1968

The Effect of Follicle-Stimulating Hormone and Testosterone Propionate on the Reproduction of the Bull Snakes, Pituophis Melanoleucus Catenifer and Pituophis Melanoleucus Affinis; Accompanied with Analysis of Changes in Blood Protein Constituents Following Stimulation

Jack Bradford
Central Washington University

180

THE EFFECT OF FOLLICLE-STIMULATING HORMONE AND TESTOSTERONE PROPIONATE ON THE REPRODUCTION OF THE BULL SNAKES, PITUOPHIS MELANOLEUCUS CATENIFER AND PITUOPHIS MELANOLEUCUS AFFINIS; ACCOMPANIED WITH ANALYSIS OF CHANGES IN BLOOD PROTEIN CONSTITUENTS FOLLOWING STIMULATION

A Thesis

Presented to

the Graduate Faculty

Central Washington State College

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Jack Bradford

August, 1968

APPROVED FOR THE GRADUATE FACULTY

_____

Phillip C. Dumas, COMMITTEE CHAIRMAN

_____

Sheldon R. Johnson

_____

Glenn W. Clark

ACKNOWLEDGEMENTS

I would like to thank the members of my committee for the time they spent with me in the construction of this thesis. I would especially like to thank Dr. Philip C. Dumas, without whose efforts this study would not have been possible. I would also like to thank Dr. Bernard L. Martin, Assistant Dean of Arts and Sciences, and those at the Data Computer Center at Central Washington State College for their assistance with the statistical analysis.

TABLE OF CONTENTS

INTRODUCTION

Most vertebrate taxonomists include as a major criterion in their concept of species, reproductive isolation. Testing for reproductive isolation in a laboratory situation could be brought about by means of artificial insemination. Such testing could enable the researcher to test for viability of resulting offspring. In addition, with reproduction on a more assured basis, genetic experimentation could be applied to a wider selection of vertebrates.

The problem when working with snakes as subjects is that, to date, no satisfactory method of predicting when a snake is in a state of reproductive readiness has been developed. Palpation for ova is a satisfactory technique for some types of reptiles but the muscular body wall of snakes makes this method non-precise at best and also the method can tell us nothing as to the reproductive state of the male.

The bull snake, Pituophis melanoleucus, was chosen for this experimentation for several reasons; (1) it is relatively abundant in Central Washington (2) it is a large snake, well able to withstand the effects of the frequent blood samplings which were necessary in this experimentation (Soter, 1955) (3) the bull snake is an oviparous snake and ova development is rapid and after dissection, obvious.

The bull snakes P. m. catenifer and P. m. affinis generally breed in the spring of the year and lay their eggs in early to mid-summer, with the young hatching in late summer to early fall. In 1967 and 1968 P. m. catenifer in Grant and Kittitas Counties, Washington,

were not observed undergoing reproductive behavior prior to 1 June. By late September to mid-October all signs of sexual maturity disappear, for instance, ova not deposited by females during the summer are nearly reabsorbed.

The bull snake undergoes seasonal reproductive cycling as do other reptiles. "During the annual reproductive cycle there is an alteration of a breeding, or reproductive, season with a more or less prolonged interval of sexual inactivity. During the sexually inactive period secretion of sex hormones is greatly reduced, the reproductive tract regresses to a juvenile condition, and the animal is anatomically, physiologically, and behaviorally incapable of reproduction." (Frye, 1967) The events that bring the reproductive tract into a state of maturity in the spring must then come about rapidly because the changes that take place probably do so in the time span of something less than 60 days after emergence from hibernation.

In as much as hormones of the endocrine system are carried by the blood stream, it would seem a reasonable assumption that as reproductive development begins and different hormones become present in the system, that these hormones would directly or indirectly alter the protein composition of the blood. If then a method could be devised to detect these hormones directly or to indirectly detect their impact on the blood proteins by means of an electrophoretic analysis, the reproductive development could be estimated with only a minimal disturbance to the animal involved.

METHODS AND MATERIALS

Specimens of P. m. catenifer were collected in Grant and Kittitas Counties, Washington, in the spring and summer of 1967 and 1968. Specimens of P. m. affinis were purchased in the fall of 1967 from the Pet Corral, Tuscon, Arizona. Each specimen was weighed, measured (snout-vent), sexed, and marked for identification by clipping ventral-caudal scutes. In August, 1967, a 28¼ inch (snout-vent) female deposited three eggs in her cage. This body length was set as a minimum and any snake this size or larger was considered sexually mature and suitable for experimentation. The specimens were caged in glass-fronted, wooden cages measuring 36x20x20 inches. The laboratory where the specimens were housed was constantly illuminated during the experimental period and temperature was regulated by thermostatically controlled electric heaters. The temperature in the cages fluctuated from 80° F at night to 94° F during the day. During the experimental period each snake was fed one mouse or one young Japanese Quail (Coturnix) on a weekly basis.

Prior to any other experimentation, the developmental condition of the testes and ovaries was determined to establish a base reference point as to reproductive maturity. To do this, each specimen underwent exploratory surgery. Each animal was anesthetized with a sodium barbituate, Seconal Sodium, at a dosage of .4mg/50gm body weight. Additional anesthetic was used when necessary. When the animal had lost its righting response it was stretched out, ventral side up. The ventral

side was then sterilized with an antiseptic solution, first alcohol and then Medi-Quick (antibacterial spray).

Placement of an incision that would expose the ovaries and testes was determined in the manner of Bragdon (1953). Working with preserved snakes it was found that the location of the gonads could be determined by association with ventral scutellation. With P. m. catenifer it was found that the females left ovary lay between scutes 38 and 62±5 (counting anterior from the anal scute) and the right ovary between scutes 57 and 91±5. The males left testis lay between scute 9 and 36±5 and the right between scutes 15 and 45±5. For P. m. affinis, the females left ovary was between scutes 52 and 74±5 and the right ovary between scutes 72 and 111±5. The males left testis lay between scutes 31 and 48±5 and the right between scutes 27 and 59±5. (These measurements were taken on animals that were not in a reproductive state. When the animals are in the reproductive portion of the cycle, the gonads are greatly enlarged.)

An incision of approximately 10 scutes in length was made at the predetermined midpoint of the left gonad. The incision was placed slightly lateral to the midventral area, along the longitudinal axis, taking care that the incision avoided the tips of the ribs which extend slightly into the lateral extremes of the ventral scutes. The skin was carefully pulled to the sides and held in place by a set of clamps. The exposed layer of muscles and connective tissue was then carefully separated and cut. The coelomic lining was then in view and it too was carefully cut. Then by careful probing along the

inner dorsolateral surface of the coelom (taking care not to disturb the other visceral organs) the gonad was exposed. The width of the gonad was recorded and the developmental condition was noted. The clamps were then removed and the incision was then closed using sterilized silk thread as suture material. (Belding Corticelli Size A thread) Sutures were placed about every other scute. The area was once again sterilized with a spray antiseptic and a spray plastic bandage was put over the entire area. The specimen was then placed in a sterilized cage for a post-operative period of about two days. By the end of the third day, the incision was showing signs of rapid healing and the snakes were alert and gaining in strength.

After initial surgery and allowing time for recovery, the animals were divided into experimental and control groups consisting of 3 experimental males, 3 control males, 4 experimental females, and 2 control females.

A blood sample was taken from each specimen at this time to determine a base reference of blood protein constituents. The blood samples were obtained by utilizing a tail-clipping technique. The tip of the tail was cut off and the snake was then held in a vertical position, head up, for about one minute. The drop of blood that collected at the tip of the tail was then collected in a capillary tube. One end of the capillary tube was then heat-sealed, using an alcohol lamp. The plasma portion of the blood was saved for analysis and the other portion of the blood was discarded.

All samples taken were analyzed immediately after the preceeding

steps. The samples were electrophoretically separated and analyzed using the Beckman Microzone Analyzer Cell and Microzone Densitometer, Model R-110. A barbituate buffer of pH 8.6 was used in all instances. The samples were stained with a dye prepared by Beckman consisting of 0.2% by wt. ponceau-S, 3% trichloroacetic acid, 3% sulfosalicylic acid in distilled water. Procedure for the electrophoretic determination as outlined in the Beckman Instruction Manual was followed. Further blood samples were taken from each animal on a weekly basis for a period of six weeks, including the initial sample. Two samples were taken for each time period for each animal and the results of these two samples were averaged.

After each animal had an initial blood sample taken, hormonal treatment began. Commercial grade follicle-stimulating hormone (FSH) was obtained from the Calbiochem Company, Los Angeles, California, and crystalline testosterone propionate (U.S.P.) was purchased from the Sigma Chemical Company, St. Louis, Missouri. The four experimental females each received 10 mg of FSH in 1cc. of .7% saline solution biweekly for a total of five treatments or 50mg. of FSH each. (Ferguson, 1966) These treatments were given on 11 May, 14 May, 19 May, 22 May, and 26 May. The hormone was administered by intraparitoneal injection. The two control females were treated biweekly with 1cc. of .7% saline solution, but no FSH. Control treatments were given on the same dates as experimental treatments and in the same manner. The three experimental males were each treated with a single dose of 1mg/gm. body weight T. P. on 13 May. This dosage was roughly extrapolated from the

work of Ferguson, 1966. The hormone was administered by putting it in a gelatin capsule and inserting the capsule under the skin in the mid-dorsolateral area about midbody. The incision in the skin was then closed with two or three sutures. The sutures in addition to closing the wound also aided in keeping the capsule in place. Control males received an empty gelatin capsule on 13 May.

