THE EFFECTS OF PURIFIED SALIVA-PAROTIN-A ON THE
DEVELOPMENT OF 9-DAY CHICK EMBRYO
FEMORA GROWING IN TISSUE CULTURE*
(STUDIES ON THE PHYSIOLOGICAL CHEMISTRY
OF THE SALIVARY GLANDS LIV†)

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In the previous work (Ito and Endo, 1956) parotin was demonstrated to have a direct stimulative action on calcification of the chick embryo femora growing in tissue culture.

From human mixed saliva a parotin-like protein, termed saliva-parotin-A, has been isolated by glacial acetic acid treatment (Ito and Okabe, 1959) and subsequently purified by the succeeding ethanol fractionation or ultracentrifugation, or both (Okabe, 1959a). And the purified saliva-parotin-A has proved almost homogeneous electrophoretically and ultracentrifugally and of extremely high biological activities as compared with parotin (Okabe, 1959b). The minimum effective dose of purified saliva-parotin-A for clearly enhancing the calcification of rabbit incisor dentine was 2 to 5 $\mu\text{g}/\text{kg}$ body weight of the animal for intravenous injection (Okabe, 1959b), while that of parotin was above 500 μg .

In this study, therefore, purified saliva-parotin-A was examined for the effects on the development of 9-day chick embryo femora cultivated by the roller-tube method without plasma, which has permitted of cultivation of the long bone for quantitative studies (Ito and Endo, 1956; Miyazaki *et al.*, 1957; Ito *et al.*, 1959; Saito, 1959; Endo, 1960; Ito *et al.*, 1960a, b).

MATERIALS AND METHODS

1. *Tissue Culture Technique*

The roller-tube culture method without plasma was used.

The medium The culture medium used in this work consisted of 1 part of chick embryo extract, 2 parts of horse serum and 7 parts of Gey's saline solution.

Chick embryo extract was prepared from whole embryos of 13 days' incubation. The embryos were crushed in a mechanical homogenizer and stored in deep freezing. Just before each cultivation or renewal of the medium, the homogenate was centrifuged at about 3,000 r.p.m. for 10 mins. and the supernatant was then ready for use.

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Sera from several horses were sterilized by passage through Seitz's filter together in order to reduce the presumable variation in concentration of the components.

Ingredients of Gey's saline solution per liter were as follows; NaCl 8.00 g, KCl 0.30 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.364 g, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.210 g, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 0.150 g, KH_2PO_4 0.025 g, Glucose 2.00 g, and NaHCO_3 0.250 g.

The explants The femora were removed from 9-day chick embryos with the aid of a binocular dissecting microscope. Adhering soft tissue was fully detached from the bones by rotating them on dry paper. The explants were placed directly on the inner surface of the glass roller-tubes. One femur was put and 1 ml of the medium was added into each tube. The tubes were incubated at 38°C while rolling 10 times per hr. by a roller-drum, which held them approximately at an angle of 5 degrees. In order to exclude the cause of variation between embryos, the femora from one side of an embryo were cultivated in the medium described above and those from another side of the same embryo in the same medium but containing purified saliva-parotin-A. The media were renewed every other day. The cultivation terminated on the 6th day.

Purified saliva-parotin-A This preparation was isolated from fresh human mixed saliva, purified and lyophilized (Okabe, 1959a).

2. Measurement and Analysis

Longitudinal growth The length of each bone was measured every day with ocular micrometer of a microscope at low magnification.

Dry weight At the end of the cultivation period the femora were removed from the roller-tubes, washed with distilled water and then dried *in vacuo*. Dry weight of each femur was obtained with microbalance.

Phosphorus After being weighed, each bone was determined of its inorganic phosphate extracted at 50°C for 3 hrs. with 3 ml of 5% trichloroacetic acid. The determination was made by Gomori's colorimetric method (Gomori, 1942).

Hydroxyproline The amount of hydroxyproline of each extracted bone was determined colorimetrically after Stegemann's procedure (Stegemann, 1958), after the bone had been hydrolyzed in a sealed tube at 150°C for 3 hrs. with 0.1 ml of 3N hydrochloric acid.

Statistical treatment All results of the experiments were statistically analyzed and the term "significant" used in this paper means that the statistically significant difference was found with certain level of significance by the Student's *t*-test, after eliminating the variation between embryos.

RESULTS

The femur from 9-day chick embryo is a cartilaginous rudiment 6~6.5 mm long, diaphysis of which has been already encapsuled by a thin perichondrium with a sign of primary ossification. During cultivation under the experimental condition described above, the femora grew to 7.5~8 mm and advanced into a developmental stage showing the entire shaft ossified in histological examination.

