QUALITATIVE AND QUANTITATIVE CHROMATOGRAPHIC DETERMINATION OF MUSCLE MYOSIN PRODUCTION IN CONTROL AND CHRONICALLY ACCELERATED CHICK EMBRYOS

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CHAPTER I

INTRODUCTION

A recent study in this laboratory to determine the effects of chronic acceleration on the growth of bone in chicken embryos has revealed an unparallel growth between control and accelerated animals (20). The bone dimensions (both length and width) of all long bones in the accelerated animals are greater during the period of development up to 18 days; after 18 days bone growth in the accelerated animals falls behind that in the control animals.

Wunder et al. (24) reported that the femurs of accelerated mice continued to grow as fast as, if not faster, than the controls during the first week of acceleration. At the same time the total body mass of the accelerated mice first decreased but returned to approximately initial body mass at the end of the first week. As is further stated, this rapid growth of bone in the experimental mice is not as pronounced at later stages of development. Wunder et al. (25) later reported that femur bones accelerated at 4 times the earth's gravity (4 G) can grow at a rate faster than the controls. These bones also show increased cross-sectional area.

As reported by Wunder et al. (24) there are at least two possible explanations for the faster bone growth as compared to the total growth in mass. These are mechanical stress and moderate starvation. He states that starvation alone does not seem a plausible explanation for this change in growth. However, it is well known that bone growth occurs in proportion to the load that the bone must carry. For this reason bone dimensions should increase in response to the mechanical stress applied to the bone by way of the attached musculature. This being the case, an increase in bone growth could perhaps reflect increased muscle mass or at least an increased ability of the muscle to contract. Wunder et al. (25) states that during acceleration mice show increases in the relative sizes of the gastrocnemius muscle as well as that of the diaphragm and heart. An increased ability to contract might indicate a change in muscle quality or quantity. More investigations are required at the present in an attempt to demonstrate the actual cause or causes for the relative increase in size of femurs (24, 25) in one case and the increase in all bones studied (20) in the other.

Recent developments concerning isolation of a purified, stable, polyribonucleotide-free myosin fraction from skeletal muscle of chickens made possible a comparative study of myosin production within the experimental and control animals. The

physical nature of the myosin molecule as well as its proposed involvment in muscle contraction made it the protein of choice in this study.

Literature

Myosin is the major structural protein in muscle. The molecular weight, size, and shape of the molecule have been determined by numerous investigators (8, 10, 11, 13, 14, 21, 23). Most eminent among these investigators are Holtzer and Lowey (10, 13), who have identified the molecule as a rod 1620 A long and 26 A thick with a molecular weight of approximately 493,000.

Actin-free myosin was first crystallized by A. Szent-Györgyi (22) in 1943. In the presence of even small concentrations of KCl myosin readily crystallizes at neutral or slightly acidic pH (pH 6.4-7.0). In outline, the procedure consists of the simultaneous precipitation of myosin and actomyosin from a muscle extract by dialysis, which renders most of the actomyosin irreversibly insoluble. The remaining actomyosin, if any, is precipitated from the redissolved myosin fraction by adjusting the ionic strength and pH, crystallization of myosin from the supernatant by dilution with water, and recrystallization by repetition of the last step (15, 22).

A somewhat older method yet one still performed by Szent-

Györgyi and others in the 1940's required the simultaneous action of adenosine triphosphate (ATP) and a high concentration of salt (0.5 M KCl). Once the myosin was dissolved and free of actin, a 0.1 M KCl solution was sufficient to keep myosin in solution (22). Both procedures involved manipulations which were difficult, loss of large quantities of material, and myosin exposure to denaturation (22).

In 1946 M. Dubuisson (6) fractioned myosin with ammonium sulfate into two different fractions which he called α myosin and β myosin. There are reasons to believe that his α myosin was actomyosin and his β myosin was a rather actin-free myosin fraction (22). The fractionation involved subjecting myosin to ammonium sulfate; actomyosin was precipitated according to the actin content at 27-40 per cent saturation. The solution was brought up to 40 per cent saturation at 0°C (pH 6.0-6.5), the precipitate separated on the centrifuge, the fluid brought up to 45-47 per cent saturation, the myosin centrifuged out, dialyzed overnight at 0°C, and precipitated by dilution. Evidence shows that myosin prepared by ammonium sulfate fractionation is more actin-free than the myosin of earlier methods. The ammonium sulfate denatures actin, in that way rendering the myosin free of actin (22). Myosin heterogeneity is likewise reduced by ammonium sulfate; such heterogeneity has been

shown to be due to the presence of true contaminants in the purified myosin (2, 3, 4, 7, 17, 22).

The preparation of myosin from chick embryos in a highly pure form was requisite to immunological, enzymatic, and radioisotopic studies of myosin synthesis during embryonic development. Perry (17) noted a lack of specificity in all previous methods employed to isolate pure myosin. Myosin is a labile protein; there was at this time no report of successful separation of myosin on such synthetic ion-exchange resins as diethylaminoethyl (DEAE)-cellulose, which had been limited to the more robust proteins (17). A general study of the activity of muscle proteins during ion-exchange chromatography had just been carried out by Perry and Zydowo (18).

