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Syntheses and Characterization of Polymeric Derivatives of the Antitumor Agent Methotrexate

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SUMMARY:

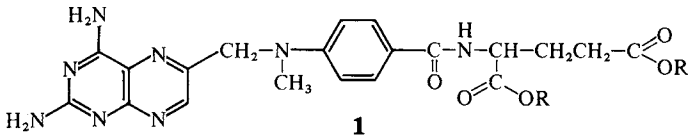
Synthesis and characterization of polymeric derivatives of the folic acid antagonist, Methotrexate, *N*-[4-(*N*-methyl-2,4-diamino-6-pteridinylmethylamino)benzoyl]glutamic acid (**1a**), a widely used antitumor agent, are described. Derivatives of poly(L-lysine), poly(iminoethylene), poly(vinyl alcohol), and carboxymethylcellulose with various contents (3–15 mole-%) of substituted repeating units were prepared by polymer-analogous amidation and transesterification of the dimethyl ester of **1a** and with *N*-cyclohexyl-*N'*-[2-(*N*-methylmorpholinio)ethyl]carbodiimide *p*-toluenesulfonate as coupling reagent. By nucleophilic addition reactions of the pteridinyl amino groups under mild conditions, **1a** was bound without cross-linking to divinyl ether-maleic anhydride (DIVEMA) copolymers of various molecular weights. This copolymer appeared to be of particular interest as carrier because of its established antitumor and immune-stimulating activity.—With the exception of the poly(vinyl alcohol) derivative, all polymers containing the **1a** residue are water soluble at the physiological pH range. Besides the dimethyl ester of **1a** used as convenient starting material for polymer-analogous reactions, further dialkyl esters of **1a** were synthesized as low molecular weight model compounds for the study of the pharmacodynamic properties of polymers, substituted with **1a**. The polymeric derivatives of **1a** were purified by membrane filtration, solvent extraction, and reprecipitation; the polymers and the dialkylesters **1b–d** were characterized by thin-layer chromatography, IR, NMR, UV and mass spectra, and elemental analysis. Preliminary results of the pharmacological studies with the DIVEMA derivatives of **1a** are also reported.

Introduction

Methotrexate, *N*-[4-(*N*-methyl-2,4-diamino-6-pteridinylmethyl amino)benzoyl]glutamic acid (**1a**), the folic acid antagonist, has been in clinical use for the treatment of various malignant as well as nonneoplastic diseases for many years[1–3]. Considerable beneficial effects are achieved with **1a** either as a single agent or in combination with other drugs, e.g. in the treatment of choriocarcinoma[4], acute childhood leukemia[5], Burkitt's Lymphoma[6], and breast cancer[7]. However, despite its wide range of clinical application, serious problems are associated with its use. Toxicity to normal tissues, particularly to bone marrow and small intestine[8], is often life threatening. Development of resistance after initial use is frequently seen[9]. **1a** shows

*) Part 16: H. Ringsdorf "Polymeric Delivery Systems" in Midland Macromolecular Monographs, B. Kostelnik, Gordon and Breach, New York, N. Y. 1978, Vol. 5.

unsatisfactory activity in animals against several solid tumors, such as Lewis Lung Carcinoma[10]. It also exhibits severe immunosuppressive effects[11], which are typically observed within the class of antimetabolic cytotoxic agents[12]. A large number of low molecular weight, structural analogs of **1a** were synthesized and investigated in recent years[13,14], but for none of these derivatives was the therapeutic index (relative antitumor action to toxic action) superior to the parent drug. The selectivity of the action of **1a** appears in large part to be due to the transport characteristics of normal sensitive tissues and tumors relative to each other[15].



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	1a	**b**	**c**	**d**
R	H	CH_3	C_2H_5	$CH_2CH_2CH_3$

As a part of the program on the synthesis and biological properties of polymeric antitumor agents[16-19], we have been interest in the preparation of polymeric derivatives of **1a** and their comparative pharmacologic properties. Alterations of the pharmacodynamic behaviour of **1a** linked to a macromolecular carrier, e.g. by a different body distribution and slow release of the drug, might be advantageous in view of the rapid excretion of **1a** after single dose administration to experimental animals and man[20,21]. A typical depot effect and prolonged plasma concentration was demonstrated recently by Chu and Whiteley for **1a** linked to albumin and dextran derivatives[22]. Coupling reactions of the glutamic acid moiety of **1a** to nucleophilic groups of natural polypeptides can yield only relatively small amounts of bound drug[23]. We have attempted to bind **1a** to synthetic carriers in higher yields and still provide solubility at physiological pH ranges for the resulting polymers. In this paper, we describe the fixation of **1a** to modified cellulose, poly(vinyl alcohol), poly(L-lysine), and linear poly(iminoethylene)[24], using carbodiimide-promoted coupling procedures, transesterification, and amidation reactions of dialkyl esters of **1a**. By a direct polymer-analogous addition reaction, **1a** was linked to a copolymer from divinyl ether and maleic anhydride (DIVEMA)[25], which itself shows activity against a variety of tumors and was approved for phase 1 clinical evaluation[26,27]. Immune-stimulating activity has also been demonstrated for DIVEMA[28,29]. By combining **1a** with this copolymer it was hoped to provide a synergism of antitumor activity coupled with maximal immune stimulation.

Of particular interest to us is the possible effect on the cellular uptake of polymer-linked **1a**. Polymeric derivatives of **1a** not amenable to spontaneous (hydrolytic) release of the drug may then be taken up into the cell only by endocytosis[30], whereas an active, carrier-mediated transport into cells has been shown for the free drug[31]. The concept of lysosomotropic agents[32,16] has been demonstrated with a DNA-Adryamicin complex as a first example which has reached the stage of clinical evaluation[32,33]. For polymer-linked derivatives of **1a**, uptake into tumor cells by endocytosis and degradation by lysosomal enzymes might lead to a more selective action with a decreased toxic effect on normal organs. These considerations together with the above mentioned possibility to combine a polymeric carrier having an immume stimulating

effect (e.g. DIVEMA) with usually immunosuppressive antitumor agents (e.g. **1a**) served as guideline for the design of the polymers described in this paper.

