

## Characterization of mouse salivary polypeptide secretion after oral administration of pilocarpine

### Caracterización de la secreción de polipéptidos salivales murinos en respuesta a la administración oral de pilocarpina

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#### ABSTRACT

Mouse salivary secretion provoked by single or multiple oral administrations of the cholinergic agent pilocarpine was characterized. An accessory ad hoc device, manipulated by a single operator, was used to collect saliva from various mice simultaneously. A single challenge by pilocarpine in the range 40-400 µg provoked a dose-dependent secretory response. The secretory response lasted for about 40 minutes and was constituted by four clearly defined stages, namely: lag (5-10 min), maximal flow (10 min), slowing (15-20 min) and postsecretory rest. In this response, usual parameters were: maximal flow rate, 30-40 µl/min; total volume of saliva, 250-350 µl/mouse and total salivary protein, about 700 µg/mouse. Temporal desensitization of the secretory response was observed within the first hour following a single stimulation by pilocarpine. However, recurrent stimulations by this agonist given at 24-h intervals produced an equally intense secretory response, thus suggesting resensitization during that period. The polypeptide composition of salivas obtained from a number of mice after a first pilocarpine stimulation were undistinguishable from each other. That salivary polypeptide pattern was also observed in a series of salivas obtained day by day from single animals stimulated at 24-h intervals by the agonist. Thus, both the characteristics of the secretory response as well as the polypeptide composition of mouse saliva after short-term or long-term challenges by pilocarpine were found to be highly consistent. Accordingly, these studies open the possibility of accomplishing a systematic molecular typing of saliva from individual living mice either from natural populations or from mice subjected to experimental laboratory conditions.

**Key words:** mouse, saliva, secretion, pilocarpine, polypeptides.

#### RESUMEN

Se caracterizó la secreción salival murina producida por administraciones orales únicas o múltiples del agente colinérgico pilocarpina. La saliva fue colectada desde varios ratones a la vez mediante un accesorio diseñado para tal fin y manipulado por un solo operador. Una simple estimulación mediante una dosis del agonista en el rango 40-400 µg provocó una respuesta secretoria dependiente de la dosis. La respuesta secretoria duró alrededor de 40 min y en ella se pudo definir cuatro etapas, a saber: lag (5-10 min), flujo máximo (10 min), enlentecimiento (15-20 min) y reposo post-secretorio. En la respuesta, los parámetros usuales fueron: velocidad de flujo máxima, 30-40 µl/min; volumen total de saliva, 250-350 µl/ratón y proteína salival total, alrededor de 700 µg/ratón. Se observó desensibilización temporal de la respuesta secretoria durante la primera hora siguiente a una estimulación simple por pilocarpina. Sin embargo, estimulaciones sucesivas a intervalos de 24 h con este agonista produjeron una respuesta secretoria igualmente intensa, lo que sugirió resensibilización en tales intervalos. La composición polipeptídica de cada una de las salivas obtenidas a partir de una serie de ratones estimulados por una vez con pilocarpina fueron indistinguibles entre sí. Tal patrón polipeptídico salival también fue observado en series de salivas obtenidas día a día a partir de ratones únicos estimulados con pilocarpina a intervalos de 24 h. De esta manera, tanto las características de la respuesta secretoria como la composición polipeptídica de la saliva murina obtenida mediante estimulaciones únicas o múltiples con pilocarpina resultaron altamente consistentes. Así, estos estudios abren la posibilidad de emprender una tipificación molecular sistemática de la saliva de ratones individuales vivos obtenidos ya sea de poblaciones naturales o de aquellos sometidos a condiciones experimentales de laboratorio.

**Palabras clave:** ratón, saliva, secreción, pilocarpina, polipéptido.

## PROLOGUE

Dr. Danko Brncic Juricic was a Master for generations of biologists. He used to hierarchize the three columns of the modern biological thinking. He impregnated his friends in the Academy, as I feel proudly I was, regardless our ancestors in cell biology or in genetics, with the fundamental view of evolution. This undelible stamp was bequeathed to us through his thoughts and on the experience of his deep scientific task carried out mostly in Chile and in the University. On his memory, my first observations on murine saliva.

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## INTRODUCTION

The interest in biology of mammal saliva has been in continuous increase during the last few years (Malamud & Tabak 1993, Martínez 1993). Areas like diagnosis (Mandel 1990, Malamud & Tabak 1993), drug and hormone monitoring (Cone 1993, Lam et al. 1991) and secretion formation (Jensen et al. 1991, Schenkels et al. 1995) in relation to human saliva have presently become the focus of active research. Animal models have proved of much help to overcome or simplify a variety of currently limiting methodological aspects in those studies (López-Solís et al. 1989, Quissell et al. 1993, Purushotham & Humphreys-Beher 1995). Stimulation of salivary secretion is an usual preparative stage in the collection procedures (Nicolau et al. 1992, Ryberg & Johansson 1995) and rat has been the preferred species in these studies (Nicolau et al. 1992, Quissell et al. 1993, Purushotham & Humphreys-Beher 1995, Ryberg & Johansson 1995). Among the stimulants, pilocarpine has been the favorite drug in comparison to a wide variety of pharmacological secretagogues such as the adrenergic agonists isoproterenol and adrenaline (Koller et al. 1992), peptidergic agonists like physalemin and substance P (Soltoff & Toker 1995) or other cholinergic agonists like carbachol (Liu et al. 1995). However, major differences in the quality of the saliva produced in response to some secretagogues have been reported (Muenzer et al. 1979, Ann et al. 1987, López-Solís et al. 1989, López-Solís et al. 1990, Bedi & Bedi 1995, Miao et al. 1995). For instance, stimulation of rats by pilocarpine or by  $\alpha$ -adrenergic agonists results in an abundant and aqueous salivary secretion in contrast to a scarce and proteinaceous saliva produced by  $\beta$ -adrenergic

