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DNA DEPENDENT RNA POLYMERASE ACTIVITY OF RAT HEART NUCLEI DURING EXPERIMENTALLY INDUCED HYPERTROPHY

By

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LIST OF ABBREVIATIONS

TCA	trichloracetic acid
PCA	perchloric acid
PEP	phosphoenolpyruvate
PK	pyruvate kinase
GSH	glutathione
ATP	adenosine triphosphate
GTP	guanosine triphosphate
CTP	cytosine triphosphate
UTP	uridine triphosphate
UTP^{H3}	tritium labelled UTP
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
t, m, r RNA	transfer, messenger, ribosomal RNA
RNA-ase	ribonuclease
DNA-ase	deoxyribonuclease
l.v.	left ventricle
w	weight
v	volume
OD	optical density
$MgCl_2$	magnesium chloride
$MnCl_2$	manganese chloride
$(NH_4)_2SO_4$	ammonium sulphate
NaF	sodium fluoride
SD	standard deviation
SE	standard error
m	moles

ABSTRACT

Nuclei from groups of twelve pooled left ventricles of rats were isolated and DNA, RNA and protein contents of the nuclear suspension were determined. RNA polymerase activity was assayed in the presence of either Mg^{++} or Mn^{++} and $(NH_4)_2SO_4$. Electronmicroscopic examination of normal nuclear preparations revealed undamaged nuclei with very little contamination.

Cardiac enlargement was produced by constricting the abdominal aorta in experimental animals. The operated animals were sacrificed at different intervals after operation between 4 hours and two weeks The "pure" nuclear suspensions of the myocardium of these animals was isolated for examination. Sham operated animals were used as control up till the third day, after this time normal animal served as control, since the difference between sham operated and normal animal did not persist beyond 3 days.

An increase in RNA, DNA and protein content of the nuclear preparation was observed in aortic constricted animals. DNA showed a delayed increase compared to the time course of changes in RNA and protein. RNA polymerase activity showed a biphasic increase following constriction in both Mg^{++} and Mn^{++}-$(NH_4)_2SO_4$ activated systems. In rats killed soon after constriction (4 hours in this study) the Mn^{++}-$(NH_4)_2SO_4$ system showed the greater percentage increase and on the 3rd-4th day after aortic constriction the increase was considerably greater in the Mg^{++} activated system.

An increase in RNA polymerase activity as early as 4 hours after aortic constriction was demonstrated in this study, among other findings. This early increase suggests that the stimulation of RNA synthesis - one of the first biochemical events in hypertrophy may play a role in translating the mechanical stress caused by increased outflow resistance into tissue growth.

INTRODUCTION

I. Introductory Remarks

Hypertrophy is defined as the state of enlargement of individual cells within a tissue or organ without an increase in cell number (Norman, 1962).

Growth of a differentiated tissue such as heart muscle is thought to occur by hypertrophy rather than by hyperplasia (increase in cell number). Experimentally induced cardiac hypertrophy is one model which may be used to investigate the compensatory growth process. Introductory remarks will be given below under subheadings of Physiological, Biochemical and Histological Studies.

The "Physiological Studies" section reviews briefly the mechanism of physiological changes in cardiac hypertrophy. Biochemical studies deal with alterations mainly in protein, RNA and DNA synthesis in an attempt to localize biochemical reactions initiating the series of metabolic changes during the development of hypertrophy. The direction of histological studies differs from the biochemical studies in that the fundamental changes have been observed on the sarcoplasmic reticulum, rather than on the nucleus.

II. Physiological Studies

A number of experiments have provided functional studies not only of heart hypertrophy but of other cardiovascular parameters which respond to a haemodynamic load. In response to a haemodynamic load many changes occur before actual hypertrophy has set in. For example, Beznak (1958) has shown that between one and two hours after narrowing cardiac output and heart rate dropped sharply and so did the reserve force of the heart. The latter was in this instance defined as the

maximum to which the cardiac output could be raised by infusion of 3,5% polyvinylpyrrolidone into the right side of the heart via the jugular vein. By the end of 24 hours, tachycardia developped and by the end of one week when the weight of the heart increased by about 30%, the cardiac output returned to normal, so did the heart rate and reserve force of the heart. Many have suggested that enlarged myocardium is capable of increased work capacity. Beznak (1958) showed that a higher maximum output and work per gram left ventricle was obtained when the hypertrophy was due to thyroxine treatment. When hearts were hypertrophied by aortic constriction, it was shown that in no case was the maximum output per gram left ventricle above that of normal rat hearts

Concerning the functional capacity of the basic contractile unit of the hypertrophied heart, most of the data supports a decrease in myocardial contractility, although there may be no change in the peak isometric developed tension of trabecular muscles (Fanburg, 1970).

The Stimulus to Hypertrophy of the Myocardium

A change in any parameter of cardiac function that leads to hypertrophy of the myocardium will be defined as the "stimulus" (Badeer, 1964). The clinical and experimental causes of cardiac hypertrophy are numerous. There have been a number of attempts to find a common mechanism which operates in inducing the myocardial change, even though the nature of the stimulus differs. The common factors which have been implicated are as follows:

1. Increased mechanical work of the myocardium

One of the major criticisms of the work hypothesis is that often the degree of hypertrophy produced is not proportional to the calculated external mechanical work. One possible reason for the discrepancy between work and hypertrophy may be the fact that cardiac work is not usually expressed in terms of fundamental muscle mechanics. (Badeer, 1964).

2. Hypertension

Most of the methods which produce experimental hypertrophy also cause hypertension. The fact that hypertension and hypertrophy are not always correlated (Beznak, 1954), however seems to indicate the participation of other factors.

3. Excessive hormonal secretion

The role of the anterior pituitary hormones in controlling the mass of heart tissue is complex. Beznak (1952) showed that hypertrophy only occurred in hypophysectomized, aorta-constricted animals if both growth hormone and thyroxin were given.

Insulin and the adrenal cortex and medulla also have an influence on cardiac growth while certain hormones (thyroxin, growth hormone) are essential for cardiac hypertrophy, there is no evidence that increased secretion of these hormones is the common factor leading to hypertrophy.

4. Ischemia of myocardium

Since so many of the conditions leading to hypertrophy are associated with hypoxia, it has been proposed by many that increased oxygen demand, relative to its supply, may be the actual stimulus that induces cell growth. Though Norman (1962) observed a significant increase in heart weight after coronary artery ligation, one can explain hypertrophy more plausibly on the basis of changes in other parameters of cardiac function (stroke, volume etc.) than on the basis of changes in myocardial nutrition.

5. Dilatation

The classical idea based on Starling's law of the heart is that the ventricles adjust in fibre length to expel whatever volume is put into them even if there is a change in inflow or diastolic pressure. This type of regulation includes the concept of change in myocardial contractility so as to provide a control of the various ventricular function curves, i.e. the first response of the heart to an increase in blood pressure due to e.g. aortic constriction, may be a change in fiber length, thus, dilatation may be a part of the early process of myocardial hypertrophy.

A theory based on dilatation causing myocardial injury was proposed by Eyster in 1927 (Norman, 1962) as follows: cardiac dilatation occurred due to the circulatory disturbances present and such dilatation injured the myocardium. The hypertrophy is resulted from this injury - but its mechanism was not suggested.

6. Increased myocardial metabolic rate per beat

Badeer (1964) proposed a hypothesis that the increase in myocardial metabolic rate per beat per unit mass of tissue constitutes a stimulus more inclusive than any other in explaining hypertrophy. This concept received additional support from observations on skeletal muscle. The question as to how an increase in metabolic rate per stroke may bring about structural changes in heart muscle in terms of biochemical alterations remains to be elucidated.

Other factors as for instance, the role of neurogenic control, tachycardia, alterations in water balance (Harwood, M.Sc. thesis), certainly participate in developing hypertrophy.

III. Biochemical Studies

1. Development of hypertrophy

The biochemical changes which occur in hearts of rabbits and dogs during hypertrophy following aortic constriction are reviewed by Meerson (1962). He classified the development of compensatory hypertrophy into three stages. In the first, or transient breakdown stage, symptoms of left ventricular insufficiency are observed with chemical changes which include decrease of the glycogen and phosphocreatine content of the myocardium. The weight of the heart increases each day. The rate of protein synthesis, as measured by incorporation of S35-methionine, doubles and RNA concentration increases 32%. The second, or "protracted stage or relatively stable hyperfunction" is characterized by hypertrophy of the muscular fibres and absence of signs of cardiac insufficiency.

The heart weight is approximately twice that of normal. In the third or "protracted stage of progressing cardiosclerosis", cardiac insufficiency develops with myocardial fibrosis, focal fatty degeneration, decrease of DNA concentration and protein synthesis. The biochemical changes accompanying hypertrophy during the "first stage" are briefly discussed in connection with the question of hypertrophy - hyperplasia in muscle tissue.

2. Hypertrophy and hyperplasia

The major questions in evaluating biochemical processes at the subcellular level are as follows: Do the biochemical changes observed in heart samples reflect changes in myocardial cells only or do other cell types contribute significantly? Which type of cells are responsible for the increased DNA content? Does the increase in DNA mean mitosis or a changed ploidy or both? How does the initiation of increased RNA synthesis take place and what kind of alteration is associated with it at the subcellular level? The major obstable is that the nuclei of muscle and connective tissue cells cannot be separated for analytical studies. Therefore, experiments were carried out using various approaches to investigate normal and hypertrophied muscle tissue.

The incorporation of tritiated thymidine was observed by autoradiography (Norman and Foster (1963). No incorporation was detected into DNA of muscle cell, but only into DNA of connective tissue cells of hypertrophied muscle. In other studies, the number of muscle and connective tissue cells was counted per unit of sectional area

under electronmicroscope. The relative proportion of the two cell types remains normal in hypertrophied conditions (Meerson et al. 1968). From these and other observations (Meessen, 1968, Grove et al. 1969) it can be concluded that both muscle and connective tissue cells participate in the cardiac growth during myocardial hypertrophy but in different ways. Since the connective tissue cells possess a high proliferative activity, they increase in number, and hence, the increased amount of DNA in the hypertrophied muscle tissue is due to the increased number of connective tissue nuclei. Evidence showing increased ploidy of muscle cells is in conclusive, but the majority of experiments indicates only a slight increase which does not account for the elevated DNA content.

In contrast, the heart muscle cell increases in size, and thus the distance between the nuclei becomes larger (Meessen, 1968). Experimental evidence for regeneration of adult cardiac muscle cells has never been obtained, i.e. no mitotic activity could be detected. Consequently, in the process of cardiac hypertrophy, hypertrophy in the true sense of the word occurs in the muscle cell. The enlargement of muscle cells with no change in number is associated with an increase in the number of connective tissue cells with no change in size. Meerson et al. (1968) estimate the alteration in the ratio of number of muscle cell nuclei to number of connective tissue nuclei as from 1;1,5 normal value to 1;3,1 180 days following aortic constriction.

One of the important factors in this discussion is the age of the animal. During the developmental heart growth in the embryonic stage, cellular division occurs in the muscle cells. This period is believed to end around the time of birth. Autoradiography has provided evidence pointing out the occurence of mitotic activity in normal hearts. MacDonald and Mallory (1959) using a single injection of H^3 - thymidine, reported approximately two to three percent of labelled myocardial nuclei in rats less than 40-60 days old. This age corresponds to 120-220 gram bodyweight on Fig. 1. Above this age, a labelled myocardial nucleus was only occasionally found and the rate of radioactive thymidine incorporation is significantly reduced. This finding suggests that any significant cellular hyperplasia that takes place in experimentally induced cardiac hypertrophy occurs in interstitial cells in rats older than sixty days (Fanburg, 1970).

3. RNA synthesis

The nucleus, forming DNA, RNA, histone and non histone proteins (including the ribosomal protein), thereby produces the cytoplasmic subcellular structures for protein synthesis and determines the type of protein. Since proteins are important structurally as well as functionally during the process of adaptation to such stress as aortic constriction, the biosynthesis of RNA which directs protein synthesis plays a very distinguished role.

The RNA in the nucleus formed by DNA-dependent RNA polymerase,

transfers the genetic information to the cytoplasm. RNA molecules are utilized during the protein synthesis in at least three forms messenger RNA, transfer RNA and ribosomal RNA (Sibatani, 1966). However, the biosynthesis of RNA and protein represents one of the most complicated control mechanisms in the cell and the precise feedback mechanism from the cytoplasmic organelles to the nucleus is still rather obscure.

4. RNA-polymerase

The enzyme reaction occurs in three steps (Georgiev, 1967) 1) DNA-enzyme attachment 2) transcription (the enzyme moves along the DNA chain synthesizing the growing RNA molecule, thus, forming DNA-enzyme-RNA complex) and 3) liberation (RNA becomes separated, and the enzyme becomes detached from the DNA chain). The newly formed (45S) RNA is subjected to ribonuclease action, the products of which are the 28S and 18S RNA molecules. They are transferred, after being combined to ribosomal protein, to the cytoplasm.

Most of the RNA synthesis occurs in the nucleolus, using the nucleolar chromatin for template, but there is extranucleolar RNA synthesis as well which, as will be discussed later, is normally repressed and is important in the early phase of hypertrophy.

No extranuclear RNA synthesis has so far been found. In the course of investigating the RNA polymerase action one has to face the problem of obtaining pure mammalian enzyme preparation, because of its peculiar association with the other nuclear components, most of which serve as part of the control mechanism for the action of RNA polymerase

(chromatin, histone and other proteins, cations etc.). Another characteristic of the polymerase which causes problems in experimental work is that the activity decreases very rapidly in preparation, although, glycerol (40%) proved to be an efficient stabilizing agent. The mechanism by which it exerts the protective action is obscure (Liao et al., 1968). These difficulties of enzyme preparation were avoided in this study by using isolated heart muscle as the source of enzyme. Consequently, the purity and yield of the latter preparation became very critical.

Another important issue which has to be taken into account in measuring the polymerase activity is the anabolic nature of both polymerase and ribonuclease. Although one kind of ribonuclease degrades RNA during digestion, there is speculation about the possible existence of more than one ribonuclease, the action of which is to produce the "active" RNA found in the cytoplasm from the originally synthesizedRNA having a long chain length. Therefore, the measured polymerase activity is an apparent activity indicating a difference of synthesis and breakdown. This polymerase - ribonuclease interrelationship is closely related to the question, "How many polymerases exist?", which will be discussed later.

IV. Possible Initial Biochemical Step Responding to an Applied Stimulus

In all growth processes the basic control mechanism must be related to DNA. Presumably, a gene of genes in the cells respond to some clue from the environment with a controlled synthesis of

RNA and protein and so adaptation results to that environment, as in this study, to match the increased demand for work by the heart (Norman, 1962). The nature of this initial stimulus is not known. Meerson et al (1963) postulated that the increase in DNA-dependent RNA synthesis is the initial and essential step in the process of activation of the genetic apparatus of the cell under conditions of what he calls increased intensity of functioning of its structures; it is an essential part of the mechanism responsible for growth of differentiated cells i.e. in the process of hypertrophy of organs.

However, evidence that the increased RNA synthesis is preceded by a particular protein synthesis is accumulating. For example, hormone induced stimulation of RNA synthesis required protein synthesis (Hamilton, et al. 1968, Steiner and King, 1966). Inhibition of protein synthesis during the first hours after aortic constriction was found to inhibit increased RNA labelling in the hypertrophying rat heart, but not in control hearts (Posner and Fanburg 1968, Fanburg and Posner, 1968), suggesting than an early stimulation of protein is a prerequisite for increased RNA synthesis. Kako and Minelli's (1968) findings also support the hypothesis that RNA synthesis may not necessarily be required for the regulation of amino acid incorporation in the heart at an early stage in the changing work level, although the precursor activity was not measured in their study.

What is the function of this new protein synthesized and where does it come from? McCallister and Brown (1969) found that the mitochondria of the hypertrophic rat heart differ morphologically from those of the normal and the differences may be related to the role of the mitochondria in the active protein synthetic process in this special condition of myocardial growth. The morphological similarity with fetal mitochondria led to the assumption that the fetal protein synthetic ability of heart mitochondria is regained in the condition of induced hypertrophy. This is supported by the finding that hypertrophying muscle cell is highly susceptible to actinomycin and puromycin which is characteristic of embryonic muscle (Fanburg, 1970).

