CALIFORNIA STATE UNIVERSITY, NORTHRIDGE

EVIDENCE OF A GASTRIN-LIKE SUBSTANCE
" IN RHINOBATUS PRODUCTUS

A thesis submitted in partial satisfaction of the
requirements for the degree of Master of Science in

Biology

by

Duane Gilbert Hansen, Jr.

January, 1973

The thesis of Duane Gilbert Hansen, Jr. is approved:

Committee Chairman

California State University, Northridge

January, 1973

To my Father, Mother, and Brother:

 a family
of which I am proud to be a member.

ACKNOWLEDGEMENTS

I am indeed grateful to many people for their help in this study.

First of all, I wish to thank the investigators and laboratory personnel of the Gastrointestinal Research Laboratory of VA Wadsworth Hospital, especially:

Dr. Morton I. Grossman, Senior Medical Investigator, for introducing me to the study of gastrin and for supporting me through several years of gut physiology research;

Dr. John H. Walsh for his advice and encouragement; and

Mr. Raymond Lichter for sharing with me his experience in laboratory and animal handling techniques.

I would also like to express my appreciation to Dr. Richard Potter, my Graduate Advisor, for the use of his laboratory and for his many hours of reviewing my research. I am also indebted to Dr. Anthony Gaudin and Dr. Peter Fischer who, as members of my Thesis Committee, improved this final manuscript with their comments and suggestions.

Finally, I wish to thank my family and friends, without whose moral support I would have found thesis writing a discouraging task.

TABLE OF CONTENTS

Acknowledgements	iv
Table of Contents	v
Abstract	vi
Introduction	1
Methods and Materials	3
Tissue Collection	
Tissue Extractions	
Rat Surgery	
Rat Bioassay	
Results	17
Conclusions and Discussion	25
Summary	28
References	29
Bibliography	
Personal Communications	
Table 1. Extraction procedure flowsheet	8
Figure 1. An <u>R. productus</u> specimen	5
Figure 2. The <u>R. productus</u> digestive tract	6
Figure 3. The rat gastric cannula	12
Figure 4. The gastric cannula surgically implanted in the test rat	13
Figure 5. The rat bioassay apparatus	16
Figure 6. Dose-response curve to pentagastrin	19
Figure 7. Time courses of the responses to a 62.5 ug/kg dose of pentagastrin	20
Figure 8. Acid response vs. volume response to pentagastrin stimulation	21
Figure 9. Dose-response curve to porcine antral extract	22
Figure 10. Dose-response curve to <u>R. productus</u> antral extract	23
Figure 11. Dose-response curve to <u>R. productus</u> upper stomach, spiral valve, and somatic muscle extracts	24

ABSTRACT

EVIDENCE OF A GASTRIN-LIKE SUBSTANCE
IN RHINOBATUS PRODUCTUS

by

Duane Gilbert Hansen, Jr.

Master of Science in Biology

January, 1973

While the stomach hormone gastrin is known to exist in several species of mammals, gastrin activity in the alimentary tracts of other chordates has not been confirmed. To determine if the gastrin hormone is strictly a mammalian feature with respect to the evolutionary process, the digestive tracts of other chordates should be analyzed for the hormone. The basic hypothesis in this study is that the gastrin molecule occurred in early chordate forms preceding the appearance of mammals and that as a result the hormone should be found in at least one other class of modern chordates.

A factor possessing gastrin activity was extracted from the Elasmobranch, Rhinobatus productus, the

shovelnose guitarfish. This gastric factor resembles porcine gastrin in its physico-chemical properties (Gregory-Tracy extraction method), its biological activity (chronic rat bioassay for gastric acid secretion), and its organ localization (extraction of serial gut sections).

If the gastrin molecule is then common to modern mammals and to other contemporary chordates, this suggests that this molecule may have occurred in the ancestral stock from which these recent chordates evolved.

INTRODUCTION

While the existence of the gastric hormone gastrin in several species of mammals is well known (Gregory and Tracy, 1964; Grossman, 1970), the presence of gastrin in any non-mammalian chordate has not been confirmed. As a result, it is still unknown whether gastrin was strictly a mammalian innovation in the evolutionary process or whether the gastrin molecule was present in mammalian predecessors. If gastrin did occur in the ancestral stock from which all modern chordates (including mammals) evolved, it is also likely to be present as an inherited characteristic in at least one other class of chordates.

