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## Association of Hypertrophy and DNA Synthesis in Mouse Salivary Glands after Chronic Administration of Isoproterenol

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ENLARGEMENT OF salivary glands induced by chronically administering isoproterenol (IPR) was first described by Selye *et al*<sup>1</sup> in 1961 in the rat and subsequently confirmed in rats and mice.<sup>2-7</sup> An increase in size of acinar cells and variations in the number of mitoses were reported. Brown-Grant,<sup>2</sup> Schneyer<sup>3</sup> and Wells<sup>4</sup> attributed the enlargement of salivary glands principally to hypertrophy. Subsequently, Barka<sup>8</sup> demonstrated that cell proliferation was a major factor in the increase in size of rat salivary gland. More recently, a sharp increase in mitotic rate was observed by Schneyer *et al*<sup>9</sup> in rat parotid 2-3 days after IPR was administered twice daily. Since mitotic activity dropped to zero after the fourth day, it was suggested that hyperplasia may not play a significant role in gland enlargement after prolonged IPR treatment. Changes in ploidy after repeated injections of IPR, first report by Schneyer *et al*,<sup>9</sup> have been confirmed in rat submaxillary gland by Radley.<sup>10</sup>

The experiments presented here were carried out to determine the relative role of each of the processes, hypertrophy and hyperplasia, in causing salivary glands in mice to enlarge after IPR was administered chronically. According to Kirby *et al*,<sup>11</sup> both optical isomers of IPR are

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quite active in stimulating DNA synthesis in salivary glands after a single dose. On the other hand, Houssay *et al*<sup>5</sup> found that *l*-isoproterenol was much more effective than the *d*-isomer in causing enlargement of the parotid gland after chronic administration. Since Kirby *et al*<sup>11</sup> hypothesized that both isomers can cause hyperplasia, but that only *l*-isoproterenol could produce hypertrophy, the effect of repeated injections of the racemic form of IPR and its optical isomers on DNA synthesis, mitotic rate and size of mouse salivary glands was also compared.

The results show that *dl*-IPR causes, in the first few days of treatment, marked stimulation of cell proliferation, followed later on by continued DNA synthesis, while mitotic activity drops progressively to zero. *l*-Isoproterenol was more effective than the *d*-isomer both in inducing cells to proliferate and in causing the salivary gland to enlarge.

### Materials and Methods

Fels A, male mice, weighing 30 g, bred in this laboratory, were used. The animals were kept on a 12-hour light and dark schedule (no light from 6 PM to 6 AM) and fed *ad libitum* until 2 hours before IPR was administered, when food but not water was removed from the cages. After IPR was injected, the mice again were given access to food. *dl*-Isoproterenol and its isomers, dissolved in water, were administered intraperitoneally once daily for the desired number of days. To determine the amount of DNA synthesis, the mice were killed 30 minutes after 0.3  $\mu$ Ci/g body weight of <sup>3</sup>H-thymidine was administered subcutaneously, unless otherwise stated. The parotid and submandibular glands were dissected free of lymph nodes and fat tissue and processed separately, as described below. For autoradiographic studies, pancreas, kidney and liver were also removed.

### Autoradiography

The organs were fixed in calcium-formalin, sectioned at 6  $\mu$  and autoradiographed as previously described.<sup>12</sup> The emulsion was Eastman Kodak NTB and exposure time, 30 days. The percentage of labeled cells was determined for each type of cell by examining 1000 cells. Mitotic rates were determined by counting the number of mitoses/2000 cells and are expressed as mitotic index, or number of cells in mitosis/1000 cells. In some experiments, colcemid (0.005  $\mu$ mole/g body weight) was used to arrest mitoses in metaphase.

### Extraction of Nucleic Acid

Parotid and submandibular glands were rapidly weighed, homogenized in 2.0 ml of 0.25 M sucrose containing 5% citric acid, and extracted by the method of Scott *et al*,<sup>13</sup> as modified by Hinrichs *et al*.<sup>14</sup> The amounts of DNA and RNA were determined by ultraviolet spectrophotometry. For RNA determination, the two-wavelength correction described by Tsanev and Markov<sup>15</sup> and Fleck and Munro<sup>16</sup> was used. The radioactivity in the DNA fraction was determined by adding 0.1- or 0.5-ml aliquots of DNA extract to 10 ml of a mixture of either toluene-ethanol-liquifluor or a Triton X-100-toluene-liquifluor. The samples were counted in a Packard Tri-Carb liquid scintillation spectrometer, at an efficiency of 33%.

