STUDIES ON THE SALIVARY GLAND HORMONES LABELED WITH I^{131}

4. STUDIES ON THE DEGRADATION OF I^{131}-LABELED PAROTIN BY SEVERAL TISSUE HOMOGENATES OF RAT*

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Recently it was reported from this laboratory as one of a series of studies on the Salivary Gland Hormones, that liver and spleen were the main and characteristic sites of localization of radioactivity after administration of I^{131} labeled Parotin to rats (Tanaka and Ito, 1958). And subsequently it was also observed that this characteristic localization of radioactivity in the liver and spleen and the rate of degradation of I^{131}-Parotin in vivo were remarkably decreased by the simultaneous injection of intact Parotin with the labeled Parotin to rats (Tanaka et al., 1958).

This report presents the results of studies on the in vitro degradation of I^{131}-labeled Parotin and other similarly radioiodinated proteins such as bovine serum albumin (B.S.A.), pituitary growth hormone (GH) and casein by several tissue homogenates of rat.

MATERIALS AND METHODS

Parotin**, B.S.A. (Armour and Co.), GH (U.S.P. reference standard) and casein (Kasein nach Hammersten, E. Merk) were labeled with radioactive iodine by the method similar to those previously reported (Tanaka et al., 1958). The purities of radioiodinated Parotin, B.S.A., GH and casein as expressed by the per cent of the total radioactivity found in trichloroacetic acid insoluble fraction were 98.5, 98.5, 90 and 97.5 respectively.

Wistar male rats, from 190 to 250g in weight, were sacrificed by exsanguination from the common carotid artery after the injection of suitable amount of heparin. Blood plasma was collected by centrifugation at low temperature. And liver, kidney, spleen, muscle, pancreas, testis, submaxillary and parotid gland were excised immediately after the exsanguination, cooled on ice, homogenized with ice cold M/10 phosphate buffer, pH 7.4 using the Potter Elvehjem homogenizer (the concentration of tissue homogenates was 7.5% at this stage). Separately, I^{131}-labeled protein was dissolved in distilled water adjusted to pH 8 (90 µg per ml) so as to avoid the loss of radioactivity from the solution. 0.5ml of this I^{131}-labeled protein solution was mixed with 1ml of the 7.5% tissue homogenates in a small test tube and aerobically incubated at 37°C. As a control, each labeled protein was incubated with M/10 phosphate buffer, pH 7.4, containing B.S.A. (1mg per ml) to stabilize the labeled protein in buffer solution.

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in the absence of homogenate proteins. The reactions were terminated by pouring an equal volume of 10% trichloroacetic acid (TCA) to each test tube at the varying times after the start of incubation (0, 1, 2, and 4 hrs.). After centrifugation, the precipitate was washed once with 2 ml of 5% TCA. Supernatants were pooled and neutralized with sodium hydroxide solution and radioactivity was assayed with a well-type scintillation counter in relation to the total radioactivity added to each test tube.

For paperchromatographic analysis, each labeled protein was dissolved in distilled water adjusted to pH 8 (1 mg per ml), and 0.5 ml of this solution was incubated with 1 ml of the 7.5% tissue homogenates at 37°C for 1 hr. The incubation mixture was centrifuged at low temperature without adding TCA solution. Thus obtained supernatant fluid was spotted on a filter paper in 2 cm width and developed in 95% ethanol and 2 N ammonia (89:11). Inorganic iodide labeled with I¹³¹ and initial I¹³¹-labeled proteins were also chromatographed simultaneously as controls. When the solvent front had reached the line of 23.5 cm distance from the original line where sample was spotted, paper was dried and cut into 24 sections, each in 1 cm width. And each section was numbered from 0 to 23, according to the Rf value respectively. Radioactivity of each section was assayed separately or in groups with a well-type scintillation counter in relation to the total radioactivity of the chromatogram.

