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EFFECT OF REAGENTS AFFECTING SPECIFIC GROUPS OF PROTEINS ON THE HAEMAGGLUTINATING ACTIVITY OF RUBELLA VIRUS

G. GAGNONI, P. CANTAGALLI, A. GRASSO, F. DIANZANI

Institute of Microbiology of the University of Siena, Siena, Italy*

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Summary. — Rubella virus haemagglutinin (HA) was treated with reagents affecting specific groups of proteins. The haemagglutinating activity was reduced or destroyed by sodium iodoacetate, N-ethylmaleimide, p-hydroxymercuribenzoate, copper sulfate, acetic anhydride and benzoylchloride indicating that sulfhydryl and amino groups are essential for the agglutination. The HA was not affected by treatment with cysteine, 2-mercaptoethanol and diazotized sulfanilic acid suggesting that unmasked disulfide and imidazole groups of histidine are not essentially involved in the mechanism of haemagglutination by rubella HA.

The inactivation of viral activities by reagents affecting specific reactive sites of proteins may support indication on the groups with which they are involved. Previous research performed by studying the effect of various chemical modifications of the viral surface has demonstrated that sulfhydryl, disulfide and amino groups may be essential for the biological activity of a number of viruses. However the susceptibility to inactivation by specific reagents can vary in various groups of viruses indicating a different superficial structure and providing some additional indication for taxonomic purposes.

In the course of recent studies on rubella virus, a proposed member of Myxovirus group, the role of some chemical groups of the viral surface on the haemagglutinating activity of this virus has been investigated. The results will be reported in the present note.

Preparation of rubella virus HA. Rubella virus was obtained from Istituto Sieroterapico Italiano, Naples, as infectious fluid from monkey kidney cell cultures. After 4 passages in hamster BHK-21 cell line, a large stock of virus was obtained by infecting BHK-21 cell cultures with an input multiplicity of 0.5 TCD_{50} per cell. Two hours later unadsorbed virus was removed and fresh medium (Eagle's medium supplemented with 2% calf serum) was added. Two days later the medium was replaced and the cultures were incubated for 5 days more without any further change of medium. Seven days after the infection the cultures were frozen and thawed 3 times and the fluid was centrifuged at 2000 rev/min for 15 minutes to remove cells and cell debris. The supernate had an infectious titre of 10^6 TCD_{50} per ml and a HA titre of 16. This preparation was then treated with tween 80 and ether as described by Norrby (1962). The HA titre increased 16 times after the treatment. The preparation was then concentrated 10 times by forced dialysis against dry Carbowax 1500 (Union Carbide) and partially purified by gel filtration on

* National Research Council, National Group of Experimental Medicine.

Sephadex G-200 (Pharmacia) using a 60 × 3 cm column. All the HA activity was recovered in the void volume. Two fractions corresponding to the peak of HA activity were collected and pooled; the content of proteins was 150 μg per ml and the HA titre was 128.

Haemagglutination was performed using a 0.5% suspension of adult pigeon red cells (Peetermans and Huygelen, 1967).

Chemicals. Sodium iodoacetate was purchased from Light & Co. p-Hydroxymercuribenzoate and N-ethylmaleimide were obtained from Sigma Chemical Co. DL-Cysteine hydrochloride was purchased from B.D.H. and 2-mercaptoethanol from Fluka. Acetic anhydride, benzoylchloride, copper sulfate and diazotized sulfanilic acid were obtained from Merck. Unless otherwise specified, all the chemicals were dissolved immediately before use in 0.15 M phosphate buffered saline, pH 7.4 (PBS).

Treatment of HA with the reagents. The effect of iodoacetate, p-hydroxymercuribenzoate, N-ethylmaleimide, cysteine and 2-mercaptoethanol was tested by mixing 0.5 ml of HA with 0.5 ml of the reagents dissolved in order to reach final concentrations of 10^{-2} and 10^{-3} M. The reaction mixtures were shaken and kept at 37° C for 30 minutes. The residual HA activity was tested immediately thereafter. It was previously determined that agglutination by rubella HA was not affected by the pretreatment of red cells with these reagents.

The effect of acetic anhydride was tested by mixing 0.5 ml of HA with 0.5 ml of 5.6 M sodium acetate and with 0.05 ml of acetic anhydride dissolved in dioxane to obtain a final concentration of 10^{-2} and 10^{-3} M. The mixtures were magnetically stirred for 2 hours at 0° C, and then dialyzed for 12 hours at 4° C against 1000 volumes of PBS.

The effect of benzoylchloride was tested in the same way as that of acetic anhydride, using 0.5 ml of HA, 0.5 ml of 0.15 M phosphate buffer at pH 7.5 and benzoylchloride dissolved in dioxane to obtain final concentrations of 5×10^{-3} and 5×10^{-4} M.

