**EXPERIMENTAL STUDY ON THE INFLUENCE OF AZO-TUBERCULIN
UPON GUINEA-PIGS INFECTED WITH TUBERCLE BACILLI
PART II. ISOLATION OF THE *o*-AMINOPHENOL AZO-TUBERCULIN
DERIVATIVE FROM "SAUTON'S SYNTHETIC-MEDIUM
OLD TUBERCULIN"**

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The preceding paper (1) of this series includes reports on the biological properties of *o*-aminophenol azo-tuberculin derivative prepared by coupling purified tuberculin (Maschmann) with diazotized *o*-aminophenol.

Due to the lamentable circumstances in Japan, which oppose a repetition of azo-tuberculin experiments with purified tuberculin on a large scale, studies were being continued in our laboratory in an effort to find more simple method for obtaining *o*-aminophenol azo-tuberculin.

The present communication records the newly devised method for preparing highly active *o*-aminophenol azo-tuberculin derivative from "Sauton's synthetic-medium Old Tuberculin" itself.

I. Isolation of *o*-Aminophenol Azo-Tuberculin Derivatives

a) Preparation of a Sauton's Synthetic-Medium Old Tuberculin:

5,600 cc of Sauton's synthetic medium* was distributed in 28 half-litre bottles, 200 cc to a bottle, and all bottles of medium were planted at a time with the human type of tubercle bacillus, strain H₂, in the usual manner. After incubation at 37°C for ten weeks, the cultures were shaken and heated in a steam sterilizer at 100°C for one hour. The bacilli were filtered from the culture liquid through filter paper. The total volume of filtrate was 3,850 cc.

The filtrate was then concentrated on the water bath to a volume of 770 cc, that is, to one-fifth the original volume of the culture filtrate. After passing through Seitz E. K. filter, 170 cc of this concentrated liquid** was withdrawn and preserved

* The formula of the Sauton's synthetic medium:

Asparagine	0.4	} (Adjusted to pH 7.2 with ammoniac.)
Citric acid.....	2.0	
Potassium monohydrogen phosphate.....	0.5	
Magnesium sulfate	0.5	
Ferric ammonium citrate	0.05	
Glycerol.....	60.0	
Distilled water	1000.0	

** The concentrated liquid was called SOT, meaning "Sauton's synthetic-medium Old Tuberculin", to fit into a scheme of abbreviated nomenclature.

for control purposes throughout the biological experiments.

The remaining 600 cc of the Sauton's synthetic-medium Old Tuberculin (SOT) was used for the experiments concerned with the correlation between azotization grade and tuberculin activity.

b) Azotization Experiments of Sauton's Synthetic-Medium Old Tuberculin (SOT) with *o*-Aminophenol Diazonium Chloride:

The remaining 600 cc of SOT was divided into six equal lots of 100 cc each, designated as Lots I to VI. To six lots of SOT, six different amounts of *o*-aminophenol diazonium chloride were added respectively, as indicated in Table 1. As further manipulations were the same in the all lots, so only the procedures employed for the isolation of *o*-aminophenol azo-tuberculin from the Lot V will be described below in detail:

1.0 g of *o*-aminophenol base was dissolved in 22 cc of N/1 HCl and diazotized with 0.63 g of sodium nitrite dissolved in 8 cc distilled water, at a temperature of 0°~5°C with starch iodide paper as indicator. After the solution showed no excess of nitrite, it was diluted with ice water to a volume of 80 cc and added to a cold mixture of 100 cc SOT (Lot V) and 30 cc N/1 NaOH. The mixture, chilled with ice, was kept weakly alkaline to litmus by adding about 10 cc N/1 NaOH.

After standing overnight at ice box temperature, the mixture was carefully acidified with N/1 HCl to a point of maximum precipitation, the pH of the solution being 4.8. The highly colored precipitate of *o*-aminophenol azo-tuberculin (=O.A.-azo-tuberculin) was removed by centrifugation.

The precipitate, after washing three times with 0.85% NaCl solution (using 50 cc in each time), we call "crude O.A.-azo-tuberculin." The three washings were combined with the supernatant.

(i) Purification of crude O.A.-azo-tuberculin derivative:

The crude O.A.-azo-tuberculin was dissolved in 20 cc of salt solution by adding the minimal amount of N/10 NaOH, some insoluble material centrifuged out, and from the dark brown supernatant the active substance (that is, O.A.-azo-tuberculin) was reprecipitated by acidifying to pH 5.0 with N/10 HCl. Solution and reprecipitation were repeated more two times. The precipitate was then dissolved in 15 cc of saline with the aid of a little N/10 NaOH, and to the solution, with stirring, three volumes of cold 60% alcohol was added and subsequently enough N/10 HCl to flocculate the material.