RESULTS

Previously it was reported that large localization of radioactivity was found in liver, kidney and spleen after the administration of I¹³¹-labeled Parotin to rats

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**Fig. 1. Effect of pH on the degradation of I¹³¹-Parotin by kidney and liver homogenates**

One ml of 7.5% homogenate was incubated with 0.5 ml of substrate solution containing 45 µg of I¹³¹-Parotin for 2 hrs. at 37°C and the designated pH.

**Fig. 2. Effect of substrate concentration on the degradation of I¹³¹-Parotin by kidney homogenate**

Incubation of 1 ml of 7.5% kidney homogenate and 0.5 ml of substrate solution containing various quantities of I¹³¹-Parotin for 2 hrs. at 37°C and pH 7.4.
(Tanaka and Ito, 1958). As the result of the preliminary studies on the \textit{in vitro} degradation of I\textsuperscript{131}-Parotin by liver, spleen and kidney homogenate, the largest degradation activity was observed in case of kidney homogenate. Therefore, the conditions for the incubation were studied mainly with kidney homogenate.

Effect of pH on the degradation of I\textsuperscript{131}-Parotin by kidney and liver homogenates was determined at eight different pH, ranging from 6.2 to 9.0 (Fig. 1). With liver homogenate gradual decrease of activity was observed with the increase of pH. In the case of kidney homogenate, however, no significant difference was seen among the pH values undertaken, except at pH 9.0. From these results, pH 7.4 was adopted, also for the purpose of avoiding the least possibility of exchange between I\textsuperscript{131} bound in organic compounds and iodide ion and the possibility of oxidation of the latter during the incubation, and furthermore for the sake of keeping the physiological conditions.

Effect of the substrate concentration on the degradation of I\textsuperscript{131}-Parotin by kidney homogenate was examined at four different concentrations ranging from 3.3 to 90 \(\mu g\) of I\textsuperscript{131}-Parotin per ml of incubation mixture. As shown in Figure 2, no significant difference was found among the four substrate concentrations. Therefore the following experiments were performed with the 30 \(\mu g\) of I\textsuperscript{131}-Parotin per ml of incubation mixture for the mere experimental convenience.

Results of the degradation of I\textsuperscript{131}-Parotin and I\textsuperscript{131}-B. S. A. by liver, kidney, spleen, testis, muscle, submaxillary gland, parotid gland, and pancreas homogenates and plasma were recorded in Tables 1, 2 and Figures 3, 4. The labeled Parotin was found to be degraded by submaxillary homogenate as rapidly as 50\% in 1 hr., 65\% in 2 hrs. and 70\% in 4 hrs. The labeled Parotin also received appreciable degradation by other tissue homogenates approximately in the following

<table>
<thead>
<tr>
<th>Time of incubation (mins.)</th>
<th>Exper. number</th>
<th>Per cent radioactivity in TAC soluble fraction</th>
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<td>Liver</td>
<td>Kidney</td>
</tr>
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* Incubation of 1 ml of 7.5\% homogenate and 0.5 ml of substrate solution containing 45 \(\mu g\) of I\textsuperscript{131}-Parotin, at 37\(^\circ\)C and pH 7.4 for the designated period

** One ml of phosphate buffer, pH 7.4, containing 1 mg of B.S.A. was incubated with 0.5 ml of the substrate solution.

*** In each experiment, tissues of 3 rats were used.
order: kidney, spleen, parotid gland, muscle, testis and liver. But as for the absolute value, they were far smaller than that of submaxillary gland homogent. Pancreas homogenate showed remarkably high activity after 4 hrs. incubation, although its activity at 1 hr. could not be distinguished from that of kidney homogenate. No significant difference was found between the values of plasma and control. On the other hand, I131-labeled B. S. A. was not degraded by any tissue homogenate except submaxillary homogenate which showed some degradation activity.

