SYNTHESIS OF CONJUGATES OF 1-(CARBOXYMETHYL)CYTOSINE AND 1-(5-O-CARBOXYMETHYL-β-D-ARABINOFURANOSYL)CYTOSINE WITH PROTEINS*

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1-(Carboxymethyl)cytosine (*Ia*), 1-(5-O-carboxymethyl-β-D-arabinofuranosyl)cytosine (*IIa*) and 5'-O-carboxymethylcytidine (*IIIa*) were transformed by treatment with acetic anhydride and 4-dimethylaminopyridine to the peracetyl derivatives *Ib—IIIb*. These products reacted with *p*-nitrophenol in the presence of N,N'-dicyclohexylcarbodiimide to give the activated esters *Ic—IIIc* which on reaction with ammonia, dimethylamine or 2-aminoethanol afforded the corresponding carboxamides *Id—IIId*, *IIe,f*. Reactions of *Ic* and *IIc* with human serum albumin and bovine γ-globulin at pH 9·2, followed by hydrolysis of the N- or O-acetyl groups at pH 9·5, gave 50% up to 64% yields of the respective conjugates *Ig*, *IIg* and *Ih*, *IIh*.

1-(β-D-Arabinofuranosyl)cytosine (Cytarabine, araC) is a known immunosuppressant and cytostaticum, inhibiting the DNA synthesis *de novo*[1-5]. Its 5'-O-acyl derivatives have analogous properties[6,7] and also its N^4-acetyl derivative has been shown to exhibit an antineoplastic activity[8]. In the connection with our study of conjugates of proteins with immunosuppressive haptenes we were interested in the synthesis of conjugates, containing the araC moiety bonded to the protein antigen, the aim being the preparation of potent and specific immunosuppressive compounds[9,10]. To this end we synthesised carboxymethyl derivative of cytosine, cytidine and araC[11]; however, because of their zwitter-ionic structure it was not possible to bind them to proteins by the method of mixed anhydrides[10,12].

In this paper we describe an alternative approach, consisting in peracetylation of the corresponding haptene. By the acetylation of the amino group of the heterocyclic base the character of the carboxyl is made more expressive: it is thus capable of transformation to the active *p*-nitrophenyl ester. The mentioned active esters were already successfully used[13] in the condensation of 1-carboxymethyl uracil with proteins; their use diminishes the danger of side-reactions of the haptene or protein which with other condensation techniques is considerable.

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The compounds *Ia–IIIa* were transformed to well soluble quaternary tetra--n-butylammonium salts which after drying were acetylated with acetic anhydride in the presence of 4-dimethylaminopyridine as catalyst[14] (nucleoside derivatives could be acetylated even without the preliminary transformation into the salts). After deionisation the acetyl derivatives *Ib–IIIb* were isolated by preparative chromatography. The UV spectra of the products differ from those of the starting compounds *Ia–IIIa* in that they exhibit a long-wavelength band at about 300 nm and a shift of the short-wavelength maximum to 250 nm. They display also typical pH-dependences of the spectra, characteristic for N-substituted cytosine derivatives. The effect of the presence of the sugar moiety manifests itself, as expected, by only a small, though distinct, shift of the absorption maxima (Table I).

In order to find conditions for the reverse hydrolysis of N-acetyl group which is decisive for the possible use of the mentioned derivatives as haptenes, we followed the course of hydrolysis of the compounds *Ib* and *IIb* in various buffer solutions by spectrophotometric measurements. (An analogous study was performed recently also for 2'-deoxycytidine 5'-phosphate derivatives[15,16]). Since the products *Ia* and *IIa* have no absorption at 300 nm (Fig. 1), we followed the absorbance drop at this wavelength. The obtained values which indicate a pseudomonomolecular reaction are given in Table II. We can expect that the O-acetyl derivative will hydrolyse at least equally well as the acetamide function; nevertheless we performed a control hydrolysis of the compond *IIb* under conditions of quantitative hydrolysis of the N-acetyl function (Table II) and found that the reaction indeed afforded practically quantitatively the expected product *IIa*.