During the experimental period notes were taken on the behavioral aspect. Each male was introduced into the cage of an experimental and a control female at least twice weekly. The male was left in the females cage for a maximum of five minutes, observations were made and the male was removed. None of the animals were allowed to copulate; rather, if the male demonstrated positive behavior, he was quickly removed after his behavior pattern had been established and noted.

Criteria for positive reproductive behavior were determined by field observations and in agreement with Goin and Goin (1962). The earliest reproductive behavior was noted on 20 May and the latest on 12 June 1968. Positive reproductive behavior for males consisted of active pursuit of the female, often times biting and holding fast to the neck area while the rest of the body underwent a jerky, shuddering movement (caudocephalic waves). Males that did not display the above behavior when introduced to a female were considered displaying negative behavior.

Reproductive behavior for the females was considered positive if she allowed the male to court her rather than actively trying to escape. Also some females underwent a jerky tic-like motion when

receptive to a male that traversed their back or touched them in any way. Attempts at active escape were considered negative behavior.

On 18 June, all specimens were sacrificed and their gonads removed and immediately weighed and measured, and examined. The gonads were weighed (wet weight) on a Mettler analytical balance (nearest 0.1 mg.), the overall length of the longest gonad and the length and width of the largest follicles were estimated with a metric ruler (nearest 0.5 mm.), and the condition of the follicles (ie. yolked-nonyolked, turgid-flaccid, etc.) was noted as in the manner of Ferguson (1966). A section of the vas deferens was made and examined microscopically for motile sperm. The smears were later fixed and stained, using alcohol fixative and Giesma stain. The sperm then was examined and measured as to overall length, head length, and head width. These measurements were made by means of an ocular micrometer (nearest .5μ). These results were compared with sperm smears taken from disected field specimens which were in a state of reproductive readiness.

RESULTS AND DISCUSSION

The initial exploratory surgery showed that none of the twelve (experimental and control) animals had begun to achieve reproductive activity before 5 May. In all cases the gonads were only a thread of tissue that had not undergone differentiation into structural organization.

Commercial grade T. P. and FSH brought about essentially the same behavioral, physiological, and morphological response in P. m. catenifer as in P. m. affinis. The results indicate that commercial gonadal extract influences the blood proteins significantly in several areas. The initial blood samples revealed a low gamma globulin level and a relatively low overall total of blood protein units. The one exception to this was specimen number 11 (see graph 12, Appendix). This specimen was believed to have contacted pneumonia because on 6 May, a watery discharge was seen coming from the mouth and nose of this animal. When the specimen had apparently recovered; its abnormally high gamma globulin level and its overall level of blood protein units decreased.

Overall protein units for experimental animals, male and female combined rose by an average factor of 2.12 (comparing initial with final values; see Table #1). The protein portion of the blood that rose the most graphically was the gamma globulin fraction which rose by an average factor of 4.72 in male and female experimentals.

Graphs 1-12 summarize the above results. The graphs depict the percentage of each protein fraction through time. These graphs

	A_1 ♂						A_2 ♀					
	C_1	C_2	C_3	C_4	C_5	C_6	C_1	C_2	C_3	C_4	C_5	C_6
B_1 Total Proteins	102	242	278	248	217	238	215	273	434	514	361	458
	228	312	177	212	236	265	104	320	317	562	383	346
	482	192	494	616	596	659	172	249	235	535	376	433
B_2 Albumin	49	94	105	82	74	82	86	83	105	65	66	65
	114	148	62	72	78	82	70	138	100	100	88	107
	91	75	43	44	42	47	41	85	73	78	86	99
B_3 Beta	2	26	33	25	8	29	66	76	138	111	100	95
	27	44	15	11	14	23	29	76	76	132	74	76
	148	38	135	166	134	43	12	36	23	114	84	73
B_4 Gamma Globulin	35	95	126	119	100	103	30	78	181	300	177	275
	37	86	90	106	123	137	22	54	100	290	188	112
	217	40	292	391	407	552	106	77	126	288	161	233

TABLE #1

Actual Protein Units

of Experimental Animals

KEY

A = Sex
- A_1 = Male
- A_2 = Female

B = Type of Blood Protein
- B_1 = Total Protein Units
- B_2 = Albumin Fraction
- B_3 = Beta Fraction
- B_4 = Gamma Globulin Fraction

C = Time
- C_1 = 6 May, 7 May, or 8 May
- C_2 = 19 May
- C_3 = 26 May
- C_4 = 2 June
- C_5 = 9 June
- C_6 = 16 June

show a massive build-up of percent composition of the gamma globulin fraction in both male and female experimental animals. In comparing this build-up of gamma globulin in the experimentals with the gamma globulin of the controls, no significant building trend in control animals was indicated. However, a slight building trend was noticed in the control males near the end of the experimental period. Specimen number 17, a control female had begun to show a rise in gamma globulin between 9 June and 16 June, possibly indicating that "natural" reproductive development was taking place.

The histogram included on the right of each graph represents the entire blood protein units as actual numbers plotted against time. The experimental females underwent a trend of building total protein units which reached a maximum 25-27 days after initial stimulation. No trend of this magnitude was noticed in the control females.

In addition to graphic representation of the levels of blood protein constituents (graphs 1-12, Appendix) a three factoral statistical analysis (see Table #2) was conducted. Factor A represents sex; A_1 males, A_2 females. Factor B represents different protein fractions; B_1 represents overall protein units, B_2 represents albumin, B_3 represents beta, and B_4 represents gamma globulin. The C factor represents time; specific dates are: C_1 6 May, 7 May, or 8 May; C_2 19 May; C_3 26 May; C_4 2 June; C_5 9 June, and C_6 16 June. Alpha 1 and Alpha 2 blood fractions were not included in this analysis because by inspection they began at a low value and changed very little through time.

Factor A represented sex, male and female. At the 5% confidence

TABLE #2

ANALYSIS OF VARIANCE

5% level of significance

SOURCE OF VARIATION	SUM OF SQUARES	DEGREE OF FREEDOM	MEAN SQUARE	F	REMARKS
A	5451.4	1	5451.40	.061	N.S.
B	1658660.7	3	552886.90	6.243	S.
C	219662.3	5	43932.46	.496	N.S.
AB	515090.9	3	171696.96	1.938	N.S.
AC	1954089.3	5	390817.86	4.413	S.
BC	300880.0	15	20058.66	.226	N.S.
ABC	3441060.0	15	229404.00	2.590	S.
ERROR	8501100.0	96	88553.13	-----	----
TOTAL	3029313.0	143	-------	-----	----

level, sex was not a significant factor. The blood proteins of the males did not vary significantly from those of the females. Testosterone propionate had essentially the same impact on the blood proteins in the males as follicle-stimulating hormone had on the females.

Factor B represents different protein fractions; the overall total of protein units, the albumin fraction, the beta fraction, and the gamma globulin fraction. At the 5% confidence level the difference in the various proteins was significant. This fact was obvious when compared either with the raw data, Table #1 or to any of the graphs.

Factor B_1-Total proteins underwent a massive build-up in experimental animals following initial stimulation. This reached a peak in about 25-28 days in females. The peak values for males were much more variable in respect to time.

Factor B_2-Experimental females demonstrated a slight to moderate rise in albumin units, usually followed by a drop to a value somewhat greater than the initial value. Males albumin varied but seemed to indicate a slight drop on the average.

Factor B_3-Beta fractions in females showed an overall building trend in close association with the build of gamma globulin. Males beta fraction indicated a slight building trend on the average.

Factor B_4-Females gamma globulin underwent a massive build-up which reached peak values 25-28 days after initial stimulation. This greatly contributed to the rise in overall protein units. On the average, males underwent a massive build of the gamma globulin fraction which reached a peak value around 16 June.

Factor C representing time, showed no significant difference at the 5% confidence level. The C factor included all C's, 1-6 for both males and females. The C factor showed no significance, due in part to variation of individual values and also due to the variation in the sex-time interaction.

Factor AB, representing the interaction of sex and type of protein involved, showed no significant difference.

Factor AC, representing the interaction of sex and time was significant at the 5% confidence level. This fact was borne out in comparing, by inspection, the AC values of Table #1. Taking for example, values A_2C_1 and A_2C_4 and comparing them, the significance of this difference is made more apparent. Also lending to this difference is the low initial protein values for the females and because of the very high "midterm" values for the females. The C_4 values were the highest values for the experimental females; their values being, 514, 562, 535 representing a rise from initial values of 215, 104, and 172 respectively.

