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CHROMIUM-PROTEIN INTERACTIONS*

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There are many conflicting reports concerning the form of the chromium responsible for chrome dermatitis. Some investigators (17, 19) incriminate the chromates (hexavalent compounds) as the cause of allergic eczematous dermatitis. Others (14, 15) attribute allergic reactions to chromic salts (trivalent compounds). Several investigators (4, 5, 7) have demonstrated cross sensitivity. Clinical and laboratory studies have presented the following paradox: Hexavalent chromium, a strong sensitizer and elicitor, does not bind with skin proteins; trivalent chromium, a poor sensitizer and elicitor, binds readily with skin proteins.

With *in vitro* studies it has been attempted to define the role of chromium in allergic eczematous dermatitis. Magnus (11) failed to demonstrate the binding of hexavalent chromium to non-skin proteins. Anderson (1) reported binding of chromium by whole skin after immersion in either hexavalent or trivalent chromium solutions, but he failed to establish the oxidation state of the bound chromium. Grey and Stirling (8) have reported that serum proteins bind only trivalent chromium and Baejer (2) has made similar observations with lung tissue. We (18) have demonstrated that skin binds only trivalent chromium, and have proposed that hexavalent chromium undergoes reduction prior to binding. This view is shared by Mikulecky (13) and Mali (12).

In our present preliminary studies we bound chromium to an extract of soluble skin proteins, measured the extent of binding, located the site of binding and prepared chromium-protein conjugates for clinical evaluation.

Materials and Methods

1. *Soluble Skin Proteins*

Human or dehaired guinea pig skin was cut into pieces, approximately 4 mm^2 in size, chilled to 17°C and homogenized in Tyrode solution in a cold Waring blendor. Approximately 1 cm^2/1 ml Tyrode solution was used; the low temperature was maintained by intermittently interrupting the homogenization and chilling the blendor cup and contents at 17°C. The homogenate was centrifuged at 35,000×g for 2 hours at 0°C and the middle layer containing the soluble protein was then removed.

2. *Methylated and Acetylated Skin Protein*

Protein was precipitated from the skin extract by the addition to ten volumes of acetone at −18°C. The precipitate was removed by centrifugation in the cold, and washed twice by suspending in cold ace-

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tone. The washed precipitate was dried under vacuum and stored at −15°C. Acetylation of amino groups was carried out by adding 33 mg of dry protein to a mixture of 0.66 ml of half saturated sodium acetate and 0.05 ml of acetic anhydride at 0°C according to the procedures of Fraenkel-Conrat (6). The carboxyl groups on 35 mg of dry protein were methylated by treatment with three ml of 0.1 N hydrochloric acid in methanol at room temperature. A 37 mg sample of the dry protein served as control. Each sample was diluted to ten ml with buffered saline and dialyzed against buffered saline to remove the reagents. Precipitated protein was removed by centrifugation and the solutions were stored at 5°C. Some precipitated protein was noted in the acetylated and methylated samples.

3. *Guinea Pig Blood Fractions*

Ten ml of guinea pig blood was centrifuged to remove the formed elements. Six ml of the resulting serum was diluted to 10 ml with buffered saline and treated with 10 ml of saturated ammunium sulfate. The precipitated globulins were removed by centrifugation, redissolved in buffered saline and reprecipitated by half saturation with ammonium sulfate. The globulins were redissolved and reprecipitated twice more, and the isolated material was stored at −5°C. The albumins were recovered from the first centrifugate by saturation with solid ammonium sulfate. The precipitated material was redissolved in buffered saline and reprecipitated by saturation with solid ammonium sulfate three times. The albumins were stored at −5°C.

4. *Carrier-Tracer Chromium Solutions*

Stock solutions of hexavalent chromium were prepared by mixing a measured volume of high specific activity $Na_2Cr^{51}O_4$ with a measured volume of standard potassium dichromate solution and diluting in a volumetric flask. Stock solutions of trivalent chromium were prepared by chemical reduction of the stock hexavalent chromium solution with sodium metabisulfite.