Later in 1959 Brahms (5) initiated studies on the chromatography of myosin. Even highly purified myosin was chromatographically heterogeneous (1, 2, 3, 4, 5, 16, 17). This heterogeneity was resolved into three chromatographically distinct myosin fractions and two other fractions with none of the characteristic properties of myosin (2, 3, 4, 12). A single homogeneous fraction has not been achieved; the heterogeneity has been attributed to a binding of polyribonucleotides with myosin which is mediated by divalent ions (2, 3, 4, 7).

Between the years 1964-1966, Baril, Love, and Herrmann (3, 4) devised a procedure whereby a purified, stable, polyribonucleotide-free myosin fraction could be obtained from skeletal muscle of chicks. The method involved extraction by pyrophosphate, differential ultracentrifugation, treatment with pancreatic ribonuclease, gel filtration of the treated extract, and chromatography on DEAE-cellulose. Electrophoresis and analytical ultracentrifugation showed such extracted myosin fractions to be homogeneous. Numerous other investigators (1, 16, 17, 18) have employed similar chromatographic techniques to obtain relatively pure myosin fractions.

The pyrophosphate extraction technique aforementioned was developed by Hanson and Huxley (9). Love (12) has since modified the technique to meet the requirements of myosin extraction. As described by Peterson and Sober (19) in 1956 myosin can be truly adsorbed and eluted like a typical protein from pyrophosphate solution of even very low ionic strength.

Pancreatic ribonuclease treatments were not employed in this study since the assumption could be made that the ribonucleoproteins would effect both groups of animals equally, unless there was unequal synthesis of ribonucleic acids or proteins within the groups. The latter gives rise to yet another problem, knowledge of which is requisite perhaps to a

fuller understanding of the problem at hand.

Statement of the Problem

The purpose of this investigation was to employ newly improved qualitative and quantitative chromatographic techniques to obtain purified myosin from 1 G and 3 G chick embryos and to determine if muscle myosin production either follows or precedes the unparallel bone growth during chronic acceleration as reported by several investigators.

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CHAPTER II

MATERIALS AND METHODS

Incubation

The fertile eggs used throughout the experiments were Strain K-137, obtained from Western Pullet Producers. All eggs upon arrival were examined for breakage; those eggs which were either broken or cracked were discarded. All unbroken-uncracked eggs were then weighed to 0.1 gram accuracy on a Harvard Trip balance. The eggs were numbered, starting with 1, in order of increasing weights. Weights and egg numbers were recorded in duplicate, once in the data book and again on the egg shell. The eggs were incubated prior to acceleration for a minimum of two days in a specially designed area of the incubator-centrifuge. Following this period of initial incubation all eggs were candled to determine fertility and stage of development. Those eggs exhibiting what was considered a good start of growth (36-48 hours) were paired by equal weights to obtain 15 pairs. Pairs were chosen so as to represent all egg sizes equally within the weight distribution. These pairs were then placed on the centrifuge; the remaining number (excluding all undeveloped eggs which were discarded) were placed back into

the bottom of the incubator-centrifuge to serve as control eggs.

In order to make reference to the incubator-centrifuge it is necessary at this point to describe those parts directly related to the experiments at hand. The incubator-centrifuge is a term describing the combination of the centrifuge and incubator. When the term incubator is used alone, it refers to that aforementioned area in the bottom of the incubator-centrifuge where eggs were kept during initial incubation and later where the control eggs were kept during acceleration. It consists of three egg crate dividers placed side to side, supported at the center by a rod which passes to the outside of the incubator-centrifuge so that by means of a handle attached to one end of that rod the eggs can be turned. The term centrifuge, when used alone, will refer exclusively to the rotor, that part of the incubator-centrifuge which is accelerated and thus carries the experimental eggs. The rotor consists of a flat, round metal plate 70 centimeters in diameter onto which are fixed around the edge 30 specially designed steel cups. These cups are lined with a cushioning layer of foam rubber.

Conditions necessary for incubation were made possible by simple component systems situated within the incubator-centrifuge. A temperature of 95-100^{o}F was maintained at all points

within the incubator-centrifuge by a thermostatically controlled set of heating coils. Proper humidity and circulation were provided by two small fans which passed a continual stream of air across a dish of water.

Both environmental factors were monitored externally by means of the following systems. Temperature was monitored through five thermisters, placed at critical points within the incubator-centrifuge and attached to a Tele-Thermometer system (Model 46 T U) made by Yellow Springs Instruments Company. Humidity was not quantitated, but the presence of condensate in a plastic cup set into the lid of the incubator-centrifuge gave indication that humidity was high enough to insure incubation. Both environmental parameters were observed daily.

Acceleration

An accelerative force of 3 G was used in each of the experimental runs. When the eggs were placed onto the centrifuge, the embryos were turned toward the center of the centrifuge. The location of the embryo within the egg had been previously determined when the eggs were candled, and a small "X" had been penciled onto the shell to mark that location.