Factor BC, the interaction of protein types and time was not significant at the 5% confidence level. This is apparent by inspection of the raw data which clearly shows that some portions of B do not change significantly through time. Also some individuals display variation through time which lends to this conclusion.

Factor ABC, the interactions of sex, types of protein fractions and time was significant. A biological significance can not be ascribed to these interactions at this stage of investigation.

Commercial grade T. P. brought about reproductive behavioral

responses in experimental males about two weeks earlier on the average than any of the control males (See Table #3). Only one experimental male demonstrated reproductive behavior in June. All other experimental males ceased to demonstrate reproductive behavior after 28 May. Control males did not demonstrate reproductive behavior before 10 June and were possibly coming into their "natural" reproductive cycle at that time. This also closely corresponds with the dates that reproductive behavior has been observed in the field in the Central Washington area.

Commercial grade extracts of FSH brought about fairly consistant receptive behavioral responses in the experimental females between 20 May and 12 June. Control females, on the other hand, had not, as of 12 June, demonstrated receptive behavior to the advances of either control or experimental males.

Autopsy (See Table #4) indicated that FSH treated females had undergone follicle growth; this follicle growth not being detectable from the exterior by palpation. Some follicles were as large as 29x14.5 mm. Average of the largest follicles for the four experimental females were as follows: 23.9x12.1, 4.5x2.3, 7.0x4.5, and 12.0x7.1 mm. Control female number 17 had just begun to undergo follicular development; the largest follicles averaging about 3.5x2.0 mm. All of the larger follicles examined were turgid and none of these showed signs of follicular astresia. Only the larger follicles appeared to be yolked. The ovaries of the treated females weighed 11.76 gm on the average, as compared to 10.63 gm for the non-treated females. While the wet weights are not appreciably different, the difference in developmental condition

KEY

Positive Reproductive Behavior +
Negative " " —
Uncertain " " ?

Dates of Observation	5-20	5-25	5-26	5-27	5-28	6-1	6-5	6-8	6-10	6-12	6-14 Motile Sperm	6-14 Follicles Developed
Specimen 12	—	+	+	+	—	+	?	?	?	—	+	
" 25	—	—	—	?	+		—	—	?	—	+	
" 26	+	—	—	?	+	—	—	?	—	—	+	
" 11	—	—	—	—	—	—	—	—	?	+	+	
" 23	—	—	—	—	—	—	?	—	—	+	+	
" 21	—	—	—	—	—	?	—	—	+	+	+	
" 0	+	—	—	+	—	—	—	—	+	+		+
" 13	—	+	—	—	—	+	—	+	+	+		+
" 18	—	—	+	—	—	+	+	—	—	—		+
" 15	—	—	—	—	—	—	—	?	—	+		?
" 17	—	—	—	—	—	—	—	—	—	—		—
" 16	—	—	—	—	—	—	—	—	—	—		—*

TABLE NUMBER 3

Reproductive Behavior

* Note: This specimen died unexplainably on 6/14/68.

TABLE #4

Autopsy Results

	Body Length (cm.)*	Body Weight (gm.)*	Ovary Weight (gm.)	Ovary Length (mm.)	Ova Size (av. mm length)	Turgid Ova	Yolked Ova	Testes Weight (gm.)	Testes Length (mm.)
Experimental Females									
Specimen No. 13	181	458	15.5	575	12.0	+	+		
Specimen No. 15	169	244	3.6	460	4.5	+	-		
Specimen No. 0	163	170	20.5	465	23.9	+	+		
Specimen No. 18	162	349	7.3	513	7.0	+	+		
Control Females									
Specimen No. 17	193	456	10.6	607	3.5	+	-		
Specimen No. 16	181	218	**	**					
Experimental Males									
Specimen No. 26	165	150						.4	267
Specimen No. 12	168	348						1.4	185
Specimen No. 25	154	151						.5	250
Control Males									
Specimen No. 21	139	135						4.3	320
Specimen No. 23	153	138						3.5	470
Specimen No. 11	159	186						.9	300

*Remained essentially the same through the testing period.
**Died before these measures could be accurately taken.

was apparent. Control females had barely developed beyond a totally undifferentiated state while the treated animals had developed ova of a size range that indicated that they were only a short time away from being full term. Also the ovarian tissue of the experimental females was extremely vascular in nature; a condition that was not noted in the control females.

Commercial grade T. P. illicited an early behavioral response with treated males and evidently an early physiological and morphological response because autopsy of experimental animals revealed their gonads to be in a state of regressed development. Since these animals had displayed positive reproductive behavior earlier, it is possible that the males had completely cycled and were regressing to the inactive state. Control males upon autopsy, however, were characterized by large-lobed, well developed testes. It would seem that these non-treated males were nearing the height of their breeding condition.

Weight of non-treated males testes average 2.87 gm while those of treated males averaged .74gm.

Examination of the contents of the vas deferens showed that motile sperm had been produced by all males. The sperm produced both by control and experimental males compared favorably with sperm produced by males which were taken directly from the field. The sperm averaged about 66μ overall, 6μ head length, and 2μ head width.

SUMMARY

Problems are encountered when trying to determine the state of reproductive readiness in snakes. A method that would measure, directly or indirectly, reproductive hormones in the blood stream would allow estimation of reproductive development with only a minimal disturbance to the animal involved.

Commercial grade hormonal extracts of testosterone propionate and follicle-stimulating hormone bring about a state of reproductive readiness in the bull snake in a relatively short time. In addition to illiciting behavioral responses and stimulating production of motile sperm and ova, these hormones greatly influence the protein constituents of the blood.

The areas of the blood which are most greatly influenced are the total protein units and the gamma globulin fraction which undergo a massive build-up following stimulation. Monitoring this building of proteins allows the researcher to predict when an animal is in a state of reproductive activity.

BIBLIOGRAPHY

Bragdon, D. E. 1952. A contribution to the surgical anatomy of the water snake, Natrix sipedon sipedon; the location of the visceral endocrine organs with reference to ventral scutellation. Anat. Rec. 1952:145-160.

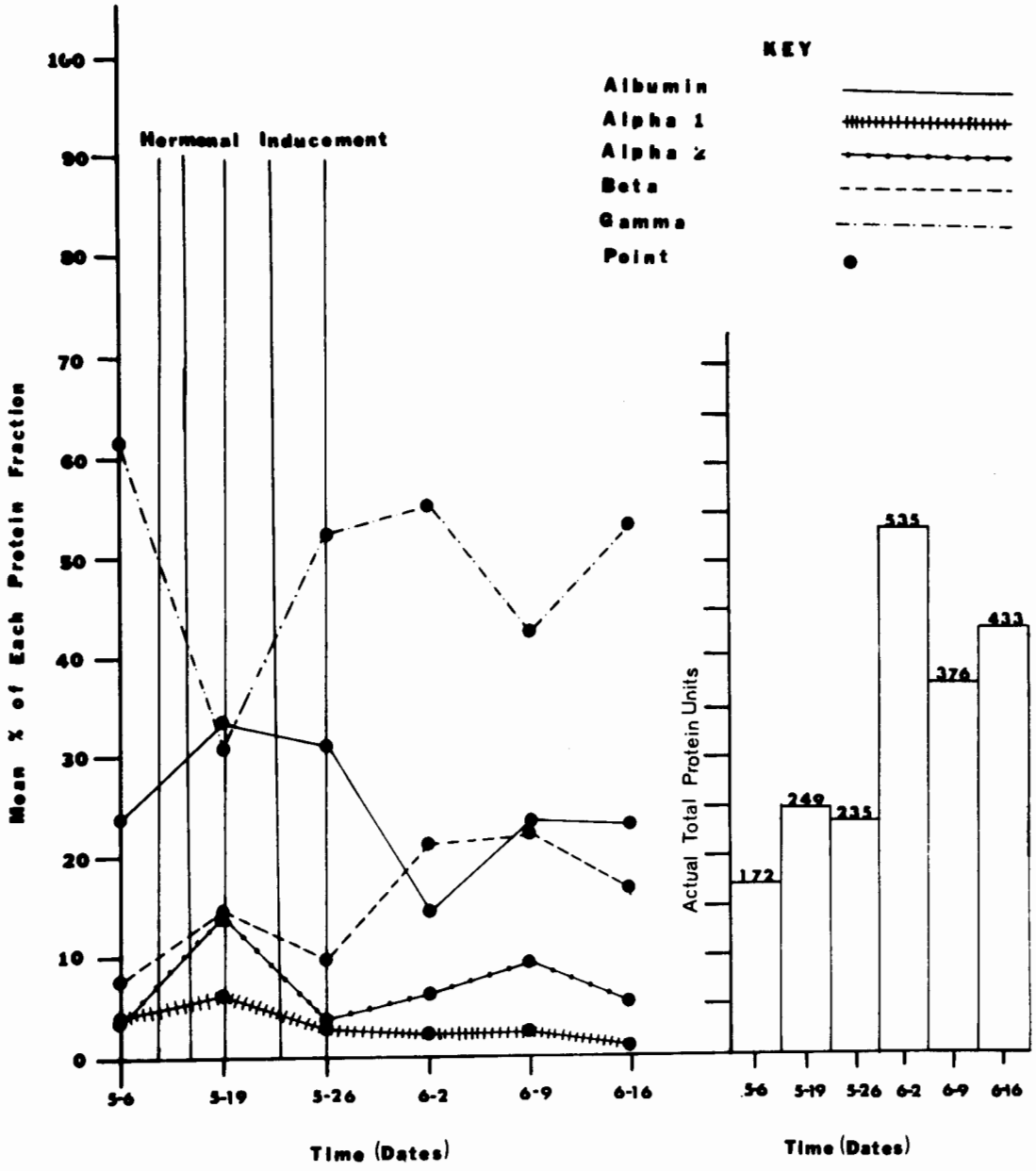
Ferguson, G. W. 1966. Effect of follicle-stimulating hormone and testosterone propionate on the reproduction of the side blotched lizard, Uta stansburiana. Copeia 1966:495-498.