Gastrin itself is a linear peptide consisting of 17 amino acid residues. It is secreted by the pyloric mucosa of the stomach of at least six species of mammals. While many of the effects of gastrin have not yet been elucidated (Grossman, 1970), this hormone is mainly characterized as a stimulator of acid secretion by the body of the stomach. An injection of gastrin given subcutaneously or intravenously into a test animal will induce a measurable increase in stomach acid secretion, and this increased secretion can be monitored in a bioassay.

Most previous studies of non-mammalian digestion have been done studying selachians (the sharks and rays). Babkin et al (1935) were unable to find evidence that

gastric secretion in elasmobranchs is under nervous control. Barrington (1942) suggested that humoral rather than nervous mechanisms are responsible for the regulation of digestive functions in the lower vertebrates. Indeed, prior to these studies, Bayliss and Starling (1903) demonstrated the existence of the intestinal hormone secretin in the dogfish, skate, and salmon; and Babkin (1933) showed that a crude preparation of secretin will stimulate an increase in pancreatic secretion in immobilized rays. The ray, then, appeared to be an excellent candidate for investigating the presence of gastrin in a non-mammalian chordate. If gastrin is present in the digestive tracts of both mammals and elasmobranchs, then gastrin was probably also present in the evolutionary ancestors from which these modern chordates developed. This study investigates the question of when the gastrin molecule appeared in chordates — at the therapsid threshold to Mammalia or at an earlier chordate stage.

The shovelnose guitarfish, Rhinobatus productus, was chosen to be the donor of digestive tissue for gastrin extraction in this study. This ray was selected since live specimens could be obtained in quantity. The entire digestive tracts of 25 rays were divided into three sections: upper stomach, antrum, and spiral valve (including duodenum as well as colon). These sections were extracted separately for gastrin and then tested

separately for gastrin activity in a chronic rat bioassay for gastric secretion. Each extract having gastrin activity was assayed for the presence of any contaminating histamine, which can also stimulate gastric acid secretion. An active substance isolated by a standard gastrin extraction procedure and found to be free from histamine would be assumed to have gastrin activity.

METHODS AND MATERIALS

Tissue Collection

Five types of tissue were collected for this investigation: porcine antral mucosa and four tissues from R. productus: upper stomach, antral region, spiral valve, and somatic muscle.

Hog antra were cut from the excised stomachs of hogs within 15 minutes after their death at the abattoir (Farmer John meat packing plant, Los Angeles). The antra were sealed in plastic bags and cooled in an ice chest packed with wet and dry ice for transport to the laboratory. There the antra were slit open, rinsed in cold tap water, and trimmed of any adhering upper stomach or duodenum. The antral mucosa was then stripped off the muscle wall, using either a scalpel or scissors. The mucosa was minced, weighed, wrapped in parafilm in 200 gram wet weight portions, and stored at -20° C until used.

The ray gut tissues were obtained from 25 R. productus

specimens captured at Seal Beach, California. A suitable stretch of beach was selected here as the collection site. A surf skiff carrying a 150-foot beach seine was rowed through the surf to a distance of about 100 yards offshore. The net was dropped parallel to the beach, and the two net ends were subsequently retrieved with the use of tow lines pulled by two dump trucks. More than 200 R. productus were captured; and 25, each measuring approximately one meter in length, were selected to provide the required tissue.

The entire gut tracts were excised from the rays on the beach; the guts were sectioned into three continuous divisions: upper stomach, antrum, and spiral valve. Boundaries of the three divisions were determined by the gross morphology of the gut and on the character of mucosal color, texture, and configuration. A single specimen of R. productus, 54.0 cm long (excluding caudal fin), had a complete digestive tract measuring 15.5 cm. This gut would typically be sectioned as 4.0 cm upper stomach, 5.0 cm antrum, and 6.5 cm spiral valve. The sections were immediately cooled in an ice chest for transport to the laboratory, where the tissues were rinsed in cold tap water. The antral mucosa was stripped from the muscle walls, minced, and wrapped in parafilm sheets. This yielded a net of 82.22 grams wet weight of mucosa. The stomach sections were minced and wrapped in parafilm sheets in 100 gram aliquots, as were the spiral valve

Figure 1. An R. productus specimen. View of right dorsal side. Ruler is in inches.