The accelerative force was measured in revolutions per minute; 3 G was equal to approximately 72 revolutions per minute. Revolutions per minute were monitored daily by means of

a Metron battery-operated tachometer (Type 25 B) made by Metron Instruments Company. The speed of the electric motor which powered the incubator-centrifuge was in turn regulated by means of a Boston Gear Radiotrol varible motor speed control (Model R 12) made by Boston Gear Works.

The nomenclature used herein to describe the vector forces of acceleration is the triaxial physiological system proposed by Clark et al. (6) and was prepared as it appears in Table I by Gell (10).

Recent investigation in this laboratory concerning the positioning of chick embryos during acceleration indicates that initially (1-13 days) all the centrifuged embryos experience a $-G_x$ acceleration, as the embryo-to-yolk mass relationship is small and the embryo remains nearer the center of the centrifuge. Between the 13-18 day period the embryo turns, approximately 90 degrees, either from left to right or from right to left, now experiencing $+G_y$ or $-G_y$ acceleration, respectively. Between the 18-19 day period the embryo must turn again approximately 90 degrees, as its mass now exceeds that of the yolk. The embryo is now at a point within the shell farthest from the center of the centrifuge and is now experiencing a $+G_x$ acceleration (22).

TABLE I

NOMENCLATURE FOR BODY ACCELERATION

Linear

Direction	Acceleration Descriptive	Physiological Standard	Vernacular Descriptive
Forward	Foward Acceleration	$+ G_x$	Eyeballs In
Backward	Backward Acceleration	$- G_x$	Eyeballs Out
Upward	Head Acceleration	$+ G_z$	Eyeballs Down
Downward	Foot Acceleration	$- G_z$	Eyeballs Up
To Right	Right Lateral Acceleration	$+ G_y$	Eyeballs Left
To Left	Left Lateral Acceleration	$- G_y$	Eyeballs Right

Preparation of Myosin Solutions

The main steps used in the preparation of muscle myosin for DEAE-cellulose chromatography follow those developed by Baril, Love, and Herrmann (2, 3, 4, 13). Fresh leg muscle (8-36 grams) was excised from 18-day chicks killed by decapitation and exsanguination (4, 8, 24). All carcasses were refrigerated for 30 minutes prior to excision of muscle (4, 8, 12, 14, 15). The muscle was separated from the developing bone by glass probe dissection and was cut, using iris transplant scissors. Fat deposits were avoided as much as possible, for there appeared to be unequal fat deposition within the two groups. Oyama (17) reported that fat deposits are lost during chronic acceleration of mice. Muscle from the embryos of both groups was pooled separately and then divided into equal wet weight portions of approximately 6.45 grams. All muscle weighings were performed on a Sartorius analytical balance. From this point the procedure for both groups was identical, so that the remaining procedure applies equally to each group. Muscle was minced to fine consistency between the blades of standard surgical scissors (4). Each portion of minced muscle was homogenized for 30 seconds, using a small tissue homogenizer made by Chemical Rubber Company. The homogenizer was operated within the refrigerator at $4^{\circ}C$; the muscle

tissue was kept at 4°C during the operation of the homogenizer by the passage of an acetone-ice water mixture (-12 to -15°C) through the homogenizer cooling jacket. The homogenate was prepared by mixing the minced muscle tissue in 10 volumes of 0.04 M KCl containing 0.0067 M K_2HPO_4, adjusted to pH of 7.0 by the addition of a few drops of concentrated H_3PO_4 (3, 4). The homogenate was stirred in a 0°C ice bath for 90 minutes atop a Sargent magnetic stirrer; the homogenate was then centrifuged at 10,000 G in the rotor No. 856 of an International High Speed Refrigerated centrifuge (Model HR-1) made by International Instruments Company (4). The supernatant was discarded. The residue was redissolved and homogenized for 30 seconds in 10 volumes of 0.02 M $K_4P_2O_7$ containing 0.001 M $MgCl_2$, adjusted to pH of 9.2 by the addition of a few drops of concentrated $H_4P_2O_7$ (3, 4). Following 90 minutes of stirring in a 0°C ice bath the homogenate was centrifuged at 30,000 G for 20 minutes in the rotor No. 856 of the International centrifuge; the residue was discarded (3, 4). In both cases the International centrifuge was precooled to 0°C. The supernatant was centrifuged on the "Ti" rotor No. 50 of a Beckman Model L Ultracentrifuge at 150,000 G for 230 minutes. The ultracentrifugate was filtered through a standard pore Kimax fritted glass funnel and concentrated to 20-30 milliliters with Sephadex

G-25 obtained from Pharmacia Fine Chemicals. The concentrate was subjected to gel filtration on Sephadex G-200, also obtained from Pharmacia Fine Chemicals. Gel filtration columns (3.1 by 30.5 centimeters) had been equilibrated with 0.02 M $K_4P_2O_7$, adjusted to pH of 8.5 by the addition of a few drops of $H_4P_2O_7$ (3, 4, 9, 21). Both columns maintained a gravity flow rate of approximately 0.9 milliliters per minute at 4°C. The Sephadex G-200 was prepared for the columns as outlined by Pharmacia and followed the procedure of Flodin and Killander (3, 4, 9, 21). Five-milliliter fractions were obtained simultaneously from the two columns and were collected on a Misco fraction collector (Model 6510 A), manufactured by Misco Scientific Microchemical Specialities Company (4, 9).