TABLE I

Ultraviolet Spectra of Cytosine and N[4]-Acetylcytosine Derivatives

Compound	pH 1		pH 7–10·5	
	λ_{max}, nm (ε_{max})	λ_{min}, nm	λ_{max}, nm (ε_{max})	λ_{min}, nm
Ia	280 (10 400)	240	275 (7 500)[a]	252
Ib	242, 307 (7 800, 9 900)	227, 267	247, 300 (12 200, 7 400)	228, 271
IIa	282 (11 200)	242	272 (7 500)[a]	250
IIb	245, 305 (10 100, 9 850)	226, 270	248, 298 (14 400, 7 500)	227, 272

[a] Also at pH 12.

The acetyl derivatives *Ib*–*IIIb* were condensed with *p*-nitrophenol in dimethylformamide in the presence of N,N′-dicyclohexylcarbodiimide and the formed *p*-nitrophenyl esters *Ic*–*IIIc* were isolated by chromatography on silica gel. In order to study the reactivity of these compounds with nucleophilic agents as protein models, we transformed the compounds *Ic*–*IIIc* by reaction with ammonia into the amides *Id*–*IIId*, and the biologically interesting derivative of araC *IIc* also into the corresponding N,N-dimethylamide *IIe* and the N-(2-hydroxyethyl)amide *IIf*. These compounds, isolated by ion-exchanger chromatography, exhibited UV spectra corresponding to cytosine derivatives and their neutral character was in accord with transformation of the carboxyl into the amide group.

However, neither of the araC derivatives *IIa*, *IId*–*f* showed any antibacterial activity towards *E. coli* B (synthetic medium with glucose) in concentrations up to 1 mg/ml of medium.

TABLE II

Halftimes ($\tau_{1/2}$, min) of Hydrolysis of Compounds *Ib* and *IIb*

Buffer solution		*Ib*	*IIb*
0·1M Sodium borate	pH 10·5	102·6	26·0
	pH 9·5	[a]	210·0
	pH 8·5	[a]	[b]
Ammonium carbonate	pH 9·5	1 980	1 440

[a] Without substantial change after 16 h; [b] 20% N-deacetylation after 24 h.

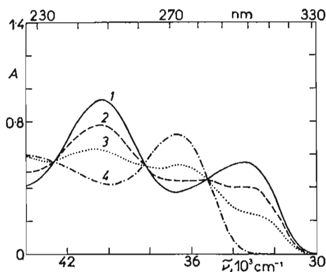
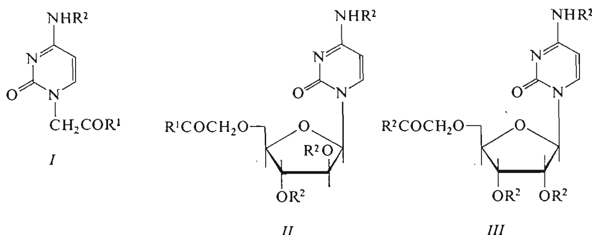


FIG. 1

Ultraviolet Spectra (pH 10·5)

1 Compound *IIb* at t_0, *2* 12 min, *3* 30 min, *4* t_∞ (compound *IIa*).

Condensation of the active esters *Ic*, *IIc* with proteins was carried out by adding a dimethyl sulfoxide solution of the active ester to a solution of human serum albumin or bovine γ-globulin in 0·2M borate buffer (pH 9·2) at room temperature[13]. Since dialysis of the low molecular weight portions proceeds slowly, the mixture was worked up by gel filtration[13]. In addition to the high molecular weight portion we isolated also a fraction of low molecular weight, which consisted of *p*-nitrophenol and the carboxylic acids *Ia* and *IIa*, formed by hydrolysis of the active esters *Ic* and *IIc*. Since the haptene probably still contained some non-hydrolysed acetyl groups, the corresponding fractions were hydrolysed for 4–5 days at pH 9·5 till their UV spectra did not change.



