Use of Urea and Salicylate to Elute Antibody from Insoluble Antigen.* (23094)

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As a part of research directed toward the use of antibodies as carriers of radioactivity for therapeutic purposes we are investigating technics of dissociating and rendering soluble an antibody bound to insoluble antigen. Methods commonly used have involved either acid ≈ pH 3.2(1), alkali ≈ pH 11(2), or heat, 60°C(3). Our recent experiments show that some hydrogen bond forming reagents, in particular urea and sodium salicylate, can also be used effectively and conveniently for this purpose. At concentrations breaking antigen-antibody bonds no denaturation or other alteration of rabbit gamma globulin has been detected.

Experimental. Elution with urea. By general methods previously described(4,5) a partially purified preparation of I^{131} labeled rabbit anti-rat Walker carcinoma 256 antibodies was obtained in which approximately 30% of the I^{131} was attached to antibodies that would bind to tumor residue on *in vitro* incubation. The test system used was to incubate with shaking 0.5 ml portions of this anti-tumor preparation with 1 ml of tumor homogenate plus buffer and normal rabbit serum for 1 hour at 37.5°C, using technics previously described(4,5). The residues obtained after centrifugation were washed with pH 8 borate buffer and the bound antibody eluted with urea under varied conditions indicated in columns 2-6 of Table I. Column 7 shows the per cent elution measured as I^{131} remaining in solution in the supernatant after centrifuging. To test specificity of the eluted labeled antibody for rat tumor compared with rabbit tissue, portions of these eluates were incubated once more with rat tumor and with rabbit kidney homogenates. By dilution the urea concentration was below 2.3%. Fixation of labeled antibody to the washed residues is shown in columns 8 and 9. For comparison, the binding to rat tumor and rabbit kidney homogenates of the I^{131} antibody preparation before absorption onto tumor and urea elution is shown as the last 3 experiments in this table. Comparison with the earlier values indicates that all conditions tested for urea elution gave a product with greater specific uptake by rat tumor and less nonspecific uptake by rabbit kidney than found for the untreated preparation. However, increased temperature and higher pH, as well as higher urea concentrations, within the range of the experiment were associated with larger yields of eluted labeled antibody. Further experiments indicated that overnight storage in 16% urea at 2-3°C did not adversely affect eluted antibody as measured in this type of experiment.

Elution with sodium salicylate. Similar experiments were made using solutions of sodium salicylate as an eluting medium, with similar results except that salicylate was active at somewhat lower concentrations; 70% of tissue-bound antibody could be solubilized by stirring it in a 10% sodium salicylate solution for 5 minutes at room temperature. The salicylate eluted antibody reacted similarly to the urea eluted antibody when tested with rat tumor and rabbit kidney homogenate, and seemed not to be harmed by overnight storage at 3-4°C in 10% salicylate solution.

In vivo degradation studies of urea and salicylate treated rabbit gamma globulin. McFarlane has presented convincing evidence (6) that a very sensitive indication of non-physiological alteration or denaturation of blood plasma proteins is an increased rate of elimination from the blood compared with similar unaltered proteins. Twenty mg of gamma globulin were therefore obtained by ammonium sulfate fractionation of normal

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TABLE I. Description of Conditions of Urea Elution of I^{131} Labeled Anti-Walker Tumor Antibodies, % Elution, and Uptake of Eluted Antibody by Tissue Homogenates.

% urea	Solvent	Vol urea, ml	Temp., °C	Elution time, min.	% elution	Uptake by Tumor homog.	Uptake by Rabbit K homog.
16	Dist. H_2O	4	23	15	56.4	50.1	5.6
32	*Idem*	"	"	"	72.2	49.6	6.4
16	"	"	"	5	51.9	49.0	5.7
"	"	"	"	"	54.1	47.9	4.5
"	"	"	37.5	"	59.8	46.3	5.9
"	"	2	23	"	41.3	47.7	4.8
"	"	6	"	"	57.6	48.8	6.3
32	"	4	"	"	71.2	47.2	5.6
16	pH 8 PO_4 buffer	"	"	"	41.5	44.4	8.4
"	9 " "	"	"	"	54.1	47.9	8.2
"	10 " "	"	"	"	73.0	48.2	6.6
"	5 c a* "	"	"	"	49.6	58.4	4.9
"	4 " "	"	"	"	37.8	58.3	2.9
32	Dist. H_2O	"	3–4	"	47.7	58.5	6.4
24	*Idem*	"	23	"	65.6	53.9	5.6
8	"	"	3–4	"	13.1		
"	"	"	23	"	19.3		
—	—	—	—	—	—	30.3	13.8
—	—	—	—	—	—	30.8	14.3
—	—	—	—	—	—	30.5	13.9