Frye, B. E. 1967. Hormonal control in vertebrates. The Macmillan company, New York, New York, pp. 122.

Goin, C. J. and O. B. Goin, 1962. Introduction to herpetology. W. H. Freeman and Company. San Francisco, California. pp. 341.

Soter, C. A. 1955. Technique for bleeding snakes by cardiac puncture. Copeia 1955:(Herp Notes).

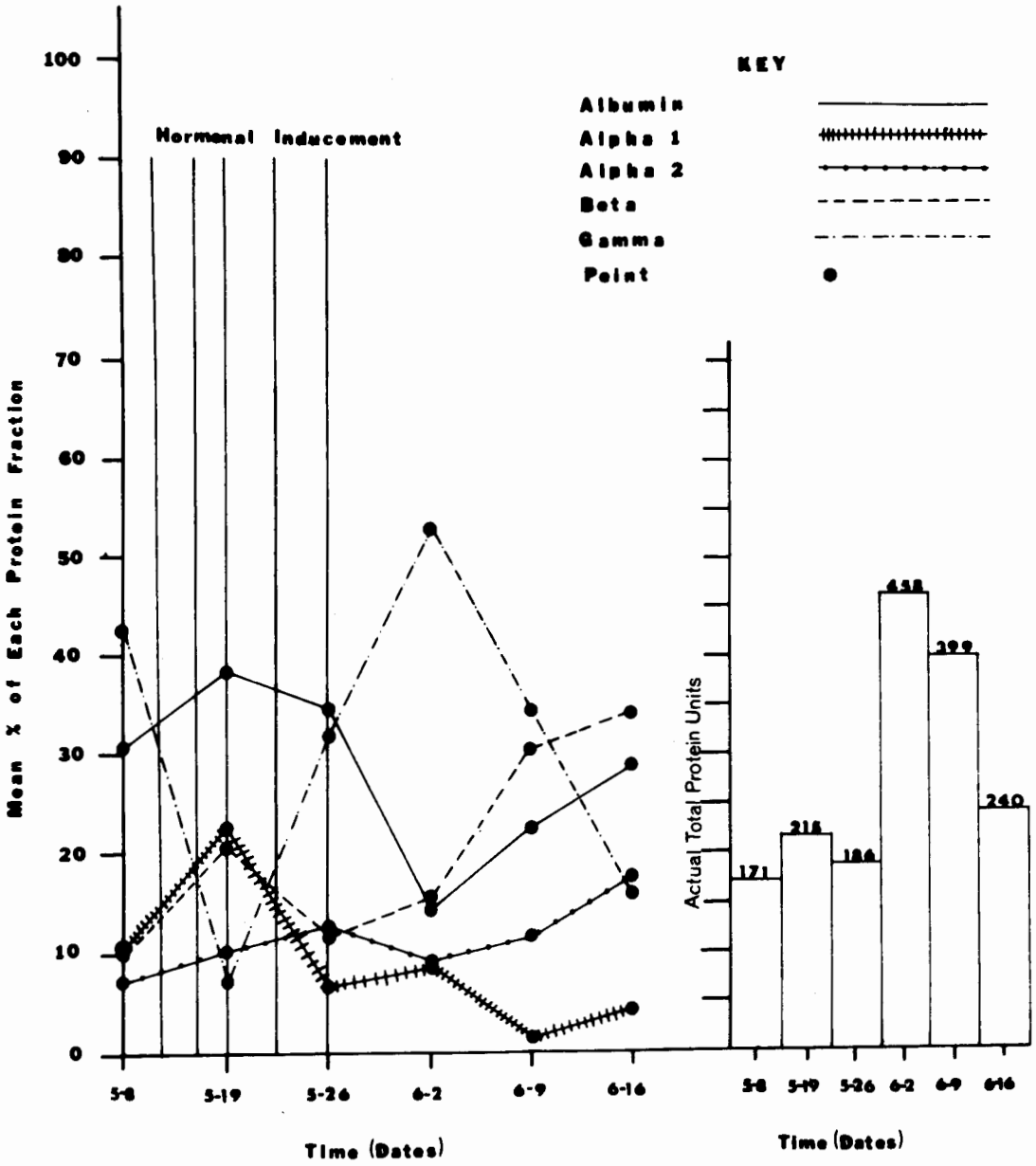
APPENDIX



GRAPH NUMBER 1

Specimen Number 13

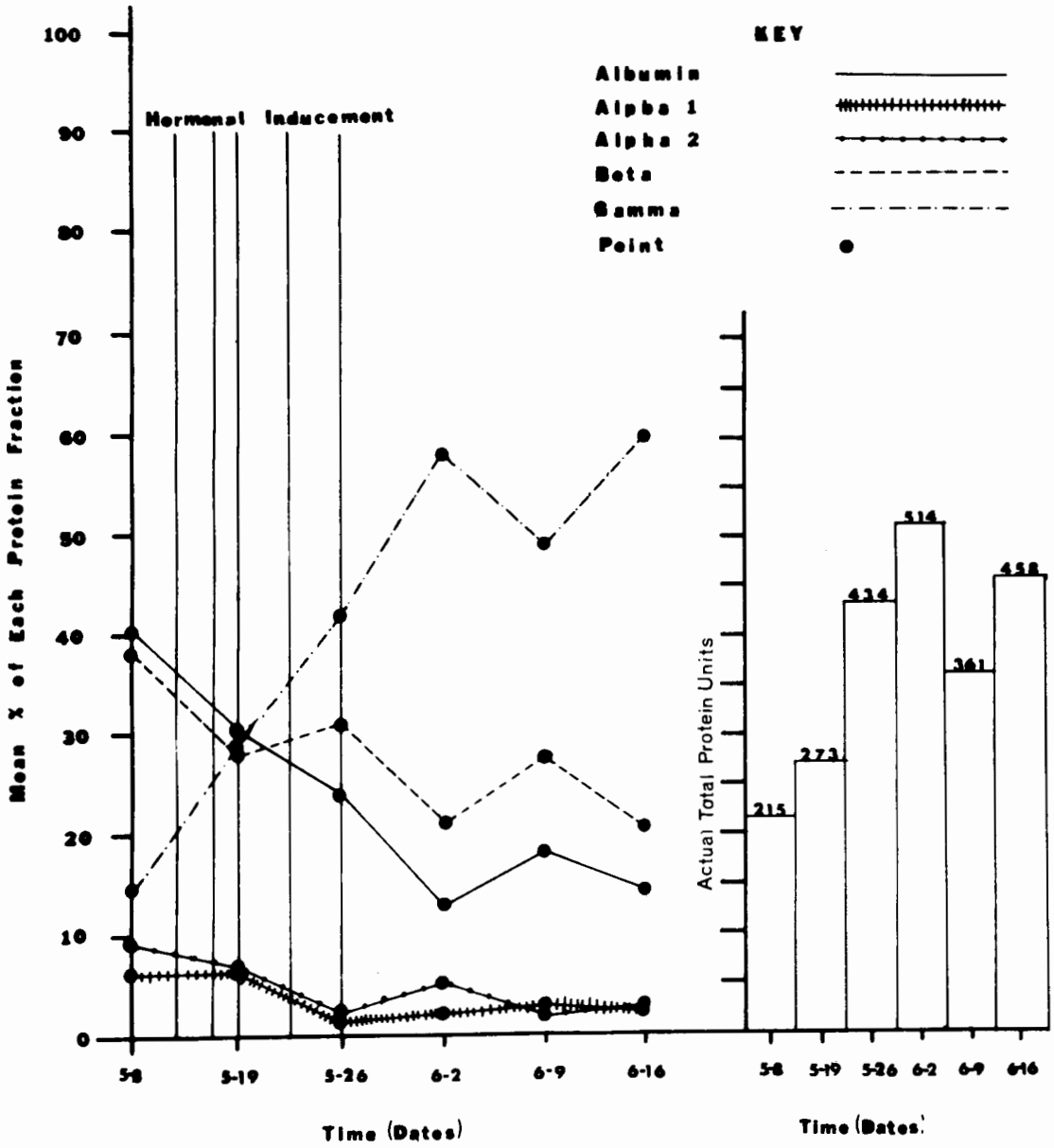
Experimental female Pituophis melanoleucus affinis