Zak et al. (1967) stressed the role of "Myofibrillar" ribosomes in the protein synthesis during the early phase of the development of hypertrophy. This might as well be the locus of production of a rate-controlling protein factor.

The "new" protein may be a new sigma factor for RNA polymerase (Travers and Burgess, 1969), a derepressor of DNA template or polymerase, an "attachement factor" for the ribosomes (stronger association of the particles to membranous structures observed by Tata and Widnell (1966) under thyroid hormone treatment) or a "stimulating factor" for RNA synthesis. (Thompson and McCarthy 1968).

Cross-circulation experiments between regenerating and normal rat liver (Buccher, 1967) gave evidence for the existence of a "humoral factor". Similarly, hypertrophy producing material,

transferred from an estrogen stimulated uterus to a normal one by supernatant fraction of the stimulated uterus caused increased RNA synthesis in normal tissue (Villee, 1970). On the other hand the existence of such a humoral factor was not confirmed by an in vitro experiment of heart microsomes (Moroz, 1967) nor is the composition of this "humoral factor" known. A hypothesis unifying these observations regarding the initial events of hypertrophy may be as follows: some factor (s), probably protein, and probably originated from nucleoli or mitochondria de-represses DNA template for extra-nucleolar RNA synthesis, the product of which is predominantly m RNA. Nucleolar RNA synthesis continues, since this is not repressed. At the same time, the same (or different) factor (s) de-inhibit RNA polymerase, which produces a greater amount of RNA.

V. Histological Studies

Meessen (1968) investigated the ultrastructure of the myocardium in cardiac hypertrophy with an electronmicroscope. He found that the new myofilaments do not structurally differ from the pre-existing ones; the most important morphologic change in the longitudinal sarcoplasmic reticulum consists in its unfolding and distension and the enlargement of its surface may occur within a few minutes; a diminution in the ratio of mitochondria to myofibrils was observed in the hypertrophied heart muscle cell; the distance between the nuclei became longer.

According to Meerson et al. (1963) the nuclei in the hypertrophied muscle tissue were enlarged but the intensity of their

staining with Feulgen reaction was either unchanged or weakened. The number of nucleoli per nucleus of the muscle cell was increased (Meerson et al. 1968) from 1,75 to 3,68 in the first stage following aortic constriction.

Fanburg (1970) has summarized the changes in subcellular structure in cardiac hypertrophy; he emphasized the increase in number of myofibrils per muscle cell and the enlargement observed in the mitochondria..

VI. The Aim of this Study

The aim of this project was to obtain a nuclear preparation from heart muscle suitable for enzymatic study and to follow the changes in RNA polymerase activity during the early phase of induced hypertrophy produced by aortic constriction. It was hoped that this may provide some clues as to the sequence of events leading to net protein synthesis and heart hypertrophy.

Until the substrates, template and co-factor requirements are well defined and the activity of ribonuclease accounted for, no "true polymerase" activity can be measured. At present only an "apparent polymerase" activity can be estimated. With this limitation in mind it was still felt useful to relate this to changes in ventricular RNA and muscle mass which occur in response to the stimulus of aortic constriction.

Nuclear DNA, RNA and protein concentrations were also determined.

METHODS

Outline of Procedure

Nuclei of control and hypertrophied cardiac muscle tissue were isolated in sucrose solution. DNA, RNA, protein concentration and content of the preparation were determined. The activity of nuclear RNA polymerase was assayed. Nuclear preparation was used as a source of the enzyme because the isolation of the pure enzyme from mammalian tissues has so far not been successful.

1. METHOD FOR ISOLATION OF HEART MUSCLE NUCLEI

Nair and al. (1967) have prepared a highly purified nuclear preparation from mammalian heart thus providing a system for the assay of DNA-dependent RNA polymerase activity. This method is a modification of the technique for the isolation of liver nuclei used by Tata and Widnell.

The present study utilizes Nair's method with minor modifications. The method is based on the isolation of nuclei in sucrose solution. Preparation of nuclei in this way gives the least possible damage to the proteins and nucleic acids. Moreover, the yield and purity of such nuclei are high. In particular, for enzymatic studies, the use of sucrose in preparation of nuclei is desirable, because this method best preserves the native state of nuclei, including enzyme proteins.

The rats of 200-220 g bodyweight were purchased (Robidoux Co., Montreal). At the time of experiments, the bodyweight was 241 ± 21 g

(SD). SE for the bodyweights calculated according to the groups operated at different times was 6,9. Animals were purchased in groups in order to keep the bodyweight similar at the time of operation. The standard error of the mean of bodyweight was so small that there was no significant difference in the results for the control groups. The 504 rats were divided into normal, aortic constricted and sham operated groups. They were fed on Master Fox cubes and water ad libitum.

The animals were killed by decapitation. The whole hearts were excised, cleared of pericardium and attached fat and rinced with ice cold homogenizing medium (0,32 M sucrose; 0,03 M $MgCl_2$; 0,05 M tris HCl pH 7,6). The left ventricles were separated, and the pooled left ventricles of twelve rats were weighed. All subsequent procedures were performed at 0-4°C.

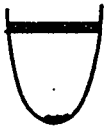
The tissue was minced and passed through a stainless steel press using a sieve of pore diameter 0,5 mm (Harward Apparatus Co.). Then the tissue was homogenized in 1:3 volume (weight/volume) of the homogenizing medium using the Potter-Elvehjem (1936) type of loose-fitting homogenizer. During homogenization a sucrose concentration of 0,32 M was used in order to avoid heating by high viscosity sucrose. The pestle was rotated by an electric drill at about 600-1,000 rpm. The technique of homogenization was standardized in such a way that three slow strokes with a glass reinforced Teflon pestle followed by three slow strokes with a simple Teflon pestle were carried out in each experiment.

The method of disrupting the cells is of particular importance

when sucrose solutions are used. The reason for this is that the nuclei are longer and more fragile in this medium and therefore, the tendency for destruction of nuclei is greater.

Then the homogenate was diluted without mixing to give a final concentration of tissue in the homogenizing medium of 1:10 (w/v) and this was followed by centrifugation for 10 minutes at 700 x g (International refrigerated centrifuge Model PR-2). The supernatant was discarded and the reddish crude nuclear pellet was resuspended in 2,4 M sucrose containing 1 mM $MgCl_2$ and tris HCl, pH 7,6 with the aid of a Teflon pestle gently rotated by hand. The volume of the heavy density sucrose was six times the tissue weight (w/v). The suspension was centrifuged for one hour at 24000 rpm in SW 25,2 rotor (Model L-2 65 Beckman ultracentrifuge).

The final nuclear pellet appears white (see electronmicrograph). This was resuspended in 1-2 ml of 0,25 M sucrose containing tris HCl pH 7,6. At the top of the ultracentrifuge tube there was a thick solid dark red layer containing myofibrils, broken mitochondria and cell debris.



2. CRITERIA FOR THE EXAMINATION OF NUCLEI

There are a number of criteria which must be satisfied before a preparation of nuclei can be regarded as adequate. The criteria serve as a basis for comparison among different preparations and this makes it possible to follow the adequacy of preparation by examining the material obtained at different stages of isolation.

The first criterion is the native, undamaged state of the preparation. This is ascertained from the morphological appearance of the preparation and by measuring the activity of exclusively nuclear enzymes.

In this study, the homogenate, the first crude nuclear pellet, the final nuclear pellet and the top layer after ultracentrifugation were examined by a phase-contrast microscope. The contamination of nuclei gradually decreases at each stage and finally the nuclei become nearly free of contamination. The top layer contained myofribril, cell debris, but nuclei could not be detected. The morphological appearance did not change even after centrifugation in heavy density sucrose.

Electronmicrographs were prepared from the pure nuclear pellet. They were prepared as follows: the pellet was fixed in gluteraldehyde and postfixed in osmiumtetroxide. It was dehydrated in graded concentrations of alcohol and imbedded in Araldite. The blocks were sectioned in Reichert ultramichrotome and the specimen was examined through an electronmicroscope(Phillips 100-B)*. There was very little cytoplasmic

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* The assistance by S. Kulczycky is gratefully acknowledged.

contamination (broken mitochondria) and the shape of nuclei was ellipsoid. With high magnification, the double envelope configuration of the nuclear membrane can be seen as in intact nuclei. Morphological appearance is a property defined with difficulty. The shape of nuclei varies according to the kind of tissue, e.g. the heart muscle nuclei appear ellipsoid. Malformation, small tops attached to the nuclei or the appearance of holes in the nuclear membrane are evidences of damage. One should also assume that the preparation is damaged whenever nuclei are observed to aggregate in clumps.

A second criterion is the purity of the preparation. This could be tested by microscopic examination as has just been described. In addition, absence of mitochondria in the preparation could be proven by the absence of succinoxidase activity which is associated exclusively with the mitochondria, but this was not tested.

The last and most precisely defined criterion for the examination of nuclei is comparison of the DNA content* of the original homogenate with that of the isolated nuclei, because the DNA is entirely located in the cell nucleus. Yields of nuclei vary depending upon the method of isolation. In the present study the recovery of total DNA is about 39%. On the following table it can be seen that the absolute amount of DNA decreased in the nuclear preparation but the DNA/RNA increased 7,3 times compared to the homogenate. This ratio DNA/RNA can be taken as a measure of the purity of the preparation.

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* DNA determination and other chemical methods are described in the following chapter.

YIELD OF NUCLEI IN THE NUCLEAR PREPARATION

	DNA µg	DNA/RNA
Homogenate	165	0,57
Nuclear pellet	42	4,2

The present study requires not only material of high purity but a certain amount of material as well. In order to obtain a sufficient amount of pure nuclei, the purity of the preparation had to be compromised to a certain degree. For this purpose, the effect of various modifications of the method of isolation on the purity of the nuclear preparation as indicated by DNA/protein and DNA/RNA, was investigated.

3. DETERMINATION OF DNA, RNA AND PROTEIN

In each experiment DNA, RNA and protein contents were determined as follows. Extraction of nucleic acids was carried out by a method originally described by Tanhauser (1945) and modified by Munro (1966). The nuclear suspension (0,6 - 1 ml) was precipitated with 8 ml of 10% TCA, centrifuged (320 X g for 20 minutes) (Lourdes centrifuge Model LCA-1) and washed twice with 10% TCA. The supernatant was decanted and the pellet mixed with 4 ml 0,3 N KOH. The latter was incubated in a water bath for one hour at 37°C. After 0,1 ml of an aliquot was taken out for protein assay, the remaining digest was treated with 1,4 N PCA.

Solubilized ribonucleotides were separated from DNA and protein by centrifugation with 5% PCA. The precipitate was further reextracted with 5% PCA. The two extracts were combined.

DNA was extracted from the residual pellet. First the pellet was incubated with 5% TCA for 30 minutes at 90°C (Dri-Thermolyne bath), followed by centrifugation and washing twice with 5% TCA. The three supernatants (3 ml) were combined and used for a DNA assay.

The determination of nucleic acids can be carried out by the methods based on the determination of one of their three main components, namely phosphoric acid, the sugar or the nitrogenous bases. Widely used and well-tested are methods using color reactions of the sugars of nucleic acids.

RNA was determined with orcinol (Dische, 1955). The color reaction is catalyzed by $FeCl_3$ in a high concentration of HCl. The sample is boiled in a double volume of the reagent for 30 minutes and the optical density (OD) is read at 570 and 675 mμ wavelength against 0,33 N PCA containing blank. Although the glucose gives a reading at both wavelength, the OD difference for glucose between them is zero. Therefore, the use of OD difference of dichromatic readings gives the nucleic acid content free of interference and the results proportional to the concentration of RNA (Dische, 1955). RNA standard was prepared from Yeast (type XI, Sigma Chemical Company). 1 mg/ml solution with 0,3 N KOH was prepared as the stock solution. The latter was diluted ten times with 0,33 N PCA (i.e. 100 μg/ml). The standard

was run each time together with samples.

DNA was determined according to the method of Burton (1968). The diphenylamine reaction of deoxypentoses produces blue color. The reagent contains diphenylamine in glacial acetic acid with H_2SO_4 and acetaldehyde. The mixture was incubated for 17 hours in a waterbath at 30°C. By dichromatic readings, interference by substances which produce colored products with the diphenylamine containing reagent can be eliminated, since the difference of OD measured at 600 and 650 mμ is zero in the case of substances other than 2-deoxyribose. The difference in OD was found to be proportional to the concentration of DNA (Dische, 1955). DNA standards were run each time, together with a blank containing 5% TCA. DNA standard was prepared from highly polymerized DNA from calf Thymus (Type V, sodium salt, Sigma Chemical Co.). After disolving the DNA in 0,3 N KOH, 10% TCA was added; the mixture was incubated for 30 minutes at 90°C, then cooled. 5% TCA was added to obtain a final concentration of 100 μg/ml.

Protein was determined by Lowry et al's (1951) method with the Folin-Phenol reagent. (phosphomolybdic-phosphotungstic). This method was chosen because of its great sensitivity, specificity and simplicity. Albumin was used as the standard and the OD was read against blank at 750 mμ (Beckman DN).

4. PURITY OF THE PREPARATION

The extent of purification is shown in the following table, which indicates that roughly 100 times purification was achieved by the isolation procedure of heart nuclei.

CHANGES IN THE DNA/PROTEIN IN THE COURSE OF ISOLATION PROCEDURE

Stages of Isolation	DNA/protein
homogenate	0,0025
first pellet	0,0108
first supernatant	no DNA
top layer	0,0024
nuclear pellet	0,2400

In this experiment, which was carried out in the initial stage of this work, dog hearts were used for convenience. In order to find an appropriate method of isolation, the purity of the preparation represented as DNA/RNA and DNA/protein, was determined at each stage of isolation. Later DNA, RNA and protein contents were determined only in the nuclear pellet.

The yield of the isolation i.e. the amount of the final nuclear pellet and consequently the purity of the preparation were greatly influenced by conditions of isolation, namely the amount of tissue, volume of homogenizing medium and high density sucrose, the concentration of the latter, the duration and speed of ultracentrifugation, the technique of homogenization and filtration etc. The relationships were examined and the results are shown in the following tables. The method finally adopted therefore used 6 times the volume of

homogenizing solution, the ratio of tissue weight/heavy density sucrose, 1:6 and the 2,4 M sucrose. When the concentration was increased above 2,4 M the purity did not improve significantly.

THE EFFECT OF DIFFERENT RATIOS OF TISSUE WEIGHT TO THE VOLUME OF HOMOGENIZING SOLUTION (w/v) ON THE PURITY OF THE NUCLEAR PREPARATION

Experiment	w/v	DNA/RNA	DNA/protein
1	1:3		0,1741
2	1:5	9,8	0,1009
3	1:7		0,2188
4	1:10	16,6	0,2582

THE EFFECT OF DIFFERENT RATIOS OF TISSUE WEIGHT AND THE VOLUME OF THE HEAVY DENSITY SUCROSE SOLUTION (w/v) ON RNA/DNA AND DNA/PROTEIN OF THE NUCLEAR SUSPENSION

Experiment	w/v	RNA/DNA	DNA/protein
1	1:3,8	0,185	0,1249
2	1:6	0,096	0,2000

CHANGES IN THE PURITY OF THE PREPARATION AS A CONSEQUENCE OF THE VARIATION IN THE CONCENTRATION OF HEAVY DENSITY SUCROSE

Experiment	Concentration of the heavy density sucrose	DNA/protein
1	2,35 M	0,0305
2	2,4 M	0,0384
3	2,45 M	0,0414

5. METHOD FOR DETERMINATION OF RNA POLYMERASE ACTIVITY

Assays were carried out following essentially the methods of Weiss (1960), Widnell et al.(1967) and Nair et al. (1967). The nuclear preparation described above served as the source of the enzyme and the DNA template.