In addition, synthetic polymeric carriers might offer one advantage over endogenous macromolecules, such as DNA or proteins, because of the greater possibility for control of reproducibility and structural variation. With biological polymers, slight structural differences may already lead to a dramatic change of the biological effect. Such effects may then be difficult to assess in chemotherapeutic experiments, as was indicated recently in ongoing pharmacologic studies with DNA-adriamycin complexes[33].

Results and Discussion

Diester derivatives of **1a** *as starting materials for polymer-analogous syntheses*

Carbodiimide-promoted coupling reactions were initially considered as a primary possibility for the fixation of the α- or γ-glutamic acid moiety of **1a** to nucleophilic groups of polymers. Because of the poor solubility of **1a** in non polar organic solvents and the problems encountered with carbodiimide reagents used in more polar solvents, N-cyclohexyl-N′-[2-(N-methylmorpholinio)ethyl]carbodiimide *p*-toluenesulfonate as a water-soluble agent was used, which, however, has an optimal reactivity at acidic pH, where the solubility of **1a** is limited[34]. As an alternative, polymer-analogous transesterification and amidation of ester derivatives of **1a** appeared appropriate. Syntheses of dialkyl esters of **1a**, as fluoroborate complexes, and their properties as substrates for aldehyde oxidase and dihydrofolate reductase were recently described by Johns et al.[13]. Derivatives in which **1a** was esterified at the glutamic acid moiety seemed further to be appropriate as low molecular weight model compounds for the pharmacodynamic behaviour of polymeric derivatives of **1a**. Among different methods, the esterification of **1a** in aqueous HCl proved to be a convenient procedure. The trihydrochlorides of the dialkyl esters **1b**, **1c**, and **1d** were obtained in high yields, and were used preferentially for the syntheses of polymeric derivatives because they are much better soluble and more stable than the corresponding free esters.

1b–d were characterized by elemental analysis, IR and NMR spectroscopy, and by field desorption (FD) mass spectrometry. Small abundances due to cluster molecular ions[35] with one and two molecules of HCl were observed in the FD mass spectra of the hydrochlorides, whereas most intense "quasimolecular ions", MH^+, were found for all compounds (s. Exptl. Part).

*N-Acylation of poly(*L*-lysine) and poly(iminoethylene) with* **1a** *or* **1b**

Previous preparation procedures with serum albumins used as polymeric carriers demonstrated the possibility of binding the glutamyl moiety of **1a** via amide bonds to amino groups of poly(α-amino acid)s[22]. However, the drug content in albumin derivatives is limited by the total number of ε-amino groups in these polymers. Even in the presence of excess carbodiimide as coupling reagent, only about 80 mg/g polymer can be bound[22]. We, therefore, used poly(L-lysine), a biodegradable poly(α-amino acid) derivative with a molecular weight of approximately

70000, which was hoped to provide the possibility of binding **1a** in amounts sufficient for pharmacokinetic studies, in a still tolerable dose range*).

For the fixation of **1a**, both the carbodiimide-coupling and the polymer-analogous amidation of the dimethylester **1b** were attempted. The latter reaction gave slightly higher yields and presented less difficulties in the purification of the coupling product. With a mole ratio of repeating units of poly(L-lysine)/**1b** = 0,2–0,35, water-soluble polymers **2** containing 12 mole-% of substituted units were obtained (s. Exptl. Part), whereas higher mole ratios led to considerable amounts of insoluble material. After extensive dialysis of **2** no free **1a** or **1b** could be detected by thin-layer chromatography. Besides the polymer at the origin of the chromatogram only traces of unknown material were detectable. However, low molecular weight impurities presumably derived from the coupling reagent were observed when the carbodiimide was used for the fixation of **1a**. The polymer was characterized by IR and UV spectroscopy. The comparison of the UV spectra of **1b** with **2** in 0,1 M HCl (Fig. 1) showed identical absorption maxima with λ_{max} at 243, 307, and 350 nm (shoulder)[13]. No interfering absorption from the polymer was observed in reference spectra at the maximum at 307 nm which was used for the quantitative determination of bound drug (s. Exptl. Part).

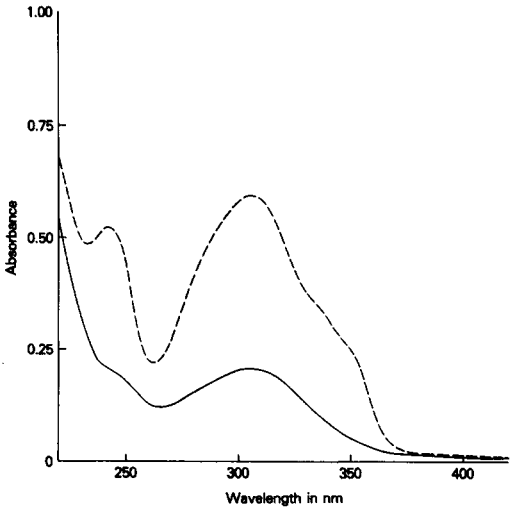


Fig. 1. UV Absorption spectra of **1b** (dashed line) and of **2** (solid line) in 0,1 M HCl