agonists (Mangos et al. 1973, Mandel & Wotman 1976, Martínez 1990). Taking into consideration that dependency, a systematic characterization of both the drug-induced secretory response in an *in vivo* animal model and the properties of the corresponding saliva could be of major interest for the development of experimental approaches in salivary research. In the same direction, the availability of procedures for collecting saliva from several animals at once would be specially useful for addressing studies in which a minimal number of animals per experimental condition is required. Also, compositional data derived from the saliva of individual animals would be highly relevant for studies, like the ones on genetics, in which the previous knowledge on individual traits are fully necessary. Needless to mention in this kind of studies the relevance of keeping alive the experimental animals after the corresponding molecular typing. In the present study we have prepared a simple accessory device which allows, under the control of a single operator, the fixation and positioning of several mice at once for the collection of their salivas. The procedure has been used both in the characterization of the salivary response produced by a single or a long-term challenge by orally-administered pilocarpine and to establish the invariance of the mouse salivary polypeptide composition during the procedure.

## MATERIAL AND METHODS

*Animals*

A/Snell male mice weighing  $22 \pm 2$  g were used when 3-4 months old. The animals had free access to foods and water and were maintained under a 12-h alternate period of light and darkness. The experiments were carried out routinely between 08:00 and 12:00.

*Secretion stimulation*

Microvolumes (5-20  $\mu$ l) of 4 % pilocarpine or dilutions of it were instilled right in the mouse mouth. In the case of multiple collection procedures, salivary secretion stimulation in the various mice was scheduled at 10-min intervals.

*Saliva collection and quantification*

Salivation was monitored continuously in the experimental animals by blotting the mouth with discs of absorbing filter paper. Once salivation

became visible, aspiration of the salivary fluid was initiated by using a micropipette fitted with a bent disposable tip. Saliva from every single mouse was accumulated in preweighed Eppendorf tubes that were maintained in ice. At the end of or during the collection procedure, the Eppendorf tubes were weighed in order to estimate the amount of collected saliva by assuming a specific gravity of 1.00 g/ml.

#### *Protein content*

Aliquots of 30  $\mu$ l of saliva were spotted onto cellulose discs, fixed in 5 % trichloroacetic acid (TCA) and washed successively in 5 % cold TCA, 5 % TCA for 20 min at 90 °C, 80 % ethanol and 3:1 (v/v) ethanol/ether. The dry discs were incubated at 45 °C in 0.25 % Coomassie blue R-250, drained and washed exhaustively in several changes of 7 % acetic acid until the background was clear. The discs were dried and eluted in 3 ml of 66 % methanol-0.25 % ammonia. Eluates were read at 610 nm in a double-beam spectrophotometer. A standard curve was prepared by including discs containing 10-50  $\mu$ g of bovine serum albumin. Blank discs contained no protein (Bramhall et al. 1969, Durham & López-Solís 1979).

#### *Protein electrophoresis*

Aliquots of whole mouse saliva containing 30  $\mu$ g of protein were mixed with sample buffer and electrophoresed in SDS-polyacrylamide slab gels (11 %) as specified elsewhere (Laemmli 1970, López-Solís & Miranda-Wilson 1986). Following the electrophoretic separation, gels were fixed overnight in 15 % isopropanol/10 % acetic acid, rinsed twice in the same solution and stained for at least 15 hours in 0.25 % Coomassie blue R-250 dissolved in 45 % methanol/10 % acetic acid. The gels were rinsed exhaustively in 10 % isopropanol/10 % acetic acid until clear background and, finally, were photographed using Ilford NTB4 film. Gels were calibrated by using the following molecular weight standards: phosphorylase b (97 kDa), phosphofructokinase (84 kDa), bovine seroalbumin (66 kDa),  $\alpha$ -amylase (55 kDa), ovoalbumin (45 kDa), carbonic anhydrase (29 kDa) and  $\alpha$ -lactalbumin (14.2 kDa).