In formulae *I*–*III*: *a*; $R^1 = OH$, $R^2 = H$
b; $R^1 = OH$, $R^2 = COCH_3$
c; $R^1 =$ O-*p*-nitrophenyl, $R^2 = COCH_3$
d; $R^1 = NH_2$, $R^2 = H$
e; $R^1 = N(CH_3)_2$; $R^2 = H$
f; $R^1 = NHCH_2CH_2OH$, $R^2 = H$
g; $R^1 =$ NH-HSA, $R^2 = H$
h; $R^1 =$ NH-BGG, $R^2 = H$

HSA human serum albumine, BGG bovine γ-globuline.

The content of the bonded haptene was determined either by direct UV measurement on the conjugates *Ig*, *IIg* and *Ih*, *IIh*, corrected for the protein absorption[17], or indirectly by determination of *p*-nitrophenol and the carboxylic acids *Ia* or *IIa* in the low molecular weight portion (also in this case the mixture was made alkaline prior to the measurement in order to remove the N-acetyl groups). The results of these two methods were very similar and are given in Table III; the yields, related to the covalently bound haptene, range between 56·8–63·8% and 49·1–59% for human albumin and γ-globuline derivatives, respectively. The degree of substitution is not markedly reflected by the UV spectrum of the conjugate because the ab-

TABLE III

Yields and Properties of Protein Conjugates

Conjugate	Molar excess of haptene applied	Yield %	Content haptene[a] %	Haptene[b] protein
Ig —2·62	20	63·8	2·62	12·8
Ig —4·93	40	58·3	4·93	23·3
IIg—4·78	20	61·0	4·78	12·2
IIg—8·55	40	56·8	8·55	22·7
Ih —0·93	20	49·1	0·93	9·8
Ih —1·95	40	51·9	1·95	20·8
IIh—1·84	20	53·2	1·84	10·6
IIh—4·01	40	59·0	4·01	23·6

[a] Based on the residue of *Ia* or *IIa*; [b] mol of the cytosine derivative per mol of the protein.

TABLE IV

Chromatography and Electrophoresis

Compound	R_F					E_{Up}
	S1	S2	S2[a]	S3	S4	
Ia	0·37	—	0·37	0	0	0·45
IIa	0·42	0·46	0·38	0	0	0·43
IIIa	0·34	0·38	—	—	—	0·45
Ib	0·55	0·63	0·49	0	0·13	0·70
IIb	0·63	0·82	0·88	0·30	0·75	0·40
IIIb	—	—	0·85	0·32	0·72	0·40
Ic	—	—	0·89	0·65	0·89	—
IIc	—	—	0·83	0·50	0·78	—
IIIc	—	—	0·83	0·49	0·81	—
Id	0·60	0·78	—	—	—	—0·18
IId	0·60	0·60	—	—	—	—0·11
IIId	0·64	0·56	—	—	—	—0·12
IIe	0·72	0·66	—	—	—	—0·16
IIf	0·67	0·64	—	—	—	—0·12

[a] TLC system.

sorption maxima of cytosine and the protein are very close. Conjugates, prepared by this method, were also analysed by gel electrophoresis: under the conditions used they were homogeneous and migrated to the anode.