After centrifugation, the precipitate obtained was redissolved in 20 cc salt solution with a small amount of N/10 NaOH, and the solution was dialyzed, in fish bladder membrane, against running water for about 24 hours. From the solution left in the dialyzing sac O.A.-azo-tuberculin was precipitated by the addition of N/10 HCl, washed with distilled water and finally dried in a vacuum desiccator.

About 142 mg of O.A.-azo-tuberculin derivative was obtained in the form of a

deeply reddish-brown, amorphous substance.

(ii) The residual part:

A mixture of the supernate and the three washings was adjusted to pH 5.4, concentrated on the water bath to the original volume of SOT (100 cc) and passed through Seitz E. K. filter. The brownish-red colored liquor thus obtained, we call "residual part."

Table 1. The degree of coupling the SOT with diazotized *o*-aminophenol and the yields of O.A.-azo-tuberculin derivatives.

No. of SOT-lot	I	II	III	IV	V	VI
<i>o</i> -Aminophenol used for azotization (in mg)	20	100	400	800	1000	1600
Yields of O.A.-azo-tuberculin derivatives (in mg)	less than 4	9	30	49	142	83

Thus, the yield of O.A.-azo-tuberculin derivatives was the greatest in the azotization experiment of No. V-lot (142 mg) followed respectively by No. VI-lot (83 mg), No. IV-lot (49 mg), No. III-lot (30 mg), No. II-lot (9 mg) and No. I-lot (less than 4 mg).

It will be noted with exception of the yield from lot VI in Table 1 that the yield of O.A.-azo-tuberculin derivatives rises in proportion to the quantity of *o*-aminophenol used for azotization.

II. Biological Properties

1. Skin Test.—

A. Experiments with O.A.-azo-tuberculin preparations.

Five O.A.-azo-tuberculin preparations, Nos. II to VI, were tested for their skin-reacting potency, using the original Sauton's medium Old Tuberculin as a standard of comparison.

Methods

1) Substance used:

(i) O.A.-azo-tuberculin preparations, Nos. II, III, IV, V, and VI.

0.5 mg of O.A.-azo-tuberculin preparation to be tested was dissolved in 5.0 cc of sterile 0.85% NaCl solution with the aid of the minimal amount of N/10 NaOH. (Thus, 0.1 cc of this solution contains 0.1 mg of O.A.-Azo-T. "S".) This original solution was diluted with sterile salt solution serially, i. e., 0.01 mg in 0.1 cc, 0.001 mg in 0.1 cc, 0.0001 mg in 0.1 cc, 0.00005 mg in 0.1 cc and 0.00002 mg in 0.1 cc.**

(ii) SOT (=The clear fluid left after removal of tubercle bacilli grown on the Sauton's medium was evaporated until reduced to one-fifth of its original volume.)

0.5 cc of the SOT was diluted with 4.5 cc of sterile 0.85% NaCl solution. (Thus, 0.1 cc

* For convenience, the abbreviated nomenclature, O.A.-Azo-T. "S", is occasionally employed to denote "*o*-aminophenol azo-tuberculin derived from the Sauton's synthetic-medium Old Tuberculin".

** We have chosen to report O.A.-Azo-T. "S" dosage as the actual amount of the preparation in mg contained in the 0.1 cc of solution received into the skin.

of this solution contains 0.01 cc (=10 "mg"*) of SOT.) This original solution was diluted serially, i.e., 1 "mg" in 0.1 cc, 0.1 "mg" in 0.1 cc, 0.05 "mg" in 0.1 cc and 0.02 "mg" in 0.1 cc.*

2) Animals:

- Guinea-pigs were infected (subcutaneously in the inner side of the left thigh) 4 weeks previously with 1 mg of human type of tubercle bacillus, strain H₂.

3) Injection of tuberculin:

The two tuberculins under comparison, O.A.-Azo-T. "S" and SOT, were injected intracutaneously into corresponding regions of the opposite flanks of tuberculous guinea-pig. On the other hand, control animal (non-tuberculous) received the following amounts of the two tuberculins, i.e., 0.1 mg of O.A.-Azo-T. "S" and 10 "mg" of SOT.

4) Reading the results:

Readings were made 24, 48 and 72 hours after injection. The figures were millimeter measurements of reaction (redness and induration of the skin).

Results

The results of this test are summarized in Table 2.