#### *Materials*

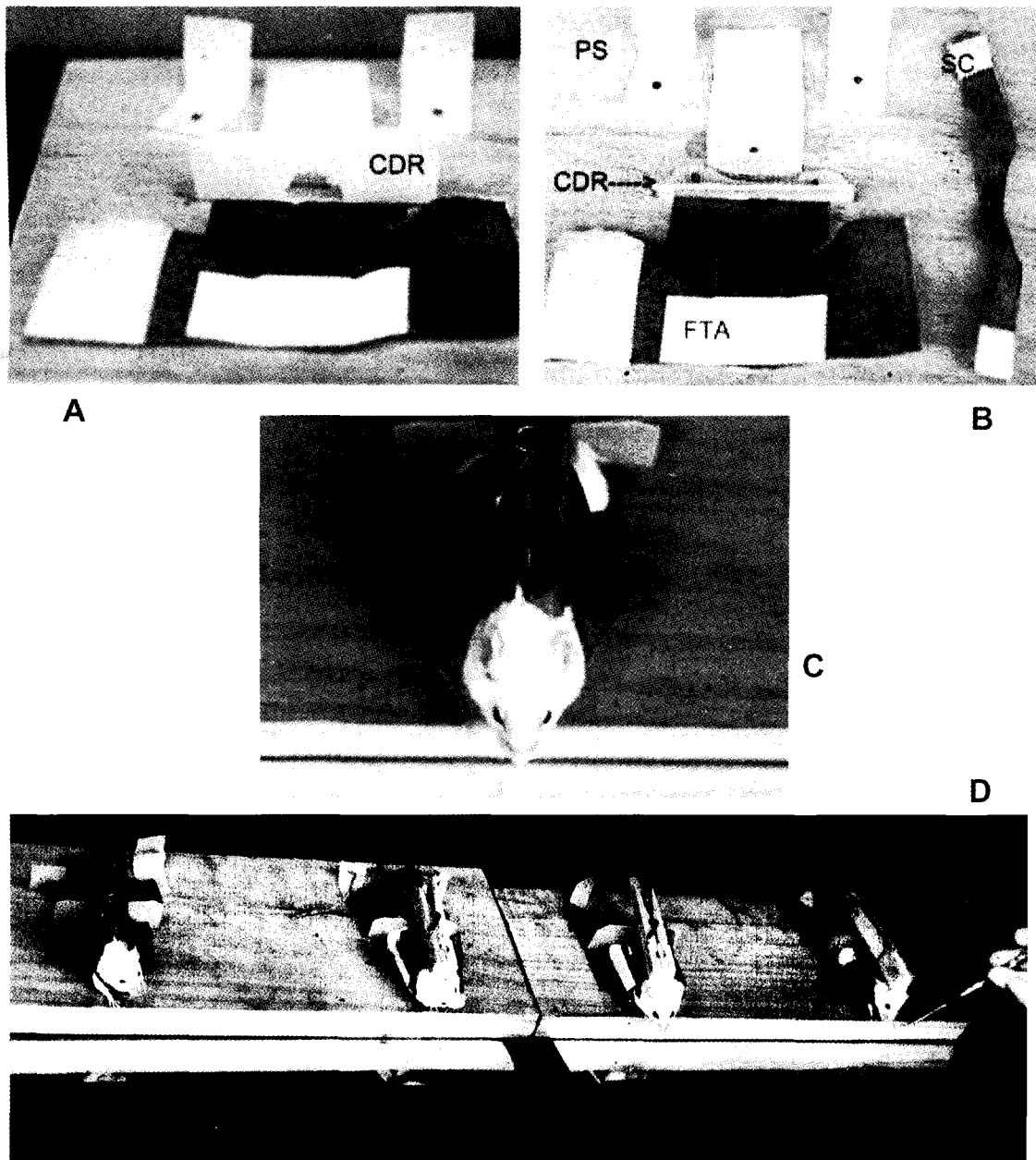
Pilocarpine in 4 % solution was obtained from pharmaceutical suppliers (Licarpin™, Saval

Laboratories, Chile). Chemicals and molecular weight standards for gel electrophoresis were acquired from Sigma, St. Louis, Missouri. Solvents used for gel processing were purchased from Merck-Chile. Cellulose discs (Whatman grade 1, 2.5 cm diameter) were obtained from Whatman, Maidstone, England.

