STUDIES ON THE ISOMER OF SUCCINIC ACID

III. ANTICANCER SCREENING TESTS ON SOME SUCCINIC ACID MODIFICATIONS

Tomoyoshi KAMATA, Tsunetoshi SUGA and Tadataka HARA

Department of Veterinary Pharmacology, Nippon Veterinary and Zootechnical College

(Received for Publication July 24, 1969)

Deodorizing crystalline substances were extracted from some fermentation products of yeast with ether under such conditions from which day-light had been shut out. They were identified as modifications of the isomers of succinic acid²⁾. Their anticancer activity has been reported³⁾.

It seems that Dupré LA TOUR⁴⁾ may have got his crystals by a chemical method. So the authors intended to get these modifications of succinic acid by some chemical methods.

In the present investigation, a practical recognition method was used to examine anticancer activity against mouse Ehrlich ascites carcinoma, but no physico-chemical method was adopted for the identification of any modification. The results of drug evaluation were expressed by the percentage of animals surviving for 50 days after inoculation with carcinoma cells. A drug which had exhibited exceeding a survival rate 70% was named an active succinate in this report.

TESTING METHOD OF ANTICANCER ACTIVITY

Mice of the ddN strain weighing 20 ± 1 g were used as experimental animals. Male and female were separated from each other. All the animals were kept in metallic cages, in which chopped shavings were spread. They were allowed to take ad libitum water, feed in pellet form produced by the Oriental Yeast Company, and a small amount of dried small sardine.

Eight animals were housed in a cage. Two cages were used as a group at one time. Each experiment was repeated two or three times. Ehrlich ascites carcinoma cells were suspended in physiological saline, and such amount of suspension as containing about four millions of them (0.2 ml) was inoculated intraperitoneally into each mouse. The inoculated mice were weighed every other day and held under observation for 50 days. At the end of this period, the surviving animals were sacrificed by the inhalation with ethylether and subjected to autopsy. A sample was also harvested on a slide glass from the ascites of each of them and stained with Giemsa stain for microscopical examination to see whether the carcinoma cells were still present in the ascites or not.

Each drug was dissolved in distilled water at a concentration of one percent. The reaction of each resultant solution was adjusted to pH 4.6 with 5 N sodium hydroxide solution. Then the solution was poured into brown-glass Earlenmeyer's flasks with a ground-glass stopper, which were stored in the ice-box.

Each drug was injected into mice by the intraperitoneal route in a dose of 25 mg per kg of body weight once a day for 7 successive days, beginning at 24 hours after the inoculation of Ehrlich carcinoma cells.

Mitomycin C, endoxan, and toyomycin were employed as control drugs in each experiment.

Mitomycin C was dissolved in distilled water at a concentration of 0.1 mg/ml and injected intraperitoneally in a dose of 1.0 mg/kg once a day for 7 consecutive days starting at 24 hours after the inoculation.

Endoxan was dissolved in distilled water at a concentration of 0.2 mg/ml and injected intraperitoneally in a dose of 2.0 mg/kg once a day for 7 consecutive days starting at 24 hours after the inoculation of Ehrlich carcinoma cells.

Toyomycin was dissolved in distilled water at a concentration of 0.05 mg/ml and injected intraperitoneally in a dose of 0.01 mg/kg once a day for 7 consecutive days starting at 24 hours after the inoculation of Ehrlich carcinoma cells.

The drugs were evaluated by the percentage of animals surviving for 50 days after Ehrlich carcinoma cells had been inoculated.

METHOD I TO OBTAIN A SUCCINIC ACID MODIFICATION

The comparative test used for ultraviolet spectrum absorbancy between the isomer of succinic acid and the ordinary succinic acid was the same as employed in the previous experiments²⁾. In the present experiments, the following phenomenon was also observed. An ordinary aqueous solution of succinic acid decreased in absorbancy with the lapse of time after it was prepared. It revealed nearly the same absorbancy as the isomer of succinic acid 24 hours after it had been prepared by dissolution in distilled water. The aqueous solution of the isomer of succinic acid obtained by the fermentation method showed no change in absorbancy. This phenomenon was observed when distilled water had been poured in to a glass receptacle, but was not when a filtered ion-exchange resin column had been used or when the sample was stored in a resin (polyethylene) receptacle for a few days.

