a]		Monomer feed composition (mol %)[b]						mol %[d]			mass %[e]		
M_2	M_3	f_1^0	f_2^0	f_3^0	Solvent	$[M]_0$[c] mol/l	Yield in 18 h (%)	F_1	F_2	F_3	M_2	$[\eta]$[f] dl/g	M_v[g] $\times 10^{-3}$
CC	—	95.1	4.9	—	2-Propanol	1.9	88	95.4	4.6	—	13.3	0.20	30
DC	—	95.2	4.8	—	2-Propanol	1.9	80	95.7	4.3	—	13.4	0.20	30
CC	DEAEC	65.0	5.0	30.0	DMF	1.0	41	80.6	10.5	8.9	25.1	0.12	14
DC	DEAEC	84.6	4.7	10.7	2-Propanol	2.0	65	89.7	5.8	4.5	17.0	0.13	17
CC	CA	82.6	11.5	5.9	Ethanol	1.0	72	83.8	10.9	5.3	27.7	0.08[h]	9
DC	CA	81.3	11.3	7.4	Benzene	3.6[i]	91	84.6	10.5	4.9	28.1	0.30[j]	40

[a]M_2—steroid monomer, M_3—ionogenic monomer.
[b]f_1^0, f_2^0 and f_3^0—mol.% VP, M_2 and M_3, respectively.
[c]Monomer mixtures also contained 1.0 mol. % AIBN; T = 65 °C.
[d]F_1, F_2 and F_3—mol.% VP, M_2 and M_3, respectively.
[e]Content of hormone residues.
[f]Intrinsic viscosity in methanol at 25°C.
[g]Viscosity-average molecular weight.
[h]DMF, 25 °C.
[i]0.7 mol. % AIBN.
[j]Ethanol, 21 °C.

Table 2. The Composition of CC Copolymers as Determined by Different Methods

Copolymer M_1-M_2-M_3	Copolymer Composition (mol %)[a]		
	F_1	F_2	F_3
VP-CC	86.2 ± 0.3[b]	13.8 ± 0.3[c]	—
	87 ± 1[d]	13 ± 1[b]	—
VP-CC-CA	72 ± 2[b]	6.0 ± 0.1[c]	22 ± 2[e]
	70 ± 4[f]	5.0 ± 0.5[f]	25 ± 3[f]

[a]The mean values with ± SD (n = 3) are given.
[b]F_1 = 100%—F_2—F_3, F_2 = 100%—F_1.
[c]Spectrophotometry.
[d]Element analysis (N).
[e]Titrimetry.
[f]NMR.

Table 3. The Influence of the Composition of Initial Monomer Feed on Weight-Average Molecular Weight M_w and Polydispersity M_w/M_n of Copolymers of VP (M_1) and CC (M_2)

M_3	f_1^0	f_2^0	f_3^0	Conversion (%)	$M_w \times 10^{-3}$	M_w/M_n
—	0.95	0.05	—	32	27	2.11
—	0.80	0.20	—	26	13	1.84
DEAEC	0.65	0.05	0.30	28	17	1.55
CA	0.65	0.05	0.30	30	15	1.64

Note: Conditions of synthesis: $[M]_0$ = 1.0 mol/l in DMF, 1.0 mol% AIBN, T = 65 °C.

containing up to 25 mol% of CA and up to 14 mol% of steroid units are water-soluble when converted to their sodium salt. The solubility threshold of DEAEC terpolymers remains unchanged upon conversion of the terpolymers to their salt form.

Hydrolytic Stability. The data on stability of copolymers VP-CC-CA and VP-CC-DEAEC in aqueous solutions at different pH are shown in Figure 1. The profiles of cortisol release from a binary copolymer VP-CC (dashed lines) are provided for comparison.

All of the copolymers showed maximum hydrolytic resistance in the pH range 5.2–7.3. In alkaline conditions, the copolymers are relatively unstable (Figure 1). Steroid was released from the copolymers completely in a 1 M sodium hydroxide solution at elevated temperature (not shown). At pH 5.2–7.3, ionogenic copolymers exhibited a slightly lower rate of steroid release (1–5%/year) than a non-ionogenic copolymer (7–15%/year). However, no significant effect of the sign of macromole-

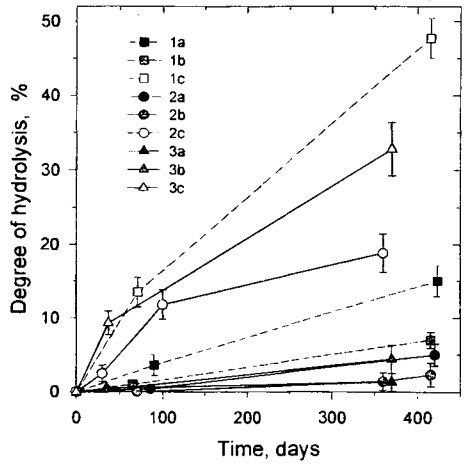


Fig. 1. The effect of pH and the copolymer structure on hydrolytic stability of cortisol polymer derivatives. 0.1% solutions of copolymers of VP-CC 95:5 (1), VP-CC-CA 72:6:22 (2) and VP-CC-DEAEC 85:6:9 (3) in a 0.01 M sodium acetate buffer (pH = 5.2) (a), 0.01 M sodium phosphate buffer (pH = 7.3) (b) and 0.05 M sodium tetraborate buffer (pH = 9.3) (c) were assayed over time for cortisol release at T = 23°C. The mean values (n = 3) are given with error bars (± SD) shown where exceed symbol size.