#### Isolation of Nuclei and Determination of the Amount of DNA/Nucleus

Parotid gland nuclei were isolated by the method of Blobel and Potter.<sup>17</sup> The number of nuclei present in the final nuclear preparation was determined in a hemocytometer. The total amount of DNA in each final nuclear preparation was evaluated by Burton's modification of the diphenylamine reaction.<sup>18</sup> The average DNA content nucleus was calculated by dividing the total amount of DNA in the nuclear preparation by the number of nuclei.

#### Chemicals

*dl*-Isoproterenol was purchased from Winthrop Laboratories, New York, New York. *l*-Isoproterenol bitartrate dihydrate and *d*-isoproterenol bitartrate were kindly donated through the courtesy of Drs. Sydney Archer and Frederick Nachod of the Sterling-Winthrop Research Institute, Rensselaer, New York. Colcemid was purchased from Ciba Pharmaceutical Company, Summit, New York. Thymidine-methyl-<sup>3</sup>H (specific activity, 14.1–21.9 Ci mM) was purchased from New England Nuclear Corporation.

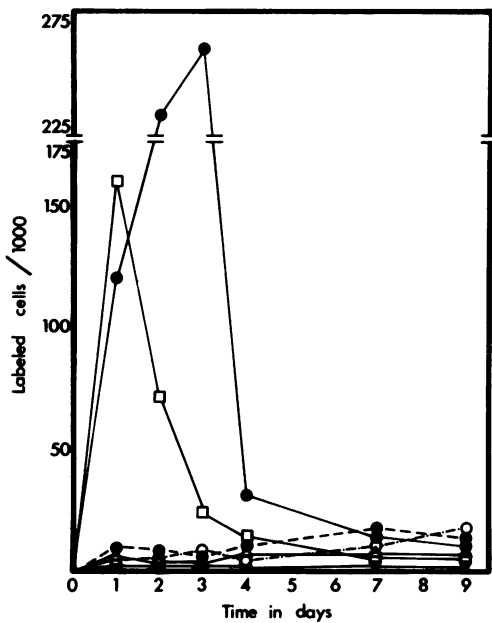
#### Results

##### Effect of Repeated Injections of *dl*-Isoproterenol on Incorporation of Thymidine-<sup>3</sup>H into DNA of Salivary Glands, Kidney, Liver and Pancreas

In these experiments, groups of mice were injected every 24 hours with *dl*-isoproterenol (0.8  $\mu$ mole/g body weight). Groups of 4 animals were killed daily, 24 hours after the last injection of the drug. Thirty minutes before killing, the animals were injected with 0.3  $\mu$ Ci/g body weight of thymidine-<sup>3</sup>H. Autoradiographic studies on the fraction of cells labeled by thymidine-<sup>3</sup>H under these conditions are shown in Text-fig 1. The stimulus for DNA synthesis was largely restricted to the salivary glands. The parotid showed the highest fraction of DNA-synthesizing cells, with a peak between the second and the third day of treatment. The submandibular gland responded less vigorously, with a maximum on the first day after the beginning of treatment. In the parotid, acinar cells were much more stimulated than interstitial cells, although the thymidine index of the interstitial cells increased slightly<sup>19</sup> at later intervals after the beginning of treatment. DNA synthesis was not stimulated under these conditions in liver or pancreas, but the thymidine index of the kidney cells increased moderately on the seventh and ninth days.