Figure 2. The R. productus digestive tract. Ventral view, dissected to show the gut tract. u.s.= upper stomach, a.= antral region, s.v.= spiral valve region.

sections. A 100 gram wet weight sample of dorsal somatic muscle from one R. productus was excised, minced and extracted by the method used for the other tissues to serve as a tissue control for the extraction procedure.

Tissue Extractions

The extraction method used here was modified from the porcine gastrin procedure described by Gregory and Tracy (1964). While no more than two extractions could be performed at any one time, they were carried out in as identical a manner as possible. A 100 gram sample of tissue was used for each extraction except in the case of the ray antral section in which the entire 82 gram collection was used, with the accompanying reduction in volumes and chemical weights needed. A flow-sheet for this extraction procedure is presented in Table 1. The procedure used is essentially that presented below.

The 100 gram frozen tissue sample was added to 600 ml of boiling distilled water. With continual stirring, the mixture was again brought to boil and allowed to boil for 30 minutes to extract the gastrin and destroy any proteases. The mixture was cooled to 15° C, and the sample was strained through a large Buchner funnel to remove the tissue pieces. To the broth was added 2.5 grams of diethylaminoethyl cellulose in "floc" form (Whatman DE-1), that had been previously washed sequentially with

Table 1. Extraction Procedure Flowsheet

50 ml portions of distilled water, 0.1 N NaOH, 0.1 N HCl, 0.1 N NaOH, and finally several hundred ml of distilled water. The "floc" was vigorously stirred into the liquid broth for three hours at room temperature. The "floc" was then filtered out through a fine silk cloth and washed with 350 ml of distilled water; all fluid was discarded, and the retained "floc" contained some proteins and any gastrin present in the tissue. The gastrin and protein were released from the "floc" by stirring with 74 ml of 0.2 N NaOH for 40 minutes. The NaOH wash was eluted through #4 Whatman filter paper, and the remaining "floc" was rinsed with an additional 12 ml of 0.1 N NaOH, which was added to the eluate already collected. The NaOH wash was adjusted to pH 7 with glacial acetic acid using a glass electrode and pH meter (Bechman) to measure pH. The total extract was cooled to 15° C, readjusted to pH 4 with glacial acetic acid, and placed in the refrigerator overnight.

The precipitate obtained after cooling was separated from the supernatant by centrifuging the mixture at 4° C in a refrigerated centrifuge (Servall) at 9,000 RPM for 10 minutes. The precipitate was suspended in 8 ml of distilled water and adjusted to pH 9.8 with concentrated NH_4OH . The volume is adjusted to 10 ml with distilled water and cooled to 20° C. Keeping the mixture at 20° C, 5.0 grams of K_2HPO_4 (Reagent grade) and 8.0 ml of 2-Propanol (Spectrometric Reagent grade) were added to the

sample, stirred using a magnetic stir plate. The mixture was stirred for 30 minutes at 20° C. After this, the suspension was centrifuged at top speed for 10 minutes in an International Clinical Centrifuge (tabletop model). After centrifuging, the mixture was separated into three layers: a lower aqueous layer, a salt cake layer, and an upper alcohol layer. The alcohol phase containing the gastrin was recovered using a Pasteur pipet. To the alcohol phase was added 8.0 ml of distilled water and an equal volume of ether. The sample was mixed well and then centrifuged for one minute at low speed in the clinical centrifuge. The ether-alcohol upper phase was pipetted off the aqueous volume now containing gastrin free from histamine (subsequent tests using paper chromatography confirmed this). The gastrin sample was washed twice more with two volumes of ether. Any remaining ether was evaporated off by stirring under a fume hood. The final gastrin extract was adjusted to pH 7.8 with 4 N HCl, and the volume made up to the working final volume (10 ml or 10 grams of tissue/ml). The final extract was divided into 2 ml aliquots and stored at -20° C.

Any extract having gastric stimulatory activity was tested for possible histamine contamination. Trace amounts of the test gastrin extracts and of histamine diphosphate were spotted separately on chromatography paper. The solvent used was a mixture of n-butanol: glacial acetic acid: pyridine: water (75:15:50:60). The chromatogram

was allowed to run and was then developed using a 0.1% ninhydrin in isopropanol solution. Any histamine would be detected as a purple spot, while the gastrin would remain colorless.