Binding from Solutions of Hexavalent and Trivalent Chromium

Four ml portions of soluble skin protein were mixed with either one ml of stock hexavalent (1.00×10^{-2} M $K_2Cr_2O_7$, 10 μ C Cr^{51}/ml) or stock trivalent (1.00×10^{-2} M $Cr_2(SO_4)_3$, 10 μ C Cr^{51}/ml) chromium solution. Control systems containing only soluble skin protein or stock chromium solution only diluted with Tyrode solution were also prepared. After standing overnight at 5°C, unbound chromium was removed by dialysis against ten 200 ml changes of buffered saline over a five day period in the cold. The efficiency of the dialysis was monitored by radioassay of the outside solutions. The dialyzed protein was then assayed from chromium by measuring the radioactivity of aliquots in a well-type scintillation counter and compared with the radioactivities of dilutions of the original stock chromium solutions. The protein content of the dialyzed protein was determined by the Lowry method (10). (These data are presented in Table 1.)

Table 1. *Binding of Chromium by Soluble Skin Protein*

mg A Cr bound/g protein solutions originally 0.002 M in $Cr_2(SO_4)_3$	mg A Cr bound/g protein solutions originally 0.002 M in $K_2Cr_2O_7$
0.126	0.0071
0.136	0.0063
0.120	—
0.112	0.0100
0.152	0.0066

Extent of Binding

Four ml portions of soluble human skin protein were mixed with 1 ml of serial dilutions of the stock hexavalent or trivalent chromium solutions. Control solutions were also prepared. After standing overnight in the cold, unbound chromium was removed by exhaustive dialysis, and the dialyzed protein was analyzed for chromium and protein (Table 2).

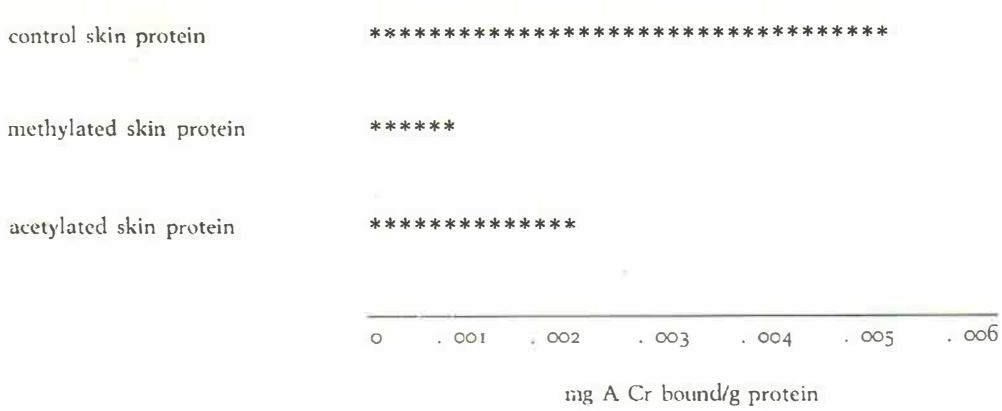

Fig. 1. Location of binding sites

Location of Binding Sites

Samples of methylated, acetylated and unmodified soluble skin protein each contained in 10 ml of buffered saline, were treated with 2 ml of stock hexavalent chromium solution and allowed to stand overnight at 5°C. Unbound chromium was removed by exhaustive dialysis against ten 200 ml changes of buffered saline over a five day period at 5°C. Aliquots of the dialyzed protein were removed for chromium and protein analysis. These data are presented in Figure 1.

Preparation of Chromium-Protein Conjugates for in vivo Evaluation

Solutions of guinea pig soluble skin protein, guinea pig serum albumin and globulin were treated with measured volumes of either stock hexavalent or trivalent chromium solution. After incubation and exhaustive dialysis in the cold, the dialyzed proteins were analyzed for chromium and protein. Guinea pigs sensitized to either trivalent or hexavalent chromium were challenged with these protein solutions.

Results and Discussion

The data in Table 1 show that the soluble skin proteins bind chromium from solutions of either hexavalent or trivalent chromium. Approximately fifteen times more chromium is bound from solutions of the trivalent form. This is in accord with Anderson's (1) and our own (18) observations with whole skin.