The effect of copper sulphate was tested by mixing 5 ml of HA with 0.01 ml of 0.1 M copper sulfate in distilled water and with 0.2 ml of 0.1 M ammonium acetate buffer at pH 8. The mixtures were kept for 30 minutes at room temperature and then assayed for HA activity.

The effect of diazotized sulfanilic acid was tested by mixing 2 ml of HA with 0.02 ml of 1% aqueous solution of sulfanilic acid and with 0.2 ml of 0.1 M ammonium acetate buffer at pH 8. After 30 minutes at room temperature, the mixtures were dialyzed for 12 hours at 4° C and then assayed for HA activity.

Results and Discussion

The results of representative experiments are shown in Table 1. The control samples were mixed with the same diluents used to dissolve the reagents and they were processed in the same way as the treated samples. All the experiments were repeated with another stock of rubella HA, kindly supplied by R.I.T. Laboratories, Génval, Belgium, and substantially the same results were obtained.

It can be seen that sodium iodoacetate, N-ethylmaleimide, and p-hydroxymercuribenzoate caused substantial inactivation of HA at both 10^{-2} or 10^{-3} M concentrations. The effect was greater with the higher concentration of the reagents. Copper sulfate caused complete inactivation of HA as did also acetic anhydride and benzoylchloride at both concentrations used. Cysteine, 2-mercapthoethanol and diazotized sulfanilic acid did not affect the HA activity.

Some characteristics of rubella virus HA suggest its proteinaceous nature (Furukawa *et al.*, 1967). The inhibitory effect of reagents affecting specific protein groups may support this view.

Haemagglutinating activity was inactivated by sodium iodoacetate, N-ethylmaleimide, p-hydroxymercuribenzoate and copper sulfate indicating that —SH groups are essential for the biological activity of HA. However, while p-hydroxymercuribenzoate and copper sulfate are considered specific

Table 1. Effect of treatments of rubella virus haemagglutinin with various reagents affecting specific groups of proteins

Reagent	Molarity	Time of reaction (minutes)	Groups specifically affected	HA titre of		Inactivation %
				control sample	treated sample	
Sodium iodoacetate	10^{-2}	30	$-SH$; $-NH_2(\alpha)$; imidazole	128	<2	>98.5
	10^{-3}	30		128	16	87.5
N-Ethylmaleimide	10^{-2}	30	$-SH$; $-NH_2(\alpha)$	128	16	87.5
	10^{-3}	30		128	32	75.0
p-Hydroxymercuribenzoate	10^{-2}	30	$-SH$	128	8	93.8
	10^{-3}	30		128	32	75.0
Copper sulfate	2×10^{-4}	30	$-SH$	128	<2	>98.5
Cysteine	10^{-2}	30	$-S-S-$ (unmasked)	128	128	0
	10^{-3}	30		128	128	0
Mercaptoethanol	10^{-2}	30	$-S-S-$ (unmasked)	128	128	0
	10^{-3}	30		128	128	0
Acetic anhydride	10^{-2}	120	$-NH_2(\alpha, \varepsilon)$; $-OH$	64	<2	>96.9
	10^{-3}	120	$-NH_2(\alpha)$; $-OH$	64	<2	>96.9
Benzoylchloride	5×10^{-3}	120	$-NH_2$	64	<2	>96.9
	5×10^{-4}	120		64	<2	>96.9
Sulfanilic acid	5×10^{-4}	30	Imidazole	64	64	0

reagents for —SH groups (Boyer, 1954; Fraenkel-Conrat, 1963), N-ethylmaleimide may react also with amino groups (Riggs, 1961) and with the imidazole group of histidine (Gundlach *et al.*, 1959). The possibility that also amino radicals are involved in the mechanism of the haemagglutination was confirmed by the evidence that the HA activity was completely destroyed by acetic anhydride and by benzoylchloride (Ragetli and Weintraub, 1962). On the contrary, the failure to inactivate the HA by diazotized sulfanilic acid may indicate that the imidazole group of histidine is not essential for the HA activity.

The lack of inhibitory effect by cysteine and mercaptoethanol indicates that the HA activity is not dependent on the integrity of unmasked —S—S— groups.

These results suggest that the mechanisms of attachment to red cells of rubella virus and of the members of Myxovirus group are different. In fact, rubella virus HA was inactivated by sulfhydryl reagents while HA of influenza (Buckland, 1960; Philipson and Choppin, 1960; Hoyle and Hana, 1966) and measles (Dianzani, 1964) viruses were not affected by such reagents. Also amino groups and the imidazole group of histidine were found essential for rubella and not for influenza virus (Hoyle and Hana, 1966). Taken together these data might suggest some implication with respect to the taxonomic position of rubella virus.

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