The reagents used were J. T. Baker reagent grade or other reagent grade chemicals with the least heavy metal contamination (1, 4, 7, 11, 15, 24, 25). Deionized glass-distilled water was used in all myosin preparative procedures (1, 4, 5, 11, 15, 24, 25). All solutions were cooled to 4°C before use. The pH of all solutions used was determined at room temperature (approximately 23°C) on a Coleman Metrion IV (Model 28C) pH meter. The electrodes were standardized at pH 7.00 and 9.00 with Coleman certified standard buffer tablet solutions.

DEAE-cellulose Chromatography

The major eluted fraction from the Sephadex columns was chromatographed on DEAE-cellulose according to the procedure developed by Love (3, 4, 13). The DEAE-cellulose purchased from BIO-RAD Laboratories was washed several times with 0.5 M KOH and 0.5 M HCl, followed by rinsing in glass-distilled water (1, 4, 16, 19, 20, 21, 23). Smaller column sizes (2.5 by 24 centimeters) were used for cellulose chromatography. The columns were poured in the usual manner after equilibration of washed material against 0.02 M $K_4P_2O_7$, adjusted to pH of 8.5 (3, 4). Eighty 5-milliliter fractions were obtained simultaneously from two chromatographic columns. Elution was effected by discontinuous, stepwise increases in ionic strength at a constant pH of 8.5. The following solutions were used for the elution of the myosin fractions: Fraction I, 0.02 M $K_4P_2O_7$; Fraction II, 0.125 M KCl-0.003 M $K_4P_2O_7$; Fraction III, 0.18 M KCl-0.005 M $K_4P_2O_7$; and Fraction IV, 0.36 M KCl-0.01 M $K_4P_2O_7$ (4). Faster gravity flow rates of approximately 1.6 milliliters per minute were obtained on the cellulose columns.

The entire procedure for myosin solution and column chromatography was carried out within the temperature range of 0-4°C (1-5, 7, 8, 11, 12, 14, 15, 16, 18, 19, 24, 25).

Optical Density Determinations

The optical densities of all 5-milliliter fractions obtained from DEAE-cellulose were determined at a wavelength of 280 millimicrons on a Beckman Model DU Spectrophotometer (2, 3, 4, 13, 16, 18, 25). The first fraction eluted contained only 0.02 M $K_4P_2O_7$ and was used to calibrate 100 per cent transmittance on the spectrophotometer. The following settings of the spectrophotometer remained constant during all the determinations: (i) a slit width of 0.125 millimeters, (ii) a hydrogen lamp setting of 0.04, (iii) a sensitivity setting of 0.5, and (iv) a phototube load resistance setting of 3. All measurements were recorded on the attached Beckman Recorder, using chart No. 93512; all per cent transmittance readings were converted to optical density values. These values were plotted against time (or 5-milliliter fractions), resulting in the major fractions (I-IV) mentioned above.

The number of control and experimental animals varied within runs and was as follows: run 1, named 3G-B4, consisted of 7 control and 25 experimental animals; run 2, named 3G-B5, consisted of 22 control and 29 experimental animals; and run 3, named 3G-B6, consisted of 33 control and 29 experimental animals. This variation was due to fluctuation in the per cent maturation of the embryos. In explanation of the names given each of the three runs, the 3G indicated that the

accelerative force was 3 times the earth's gravity. The B4, B5, and B6 indicated a specific run at the 3 G level; the B named the series, which was named after a color. In this case that color was black.

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CHAPTER III

RESULTS

Muscle myosin was put into solution, chromatographed, and measured in each of three experimental runs. There was variation in the number of embryos within runs. Consequently, there was also variation in the quantity of muscle collected within runs. The 3G-B4 run produced 6.8439 grams of control muscle and 21.6073 grams of experimental muscle. The 3G-B5 run produced 24.9719 grams of control muscle and 24.6996 grams of experimental muscle. The 3G-B6 run produced 25.1012 grams of control muscle and 36.8918 grams of experimental muscle. These muscle quantities were divided within runs into equal wet weight portions of approximately 6.45 grams. The number of equal wet weight portions within each run also varied and was as follows: 3G-B4, 1 control and 3 centrifuge; 3G-B5, 3 control and 3 centrifuge; and 3G-B6, 3 control and 4 centrifuge. For reason of time required for myosin solution and DEAE-cellulose chromatography, results were obtained on only 6 control and 5 centrifuge portions.