## RESULTS

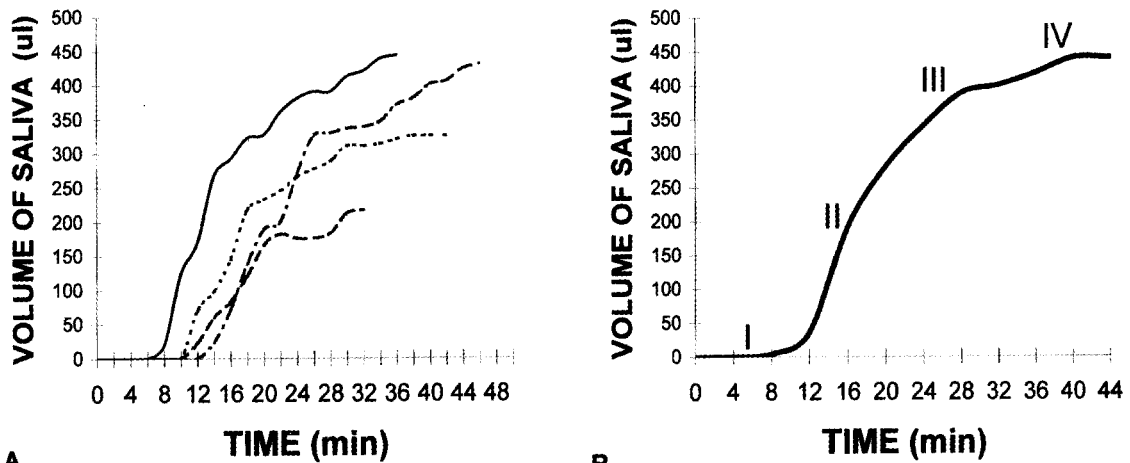
#### *Device for saliva collection*

A collection device was designed considering that the donor mouse should be held with no harm or stress, that whole saliva should drain preferentially toward the outside of the mouth, and that collection should be carried out continuously with minimal loss by trained personnel. The first aim was achieved by holding the mouse firmly and gently by using various leather and plastic pieces that were fixed to a solid platform (Fig. 1). The mouse is firstly positioned against a rigid plastic vertical sheet (CDR) that avoided the sliding of the animal backwards (Fig. 1A). A hole through this piece allowed to keep the mouse tail in a straight position close to the platform with the aid of an adhesive tape. A second part of the device consists in a leather rectangle (FTA) fixed by its center to the platform. This piece is necessary to wrap both the chest and the abdomen of the mouse leaving free the four extremities (Figs. 1B and 1C). On both ends of the leather rectangle two pieces of attached adhesive fabric (Bellcro™) allow the regulation of the fastening pressure. A third part of the device is a leather strip (SC) with a piece of adhesive fabric on both ends. This strip is passed between the chest and the jaw in order to keep the head distant from the upper extremities. Fourthly, a wood clamp is used to pull upwards the fur of the upper part of the head/neck interphase and so to keep open the mouse mouth (Fig. 1C). The wood clamp is kept horizontally by tying its distal ends to two rigid plastic pieces that are fixed to the platform (see Fig. 1A). Once the animal is installed, its head surpasses the border of the platform in such a way that the access to its mouth by the operator is unimpeded. To facilitate the collection procedure, the device includes two final accessories, namely, a mirror and a wedge. The mirror is positioned underneath the platform and protruded from it in such a way that both the mouse mouth and its saliva can be readily seen from above by the operator. The wedge is positioned between the mirror and the platform and is used to inclinate the platform (30 °C) in such a way that the animal reaches an



*Fig. 1.* Accessory device for multiple mouse saliva collection. (A) Frontal view of the slide stopper. At the bottom, two vertical rigid plastic pieces to which the arms of the wood clamps are tied by means of strings. (B) Major components of the holding device. PS, supporting platform; FTA, thoraco-abdominal band; SC, neck belt and CDR, slide stopper. (C) Details of the holding of a single mouse. The head is held by pulling upwards the fur of the head/neck interphase by means of a wood clamp and by passing a leather strip (SC) between the chest and the jaw. The wood clamp is positioned in parallel to the mouse body. (D) Panoramic view showing four mice during a collection procedure. Saliva is being aspirated from the mouse on the right by means of a bent disposable tip attached to a syringe.

Dispositivo para la colección múltiple de saliva murina. (A) Vista frontal de la lámina antideslizante. Al fondo, dos piezas plásticas rígidas verticales a las que se amarran ambos brazos de la pinza de madera. (B) Componentes principales del sistema de sujeción. PS, base de soporte; FTA, banda toraco-abdominal; SC, correa para el cuello y CDR, lámina antideslizante. (C) Detalles de la sujeción de un ratón. La cabeza es mantenida mediante tracción de la piel hacia arriba en la interfase cuello/cabeza mediante una pinza de madera y pasando una tira de cuero (SC) por debajo de la mandíbula. La pinza de madera se dispone en paralelo al cuerpo del ratón. (D) Vista panorámica de 4 ratones durante un procedimiento de recolección. En el ratón del costado derecho la saliva está siendo aspirada mediante una punta de micropipeta doblada que ha sido adaptada a una jeringa.



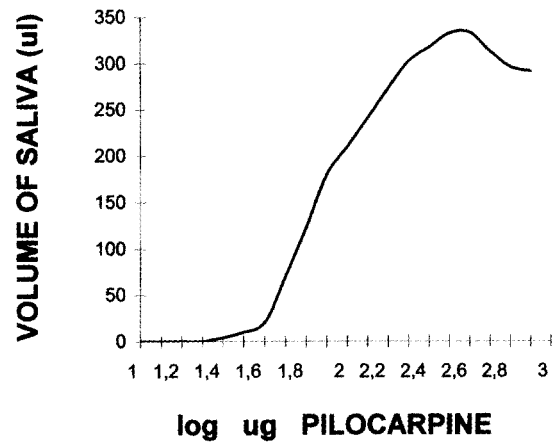
**Fig. 2.** Time course of salivary secretion in mice orally stimulated by pilocarpine. (A) A single oral administration of 400  $\mu\text{g}$  pilocarpine in 10  $\mu\text{l}$  was given at time zero to a group of 4 mice positioned on the device described in the previous figures. Saliva from each animal was continuously collected and quantified gravimetrically as described under Material and Methods. (B) The usual four stages of the pilocarpine-induced salivary response displayed by every single animal, namely, I, lag; II, maximal rate; III, slowing or retardation and IV, post-secretory rest, are indicated on the representative curve.

Curva de progreso de la secreción salival en ratones estimulados oralmente por pilocarpina. (A) En el tiempo cero se administró 400  $\mu\text{g}$  de pilocarpina en 10  $\mu\text{l}$  a cada uno de 4 ratones dispuestos en el dispositivo descrito en las figuras anteriores. La saliva de cada animal fue colectada y cuantificada gravimétricamente en forma continua, de acuerdo a lo descrito en Materiales y Métodos. (B) Las 4 etapas de la respuesta salival inducida por pilocarpina en animales individuales, a saber, I, lag; II, velocidad máxima; III, entlentecimiento y IV, reposo post-secretorio, son indicadas en la curva representativa.

equivalent inclination downward. That inclination helps to orientate the flow of saliva out of the mouth. Positioning of the animal in the device may take no longer than 1 minute. The device allows collection of whole saliva from single animals but it can be readily expanded to allow collection from several animals at once (Fig. 1D). In our experience, trained personnel could manage the collection of whole saliva from as many as 8 mice simultaneously.