EXPERIMENTAL

Melting points were determined on a Boetius melting point apparatus and are uncorrected. Unless stated otherwise, the solutions were taken down at 40°C/2 kPa and the compounds dried over phosphorus pentoxide at 13 Pa. The human serum albumin was obtained from Forschungsinstitut für Impfstoffe, Dessau (G.D.R.), bovine γ-globulin from Ferak (West Berlin). Paper chromatography was carried out on a paper Whatman No 1 in the system S1, 2-propanol--conc. ammonia–water (7 : 1 : 2), S2, 1-butanol–acetic acid–water (5 : 2 : 3); thin layer chromatography on Silufol UV_{254} plates (Kavalier, Votice, Czechoslovakia) in the system S2, S3, chloroform–ethanol (9 : 1) and S4, chloroform–ethanol (4 : 1). Preparative chromatography was performed on loose layers (30 × 16 × 0·3 cm) of silica gel, containing fluorescence indicator (Kavalier, Votice, Czechoslovakia). Paper electrophoresis was carried out on a paper Whatman No 3 MM (20 V/cm, 1 h) in the buffer E1, 0·1M triethylammonium hydrogen carbonate, pH 7·5. The R_F and electrophoretic mobility values are given in Table IV. For electrophoresis on an agarose gel see ref.[18]. Preparative gel chromatography was carried out on a column (100 × 2 cm) of Sephadex G 50 (Pharmacia, Sweden) in ammonium carbonate pH 9·5 (10·0 g of ammonium carbonate in 5 l of water, adjusted with aqueous ammonia to pH 9·5), elution rate 1·2 ml/min, 3 ml fractions (the elution was monitored continuously by a Uvicord instrument). The UV spectra were taken on a Specord UV–VIS (Carl Zeiss, Jena, G.D.R.) spectrophotometer in aqueous solutions, quantitative measurements were performed on a Spectromom 203 instrument (Hungarian Optical Works, Budapest, Hungary).

1-Carboxymethyl-N^4-acetylcytosine (*Ib*)

1-Carboxymethylcytosine (*Ia*, see ref.[12]) (0·85 g; 5 mmol) was dissolved in a solution of tetra--n-butylammonium hydroxide (5 mmol), the solution was taken down *in vacuo*, the residue codistilled with ethanol and acetone and dried *in vacuo*. Acetic anhydride (15 ml), followed by 4-dimethylaminopyridine (0·25 g), was added, the mixture was stirred until it became homogeneous, set aside at room temperature overnight with exclusion of moisture and taken down at 40°C/13 Pa. The residue was thrice codistilled with toluene, dissolved in 50% pyridine (50 ml) and applied to a column of Dowex 50X8 in pyridine cycle (250 ml). The column was washed with 50% pyridine (300 ml), the eluate taken down *in vacuo*, the residue codistilled successively with water and ethanol and then chromatographed on a column (70 × 4 cm) of microcrystalline cellulose (Macherey-Nagel, F.R.G.) in 70% aqueous 2-propanol. Fractions, containing the compound *Ib*, were combined, taken down *in vacuo* and the residue crystallised from ethanol (with addition of ether); yield 70% of *Ib*. The analytical sample was purified by thin-layer chromatography (*vide supra*) in the system S4 (elution with methanol). M.p. 212–215°C. For $C_8H_9N_3O_4$ (212·2) calculated: 45·50% C, 4·29% H, 19·90% N; found: 45·34% C, 5·05% H, 19·49% N.

1-(5-O-Carboxymethyl-2,3-di-O-acetyl-β-D-arabinofuranosyl)-N^4-acetylcytosine (*IIb*)

a) A mixture of the compound *IIa* (see ref.[11]) (0·60 g; 2 mmol), 4-dimethylaminopyridine (0·40 g) and acetic anhydride (20 ml) was stirred at room temperature for 5 h, taken down at 40°C/13 Pa and the residue codistilled three times with toluene. The residue was dissolved in 50% pyridine

(25 ml) and set aside for 1 h, the pyridine was evaporated *in vacuo* and the residue after coevaporation with ethanol chromatographed on two loose layers of silica gel in the system S3. The product band (R_F 0·3—0·4) was eluted with methanol and the eluate taken down *in vacuo*. The crystalline residue was mixed with methanol (5 ml), filtered and the filtrate treated with ether (50 ml). The crystalline product was filtered and dried *in vacuo*, yield 60%, m.p. 156—157°C. For $C_{17}H_{21}N_3$. O_{10} (427·4) calculated: 44·77% C, 4·95% H, 9·83% N; found: 44·31% C, 5·21% H, 9·35% N.