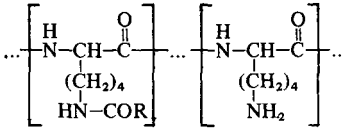
As a further polymeric carrier appropriate for the fixation of **1a** by (glutamic) amide groups, poly(iminoethylene) was of particular interest due to its own potential cytotoxic properties[36]. For this reaction, a recently synthesized linear poly(iminoethylene) of relatively low molecular weight[24] was available. A derivative with **1a** linked to this polymer by non-cleavable, tertiary

*) Expressed in equivalent amounts of polymeric derivatives of **1a**, referring to the quantity residues of **1a** in the polymers. Concentrations of **1a** residues in these polymers are given in mole-% which means:

$$\frac{100 \times [\text{Substituted repeating units of poly(L-lysine)}]}{[\text{Repeating units of unsubstituted poly(L-lysine)}]}$$

amide groups was hoped to be a useful model to show an eventual direct interaction with dihydrofolate reductase, the target enzyme for the action of **1a**[3], although its low molecular weight might critical to effect cellular uptake by endocytosis. Moreover, the high reactivity of linear poly(iminoethylene) provides various possibilities to modify its toxicity by polymer-analogous derivatization as could be shown in preliminary experiments[37].

Due to the high solubility of the polymer in acidic aqueous medium, the reaction with the water soluble *N*-cyclohexyl-*N'*-[2-(*N*-methylmorpholinio)ethyl]carbodiimide *p*-toluenesulfonate proved to be convenient for the fixation of **1a**.



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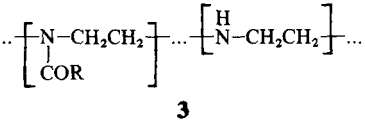

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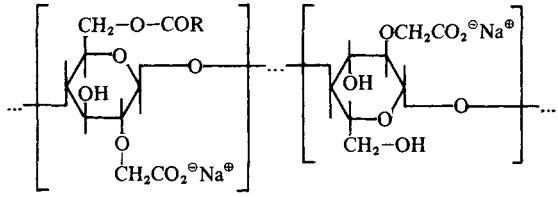

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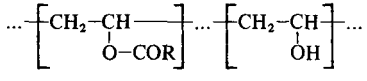

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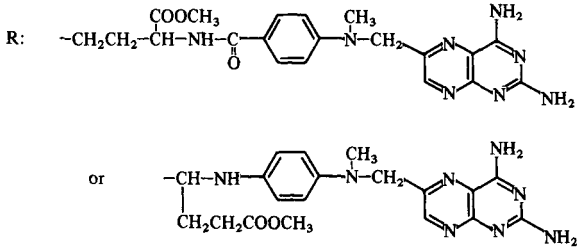


After purification by dialysis against a membrane with an exclusion size of ca. 1000, polymer **3** was examined by thin-layer chromatography and did not contain free **1a**. It was further characterized by IR and UV spectra. The content of substituted repeating units in **3** was determined spectroscopically to be 13 mole-% using λ_{max} at 307 nm (0,1 M HCl). Due to the presence of the remaining free carboxyl groups of **1a** which lead to a partial "zwitterion" structure of the polymer, solubility in various physiologic neutral and slightly basic buffer solutions was found, whereas poly(iminoethylene) is insoluble in aqueous medium at pH > 6. No appreciable aggregation due to cross-linking occurred during the polymer-analogous reaction, which might be explained by a steric effect preventing a bifunctional reaction of the glutamyl moiety of **1a**.

O-Acylation of carboxymethylcellulose and poly(vinyl alcohol) with **1b**

The polymer-fication of **1a** as (glutamyl)-ester could provide a depot effect and prolonged plasma concentration of the drug, both in systematic and eventually in their direct application in localized tumor tissues. Previous studies on the degradation and metabolism of polymer-linked drugs have shown that ester groups attached to various biostable polymer chains are amenable to enzymatic hydrolysis[38]. To study the pharmacodynamic effect of esterification of **1a** with nondegradable macromolecules, poly(vinyl alcohol) and a partially carboxymethylated (30%) cellulose derivative were used as carriers.

Only small amounts of **1a** could be attached to poly(vinyl alcohol) without crosslinking, by transesterification of **1b**. The reaction product **5** contained approximately 3 mole-% of substituted repeating units and showed poor solubility in basic aqueous solution. Considerable amounts of insoluble material were formed when higher amounts of **1b** and prolonged reaction times were used to increase the content of bound drug.

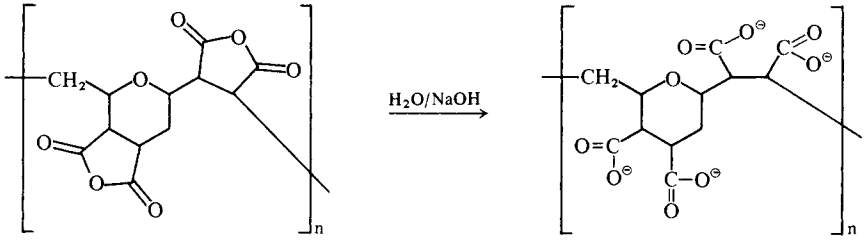
Various preparation procedures were recently applied to the acid-catalyzed transesterification of carboxymethylcellulose[39]. For the fixation of **1a** to this polymer, the transesterification of the trihydrochloride of **1b** in water/DMF at 50°C with trifluoroacetic acid catalysis gave polymer **4** (sample A) which had a UV absorption spectrum identical to that of **1b** and a content of substituted repeating units of 12,9 mole-% (UV, at $\lambda_{max} = 303$ nm). A slightly higher content (14,8 mole-%) was achieved for sample B when the esterification was carried out in a DMF suspension at 60°C. However, attempts failed to obtain water-soluble polymers with higher contents of substituted repeating units using elevated temperatures (100°C) and longer reaction times. The resulting polymers were insoluble in water and only slightly soluble in DMF, which might be caused by crosslinking reactions or by partial cleavage of carboxymethyl groups during the reaction. Both samples of polymer **4** were water-soluble at pH 8, but their solubility was somewhat lower than that of unsubstituted carboxymethylcellulose.

