ON THE HEMOLYTIC ACTION OF *o*-AMINOPHENOL AZO-PROTEIN DERIVATIVES

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Our preliminary paper appeared in October, 1948 (1) reported, in so far as we knew, the first experimental evidence that O.A.-azo-tuberculin† gives powerful tuberculin skin reaction in tuberculous guinea-pigs, and has very low toxicity for guinea-pigs infected with a human strain of tubercle bacilli.

While engaged in the study of the pharmacological action of O.A.-azo-tuberculin derivatives, we have recently noticed a curious phenomenon indicating that O.A.-azo-tuberculin dissolves the red blood cells.††

In view of this finding, it was also of interest to study the effect of various aminophenol azo-protein derivatives upon red blood cells. For this purpose, a number of azo-protein derivatives were prepared and tested for their hemolytic activity, and the results are reported in the present communication.

METHODS, MATERIALS AND TERMINOLOGY

I. Materials tested for their hemolytic activity.—

A. Azo-proteins:

* 1. <i>o</i>-Aminophenol azo-albumin
(=2-Hydroxyphenyl-azo-albumin) ** 2. <i>p</i>-Aminophenol azo-albumin ** 3. Sulfanilic acid azo-albumin ** 4. Anthranilic acid azo-albumin * 5. <i>o</i>-Aminophenol azo-albumin (prepared from horse serum) * 6. <i>o</i>-Aminophenol azo-globulin (prepared from horse serum) * 7. <i>o</i>-Aminophenol azo-albumin egg (prepared from Egg Albumin "Merck") * 8. <i>o</i>-Aminophenol-β-d-glucoside azo-globulin (prepared from horse serum globulin) | } | (prepared from Albumin sanguinis
"Kahlbaum") |
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† The term, O.A.-azo-tuberculin, is employed to denote "*o*-aminophenol azo-tuberculin (= *o*-hydroxyphenyl-azo-tuberculin)", an azo-tuberculin derivative obtained by coupling diazotized *o*-aminophenol with purified tuberculin.

†† The detailed data will be published in the near future.

* S. Koshimura will make a separate report on the chemical phases of these preparations, as soon as the work to clarify the correlation between the diazotization grade of the protein and the grade of hemolytic power of the protein diazotized is completed.

** *p*-Aminophenol azo-albumin, sulfanilic acid azo-albumin and anthranilic acid azo-albumin were prepared according to the Landsteiner and Lampl's procedures (Biochem. Z., 86, 343, 1918; Z. Immunit.-forsch., 26, 293, 1917). [cf. J. Marrack; The Chemistry of Antigens and Antibodies, Med. Res. Council, London, Spec. Rep. No. 194. 1938.]

- * 9. 4-Bromo-2-aminophenol azo-protein
 - * 10. 3-Amino-4-hydroxybenzenesulfonic acid azo-protein
 - * 11. 4-Hydroxy-2-aminophenol azo-protein
 - * 12. 4-Methyl-2-aminophenol azo-protein
 - * 13. 5-Nitro-2-aminophenol azo-protein
 - * 14. 3-Amino-4-hydroxybenzenecarbonic acid azo-protein
 - * 15. 7-Amino-8-hydroxyquinoline-5-sulfonic acid azo-protein
- (prepared from fresh horse serum)
- B. Sources of proteins:
- 16. Albumin sanguinis "Kahlbaum"
 - 17. Albumin (prepared from horse serum)
 - 18. Globulin (prepared from horse serum)
 - 19. Egg Albumin "Merck"
- C. Miscellaneous:
- 20. *o*-Aminophenol
 - 21. *p*-Aminophenol
 - 22. Sulfanilic acid
 - 23. *o*-Aminobenzoic acid (Anthranilic acid)
 - 24. Saponin (Saponin "Merck" has been used throughout for control.)

II. Original solution of the materials tested.—

The materials to be tested were all diluted with 0.85% NaCl solution.

Protein materials:

20.0 mg of the azo-protein derivative (undiazotized protein also) to be tested was dissolved in 1.8 cc of 0.85% salt solution with the aid of one or two drops of normal sodium hydroxide and the solution is then neutralized with dilute hydrochloric acid. This is then brought to 2.0 cc by the addition of 0.85% salt solution and adjusted to pH of 7.0—7.8 with the aid of indicators, as specified on Table 2. (The excess of the NaOH or HCl solution must be rigidly avoided.) Thus the concentration of the original solution was 1:100.

Non-protein materials:

20.0 mg of *o*-aminophenol (*p*-aminophenol also) was dissolved in 1.0 cc of 0.85% salt solution.

20.0 mg of sulfanilic acid (anthranilic acid also) was dissolved in 2.0 cc of 0.85% salt solution with the aid of a small amount of normal sodium hydroxide.

III. Test for hemolytic power.—

The method is the same as that described in the earlier reports (2)—(5): Serial dilutions of the material to be tested were made in 0.85% NaCl solution (the total volume of the each test tube was 1.0 cc) and 1.0 cc of 1% suspension of 3 time washed rabbit's red blood cells was added to each tube. All tubes were thoroughly shaken, placed in an incubator at 37°C for 2 hours, and then placed in an ice-box for 22 hours.

EXPERIMENTAL DATA

Table 1 represents the results of hemolysis experiments carried out with *o*-aminophenol azo-albumin prepared from Albumin sanguinis "Kahlbaum", *o*-aminophenol, Albumin sanguinis "Kahlbaum" and saponin.