Figure 1 compares the mean values of egg weight, embryo weight, and muscle wet weight of the control and experimental

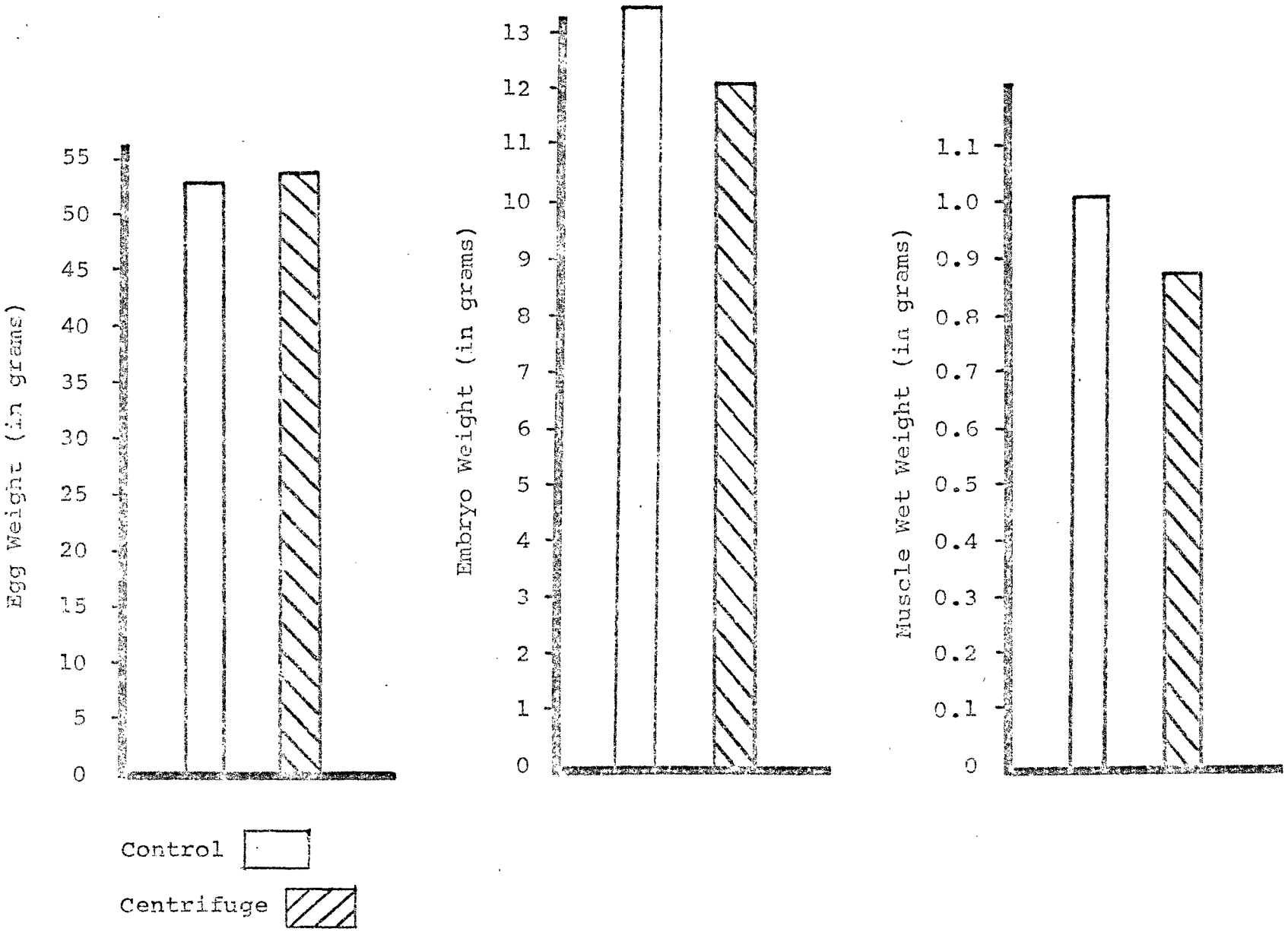

Fig. 1--Comparison of egg weight, embryo weight, and muscle wet weight means for control and experimental 18-day chick embryos.

18-day chick embryos. Examination of this figure reveals the similarity between the egg weight means of the two groups. The numerical values for the control and experimental group were 52.35 and 52.69, respectively. The results of an analysis of variance for a one-way classification (Table II) indicate there is no significant difference between the egg weight means.

TABLE II

ANALYSIS OF VARIANCE OF THE EGG WEIGHT MEANS OF THE CONTROL AND EXPERIMENTAL 18-DAY CHICK EMBRYOS

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
Between	1	31.02	31.02	
Within	143	1,831.84	12.81	2.42
Total	144	1,862.86		

A comparison of the control and experimental embryo weight means in Figure 1 indicates that the control embryos are greater in mass. The embryo weight means of the control and experimental groups were 13.6167 and 12.0266 grams, respectively. This difference had been reported previously, following investigation in this laboratory concerning the

effects of chronic acceleration on embryo weight (1). The F value for the comparison of embryo weight means (Table III) is significant at the P = .001 level, a high level of significance. An analysis of variance could not be performed on the mean values of muscle wet weight. The quantity of muscle available represented the pooled amount of muscle from all embryos within each group of the three runs. Muscle weights per embryo were not at hand.