##### Effect of *dl*-Isoproterenol and its Optical Isomers on Stimulation of DNA Synthesis in Salivary Glands

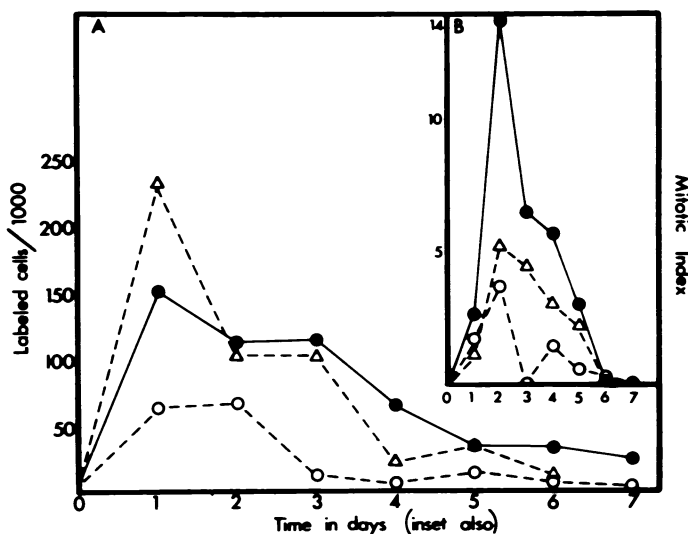
In these experiments, groups of mice were injected with the racemic form of IPR or with its optical isomers, *l*- and *d*-isoproterenol daily, at a dose level of 0.5  $\mu$ mole/g body weight. As in the previous experiment, the animals were killed at various intervals, but always 24 hours after the last injection of IPR. Thirty minutes before killing, the animals were



TEXT-FIG 1.—Percentage of labeled cells in mouse salivary glands, kidney, liver and pancreas after repeated injections of *dl*-isoproterenol (IPR). Mice received *dl*-IPR (0.8  $\mu$ mole/g body weight) daily and were killed 24 hours after last injection of drug at time intervals after the first injection of IPR, indicated on the abscissa. Thirty minutes before killing, thymidine- $^3$ H (0.3  $\mu$ Ci/g body weight) was injected subcutaneously. Parotid gland (acinar ●—●, interstitial ●- - -●); submandibular gland, acinar (□—□); kidney, tubular cells (○—○); liver, parenchymal cells (△—△); pancreas, acinar cells (▽—▽). Each point represents mean of 4 animals.

injected with thymidine- $^3$ H. The results are shown in Text-fig 2 to 4. Text-fig 2 shows the effect of *dl*-IPR and its optical isomers on the fraction of labeled cells in parotid gland, as well as the mitotic index determined 24.5 hours after the last injection of IPR. It is evident that *l*-IPR and *dl*-IPR were much more effective in stimulating cell proliferation in parotid, under these conditions, than *d*-IPR. The maximum stimulation, at this dose level, was observed at 1 day after the beginning of treatment, but a considerable amount of cellular proliferation, as measured by thymidine- $^3$ H incorporation, could still be detected up to the fifth day after treatment was begun. On the sixth and seventh days, the thymidine index was very low, almost near control levels. The mitotic index reached a peak on the second day after treatment was begun, and it returned to control levels by the sixth day. The results obtained with the submandibular gland, shown in Text-fig 3, were essentially similar, with *d*-IPR being much less effective than *dl*- or *l*-IPR in stimulating DNA synthesis. However, at variance with the parotid, the mitotic index, although clearly depressed, did not return to normal by 6 or 7 days after the beginning of treatment.

Text-fig 4 presents a different parameter of the same experiment described in Text-fig 2. Instead of the autoradiographic determination of the thymidine index, Text-fig 4 gives the specific activity of DNA of parotid gland 24 hours after the last injection of IPR and at various intervals