It will be noted from this table that *o*-aminophenol azo-albumin showed hemolytic activity up to a dilution of 1:50,000 at the end of 24 hours, while neither *o*-aminophenol nor Albumin sanguinis "Kahlbaum" showed any appreciable hemolytic activity. In the experiment with saponin, the hemolytic effect was obtained up to a dilution of 1:500,000.

Table 1. The effect in vitro of *o*-aminophenol Azo-protein, *o*-aminophenol, albumin and saponin upon erythrocytes.

Material tested [pH of the original solution]	Readings taken after	Dilutions of materials												
		50	100	200	500	1,000	2,000	5,000	10,000	20,000	50,000	100,000	200,000	500,000
<i>o</i> -Aminophenol azo-albumin, prepared from albumin sanguinis "Kahlbaum" [pH=7.4]	30 min.	•	•	•	###	###	—	—	—	—	—	—	•	•
	1 hr.	•	•	•	###	###	###	+	—	—	—	—	•	•
	2 hr.	•	•	•	###	###	###	###	+	—	—	—	•	•
	24 hr.	•	•	•	###	###	###	###	###	+	—	—	•	•
	30 min.	•	•	•	—	—	—	—	—	—	—	•	•	•
<i>o</i> -Aminophenol [pH=7.8]	1 hr.	•	•	•	—	—	—	—	—	—	—	•	•	•
	2 hr.	•	•	•	—	—	—	—	—	—	—	•	•	•
	24 hr.	•	•	•	—	—	—	—	—	—	—	•	•	•
	30 min.	—	—	—	—	—	•	•	•	•	•	•	•	•
Albumin sanguinis "Kahlbaum" [pH=6.2]	1 hr.	—	—	—	—	—	•	•	•	•	•	•	•	•
	2 hr.	—	—	—	—	—	•	•	•	•	•	•	•	•
	24 hr.	—	—	—	—	—	•	•	•	•	•	•	•	•
	30 min.	•	•	•	•	###	###	###	###	###	###	###	+	—
Saponin "Merck" [pH=6.0]	1 hr.	•	•	•	•	###	###	###	###	###	###	###	+	—
	2 hr.	•	•	•	•	###	###	###	###	###	###	###	+	—
	24 hr.	•	•	•	•	###	###	###	###	###	###	###	+	—

Notes: 1 cc of 1% washed rabbit's red cells suspension was added to 1 cc of the graduated dilutions of the material.

indicates complete hemolysis; ##, + and ± indicate partial hemolysis; — indicates no hemolysis.

Similar results were obtained in the experiments with *o*-aminophenol azo-albumin prepared from horse albumin, albumin prepared from horse serum, and *o*-aminophenol.

Table 2 represents the summaries of a large number of such comparative hemolysis experiments carried out upon 15 azo-protein derivatives, 4 proteins, and *o*-amino-

[illegible]

phenol, *p*-aminophenol, sulfanilic acid, anthranilic acid and saponin, in relation to their structural formulas. Special attention may here be directed to following points:

1. *o*-Aminophenol azo-albumin, *o*-aminophenol azo-globulin, *o*-aminophenol azo-albumin egg and 4-bromo-2-aminophenol azo-protein were all found to have distinct hemolytic activity.

By contrast, 3-amino-4-hydroxybenzenesulfonic acid azo-protein, 4-hydroxy-2-aminophenol azo-protein, 4-methyl-2-aminophenol azo-protein, 5-nitro-2-aminophenol azo-protein, 3-amino-4-hydroxybenzenecarbonic acid azo-protein and 7-amino-8-hydroxyquinoline-5-sulfonic acid azo-protein, all do not share the hemolytic properties of the above-mentioned *o*-aminophenol azo-protein derivatives.

2. *p*-Aminophenol azo-albumin sulfanilic acid azo-albumin and anthranilic acid azo-albumin, all contrasted strongly with *o*-aminophenol azo-proteins in failing to cause hemolysis.

It is also particularly noteworthy that *o*-aminophenol- β -D-glucoside azo-globulin was found to be entirely without hemolytic effect.

3. Note that no hemolysis was observed in any of the series of dilutions made from the undiazotized proteins, namely Albumin sanguinis "Kahlbaum", Egg albumin "Merck", horse serum albumin and horse serum globulin. Moreover simple organic compounds such as *o*-aminophenol, *p*-aminophenol, sulfanilic acid and anthranilic acid, were all proved non-hemolytic.

Thus, as a result of this limited number of experiments, it may, at least, be said that when *o*-aminophenol is diazotized and linked to protein, which is in itself devoid of hemolytic power, the resulting product acquires a new property, i.e., the ability to lyse erythrocytes, as the same was the case with tuberculin.

Up to August, 1948, we could offer no explanation for this curious phenomenon. Very recently, it has however been shown by S. Koshimura that both *o*-aminophenol azo-histidine and *o*-aminophenol azo-tyrosine have a fairly high hemolytic power,[†] and from these observations it now becomes, at least, to be supposed that the presence of the intramolecularly situated groups of *o*-aminophenol azo-histidine and *o*-aminophenol azo-tyrosine is essential condition for the hemolytic property possessed by the above-mentioned *o*-aminophenol azo-protein derivatives. Further work is in progress to elucidate this question.

[†] In connection with these data, the work which was done by T. Okami (6) of this laboratory is particularly noteworthy: He found that 3,3',5,5'-tetrabromo-2,2'-dihydroxy-azobenzene [I] possesses about the same hemolytic activity as does saponin, and that 2,2'-dihydroxy-azobenzene [II] itself is a slightly hemolytic agent.

CONCLUSION

Evidence has been presented for the phenomenon of acquiring hemolytic property of proteins by coupling them with diazotized *o*-aminophenol.

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