### Table 2. Degradation of I131-bovine serum albumin by several tissue homogenates of rat*

<table>
<thead>
<tr>
<th>Time of incubation (mins.)</th>
<th>Exper. number</th>
<th>Liver</th>
<th>Kidney</th>
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<th>Testis</th>
<th>Muscle</th>
<th>Submax. gland</th>
<th>Parotid gland</th>
<th>Pancreas</th>
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* Incubation of 1 ml of 7.5% homogenate and 0.5 ml of substrate solution containing 45 μg of I131-bovine serum albumin, at 37°C and pH 7.4 for the designated period.
Although submaxillary and parotid gland belong to the salivary glands, these two glands exhibited quite different characters in degrading \( ^{131}\)-Parotin and \( ^{131}\)-B.S.A. by their homogenate. These were re-examined using another five rats separately, and again the marked difference was confirmed between submaxillary and parotid glands (Fig. 5).

As shown in Figures 6 and 7, which express the results of the degradation of \( ^{131}\)-GH and \( ^{131}\)-casein by several tissue homogenates, submaxillary homogenate exhibited extremely strong activity at 1 hr.’s incubation (\( ^{131}\)-GH, ca. 90%; \( ^{131}\)-casein, ca. 100%), while other tissue homogenates have indicated some appreciable degradation activity.

Because the higher radioactivity is essential for quantitative paperchromatographic analysis, effect of the higher substrate concentration on the degradation of \( ^{131}\)-Parotin was primarily examined with liver, submaxillary and pancreas homogenates. As seen in Figure 8, only a slight decrease of activity was observed even at the highest substrate concentration (900 \( \mu g \) per ml). Therefore, the
DEGRADATION OF LABELED PAROTIN IN VITRO

Liver Submaxillary gland Pancreas

Fig. 8. Effect of higher substrate concentration on the degradation of I^{131}-Parotin by liver, submaxillary gland and pancreas homogenate
Incubation of 1 ml of 7.5% homogenate and 0.5 ml of substrate solution containing various quantities of I^{131}-Parotin for 2 hrs. at 37°C and pH 7.4

Figure 9 shows the typical results of the quantitative paperchromatographic analysis of the supernatant fluid of each incubation mixture. In the case of unincubated I^{131}-proteins, almost all radioactivity was found in the region of section No. 0, that is around the original line (Fig. 9-a). And iodide ion labeled with I^{131} was appeared in the region of section No. 15 to 17 (Fig. 9-b). In the case of I^{131}-proteins incubated with several tissue homogenates, there was some spreading of radioactivity on papergram, showing different Rf values. Those results of chromatography seemed to be in good accordance with the rate of degradation of I^{131}-proteins indicated by TCA-treatment of the incubation mixtures (Fig. 9-c~9-f). In the region of section No. 14 to 18 where radioactive iodide ion was developed, no appreciable increase of radioactivity was found throughout all the paperchromatography performed with the supernatant fluid of each incubation mixture. From these results, it was found that the degradation of I^{131}-proteins by several rat tissue homogenates, including submaxillary gland homogenate, was not due to the deiodination.

DISCUSSION

Besides by measuring the increase of the radioactivity in TCA soluble fraction of the incubation mixture, several workers have demonstrated that I^{131}-insulin or I^{131}-glucagon was proteolytically degraded by rat liver extract, by measuring the increase of non-protein nitrogen (Tomizawa et al., 1955), by autoradiopaperchromatography of TCA soluble fraction (Mirsky et al., 1955), and by measuring the increase of the tyrosine concentration of the non-protein fraction (Narahara and Williams, 1957). Through quantitative estimation of radioactivity of the paperchromatograms of the supernatant fluid of the incubation mixture, it was found that the degradation of I^{131}-Parotin and other I^{131}-proteins by several tissue homogenates was not due to deiodination. And furthermore it was strongly suggested that the degradation of the labeled proteins by several tissue homogenates was due to proteolytic degradation because of the increased radioactivity in the TCA soluble fraction of the incubation mixture as well as the increased radio-
Fig. 9. Typical results of the quantitative paperchromatographic analysis of the supernatant fluid of incubation mixture

Ordinates: Per cent of total radioactivity of each papergram found in each section or group of sections.