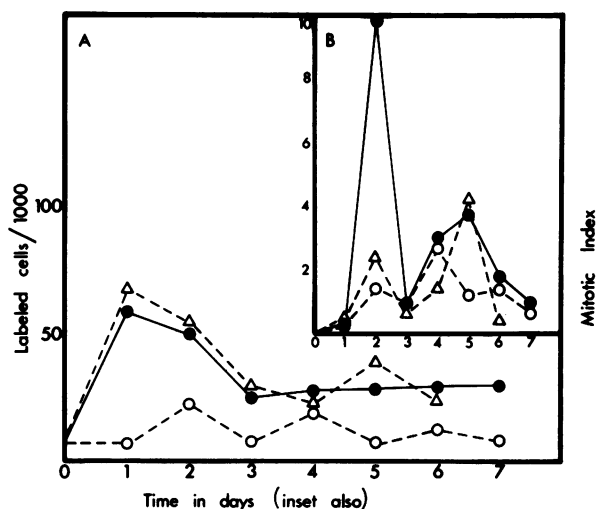
TEXT-FIG 2.—Percentage of labeled cells (A) and mitotic index (B) in mouse parotid gland after repeated injections of equimolar doses of *dl*- (●—●), *l*- (△---△) or *d*-isoproterenol (○---○). Mice were injected daily with *dl*-, *l*- or *d*-isoproterenol (0.5 μmole/g body weight) and killed 24 hours after last injection of the drug at times, after first injection, indicated on the abscissa. Thirty minutes before killing, thymidine-<sup>3</sup>H (0.3 μCi/g body weight) was injected subcutaneously. Each point represents mean of 4 animals.

after repeated daily injections of *dl*-IPR or its optical isomers. As in the autoradiographic studies, the incorporation of thymidine-<sup>3</sup>H into DNA was much higher after *dl*- or *l*-IPR than after *d*-IPR was administered. The specific activity of DNA reached a peak on the first and second days of treatment and then began slowly to decrease without, however, falling to normal levels. A similar pattern, not shown, was found in the submandibular gland.

It should be emphasized at this point that the thymidine index and the specific activity of DNA were determined at fixed intervals—*ie* 24 hours after the last injection of IPR. Since Whitlock *et al*<sup>20</sup> and Radley<sup>21</sup> have shown that repeated injections of IPR cause a shortening of the prereplicative phase, it is possible that the decreased thymidine index and specific activity of DNA observed in these experiments may be more apparent than real and may be due, in fact, to a shifting of the peak of response to the IPR stimulus.

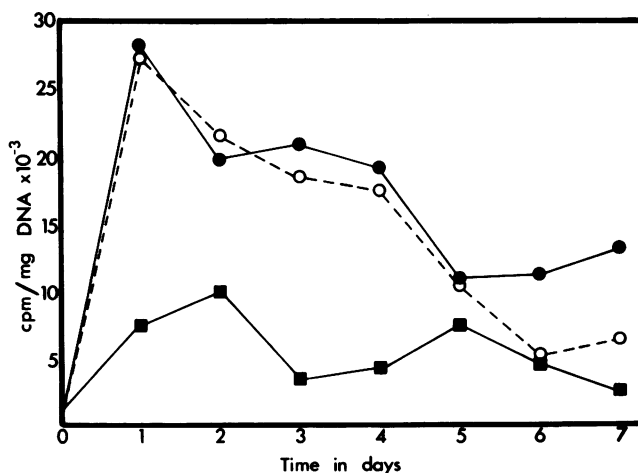
**Effect of Repeated Injections of *d*-Isoproterenol and its Optical Isomers on Weight of Salivary Glands**

In Text-fig 5, the effects of *dl*-IPR and its isomers on the weight of parotid gland are shown. On the fifth day of treatment, the weight of the