Acylation of **1a** *by divinyl ether-maleic anhydride (DIVEMA) copolymers*

Previous pharmacological investigations with DIVEMA copolymers[26,27,29] established that both antitumor activity and immune-potentiating effect are strongly dependant on the molecular weight and the molecular weight distribution of the polymer. Moreover, DIVEMA showed activity against various transplantable tumors in experimental animals, e.g. Lewis Lung tumor and Adenocarcinoma 755[26], which are partially resistant to **1a**. It was, therefore, of interest to bind **1a** to DIVEMA copolymers of different molecular weight ranges and with various contents of the drug, in order to evaluate a possible immune-stimulating or synergistic antitumor effect of the resulting polymers, as discussed in the introduction. For the fixation of **1a**, several molecular weight species of DIVEMA (**6**) were used. Their molecular weight distribution was determined by gel permeation chromatography after the conversion of the anhydride groups to the methyl esters[27,40].

Reaction conditions used for the preparation of samples A–K of the DIVEMA-polymers and the contents of substituted repeating units obtained in different procedures of synthesis and purification are summarized in Tab. 1. Because of the poor solubility of **1a** in aprotic, nonpolar solvents, the reactions were carried out in DMSO and DMF, although the latter solvent was reported to cause hydrolysis of the anhydride groups of the polymer[25]. Alternatively,

a mixture of acetone/DMSO was found to be an appropriate solvent. With reaction times of 24–96 h considerable differences in the contents of substituted units in the products were obtained, ranging from 3 mole-% to more than 40 mole-%. These differences were obviously to be expected because of the varying anhydride contents in the different samples of DIVEMA (s. Tab. 2, Exptl. part) which are hydrolyzed slowly, due to the uptake of water from ambient. On the other hand, spontaneous hydrolysis of **1a** might occur when the poly-anhydride **6** is transferred into the water-soluble poly-carboxylate form **7**, as was indicated by a comparatively low contents of substituted units found for the samples A–F of DIVEMA polymers (Tab. 1) after extensive purification by dialysis in dilute basic medium.



6 **7**

Relatively higher amounts of **1a** could be bound when a sample of DIVEMA containing almost 100% anhydride (sample G) was used and hydrolysis of the poly-anhydride form during the purification was avoided. The samples G–K of DIVEMA partially substituted with **1a** were purified by repetitive reprecipitation and solvent extraction. No free **1a** or other impurities could be detected on thin-layer chromatography. They were further characterized by IR and UV spectroscopy. The IR spectrum of copolymer sample G with a content of substituted units of 25 mole-% (Fig. 2) exhibits intense carbonyl-anhydride and -amide vibrations at 1850, 1775, and 1640 cm^{-1}. The band at 1720 cm^{-1} (free carboxyl) is attributed to the glutamyl moiety of **1a** and to the free carboxyl groups formed during the reaction of the anhydride moieties of DIVEMA (**6**) with the amino groups of **1a**. The UV spectrum of DIVEMA

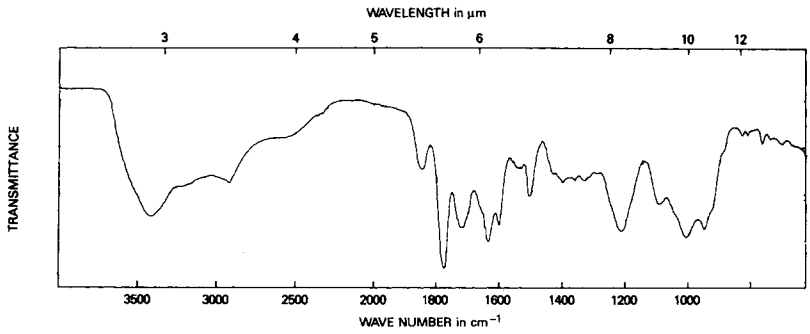


Fig. 2. IR Spectrum of sample G of DIVEMA copolymer partially substituted with **1a**

Tab. 1. Conditions for the acylation of **1a** with DIVEMA copolymers and characterization of the resulting products

Sample no.	Mole ratio DIVEMA: **1a**[a]	Reaction time in h	Solvent	**1a** Residue in the copolymer		Molecular weight[d]	
				content[b] in mole-%	form[c]	$\bar{M}_n$	$\bar{M}_w$
A	3,1 :1	27	DMF	11	C	20000	83500
B	3,46:1	24	DMF	3	C	9100	34300
C	3,37:1	48	DMF	3,5	C	20000	83500
D	3,55:1	24	Acetone/DMSO	3,5	C	9600	18100
E	3,5 :1	24	Acetone/DMSO	4	C	4400	9300
F	3,5 :1	24	DMSO	6,1	C	15500	30000
G[e]	3,1 :1	72	Acetone/DMSO	25,2	A	24000	
H	3,1 :1	72	Acetone/DMSO	20	A	24000	
I	1,9 :1	96	Acetone/DMSO	42,6	A	24000	
K	3,5 :1	72	Acetone/DMSO	16,6	A	15500	30000

[a] Related to the amount of repeating units in DIVEMA.
[b] 100 × [Substituted repeating units of DIVEMA]/[Repeating units of unsubstituted DIVEMA], determined by UV spectroscopy at 303 nm in 0,1 M NaOH.
[c] A = anhydride form; C = carboxylate form.
[d] Determined for the unsubstituted DIVEMA copolymers (cf.[27,40]).
[e] National Institutes of Health Screening test No: NSC 282447.

sample G partially substituted with **1a** (Fig. 3) exhibits the absorption maxima (258, 303, 370 nm, in 0,1 M NaOH) identical with the free drug **1a**. The maximum at 303 nm was used for the spectroscopic determination of contents of substituted units in the DIVEMA-samples.