The enzyme assay is based on the principle that the activity of the enzyme is most easily measured by determining the incorporation of a radioactive ribonucleotide triphosphate into RNA. In this study, incorporation of UTP labelled by tritium into RNA was measured. The enzyme activity was expressed in three ways as follows:

μμ moles of UTP^{H3} incorporated into RNA per a) mg of DNA

b) g left ventricle

c) left ventricle

For the calculations of b) and c), the UTP^{H3} incorporated into

RNA in μ mole of the whole nuclear suspension was divided by the weight of the pooled left ventricles in grams and by the number of left ventricles used for each experiment, respectively. This calculation involves the assumption that the yield of the individual nuclear preparations is constant.

Mg^{++} or Mn^{++} and $(NH_4)_2\ SO_4$ existing in an incubating medium influences greatly the behaviour of RNA polymerase (Nair, et al. 1967, also see Discussion). Therefore, two kinds of incubation mixture have also been used in the present work. System I contains Mg^{++} (Mg^{++} activated reaction) and System II contains Mn^{++} and $(NH_4)_2\ SO_4$ (Mn^{++} activated reaction) as shown in the following tables.

I.

Tris - HCl buffer	56		μ moles (pH8.1)
$Mg\ Cl_2$	2,5		"
GSH	10		"
PEP	5		"
PK	10	mg	
NaF	3		"
GTP	0,4		"
CTP	0,4		"
ATP	0,4		"
UTP^{H3}	0,024		"

The composition of the incubation medium for the Mg^{++}-activated polymerase (System I) assay, (final volume of 0,5 ml).

II.

Tris - HCl buffer (pH 7.5)	50	µ moles
$Mn\ Cl_2$	2	"
$(NH_4)_2\ SO_4$	300	"
(pH adjusted to 7.5 with NH_4OH)		
PEP	5	"
PK	10 mg	
CTP	0,4	"
GTP	0,4	"
ATP	0,4	"
UTP^{H3}	0,024	"

The composition of the incubation medium for the Mn^{++} activated reaction (System II). (final volume of 0,5 ml).

The specific activity of UTP^{H3} was 236 µc/ µmole diluted from the original value of 23600 µc/ µmole. 3 ml of absolute alcohol was added to the 2 ml of UTP^{H3} solution containing 0,0417 µmoles (24 µg) of $UTPH^3$. A 100 times dilution was obtained by the addition of 4,17 µ moles (2594 µg) non-labelled UTP (trinatrium salt, Boehringer, Mannheim GmbH). To 0,5 ml of incubation mixture 0,1 ml of nuclear

suspension corresponding to 30,4 ± 2,5 (SE) µg DNA (222 ± 18 µg protein) was added. The incubation was carried out in a Dubnoff metabolic shaking incubator at 37°C. Figure A shows that the duration of incubation in System I is not useful after 15 minutes. In most of the experiments the reaction was stopped after 15 minutes. After 30 minutes of incubation, increases in incorporation were not linear with the time. For this reason the incubations were carried out 5;15 and 30 minutes in System I, and for 5 and 10 minutes in System II. In System II the enzyme activity decreases after 20 minutes of incubation.

For a blank, which was run for each experiment, the reaction was stopped at zero time.

An attempt was made to increase the apparent activity of polymerase by lowering the temperature, assuming that it may suppress the ribonuclease activity. But as shown on the following table, this was not the case. The apparent activity of polymerase in the Mg^{++} activated system decreased to 71% at 30°C compared to the activity at 37°C.

EFFECT OF TEMPERATURE ON RNA POLYMERASE ACTIVITY IN THE Mg^{++} ACTIVATED SYSTEM

Sample	Incubation time in minutes	Temperature	dpm	μμ mole ATP C^{14} incorp. per DNA
1	5	30°	235	130
2	15	30°	396	200
3	5	37°	335	180
4	15	37°	516	280

Consequently, a temperature of 37°C was adopted in the rest of my experiments. Reactions were terminated by the addition of 5 ml of 0.5 N, $HClO_4$ at 0°. 1 mg Yeast RNA in aqueous solution at pH^7 was added as carrier. The precipitate was collected after standing 1 hr at 0° by centrifugation at 4000 rpm (2000 X g) for 10 min. (Model PR-2 International refrigerated centrifuge) and was washed twice with 5 ml of ice cold 0,2 N PCA. This was again centrifuged (Lourdes LCA-1, 10,3000 X g, 15 min) and washed twice with 4 ml ethanol-ether (3:1, v/v). The RNA was then extracted from the precipitate with 2 ml of 10% NaCl in 0,05 M Tris-HCl (pH 7.5) containing 0,5 mg of carrier Yeast RNA/ml at 100° (Temp-Blok Module tube-heater) for 30 min. The precipitate was reextracted for 15 min with 2 ml of the above solution and the pooled supernatants were cooled to 0°. The RNA was precipitated

with two volumes of cold absolute ethanol and kept overnight at -20°. The pellet was dissolved in 1 ml of distilled water and transferred with a Pasteur pipette into counting vials, containing 10 ml of Bray solution* (Bray, 1960).

Samples were counted in a three channel Nuclear Chicago Liquid Scintillation Counter Mark I with a computer, Model 6860.

The "counting sample" contains the radioactive material in a solvent containing scintillation solutes. The scintillation solution converts to light the energy of the primary particle emmitted by the radioactive sample and the phototube responds to the light energy by producing a current pulse which is then amplified and fed into a pulse height analyzer. The latter compares the signal with reference voltages. This is possible since scintillation counting is a proportional counting method, i.e. the magnitude of the output signal from the detector is proportional to the energy given up to the detector by primary particles. A discriminator window selects signals which fall between two pre-selected voltages i.e. two energy levels.

Disintegration of a radioactive material does not occur at evenly spaced time intervals but have a random distribution in time, and the number of disintegrations in any particular minute may vary from the number in any other minute. The "true" counting rate can

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* Bray: 1.8 gr Omnifluor, 60 g naphtalene, 100 ml methanol, 20 ml ethylenglycol, dioxan up to 1 l.

only be obtained by counting for longer intervals i.e. calculating the average value of the series or one-minute counts.

The standard error of the counting rate, R, is calculated by dividing the square root of the total number of counts by the counting time $SE=\frac{\sqrt{n}}{t}$. The SE of counts calculated in this study was ± 5,6; the mean of counts obtained: n = 56094, during 40 minutes of counting time. A minimum of 10000 counts is required, which gives 1% of relative SD. $(\frac{\sqrt{n}}{n})$ and "confidence" of 68% in the computed value.

Principle of channels ratio methods

The counting efficiency was determined from a quench correction curve (Fig. B) based on the channels ratio method. Anything which prevents the light reaching the photomultiplier tube is called a quencher. This decreases the counting efficiency. The principle of this method is that the distribution of pulse heights produced by radioactive disintegration over two pre-selected channels varies depending on the degree of quenching. The determination of counting efficiency can be best calculated from a linear (or quasi-linear) curve. This can be achieved by appropriate setting of the discriminator. The quench correction curve should cover the efficiencies to be found in variously quenched samples. A set of quenched standards was used for this purpose. From the known dpm for each standard in the quenched series and from the observed counts per minute (cpm), the percent efficiency is calculated. This is plotted against the channels ratio for each standard to give a quench correction curve (see Fig. B).

6. CHECKING OF THE EXTRACTION METHOD

Each step of the washing and extraction of labelled RNA was checked as follows: a known amount of Yeast RNA was passed through the whole extraction procedure to find out the effectiveness of RNA extraction. Standing 1 hour at 0° was found essential for a good recovery.

After the precipitation with ether-ethanol, the centrifugation did not separate perfectly the pellet from the supernatant unless a very strong centrifugation (10300 X g) was applied.

The ability of NaCl to extract RNA was checked by adding increasing amounts of RNA to the same volume of NaCl. It was found that the added RNA was extracted proportionally (next table).

INCREASING AMOUNT OF RNA EXTRACTED BY A STABLE VOLUME OF 10% NaCl

Amount of RNA added	OD diff. of extracted RNA
0,5 mg	1179
1 mg	2226
1,5 mg	3300
2 mg	4146

For complete recovery study, 2 mg/ml of aqueous RNA solution was prepared (RNA grade VI from Torula Yeast, Sigma Chemical Corporation). Three samples were precipitated with 0,5N PCA, and washed twice with

0,2 N PCA and with an ethanol-ether mixture. RNA was determined according to Dische (1955) from a sample at this stage (sample 1) and after NaCl extraction followed by precipitation with ethanol (sample 2). Sample 3 differs from sample 2, only in the precipitation procedure by ethanol. An attempt was made to complete the precipitation by lowering the pH previously by 1N acetic acid. As is shown on the table, the recovery was satisfactory. Adjustment of pH at the NaCl extraction stage was not required for quantitative precipitation.

RECOVERY OF RNA DURING THE COURSE OF RNA POLYMERASE DETERMINATION

Sample	mg of RNA
1	0,9
2	1,04
3	1,02

The final precipitation with ethanol requires ethanol at -20^0 and the precipitation is complete after standing overnight. The separation of the pellet requires centrifugation at 4500 rpm (PR-2 International refrigeration centrifuge) for 15 min.

The possible loss in radioactivity in the course of enzyme assay was estimated as follows. RNA content of the supernatants was determined after the washing steps. The supernatant before extraction with NaCl contained no measurable RNA, nor did the final supernatant

after ethanol precipitation.

The radioactivity of supernatants was also checked and gave the same conclusion: the radioactivity gradually disappears before the extraction during the washing processes and none is left in the final supernatant.

The sample with 0 time of incubation served as a control in each experiment, and this also shows the background radioactivity.

The final pellet was dissolved by distilled water, since NCS* + POPOP solution with the sample formed was found to be very cloudy, thereby, resulting in a high quenching. Even with the Bray solution, the mixture appeared cloudy. This would further lower the already low efficiency, resulting in a decreased accuracy. To verify this effect, the actual count per minute and the calculated incorporation per mg DNA were compared in the cloudy Bray solution and in the clear one. The clear solution was obtained by re-centrifugating the final pellet dissolved in water. The dissolved pellet was centrifuged for 10 minutes at 2500 rpm (PR-2 International refrigerated centrifuge), 1 ml from 1,5 ml of clear supernatant was counted together with the sample in which the dissolved pellet was transferred directly into Bray solution. The pellet after centrifugation also was counted. As is shown on the following table there was no need for re-centrifugation and the sample can be counted directly. The pellet was free of radioactivity suggesting that all the activity was obtained in the supernatant.

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* a liquid scintillation solubilizer (Nuclear - Chicago).

Mg act. system				
Sample	Incub. time	extra treatment	Cpm	µµ m UTP^{H2}/DNA
1	0 min			
2	15 min		786	243
3	15 min	recentr. super.	737	236
4	15 min	recentr. pellet		0,77
5	20 min			259

The effect of recentrifugation of pellet dissolved in water

7. VALIDITY OF POLYMERASE ASSAY METHOD

The first series of experiments was carried out in an attempt to demonstrate the validity of the method, i.e. to identify the final product as RNA and to investigate various conditions for incubation.

The following table shows the almost complete suppression of apparent enzyme activity by RNA-ase. In the Mg^{++} activated system the activity dropped to 3%, in the Mn^{++} activated system up to 5%.

EFFECT OF RNA-ase ADDITION IN THE INCORPORATION INTO THE Mg^{++} AND Mn^{++} ACTIVATED SYSTEM

Sample	Incub. med.	Incubation	$\mu\mu$ mole UTP^{H3}/DNA
1	Mg^{++} complete	15	2362
2	act. +RNA-ase	15	84
3	Mn^{++} complete	10	4788
4	act. +RNA-ase	10	235

Another evidence for true RNA synthesis is that the process is strongly dependent on each of 4 nucleotides triphosphate. It was shown that omission of any one nucleotide triphosphate causes a marked reduction of the incorporation of labelled nucleotide. The following table shows the effect of CTP omission on the incorporation in the Mg^{++} activated system during 15 min. of incubation.

EFFECT OF THE OMISSION OF CTP FROM SYSTEM I ON THE ENZYME ACTIVITY

Sample	Medium	μμ mole UTP^{H3}/DNA
1	complete	1693
2	CTP omitted	405

It seems that even without additional nucleotide there is low rate of RNA synthesis from the pre-existing nulceotide in the nuclear preparation. However, this is only 24% compared to the synthesis in the complete system. A similar conclusion was drawn from the experiment in which UTP was omitted.

The next very important requirement for the incorporation of labelled nucleotide into RNA is the presence of DNA. Since the source of DNA in this study is the nuclei themselves, the DNA dependency can be proved only by the addition of DNA-ase. The apparent activity was reduced to 30% during 10 min of incubation in Mn^{++} activated system in the presence of DNA-ase.

EFFECT OF DNA-ase ADDITION ON THE RNA POLYMERASE ACTIVITY

Sample	incub. medium	μμmole C^{14} UTP/mg DNA
1	complete	1110
2	complete + 25 ug DNA-ase	365

In contrast, an addition of DNA did not increase the enzyme activity. It was thought then that the nuclear membrane may not be permeable to DNA and the addition of deoxycholate may change the membrane permeability, but the presence of the deoxycholate did not alter this conclusion. This is shown on the next table.

EFFECT OF DNA ADDITION INTO SYSTEM I (Mg^{++} ACTIVATED) AND II (Mn^{++} + $(NH_4)_2$ SO_4 ACTIVATED) WITH AND WITHOUT DEOXYCHOLATE

Sample	Incub. time in min.	Incub. med.	μμ mole UTP^{H3}/DNA
1	5	System I complete	348
2	15	complete	753
3	15	complete + DNA	665
4	15	complete + DNA + deoxycholate	382
5	5	System II complete	1194
6	10	complete	1586
7	10	complete + DNA	1595
8	10	complete + DNA + deoxycholate	942

As is shown later "Results", differences in the DNA content of the control heart nuclei did not influence the RNA polymerase activity, providing further support to the above results.

The enzymatic incorporation of ribonucleotide into RNA requires the presence of divalent cations. At low concentrations both magnesium and manganese can activate the system, but at higher concentrations both become inhibitors (Weiss, 1960). The presence

of magnesium is essential in the Mg^{++} activated system and its omission reduces the enzyme activity to 3,2% as shown in the table below.

EFFECT OF THE OMISSION OF Mg^{++} ON Mg^{++} ACTIVATED SYSTEM

Sample	Incub. time	Medium	μμmole UTP^{H3}/DNA
1	15	complete I	1693
2	15	-Mg Cl_2	56

However, the addition of Mg^{++} to the Mn^{++} activated system, which should contain some Mg^{++} pre-existing in the nuclei, did not influence the enzyme activity, as is shown in the following table.

EFFECT OF THE ADDITION OF Mg^{++} ON THE Mn^{++} ACTIVATED SYSTEM

Sample	Incub. time	Medium	μμ mole UTP^{H3}/DNA
1	10	complete II	4788
2	10	complete + 0,5 mg $MgCl_2$	4596

8. RIBONUCLEASE DETERMINATION

To investigate the effect of different incubation mixtures and the incorporation of labelled nucleotide into RNA, an attempt was made to approach the question from the direction of ribonuclease assay.

The enzyme was determined according to a simple modification of Shortman's method. The buffer solution contained 0,25 M sucrose, 0,025 M KCl; 0,005 M $MgCl_2$ and 0,05 M Tris HCl (pH 7.6). The OD's of soluble nucleotides in the supernatant after acid-alcohol treatment should be proportional to ribonuclease activity. The activity is calculated in percentage compared to the standard value.

Ribonuclease activity was determined from the nuclear preparation at zero time and after 10 minutes of incubation. Sample I was incubated without medium, sample 2 in the presence of Mg^{++} and sample 3 in the presence of Mn^{++}. Standard enzyme was Ribonuclease-A from bovine pancreas, protease free, type X-A in 0,2 M Phosphate buffer, pH 6.4, with 100 Kunitz units/ mg protein activity (Sigma Chemical Corporation). The protein concentration of the standard was 10 mg protein/ ml.

To the buffer solution, substrate RNA, purified from Yeast RNA

Type XI (Sigma Chemical Corporation)* was added (the control run). The Standard contained in addition 0,1 ml of the Ribonuclease mentioned above, and the samples contained 0,1 ml of nuclear suspension.