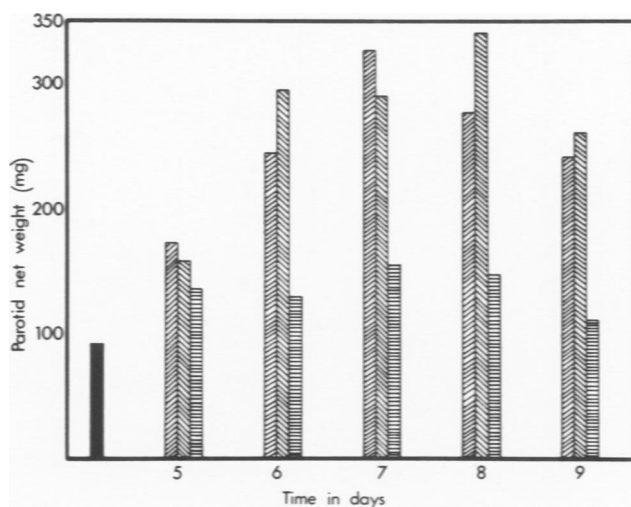


TEXT-FIG 3.—Percentage of labeled cells (A) and mitotic index (B) in mouse sub-mandibular gland after repeated injections of equimolar doses ( $0.5 \mu\text{mole/g}$  body weight) of *dl*- ( $\bullet$ — $\bullet$ ), *l*- ( $\Delta$ — $\Delta$ ) or *d*-isoproterenol ( $\circ$ — $\circ$ ). For details of experimental conditions, see Text-fig 2. Each point represents mean of 4 animals.

parotid gland was approximately the same in the 3 experimental groups, which was about twofold above control levels. After the sixth day, the weight increased sharply in mice receiving *dl*- or *l*-IPR, whereas *d*-IPR did not produce any further increase. Changes in weight of the sub-



TEXT-FIG 4.—Specific activity of DNA from mouse parotid gland after repeated injections of equimolar doses ( $0.5 \mu\text{mole/g}$  body weight) of *dl*- ( $\bullet$ — $\bullet$ ), *l*- ( $\circ$ — $\circ$ ) or *d*-isoproterenol ( $\blacksquare$ — $\blacksquare$ ). Details of experimental conditions are given in Text-fig 2. Each point represents mean of 4 animals.



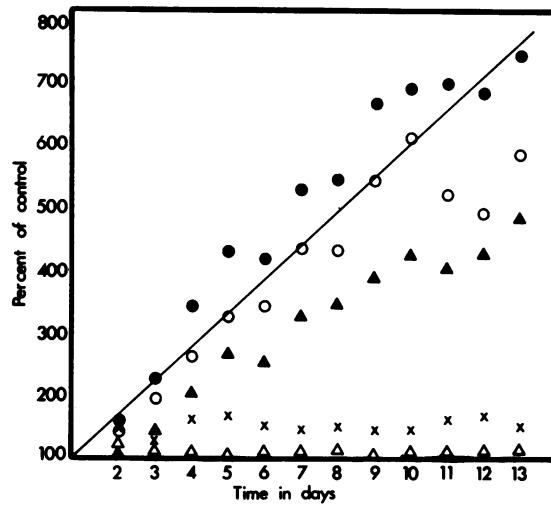
TEXT-FIG 5.—Weight of mouse parotid gland after repeated injections of equimolar doses ( $0.15 \mu\text{mole/g}$  body weight) of *dl*- (▨), *l*- (■) or *d*-isoproterenol (▩). Untreated mice (■). For details of experimental conditions, see Text-fig 2. Each value is mean of 4 animals.

mandibular gland induced by chronic administration of *dl*-IPR and its isomers (not shown) were similar to those of parotid.

#### Effect of Repeated Injections of *dl*-Isoproterenol on Parotid Gland

Since the first experiments confirmed that, in terms of DNA synthesis and cell division, salivary glands are definitely the organs most responsive to repeated injections of IPR, that the parotid is more responsive than the submandibular gland and that *dl*- and *l*-IPR are clearly more effective than *d*-IPR, subsequent studies were limited to the effect on the parotid gland of repeated injections of *dl*-IPR.

Text-fig 6 shows the effect on the parotid gland of repeated injections of *dl*-IPR ( $0.3 \mu\text{mole/g}$  body weight). In these experiments, the animals were injected repeatedly with *dl*-IPR and killed at the intervals indicated on the abscissa after the first injection of IPR. The ordinate gives the values expressed in percent of control values. It may be noted that the DNA concentration of the salivary gland did not change appreciably during a period of 13 days of treatment. The RNA/DNA ratio increased slightly in the first few days of treatment but then remained constant. On the other hand, the weight of the parotid, the total amount of DNA/gland and the total amount of RNA/gland continued to increase steadily for at least 10 days after treatment was begun. The question was then raised whether DNA continued to be