It seems to the authors that the decrease in absorbancy may suggested the transformation of the molecular formula in the dissolved substance. The cause for the transformation would be attributed to the change in value of pH of the water induced by carbon dioxide in the air dissolved in the water.

Then the pH was measured in some kinds of water. When distilled water was poured in a glass receptacle, its pH changed in value gradually after distillation at room temperature.

The values of pH measured were as follows.

Immediately after distillation		pH
		7.0
after	5 minutes	6.8
after	30 minutes	6.5
after	3 hours	6.0
after	24 hours	5.6
after	72 hours	5.4
after	96 hours	5.4

When distilled water was poured in a resin receptacle, its pH changed in value in the following manner.

Immediately after distillation	pH	7.0
after 5 minutes		6.8
after 30 minutes		6.6
after 3 hours		6.4
after 24 hours		6.2
after 72 hours		6.0
after 96 hours		6.0

When water was passed on the ion-exchange resin column and stored in a resin receptacle, its pH changed in value in nearly the same manner as mentioned above.

Carbon dioxide gas was blown into the three kinds of water mentioned above at 10°C. The value of pH changed in the following manner.

CO ₂ gas blow into water	Beginning	For after 2min.	For after 10min.	For after 20min.
(a) Distilled water stored in a glass receptacle	5.4	4.4	3.5	2.9
(b) Distilled water stored in a resin receptacle	6.0	4.6	4.4	4.4
(c) Water passed on an ion exchange resin column and stored in a resin receptacle	6.0	4.8	4.6	4.5

Then pH values were measured in the three specimens, (a) to (c), after 24 hours' storage at 10°C. They were as follows:

2.0 in (a), 5.8 in (b), and 6.0 in (c).

Ordinary succinic acid was dissolved at a concentration of one per cent immediately after carbon dioxide gas had been blown into the three kinds of water. By the CO₂ gas, the pH was lowered to 4.5 at 10°C. The specimens were stored at a temperature below 10°C for 24 hours.

When the concentration was higher than one per cent, no variation could be observed clearly in ultraviolet absorbance. The pH values of the solutions prepared from specimens (a), (b), and (c) were 2.6, 2.9, and 3.1, respectively, 24 hours after preparation.

Then hydrochloric acid (HCl) and acetic acid (CH₃COOH) were used in place of carbon dioxide gas. Some portions of the distilled water, pH 5.4, stored in the glass receptacle was adjusted to pH 5.0 (d), 4.5 (e), 4.0 (f), and 3.5 (g) with HCl and CH₃COOH, with respectively.

After that, ordinary succinic acid was dissolved in each of these portions at a concentration of one per cent. The resulting solutions were stored at 10°C for more than 24 hours.

Part of some distilled water was stored in resin receptacles (i) and (k). The remaining part of water was filtered through an ion-exchange resin column and stored in resin receptacles (j) and (l). The pH values were adjusted to 4.5 with HCl in receptacles (i) and (j), and with CH₃COOH in (k) and (l). Ordinary succinic acid was dissolved in the same manner as mentioned above. The pH values of the resulting solutions were adjusted to 4.5 with 5 N sodium hydroxide (NaOH) solution at the time of injection.

METHOD II TO OBTAIN A SUCCINIC ACID MODIFICATION

As reported in the previous report³⁾, mice inoculated with Ehrlich ascites carcinoma cells showed a prolonged time of survival when injected with some samples

of ordinary succinic acid commercially obtained. When fractional crystallization was repeated five or six times with ordinary succinic acid and ether, the resultant hardly soluble fraction exhibited a higher anticancer activity than any other fraction.

Accordingly, it was presumed that a small amount of active form might have been included in the ordinary succinic acid used.