b) A suspension of the compound *IIa* (see ref.[11]) (1·0 g; 3·3 mmol) in water (50 ml) was titrated with 1M solution of tetra-n-butylammonium hydroxide to pH 7·0, the solution taken down *in vacuo*, the residue codistilled with ethanol and dried *in vacuo*. Acetic anhydride (25 ml) and 4-dimethylaminopyridine (0·25 g) were added, the mixture was stirred until it became homogeneous, set aside overnight and taken down at 40°C/13 Pa. The residue was codistilled with toluene (3 × 20 ml) under the same conditions, dissolved in 20% pyridine (20 ml) and applied to a colum, of Dowex 50X8 in the pyridinium form. The column was washed with 20% pyridine (250 ml), the eluate taken down *in vacuo*, the residue codistilled with water and chromatographed on one plate of silica gel in the system S4. The product was eluted with methanol and crystallised from ethanol (with addition of ether), affording 0·63 g (45%) of compound *IIb*.

N^4,2',3'-Triacetyl-5'-O-carboxymethylcytidine (*IIIb*)

A suspension of the compound[11] *IIIa* (0·55 g; 1·83 mmol) in water (25 ml) was neutralised with a solution of tetra-n-butylammonium hydroxide to pH 7·0 and worked up as described for the compound *IIb* under *b*); yield 0·35 g (45%) of *IIIb*. For $C_{17}H_{21}N_3O_{10}$ (427·4) calculated: 44·77% C, 4·95% H, 9·83% N; found: 44·85% C, 5·38% H, 10·49% N.

1-(Carboxymethyl)-N^4-acetylcytosine *p*-Nitrophenyl Ester (*Ic*)

N,N'-Dicyclohexylcarbodiimide (0·45 g; 2·2 mmol) was added to a solution of the compound *Ib* (0·42 g; 2 mmol) and *p*-nitrophenol (0·31 g; 2·2 mmol) in dimethylformamide (8 ml) and the mixture was set aside at room temperature overnight under exclusion of moisture. Dioxane (8 ml) was added, the precipitate filtered off, the filtrate taken down, the residue codistilled with toluene (2 × 10 ml) at 40°C/13 Pa and chromatographed on one plate of silica gel (*vide supra*) in chloroform–ethanol (94 : 6) mixture. The product band (R_F 0·4—0·5) was eluted with methanol, the eluate taken down *in vacuo*, the residue dissolved in a minimum amount of chloroform and the product precipitated with light petroleum. Yield 45%, m.p. 110—114°C. UV spectrum (methanol): λ_{max} 247, 300 nm. For $C_{14}H_{12}N_4O_6$ (332·2) calculated: 50·60% C, 3·64% H, 16·86% N; found: 48·98% C, 4·07% H, 15·82% N.

1-(5-O-Carboxymethyl-2,3-di-O-acetyl-β-D-arabinofuranosyl)-N^4-acetylcytosine *p*-Nitrophenyl Ester (*IIc*)

N,N'-Dicyclohexylcarbodiimide (0·34 g; 1·65 mmol) was added to a solution of the compound *IIb* (0·64 g; 1·5 mmol) and *p*-nitrophenol (0·23 g; 1·65 mmol) in dimethylformamide (5 ml) and the mixture was worked up as described for the compound *Ic*. The crude product was purified by chromatography on two plates of silica gel in chloroform–ethanol (94 : 6) mixture, the product band (R_F 0·3—0·4) was eluted with methanol, the eluate taken down and the product precipitated from chloroform with petroleum ether; yield 50%, m.p. 125—129°C. UV spectrum (methanol): λ_{max} 246, 298 nm. For $C_{23}H_{24}N_4O_{12}$ (548·5) calculated: 55·19% C, 4·83% H, 11·20% N; found: 54·56% C, 5·30% H, 10·22% N.