Rat Surgery

The laboratory rat was chosen as the bioassay test animal for three reasons: 1) it is easily handled and cared for; 2) it requires a smaller absolute amount of gastrin to obtain maximal gastric secretion than does the dog or cat (Adashek and Grossman, 1963; Emas et al, 1968); and 3) the rat's sensitivity to histamine, as affecting gastric secretion, is much less than the dog's (Adashek and Grossman, 1963; Emas et al, 1968).

Six Sprague-Dawley strain female rats weighing between 336 and 404 grams were prepared with Pavlov type gastric fistula cannulas according to the surgical procedure of Komarov et al (1963). The surgical stainless steel cannula was 10 to 12 mm in length with a beveled flange at each end. The inner bore of one flange was threaded to permit the insertion of a removable machine screw which acted as a stopper to close the cannula between experiments. Prior to surgery, the test rat was anesthetized with ether and strapped ventral side up to a surgery board. The rat's abdomen was washed with soap and water and shaved with an electric clipper. An incision about 20 mm in length was made following the midline of

Figure 3. The rat gastric cannula. Two shown: (right) a cannula closed with a machine screw; (left) an open cannula, with removed screw and plastic drain tube to be inserted. Top syringe is fitted for air-flushing cannula.

Figure 4. The gastric cannula surgically implanted in the test rat. The machine screw, which acts as a stopper between experiments, is secured in the exposed cannula flange. Photo taken about nine months after surgery.

the upper abdomen. The most dependent part of the stomach was then drawn up through the incision. A tiny puncture was made in the body of the rat stomach as close to the greater curvature as possible but still far enough away from the antrum to prevent mechanical stimulation of that secretory gland. A loose purse-string suture was placed around the edges of the puncture. The unthreaded end of the cannula was slipped into the opening and secured by tightening the suture. A piece of omentum was wrapped around the cannula shaft to insure a watertight seal. The cannulated stomach was replaced in the abdominal cavity with the threaded flange of the cannula extending to the outside of the abdomen. The initial incision was then closed around the cannula using interrupted sutures in two steps: first for the muscle layers, and second for the skin.

Rat Bioassay

Before each test the rats were fasted from 14 to 18 hours, during which time only drinking water was supplied. Coprophagia was prevented by cleaning their cages prior to the start of the fast. Each animal was placed in a Bollman restraining cage (Bollman, 1948) modified with a platform on which the rat could rest during the experiment. The cannulas were opened, and the stomachs were repeatedly irrigated with warm tap water (2 - 3 ml) and allowed to drain. A short plastic tube fitted with a

rubber collar gasket of my own design was threaded into the interior of each cannula, and this tube drained all the gastric contents into a graduated 15 ml nalgene centrifuge tube (Figure 5). The cannulas were flushed with air prior to each collection by means of a short length of plastic tubing affixed to an air-filled syringe. Secretion was collected continuously for 15 minute intervals. The volume of the secretion was recorded, and the total 15 minute collection was titrated to a phenolphthalein endpoint with 0.037 N NaOH using a 5.00 ml microburette.

In order to prevent stress in the animal from repeated skin punctures, a 26-gauge hypodermic needle was inserted under the skin on the back of each animal and allowed to remain there for the duration of the experiment. Before the collection of the first basal, 0.4 ml of isotonic saline was injected to create a drug administration pocket under the skin to prevent any stress due to differences in injection volumes. To prevent a back-drainage of test injections, the needles were plugged typically with parafilm between injections after the removal of the syringes.

After a one hour drainage period, basal collections were taken, since by that time the secretions had stabilized at about 0.3 ml/15 minute interval. The test injection was administered, followed by a 0.1 ml saline flush. Volumes injected ranged from 0.08 ml to 0.55 ml.

Figure 5. The rat bioassay apparatus. The rat, its cannula open and draining into a collection tube, is restrained in a modified Bollman cage. An injection needle has been inserted under the skin on its back.

The gastric secretion was recorded until the secretion returned to basal volume, usually after one hour (Figure 7). The next injection would then be given; usually four test injections were given to each rat in a single day.

The injection order of the test substances and dose amounts were determined at random by Latin square techniques (Butt, 1967). Pentagastrin (ICI 50,123) was administered in doses of 15.6, 31.2, and 62.5 $\mu\text{g/kg}$ rat. Tissue doses were expressed as grams tissue weight (following Adashek and Grossman, 1963) and administered in amounts of 1.25, 2.50, and 5.00 g/animal (i.e., 0.42, 0.83, and 1.67 g/kg rat). Saline injections (0.4 ml) served as a control.