GRAPH NUMBER 2

Specimen Number 15

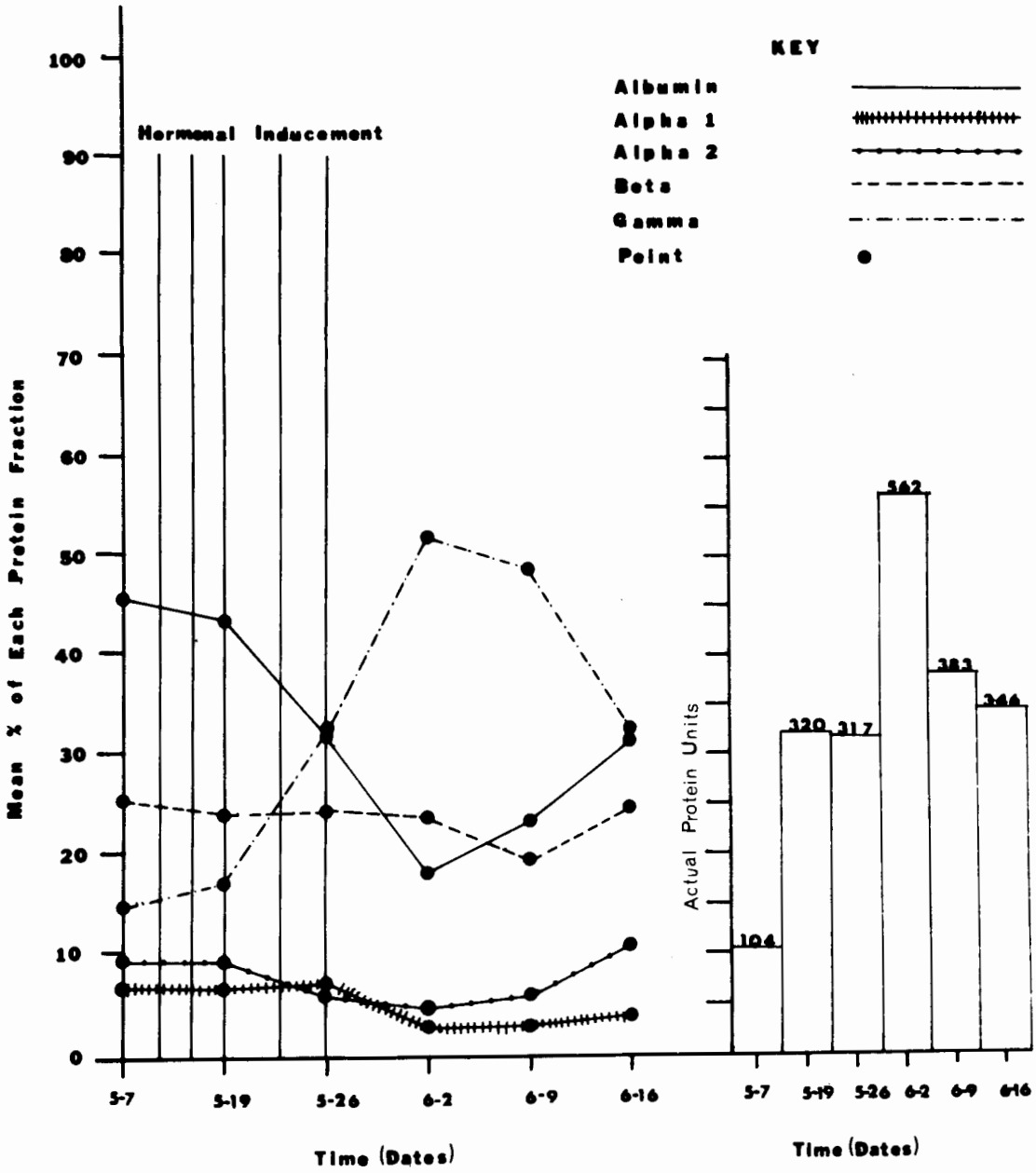
Experimental female Pituophis melanoleucus affinis



GRAPH NUMBER 3

Specimen Number 0

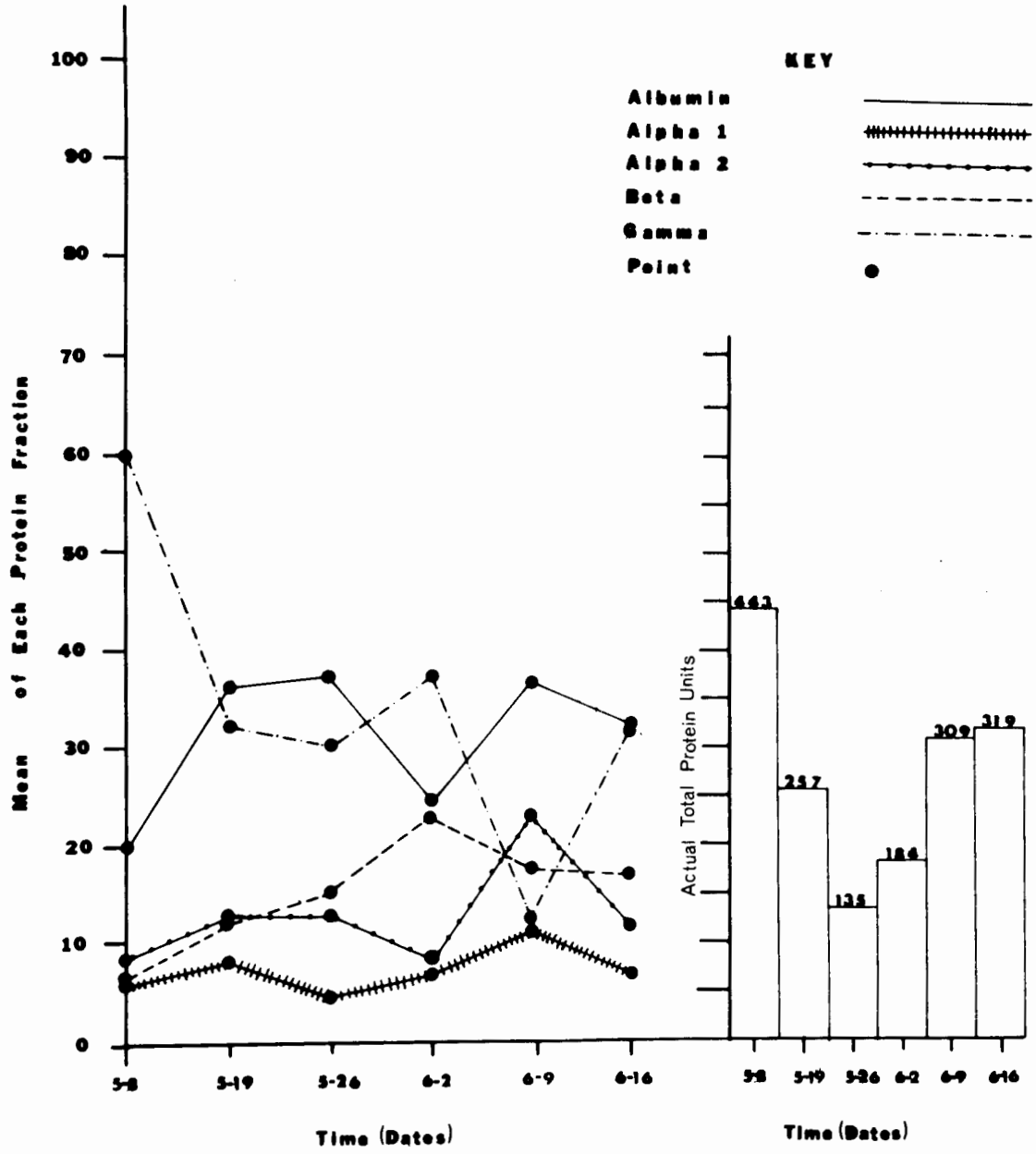
Experimental Female Pituophis melanoleucus catenifer



