ON THE OCCURRENCE OF A PAROTIN-LIKE SUBSTANCE
(SALIVA-PAROTIN) IN THE HUMAN SALIVA
(STUDIES ON THE SALIVARY GLAND HORMONES. XXVII*)

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SEVEN FIGURES

Ogata claims in his "salivary gland endocrine theory" that the epithelial cells of the serous tubules ("Streifenstück") in the salivary ducts absorb a portion of saliva with their specific function into oral cavity and increte it into the general circulation via the lymph cavity surrounding them. Thus the increted saliva reveals its hormone-like stimulating action on the mesenchymal tissues. Based on this theory, Ito and Mizutani (1952) succeeded in isolating a new protein, which had been surmised as the salivary gland hormone as claimed by T. Ogata, in crystalline form from bovine parotid glands, and named it "Parotin".

Parotin or parotin-like substance should exist in the human saliva, if parotin is really the salivary gland hormone itself. Indeed, Ogata, Ito and Okabe (1944) previously reported that a substance, which possessed the action of decreasing the serum calcium level in rabbits and of promoting the growth of teeth in rats and mice, existed in human saliva. In the work the following three fractions were separated and their biological activities were examined: Fraction A, precipitated out from the human saliva by the addition of an equal volume of ethanol and one-half volume of ether, the fraction B, a precipitate which was obtained by further adding one-half volume of ether to the supernatant of the precipitate A, and the fraction C, the final concentrate of the mother liquor of precipitate B at low temperature. The biological and amylase activities of these fractions are shown in Table I.

Table I.

Fraction	Yield	Dose, mg./2 kg., subcut. inj.	Mean rate of serum-Ca decrease (3 rabbits), %	Activity Promoting growing tooth in rabbits	Amylase activity
A	0.13	7.5	15.4	++	strong
B	0.01	0.6	13.1	+++	slightly weak
C	35.0	59.0	9.8	+	

It was also found that the active principle was salted out from the saliva by saturation of neutral salts, especially, the precipitate which was salted out by

Received for publication July 13, 1955.

* XXVI: Y. Ito, *et al.*: J. Pharm. Soc. Japan **75**: 63 1955; Chem. Abstr.: **49**, 6336, 1955; cf. "Biochemical Studies on Salivary Gland Hormone" reviewed by Y. Ito in *Endocrinologia Japonica*: **1**: 1—50, 1954. (in English)

saturation of sodium chloride had a very strong activity per unit weight, but its yield was very low. Consequently, this behavior could not be applied for isolating or purifying the active principle from the human saliva.

This paper deals with the improved procedure for obtaining the active substance from the human saliva in a pure form and in a good yield.

The acetone-dried precipitate which was settled out with acetone in the concentration between 30 and 80% from the human saliva was extracted with water at pH 8.0 and precipitated at an isoelectric point. The precipitation at isoelectric point was repeated several times and the final precipitate was extracted with saline and then precipitated with acetone in 50% concentration at pH 4.6. The precipitate thus obtained had a powerful parotin-like biological activities and was named "Saliva-parotin".

EXPERIMENTAL AND CONSIDERATIONS

Preparation of Saliva-parotin

Materials: The saliva of healthy adults, secreted naturally without giving any irritation by medication or others, was collected and mixed.

Preparation of crude powder, S-precipitate: A small quantity of acetone was added to the mixed saliva (pH 7.0~7.2) to make 30% acetone concentration (v/v%). The precipitate here formed was discarded by centrifugation and further quantity of acetone was added with stirring to the clear supernatant to make 75~80% acetone concentration. The white precipitate was collected by centrifugation and dried in a vacuum desiccator over P_2O_5 after washing with acetone. This powder was named "S-precipitate", whose yield was 0.2~0.23 g./100 cc. saliva.

Purification of S-precipitate: One part of S-precipitate was extracted with one hundred parts of water at pH 8.0 as shown in Fig. 1. By centrifuging, the clear supernatant was separated and adjusted with small quantity of 0.1N HCl to pH 4.5~4.6 (B.C.G.) and allowed to stand overnight in a refrigerator. The precipitate formed was centrifuged and dried in vacuum after washing with acetone. This dried powder was called "S-1 Fraction", and the yield was about 25% of S-precipitate.