In the calculation of results, gastric acid output was expressed as μeq of H^+ /15 minutes. Only the maximum acid output/15 minute interval was used to represent the response to the test dose regime. Response means and standard error of means were calculated for all doses and basals. Significance of differences was determined by the use of the t test for unpaired values.

RESULTS

Graded doses of pentagastrin produced graded gastric secretory responses (Figure 6) with the maximal mean response occurring at a dosage of 62.5 $\mu\text{g/kg}$. However, half this dosage (31.6 $\mu\text{g/kg}$) elicited a response not

statistically different from this peak response ($p > 0.50$). The time courses of responses to some test injections (Figure 7) suggest that peak secretions are reached generally within 30 minutes after the injection of the test substances. Increased gastric acid output was generally accompanied by increased gastric volume output (Figure 8).

Porcine gastrin also produced significant responses with the maximal mean response at 2.50 g/rat (Figure 9). This response was 23% higher than the pentagastrin maximum mean ($p < 0.05$). Both pentagastrin and porcine gastrin caused submaximal responses when supramaximal doses were given.

A significant response was observed to a 5.00 g/rat dosage of R. productus antral extract ($p < 0.05$) (Figure 10). This response was not due to injection volume stress, since a similar volume of saline injected did not cause such a secretion (Figures 6, 9, 10 and 11). The response to 5.00 g ray antral extract (Figure 10) is about equal to the response to 1.25 g porcine antral extract (Figure 9). The relative potency of the R. productus extract to the porcine extract is therefore about 25 per cent.

Doses of up to 5.00 g/rat of R. productus upper stomach, spiral valve, or somatic muscle extracts (Figure 11) failed to produce responses significantly different than the saline control ($p > 0.10$).

Figure 6. Dose-response curve to pentagastrin. Each point represents mean $\pm$ standard error of six tests in four rats.

Figure 7. Time courses of the responses to a 62.5 μg/kg dose of pentagastrin. Each point ± standard error reflects six experiments in four rats.

Figure 8. Acid response vs. volume response to pentagastrin stimulation. Points reflect tests done in four rats; each point is a single injection.

Figure 9. Dose-response curve to porcine antral extract. Each point represents the mean $\pm$ standard error of three tests in three rats.

Figure 10. Dose-response curve to R. productus antral extract. Each point represents the mean $\pm$ standard error of three tests in three rats.

Figure 11. Dose-response curve to *R. productus* upper stomach, spiral valve, and somatic muscle extracts. Each point represents the mean $\pm$ standard error of three tests in three rats.

CONCLUSIONS AND DISCUSSION

A factor having gastrin activity is present in the antral mucosa of the ray Rhinobatus productus. This conclusion was reached in view of the gastric stimulating properties of the antral extract in the rat bioassay (Figure 10) and the extract's freedom from histamine contamination (paper chromatogram). Whether this gastrin factor is functional in gastric secretion in the ray is not known. The important finding of this report is that a molecule having gastrin activity is present in Rhinobatus.

The experimental steps used in reaching this conclusion are considered valid. The chronic rat bioassay was indeed operative and was calibrated with pentagastrin. The work by Tumpson and Johnson (1969) was originally employed as a guideline for determining doses of pentagastrin. Their report that 62.5 $\mu\text{g/kg}$ rat produced the maximum secretory response was confirmed. The extraction method used in this study was also successful. Porcine antral mucosa, known to contain gastrin did yield gastrin by this procedure; porcine gastrin presence was confirmed by the rat bioassay. Also the extracts were free from histamine, as indicated by paper chromatography. Furthermore, the gastrin factor in Rhinobatus was localized in the antrum of the ray's stomach; porcine gastrin has been isolated from

the antral mucosa of hogs (Gregory and Tracy, 1964). Only the ray antral extract stimulated the rat to a significant extent. Attempts to detect gastrin from the other tissues were fruitless.

The question can be asked "Why should the ray antral extract have a different gastric potency than the porcine extract?" Similar potency differences have also been reported for gastrin preparations from several mammalian species (Grossman, 1970). The answer to this question involves three considerations: 1) the yield may have varied between preparations since extractions had to be carried out on different days; 2) the concentrations of gastrin per gram of tissue may differ between species; and 3) the individual gastrin molecules isolated from different species may have different activities. Any or all of these factors may be operating with respect to the question of extract potency difference. Grossman (1970) reported that the structures of gastrin molecules isolated from six species of mammals differ in their amino acids at only one or two positions. Walsh and I (unpublished studies) compared antral extracts from Rhinobatus productus and hog for antibody-binding ability in a gastrin radioimmunoassay. Our results suggest that if the test gastrin and the known antibody bind together on a one-to-one basis, then the porcine extract has a greater number of gastrin molecules per gram tissue than does the ray extract.