TABLE III

ANALYSIS OF VARIANCE OF THE EMBRYO WEIGHT MEANS OF THE CONTROL AND EXPERIMENTAL 18-DAY CHICK EMBRYOS

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
Between	1	201.5357	201.5357	
Within	143	1,034.6429	7.2352	27.85***
Total	144	1,236.1786		

*** Random probability of difference between groups less than 0.1 per cent.

The mean optical density values for the eighty 5-milliliter chromatographic fractions within runs 3G-B4, 3G-B5, and 3G-B6 appear in Table IV. These mean values are plotted against time (or 5 milliliter fractions) in

TABLE IV

MEAN OPTICAL DENSITY VALUES OF CONTROL AND EXPERIMENTAL 18-DAY CHICK MUSCLE MYOSIN

Tube No.	Optical Density		Tube No.	Optical Density	
	Control	Centrifuge		Control	Centrifuge
1	.00883	.01040	41	.05650	.09020
2	.01216	.01360	42	.09916	.11180
3	.01916	.01320	43	.13033	.15220
4	.01983	.01300	44	.13033	.13460
5	.01933	.02600	45	.16416	.14440
6	.01800	.03000	46	.19366	.17660
7	.01916	.02780	47	.23150	.23180
8	.02633	.04440	48	.25100	.26020
9	.04450	.04480	49	.29300	.32400
10	.05400	.08100	50	.32000	.38420
11	.07216	.08140	51	.36750	.43560
12	.07550	.09666	52	.44566	.49420
13	.07950	.12040	53	.48383	.44320
14	.11250	.16320	54	.49816	.48460
15	.13883	.21260	55	.48433	.56580
16	.19483	.21220	56	.37816	.34320
17	.21783	.23260	57	.25600	.17760
18	.21766	.10840	58	.13116	.07460
19	.17033	.09260	59	.07066	.04020
20	.08316	.02240	60	.04600	.04640
21	.05450	.03260	61	.04683	.08260
22	.02300	.03340	62	.06716	.17800
23	.01700	.03440	63	.12700	.24480
24	.02150	.06320	64	.23500	.28860
25	.03333	.10200	65	.40100	.40880
26	.06966	.11880	66	.45316	.50200
27	.11616	.11320	67	.57916	.58020
28	.14450	.17440	68	.65900	.63180
29	.11050	.06800	69	.73516	.72840
30	.07833	.03860	70	.80816	.79560
31	.06366	.02800	71	.71050	.78840
32	.03083	.02800	72	.73033	.64760
33	.01950	.03820	73	.66000	.54840
34	.02350	.04240	74	.52966	.37000
35	.02200	.03100	75	.47216	.31160
36	.02800	.03980	76	.32500	.17520
37	.03016	.03840	77	.23466	.09220
38	.03033	.04060	78	.10366	.06480
39	.02483	.05840	79	.05216	.05660
40	.03300	.06280	80	.01850	.04540