Incubation proceeded at 37° for 30 min in a Dubnoff metabolic shaking incubator. The reaction was terminated by 76% ethanol containing IN HCl. After centrifugation at 2000 X g for 30 min (PR-2), the supernatant was diluted 10 times and the OD read on Beckman DU at 257 mμ .

9. AORTIC CONSTRICTION

This method is essentially that of M. Beznak (1955).

The rats were anaesthetized with ether, an incision made in the abdomen and the viscera were displaced. The constriction ring was then slipped under the aorta just below the diaphragm, and narrowed with forceps. The viscera were replaced and the incision sutured.

The rings were made by winding silver wire around a hypodermic needle of suitable diameter (0,8 mm). The spiral was then sawed longitudionally with a very thin blade to give a number of separate rings which are opened slightly and filed smooth under a magnifying glass.

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* RNA was dissolved in water, precipitated by 76% ethanol, containing IN HCl, centrifuged for 30 min at 2000 X g in PR-2 (International Refrigerated Centrifuged). The precipitate was dissolved in distilled water and pH adjusted to 7.

10. SHAM OPERATION

The sham operation was also carried out under ether anesthesia. Skin on the abdomen was incised, the viscera were displaced, the aorta just below the diaphragm was separated free. The viscera were then replaced and the skin wound sutured. The animals were sacrificed at various times after operation.

Figure A

The Activity of RNA Polymerase of Heart Muscle Nuclei in the Presence of Mg^{++} or Mn^{++} after Various Durations of Incubation

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The enzyme activity is expressed as incorporation of UTP-H^3 into RNA in μμmole/mg DNA (ordinate).
The composition of the incubation mixture is described on p. 27.
For each experiment twelve left ventricles were used.

● represents System I. (Mg^{++} activated)

◉ represents System II. (Mn^{++} activated)

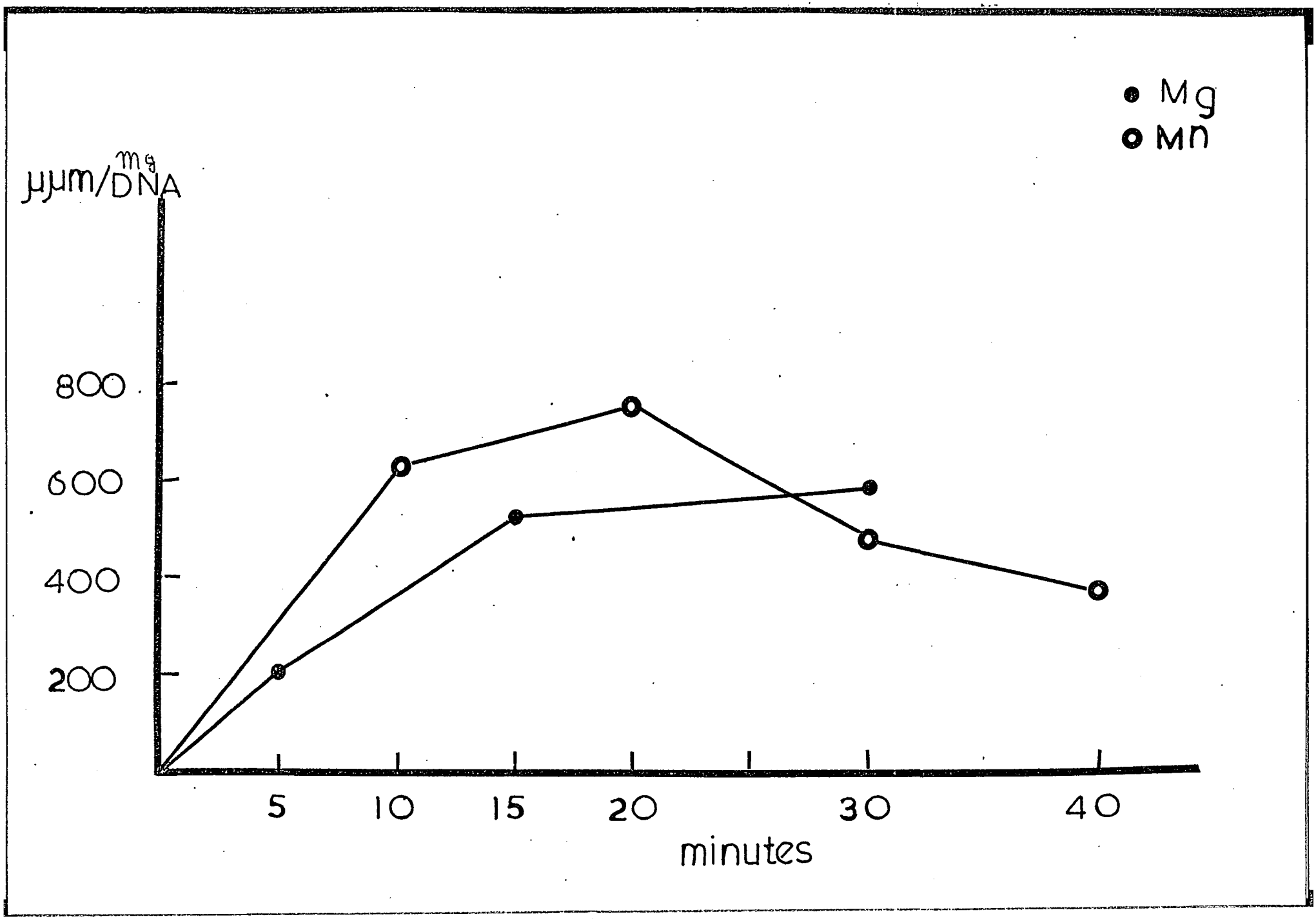
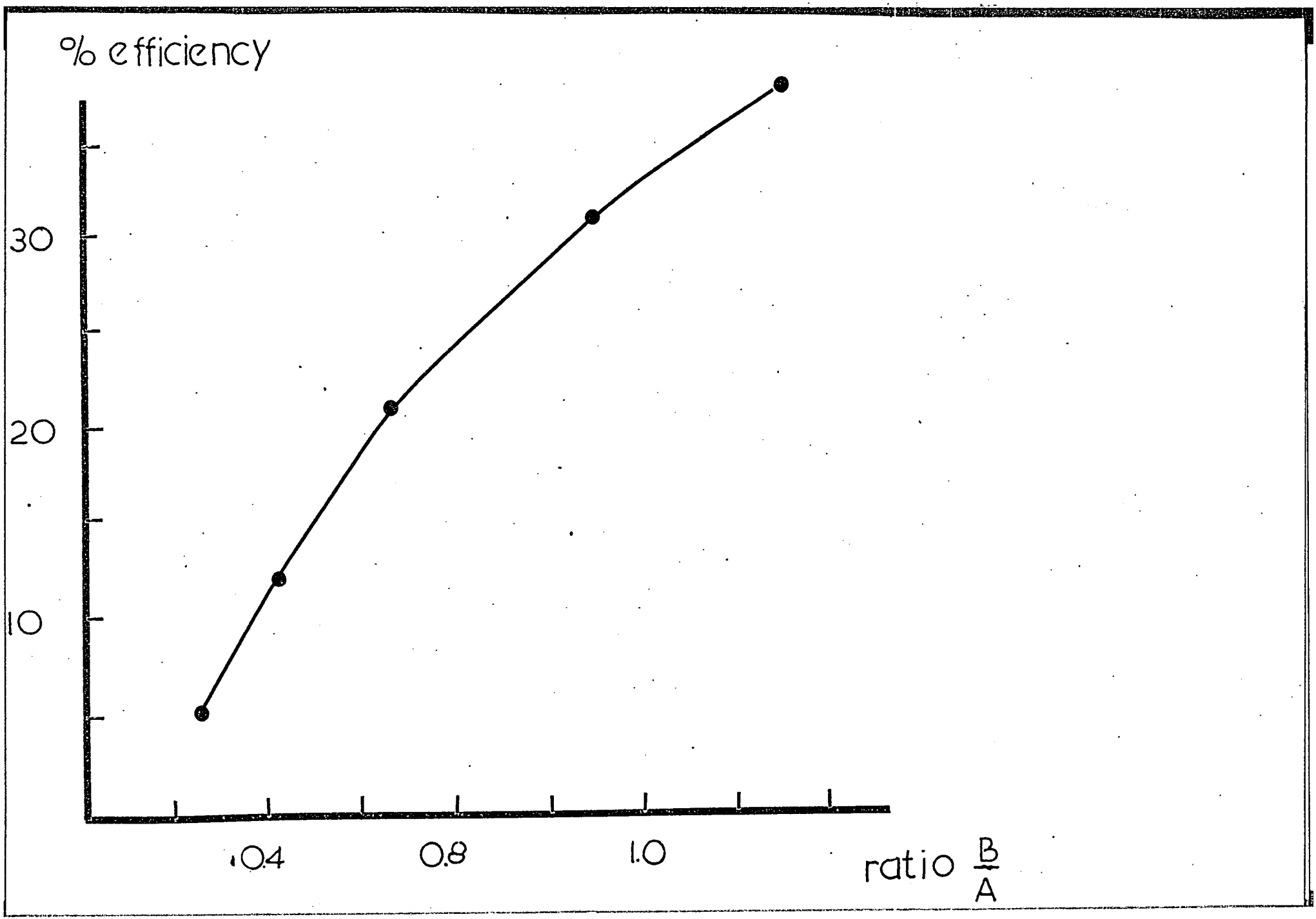

Mg
Mn
μμm/mg DNA
800
600
400
200
5
10
15
20
30
40
minutes

Figure B

Typical Tritium Channels Ratio Quench Correction Curve

The ordinate shows the percentage efficiency.

The abscissa indicates the ratio $\frac{\text{channel BCPM}}{\text{channel ACPM}}$


% efficiency
30
20
10
.0.4
0.8
1.0
ratio B/A

RESULTS

1. Control Values

At the time of the operation (described in "Methods") the body weight of the rats was 241 ± 21 g. The number of animals used was over 500. This range of weight corresponds to the period of most rapid growth, as is shown on the growth curve (Fig. 1).

The RNA polymerase activity of control heart muscle nuclei is quite reproducible in this range of bodyweight greatly differs from the above range. The values are comparable to those obtained by Nair et al.(1968). In their work the enzyme activity during 5 min incubation was 195 $\mu\mu$moles/mg DNA (in my preparation 192) in system I and 280 in system II. A higher activity was obtained in system II in my work (502). Since the rate of the reaction was not linear with time, in most of the cases 15 and 10 minutes values were used in the Mn++ and Mg++ activated systems, respectively (Fig. A).

Although experiments were carried out up to 14 days after the aortic constriction, most of them took place within 6 days after the operation.

The intervals between the time of the operation and the time at which measurements were taken, did not influence the measurements of RNA polymerase activity in control animals in spite of the fact that this was the period in which the normal growth is significant. This is indicated by the SE of the mean values obtained from normal animals in the weight range described. 268±22.3 and 912±149 $\mu\mu$ moles

UTP ^{H3}/mgDNA are the values of enzyme activity in system I and II, respectively, during 15 and 10 minutes of incubation. Thus it was not necessary to distinguish between the normal growth and the cardiac enlargement in response to hypertrophy as the factor affecting RNA polymerase activity.

However, the early changes from a few hours to a few days after the operation could partly be due to the non-specific stress of the operation rather than the specific effect caused by narrowing the aorta. In order to differentiate between these two responses, sham operated animals were examined (Fig. 2). Nuclear RNA polymerase activity of these animals increased. The increase was parallel in both the Mg^{++} and Mn^{++} activated systems, showing peak activity at the 12th hour following constriction. On the third day the enzyme activity of sham operated animals dropped back to the normal range. Therefore, in the experiments carried out between 4 hours and 2 days following constriction, the enzyme activity of corresponding sham operated animals was determined and subtracted from the enzyme activity of aortic constricted animals. From the third day after the operation, the mean value of normal animals was subtracted from values of operated animals.

The fact that RNA polymerase activity is DNA-dependent raises the following question. Does the absolute amount of DNA in the nuclear preparation obtained from the left ventricle influence the measured enzyme activity?

Though the purity of the nuclear preparation was high and reproducible in the control experiments using normal and sham operated animals judging from the DNA/protein ratio (0.1417 + 0.0098SE), the absolute amount of DNA content in the whole suspension varied from 280 to 620 µg in control experiments (Fig. 3). However, when the same amount of DNA was obtained from control and aortic constricted animals, each time the results of operated animals showed a much higher incorporation, except at 8 hours when the enzyme activity temporarily declines to the normal range.

A similar relationship was found between the RNA content of the whole suspension and the incorporation (Fig. 4). The incorporation into RNA in the control ventricles remained below the values obtained from aortic constricted animals. The total UTP^{H3} incorporated into the nuclear RNA varied between 120 and 350 µµmoles in the control preparation. These figures illustrate the results of the Mg-activated system. The same conclusion was obtained using the Mn-activated system. Thus, the apparent polymerase activity appears to be influenced by the absolute number of nuclei expressed as the increased amount of DNA and RNA. However, the increase in enzyme activity after aortic constriction is not due to the increased number of nuclei obtained, i.e. the incorporation per DNA content was always above the control values.

The apparent polymerase activity in the Mn^{++} - $(NH_4)_2SO_4$ activated reaction measured after 5 minutes of incubation was 260% if the activity of the Mg^{++} activated system after 5 min was expressed as 100% (Table I). The value was approximately 500% after 10 min., 100% being an extrapolated value from the activity measured in the Mg^{++} activated system after 15 min.

One of the possible explanations of these differences between the two systems was the effect of ammonium salt on the ribonuclease activity.

The table below shows that, in the Mg^{++} activated system, the ribonuclease activity is 152% of that found in the Mn^{++} activated system. Though the ribonuclease activity is higher in the presence of Mg^{++} the results suggest the role of ribonuclease activity in polymerase assays in both systems.

RIBONUCLEASE ACTIVITY IN THE TWO SYSTEMS DURING 10 MIN OF INCUBATION IN 0,1 ML OF NORMAL NUCLEAR SUSPENSION

Sample	Incubation Medium	µg DNAin 0,1 ml susp.	RNA-ase act. Kunitz unit	RNA-ase act. µg DNA
1	without	13,4	2,8	209
2	System I	15,6	29	1859
3	System II	17,9	21,8	1218

The chemical analysis of control (normal and sham operated) animals is shown on Table II. For each enzyme assay 0,1 ml of nuclear suspension was used; that is the reason why the chemical values were calculated to 0,1 ml of suspension as well as to the total nuclear preparation. DNA, RNA and protein contents of the nuclear suspension were determined.

The absolute values obtained by chemical analyses can not be compared because the number of left ventricles used in one determination (10-12 lv.) and the pooled weight of the ventricles, even using the same number of ventricles, varied to a considerable extent. The results can be compared if expressed per left ventricle (l.v.) or per gram l.v. and as a ratio of DNA/protein or DNA/RNA. DNA/l.v. was 39 ± 2,7 µg (SE); DNA/g l.v. was 65,9 ± 3,68 µg; RNA/l.v. was 5,5 ± 0,28 µg; RNA/g l.v. was 9,5 ± 0,69 µg and protein/g l.v. was 496 ± 31 µg. The ratio DNA/protein was 0,1417 ± 0,0098 and DNA/RNA was 7,3 ± 0,73.

2. Hypertrophy

In this study the extent of hypertrophy over different time intervals after constriction was not measured on individual left ventricles; the total weight of the 12 pooled left ventricles was used to calculate the average weight of one left ventricle. On the second day after aortic banding only a slight increase in left ventricular weight was observed, on the 4th day a 27% increase was observed and on the 11th day a 55% increase.

It was indicated in the work of Beznak (1954) and noted by others, that the size of the rat at the time of constriction has a great influence on the extent of cardiac enlargement. The hypertrophy is greater in the smaller rats. It should be mentioned here that in a few experiments the measured polymerase activity showed a relatively small increase after the narrowing of the aorta.

These low activity results are not plotted on the graphs . The reason for this smaller increase may be due to the fact that a slight change in the mean bodyweight at the time of the operation could have caused a different degree of hypertrophy and consequently a different enzyme activity. However, this slight change in body-weight did not influence the normal results.