TEXT-FIG 6.—Percentage increase of mouse parotid weight ( $\blacktriangle$ ), DNA content ( $\circ$ ), RNA content ( $\bullet$ ), DNA concentration ( $\triangle$ ), and RNA/DNA ratio (X) after repeated injections of *dl*-isoproterenol (0.3  $\mu$ mole/g body weight). All values are expressed as % of control untreated animals (control = 100%). Mice were given IPR daily and killed 24 hours after the last injection of the drug at the times, after the first injection, indicated on abscissa. Each point represents mean of 3 animals.

synthesized even after the sixth day from the beginning of treatment, when the thymidine index and the amount of thymidine- $^3\text{H}$  incorporated into DNA at 24 hours after the last injection of isoproterenol seemed to indicate a marked decrease in the extent of cellular proliferation.

#### Continued Synthesis of DNA in Late Stages of Chronic Treatment with Isoproterenol

Because of variations from animal to animal and the uncertainties of the chemical method, an isotope dilution method was devised to determine whether DNA was being synthesized 6 days or more after treatment with IPR was begun. For these experiments, the animal were first injected with IPR and their salivary glands were labeled with seven injections of thymidine- $^3\text{H}$  (0.06  $\mu$ mole/g body weight) at 2-hour intervals. The time period during which thymidine- $^3\text{H}$  was injected covered a span from 20 to 32 hours after the first injection of IPR. Since this is the peak period of DNA synthesis, it was presumed that the glands were extensively and uniformly labeled. Beginning 48 hours after the first injection of IPR, the mice were injected daily with isoproterenol (0.3  $\mu$ mole/g body weight). On the sixth day, a group of animals was killed and the specific activity of DNA in the salivary gland was determined. Similar groups were killed at other intervals, as indicated in Table 1. The results indicate that, between day 6 and day 11, the spe-



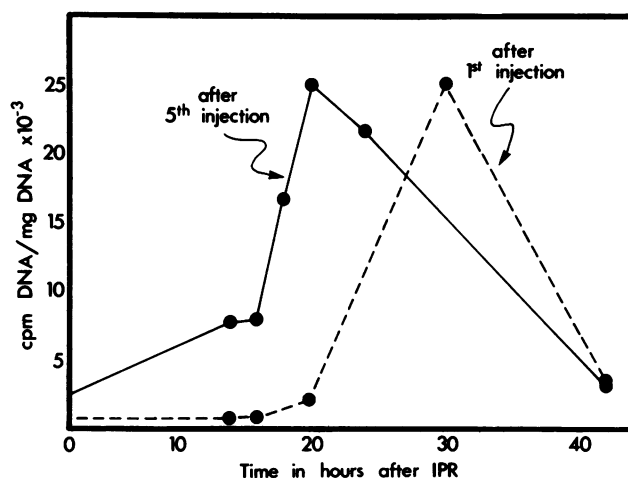
Table 1. Dilution of Specific Activity of DNA from Mouse Parotid Caused by Chronic Treatment with Isoproterenol

No. of injections of IPR	Time interval between first IPR injection and killing (days)	Mean weight parotid (mg)	Total amount of thymidine- <sup>3</sup> H/gland ( $\mu$ mole)	cpm DNA / mg DNA $\times 10^{-3}$ (mean range)	DNA	
					mg/gland	$\mu$ g / 100 mg wet wt
TREATMENT GROUP A						
1	6	62	0.037	81.9 $\pm$ 12.6	.221	356
1	12	65	0.037	78.8 $\pm$ 10.1	.233	359
TREATMENT GROUP B						
5	6	218	0.033	42.1 $\pm$ 9.1	.734	338
8	9	281	0.033	31.0 $\pm$ 1.4	.980	345
10	11	352	0.033	25.0 $\pm$ 3.8	1.406	393
11	12	339	0.033	26.4 $\pm$ 2.5	1.191	343
12	13	349	0.033	28.7 $\pm$ 4.3	1.111	317

Both groups received, at time zero, dl-isoproterenol (0.6  $\mu$ mole/g body weight) then 20 hours later seven injections of TdR-<sup>3</sup>H (0.06  $\mu$ Ci/g body weight per injection) at 2-hour intervals, from 20 to 32 hours after IPR. Group A mice served as controls for treatment group B and received no further treatment. Group B mice received dl-IPR (0.3  $\mu$ mole/g body weight) daily beginning 48 hours after the first injection. Each value represents the mean of 3 or 4 animals.