*Time-course of the salivary secretory response*

Continuous monitoring of salivary secretion in individual mice ( $n = 4$ ) from the time of pilocarpine administration (time zero) onwards shows that the flow of saliva remains unaltered until at least 6-12 min (Fig. 2A). After that lag period, an abrupt increase in the salivary flow to rates of about 30  $\mu\text{l}/\text{min}$ , was observed. The flow of saliva displayed a constant maximal rate until  $20 \pm 3$  min and was followed by a slow but continuous decrease until  $32 \pm 5.5$  min. By that time, no more secretory response could be appreciated. No significant differences between individual mice were observed with regard to both shape of the response curve and timing of the various stages



**Fig. 3.** Dose-dependency of the pilocarpine-induced salivary secretion. At time zero, pilocarpine was orally administered in a total volume of 10  $\mu\text{l}$  at a dose in the range 20-800  $\mu\text{g}/\text{mouse}$ . Saliva produced by each animal in a 30-min period was quantified gravimetrically. Averages from triplicates are shown. Standard deviations are less than 15 % of the corresponding averages.

Secreción salival inducida por pilocarpina: dependencia de la dosis. En el tiempo cero, por vía oral se administró 20-800  $\mu\text{g}/\text{ratón}$  de pilocarpina en un volumen total de 10  $\mu\text{l}$ . La saliva producida por cada animal en un período de 30 min fue medida gravimétricamente. Los valores señalados corresponden a promedios de triplicados. Las desviaciones estándar fueron menores de 15 % de los promedios correspondientes.

(Fig. 2A). Thus, in the mouse response to a single challenge by orally-administered pilocarpine at least 4 clearly-defined stages could be identified, namely, stage I or lag period; stage II or maximal flow rate; stage III, retardation and stage IV, postsecretory rest (Fig. 2B).

#### *Dose-response relationship of the salivary secretory response to pilocarpine*

The oral administration of a single dose of pilocarpine over the range of 40-400  $\mu\text{g}$  resulted in a dose-dependent increase in the volume of salivary secretion in a 30-min period. No response was detected after the oral administration of doses of pilocarpine lower than 40  $\mu\text{g}$ . Doses over 400  $\mu\text{g}$  provoked a significant decrease in the rate of salivation (Fig. 3). The substitution of the oral route of administration by the intraperitoneal injection of equivalent doses of pilocarpine produced no change in the total response or in the dose-dependent relationship (data not shown).

#### *Effect of repetitive administrations of pilocarpine on the salivary secretory response*

Considering that the decrease in the rate of salivary secretion observed in stage III after a simple stimulation by pilocarpine might be due to the catabolism of the agonist, experiments were designed in order to analyze the secretory effect of additional administrations of pilocarpine given at time-intervals in which the rate of salivary secretion was either near to decrease or had frankly decreased. Thus, after a first oral administration of pilocarpine and at a time around the end of stage II and the beginning of stage III (20-25 min after pilocarpine) the animals were restimulated by a new administration of pilocarpine. A second restimulation by pilocarpine was provoked at 20-25 min later, that is, at a time around the end of stage III or at stage IV of the secretory response to the first stimulation by pilocarpine. Monitoring of saliva secretion was continuously carried out at regular 5-min intervals for as long as 1.5 h. Under these conditions, the flow of saliva after the second or the third pilocarpine administrations remained almost unaffected in reference to the secretory response produced by a single stimulation by pilocarpine, that is, the presence of pilocarpine during the second or during the third administration of pilocarpine was not a sufficient condition for provoking a recognizable salivary secretory response (Fig. 4).

#### *Salivary secretory responsiveness to a daily chronic administration of pilocarpine*

The lack of responsiveness to additional administrations of pilocarpine that was described in the previous section led us to analyze the secretory effect of a series of pilocarpine administrations given at long intervals. To that end, groups of mice were stimulated by oral administrations of pilocarpine at 24-h intervals during a 4-day period and the saliva produced in the 30-min period following each stimulation was measured. Under this plan, every animal displayed a marked salivation in response to each one of the four pilocarpine administrations. Furthermore, no diminution in the intensity of the secretory response

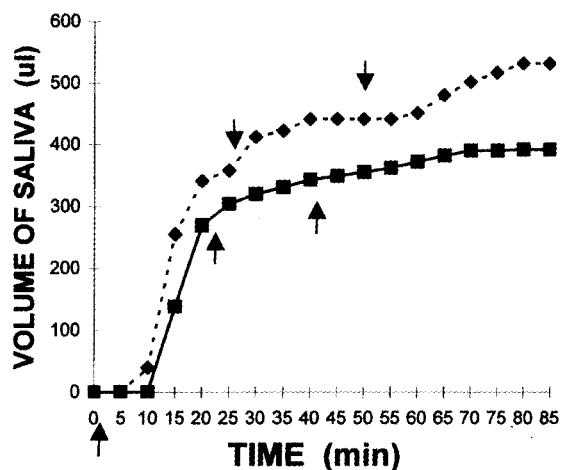


Fig. 4. Effect of recurrent oral administrations of pilocarpine on the mouse salivary secretion. At time zero, pilocarpine was orally administered at a dose of 400  $\mu\text{g}$  in a total volume of 10  $\mu\text{l}$ . Saliva was collected and quantified continuously. At about minutes 20-25 and 40-50 (arrows) every mouse received a second and a third identical dose of pilocarpine. Collection and quantification of saliva was continuously carried out for about 1.5 h. Note on the curves corresponding to two mice from a single representative experiment the minor secretory effect of the second and the third pilocarpine administrations.