* c a = citric acid.

rabbit serum, trace labeled with I^{131}, and divided into 3 portions. One portion was diluted with an equal volume of saline, one with an equal volume of 20% sodium salicylate, and one with 64% urea. After stirring 5 minutes at room temperature the solutions were centrifuged and the supernatants dialyzed for 17 hours against saline. After centrifuging, portions of each solution were injected intravenously into 2 rabbits and 2 rats. Fig. 1 shows the relative radioactivity of blood taken from the 6 rabbits, with the first blood samples, taken 1.5 hours after injection, given a normalized value of 100. Fig. 2 shows the relative radioactivity of the injected rats, as measured in terms of an I^{131} standard with an

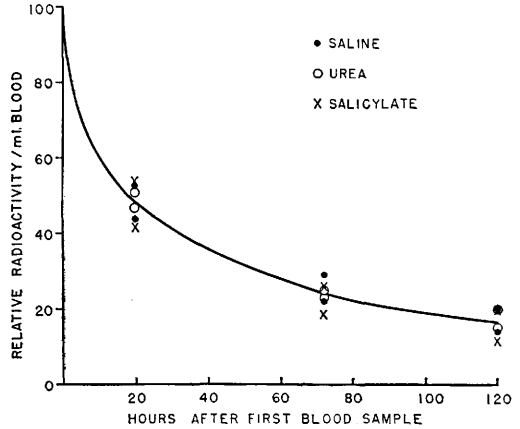

FIG. 1. Relative radioactivity of blood in rabbits after intravenous injection of saline, salicylate, or urea treated I^{131} normal rabbit gamma globulin. Initial blood sampling was made 1½ hrs after injection and this is taken as zero time.

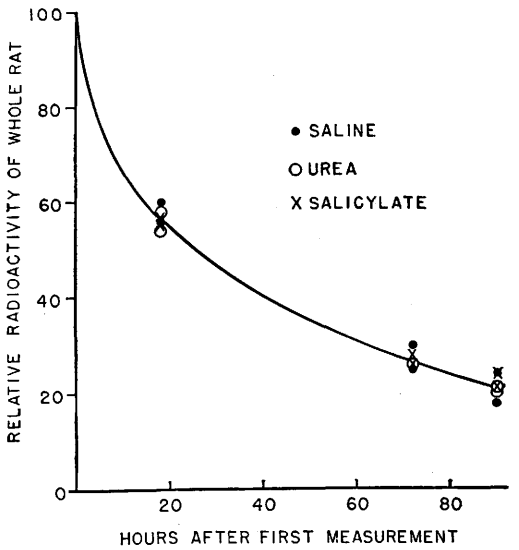

FIG. 2. Relative radioactivity of rats after intravenous injection of saline, salicylate, or urea treated I^{131} normal rabbit gamma globulin. Initial radioactivity determination was performed 1 hour after injection and this is taken as zero time.