3. Changes in Protein Concentration During the Development of Hypertrophy.

The concentration is defined as μg protein found in the nuclear suspension related to 1 g wet weight of left ventricle. The changes in protein concentration after aortic constriction are shown on Figure 5. It was increased up to maximum 50% (750 μg/g left ventricle) on the second day and is still elevated by 15% after 10 days following aortic constriction. Obviously, the increased protein concentration in the nuclear suspension represents only a fraction of the increase in protein synthesis occurs in the whole cell during hypertrophy.

4. Changes in RNA Concentration and RNA Content After Aortic Constriction.

RNA concentration means in this work the RNA measured in the nuclear suspension in μg per 1 g wet weight of left ventricle. RNA content is defined as μg RNA found in the nuclear suspension calculated to one left ventricle.

An increase in both RNA concentration (Fig 6) and content (Fig 7) in the nuclear suspension prepared from left ventricles has been observed within 24 hours after aortic banding. Maximal increases were seen by the third day after aortic constriction. The concentration was 18 μg/g (90% increase) and the content was 11,5 μ g/left ventricle (+110%) that time. The RNA concentration and left ventricular RNA content remained elevated for at least 14 days.

5. Changes in DNA Metabolism

The definition of concentration and content is described previously. The changes in DNA concentration and content of the nuclear suspension are plotted on Figure 8. They both decrease a few hours after the operation and increase on the third day as compared to the control.

The DNA concentration returns to that obtained in control animals about the 10th day. At this time the DNA content is still 20% elevated.

It can be concluded from the changes in protein, RNA and DNA concentration, that the DNA/protein and DNA/RNA ratio should decrease following the operation. (Fig 9 and 10). Their values decrease within 12 hours after narrowing and DNA/protein remained 22% and DNA/RNA 35% below the control values.

The chemical determinations of control animals are shown Table II and the values of constricted animals are shown in Table III.

6. The Activity of Nuclear RNA Polymerase in Hypertrophying Heart Muscle Nuclei

The activity of RNA polymerase was determined on the 4th, 8th, 12th and 17th hours, and 2, 3, 4, 6, 10, 11, and 14 days following aortic constriction.

The mean values of two or three experiments are shown in Table IV. Duplicate assays were used in the Mg^{++} system after 15 min. and in the Mn^{++} system after 10 min. of incubation. For each experiment 12 left ventricles were pooled. Because of the importance of the early changes more groups of animals were studied in the early than the later period. Hence, standard error could not be calculated. It was assumed that by pooling the twelve left ventricles, a biological average could be obtained.

The results are expressed in three ways: the enzyme activity expressed as $\mu\mu$moles of labelled nucleotide incorporated into RNA a) per mg DNA, b) per left ventricle and c) per gram of left ventricle. To evaluate the specific changes in enzyme activity due to aortic constriction the results were expressed as changes over values obtained from control animals (Fig 2, Table I and V).

The values after 15 min. of incubation in system I (Mg^{++}) and after 10 minutes in system II (Mn^{++}) are plotted in Figure 11 and 12, respectively. The changes in enzyme activity show a similar time course when they are expressed in any of the three ways. Each point represents the mean values of 2 - 3 experiments, over control values. Until the 2nd day, sham operated animals were used as controls while normal.

animals served as controls after the 3rd day. The increase in RNA polymerase activity is shown onee more in Figure 13 (System I) and 14 (System II), in which the activity was expressed as $\mu\mu$ mole UTP^{H3} incorporated/mg DNA for simplicity. In system I the enzyme activity increased to 1187 from 550 (sham) as early as 4 hours after aortic constriction. After this a decrease was observed: at 8 hours the activity was 250 (the value is 320 from sham). The second peak activity was found on the fourth day: 1924 while the control was only 268. These absolute values (Table IV) are not shown on the graphs, which illustrate the changes above control values (Table V). Two weeks following the operation the activity is still 522 above the control.

In system II (Fig. 14) the increase in activity after 4 hours is 1486 above the control, i.e. the absolute value was 2363, the sham operated animal showing an activity of 877. The activity diminished at 8th hour to 847 (sham was 590). The highest activity was demonstrated on the third day; 2836 above the control value (912). This was followed by a great decrease, though 14 days after the constriction the activity was still 519 above the control value.

The values shown on Fig. 13 and 14 are summarized in Table V.

On comparing the above results the reactions in the two systems show similar changes as follows: after the early increase occurred at 4 hour, a decrease occurs. This is followed by an increase reaching the highest activity on 3-4th day. Even two weeks after the operation the values are above those obtained from control animals.

However, the absolute values obtained were higher in the Mn^{++} dependent reaction at each time investigated. The difference between them was 1176 at 4 hour, 2364 on the third day, after that it becomes smaller: on the 4th

day it becomes 827 and 10 days after the constriction, only 518. The highest value in system II was 1824 above the highest value obtained in system I.

To compare the effect of aortic constriction on the Mg^{++} and Mn^{++} activated system, the above data were plotted in percentage over control values in Figure 15. The percentage was calculated from the values expressed as $\mu\mu$ mole UTP^{H3}/mg DNA.

In both system a substantial increase was detected as early as 4 hours after constriction (3 experiments showed very similar changes). The increase was 109% in system I and 169% in system II. After a decline a gradual increase occurred, lasting until the third-fourth day. The peak activity was 618% in system I and only 310% in system II above controls. This was followed by a decline which did not return to control values even after two weeks. The RNA polymerase activity was still 195% elevated in the Mg^{++} activated system and 56% in the Mn^{++} activated system.

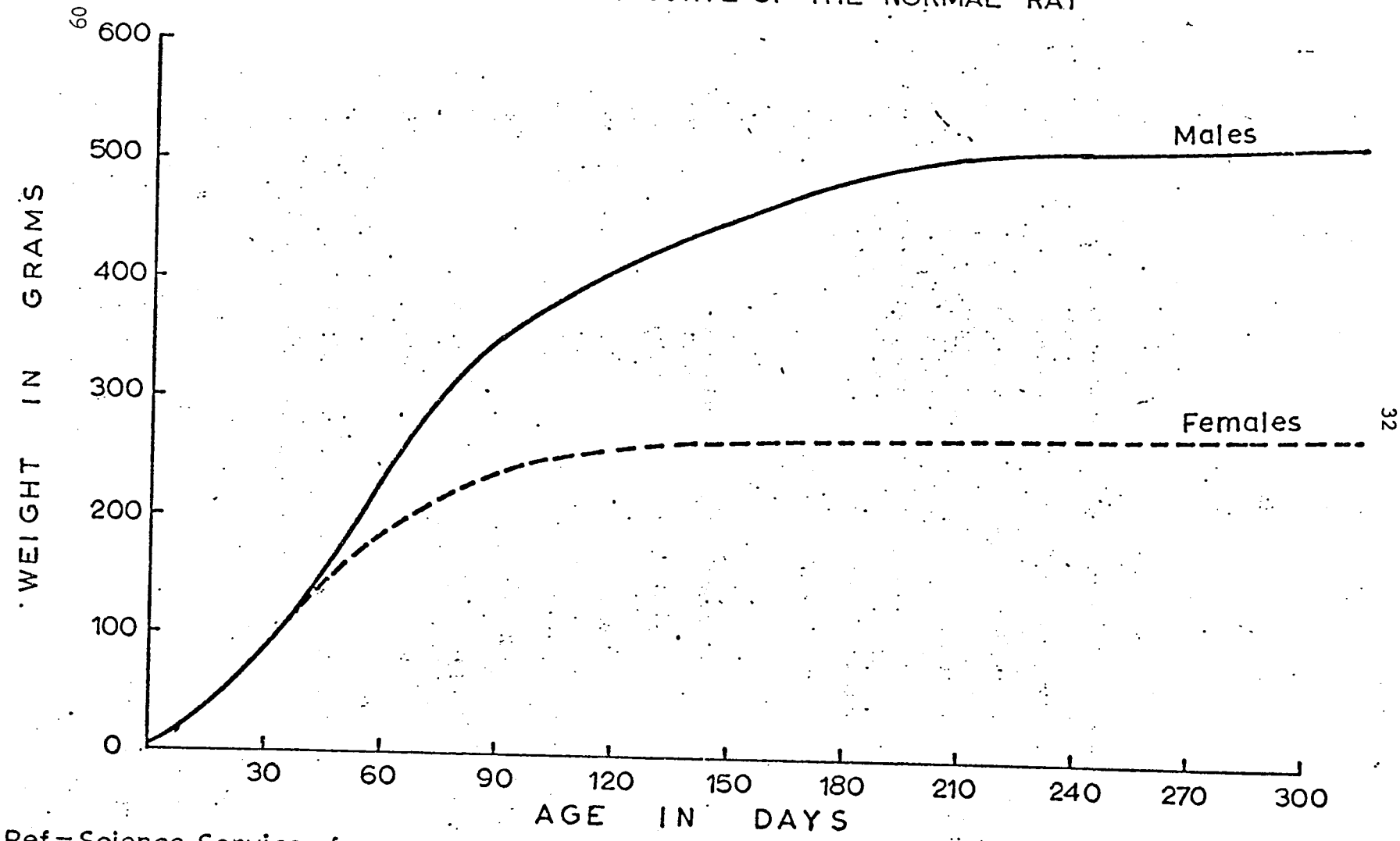
A comparison between the two systems reveals that the early, 4 hour increase is an exception in that the elevation in system II is 60% more than in system I. In the rest of the periods studied, the activity in the Mn^{++} activated system showed much smaller response to the constriction than the Mg^{++} activated system, though the time course appears to be similar.

An attempt was made to approach the problem dealing with the changes in incorporation of labelled nucleotide into RNA following aortic constriction by determining the ribonuclease activity in

addition to RNA polymerase assay. This attempt was not quite successful, as no activity was detected at the beginning of the incubation in the nuclear preparation. If the ribonuclease activity was determined after incubation in the presence of Mg^{++} or Mn^{++} (see "Methods"), interesting results may have been obtained.

FIGURE 1.

GROWTH CURVE OF THE NORMAL RAT



Ref – Science Service of
Canadian Breeding Laboratories

Figure 2.

Changes with Time in RNA Polymerase Activity after Sham Operation

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Enzyme activity is expressed as incorporation of UTP-H^3 into RNA in μμmole/mg DNA (ordinate). The duration of incubation in System I is 15 minutes; in System II 10 minutes. Abscissa shows the intervals between the sham operation and enzyme assay.

● represents system I.

◎ represents system II.

represents the mean and SEM of the control values.

Dots represent one experiment each consisting of 12 left ventricles.

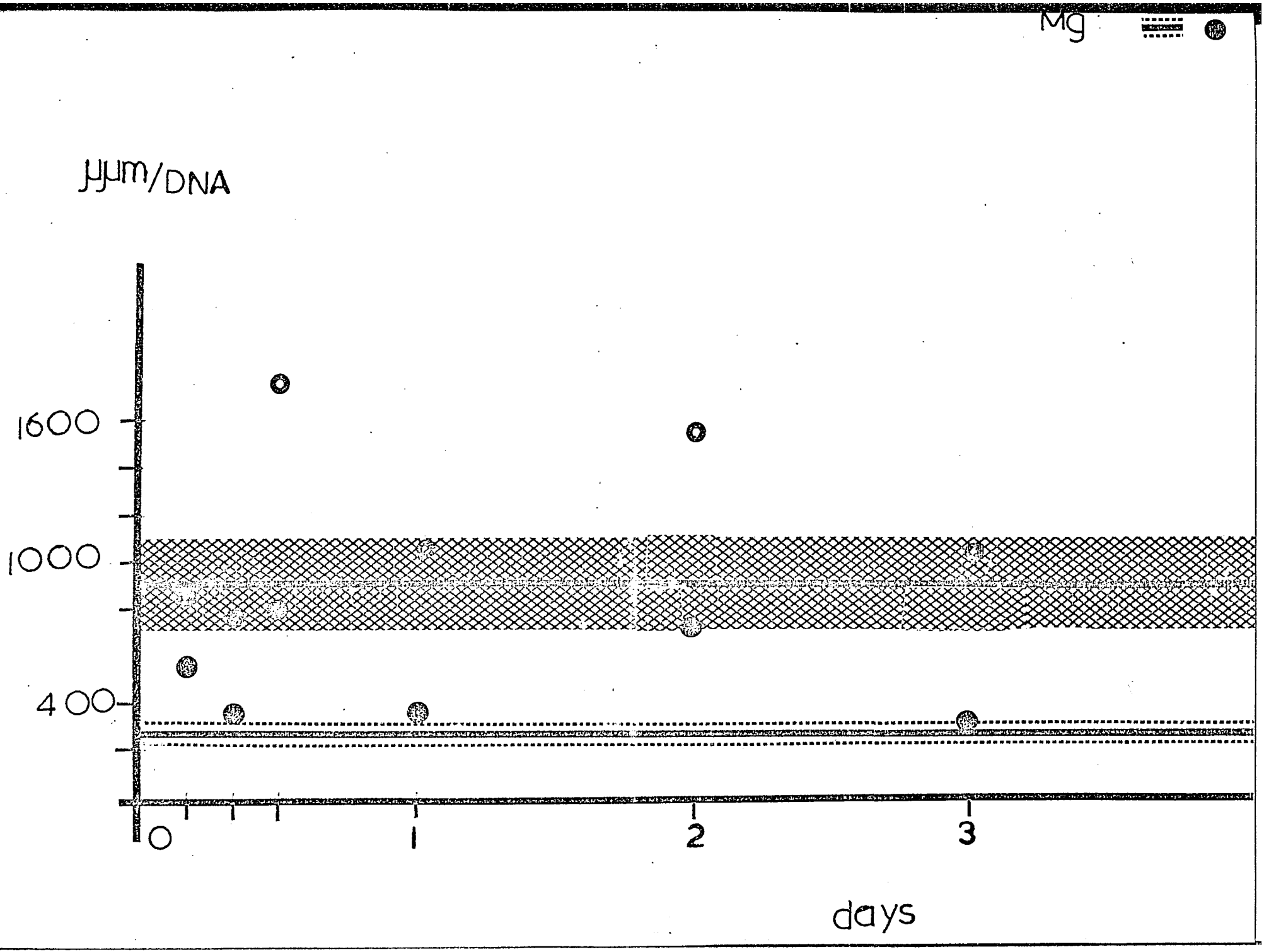

Mg
μμm/DNA
1600
1000
400
0
1
2
3
days

Figure 3.