One part of S-1 Fraction was dissolved in one hundred parts of water at pH 8.0 and insoluble substances were removed by centrifuging. By the addition of 0.1N HCl to the clear supernatant to the isoelectric point (pH 5.4, C.P.R.), a precipitate formed. After allowing the mixture to stand in a refrigerator overnight, it was centrifuged, washed with acetone and dried in a vacuum. This dried residue was named "S-2 Fraction", whose yield was about 80% of S-1 Fraction.

One part of S-2 Fraction was extracted with about one hundred parts of 0.1~0.2M sodium chloride solution at an almost neutral reaction and was filtered in a refrigerator (it was difficult to filter). The pH of the filtrate was adjusted to pH 4.6 with 0.1N HCl and some quantity of acetone was added with stirring to make 50% acetone concentration (v/v%). It was allowed to stand overnight, centrifuged and dried under reduced pressure after washing with acetone. This was named

"Saliva-parotin", the yield of which was about 18% of S-2 Fraction and about 10 mg. from 100 cc. of the saliva.

Fig. 1. Flowsheet of the isolation of saliva-parotin from human saliva

Physico-chemical Properties of Saliva-parotin

Saliva-parotin is a white powder, easily soluble in alkaline solution. Its isoelectric point is pH 3.4 and nitrogen content is 9.8%.

It gives all the color reactions of proteins, *e. g.* biuret, ninhydrin, xanthoprotein, Folin's, Sakaguchi's and Molisch's reactions, etc., but the Millon's reaction is not distinct. It does not coagulate in aqueous solution by heating, but precipitates with picric acid, trichloroacetic acid, phosphotungstate, metaphosphate, sulfosalicylic acid, lead acetate and sublimate.

The electrophoretic patterns of its 1% solution in 0.2M borate buffer solution

Fig. 2. Electrophoretic pattern of Saliva-parotin preparation
pH 9.78, 0.2M borate buffer, 15°C, 30 min., 7 mA.; Purity: 72.7%
(by ascend.), 84.2% (by descend.)

at pH 9.78 are as shown in Fig. 2 and its purity was calculated to be 72.7% from the ascending pattern.

Characteristic polarograms were obtained for the preparation of Saliva-parotin with 72.7% of purity in 0.25N ammonium chloride (4 cc.) and 0.2N ammonium hydroxide (5 cc.) buffer solution, in the presence of Co^{2+} as cobaltous chloride (1 cc. of 0.01M CoCl_2) or Co^{3+} as hexammine-cobalt (III) chloride (1 cc. of 0.01M $[\text{Co}(\text{NH}_3)_6]\text{Cl}_3$) in a range of $-0.8 \sim -2.0$ Volt. The polarogram consisted of three waves with each half-wave potential as listed in Table II. In the case of Co^{3+} the third wave was the maximum. Of these three waves, as reported in the case of parotin (Ito, *et al*, 1955), it is considered that the first one is due to the reduction of Co^{2+} or Co^{3+} , the third one may be due to reduction of $-\text{S}-\text{S}-$ groups in protein molecule and the second wave is specific to the saliva-parotin protein.

Table II.

Preparation	E 1/2 (Standard calomel electrode)			
	Co(II)-electrolyte		Co(III)-electrolyte	
	Second Wave	Third Wave	Second Wave	Third Wave
Saliva-parotin	-1.36V.	-1.57V.	-1.36V.	-1.57V.
Parotin	-1.35V.	-1.57V.	-1.35V.	-1.60V.

To one part of saliva-parotin, fifty parts of 20% HCl was added and the mixture was hydrolyzed by heating at $105 \sim 115^\circ\text{C}$ for twelve hours in a sealed tube. The resulting hydrolyzate was centrifuged and the supernatant was evaporated to dryness on a water bath. HCl was removed by drying in a vacuum desiccator over P_2O_5 and NaOH. The dried residue was dissolved in a few drops of formic

Fig. 3. Two-dimensional paper partition chromatogram of the HCl-hydrolyzate of Saliva-parotin.