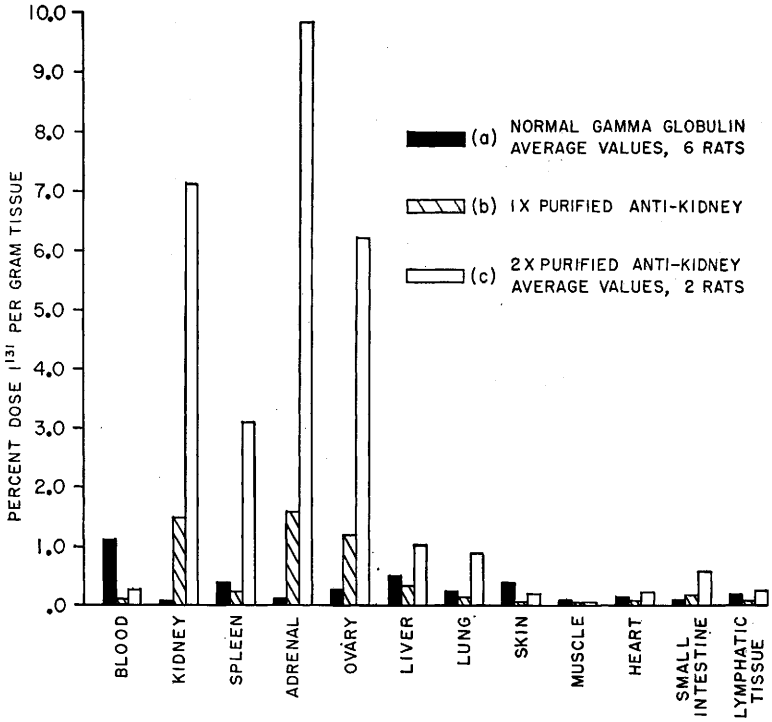

FIG. 3. Distribution of I^{131} in perfused rats following intravenous injection of labeled rabbit serum protein fractions. (a) 4 days after injection of labeled gamma globulin from a normal, non-immunized rabbit, (b) 8 days after injection of a preparation isolated by salicylate elution, before iodination, from serum of rabbits immunized against rat kidney, and (c) 5 days after injection of a fraction of the above preparation, once more purified, after iodination, using salicylate elution.

external scintillation counter, with the radioactivity of each animal, measured shortly after injection, given a value of 100. Determination of radioactivity of various tissues, made after sacrifice by saline perfusion after 90 hours, showed no significant differences between groups. Average values for all 6 animals are shown in Fig. 3. The data indicate that treatment neither with urea nor sodium salicylate has adversely affected gamma globulin as measured by the rate of elimination from the blood in rabbits or by excretion in rats.

In vivo studies with anti-rat kidney gamma globulin. Other experiments indicate that these reagents may be useful in purification of *in vivo* localizing anti-organ antibodies. In preliminary experiments, I^{131} labeled anti-rat kidney gamma globulin prepared in rabbits was absorbed on rat kidney homogenate. The labeled antibodies were then eluted with 10% sodium salicylate and, after saline dialysis, injected intravenously into rats. It was found that this antibody preparation had a several fold larger portion of I^{131} attached to antibodies that would localize in rat kidney than in the initial labeled but unpurified preparation. A similar technic was used on previously unfractionated anti-rat kidney rabbit serum. The following experiment indicates that it is possible to obtain from such an antiserum a fraction rich in rat kidney localizing antibodies. The washed residue from a homogenate of 1.7 g of rat kidney was twice extracted for 5 minutes at room temperature with 55 ml of 20% sodium salicylate. This washed, extracted residue was then incubated with stirring at 37°C for 1 hour with 17 ml of a pooled serum specimen from rabbits immunized against rat kidney. This mixture was then centrifuged, the residue washed twice with saline, then eluted for 5 minutes at room temperature by stirring with 15 ml of 10% sodium salicylate. After cen-

trifuging the supernatant was dialyzed against 15% Dextran to reduce volume, then against a pH 8 borate buffer, and the protein therein trace labeled with I^{131}. Fig. 3 shows the I^{131} distribution found in a perfused rat 8 days after intravenous injection of a portion of this labeled material. Kidney localization at 1 day was 1.82% dose per gram. The kidney localization is several fold higher than would be expected with labeled gamma globulin isolated from the same original pool of immune rabbit serum. This indicates that a higher portion of the protein isolated by this absorption-salicylate elution procedure is anti-kidney antibody than is obtained by a simple gamma globulin isolation. The high localization of I^{131} in rat adrenal and ovary is also characteristic of anti-rat kidney antibodies purified by other methods(5). A second experiment was carried out with this once purified I^{131} labeled preparation to separate labeled anti-kidney antibody from non-specific material labeled because it was present in the initial antibody preparation. Three ml of this material were incubated with a washed and salicylate eluted homogenate derived from 0.4 g of rat kidney, and the absorbed antibody again eluted with 5 ml of 10% sodium salicylate. This eluate contained 6.5% of the initial I^{131}. Salicylate was removed by dialysis. Fig. 3 also shows the average radioactivity present in 2 rats sacrificed by perfusion 5 days after intravenous injection of portions of this twice purified preparation. The I^{131} content of corresponding organs from the 2 rats showed good agreement. The individual kidney values were 7.3 and 6.9. The kidney value for a rat sacrificed at 1 day was 7.7. This second purification, carried out after I^{131} labeling, has thus resulted in a product with a 5.5 fold increase in kidney localizing tendency. It contains about 35% of the I^{131} attached to kidney localizing antibody of the initial iodinated preparation. Later duplicate experiments using either sodium salicylate or urea as eluting agents have given closely similar results.