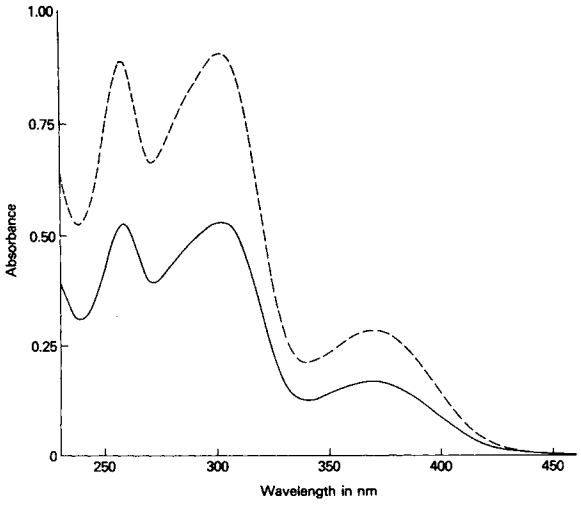


Fig. 3. UV Absorption spectra of **1a** (dashed line) and sample G of DIVEMA copolymer (solid line) in 0,1 M NaOH. Recent investigations indicate, that the polymer contains mainly hydrolyzed **1a**, under these conditions[42]

The question of an eventual spontaneous release of **1a** is of importance with regard to the biological activity of these substituted DIVEMA polymers. The linkage of **1a** to the polymer occurs at the 2- or 4-amino groups of the pteridine ring, most likely by either group because of the little, if any, differences in their chemical reactivity. No crosslinking due to reaction of both amino groups was observed during the preparation of the substituted DIVEMA polymers. Since, on the other hand, the 4-amino group is an essential binding site for the drug to dihydrofolate reductase[14], a loss or reduction of the biological activity of the polymer must be considered unless its degradation to free **1a**. Thus, the conditions for the preparation of the biologically active carboxylate form of substituted DIVEMA might be important because of the pH-dependence which must be expected for the cleavage of the drug from the polymer backbone. For the hydrolytic release of **1a** under basic conditions a neighboring group effect of the carboxylate anions must be considered. This is consistent with the instability of the polymer observed under basic conditions and during purification by dialysis as mentioned above. The spontaneous and possible enzymatic hydrolysis is currently under study within pharamacologic investigations of the substituted DIVEMA polymers.

Preliminary pharmacological results[41]

The DIVEMA copolymer substituted with **1a** (Tab. 1, Sample G) was evaluated for enzyme (dihydrofolate reductase) inhibitory activity in vitro and antitumor activity in vivo. (National Institutes of Health Screening Test No. NSC 282447)). It shows inhibition of dihydrofolate reductase and cytotoxicity to L1210 lymphoid leukemia cells in vitro similar to free **1a**. However, the polymer has significant antitumor activity against L1210 leukemia in mice, with a maximum increase in life span greater than that of free methotrexate **1a** or the combination DIVEMA (**7**) and **1a**. DIVEMA (**7**) alone was ineffective against L1210 leukemia. Long-term survivors (10–30%) are only obtained after treatment with NSC 282447. This polymer also shows activity, in both daily and intermittent treatment schedules, against murine Lewis lung carcinoma which is resistent to free methotrexate (**1a**). In addition to the established immune stimulation by DIVEMA (**7**) pharmacokinetic and toxicity studies with NSC 282447 indicate sustained plasma levels of **1a** was one reason for the potentiated antitumor effect in vivo. Pharmacologic and biochemical studies will be reported in detail elsewhere[42].

Experimental Part

Materials

A sample of Methotrexate (**1a**, NSC 740)*) contained approximately 10% water, which was removed to a great part by extensive drying at 50°C i. vac. over P_2O_5 in the dark.

Poly(L-lysine hydrobromide) with a molecular weight of approximately 70000 was a commercial sample (from Ferak AG, Berlin, West Germany) and also poly(vinyl alcohol) (molecular weight: 72000; from Merck AG, Darmstadt, West Germany). Linear poly(iminoethylene)**) with an estimated molecular weight of 2000–5000[24] was reprecipitated from methanol/acetone prior to use. Water soluble sodium

*) Obtained from the Drug Development Branch, National Cancer Institute, NIH, Bethesda, Maryland, USA.

**) Prepared by Dr. S. Kobayashi, Kyoto University, Japan.

salt of carboxymethylcellulose ($\bar{M}_w$ 300000–350000*) was reprecipitated from H_2O/acetone. All samples of divinyl ether-maleic anhydride (DIVEMA)** copolymers were kept in a vacuum desiccator over $CaCl_2$.

Dimethyl N-[4-(N-methyl-2,4-diamino-6-pteridinylmethylamino)benzoyl]-L-glutamate (**1b**)

A. *Trihydrochloride:* 2,1 g (4,62 mmol) of **1a** were dissolved in 150 ml of 2 M methanolic HCl and heated to 60 °C for 30 min. The reaction mixture was stirred 20 h at room temperature and finally heated again to 60 °C for 30 min. After evaporation of the solvent i. vac., an oily residue was obtained, which was dissolved in 100 ml of 2 M methanolic HCl. The above procedure was repeated twice. The remaining oil was stirred for 2 h in 150 ml of anhydrous methanol, the solvent evaporated, and the semi-crystalline product was suspended twice in 80 ml of acetone, followed by removal of the solvent. A light-brown crystalline powder was obtained after removal of excess HCl by drying 48 h i. vac. over NaOH. Yield: 2,5 g (94%).