Relationship between the DNA Content of Nuclear Preparation and the Incorporation of UTP-H^3 into RNA in the Mg^{++} Activated System after 15 Minutes of Incubation

---

The ordinate shows the amount of labelled nucleotide incorporated into RNA in μμmole; the abscissa shows the DNA content in μg. Both expressed as per nuclear suspension.


represents the control animals (sham and normal)


represents the narrowed animals

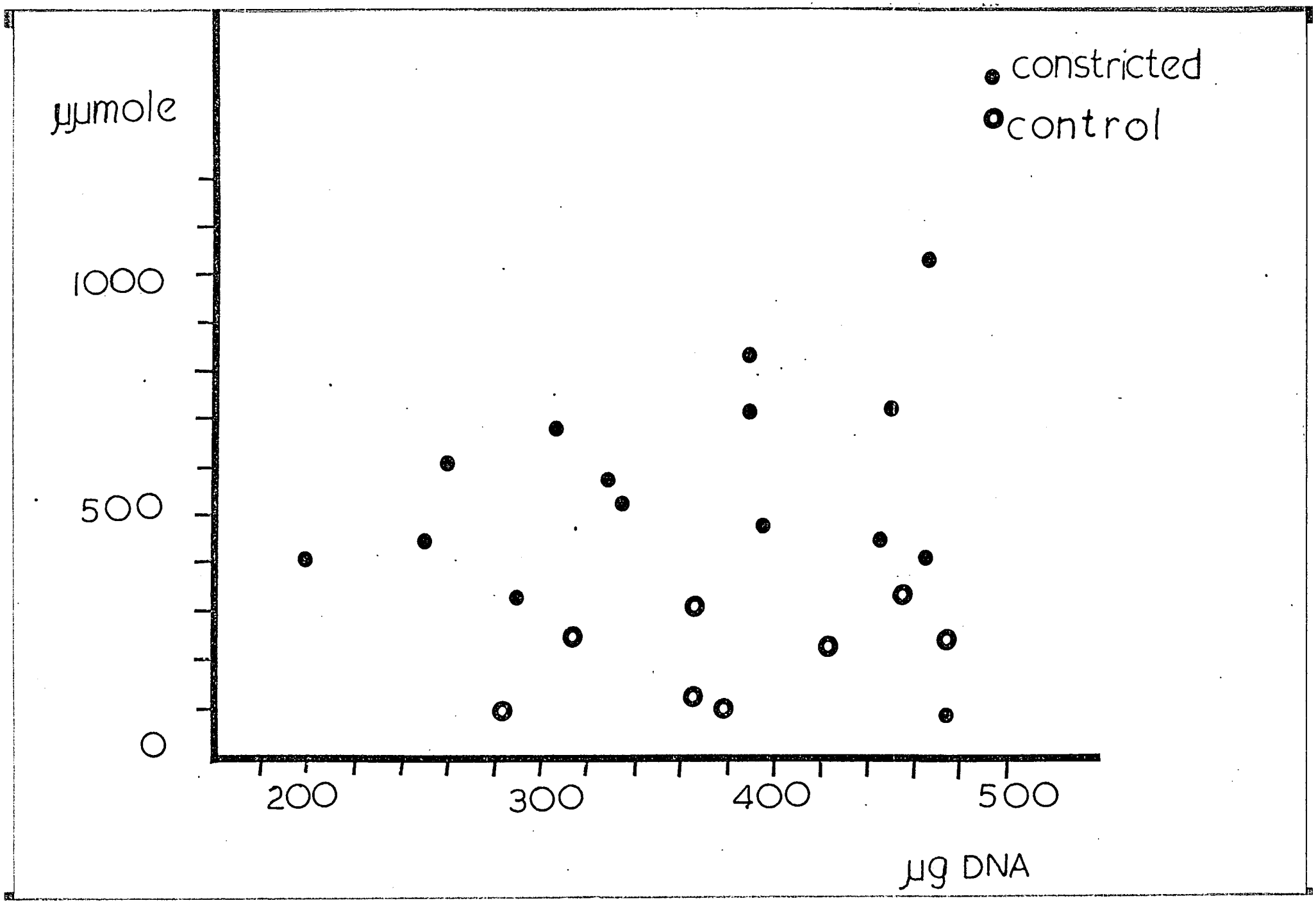

μμmole
1000
500
0
200
300
400
500
μg DNA
constricted
control

Figure 4.

Relationship between the RNA Content of Nuclear Preparation and the Incorporation of UTP-H^3 into RNA in the Mg^{++} Activated System during 15 Minutes of Incubation

---

The abscissa represents the total nuclear RNA content in μg/ nuclear suspension.
For further explanation see legend to Figure 3.

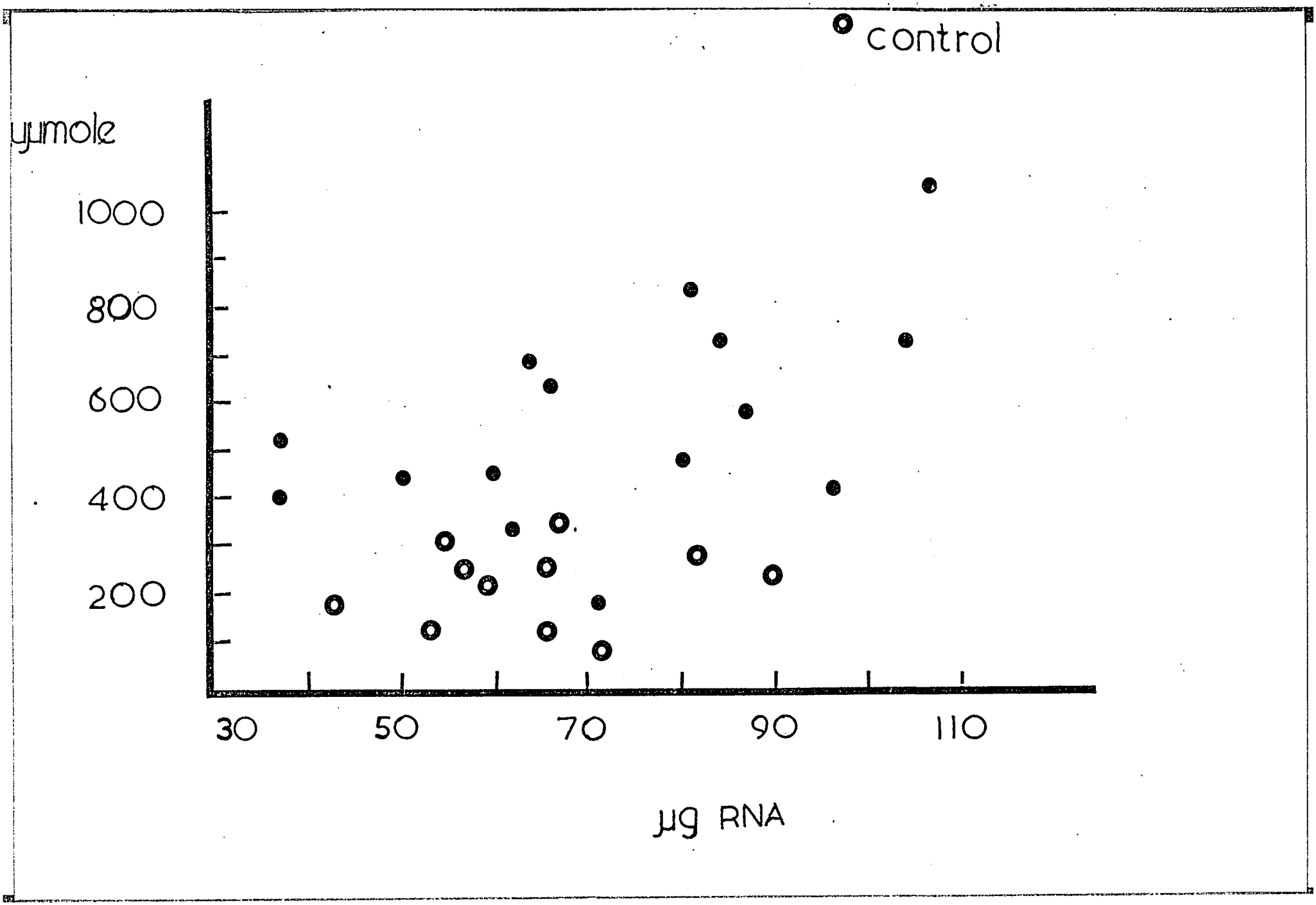

control
μmole
1000
800
600
400
200
30
50
70
90
110
μg RNA

Figure 5.

Changes in Protein Concentration of the Nuclear Suspension after Aortic Constriction

The abscissa indicates the intervals between the aortic constriction and chemical determination in days. The ordinate represents the protein concentration in μg/g net weight of the left ventricle. The values are the means of 2 to 3 experiments. Mean and SEM of the control are shown in the Figure.

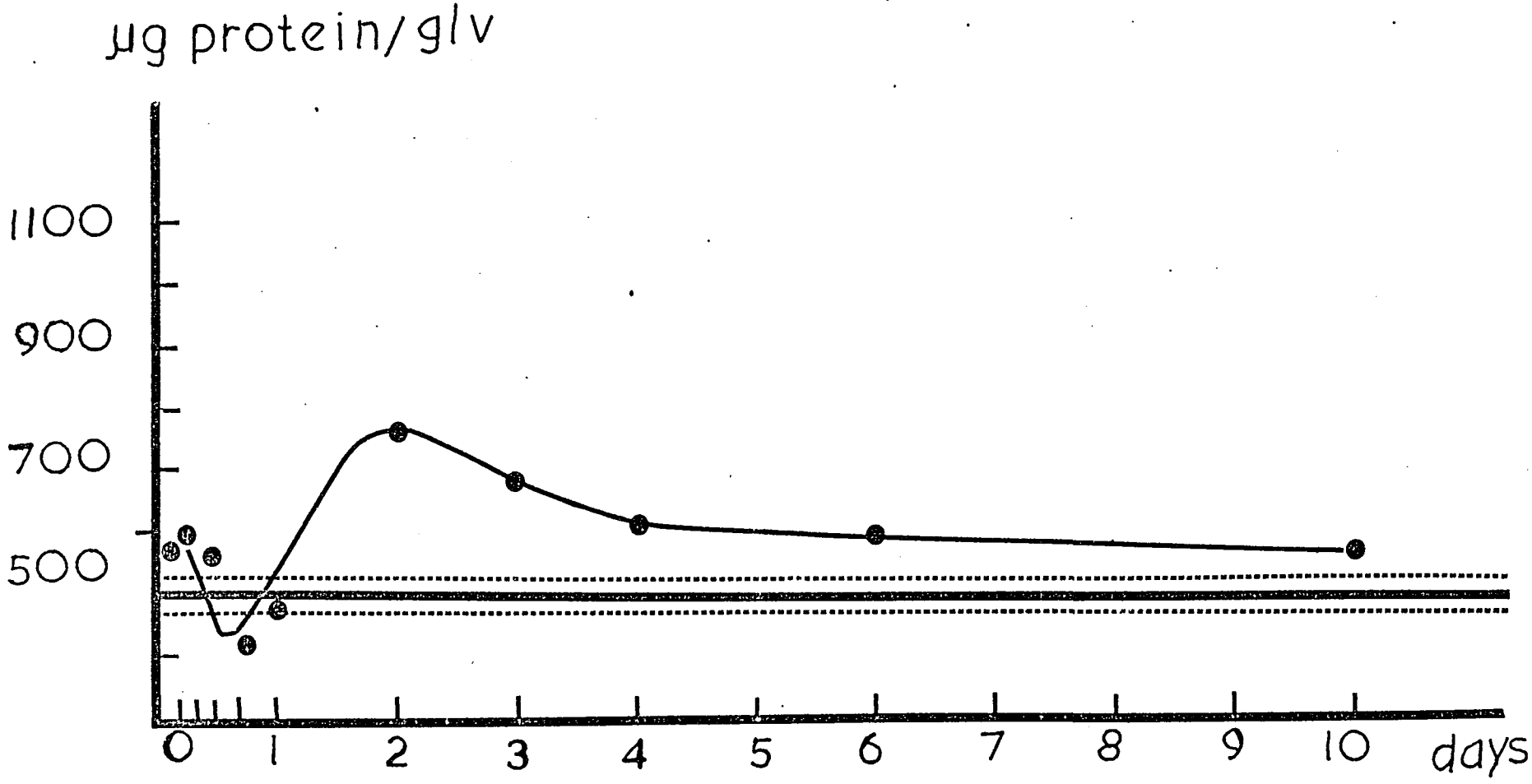

µg protein/glv
1100
900
700
500
0
1
2
3
4
5
6
7
8
9
10
days

Figure 6.

Changes in RNA Concentration in the Purified Nuclear Preparation after Aortic Constriction

Ordinate shows the concentration expressed as μg nuclear RNA/g left ventricle. Abscissa shows the days following operation. Mean and SEM of control are indicated on the figure.

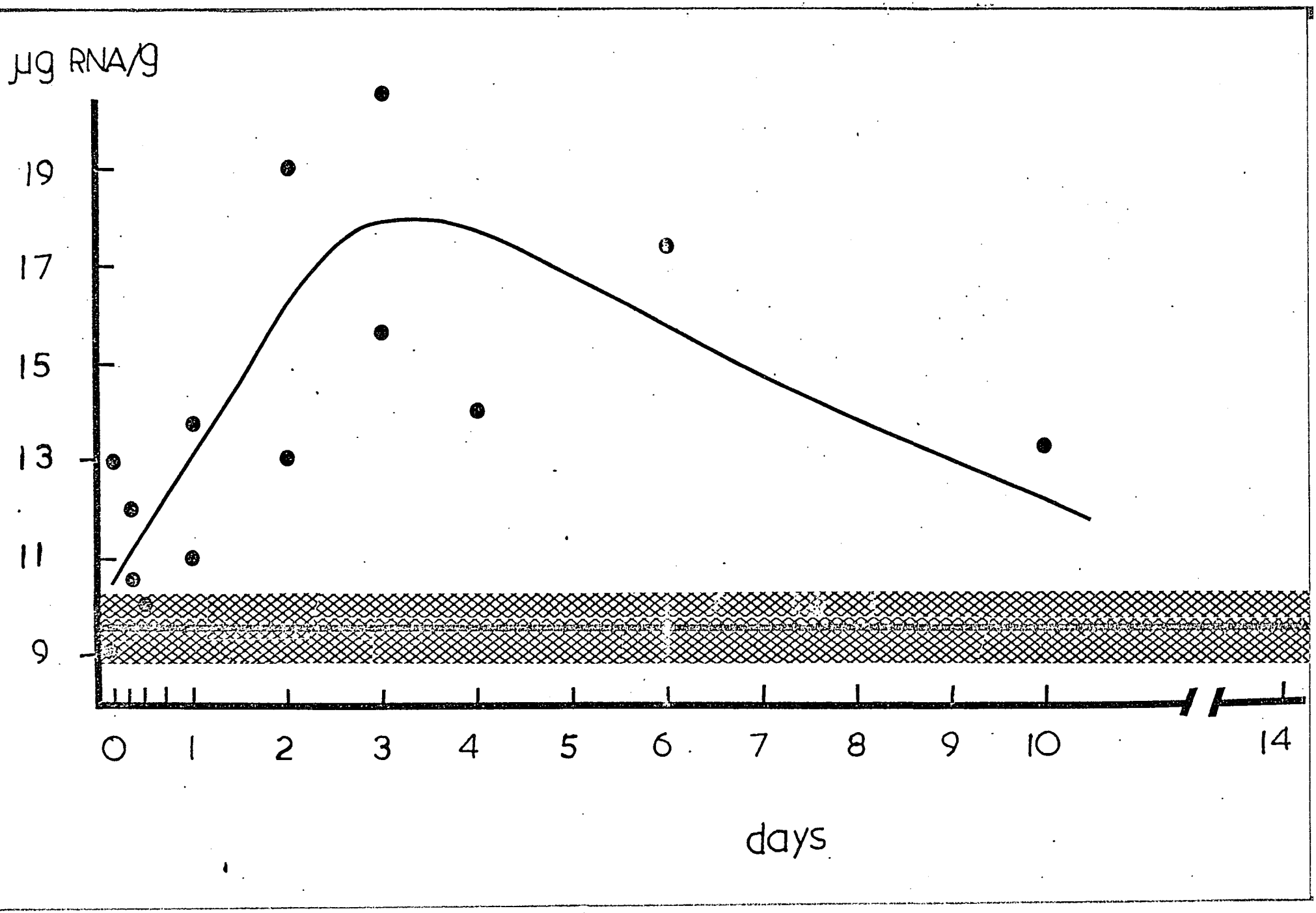

µg RNA/g
19
17
15
13
11
9
0
1
2
3
4
5
6
7
8
9
10
14
days

Figure 7.

Changes in RNA Content after Aortic Constriction

The ordinate shows the RNA content in μg of the nuclear suspension, calculated for one left ventricle. Each dot represents one experiment which includes 12 hearts.
For further explanation see legend to Figure 5.