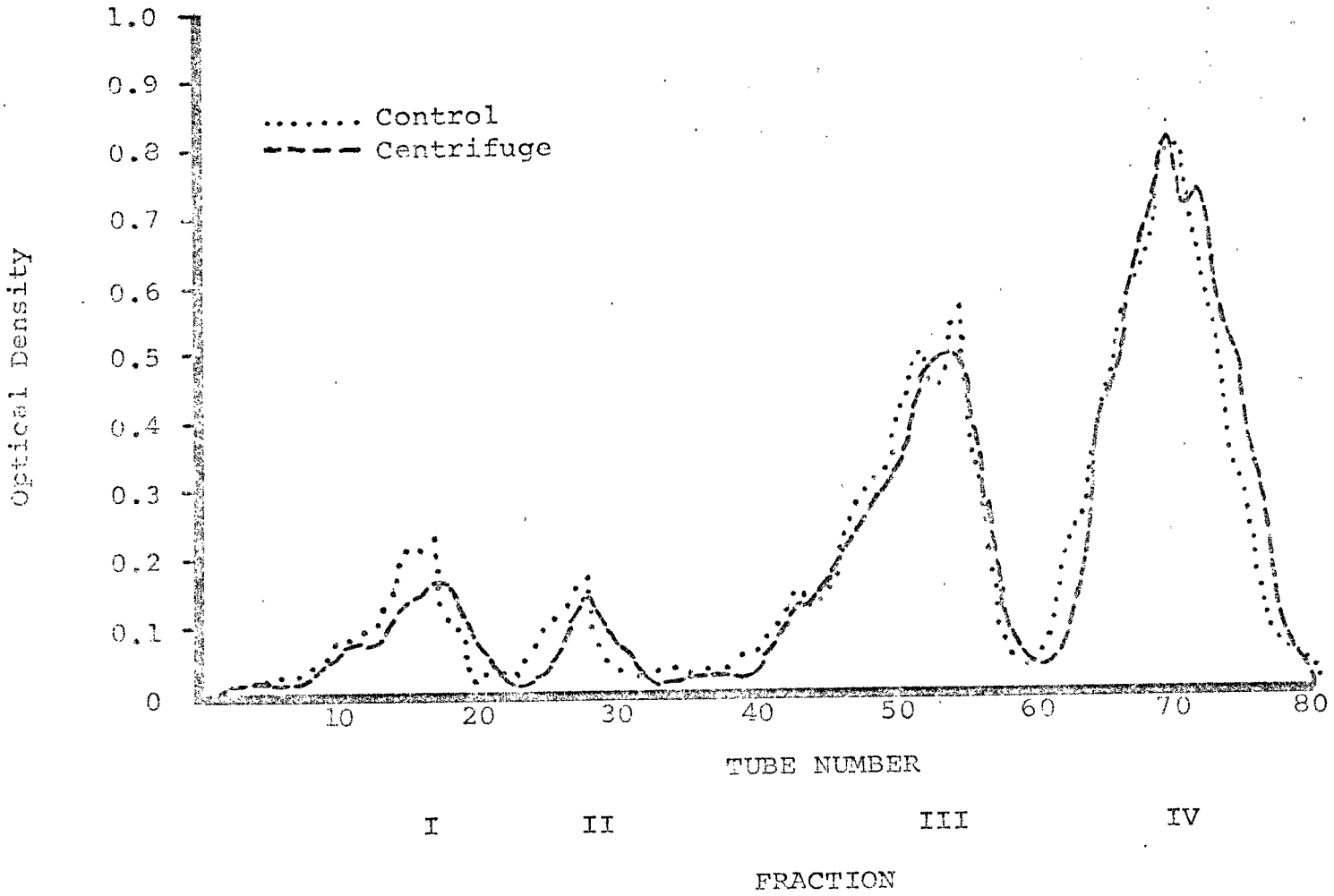

Fig. 2--Effects of chronic acceleration on elution time and optical density values of chromatographic fractions I-IV.

Figure 2. The elution time for each 5 milliliter fraction was 3 minutes. Examination of this figure indicates that the elution solutions separated four fractions on DEAE-cellulose. The area under the peaks increases in proportion to the quantity of myosin or myoglobin in those fractions.

An analysis of variance for a one-way classification (Table V) indicates there is no significant difference between the area means. Further analyses of variance on individual fractions and on the combined myosin fractions II-IV indicate no significant differences between these area means as well. Those F values are as follows: Fraction I, F = .0392; Fraction II, F = 2.1142; Fraction III, F = .0246; Fraction IV, F = .2857; and combined Fractions II-IV, F = .0120.

TABLE V

ANALYSIS OF VARIANCE OF THE FRACTION PEAK MEANS OF CONTROL AND EXPERIMENTAL MUSCLE MYOSIN

Source	Degrees of Freedom	Sum of Squares	Mean Square	F
Between	1	.0002	.0002	
Within	889	42.4102	.0477	.0044
Total	890	42.4104		

Qualitative examination of Figure 2 reveals no apparent significant change concerning the molecular configuration of the protein molecule. Elution was effected similarily by all elutions solutions within the control and experimental groups.

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CHAPTER IV

DISCUSSION

Investigations by Wunder et al. (3, 4), Redden (2), and other investigators have shown an unparallel bone growth in control and chronically accelerated animals. Wunder and his colleagues suggest at least two possible explanations to account for the faster bone growth as compared to the total body mass: moderate starvation and mechanical stress (3). In the case of moderate starvation the initial decrease in body mass can be largely attributed to a lowered food consumption during the first week of centrifugation. Bone development during this period does not show a comparable retardation, resulting in an increase in the bone mass to body mass relationship. Although starvation could account for the change in this relationship, it alone does not seem a plausible explanation for the actual change in bone cross sectional area that occurs simultaneously. On the other hand, mechanical stress could in part account for the increased bone growth in the centrifuged animals. This should be valid if Wolffe's law is followed in chronic acceleration studies. The law proposes that bone grows in proportion to the load that the bone must

carry. The artificial gravitational field (3 G) could certainly increase the mechanical load on the bone. If the experimental form is a tetrapod (mouse, turtle, etc.) as used by Wunder et al. and other investigators, Wolffe's law should be valid. The increased gravitational field (3 G) confronting the experimental form increases the work that must be done to secure food, to maintain posture, and to make other body movements. This increase in work results in an increased mechanical stress applied to the bone. If mechanical stress is the answer to the increased bone growth in experimental animals that is reported by several investigators, then Wolffe's law is followed.

In the case of the investigation cited herein the experimental form was the chick embryo. This was an embryonic form, one that was developing a skeletal and a muscular system simultaneously. Nevertheless, there was evidence of unparallel bone growth in the control and experimental animals. Explanation of the cause or causes in this case is even more difficult.