Whether this reflects concentration or yield differences is unknown.

This study concludes that a gastrin factor can be isolated from both a mammal and an elasmobranch. In addition, a gastrin factor has been isolated from teleosts by Dockray (personal communication), and an antral extract from the frog Rana catesbeiana has shown positive results in a gastrin radioimmunoassay of Walsh and Moore (personal communication). If the presence of the gastrin molecule is so common to this many different classes of chordates, it is certainly not solely a mammalian innovation in evolution.

Therefore, we suggest that gastrin (or the biosynthetic pathways to produce gastrin) was present in the common chordate ancestor from which modern chordates evolved. This conclusion answers my initial question: when did gastrin appear? The next step of investigation would be to assay a cyclostome or even a protochordate for gastrin, but, as Jarvik (1968) has pointed out, there is still some question as to the relative advancement of gnathostomes over cyclostomes. Future studies may show that gastrin appeared in animals even before the now suggested common chordate ancestor.

SUMMARY

A factor having gastrin activity can be extracted from the antral mucosa of an elasmobranch, Rhinobatus productus, and from the antral mucosa of a mammal, the hog. This suggests that gastrin (or the genetic information to produce gastrin) was present in the common chordate ancestor from which these modern chordates evolved.

REFERENCES

Bibliography

- Adashek, K. & Grossman, M.I. (1963) Response of rats with gastric fistulas to injection of gastrin. Proc. Soc. Exptl. Biol. Med. 112, 629-631.
- Babkin, B.P. (1933) Further studies on the pancreatic secretion in the skate. Contrib. Can. Biol. & Fisheries (N.S.) 7, 1.
- Babkin, B.P., Chaisson, A.F., & Friedman, M.H.F. (1935) Factors determining the course of the gastric secretion in elasmobranchs. J. Biol. Board Can. 1, 251-259.
- Barrington, E.J.W. (1942) Gastric digestion in the lower vertebrates. Biol. Rev. 17, 1-27.
- Bayliss, W.M. & Starling, E.H. (1903) On the uniformity of the pancreatic mechanism in vertebrata. J. Physiol. (London) 29, 174-180.
- Bollman, J.L. (1948) A cage which limits the activity of rats. J. Lab. & Clin. Med. 33, 1348.
- Butt, W.R. (1967) pp. 367-371 in Hormone Chemistry, D. Van Nostrand Co. Ltd, London.
- Emas, S., Billings, A. & Grossman, M.I. (1968) Effects of gastrin and pentagastrin on gastric and pancreatic secretion in dogs. Gastroenterology 3, 234-240.
- Gregory, R.A. & Tracy, H.J. (1964) The constitution and properties of two gastrins extracted from hog antral mucosa. Gut 5, 103-117.
- Grossman, M.I. (1970) Gastrin and its activities. Nature 228, 1147-1150.
- Jarvik, E. (1968) pp. 497-528 in Nobel Symposium 4: Current Problems of Lower Vertebrate Phylogeny, Almquist & Wiksells Boktryckeri AB, Uppsala.
- Komarov, S.A., Bralow, S.P. & Boyd, E. (1963) A permanent rat gastric fistula. Proc. Soc. Exptl. Biol. Med. 112, 451-453.

Tumpson, D.B. & Johnson, L.R. (1969) Effect of secretin and cholecystokinin on the response of the gastric fistula rat to pentagastrin. Proc. Soc. Exptl. Biol. Med. 131, 186-188.

Personal Communications

Graham J. Dockray, M.D.
Dept. of Physiology
University of Liverpool
Brownlow Hill
P.O. Box 147
Liverpool L69 3BX
England

John H. Walsh, M.D. & Duane G. Hansen
VA Wadsworth Hospital Center
Bldg. 115 Rm. 115
Los Angeles, California 90073

John H. Walsh, M.D. & Elizabeth Moore
c/o Dr. John Forte
Dept. of Physiology -- Anatomy
University of California, Berkeley
Berkeley, California 94720