GRAPH NUMBER 4

Specimen Number 18

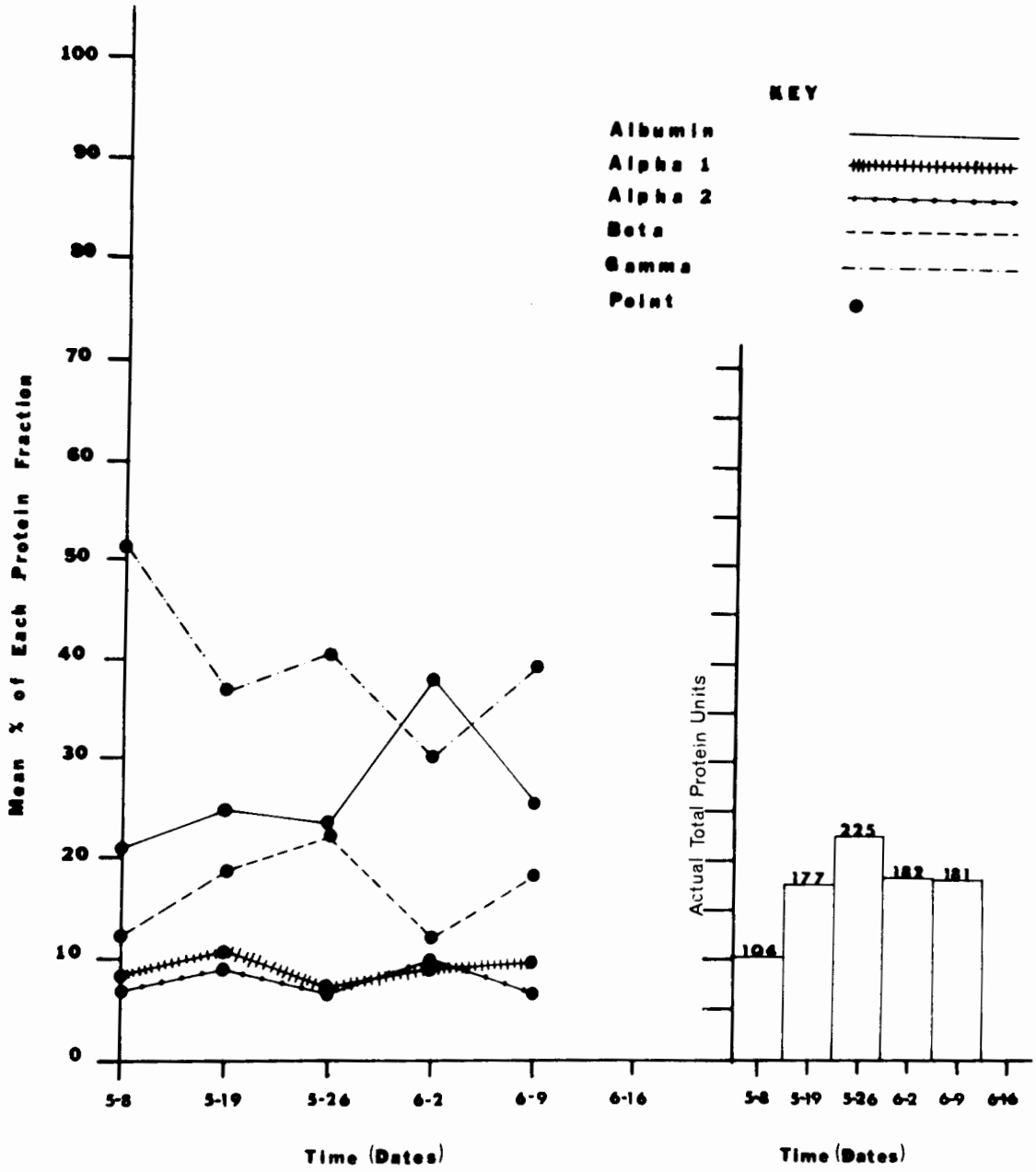
Experimental female Pituophis melanoleucus affinis