1. Gly, 2. Ala, 3. Val, 4. Leu, 5. Phe, 6. Tyr, 7. Asp, 8. Glu, 9. Lys, 10. Arg, 11. His, 12. Cys, 13. Met, 14. Ser, 15. Thr, 16. Pro.

acid and was submitted to paper partition chromatography with No. 50 and 31 Toyo Roshi filter paper by the two-dimensional ascending method, using N butanol-acetic acid-water (4:1:2) and phenol-0.1% ammonia (4:1) as the developing agents and ninhydrin as the coloring reagent. Fig. 3 shows the result of two-dimensional paper partition chromatogram with HCl-hydrolyzate. This result reveals that the HCl-hydrolyzate of saliva-parotin contains sixteen amino acids, namely, glycine, alanine, valine, leucine, phenylalanine, tyrosine, aspartic acid, glutamic acid, lysine, arginine, histidine, cystine, methionine, serine, threonine, and proline. In addition to these it was shown by the two-dimensional paper partition chromatography to contain tryptophan in the alkaline-hydrolyzate of saliva-parotin.

Biological Action of Saliva-parotin

Parotin was shown to have a variety of biological actions in many experimental and clinical studies. Of these, the growth promoting action on the hard tissues (Ishii, 1945; Sato, 1953; Huse, 1953, 1955), effects on inorganic substances in the blood (especially on calcium) (Ogata and Ito, 1944), effect on the number of circulating leucocytes (Takaoka, *et al.*, 1952), action of raising bone marrow temperature (Tasaka and Takatani, 1952) and the relationship with other endocrine organs are the most fully investigated.

Of the biological action of saliva-parotin, the foregoing actions were tested with experimental animals. The results were the same as the action of parotin, and moreover, it was proved that the activity per unit weight was far stronger in saliva-parotin than that of parotin.

(1) *Effect on the serum calcium level in rabbits:* Human saliva itself has the activity of decreasing the level of serum calcium by intravenous injection in rabbits.

Two kinds of S-precipitate were made, the one of which was prepared on the day the saliva was collected and the other was prepared after allowing the saliva to stand for one week in a room temperature (15~18°C). As shown in Table III, there was found no great difference between them in the serum Ca-decreasing activity by intravenous injection of 1 mg./kg. (containing insoluble matter), and the mean values were 17% and 19%, respectively.

Even in the S-precipitate obtained not only from the human saliva but also from bovine, dog and cat saliva, a marked decreasing activity was found.

The more the S-precipitate was purified, the greater the activity of decreasing the serum calcium level was, a preparation with 72.7% purity showed the average of 10.68% of serum Ca-decrease rate in six animals by intravenous injection with only 50 μ g per kg. body weight in rabbits and the activity was almost in proportion to the doses.

Male rabbits over 2 kg. body weight were injected intravenously with the sample solution in proportion to their body weight after fasting for 24 hours. During eight hours after injection the blood was drawn three times at certain intervals and the lowest calcium level was measured. The percentage of the maximum decrease against the original calcium level before injection was taken as the calcium decrease rate. About 4 cc. or less of blood but not exceeding 4 cc. was drawn from auricular vein and two measurements of the calcium level in two

cc. each of the serum were made according to the Kramer-Tisdall and Clark-Collip modified method.

Table III.

Fractions	Dose (intravenously) mg./kg.	No. of rabbits used	Mean rate of Ca-decrease %	Concentration of solution*
S-Precipitate (prepared on the day saliva collected)	1.0	5	16.93	0. 2%
S-Precipitate (prepared after allowing to stand for 6 days)	1.0	5	19.37	0. 2%
S-1	1.0	3	21.40	0.33%
	0.5	3	18.89	
S-2	0.5	6	18.96	0. 2% ²
	0.3	3	17.58	
Saliva-parotin	0.15	5	13.60	0.05%
	0.05	6	10.68	0.01%

* The solution was prepared by dissolving the preparations in water at pH 8.0 and the insoluble matter was centrifuged off.