Succinic acid is synthesized usually from maleic acid or fumaric acid by hydrogenation under high pressure and at high temperature. The hydrogenation to maleic acid was examined by the authors under normal pressure (760 # mmHg) in the presence of a catalyzer. Pure ethanol was used as solvent, although some polarized solvent was available. After continual hydrogenation for 20 hours, the catalyzer was removed and ethanol evaporated under reduced pressure. Then crystals were got. They were dissolved in hot ether and subjected to fractional recrystallization once. The presence of double bonds was verified by the potassium permanganate reaction. The crystals obtained were identified as those of succinic acid from the results of infra-red spectrum absorption.

A sample was dissolved in distilled water at a concentration of one per cent. The resultant solution was adjusted to pH 4.5 with 5 N NaOH at the time of injection.

METHOD III TO OBTAIN A SUCCINIC ACID MODIFICATION

It was suggested that such metallic double-cation as Ca, Mg, Mn, and Fe might be combined with succinic acid to produce a ring form, and that at the same time, two carboxyl radicals might change their position from transform to cis-form. So, at first, some commercially obtainable calcium succinate was examined for the presence of anticancer activity on mouse Ehrlich carcinoma. As a result, it was found to have an ability to prolong the survival time of the injected mouse to some extent.

From the results mentioned above, an attempt was made at the authors' laboratory to get active succinic salt. In this attempt, the following phenomenon was observed. When more than five parts of ordinary succinic acid in crystal form were melted in an aqueous suspension of calcium carbonate, the white suspension was reduced in quantity and changed finally into a transparent solution at room temperature.

After that, the following mixtures were prepared.

	Calcium carbonate		Succinic acid	H ₂ O
(1)	0.1 mol	+	0.1 mol	1.0 l
(2)	0.1 mol	+	0.2 mol	1.0 l
(3)	0.1 mol	+	0.3 mol	1.0 l
(4)	0.1 mol	+	0.4 mol	1.0 l
(5)	0.1 mol	+	0.5 mol	1.0 l

Of these mixtures, (1) to (5), (1) was not made to a solution, (2) was dissolved at 100°C, and the others were at room temperatures. Each of mixtures was filtered through filter paper. The resultant solutions were evaporated at 100°C, and the others were at room temperatures. Each of mixtures was filtered through filter paper. The resultant solutions were evaporated at 100°C and white powder remained. The powder obtained from each mixture was named as follows.

Mixture (1) : Ca. 1. Su.

(2) : Ca. 2. Su.

(3) : Ca. 3. Su.

(4) : Ca. 4. Su.

(5) : Ca. 5. Su.

Commercially obtainable calcium succinate: Ca. Su.

Each powder was dissolved in distilled water at a concentration of one percent. It showed the following pH value.

Ca. Su.	1 % suspension	5.79
Ca. 1. Su.	1 % suspension	5.63
Ca. 2. Su.	1 % solution	4.80
Ca. 3. Su.	1 % solution	4.40
Ca. 4. Su.	1 % solution	4.10
Ca. 5. Su.	1 % solution	3.85

Each solution was tested for anticancer activity against Ehrlich ascites carcinoma by the same method as mentioned above.

Then, each powder was washed with ethanol three times and divided into a soluble fraction and residue. When the ethanol was evaporated under reduced pressure, the following samples were obtained.

Original	residue	ethanol-soluble fraction
Ca. Su.	R. Ca. Su.	0
Ca. 1. Su.	R. Ca. 1. Su.	0
Ca. 2. Su.	R. Ca. 2. Su.	E. Ca. 2. Su.
Ca. 3. Su.	R. Ca. 3. Su.	E. Ca. 3. Su.
Ca. 4. Su.	R. Ca. 4. Su.	E. Ca. 4. Su.
Ca. 5. Su.	R. Ca. 5. Su.	E. Ca. 5. Su.

Each powder was dissolved in distilled water at a concentration of one per cent and subjected to a scrutiny test.

The pH values of the resulting solutions were as follows.

R. Ca. Su.	6.3	0	
R. Ca. 1. Su.	6.7	0	
R. Ca. 2. Su.	4.6	E. Ca. 2. Su.	2.9
R. Ca. 3. Su.	4.7	E. Ca. 3. Su.	2.9
R. Ca. 4. Su.	4.7	E. Ca. 4. Su.	2.9
R. Ca. 5. Su.	4.6	E. Ca. 5. Su.	2.7

The ethanol-soluble fraction was adjusted to pH 4.5 with 5N NaOH at the time of injection.