- 1) O.A.-azo-tuberculin preparations, Nos. II, III, IV, V and VI, all were found to have approximately equal potency in eliciting the specific skin reaction in tuberculous guinea-pigs; a dose of 0.01 mg of the O.A.-azo-tuberculin preparations gave a definite positive skin reaction, and a dose of 0.000,1 mg a well marked redness at the site of injection.
 - 2) In the normal animals, even when such a large dose as 0.1 mg was injected none of the above-mentioned O.A.-azo-tuberculin preparations caused and appreciable redness at the site of injection.
 - 3) It is clear from the figures that the doses from 0.001 mg to 0.000,1 mg of the O.A.-azo-tuberculin preparations, Nos. II to VI, are equivalent in skin-reacting potency to 0.1 "mg" SOT.**
 - 4) In regard to the type of skin reaction, it may at least be said that there could not be observed any distinction between the II-, III-, IV-, V- and VI-preparations, and the SOT.
- B. Experiments with Residual Parts.

Six residual parts are also tested for their skin-reacting potency, controlling with the original SOT.

Methods

1) Substance used:

- (i) The residual parts, Nos. I, II, III, IV, V and VI.
- (ii) SOT.

The sample to be tested was diluted with sterile salt solution serially, i.e., 0.01 cc in

* In this experiment, the figures "mg" given for SOT indicate the amount of original SOT in mm³ contained in the 0.1 cc of solution received into the skin.

** The activity of O.A.-Azo-T. "S" on adults was investigated by Prof. Dr. M. Kakishita and his co-workers who stated that 0.000,05 mg of O.A.-Azo-T. "S" is equivalent in skin-reacting potency to 0.05 "mg" (=0.1 cc of 1/2,000) Koch's Old Tuberculin.

0.1 cc, 0.001 cc in 0.1 cc and 0.000,1 cc in 0.1 cc.

2) Animals:

Guinea-pigs were infected 4 weeks previously with 1 mg of human type of tubercle bacillus, strain H₂.

3) Injection of tuberculin:

The two tuberculins under comparison, residual part and SOT, were injected intracutaneously into corresponding regions of the opposite flanks. Control animal (non-tuberculous) received 0.01 cc of a residual part and 0.01 cc of SOT.

3) Reading the results:

Readings were made 24, 48 and 72 hours after injection.

Results

This group of tests are shown in Table 3. The significant results were as follows:

No. I-, No. II- and No. III-residual parts were of approximately equal strength which may be compared with the potency possessed by the original SOT, but both the residual parts, Nos. IV and V, compared with the above-mentioned three parts were very weak, causing a weak reaction in the most sensitive guinea-pig. The residual part VI was found to have lost its skin-reacting activity completely.

It will be noted from this and the above-mentioned experiments that the skin-reaction producing activity of the residual parts is in inverse proportion to the quantity of *o*-aminophenol used for azotization of the original SOT.

2. Lethal Test.—

The results summarized in the Tables 4 and 5 include those secured with the O.A.-azo-tuberculin preparations, Nos. III to V, the residual parts, Nos. II to VI, and the SOT.

It will be noted from these experiments that intraperitoneally in a salt solution of the O.A.-azo-tuberculin preparations under consideration the minimal lethal dose for tuberculous guinea-pigs (250~270 g of body weight) seems to be laid between 3 and 5 mg, and that a dose of 2 cc of the II-, III- and IV-residual parts on the other hand was found to be the minimal lethal dose, whereas a 2 cc dose of the V- and VI-residual parts not lethal to tuberculous animals. The original SOT caused death of tuberculous animals when injected intraperitoneally even in doses of 0.2 to 0.5 cc.

Thus, as contrasted with our first report (1), the O.A.-azo-tuberculin derived from the Sauton's synthetic-medium Old Tuberculin appeared to show a general toxicity for tuberculous guinea-pigs to a slightly greater degree than did the O.A.-azo-tuberculin derived from the Maschmann's purified tuberculin; this might be owing to the contamination of the derivatives used in our later experiment.

Whether the biological properties of the *o*-aminophenol azo-tuberculin derivatives ("S") are due to one or to two substances has not yet been conclusively proved, but, from our works, it may, at least, be said that the active principle of tuberculin appears to be transformed into the easily precipitable *o*-aminophenol azo-derivative

without being impaired its original activity.