Figure 1 shows the effects of purified saliva-parotin-A and of a biologically non-active fraction discarded during purification of the substance on the elongation of the bone.

At the lowest concentration of purified saliva-parotin-A, 0.01 $\mu\text{g}/\text{ml}$, no difference was found at all (Fig. 1-a). At concentrations of 0.05 and 0.1 $\mu\text{g}/\text{ml}$ a slightly stimulative tendency was observed, though it was not significant statistically (Fig. 1-b and -c). With increasing concentration of purified saliva-parotin-A, however, the effect thoroughly reversed and the inhibitory effects at concentrations of 10 and 50 $\mu\text{g}/\text{ml}$ were found significant and highly significant respectively (Fig. 1-

Fig. 1. The effects of purified saliva-parotin-A and a non-active fraction on the longitudinal growth of 9-day chick embryo femora growing *in vitro*

— : mean length of the control bones

--- : mean length of the bones treated with purified saliva-parotin-A or a non-active fraction of the following concentrations a—0.01 $\mu\text{g/ml}$; b—0.05 $\mu\text{g/ml}$; c—0.1 $\mu\text{g/ml}$; d—0.5 $\mu\text{g/ml}$; e—1.0 $\mu\text{g/ml}$; f—10 $\mu\text{g/ml}$; g—50 $\mu\text{g/ml}$; h—50 μg of a non-active fraction per ml

f and -g). By the addition of the non-active fraction, on the contrary, significant retardation of the bone elongation was not observed at all even at a concentration of 50 $\mu\text{g/ml}$ (Fig. 1-h).

Figure 2 shows the effects on the dry weight and the amount of hydroxyproline. The effect on the amount of phosphorus is presented in Figure 3. Each bar in both Figures represents the mean for a single determination of 9 to 14 femora, the absolute value of each bone being expressed as a percentage of that of the control bone from the same embryo.

Fig. 2. The effects of purified saliva-parotin-A and a non-active fraction on the dry weight and the amount of hydroxyproline of 9-day chick embryo femora growing *in vitro*

The vertical line at the top of each bar represents $\pm$ the standard error of the mean. The dry weight per femur (plain bars); the amount of hydroxyproline per femur (solid bars).

Fig. 3. The effects of purified saliva-parotin-A and a non-active fraction on the amount of phosphorus of 9-day chick embryo femora growing *in vitro*

The vertical line at the top of each bar represents $\pm$ the standard error of the mean.

In the dry weight and the amount of hydroxyproline, no clear directed change could be found because of a considerable fluctuation of the estimates within concentrations ranging from 0.01 to 10 μg of purified saliva-parotin-A per ml of the medium, though only at a concentration of 1.0 $\mu\text{g}/\text{ml}$ was a significant decrease of the dry weight found (Fig. 2). As for the amount of phosphorus, however, the value increased evidently over a wide range of concentration of purified saliva-parotin-A from 0.01 to 1.0 $\mu\text{g}/\text{ml}$ (Fig. 3). At concentrations of 0.01 and 0.5 $\mu\text{g}/\text{ml}$ an increase in phosphorus was found of high statistical significance in spite of no effect on the growth in length of the bone as shown in Figure 1. This stimulative tendency persisted even at a concentration of 1.0 $\mu\text{g}/\text{ml}$, where a occasional significant decrease was observed in the dry weight of the bone as illustrated in Figure 2. At a concentration of 50 $\mu\text{g}/\text{ml}$, however, purified saliva-parotin-A resulted in marked decrease in all the estimates, while at the same concentration the non-active fraction showed no significant effect both on the dry weight and on the amount of hydroxyproline but a increasing effect

Table 1. The effects of purified saliva-parotin-A and a non-active fraction on the amount of phosphorus, amount of hydroxyproline and dry weight of the 9-day chick embryo femora growing *in vitro*, and analysis of the difference

Concentration of purified saliva-parotin-A ($\mu\text{g/ml}$)	No. of pairs	Phosphorus, (hydroxyproline and [dry weight]) ($\mu\text{g/femur}$)		Variance of the difference	Level of significance
		Control	Treated		
0.01	9	12.6	13.2	0.4411	$P < 0.025$
		(13.4)	(12.1)	—	—
		[1084.0]	[1058.1]	—	—
0.1	11	11.9	13.4	0.8456	$P < 0.001$
		(12.6)	(12.7)	—	—
		[981.0]	[1005.5]	—	—
1.0	10	13.8	14.2	—	—
		(13.3)	(13.2)	—	—
		[1043.6]	[989.9]	[5453.4]	$[P < 0.05]$
50.0	12	14.4	13.1	0.5975	$P < 0.001$
		(16.8)	(14.0)	(10.51)	$(P < 0.01)$
		[1018.6]	[913.6]	[1545.7]	$[P < 0.02]$
50.0 (Non-active fraction)	11	12.4	13.0	0.6015	$P < 0.05$
		(16.3)	(14.7)	—	—
		[1055.8]	[1022.8]	—	—

of statistical significance on the amount of phosphorus.