It is possible that during the first 13 days of acceleration the embryo may not encounter a significant increase in the force of gravity (2). At this time the embryo to yolk mass relationship is small; the embryo actually "floats" atop the more abundant and dense yolk and embryonic fluids. During

this period there should be damping of the acceleration effect followed by a comparable reduction in the effects on bone growth. Another factor which must be considered is the position of the embryo with respect to the gravitational field. At this time the embryo encounters backward acceleration as it faces away from the center of the centrifuge ($- G_x$).

Between the 13-18 day period the embryo turns from left to right or from right to left, from 0 degrees through 90 degrees (0 degrees being the position of the embryo at day one or facing away from the center of the centrifuge). During this period of incubation and acceleration the embryo mass approaches and exceeds the mass of the yolk. The embryo should encounter the increased accelerative force from the side ($+ G_y$ or $- G_y$). The embryo could make movements of adjustment to compensate for the increased gravitational field, although this has not been observed. Furthermore, during the 18-19 day period the embryo continues to turn in the field of gravity, assuming now a position 180 degrees from its early embryonic position (2). Its mass now exceeds that of the yolk; the embryo is forced to occupy a position within the shell farthest from the center of the centrifuge. The embryonic fluids begin to disappear and the yolk sac begins to retract. The embryo at this point is subjected to the full force of the increased gravitational field ($+ G_x$), the effects

of which should be reflected by the physiology of the embryo. More compensatory movements could be made by the embryo, increasing again the mechanical stress applied to the bone and muscle. It is known that the embryo moves to the air sac during this period to satisfy respiratory requirements.

If bone growth in the chick embryo follows Wolffe's stress phenomenon, a discernible change should occur in the load carried by the bone and muscle at some time during the 13-19 day period.

An examination of Figure 1 indicates both an increased embryo weight and muscle wet weight in the control group. Egg weight means in this figure show that these results can not be explained by a larger set of control eggs. There is no significant difference between the egg weight means of the control and accelerated groups. The embryo weight mean of the control group is larger; there is a highly significant difference between the embryo weight means of the two groups. More important, however, is the fact that there is a greater yield of muscle wet weight in the control group. Surprisingly, there is apparently no significant difference between the grams of muscle wet weight collected per gram of embryo weight. The values for the control and experimental groups are .07908 and .07216, respectively. Consequently, the increased muscle wet weight in the control group can be

attributed only to the fact that the control embryos were larger. No significant increase in muscle mass per embryo followed chronic acceleration.

A comparison of the plots of myosin fractions from the control and experimental groups in Figure 2 reveals the similarity between the myosin chromatographed in the two groups. Four chromatographic fractions were separated on DEAE-cellulose. These fractions have been characterized by Baril and his associates (1). Fraction 1 consists mainly of myoglobin. Fractions II-IV have been characterized as myosin-like fractions, with such characteristic properties of myosin as actin-binding and a high adenosine triphosphatase activity mediated by Ca^{++}. There was no significant difference in the fraction peak means of the control and experimental groups (Table V). This precludes any possibility that there might have been increased muscle myosin production in the accelerated animals. No significant change in myosin quantity per gram of muscle followed chronic acceleration.

Since one of the characteristics of DEAE-cellulose is separation of molecules of different size and configuration, a change in the molecular qualities of myosin should evoke changes in myosin elution on columns of DEAE-cellulose. Qualitatively the plots show no apparent significant change

in the myosin elution qualities following chronic acceleration. In relation to time the elution of the four major fractions was approximately the same. The shape of the plots was similar; the altitudes, slope, and base of all curves were approximately equal. The fact that these qualities are similar precludes any major change in the configuration of the myosin molecule. Both the shape and size of the myosin molecule have apparently remained unaltered following chronic acceleration.

The results of this investigation indicate that the increase in bone dimensions reported in embryonic acceleration studies of chicks may not be due to mechanical stress applied to the bone by increased muscle tone or muscle mass. An increase in muscle myosin content following chronic acceleration should have been requisite to explain the increase in bone growth using Wolffe's law. The results of this investigation do not, however, preclude the possibility that the increased bone growth observed in the studies on chronic acceleration of tetrapods might be produced in part or _in toto_ by mechanical stress. In turn, the latter might well be caused by increased protein (myosin) content of the muscle or some other factor that would enhance the contractile ability of the musculature. It must be borne in mind that in this investigation the accelerated form was a chick embryo,

a form found submerged in embryonic fluids and upheld by the yolk the greater part of its life, conditions which would perhaps simulate a near weightless state (2). This form should not be expected to respond in the same manner as the tetrapod form used by other investigators.

The results of the bone growth studies in this laboratory indicate the eighteenth to nineteenth day to be the time at which the bone dimensions are equal in the two groups (2). Unparallel growth precedes and follows this period of of incubation and acceleration. Additional investigations need to be done to compare the myosin content of the muscle at 15, 16, 17, 19, 20, and 21 days of incubation in the control and accelerated chicks. A similar study should be carried out, using tetrapods to further substantiate these findings.

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