N^4,2′,3′-Triacetyl-5′-O-carboxymethylcytidine *p*-Nitrophenyl Ester (*IIIc*)

N,N′-Dicyclohexylcarbodiimide (0·11 g; 0·55 mmol) was added to a solution of the compound *IIIb* (0·21 h; 0·5 mmol) and *p*-nitrophenol (0·08 g; 0·55 mmol) in dimethylformamide (2 ml). Further work-up procedure was the same as described for the compound *IIc*; yield of *IIIc* 45%, m.p. 132–136°C. UV spectrum (methanol): λ_{max} 246, 298 nm. For $C_{23}H_{24}N_4O_{12}$ (548·5) calculated: 55·19% C, 4·83% H, 11·20% N; found: 55·20% C, 5·08% H, 11·03% N.

1-(Aminocarbonylmethyl)cytosine (*Id*)

A solution of the compound *Ic* (0·11 g; 0·3 mmol) in 30% methanolic ammonia (3 ml) was set aside for 16 h at room temperature and then taken down *in vacuo*. The residue was taken up in water (5 ml), acidified with hydrochloric acid to pH 4 and extracted with ether (3 × 5 ml). The aqueous layer was applied to a column of Dowex 50X8 (H^+ form; 25 ml) and eluted first with water till the UV absorption and conductivity disappeared and then with 3% ammonia. The UV-absorbing ammonia eluate was taken down *in vacuo* and the residue, dissolved in water (5 ml), applied to a column of Dowex 1X2 (acetate form, 25 ml). Elution with water and evaporation of the UV-absorbing eluate afforded a product which was precipitated from methanol (2 ml) by addition of ether (50 ml). The yield of chromatographically pure product *Id* was 40·2 mg (79·2%); UV spectrum (pH2): λ_{max} 278 nm. For $C_6H_8N_4O_2$ (169·2) calculated: 42·85% C, 4·79% H, 33·32% N; found: 43·23% C, 4·90% H, 33·03% N.

1-(5-O-Aminocarbonylmethyl-β-D-arabinofuranosyl)cytosine (*IId*)

The title compound was prepared from *IIc* (0·11 g; 0·2 mmol) using the procedure described for *Id*; yield 60%, m.p. 142–146°C. UV spectrum (pH2): λ_{max} 278 nm. For $C_{11}H_{16}N_4O_6$ (300·3) calculated: 43·99% C, 5·37% H, 18·66% N; found: 44·98% C, 5·76% H, 19·12% N.

5′-O-Aminocarbonylmethylcytidine (*IIId*)

Prepared from the compound *IIIc* (0·11 g; 0·2 mmol) analogously as described for the compound *Id*; yield 49·3 mg (82%). UV spectrum (pH2): λ_{max} 278nm. For $C_{11}H_{16}N_4O_6$ (300·3) calculated: 43·99% C, 5·37% H, 18·66% N; found: 44·33% C, 6·31% H, 18·08% N.

1-(5-O-Dimethylaminocarbonylmethyl-β-D-arabinofuranosyl)cytosine (*IIe*)

A solution of the compound *IIc* (0·11 g; 0·2 mmol) in 2·5M ethanolic dimethylamine (3 ml) was set aside at room temperature overnight and the product was isolated as described for the compound *Id*; yield 38·1 mg (58%) of chromatographically homogeneous compound *IIe*. UV spectrum (pH2): λ_{max} 278 nm. For $C_{13}H_{20}N_4O_6$ (328·3) calculated: 47·56% C, 6·14% H, 17·07% N; found: 48·33% C, 6·31% H, 18·08% N.

1-[5-O-(2-Hydroxyethyl)aminomethyl-β-D-arabinofuranosyl]cytosine (*IIf*)

2-Aminoethanol (0·3 ml) was added to a solution of *IIc* (0·11 g; 0·2 mmol) in dioxane (2·5 ml), the mixture was set aside at room temperature overnight, taken down *in vacuo* and the residue was codistilled with toluene. The work-up procedure was analogous to that described for *Id*. The obtained product was then chromatographed on a column of microcrystalline cellulose in 70% aqueous 2-propanol (see the preparation of *Ib*) and the product-containing fractions combined and taken down *in vacuo*. The crystalline product was obtained by precipitation from

methanolic solution (2 ml) with ether; yield 61·4 mg (89%) of *IIf*. UV spectrum (pH2): λ_{max} 278 nm. For $C_{13}H_{20}N_4O_7$ (344·3) calculated: 45·34% C, 5·85% H, 16·27% N; found: 45·64% C, 5·71% H, 16·07% N.