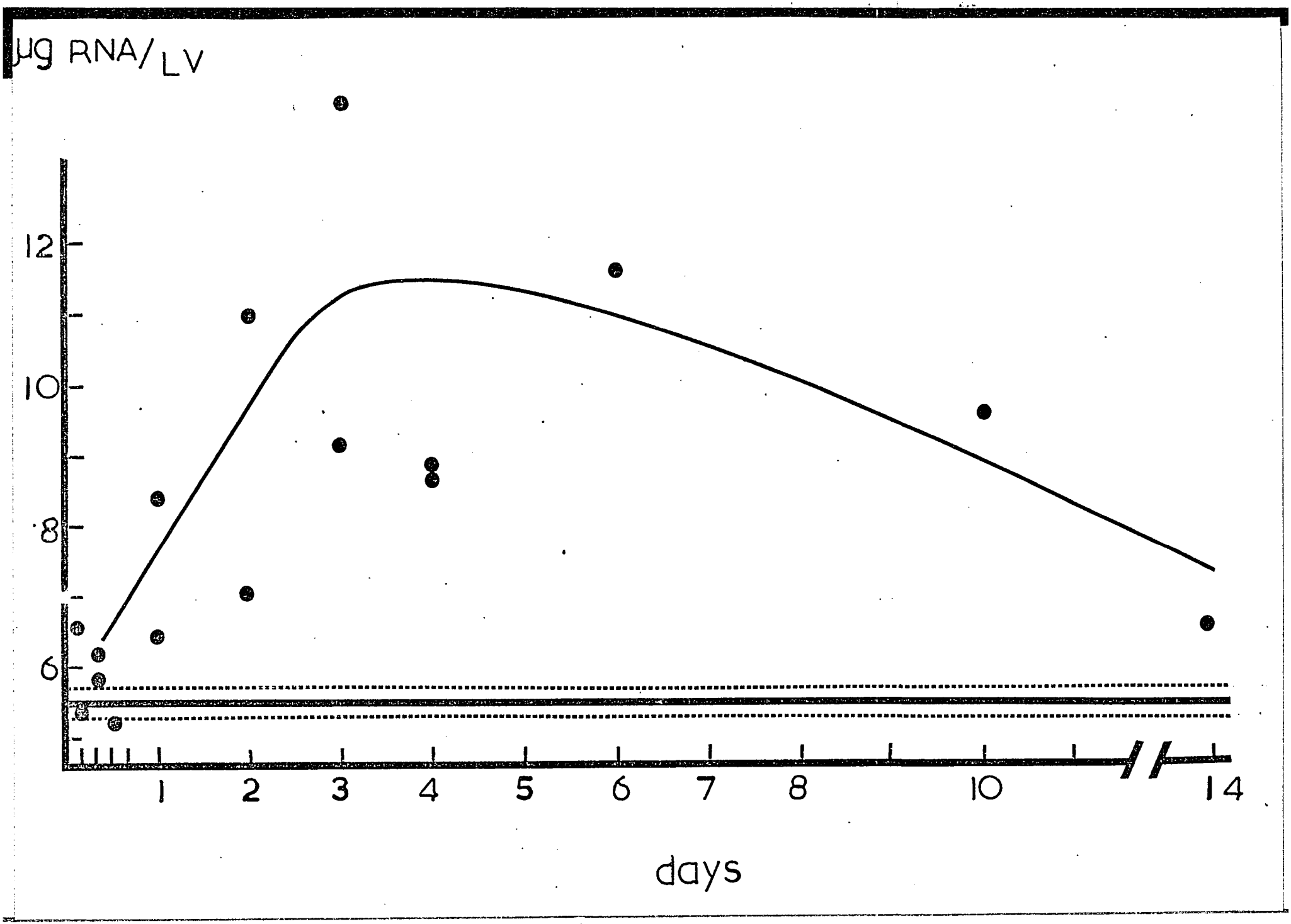

µg RNA/LV
12
10
8
6
1
2
3
4
5
6
7
8
10
14
days

Figure 8.

Changes in DNA Concentration and Content of the Nuclear Suspension prepared from Hypertrophying Left Ventricles

The ordinate indicates both the DNA concentration (epxressed μg/g wet tissue) and content in the nuclear suspension calculated for one left ventricle (in μg).
For further information see legend to Figure 5.

▲ concentration

● content

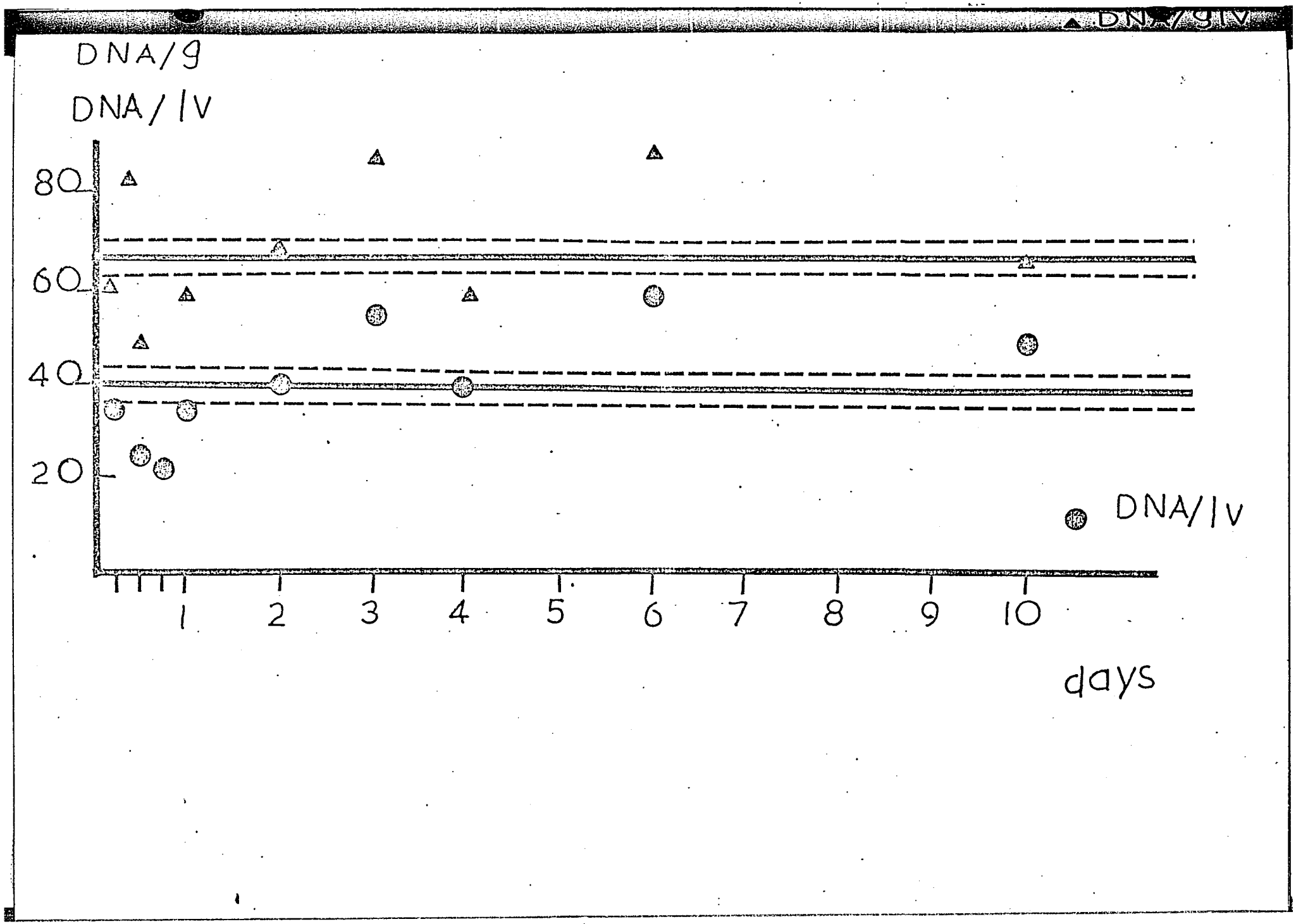

DNA/g
DNA/lv
80
60
40
20
DNA/lv
1
2
3
4
5
6
7
8
9
10
days

Figure 9.

Changes in DNA/Protein during the Development of Hypertrophy.

Each point represents the mean value of 2 - 3 analyses. Ordinate shows the ratio DNA/protein, and abscissa days after operation. Mean and SEM of the control are shown.

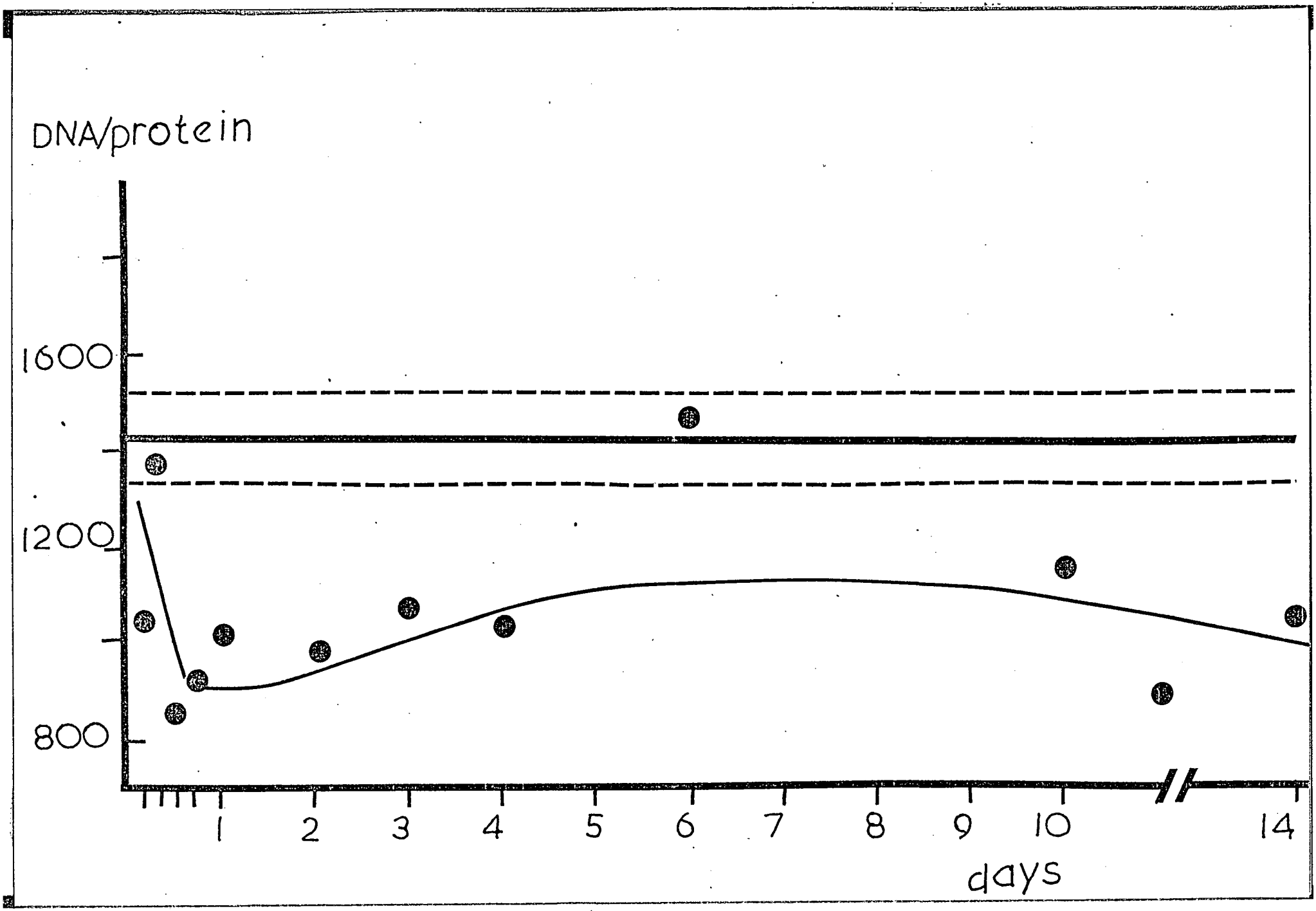

DNA/protein
1600
1200
800
1
2
3
4
5
6
7
8
9
10
14
days

Figure 10.

Changes in DNA/RNA during the Development of Hypertrophy

Ordinate shows the ratio DNA/nuclear RNA; abscissa shows the intervals between the operation and chemical analysis. Each point represents the mean value of 2 - 3 determinations.

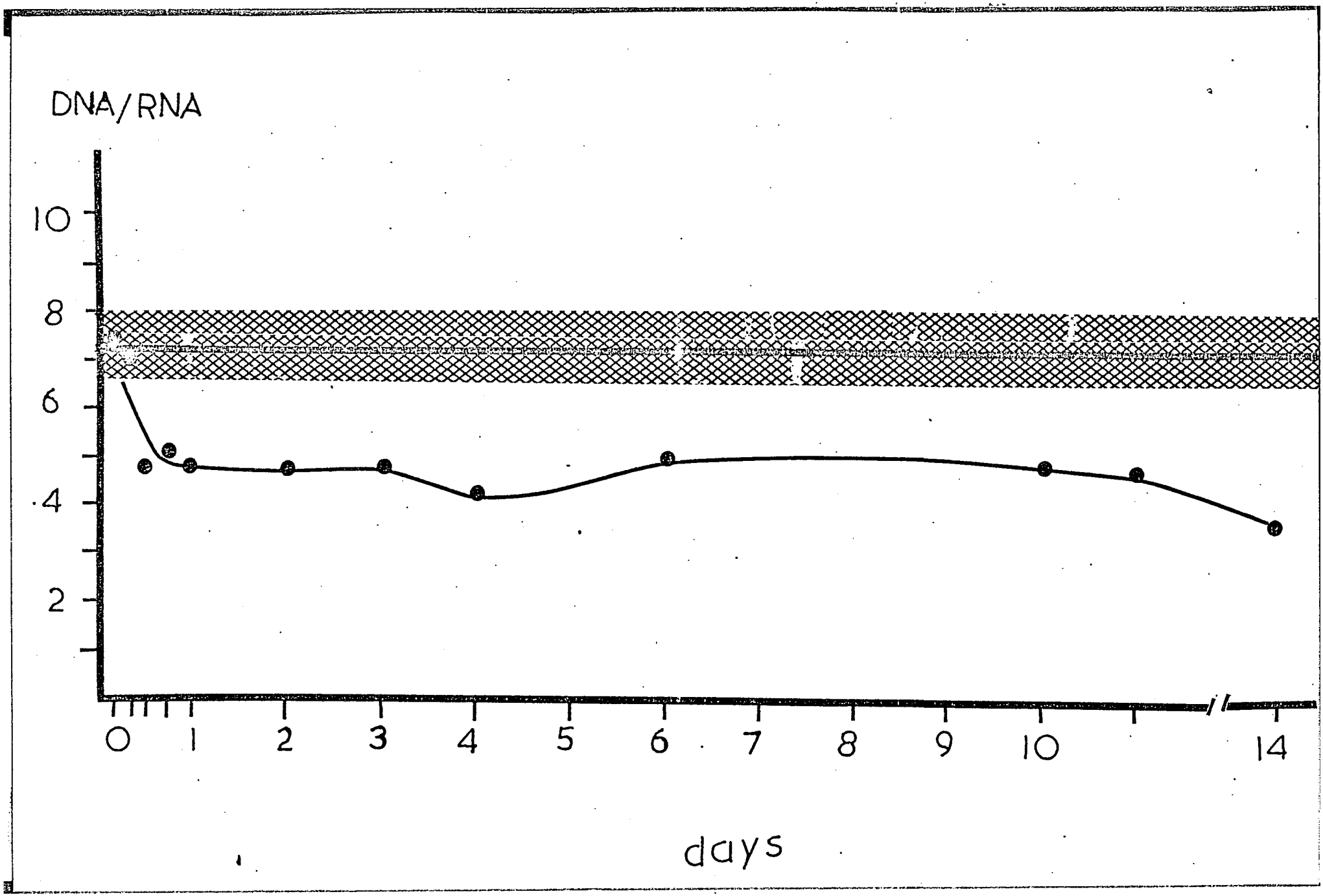

DNA/RNA
10
8
6
4
2
0
1
2
3
4
5
6
7
8
9
10
14
days

Figure 11.

Time-course of Changes in the Mg^{++} activated RNA Polymerase Activity following Aortic Banding

---

Data are expressed as mean values above control. The abscissa shows the intervals between the constriction and the enzyme determination. The ordinate represents the enzyme activity expressed in three ways, corresponding to (a), (b) and (c) on Table I.

▲ μμm UTP-H^3 incorporated/left ventricle

● " " " /g left ventricle

○ " " " /mg DNA

Each dot represents the mean value of 2 - 3 experiments.