The absolute values of a part of the percentage ones just referred to, along with their statistical analyses, are presented in Table 1.

Evidences were presented that purified saliva-parotin-A increased selectively the amount of phosphorus of the bone by some direct means whereas its overdoses suppressed the over-all growth of the bone.

DISCUSSION

The results just described show that purified saliva-parotin-A exerts a direct action on the isolated femur of 9-day chick embryo *in vitro*. The authors (Ito and Endo, 1956), using the tissue culture method, demonstrated the effectiveness of parotin in promoting the growth in length and calcium deposition of chick embryo femora, and assumed that parotin might directly stimulate some sites of development of hard tissues *in vivo*. Parotin is an active substance of protein nature isolated from the bovine parotid gland, and saliva-parotin-A is almost homogeneous protein of much smaller molecular weight obtained from human mixed saliva. A comprehensive review on parotin and parotin-like substances has recently been published (Ito, 1960).

From the results as shown in Figure 1, a tendency can be affirmative that the elongation of 9-day chick embryo femora *in vitro* was enhanced slightly, though not significantly, by the addition of purified saliva-parotin-A of low concentrations, while the effect thoroughly reversed at much higher concentrations.

As shown in Figure 2, however, no clear tendency was exhibited both in the

Fig. 4. The effects of purified saliva-parotin-A and a non-active fraction on the hydroxyproline content, expressed as the value per unit dry weight of 9-day chick embryo femora growing *in vitro*.

The vertical line at the top of each bar represents $\pm$ the standard error of the mean.

dry weight of the bone and in the amount of hydroxyproline per femur, except that marked decrease was shown in the 2 estimations at the highest concentration, 50 $\mu\text{g/ml}$.

For examining the effect of purified saliva-parotin-A on the hydroxyproline content of the bone in more detail, the relative amount of hydroxyproline per residual dry weight of the bone was presented in Figure 4. The residual dry weight obtained by deducting 5 times the amount of phosphorus from the dry weight of the bone, may be expected roughly to correspond to the dry weight of cartilage matrix alone. The amount of hydroxyproline per residual dry weight estimated here, therefore, could be considered probably to reflect the degree of formation of the cartilage matrix, though a considerable amount of collagen has been found to dissolve into the trichloroacetic acid solution during extraction of deposited calcium phosphate.

Considering these points, the results as shown in Figure 4 may be taken to indicate that purified saliva-parotin-A exerted no influence on the formation of cartilage matrix of the bone except in a concentration as high as 50 $\mu\text{g/ml}$.

The results presented in Figure 3 and Table 1, on the contrary, confirmed a profound effect of purified saliva-parotin-A in increasing the amount of phosphorus of the bone over a wide range of concentration far beyond the optimal one for elongation, though the decrease of high significance was observed at the highest concentration, 50 $\mu\text{g/ml}$.

As for the non-active fraction, however, it caused no significant decrease in the dry weight and the amount of hydroxyproline and moreover significant increase in the amount of phosphorus even at a concentration of 50 $\mu\text{g/ml}$, at which purified saliva-parotin-A exerted a highly significant effect in decreasing all the estimates as well as in retarding the elongation as mentioned above (Figs. 1, 2, 3, 4, and Table 1). The non-active fraction just referred to is a lyophilized preparation of the upper layer of the saliva-parotin-A solution divided into 3 portions after ultracentrifugation at 181,220 g for 5 hrs., designated as Fraction 1-A in the original paper (Okabe, 1959a), and purified saliva-parotin-A used in the above

experiments is a lyophilized preparation of the lower layer of the solution. When administered intravenously at a dose of $300\text{ }\mu\text{g/kg}$ body weight in rabbits, on the one hand, the non-active fraction could not show the serum calcium decreasing activity, which has been employed for the quantitative estimation in bioassay of parotin and parotin-like substances. Purified saliva-parotin-A, on the other hand, exhibited a considerable degree of the activity even at a dose of $1\text{ }\mu\text{g/kg}$ body weight. Nevertheless, the non-active fraction may be suspected of slightly contaminating with the active fraction, for complete purification can not be expected theoretically from such procedures as dividing the ultracentrifuged protein solution into several portions and then pipetting each portion for lyophilization. Accordingly, the significant increase in the amount of phosphorus of the bone by the addition of the non-active fraction was probably due to the concomitant active fraction, as purified saliva-parotin-A increased the amount significantly even at concentration as low as $0.01\text{ }\mu\text{g/ml}$.