Hydrolysis of Compounds *Ib* and *IIb*

A concentrated solution of *Ib* or *IIb* in water was quickly added to the appropriate buffer till A_{300} ~0·80 for *Ib* and A_{298} ~0·80 for *IIb*. Time dependence at these wavelengths was followed at 25°C. The following buffers were used: 0·1M sodium borate–boric acid pH 7·5, 8·5, 9·5 and 10·5. The results are given in Table II.

A solution of *IIb* (10 mg) in 0·1M borate buffer, pH 10·5 (0·5 ml) was allowed to stand for 15 h at room temperature. Chromatography on silica gel in chloroform–methanol (7 : 4) showed the presence of *IIa* (R_F 0·05), trace of the starting *IIb* (R_F 0·87) and a compound of R_F 0·66 (probably the O-acetyl derivative).

Preparation of Conjugates with Human Serum Albumin

a) *With compound* Ia: A solution of 6·6 mg (20 µmol) or 13·3 mg (40 µmol) of *Ic* in 0·5 ml of dimethyl sulfoxide was added to a solution of human serum albumin (69 mg; 1 µmol) in an 0·2M borate buffer pH 9·2 (2 ml) and the mixture was set aside for 24 h room temperature. A part (1 ml) of the mixture was applied on a column of Sephadex and eluted with ammonium carbonate buffer pH 9·5. The high molecular weight fractions (15–26) were combined, adjusted to an appropriate volume by addition of the same buffer, and analysed. From the stock solution the conjugate *Ig* was isolated by lyophilisation. The yields and properties are given in Table III.

The low molecular weight fractions (40–62) were combined, pH adjusted with 0·1M sodium hydroxide to 12 and, after standing overnight at room temperature, the solution was analysed for *p*-nitrophenol and *Ia*.

b) *With compound* IIa: The experiment was carried out analogously as described for the preparation of *Ig*, using 11·0 mg (20 µmol) or 21·9 mg (40 µmol) of *IIc*. The work-up procedure was the same as described under *a*). The properties and yield of *IIg* are given in Table III.

Preparation of Conjugates with Bovine γ-Globulin

A solution of 10 or 20 µmol of *Ic*, or 10 or 20 µmol of *IIc*, in dimethyl sulfoxide (0·5 ml) was added to a solution of bovine γ-globulin (80 mg; 0·5 µmol) in an 0·2M borate buffer pH 9·2 (2 ml). The work-up procedure was the same as described for the conjugates *Ig* and *IIg*, yields and properties of thus-obtained conjugates *Ih* and *IIh* are listed in Table *III*.

Determination of Covalently Bonded Haptene Content in the Conjugates

a) *By direct measurement*: The content was calculated from the difference between the absorbance at absorption maximum of the analysed derivative (pH 9·5) and the absorbance of the same protein solution at the same wavelength. For the calculation we used extinction coefficient values, given in Table I.

b) *Indirectly*: A stock solution of the low molecular weight fraction after the gel filtration (*vide supra*) was measured at the wavelength of maximum of the corresponding haptene *Ia*, *IIa* and the concentration was calculated using the extinction coefficient of the haptene (Table I) after correction for the absorption of *p*-nitrophenol at the given wavelength ($\varepsilon_{272} = 2450$, $\varepsilon_{275} = 2250$); the *p*-nitrophenol concentration was determined separately by measurement

at 400 nm ($\varepsilon_{400} = 18000$). The difference between the original amount of *Ic* or *IIc*, employed in the reaction, and *Ia* or *IIa*, determined in the low molecular weight fraction, corresponds to the covalently bonded haptene.

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