Abscissas: Section number (—: indicates sum of sections)
a: $^{131}\text{I}$-Parotin non-incubated (control).
b: Iodide ion labeled with $^{131}\text{I}$.
c: $^{131}\text{I}$-Parotin incubated with submaxillary gland homogenate for 1 hr. at 37°C and pH 7.4.
d: $^{131}\text{I}$-Parotin incubated with liver homogenate.
e: $^{131}\text{I}$-growth hormone incubated with submaxillary gland homogenate.
f: $^{131}\text{I}$-casein incubated with submaxillary gland homogenate.
DEGRADATION OF LABELED PAROTIN IN VITRO

I$^{131}$-GH  I$^{131}$-casein

Fig. 10. Effect of thermal inactivation of liver homogenate on the degradation of I$^{131}$-growth hormone and I$^{131}$-casein

Thermal inactivation was carried out by heating the homogenate in boiling water for 10 mins.

A: Incubated with non-heat-treated homogenate for 1 hr. at 37°C and pH 7.4.
B: Incubated with heat-treated homogenate similarly.
C: Control, incubated without homogenate.

An enzyme or enzyme system which is responsible for the degradation of I$^{131}$-labeled Parotin by submaxillary homogenate may be non-specific or may have rather broad specificity, because radioiodinated GH and casein were similarly degraded by submaxillary homogenate although I$^{131}$-B.S.A. remained almost untouched. The degradation of I$^{131}$-proteins by submaxillary or liver homogenate was almost completely inhibited after heating the homogenate in boiling water for 10 mins. (Figs. 10 and 11).

Junqueira et al. have reported the catheptic activity in submaxillary gland (Rothschild and Junqueira, 1951; Junqueira and Rothschild, 1953), and the lysozymic activity in saliva has been observed by many workers (Gialdroni and Libretti, 1951; Igari, 1953, Chauncey et al., 1954). Recently Shafer et al. have reported on the proteolytic enzyme activity of submaxillary gland in relation to the hypophysectomy and thyroxine or testosterone treatment (Shafer and Muhler, 1955; Shafer et al., 1956). The relation between the proteolytic activity in submaxillary gland observed in the activity in the higher Rf regions than the original line where I$^{131}$-protein was found to remain following the paperchromatography of the supernatant fluid of the incubation mixture. Concerning the direct deiodination of iodine bound to organic compounds, Kirkwood and co-workers (Fawcett and Kirkwood, 1954; Fawcett et al., 1954) reported that diiodotyrosine were deiodinated by salivary glands, however, Chaikoff and his school (Tong et al., 1954, 1955) were against this theory. The results of the present study revealed that I$^{131}$-labeled proteins were not deiodinated by salivary gland homogenates, although presumably proteolytic degradations were observed.

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present study and the catheptic activity, lysozymic activity or proteolytic activity reported by Shafer et al. is not yet clear. With regards to the nature of the proteolytic enzyme or enzymes in submaxillary gland, a series of experiments are now in progress in this laboratory.

Recently Ito and Okabe (1955) have reported the occurrence of a parotin-like substance with lower molecular weight (Saliva Parotin) in human saliva. In this connection, the enzyme system keeping the strong proteolytic activity in submaxillary gland is of interests, concerning the formation or secretion of biologically active principles at salivary glands.

SUMMARY

The in vitro degradation of I\(^{131}\)-labeled Parotin as well as similarly labeled bovine serum albumin, pituitary growth hormone, and casein by several tissue homogenates of rat, was studied by measuring the increase of the radioactivity in the non-protein fraction of the incubation mixture and also by employing the quantitative paperchromatographic analysis.

I\(^{131}\)-labeled Parotin, growth hormone and casein were degraded with remarkable rapidity by submaxillary gland homogenate. And some appreciable degradation of these three radioiodinated proteins was observed in case of kidney, spleen, parotid gland, muscle, testis, liver and pancreas homogenates.

By paperchromatographic analysis, the degradation of the labeled proteins by submaxillary gland homogenate and other tissue homogenates was not found to be deiodination but presumably proteolytic degradation.

ACKNOWLEDGMENTS

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REFERENCES