IR (KBr): 1685 (s; ν_{co} amide) and 1725–1740 cm^{-1} (s; ν_{co} ester).

NMR [$^2H_6$] DMSO: $\delta = 3{,}60$ and 3,65 (2s; CH_3 of ester). FD-MS: *m/e* 483 (MH^+; 100%), 468 (90), 455 (10), 390 (11), 191 (13), 177 (24).

$C_{22}H_{29}Cl_3N_8O_5$ (591,9)	Calc.	C 44,64	H 4,94	N 18,93
	Found	C 44,79	H 5,12	N 18,82

B. *Free ester* **1b**: 600 mg (1,01 mmol) of the trihydrochloride of **1b** were dissolved in 15 ml freshly distilled DMF at 5 °C and 0,5 ml (5 mmol) of anhydrous triethylamine was added in small portions. The mixture was stirred 20 min and the product precipitated with 50 ml of diethyl ether. The product was filtered, washed extensively with water and small portions of methanol, and dried i. vac. over P_2O_5. Hygroscopic yellow powder; yield: 380 mg (78%).

IR (KBr): 1720 cm^{-1} (s; ν_{co} of ester).

FD-MS: *m/e* 483 (MH^+; 100%), 482 (12).

$C_{22}H_{26}N_8O_5$ (482,5)	Calc.	C 54,76	H 5,43	N 23,22
	Found	C 53,52	H 5,20	N 22,60

Diethyl N-[4-(N-methyl-2,4-diamino-6-pteridinyl-methylamino)benzoyl]-L-glutamate (**1c**)

A. *Trihydrochloride:* 700 mg (1,54 mmol) of **1a** were dissolved in 80 ml of 2 M ethanolic HCl, and the procedure described for **1b** was repeated three times. Final evaporation of the solvent gave an oily residue which was suspended 2 h in 50 ml ethanol at room temperature. After concentration to 20 ml, the product was precipitated in 150 ml of diethyl ether, filtered and suspended in 20 ml of ethanol. The solvent was removed and the product dried 48 h i. vac. over NaOH; yellow, hygroscopic powder. Yield: 780 mg (96%).

IR (KBr): 1720–1740 cm^{-1} (s; ν_{co} of ester).

NMR ([$^2H_6$] DMSO); $\delta = 1{,}2$ (2t; CH_3 of ester) and 4,2 (m; CH_2 of ester).

FD-MS: *m/e* 511 (MH^+; 100%), 510 (37), 483 (71), 455 (55), 326 (21), 325 (22).

$C_{24}H_{33}Cl_3N_8O_5$ (616,9)	Calc.	C 46,50	H 5,37	N 18,08
	Found	C 46,01	H 5,46	N 19,23

B. *Free ester* **1c**: 300 mg (0,48 mmol) of the trihydrochloride of **1c** were dissolved in 10 ml of freshly distilled DMF and treated as described for the preparation of **1b**. Yellow, highly hygroscopic product. Yield: 230 mg (93%).

IR (KBr): 1720 cm^{-1} (s; ν_{co} of ester).

FD-MS: *m/e* 511 (MH^+; 100%), 510 (10).

$C_{24}H_{30}N_8O_5$ (510,6)	Calc.	C 56,45	H 5,93	N 21,95
	Found	C 55,75	H 6,37	N 19,80

*) From Dr. E. Perplies, Kalle/Hoechst AG, Wiesbaden, West Germany.

**) From Dr. D. S. Breslow, Hercules Inc. Wilmington, Delaware, USA.

Trihydrochloride of dipropyl N-[4-(N-methyl-2,4-diamino-6-pteridinylmethylamino)benzoyl]-L-glutamate (**1d**): 1 g (2,2 mmol) of **1a** was dissolved in 140 ml 2 M 1-propanol/HCl, and the procedure described for **1b** was carried out three times. The oily product was suspended two times with 50 ml 1-propanol, concentrated, precipitated in 150 ml diethyl ether and filtered. Removal of excess HCl by 48 h drying over NaOH i. vac. gave a brown-yellow crystalline powder. Yield: 1,2 g (84%).

IR (KBr): 1725 cm^{-1} (ν_{co} of ester).

NMR ([$^2H_6$] DMSO): $\delta = 0{,}85$–1,1 (t; CH_3 of ester) and 3,85–4,2 (m; CH_2 of ester).

FD-MS: *m/e* 611 $(M+2HCl)^+$ (4%), 574 $(M+HCl)^+$ (8), 539 (MH^+) (100), 497 (37), 364 (21), 324 (12), 177 (13).

$C_{26}H_{37}Cl_3N_8O_5$ (648,0)	Calc.	C 48,19	H 5,76	N 17,29
	Found	C 46,74	H 5,90	N 16,54

N-Acylation of poly(L-lysine) with dimethyl ester **1b**: 100 mg (0,52 mmol of repeating units) poly(L-lysine hydrobromide) was dissolved in 8 ml water, then 0,2 ml redistilled triethylamine was added dropwise and the pH of the solution was adjusted to 9. 50 mg (0,11 mmol) trihydrochloride of **1b** was added at 5 °C, and the solution saturated with nitrogen (5 min). The reaction mixture was stirred 20 h under nitrogen at room temperature, and finally heated 2 h to 60 °C. A yellow powder precipitated upon treatment with 50 ml of 0,5 M NaOH at 5 °C. The product was purified by filtration in 3 × 80 ml of 0,05 M HCl against a cellulose membrane (from Berghof, Tubingen, West-Germany, exclusion size approximately 1000). After concentration and precipitation with 1 M NaOH, 65 mg of a yellow polymer were obtained, which did not show free **1a** on thin-layer chromatography; ninhydrine-positive material was detected only at the origin. It was soluble in neutral and acidic aqueous solution, and insoluble at pH 8.