GRAPH NUMBER 5

Specimen Number 17

Control female Pituophis melanoleucus affinis

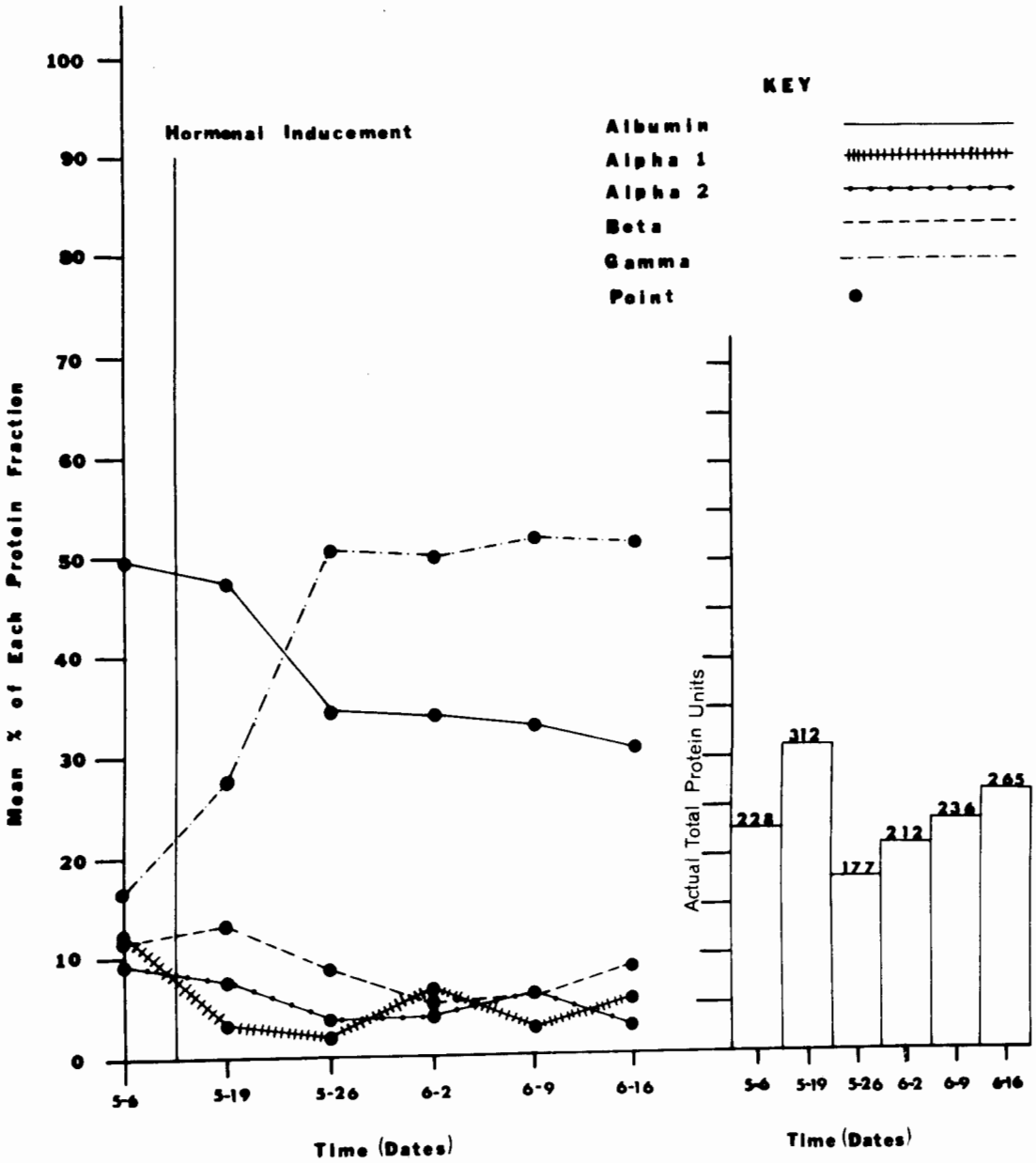


GRAPH NUMBER 6

Specimen Number 16

Control female Pituophis melanoleucus affinis

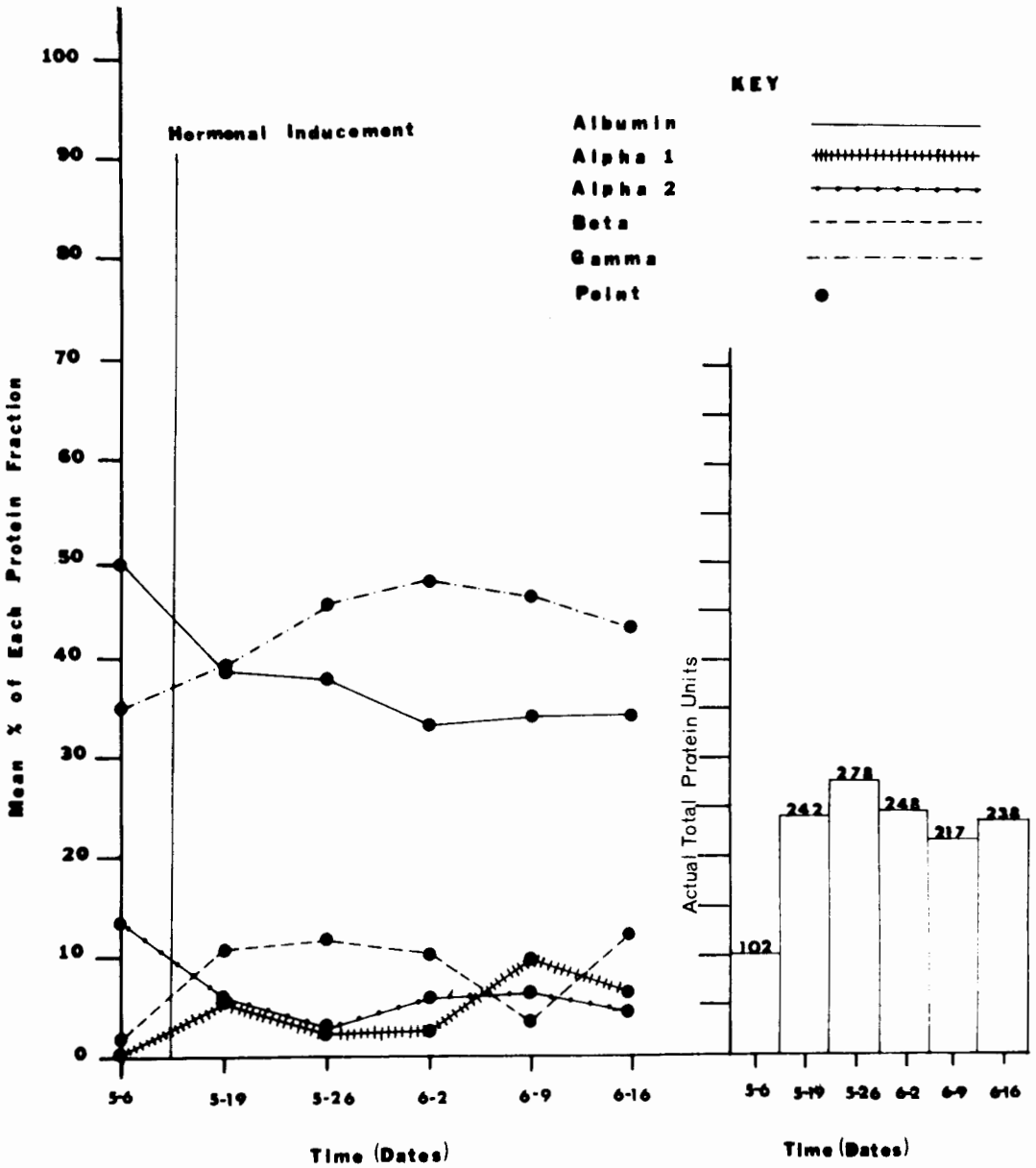
Note: This specimen died unexplainably on 6/15/68.



GRAPH NUMBER 7

Specimen Number 26

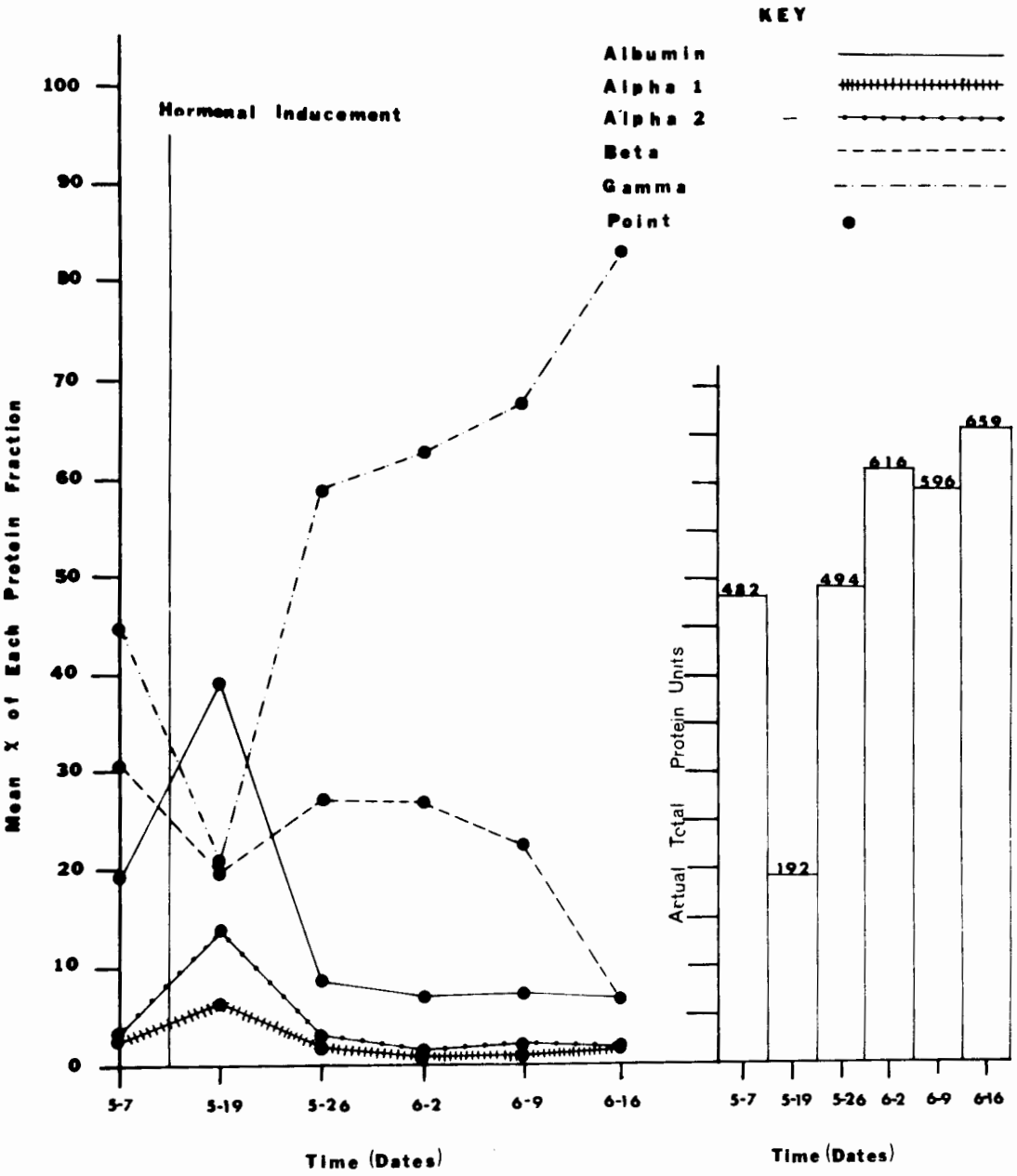
Experimental male Pituophis melanoleucus catenifer



GRAPH NUMBER 8

Specimen Number 12

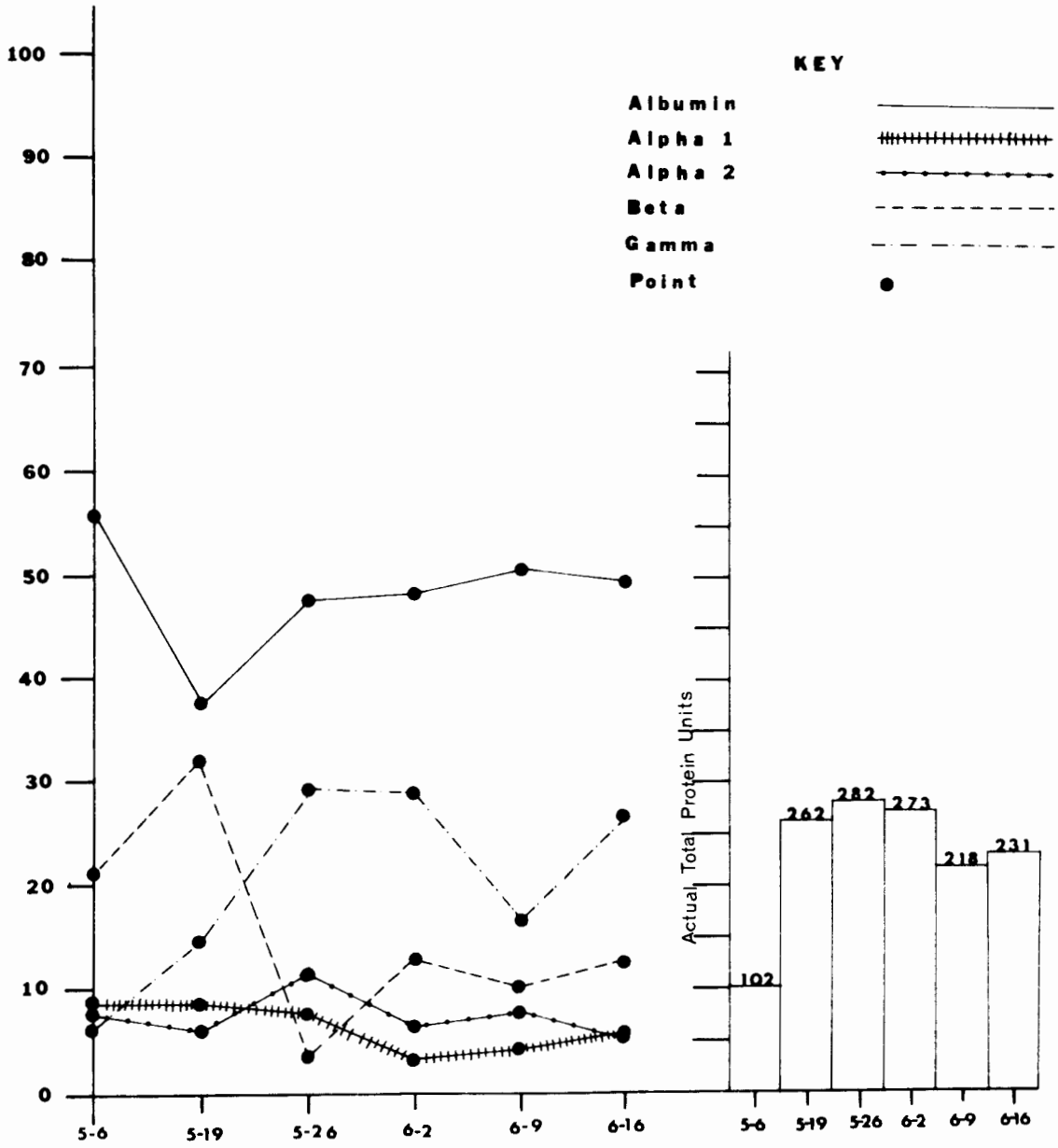
Experimental male Pituophis melanoleucus affinis