Table 4. Anti-Inflammatory, Catabolic, and Immunosuppressive Activity of Polymer Analogs of Glucocorticoids[a]

Substance	Copolymer composition (mol %) M_1	M_2	M_3	$M^b \times 10^{-3}$	"Formalin edema" (% to control)	"Cotton wool granuloma" (mg)	Rate of mass increase (%)	Rate of body length increase (%)	Thymus mass (mg)
Control	—	—	—	—	100 ± 12	139 ± 9	109 ± 9	136 ± 6	157 ± 7
Dexamethasone	—	—	—	0.39	75 ± 8	90 ± 7	60 ± 4	45 ± 3	39 ± 2
VP-DC	95.7	4.3	—	30	78 ± 10	76 ± 5	99 ± 5	126 ± 5	133 ± 5
VP-DC-DEAEC	89.7	5.8	4.5	17	70 ± 7	83 ± 6	104 ± 5	132 ± 6	109 ± 6
VP-DC-CA	89.4	5.5	5.1	19	75 ± 8	97 ± 6	102 ± 7	130 ± 6	110 ± 5
Cortisol	—	—	—	0.36	71 ± 8	90 ± 6	60 ± 3	45 ± 4	39 ± 3
VP-CC	95.3	4.7	—	9	56 ± 10	51 ± 5	98 ± 5	129 ± 5	69 ± 3

[a]The mean values with ± SD (n = 10–12) are given.
[b]Formula weight and viscosity-average molecular weight are specified for steroids and copolymers, respectively.

cule charge on the stability of the polymer-steroid ester bond was observed (Figure 1). The rate of cortisol release represented by a semilogarithmic plot shows a linear dependence in the early stages of hydrolysis:

$$\ln \frac{[\text{polymer-bound steroid}]_0}{[\text{polymer-bound steroid}]_0 - [\text{released steroid}]} = k_h t$$

An apparent first-order rate constant of hydrolysis k_h ranges from 10^{-10} to 10^{-8} s^{-1} at pH 5.2–9.3 and room temperature. The average activation energy of the reaction in the temperature range 23–75 °C and pH range 5.2–9.3 varies from 60 to 100 kJ/mol (data not shown).

PHARMACOLOGY

In the model of formalin-induced mouse paw edema, the polymer derivatives of steroids suppressed the exudative phase of inflammation, decreasing the edema by 22–44% ($p_c < 0.001$, $p_s > 0.05$). Their anti-inflammatory activity in this model did not differ from the effect of free steroids, which decreased the edema by 25–29% with respect to control ($p_c < 0.001$) (Table 4). In the model of "cotton wool granuloma", non-bound glucocorticoids suppressed the inflammatory reaction by 35% ($p_c < 0.001$) (Table 4). The activity of dexamethasone polymer derivatives was comparable to that of free steroids. They suppressed the proliferative reaction to a foreign body by 30–45% compared to a control ($p_c < 0.001$, $p_s > 0.05$). The polymer analog of cortisol suppressed the proliferation by 63% with respect to a control ($p_c < 0.001$, $p_s < 0.05$), a value 1.8 times stronger than that for cortisol (Table 4). In both models, the anti-inflammatory activity of ionogenic copolymers of DC did not differ statistically from that of a non-ionogenic copolymer VP-DC (Table 4).

The data on antishock activity of some of the copolymers tested using the "declamping shock" model are shown in Figure 2. In control tests, all animals died. The average life-time after removing the tourniquet was 20 hrs. Dexamethasone and cortisol administered in the equivalent doses increased the average life-time of animals by 1.5–2 times ($p_c < 0.001$), to 38 and 28 hrs, respectively. They decreased lethality by 5–10% ($p_c < 0.05$). The polymer conjugates of these hormones decreased lethality by 50–55% ($p_c < 0.001$, $p_c < 0.001$) and increased the average life-time of animals by more than three times ($p_c < 0.001$, $p_s < 0.001$), to 66 and 67 hrs, respectively.