(2) *Effects on the calcification of rabbit incisor dentine:* The Okada-Mimura's time-marking method by the lead acetate vital staining (Okada and Mimura, 1938) was applied. Male rabbits weighing approximately 2 kg. which had been maintained on a uniform diet were used. One per cent lead acetate solution (2~3 mg./kg. as lead acetate) was accurately injected into the auricular vein every two or four days and in this way it is possible to mark the time on their incisors. In the meantime, a single injection with the test solution was made intravenously or subcutaneously. Two or three days after the last injection of lead acetate, they were killed. Then, the incisors of the mandible were taken off, fixed in formaldehyde solution, removed calcium by immersion in 0.2N HCl saturated with H₂S, and dyed with hematoxylin after preparing a frozen section.

In the dentine, the annular concentric rings were visible. The tint of such rings by hematoxylin staining changed in proportion to the strength of calcification. Therefore, from this fact, the strength of calcification and the speed of dentine formation can be measured.

According to this procedure, the promotion of calcification was found to continue for three days after a single injection of 0.5 mg./kg. of S-2 Fraction (Fig. 4). In the case of saliva-parotin with 72.7% purity in the dose of 0.2 mg./kg., the promoting activity continued for as long as approximately eight days, but the speed of dentine formation was unchanged (Fig. 5).

(3) *Effect on the number of circulating leucocytes in rabbits:* Takaoka, *et al.* (1952) found that an injection of 10 mg./kg. of parotin decreased the number of circulating leucocytes very rapidly within the first two to three hours after injection, but subsequently increased in number remarkably in the next five to eight hours and reached the maximum in eight to ten hours after the injection, this tendency lasting for even twenty four hours.

Saliva-parotin possesses a similar action to that of parotin, but it is stronger

Fig. 4. Effect of S-2 Fraction on the calcification of rabbit incisor dentine. A transverse section of a rabbit incisor dentine gilded and dyed with hematoxylin. From left to right, D: Dentine, S: Corresponding to the portion after injection of 0.5 mg./kg. of S-2 Fraction. Figures 1, 2, 3 and 4 indicate the precipitation time of lead acetate, of which interval is 4 days. After administration, only blue stripes were found between 2 and 3, pale stripes grown in the day time had faded, and returned to the normal condition between 3 and 4 as between 1 and 2.

Fig. 5. Effect of Saliva-parotin on the calcification of rabbit incisor dentine. A transverse section of a rabbit incisor dentine gilded and dyed with hematoxylin. From left to right, D: Dentine, S: Corresponding to portion after injection of 0.2 mg./kg. of saliva-parotin. Figures 1, 2, 3 and 4 show the precipitation of lead acetate, of which interval is 3 days. After administration, only blue stripes were shown in two layers; one line is between 2 and 3, and another between 3 and 4. Pale stripes grown in the day time had faded, but the action continued for about 8 days.

compared with parotin. With the intravenous injection of saliva-parotin of 0.1~0.2 mg./kg. of dose, as shown in Fig. 6, it decreased about 70% two hours after injection, but after four hours, the value returned to that before injection, and increased rapidly after that to the maximum (160~260% of the value before injection) in ten to twelve hours. It continued to increase even after twenty four hours at the rate of 40~80%.

The number of leucocytes was calculated by Thoma-Zeiss method by using male rabbits fasted for twenty four hours before testing. Fresh blood was determined on two samples at any time of drawing blood, and the mean of the two values was calculated.

Fig. 6. Effect of saliva-parotin and parotin on the number of circulating leucocytes in rabbits

.....0.25 mg./kg. Saliva-parotin
0.10 mg./kg. Saliva-parotin
15.0 mg./kg. Purified Parotin

(4) *Effect on the bone marrow temperature:* Tasaka, *et al.* (1951) reported that the temperature of the bone marrow in rabbits showed remarkable selective rise after the injection of parotin and the rise was sustained for a long time.

Saliva-parotin possesses a similar action to that of parotin. When 0.1 mg./kg. was intravenously injected to rabbits, the bone marrow temperature rose about 0.7~1.0°C. With 0.25 mg./kg. dose, the degree of the rise was further remarkable, but with 1.0 mg./kg. dose, inversely the fall of the bone marrow temperature resulted and the tendency of fall was noticed in the liver and body temperature.