Discussion. Urea and sodium salicylate have been widely studied because of their solubilizing action on proteins and their ability in strong solutions to modify certain proteins in a way usually described as "denaturation"(7). In weaker solutions these substances affect some proteins in a manner often termed "reversible denaturation" since the proteins are apparently regenerated after the removal of the urea or salicylate(8).

Kleinschmidt and Boyer(9), Coburn(10), and Friend(11) found that 2 M urea, 0.75 M salicylic acid, and 1 M sodium salicylate respectively inhibited the precipitation of soluble protein antigens with their specific antisera, and also dissolved preformed antigen-antibody precipitates. The effect of these agents in dissociating antigen from antibody has been little studied. Kleinschmidt and Boyer(9) reported electrophoresis showed only 5% dissociation of dissolved specific precipitates of egg albumin and its antibody. Gostev(12) apparently found urea active in rupturing the bonds between specific antibody and bacterial structure. Lepper *et al.* (13) suggested that a protective action of salicylates in rabbits against anaphylactic death might be due to a direct effect on the combination of antigen and antibody.

It is generally suggested that the effect of these reagents on protein is to rupture hydrogen bonds, and often thus cause unfolding of the protein. Studies on horse diphtheria antitoxin treated with 20% urea showed the antibody activity to be unaffected(14). Apparently also concentrations of these reagents sufficient to break antigen-antibody bonds, perhaps largely of a hydrogen bond nature, do not affect the integrity of rabbit gamma globulin molecules.

Summary. Solutions of 16-32% urea and of 10% sodium salicylate were used to separate I^{131} labeled rabbit anti-rat organ antibodies from insoluble rat organ residues acting as antigens. Following removal of these eluting agents, the antibody retained its specificity for rat organs as measured by *in vivo* and *in vitro* technics. Measured by *in vivo* technics, rabbit gamma globulin was resistant to the altering or denaturing effect of these reagents.

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Use of I^{131} Labeled Oleic Acid in Study of Gastrointestinal Function.* (23095)

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Previous work using I^{131} labeled neutral fat suggested that use of a radioactive labeled fatty acid could prove valuable in studying gastrointestinal function. This report concerns use of I^{131} labeled oleic acid in the further investigation of certain physiological aspects of digestion and absorption in normal and abnormal subjects.

Procedure. Human subjects. Thirteen humans with no evidence of abnormality related to the digestive tract were used as controls. After 4 hours of fasting, each was given a gelatin capsule containing 0.5 cc of oleic acid which included 25 μc of I^{131} labeled oleic acid. They were then given 20 drops of Lugol's solution and 3 gelatin capsules containing barium sulfate powder. This was taken with small amounts of water. A fasting state was then maintained for the following 6 hour period. Two ml venous blood samples were drawn at hourly intervals at second through sixth hour following ingestion of the capsules. At the third hour fluoroscopic examination of the abdomen was made to determine gastric emptying and location of the barium. The subjects were allowed to return to their normal eating habits after the 6 hour period. All feces passed within 48 hours after the test was collected in individual containers. Blood samples were analyzed for radioiodine content and total radioiodine blood levels were determined and expressed as percentage of ingested material as described by Baylin *et al.* (1). Fecal samples were individually ana-

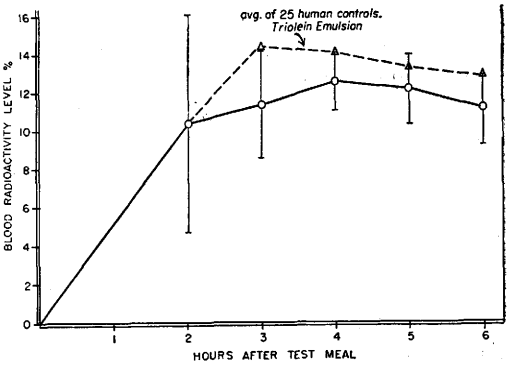

FIG. 1. Blood radioactivity levels (avg of 13 human controls with standard deviation).

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