CONCLUSIONS

1. A very simplified method is described whereby *o*-aminophenol azo-tuberculin derivative may be prepared from the concentrates of the clear fluid left after removal of tubercle bacilli grown on the Sauton's synthetic medium with *o*-aminophenol diazonium chloride.
2. This work demonstrates that the *o*-aminophenol azo-tuberculin derived from the Sauton's synthetic-medium Old Tuberculin are very active in skin test for tuberculous guinea-pig.
3. In normal guinea-pig, the derivative causes no skin reaction.

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REFERENCES

- 1) Ito, R. and Koshimura, S.: Experimental study on the influence of azo-tuberculin upon guinea-pigs infected with tubercle bacilli (Preliminary report). Jap. Med. J., 1, 427, 1948.
[cf. Ito, R., Koshimura, S. and Fujita, S.: On the hemolytic action of *o*-aminophenol azo-protein derivatives. Jap. Med. J., 2, 130, 1949.]
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Table 2. Comparative skin tests with O.A.-azo-tuberculin derivatives and SOT.

Guinea-pig			O.A.-azo-tuberculin					SOT			
No.		Body weight (g)	No. of prepa-ration	Dose (mg)	Results taken after			Dose ("mg")	Results taken after		
					24 hr.	48 hr.	72 hr.		24 hr.	48 hr.	72 hr.
Tuberculous	150	320	II	0.01	18×20	15×16 (h)	8×10 (h)	10.0	20×21	16×17 (h)	15×16 (h)
	151	370		0.001	17×18	14×17	12×14	1.0	18×20	17×19	13×16
	152	335		0.000,1	11×12	7×8	2×2	0.1	14×15	8×9	2×3
Non-tuber-culous		430		0.1	—	—	—	10.0	—	—	—
Tuberculous	153	330	III	0.01	30×30 (h)	32×35 (n)	20×22 (n)	10.0	30×30 (h)	30×34 (n)	30×30 (n)
	154	345		0.001	10×14 (h)	9×10 (h)	7×12 (n)	1.0	13×16 (h)	17×17 (h)	12×14 (n)
	155	280		0.000,1	11×12	8×9	5×6	0.1	15×20	10×10	10×10
	156	330		0.000,05	8×10	2×4	—	0.05	11×15	5×6	—
	157	340		0.000,02	11×11	3×3	—	0.02	15×15	9×9	—
Non-tuber-culous		430		0.1	—	—	—	10.0	—	—	—
Tuberculous	158	375	IV	0.01	23×25	18×20	18×20	10.1	24×25	20×20	18×19
	159	320		0.001	12×12	8×9	7×7	1.0	12×14	11×12	3×4
	160	340		0.000,1	13×16	8×9	6×7	0.1	23×26	12×14	10×10
Non-tuber-culous		375		0.1	—	—	—	10.0	—	—	—
Tuberculous	161	420	V	0.01	10×11	12×12	2×2	10.0	14×16	14×17	2×3
	162	460		0.001	11×12	12×13	6×6	1.0	15×17	16×17	9×10
	163	305		0.000,1	14×14	9×9	2×2	0.1	16×18	12×15	11×12
Non-tuber-culous		350		0.1	—	—	—	10.0	—	—	—
Tuberculous	164	300	VI	0.01	35×35 (h)	25×35 (n)	20×25 (n)	10.0	28×30 (h)	33×34 (n)	24×25 (n)
	165	340		0.001	10×10	4×5	4×5	1.0	12×16	11×14	7×9
	166	315		0.000,1	12×13	5×8	2×3	0.1	16×20	10×11	3×4
Non-tuber-culous		375		0.1	—	—	—	10.0	—	—	—

Remarks: (h) = Ecchymosis, (n) = Necrosis.

18×20 = Diameters of redness and induration in mm.

— = No trace of redness of induration at the site of injection.

Table 3. Comparative skin tests with the residual parts and SOT.