IR (KBr): 1640–1650 cm^{-1} (b) (ν_{co} of amide).

Content of acylated repeating units: 12 mole % (by UV Spectroscopy at 307 nm in 0,1 M HCl).

Found C 36,85 H 5,94 N 13,59.

N-Acylation of poly(iminoethylene) with diacid **1a**: 100 mg (2,2 mmol of repeating units) of linear poly(iminoethylene) were dissolved in 12 ml of water with the pH adjusted to 2 (0,1 M HCl). 200 mg (0,44 mmol) of **1a** were dissolved in 10 ml of water/DMSO (volume ratio 1:1), and the pH of the mixture finally adjusted to 3 (0,1 M HCl). 210 mg (0,5 mmol) of *N*-cyclohexyl-*N'*-[2-(N-methylmorpholinio)ethyl]carbodiimide *p*-toluenesulfonate (from Merck AG, Darmstadt, West Germany) was dissolved in 5 ml of water and added to the solution. The reaction mixture was stirred 24 h at room temperature under nitrogen. The product was precipitated in 150 ml of acetone, redissolved in 0,1 M NaOH and purified by diafiltration (membrane exclusion size 1000, N_2, 3 × 70 ml). The pH of the concentrated solution was then adjusted to 7, and the product precipitated in acetone and dried over P_2O_5 i. vac. 140 mg of a yellow product were obtained, which showed, on thin-layer chromatography, ninhydrine-positive material only at the origin. Small amounts of material with absorption at 254 nm showed mobility but different R_f values than that of free **1a** and are assumed to be oligomers.

IR (KBr): 1640–1660 cm^{-1} (w; ν_{co} of amide).

Content of acylated repeating units: 13 mole-% (by UV Spectroscopy at 307 nm in 0,1 M HCl).

Found C 41,14 H 6,19 H 20,47

Poly(iminoethylene):

Found C 35,06 H 8,13 N 19,28

O-Acylation of poly(vinyl alcohol) with dimethyl ester **1b**: 120 mg (2,73 mmol of repating units) of poly(vinyl alcohol) were suspended in 5 ml of DMSO, and a solution of 500 mg (0,85 mmol) of trihydrochloride of **1b** in 15 ml of DMSO was added dropwise. After addition of 5 µl of trifluoroacetic acid, the reaction mixture was stirred 96 h at room temperature. The product was precipitated in 200 ml of acetone and reprecipitated twice with DMF/acetone. It was then three times extracted for 72 h with 500 ml of acetone in a soxhlet extractor, which gave a yellow polymer free of **1a** and **1b** on thin-layer chromatography. Yield: 140 mg. The polymer is soluble in 0,1 M NaOH and DMSO, and insoluble in water and aqueous acids.

IR (KBr): 1705 cm^{-1} (w; ν_{co} of ester).

Content of acylated repeating units: 4 mole-% (by UV Spectroscopy at 303 nm in 0,1 M NaOH).

Found C 59,03 H 9,28 N 0,63.

Acylation of sodium carboxymethylcellulose with dimethyl ester **1b**

A. *Acylation in water (sample A):* 430 mg (ca. 1,65 mmol of repeating units) of partially (approximately 30%) carboxymethylated cellulose were dissolved in 35 ml of water, and 500 mg (0,84 mmol) of trihydrochloride of **1b** dissolved in 25 ml of water/DMF (volume ratio 1:3) were added. After addition of 50 µl of trifluoroacetic acid, the reaction mixture was stirred 96 h at 50°C. The product was precipitated in 300 ml of acetone and extracted 24 h with 2 × 200 ml of methanol in a soxhlet. Diafiltration in 0,02 M NaOH (2 × 300 ml, membrane exclusion size 10000) and lyophilization gave a final yield of 380 mg. The yellow polymer showed a small amount of UV-fluorescent, mobile material on thin-layer chromatography, but no free **1a** or **1b** was detectable by UV (254 nm) and ninhydrine reaction.

Content of acylated repeating units: 12,9 mole-% (by UV Spectroscopy at 303 nm in 0,1 M NaOH).
Found C 38,98 H 5,32 N 3,13

Sodium carboxymethylcellulose: Found C 33,55 H 5,18

B. *Acylation in DMF suspension (sample B):* 1,0 g (ca. 4,1 mmol of repeating units) of partially (approximately 30%) carboxymethylated cellulose were suspended in 10 ml of freshly distilled DMF. A suspension of 500 mg (0,84 mmol) of trihydrochloride of **1b** in 10 ml of DMF, and 0,1 ml of trifluoroacetic acid were added and the reaction mixture was stirred at 60°C for 5 days. The product was precipitated in 300 ml of acetone, extracted 24 h with 2 × 200 ml of methanol and then 72 h with 3 × 400 ml of acetone in a soxhlet, and finally dialyzed in 0,02 M NaOH. After lyophilization, 1,1 g of the purified polymer were obtained, which did not show free **1a** or **1b** on thin-layer chromatography. The yellow product was soluble in alkaline aqueous solution (pH 8) and DMSO, and insoluble in methanol, acetone and DMF.

Content of acylated repeating units: 14,8 mole-% (by UV Spectroscopy at 303 nm in 0,1 M NaOH).
Found C 38,96 H 4,98 N 2,78

Substitution of **1a** *with divinyl ether-maleic anhydride (DIVEMA) copolymers*

As typical examples for the preparation of DIVEMA substituted with **1a**, in the anhydride and carboxylate form, syntheses and purifications of a sample A (carboxylate form) and of a sample G (anhydride form) are described. The reaction conditions for the preparation of samples A–K are given in Tab. 1. Tab. 2 summarizes the elemental analyses for all polymeric samples, together with the analytical data and the anhydride of the starting DIVEMA copolymers.