cific activity of parotid DNA decreased. This decrease in the specific activity of parotid DNA was accompanied by a proportional increase in the total amount of DNA/gland, while the total amount of DNA-bound radioactivity/gland remained constant. After the eleventh day, both the total amount of DNA/gland and the specific activity of parotid DNA remained constant, indicating that, after the eleventh day, no further DNA synthesis occurred. This in agreement with the results shown in Text-fig 6. Notice that without chronic IPR treatment (group A mice), both the total amount of DNA/gland and the specific activity of DNA remained constant from day 6 to day 12.

The possibility was then investigated that the peak of DNA synthesis may shift during chronic treatment with isoproterenol. The results of these investigations are shown in Text-fig 7, in which the response of mouse parotid was studied after one or five injections of IPR. The mice consisted of 2 groups: the first received only one injection of IPR, the second group received five injections of IPR. After the fifth or the only injection of isoproterenol, the mice were injected with thymidine-<sup>3</sup>H at various intervals indicated on the abscissa of Text-fig 7, and killed 30 minutes later. As was known from the experiments of Barka<sup>22</sup> in rats, and Baserga<sup>23,24</sup> in mice, after a single injection of IPR, increased DNA



TEXT-FIG 7.—Specific activity of DNA from mouse parotid gland after the fifth injection (●—●) or after single injection (●---●) of *dl*-isoproterenol. Mice received *dl*-IPR (0.3  $\mu$ mole/g body weight) either once only or daily for 5 days. Beginning 14 hours after last injection, they were given thymidine-<sup>3</sup>H (0.3  $\mu$ Ci/g body weight) at intervals indicated on abscissa and killed 30 minutes later. Each point is mean of 4 animals.

synthesis does not begin until the twentieth hour after the drug is administered. However, in animals receiving five injections of IPR, increased DNA synthesis already was detectable at 14 hours after the last injection of IPR, with a peak at 20 hours, whereas the peak for animals injected only once was about 28 hours.

A further confirmation that DNA synthesis continues even after six or seven injections of IPR is given by the results shown in Table 2, where the amount of DNA/nucleus in parotid glands was investigated. The results showed that, in control mice, the amount of DNA/parotid nucleus was roughly 10 pg. The amount increased to 18 pg/nucleus in animals receiving four injections of IPR and reached a peak of 28 pg/nucleus in mice receiving a treatment of 10 daily injections of *dl*-IPR. If the amount of DNA/nucleus progressively increases during chronic treatment with *dl*-IPR, it is reasonable to assume that this continued synthesis of DNA is not accompanied by cell division. Lack of mitotic figures by 6 days after IPR treatment was begun has been previously shown in Text-fig 2. The presence or absence of mitosis was investigated further and the results are shown in Table 3. In order to arrest mitoses in metaphase, colcemid (0.005  $\mu$ mole/g body weight) was injected 28 hours after a single dose of IPR (0.3  $\mu$ mole/g body weight) was administered and the mice were killed 4 hours later. After four or 10 injections of IPR (0.3  $\mu$ mole/g body weight), colcemid was administered 18 hours

Table 2. Amount of DNA/Nucleus in Parotids of Mice Repeatedly Injected with Isoproterenol

Experiment No.	No. of animals	IPR treatment (days)	Total amount of DNA ( $\mu\text{g}$ ) in FNP*	Total No. of nuclei in FNP* ( $\times 10^{-6}$ )	Amount of DNA/nucleus ( $10^{-12}\text{g}$ )
1	6	None	70	6.3	11.1
2	6	None	190	23.4	8.1
3	6	None	70	6.6	10.6
					9.9†
4	4	4	40	2.2	18
5	4	4	57.5	3.1	18.8
					18.4†
6	2	10	97.5	3.3	29.2
7	2	10	197.5	7.5	26.3
8	2	10	200.0	7.1	28.2
					27.9†

\* FNP, final nuclear preparation.