Guinea-pig			Residual part					SOT			
No.		Body weight (g)	No.	Dose (cc)	Results taken after			Dose (cc)	Results taken after		
					24 hr.	48 hr.	72 hr.		24 hr.	48 hr.	72 hr.
Tuberculous	170	350	I	0.01	14 × 16	11 × 12	—	0.01	16 × 16	14 × 14	—
	171	425		0.001	19 × 20	25 × 25	17 × 22	0.001	18 × 22	25 × 26	22 × 22
	172	400		0.000,1	10 × 12	10 × 10	—	0.000,1	12 × 15	10 × 10	—
Non-tuberculous		370		0.01	—	—	—	0.01	—	—	—
Tuberculous	173	355	II	0.01	22 × 22	21 × 26 (h)	11 × 12 (h)	0.01	22 × 27	23 × 26 (h)	14 × 16 (h)
	174	440		0.001	16 × 18	14 × 16	7 × 9	0.001	24 × 30 (h)	20 × 20 (h)	10 × 10 (h)
	175	465		0.000,1	19 × 22	12 × 14	—	0.000,1	22 × 26	18 × 19	4 × 4
Non-tuberculous		370		0.01	—	—	—	0.01	—	—	—
Tuberculous	176	390	III	0.01	22 × 25	17 × 18	12 × 16	0.01	20 × 22	22 × 22	18 × 22
	177	465		0.001	16 × 16	14 × 14	13 × 14	0.001	15 × 20	14 × 16	14 × 14
	178	370		0.000,1	13 × 16	10 × 14	4 × 4	0.000,1	13 × 14	10 × 15	12 × 13
Non-tuberculous		340		0.01	—	—	—	0.01	—	—	—
Tuberculous	179	320	IV	0.01	11 × 12	7 × 9	—	0.01	12 × 14	12 × 15	10 × 14
	180	345		0.001	13 × 14	12 × 14	3 × 4	0.001	22 × 24	20 × 24 (h)	12 × 14 (h)
	181	370		0.000,1	—	—	—	0.000,1	14 × 17	10 × 11	2 × 3
Non-tuberculous		480		0.01	—	—	—	0.01	—	—	—
Tuberculous	182	305	V	0.01	3 × 4	—	—	0.01	12 × 14	13 × 14	5 × 7
	183	390		0.001	—	—	—	0.001	17 × 20	15 × 15	12 × 13
	184	410		0.000,1	5 × 7	2 × 2	—	0.000,1	22 × 24	12 × 13	11 × 12
Non-tuberculous		350		0.01	—	—	—	0.01	—	—	—
Tuberculous	185	340	VI	0.01	—	—	—	0.01	18 × 20 (h)	22 × 24 (n)	14 × 15 (n)
	186	330		0.001	—	—	—	0.001	13 × 18 (h)	24 × 25 (h)	12 × 13 (n)
	187	320		0.000,1	—	—	—	0.000,1	10 × 12	7 × 9	2 × 3
Non-tuberculous		430		0.01	—	—	—	0.01	—	—	—

See remarks of Table 2.

Table 4. Lethal tests with O.A.-azo-tuberculin derivative.

- 1) Substance used: O.A.-azo-tuberculin preparations, Nos. III, IV, V and VI.
20.0 mg of O.A.-Azo-T. "S" to be tested was dissolved in 10.0 cc of sterile salt solution with the aid of a little N/10 NaOH.
Thus, 1.0 cc of this solution contains 2.0 mg of O.A.-Azo-T. "S".
- 2) Animals: Guinea-pigs were infected 6 weeks previously with 1 mg of human type of tubercle bacillus, strain H₂.
- 3) Injection of substance: Intraperitoneally.

O.A.-azo-tuberculin		Tuberculous guinea-pig		Results
No. of preparation	Dose (mg)	No.	Body weight (g)	
III	5	251	310	D ₄
	4	252	355	D ₄
	2	253	420	L
		254	400	L
IV	10	255	310	D ₅
		256	410	D _{<24}
	5	257	330	D ₅
V	10	258	350	D ₅
		259	340	D ₄
	5	260	420	D ₅
		261	380	L
	2	262	290	L
		263	235	D _{<21}
VI	10	264	340	D ₅
		265	370	D ₅
	2	266	345	L
		267	320	L

Remarks: D₄ = Died in 4 hours, D_{<24} = Died within 24 hours.
L = Lived.

Table 5. Lethal tests with residual part and SOT.

- 1) Substance used :
 - a) The residual parts, Nos. II, III, IV, V and VI.
 - b) SOT.
- 2) Animals: Guinea-pigs were infected 6 weeks previously with 1mg of human type of tubercle bacillus, strain H₂.
- 3) Injection of substance: Intraperitoneally.

	Substance tested		Tuberculous guinea-pig		Results
	No.	Dose (cc)	No.	Body weight (g)	
Residual parts	II	2	268	305	D ₉
			269	350	L
		1	270	300	D ₆
			271	295	D ₆
	III	2	272	315	L
			273	320	D ₆
	IV	2	274	300	D _{<24}
			275	330	D _{<24}
	V	2	276	330	L
			277	325	L
	VI	2	278	375	L
			279	305	L
SOT	1	1	286	350	D _{<24}
			287	330	D _{<24}
	0.5	0.5	288	440	D _{<24}
			289	405	D _{<24}
	0.2	0.2	292	365	D _{<24}
			293	310	L

See remarks of Table 4.