Sample A (s. Tab. 1): 1 g (ca. 3,4 mmol of repeating units) of DIVEMA (approximately 15% anhydride) (s. Tab. 2) was dissolved in 10 ml of freshly distilled DMF and 500 mg (1,1 mmol) of **1a** dissolved in 8 ml of DMF were added dropwise with stirring at room temperature. The reaction mixture was then stirred 24 h at room temperature and finally heated 3 h to 50°C. The product was precipitated in 250 ml of methylene chloride, filtered, washed with methanol and dried over night i. vac. The crude product was hydrolyzed to the polycarboxylate and filtrated in 200 ml and subsequently 4 × 100 ml of 0,05 M NaOH (BM 10 membrane, from Berghof, Tübingen, West Germany, exclusion size 1000). Only trace amounts of free **1a** were then detectable in the filtrate. The polymer was precipitated in 150 ml of acetone, washed with acetone and dried i. vac. over P_2O_5; yield: 1,1 g.

IR (KBr): 1640 (w; ν_{CO} of amide) and 1720 cm^{-1} (s; ν_{COOH}).

Content of substituted repeating units: 11 mole-% (by UV Spectroscopy at 303 nm in 0,1 M NaOH).

Sample B (s. Tab. 1): 1 g (ca. 3,75 mmol of repeating units) of DIVEMA (99% anhydride, s. Tab. 2) was dissolved in 20 ml of redistilled acetone and a solution of 550 mg (1,21 mmol) of **1a** in 15 ml of DMSO added dropwise at room temperature. After initial heating to 45°C (4 h), the clear, orange-red reaction mixture was stirred 72 h at room temperature. The product was precipitated in 300 ml of methylene chloride, filtered and reprecipitated with acetone/DMF/methylene chloride. It was then extracted 48 h with 2 × 400 ml of methylene chloride in a soxhlet. Final purification was obtained by threefold dissolution in acetone (70 ml), removal of acetone-insoluble material, and precipitation in methylene chloride. Yield: 1 g. The polymer did not show free **1a** on thin-layer chromatography, with ninhydrine-reactive material only at the origin. It is soluble in water (after hydrolysis to the polycarboxylate), acetone, DMF, DMSO, insoluble in methylene chloride and diethyl ether.

IR (KBr): S. Fig. 2.

Content of substituted repeating units: 25,2 mole-% (by UV Spectroscopy at 303 nm in 0,1 M NaOH).

Tab. 2. Elemental analyses of DIVEMA copolymers and its derivatives partially substituted with **1a**

Sample	DIVEMA copolymers[a]			DIVEMA partially substituted		
	C	H	content of anhydride in %[b]	C	H	N
A	48,53	4,28	15[c]	46,49	5,21	4,38
B	49,82	4,01	33[c]	28,99	3,06	0,30
C	48,53	4,28	15[c]	29,71	4,34	0,30
D	52,04	3,89	63[d]	34,88	3,69	0,30
E	51,96	3,96	63[d]	33,74	3,80	0,30
F	53,04	3,75	82[d]	37,12	4,03	1,69
G	53,56	3,61	99[d]	44,36	4,78	5,01
H	53,56	3,61	99[d]	45,79	5,13	5,07
I	53,56	3,61	99[d]	46,06	5,25	8,37
K	53,04	3,75	82[d]	47,43	5,18	9,49

[a] See Tab. 1 for molecular weight characteristics.
[b] Determined before substitution reactions.
[c] Estimated by elemental analysis.
[d] Determined by morpholine titration.

Analytical Procedures

Thin-layer chromatography: Precoated 0,25 mm Silicagel G_{F254} plates (5 × 20 cm, from Merck AG, Darmstadt, West Germany) in saturated chambers were used, with the solvent systems 1-butanol/glacial acetic acid/water (volume ratio 10:3:1) and methanol/acetone/ethyl acetate (volume ratio 10:10:1). Absorption was observed at 254 and 366 nm. A ninhydrine spray reagent (from Merck AG, Darmstadt, West Germany) was used for the detection of free amino groups.

Membrane filtration: Filtrations were carried out in a stirred 400 ml polycarbonate filtration cell (from Berghof GmbH, Tübingen, West Germany) at 3–4 atm under nitrogen. Cellulose membranes (from Berghof GmbH) with exclusion sizes of 1000 and 10000 were used, which are stable against the dilute acidic and basic solutions used for purification of the polymers.

IR Spectroscopy: IR spectra were determined as KBr disks (mass ratio 1:70) with a Perkin-Elmer 257 grating IR spectrometer.

NMR Spectroscopy: ^1H NMR Spectra were recorded with a 60 MHz "Jeol Minimar" spectrometer in 10% [$^2H_6$] DMSO solutions, with tetramethylsilane as internal standard.

UV Spectroscopy: UV spectra were recorded with a Beckman model 256 spectrometer. Contents of substituted repeating units in the polymeric derivatives of **1a** were determined in 0,1 M NaOH at $\lambda_{max} = 303$ nm, and in 0,1 M HCl at $\lambda_{max} = 307$ nm using corresponding calibration curves for **1a** and dialkyl esters of **1a**. Control measurements with the unsubstituted polymers were carried out to exclude interfering absorptions at the wavelengths used.

Mass spectrometry: FD mass spectra of the dialkyl esters **1b–d** were reocrded with a Varian MAT CH 711 spectrometer equipped with a dual electron impact/field desorption ion source. The compounds were dissolved in DMSO and coated on activated tungsten (10 µm) field ion emitters by the "emitter-dipping technique"[36]. The spectra were recorded by an on-line computer (Varian 620i) on magnetic tape. Experimental conditions were: 11 kV potential between emitter anode/kathode, 200 °C ion source temperature, 800 (10%) resolution.

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