Solutions of trivalent chromium at concentrations as low as 2×10^{-3} M caused precipitation of protein. The original solutions of the soluble skin protein contained 1000–1500 μg protein/ml. After treatment with the trivalent chromium, the soluble protein concentration was only 700–800 μg/ml. In the case of solutions treated with hexavalent chromium, the soluble protein concentration was 1000–1200 μg/ml. The denaturation of protein by trivalent chromium has been described by Clark (3).

The data in Table 2 indicate increasing binding of chromium with increasing potassium dichromate concentration, but little or no change in the extent of binding of chromium from solutions of varying tri-

Table 2. *Saturation of Binding Sites*

initial chromium concentration, M	mg A Cr bound/g protein from $Cr_2(SO_4)_3$	mg A Cr bound/g protein from $K_2Cr_2O_7$
0.003	0.108, 0.100	0.0145, 0.0149
0.002	0.135, 0.105	0.0074, 0.0063
0.001	0.090, 0.100	0.0024, 0.0029

valent chromium content. We interpret this as a demonstration of saturation of the binding sites on the protein molecule in the case of trivalent chromium. Pierce and Stemmer (16) have demonstrated saturation of human serum albumin with trivalent chromium. Their saturation value is given as 17.28 moles of chromium bound/mole of protein. This corresponds to approximately 0.27 mg A Cr/g protein. Our value for soluble skin protein is 0.106 mg A Cr/g protein. From Table 2 it is apparent that in the binding from solutions of hexavalent chromium no saturation level was reached.

Blocking of amino groups by acetylation and carboxyl groups by methylation decreases the binding of chromium (Fig. 1). The decrease is much more marked in the case of carboxyl blocking, and is interpreted as an indication that both the free carboxyl groups and the free amino groups of the protein are involved to different extents in the binding of chromium. It is possible, however, that the differences in binding are due to denaturation. Pierce and Stemmer (16) have attributed the binding of chromium to serum albumin to interactions at the carboxyl groups, while Anderson (1) has reported that methylation causes an increase in the binding of chromium from solutions of both the trivalent and hexavalent compounds.

Skin and serum proteins conjugated with hexavalent and trivalent chromium were utilized in studies of delayed hypersensitivity to chromium in guinea pigs (9). Attempts were made to sensitize guinea pigs by the injection of the chromium-protein complexes with Freund's adjuvant. It was not possible to achieve this sensitization, though this could be done regularly with equivalent amounts of unbound chromium. Moreover, animals with proven sensitivity to potassium dichromate and chromic chloride were also unreactive to chromium-protein complexes. Apparently *in vitro* conjugation to thes proteins inhibits the antigenic potential of the chromium ion. The experiments are currently being extended, employing complexes of chromium with the synthetic polypeptides poly-L-lysine and poly-L-glutamic acid. The former peptide contains an epsilon amino group in each unit while poly-L-glutamic acid contains free carboxyl groups. We hope to demonstrate thereby which chromium protein linkage, if either, is antigenically more significant.

SUMMARY

Soluble skin protein binds chromium from solutions of either the hexavalent or the trivalent forms. More chromium is bound from solutions of the trivalent compounds. Saturation of the binding sites on the protein molecule was achieved with solutions of trivalent chromium at concentrations of 1.0×10^{-3} to 3.0×10^{-3} M, and the saturation value corresponds to 0.106 mg A Cr/g protein. In the binding of chromium free carboxyl groups are involved to a greater extent than free amino groups.

The binding of chromium by the carboxyl groups of soluble skin protein should involve the cationic trivalent chromium ions. The negatively charged hexavalent ions should be electrostatically repelled by the negative carboxyl groups. Assuming this to be the case, binding from solutions of trivalent chromium is to be expected; binding from solutions of hexavalent chromium should not take place. It is possible, however, that reduction of the hexavalent chromium by the sulfhydryls of the skin protein produces some trivalent chromium for binding. At this time, we are unable to establish if the oxidation of the skin protein or the binding of chromium or perhaps both are responsible for chrome dermatitis. Certainly all three possibilities must be considered.

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