RESULTS

The scrutiny test of each sample gave the following results.

Sample	Number of cages	Day of occurrence of first death	Mean No. of surviving days	% of mice surviving 50 days
Untreated control	4	16	20.8	0
Commercially obtainable succinic acid	4	15	20.2	0
Mitomycin C	4	15	—	25
Endoxan	4	15	—	20

Toyomycin	4	14	—	40
Method I (a)	4	36	—	90
(b)	4	25	—	60
(c)	4	18	—	25
(d)	4	38	—	74
(e)	4	46	—	95
(f)	4	48	—	96
(g)	4	33	—	80
(h)	4	44	—	94
(i)	4	26	37	0
(j)	4	16	22	0
(k)	4	24	35	0
(l)	4	17	22	0
Method II	4	33	—	85
Method III Ca. Su.	6	22	—	12
Ca. 1. Su.	6	8	21	0
Ca. 2. Su.	6	14	—	28
Ca. 3. Su.	6	39	—	67
Ca. 4. Su.	6	46	—	80
Ca. 5. Su.	6	33	—	74
R. Ca. Su.	6	21	41	0
R. Ca. 1. Su.	6	11	29	0
R. Ca. 2. Su.	6	12	29	0
R. Ca. 3. Su.	6	14	—	42.8
R. Ca. 4. Su.	6	17	—	40
R. Ca. 5. Su.	4	11	—	25
R. Ca. 2. Su.	4	17	—	50
R. Ca. 3. Su.	4	16	—	75
R. Ca. 4. Su.	4	31	—	80
R. Ca. 5. Su.	4	18	—	60

DISCUSSION

BERTHELOT and GAUDECHON¹⁾ reported that the ultraviolet spectrum of succinic acid dissolved in water had been reduced in absorbancy by the carbon dioxide gas dissolution, but they did not refer to the water properties or the transformation of the molecular formula of succinic acid. Samples (a), (e), (f), (g), and (h) gave rise to a high survival rate. They had been prepared in distilled water and stored in glass vessels. No active powder, however, could be obtained from the solutions prepared by method I even when the water was evaporated by simple method. None of those samples were stable to heat or light. When their pH was adjusted in such manner as to exceed 5.0, they lost their activity, which, once lost, could never be recovered.

A sample was got in crystal form by method II, but its solution had the same properties as mentioned above. It took a very long time for the hydrogenation. Ca. 4. Su. powder presented a comparatively high activity. Its aqueous solution was stable to heat and light, but its activity was lost at a pH value exceeding 5.0. Ca. 4. Su. could be prepared, however, by a simple process. A one percent solution of it was not so acidic in reaction. Ca. 4. Su. is not a drug to show satisfactory activity, but it will play a sufficient role in practical use.

REFERENCES

- 1) BERTHESLOT, D. and GAUDECHON, H. (1911): *C. R. Acad. Sci., (Paris)*, **152**, 262~265.
- 2) HARA, T. (1967): *Jap. vet. Sci.*, **29**, 55~70.
- 3) HARA, T. (1967): *Jap. vet. Sci.*, **29**, 117~131.
- 4) LA TOUR, D. (1931): *C. R. Acad. Sci., (Paris)*, **193**, 180~182.

コハク酸の異性体に関する研究

III. 制癌作用によるコハク酸の活性化試験

鎌田知能・管 恒利・原 忠孝

日本獣医畜産大学家畜薬理学教室

(昭和44年7月24日受付)

魚臭に対する脱臭作用, あるいは mouse の Ehrlich 腹水癌に対して制癌作用を有しない市販のコハク酸を特定の条件下で処理することによって, 制癌作用を有する活性型に変化せしめる方法に関する報告である。即ち

1. 蒸溜水に CO_2 ガスを飽和せしめ, これに低濃度でコハク酸を溶解せしめて 10°C 以下で24

時間以上放置して得る。

2. マレイン酸に常温, 常圧で水素添加して得る方法。

3. コハク酸溶液に炭酸カルシウム, または水酸化カルシウムを添加して得る方法。
などである。