(5) *Effect on the blood pressure:* In order to confirm that the saliva-parotin was not identical with kallikrein, effect of the saliva-parotin on the blood pressure was tested in phenobarbital-anesthetized dog with various fractions from the human saliva mentioned above, *viz.* S-1 and S-2 Fraction and saliva-parotin.

The results are shown in Fig. 7. In the case of S-1 and S-2 Fractions, the fall of the blood pressure was observed, but saliva-parotin had entirely no effects.

Fig. 7. Effect of saliva-parotin on the blood pressure of phenobarbital anesthetized dog.

S: Kallikrein, K. E. = Rabbit Unit; S-2: S-2 Fraction, 4 mg./cc.;
S-P: Saliva-parotin, 2 mg./cc.

It was considered that this depressing action was due to the contaminating kallikrein in S-1 and S-2 Fractions because 2~4 units of kallikrein is said to be contained in 1 cc. of the saliva. This results indicate that saliva-parotin is not identical with kallikrein.

SUMMARY

By purifying the dried powder, which was precipitated from the human saliva by the addition of acetone, as shown in Fig. 1, parotin-like protein was isolated and named "Saliva-parotin".

Its isoelectric point was pH 3.4 and its purity in the electrophoretic analysis was 72.7% (by the ascending).

It gave all the color reactions of proteins and precipitated with acids or heavy metals, but did not coagulate in aqueous solution by heating.

By paper partition chromatography of the hydrolyzate of saliva-parotin seventeen kinds of amino acids were identified.

Saliva-parotin possesses an entirely the same biological action as parotin; decrease of serum calcium level, promotion of calcifying the incisor dentine, increase

of the number of circulating leucocytes and rise of the bone marrow temperature were clearly observed in rabbits. The fall of the blood pressure in an anesthetized dog was recognized with an insufficiently purified preparation, but not in the case of the preparation with 72.7% purity.

Acknowledgement. The authors greatly appreciate of the cooperation of Dr. H. Takatani, Tasaka Internal Medical Department, University of Tokyo, Prof. S. Huse and Mr. N. Matsuda, Department of Dental Surgery, School of Medicine, Gunma University, Mr. H. Takigawa, Endocrinological Laboratory, School of Medicine, Gunma University, and Mr. Y. Morioka, Teikoku Hormone Manufacturing Co., and co-workers in this study.

REFERENCES

- Clark, E.P. and J.B. Collip : J. Biol. Chem. **63** : 461, 1925.
Huse, S.: Nihonrinshoo **11** : 20, 1953 (In Japanese); Soogoo Rinshoo, Suppl. Issue, 1955 : 94.
(In Japanese)
Ishii, Z.: J. Pathology, Japan **3** : 103, 1945. (In Japanese)
Ito, Y. and A. Mizutani : J. Pharm. Soc. Japan **72** : 244, 1952.
Ito, Y. and S. Okabe : Address in the Diamond Jubilee of the Pharmaceutical Society of Japan, 1955.
Ito, Y., H. Takigawa, K. Ono, S. Miyashita and S. Ota : J. Pharm. Soc. Japan **75** : 63, 1955.
(In Japanese)
Ogata, A. and Y. Ito : J. Pharm. Soc. Japan **64** : 146, 332, 1944.
Ogata, A., Y. Ito and A. Mizutani : J. Pharm. Soc. Japan **64** : 377, 1944.
Ogata, A., Y. Ito and S. Okabe : J. Pharm. Soc. Japan **64** : 79, 1944.
Okada, M. and T. Mimura : Jap. J. Med. Sci., IV, Pharmacology **6** : 166, 1938.
Sato, T.: Gunma J. Med. Sci. **2** : 183, 1953.
Takaoka, Y., T. Yamaguchi and K. Kosaka : Tohoku J. Exptl. Med. **57** : 9, 1952.
Tasaka, S., O. Takatani and K. Narita : Tokyo J. Med. Science **59** : 227, 1952. (In Japanese)