The results concerning the catabolic and immunosuppressive activity of copolymers are shown in Table 4. After a two week administration of cortisol or dexamethasone, the rate of mass increase dropped by 45% ($p_c < 0.001$) and that of body length increase by 67% ($p_c < 0.001$) as compared to control values. Unlike the non-bound steroids ($p_s < 0.001$), none of the investigated polymers influenced the physical development of young animals ($p_c > 0.05$). The copolymers caused a 15–56% thymus hypotrophy ($p_c < 0.001$) whereas free hormones suppressed thymus mass by 75% ($p_c < 0.001$) compared to a control. Thymus involution by ionogenic copolymers of DC was by 18% stronger than by a copolymer VP-DC.

DISCUSSION

Water-soluble binary non-ionogenic and tertiary ionogenic copolymers of CC and DC were prepared by radical copolymer-

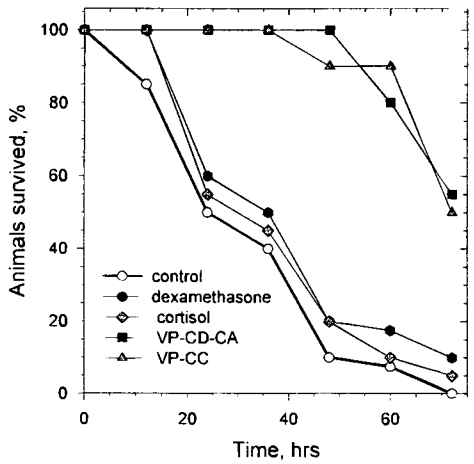


Fig. 2. Antishock activity of glucocorticoid polymer analogs in the "declamping" shock model. Copolymer VP-CD-CA (89.4:5.5:5.1) with viscosity-average molecular weight $M_v \approx 19$ kDa and copolymer VP-CC (95.3:4.7) with $M_v \approx 9$ kDa were administered as described under Materials and Methods. 40 mice were used in a control group or when dexamethasone was administered. 20 mice were used in other groups each. The percent of animals survived after tourniquet removal was surveyed over time.

ization of appropriate crotonic monomers with VP. Their structures were tentatively analyzed with respect to composition and molecular-weight characteristics. The structure of the steroid monomers (9) and the complete removal of the steroid groups from polymers at high pH suggest that all steroid fragments are incorporated into polymers via crotonic moieties esterified in position 21 of the glucocorticoid molecule.

The ionization of macromolecules of terpolymers in aqueous solution results in a much higher solubility threshold of ionogenic polymers (11–14 mol.% steroid groups) in comparison with non-ionogenic copolymers (9). We limited the study of hydrolytic stability of terpolymers to cortisol derivatives as a lesser hydrolytic resistance was reported (5) for polymer derivatives of cortisol than for dexamethasone conjugates of similar structure. A high hydrolytic resistance ($k_h \approx 10^{-10}$ s^{-1}) of both binary and tertiary copolymers at pH 5.2–7.3 and room temperature suggests considerable shielding of the ester bonds in the steroid monomer units by hydrophobic fragments of the polymer chain and by scissed steroid molecules. This, in part, is supported by a decrease in intramolecular mobility of copolymer chains in aqueous solutions (unlike methanol) with increasing steroid content in the copolymers (9). This is also accompanied by an increase in the relative compactness of the macromolecules (9). These data suggest strong hydrophobic interactions between steroid groups. It is possible that there is shielding of hydrolyzable bonds in steroid units by adjacent VP units (5). From the hydrolytic stability data one can expect that, under physiological conditions, the modified glucocorticoid hormones can remain polymer-linked in the process of their delivery and interaction with target cells.

In animal models, the investigated copolymers exhibit a pronounced anti-inflammatory activity comparable to that of glucocorticoid preparations. In addition, the copolymers showed a considerably higher antishock effect in comparison with that of initial steroids. These results may be due to the interaction between the modified hormones and their receptors localized in the plasma membrane of target cells. Particularly, the cortisol-specific binding sites were detected on the outer surface of plasma membrane of thymocytes (18). Moreover, linking of the steroid to the polymer backbone can result in a high local steroid concentration. This concentration may contribute to the observed high specific activity of the modified hormones.

On the other hand, the systemic toxicity typical of glucocorticoid preparations is absent or strongly weakened for polymer esters of these hormones. One possible reason for this observed change in the spectrum of glucocorticoid action may be reduced uptake of hormone-polymer conjugates, since polymer transportation across the plasma membrane is hindered. Indeed, excessive glucocorticoids are believed to exert negative side effects largely by a mechanism involving cell penetration (1).

In conclusion, glucocorticoids linked to a hydrophilic polymer at position 21 through hydrolytically resistant ester bonds retain some of their specific physiological effects. The sharp concomitant reduction of undesirable (hormonal) side effects may be due to the separation of regulatory functions of these substances at plasma membrane and subcellular levels.

The copolymers obtained are of interest as an instrument for locating glucocorticoid receptors in the body and for studying the molecular mechanism of glucocorticoid hormone action. Analogous polymer derivatives having optimal molecular weight characteristics can also be used for the development of anti-inflammatory and antishock glucocorticoid preparations with improved pharmacological properties.

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