Efecto de administraciones orales recurrentes de pilocarpina sobre la secreción salival murina. En el tiempo cero, a dos ratones se administró 400  $\mu\text{g}$  de pilocarpina por vía oral en un volumen total de 10  $\mu\text{l}$ . La saliva fue colectada y cuantificada continuamente. Alrededor de los minutos 20-25 y 40-50 (flechas) cada ratón recibió una segunda y una tercera dosis idéntica de pilocarpina. La colección y cuantificación de la saliva se efectuó continuamente durante alrededor de 1,5 h. Nótese en las curvas correspondientes a dos ratones de un experimento representativo el efecto menor de las segundas y terceras administraciones de pilocarpina.

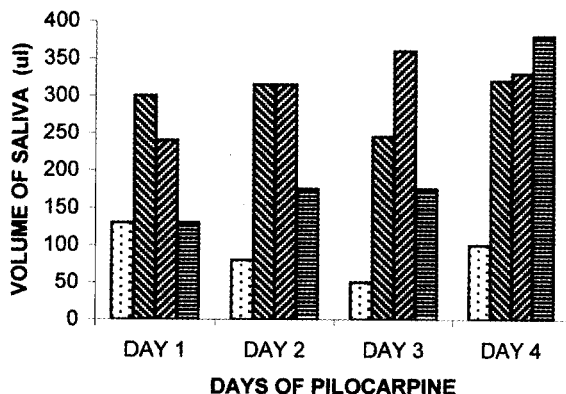


Fig. 5. Effect of daily oral administrations of pilocarpine on the mouse salivary secretion. Pilocarpine was orally administered (400 µg/mouse) at 24-h intervals during 4 days to a group of 4 mice. Following each daily pilocarpine administration, saliva from individual mice (single bars) was collected during a 30-min period. In the representative experiment, the total volumes of saliva produced after each successive pilocarpine stimulation by the whole group of mice as well as the secretory response displayed by each mouse were roughly constant. Note, however, some marked differences in the intensity of the secretory response exhibited by different mice of the group.

Efecto de administraciones orales diarias de pilocarpina sobre la secreción salival murina. Se administró pilocarpina por vía oral (400 µg/ratón) a intervalos de 24 h durante 4 días a un grupo de cuatro ratones. Después de cada administración diaria de pilocarpina, la saliva de ratones individuales (barras) fue colectada durante 30 min. En el experimento representativo, los volúmenes totales de saliva producidos por el grupo completo de ratones después de cada estimulación por pilocarpina, como también la respuesta secretoria exhibida por cada ratón, fueron aproximadamente constantes. Nótese, sin embargo, algunas diferencias marcadas en la intensidad de la respuesta secretoria exhibida por diferentes ratones del grupo.

exhibited by each individual mouse during the four-day treatment with pilocarpine was observed (Fig. 5). Thus, mice fully recovered the ability to respond to pilocarpine within the 24 hours following each new stimulation with the same agonist.

*Yield of salivary secretory protein after a single oral administration of pilocarpine*

Considering that salivary protein is secreted by exocytic mechanisms which have been mostly associated to the stimulation of β-adrenergic re-

ceptors and that pilocarpine is a cholinergic agonist acting quite likely at the level of the superior cervical ganglion, experiments were designed in order to measure salivary protein secretion and the protein concentration in saliva after the oral administration of pilocarpine. To this aim, ten

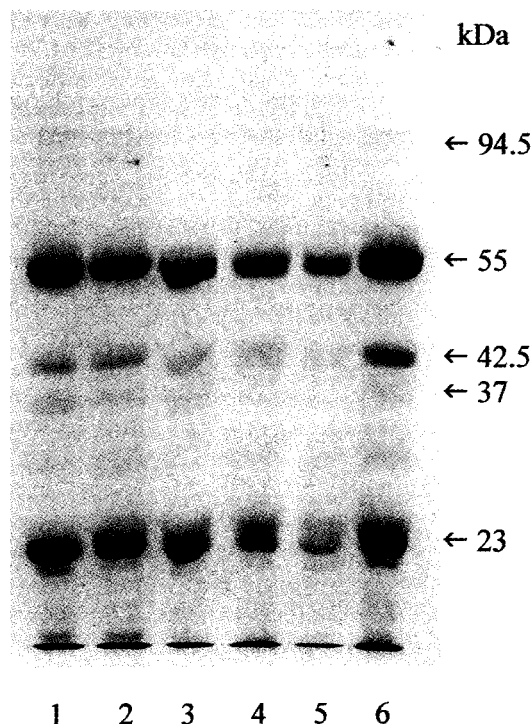


Fig. 6. Polypeptide composition of saliva produced in response to a single oral administration of pilocarpine. At time zero, pilocarpine was orally administered at a dose of 400 µg/mouse in a total volume of 10 µl and saliva was collected during a 30-min period. Aliquots of saliva from individual mice containing 30 µg protein were subjected to electrophoretic analysis as described under Material and Methods. In the gel each track corresponds to the saliva from a different mouse. Note the constancy in the polypeptides present in the saliva from the whole group of animals. Arrows on the right indicate molecular weight of polypeptides that are normally present in the pilocarpine-induced saliva.