GRAPH NUMBER 9

Specimen Number 25

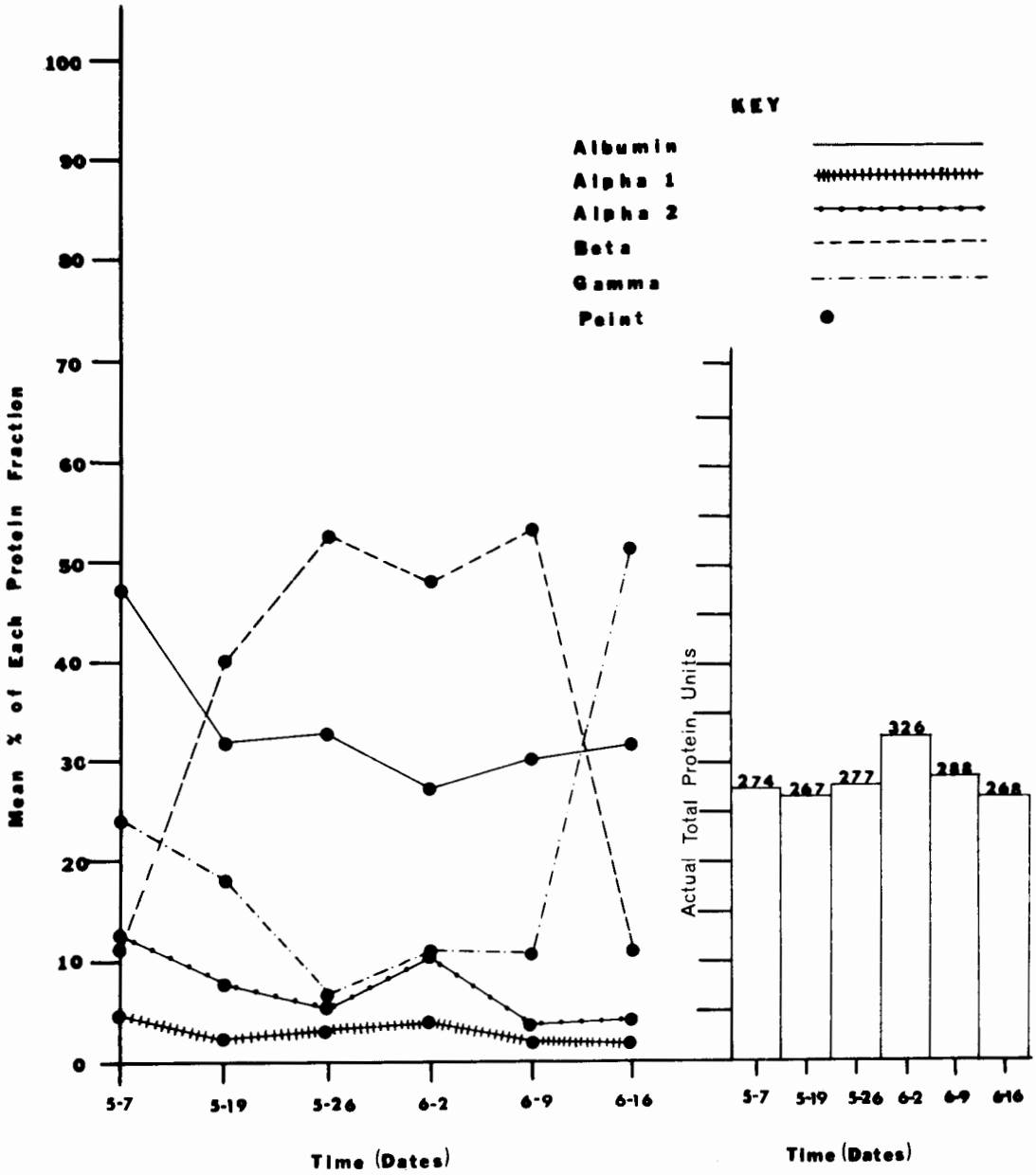
Experimental male Pituophis melanoleucus catenifer



GRAPH NUMBER 10

Specimen Number 21

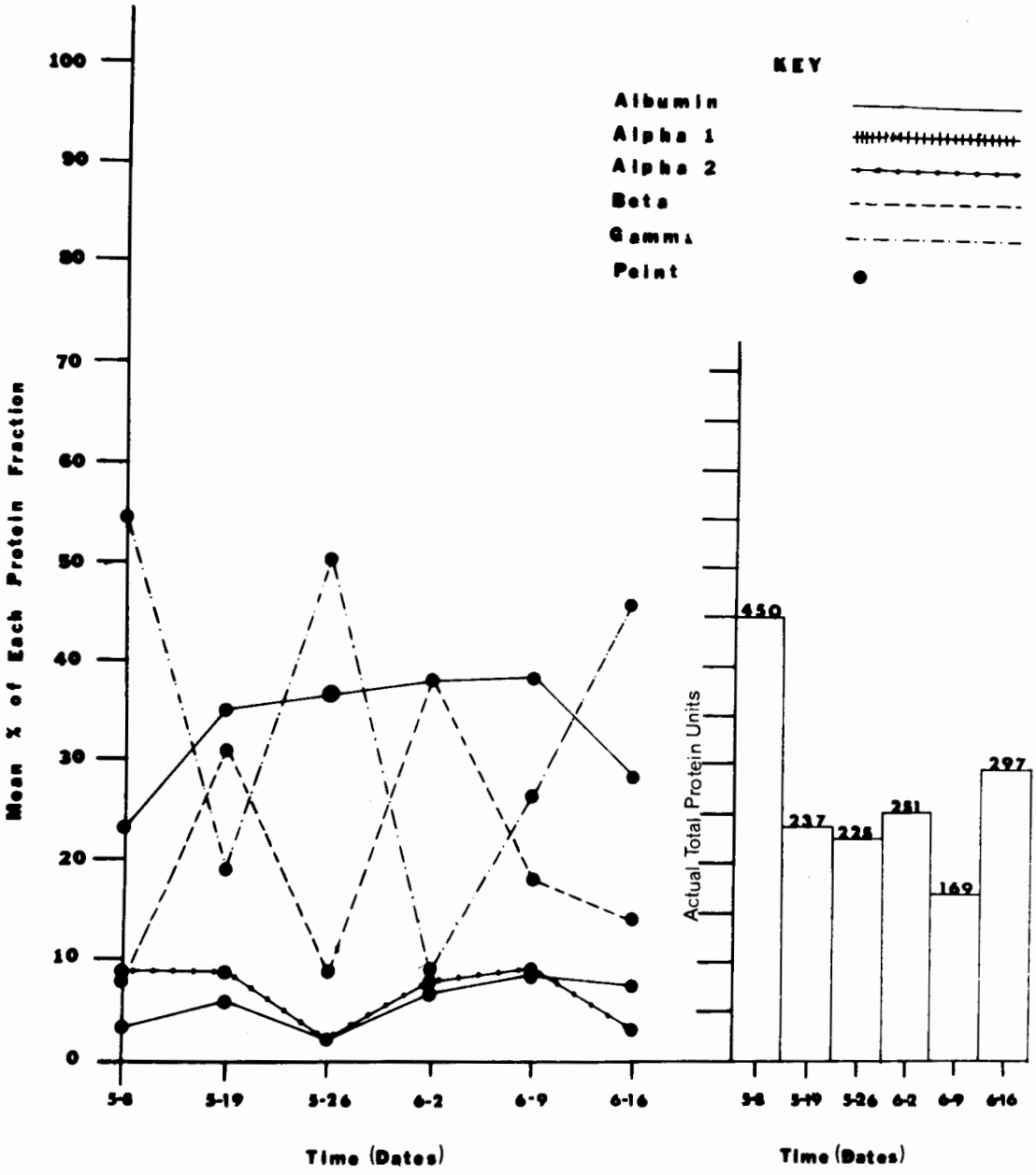
Control male Pituophis melanoleucus catenifer



GRAPH NUMBER 11

Specimen Number 23

Control male Pituophis melanoleucus catenifer



GRAPH NUMBER 12

Specimen Number 11

Control male Pituophis melanoleucus affinis