† Mean value.

after the last injection of IPR and the animals were sacrificed 8 hours later. The results confirmed our previous findings that the mitotic index was high in the early phase of IPR treatment but that, at later intervals, the number of mitoses progressively decreased to zero.

### Discussion

Our results demonstrate that both processes, hyperplasia and hypertrophy, are involved in the enlargement of mouse salivary glands caused by chronic administration of IPR.

Each process has a definite time interval during which it has a most conspicuous role. In the first 3 days of treatment with IPR, cell proliferation appears to be the main mechanism by which the glands respond to IPR. Both DNA synthesis, expressed in terms of thymidine index and specific activity of DNA, and mitotic rate increased parallelly. Subsequently, at least in the parotid, mitotic activity dropped to zero by 6 days, indicating that hyperplasia was not involved significantly in

Table 3. Mitotic Index in Mouse Parotid Gland After Treatment with Colcemid

Experiment No.	No. of animals	IPR treatment (days)	Total No. of cells counted per animal	No. of mitoses
1	3	1	1000	45 $\pm$ 9.6
2	3	4	1000	25 $\pm$ 0.9
3	4	10	2000	0 $\pm$ 0

Details of the experimental conditions are given in the text.

gland enlargement after prolonged treatment with IPR. DNA synthesis, however, continued until the ninth to eleventh day, when the increase in weight of the gland was nearly maximal.

The continued stimulation of DNA synthesis in parotid gland, in the absence of cell division, is demonstrated by the following findings: (1) an increased total amount of DNA/gland; (2) a decreased specific activity of prelabeled DNA when IPR treatment is continued between day 6 and day 12 after the first stimulatory injection and (3) the increased ploidy of the parotid nuclei. Thus, the late phase of IPR treatment (from day 6 to day 11) is characterized both by continued DNA synthesis and by an increased cell mass, as suggested by the increased wet weight of the gland, and confirmed by the continued increase in total RNA and the unchanging ratio of RNA/DNA.

The increased size of the gland, without cell proliferation, indicates that hypertrophy is the dominant cytologic event of this phase of treatment with IPR. However, the intriguing finding of continued synthesis of DNA contrasts with the classic definition of hypertrophy and raises an interesting philosophic question. Even in modern textbooks of pathology, hypertrophy is defined as an adaptive process in which "although this progressive increase in the mass of the functional cytoplasm may continue until the cell is many times larger than normal, reduplication of DNA and mitosis do not occur."<sup>25</sup> Our findings seem to indicate that a redefinition of hypertrophy may have to be considered, especially in view of other recent findings in the literature indicating that increased cell size may be associated with polyploidy.<sup>26-28</sup> Furthermore, these findings raise the possibility that hypertrophy and hyperplasia may share, to a large extent, common biochemical events, at least up to the completion of DNA synthesis—that is, at the S/G<sub>2</sub> boundary.<sup>29</sup> In other words, in some instances, the hypertrophic cell would simply represent a G<sub>2</sub>-blocked cell, as happens already under physiologic conditions in a number of tissues.<sup>30-31</sup> Thus, DNA synthesis and cell division, on one side, and DNA synthesis and polyploidy, on the other, could be regarded as different expressions of the same process.

Quite obviously, these tentative conclusions must be extended to a variety of tissues and situations before general rules can be proposed.

### Summary

Chronic administration of *dl*-isoproterenol (IPR) caused a continued synthesis of DNA in mouse parotid, associated with a progressive enlargement of the gland. The marked stimulation of cell proliferation observed in the first 3 days of treatment with IPR indicated that hyper-

plasia plays a major role in enlargement of the gland at this time. After the fifth day of treatment, the mitotic rate dropped to zero, indicating that hypertrophy becomes the dominant cytologic event in the continuing enlargement of the gland. However, DNA synthesis continued even after the fifth day, resulting in hyperploidy of parotid cells.

The effect of administering the optical isomers of *dl*-isoproterenol was also compared. *l*-Isoproterenol was more effective than the *d*-isomer, both in inducing DNA synthesis and in causing the salivary glands to enlarge.

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