Composición polipeptídica de la saliva producida en respuesta a una administración simple de pilocarpina. En el tiempo cero, por vía oral se administró pilocarpina (400 µg/ratón en 10 µl) y se colectó saliva durante 30 min. Alícuotas de saliva de ratones individuales correspondientes a 30 µg de proteína fueron sometidas a análisis electroforético según se describe en Materiales y Métodos. Cada carril del gel corresponde a un ratón diferente. Nótese la constancia en los polipéptidos presentes en la saliva de todos los animales del grupo. Las flechas al costado derecho señalan los pesos moleculares de polipéptidos normalmente presentes en la saliva inducida por pilocarpina.

mice were stimulated by a single oral administration of pilocarpine and the saliva collected in the following 30 min was used to quantify the secretory response both in terms of salivary volume and amount of secretory protein. Protein concentration in the saliva secreted by each animal was measured and the mass of protein was calculated as the product of the volume of saliva by the corresponding protein concentration. Thus, in a 30-min period the average volume of collected whole saliva per mouse was  $323 \pm 105 \mu\text{l}$  and the average protein concentration of those salivas was  $2.41 \pm 0.51 \text{ mg/ml}$ . Accordingly, in the 30-min period following the oral administration of  $400 \mu\text{g}$  of pilocarpine the present procedure allowed a collection of over  $800 \mu\text{g}$  of protein per mouse, that is, in a single collection procedure applied simultaneously to a group of ten pilocarpine-stimulated mice a total amount of 8 mg of salivary protein was collected.

*Polypeptide composition of the whole saliva collected from individual mice after a single stimulation by pilocarpine*

Salivas collected from six different mice during the 30 minutes following a single stimulation by pilocarpine were subjected to a standard electrophoretic separation in SDS-polyacrylamide gels and were visualized by Coomassie blue staining. As expected, two major electrophoretic bands (Mr 55 and 22 kD) were readily visible. Also, a few polypeptide bands were identified in each one of the six salivas (101-110 kD triplet; 40, 35 and 25 kD) (Fig. 6). Basically, no differences were observed in the whole group of salivas in such a way that a representative polypeptide composition or polypeptide pattern for the saliva of these animals obtained after a single pilocarpine stimulation could be established.

*Polypeptide composition of whole saliva collected from individual mice during a chronic daily treatment with pilocarpine*

Considering that the magnitude of the salivary secretory response was roughly constant during the chronic administrations of pilocarpine given at 24-h intervals, experiments were designed in order to assess whether the polypeptide composition of saliva was altered or whether it remained unchanged after that treatment. As shown in Fig. 7, the polypeptide composition of saliva collected at various days of the chronic pilocarpine treatment exhibited the same group of polypeptides and with the same intensity

than those observed in the saliva of mice subjected to a single pilocarpine stimulation. Such a constancy would indicate that saliva collected after a single or after a chronic stimulation by pilocarpine are

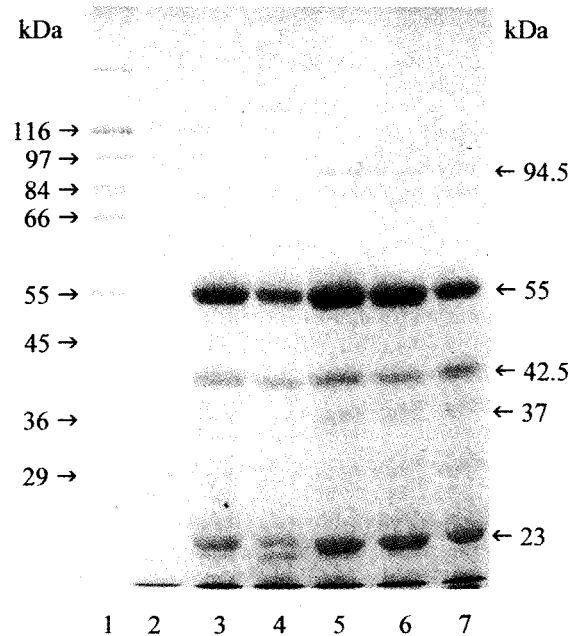


Fig. 7. Polypeptide composition of saliva produced in response to a chronic daily oral administration of pilocarpine. Pilocarpine was orally administered at 24-h intervals for 1 to 5 days ( $400 \mu\text{g}/\text{mouse}/\text{day}$ ). Saliva was collected during the 30-min period following each stimulation. Aliquots of saliva from individual mice containing  $30 \mu\text{g}$  protein were subjected to electrophoretic analysis as described under Material and Methods. The representative gel shows the polypeptide composition of saliva samples from a single mouse obtained at various successive days starting at day 1 (track 3). Note the constancy in the polypeptides present in the series of saliva samples. Arrows on the right have the same meaning as in the previous figure. On the left molecular weight standards indicated in Material and Methods (tracks 1 and 2).

Composición polipeptídica de la saliva producida en respuesta a una estimulación oral crónica diaria de pilocarpina. Se administró pilocarpina por vía oral a intervalos de 24 h durante 1 a 5 días ( $400 \mu\text{g}/\text{ratón}/\text{día}$ ). La saliva fue recolectada en los 30 min siguientes a cada estimulación. Alícuotas de saliva de ratones individuales correspondientes a  $30 \mu\text{g}$  de proteína fueron sometidas a análisis electroforético según se describe en Materiales y Métodos. El gel representativo muestra la composición polipeptídica de muestras de saliva de un mismo ratón obtenidas en días sucesivos a partir del día 1 (carril 3). Nótese la constancia en los polipéptidos presentes en la serie de muestras de saliva. Las flechas en el costado derecho tienen el mismo significado que en la figura anterior. A la izquierda, marcadores de peso molecular indicados en Materiales y Métodos.



undistinguishable from each other by their polypeptide composition.