The phosphorus determined here must derive from calcium phosphate constituting almost all the bone salt deposited during cultivation, and thus must indicate, as a first approximation, the degree of calcification of the bone. And, indeed, with the aid of histological examinations and concurrent chemical determinations, calcification of the chick embryo femur grown under much the same condition has been ascertained to be remarkably affected by the addition of various chemical agents into the medium (Miyazaki *et al.*, 1957; Saito, 1959). Cultivating the bone in the media varying in concentration of the components, moreover, the authors have recently observed a close association between the amount of phosphorus chemically determined and the degree of calcification histologically examined (Endo, 1960, Ito *et al.*, 1960a).

From these points, it would be asserted that purified saliva-parotin-A exerts a direct action *in vitro* especially upon the calcification of the bone and probably upon the elongation through some biological activity in itself, the action being stimulatory at low concentration and inhibitory at high concentration.

Furthermore, it should be noted that the aforementioned effects of purified saliva-parotin-A upon the development of the bone are in complete agreement with those previously reported for parotin by the authors (Ito and Endo, 1956), except that the optimal concentration of parotin in promoting the calcium deposition as well as the elongation was as much higher as $150\text{ }\mu\text{g/ml}$. And this difference of the optimal concentration is fairly consistent with that of the minimum effective doses per kg body weight by intravenous injection in rabbits as follows: above $500\text{ }\mu\text{g}$ for parotin and $2\sim5\text{ }\mu\text{g}$ for purified saliva-parotin-A in promoting the calcification of incisor dentine, and 1 mg for the former and $5\sim10\text{ }\mu\text{g}$ for the latter in decreasing the serum calcium level. Calculating from the doses above, moreover, the effective concentration of purified saliva-parotin-A in the culture medium (0.01 to $0.5\text{ }\mu\text{g/ml}$) would be regarded as within its physiologic level in plasma, though overdosage phenomena have ordinarily been dealt with in tissue culture, in which much larger amounts of hormones than physiologic level were required.

From these considerations, purified saliva-parotin-A might be supposed to have a direct action on the growth, especially on the calcification, of hard tissues

also in living animals, and moreover an intimate interrelationship might be assumed to exist between the activity of these 2 substances in stimulating development of hard tissues and their rabbit serum calcium decreasing activity, estimation of which has been employed as a bioassay method for parotin and parotin-like substances.

SUMMARY

The effects of purified saliva-parotin-A on the development of 9-day chick embryo femora growing *in vitro* were studied. Pair-mate bones were cultivated for 6 days by the roller-tube culture method without plasma. One of each pair was grown in the medium consisting of 1 part of chick embryo extract, 2 parts of horse serum and 7 parts of saline solution, and another in the plus-purified saliva-parotin-A medium.

The longitudinal growth of the bones was determined from measurement of the over-all length recorded every day throughout the cultivation period. The dry weight of the bones was also obtained. The degree of calcification of the bones was estimated from determination of the phosphorus deposited as bone salt. Finally the formation of cartilage matrix was evaluated from estimation of the amount of hydroxyproline in the acid hydrolysate of the bones.

These experiments resulted in the following; (a) on the longitudinal growth of the bones, purified saliva-parotin-A exhibited a slightly stimulative tendency at concentrations of 0.05 and 0.1 $\mu\text{g/ml}$, (b) on the dry weight and the amount of hydroxyproline, no clear directed change was found within the concentrations ranging from 0.01 to 10 $\mu\text{g/ml}$ and a marked decrease was observed at concentration of 50 $\mu\text{g/ml}$, (c) on the amount of phosphorus, however, evident increase was found over a wide range of concentration from 0.01 to 1.0 $\mu\text{g/ml}$, while at concentration of 50 $\mu\text{g/ml}$ a marked decrease was seen.

From these findings it was suggested that purified saliva-parotin-A has a direct stimulative effect selectively upon calcification of the bone under certain optimal condition, though the overdoses of it retard the over-all growth of the bone.

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