#### DISCUSSION

The present study characterizes both the conditions for the collection of mouse whole saliva after the oral administration of pilocarpine and some features of that secretory fluid. Collections were carried out with the aid of an ad hoc device which allowed the collection of saliva from several mice simultaneously.

A single oral administration, rather than the conventional intraperitoneal injection, of a dose of pilocarpine in the range 40-400  $\mu\text{g}$  provokes in a dose-dependent manner a salivary secretory response that lasts for about 40-45 min. In this response, four well-defined stages can be identified, namely, (a) a lag period, since the time of pilocarpine administration until the onset of salivation, (b) a period of maximal and constant salivary flow, in which the slope of the response indicates the production of about 20  $\mu\text{l}/\text{min}$  of whole saliva, (c) a retardation period, corresponding to a progressive decrease in the rate of salivation and (d) a postsecretory rest, in which no more pilocarpine-induced salivation can be observed. Thus, after oral pilocarpine the productive period for saliva collection covers about 25-30 min. Protein concentration in that saliva was about 2-3 mg/ml, that is, about 2- to 3-fold more concentrated than human whole saliva (Morales & López-Solís 1998). Qualitatively, the polypeptide composition of the saliva collected from individual mice was found to be a constant feature in all the pilocarpine-stimulated mice, thus suggesting the existence of a molecular pattern in the saliva of these animals. Strain in-breeding, that is, the production of animals by repetitive matings of siblings for over 40 years, might explain such an observation. With the present methodological approach, the analysis of the salivary polypeptide composition in individual mice from natural populations would be highly illustrative with regard to the possible occurrence of genetic polymorphism in that salivary trait.

The intensity of the mouse salivary response occurring after a single pilocarpine administration remains basically unaltered when the agonist is readministered one or more times within the retardation or the rest periods, that is, no significant increase in the rate of salivary secretion during those secretory stages was observed after additional administrations of pilocarpine. In our view, this fact would indicate that once secretion has been triggered by pilocarpine, desensitiza-

tion mechanisms of that response, other than the catabolism of the agonist, start to operate (Harper & Brooker 1978, Harper 1988, Quissell et al. 1992). Since the secretory responsiveness to successive pilocarpine administrations was found to be fully restored when restimulation occurred at 24-hour intervals, resensitization mechanisms or the inactivation of the desensitization mechanisms should operate within those periods.

Under conditions of chronic daily stimulations by pilocarpine, the pattern of salivary polypeptide composition remained unchanged. This result is in open contrast with the one observed after the administration of other salivary stimulants, such as some  $\beta$ -adrenergic agonists, which in various rodent species induce the appearance of new secretory polypeptides (López-Solís et al. 1993, Ann et al. 1997). However, our observation is in full concordance with the fact that no pilocarpine-induced salivary polypeptide has been described in rodent species.

Saliva collection in the present study was carried out by considering the various above-mentioned characteristics of the pilocarpine-induced secretory response. Thus, within the 30-min period following an oral administration of 400  $\mu\text{g}$  of pilocarpine to a single mouse, about 300  $\mu\text{l}$  of saliva and 800  $\mu\text{g}$  of salivary protein are currently collected. This is a clearly sufficient amount of protein for most of the usual general analytical procedures (López-Solís & Miranda-Wilson 1986, Schwartz et al. 1995, Veerman et al. 1996). In other words, mice could be typed individually regarding their salivary polypeptide composition. Preliminary studies from our laboratory have shown the existence of variants regarding the salivary polypeptide composition, thus opening the possibility of addressing studies on "experimental sialogenetics". On the other hand, the pattern character of the salivary polypeptide composition observed in the experimental mice would allow to address some preparative procedures, such as the purification of salivary polypeptides, by means of the combination of salivas produced by several mice after a single pilocarpine-stimulation. Likewise, taking into consideration the constancy in the polypeptide composition of mouse salivas collected at various days of the chronic daily treatment with pilocarpine and the fact that each one of that series of salivary responses along the pilocarpine treatment are equally intense, the daily accumulation of those salivas would also allow to increase the mass of collected salivary protein or the mass of specific polypeptides normally present in mouse saliva. This is specially relevant in considering that every mouse might be maintained under a continu-

ous schedule of once-a-day stimulations by pilocarpine and whole saliva collections. As a corollary of these considerations, the number of mice that need to be subjected to a chronic pilocarpine stimulation in order to produce a definite amount of salivary protein can be reduced in a reverse relationship to the days of treatment with that agonist. This kind of balance should be necessarily taken into account in the planning of in animal experiments.

Finally, in the present study saliva from groups of animals was collected with the valuable aid of a device that was specifically designed for that purpose. Animals were held in a quite comfortable position and anesthesia was unnecessary. Usually, a single operator could be encharged of the procedure involving the simultaneous collection of saliva from as many as 8-10 animals and lasting less than 1 h. Besides time saving and the possibility of organizing the experiments with a sufficient number of specimens, the use of the device allowed fidelity in the reproduction of the experimental conditions when multiple collection procedures were carried out. As a whole, these various relevant aspects of the collection procedure would make more feasible some kinds of studies in which the number of mice per experimental group, the number of experimental groups to be compared and, certainly, the maintenance of the typed animals alive, constitute an unresignable methodological need.

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