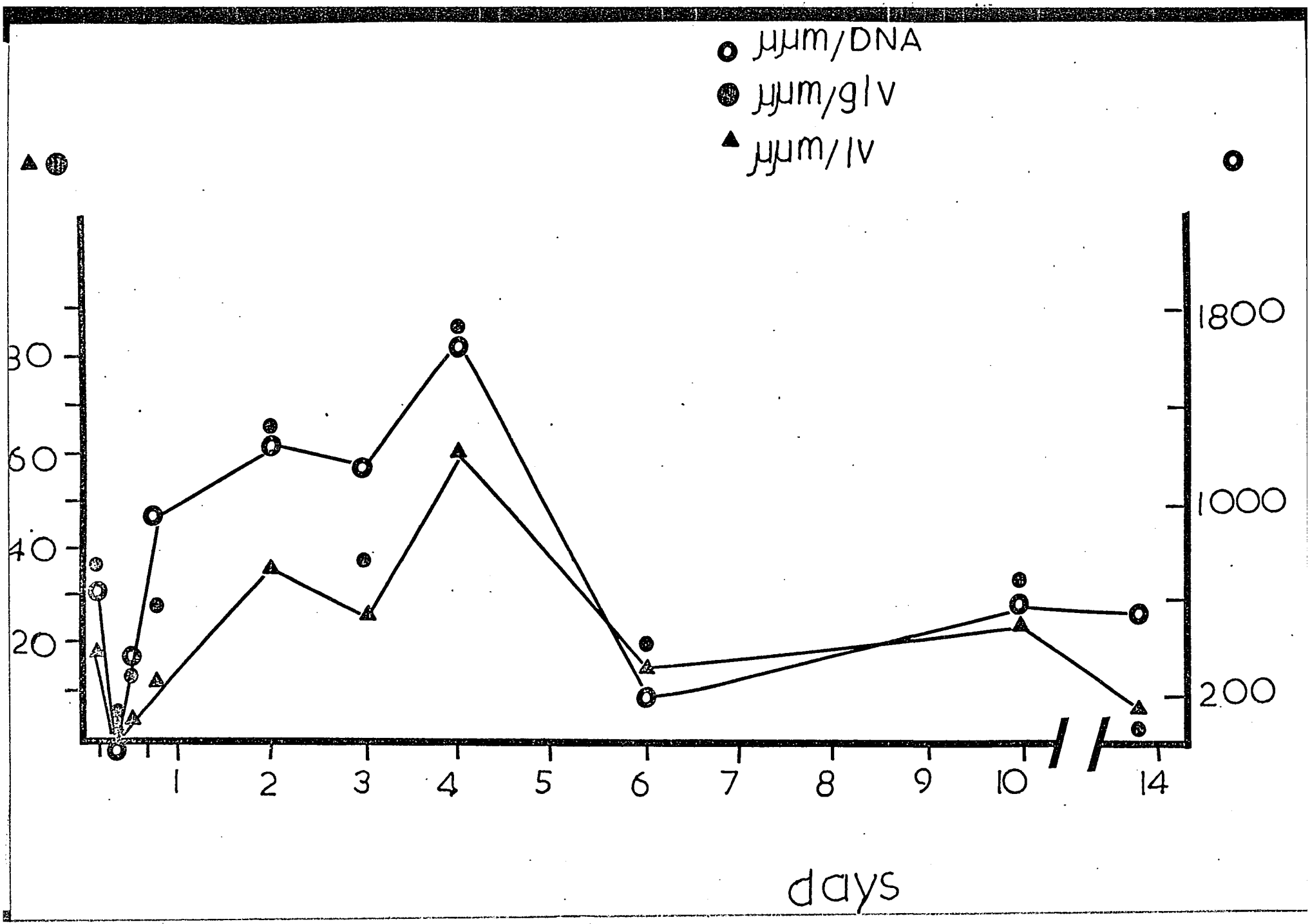

μμm/DNA
μμm/gIV
μμm/IV
80
60
40
20
1800
1000
200
1
2
3
4
5
6
7
8
9
10
14
days

Figure 12.

Changes over Time in the Mn^{++} activated RNA Polymerase Activity following Aortic Banding

---

For explanation, see legend to Figure 11.

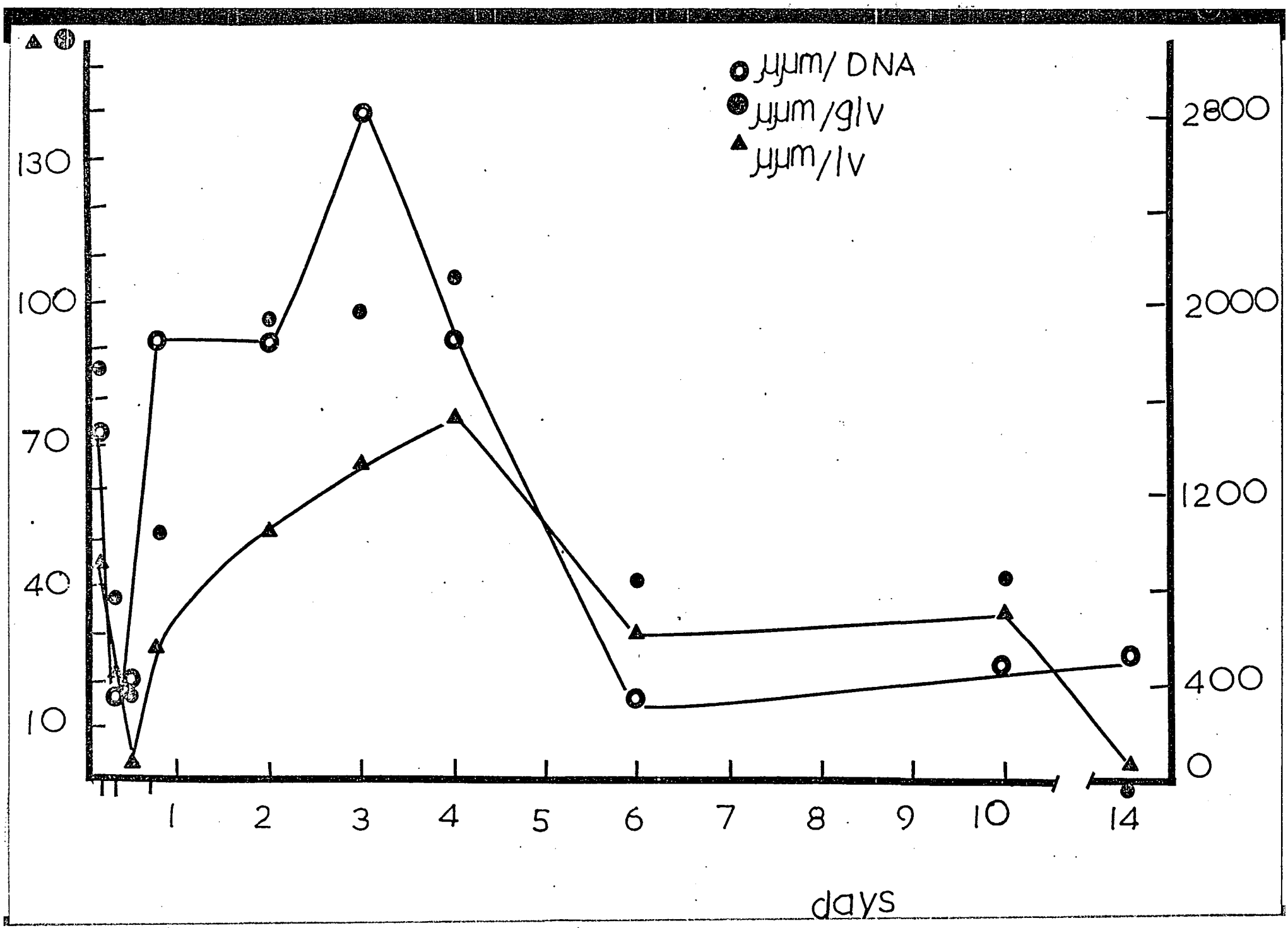

μμm/ DNA
μμm/glv
μμm/lv
130
100
70
40
10
2800
2000
1200
400
0
1
2
3
4
5
6
7
8
9
10
14
days

Figure 13.

Changes in RNA Polymerase Activity measured in System I following Aortic Constriction

---

Explanation appeared in legend to Figure 11.
The activity in this Figure is expressed in one way: μμmole UTP-H^3 incorporated into RNA/mg DNA. Each column represents the mean value of 2 - 3 assays. For each assay 12 left ventricles were pooled.

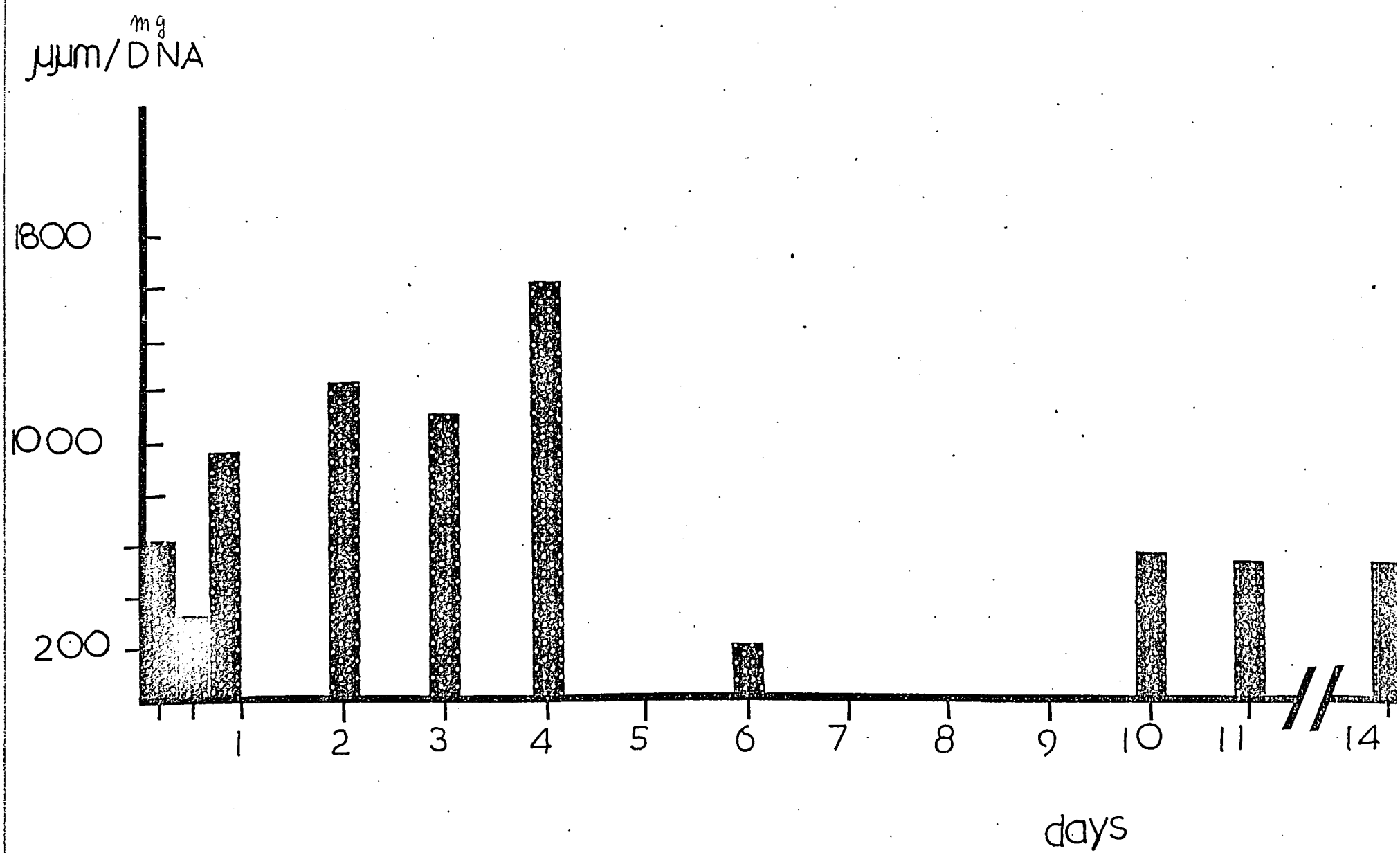

μμm/ mg DNA
1800
1000
200
1
2
3
4
5
6
7
8
9
10
11
14
days

Figure 14.

Changes in RNA Polymerase Activity measured in System II.

For explanation, consult legend to Figure 13.

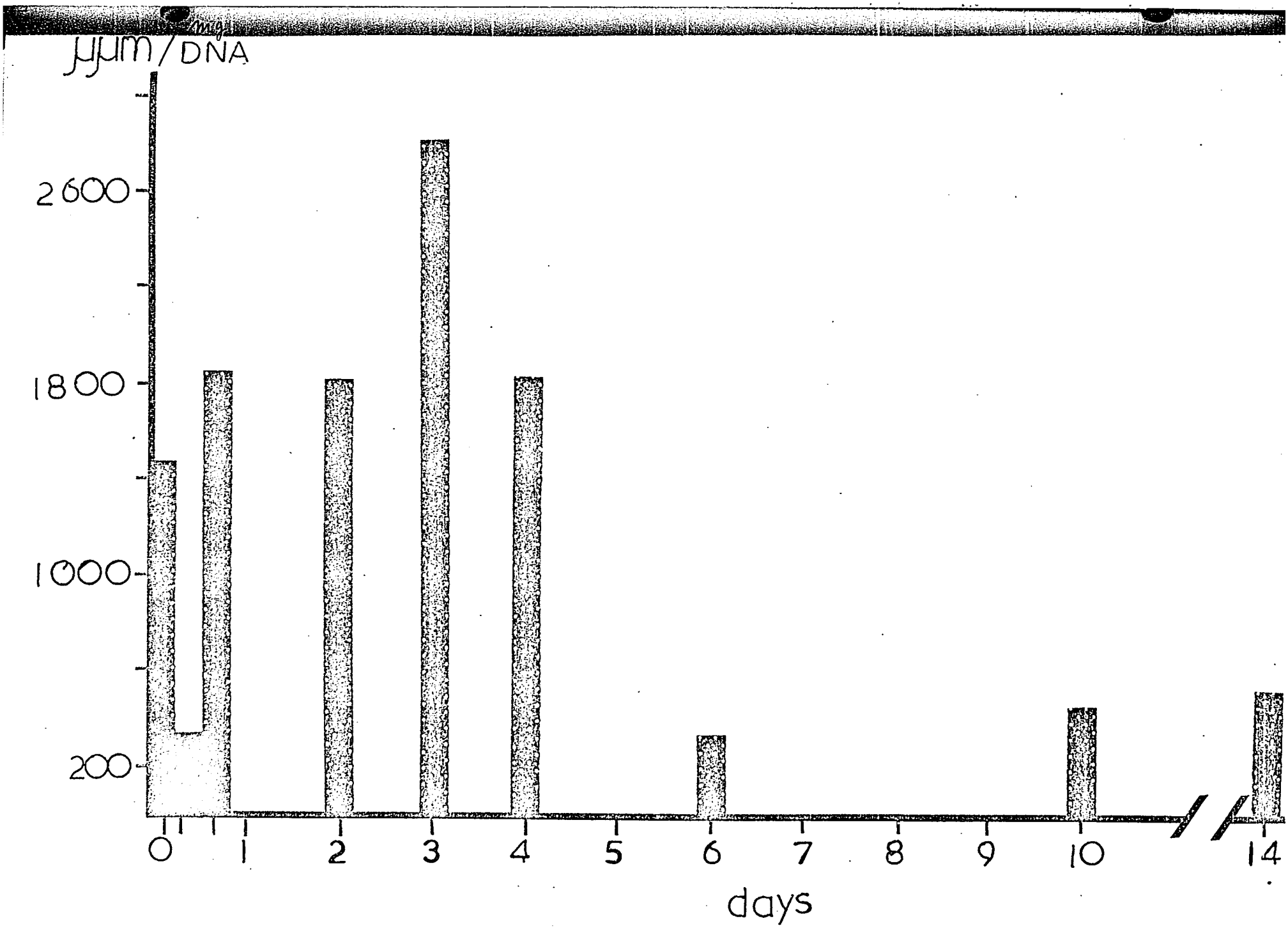

μμm/mg DNA
2600
1800
1000
200
0
1
2
3
4
5
6
7
8
9
10
14
days

Figure 15.

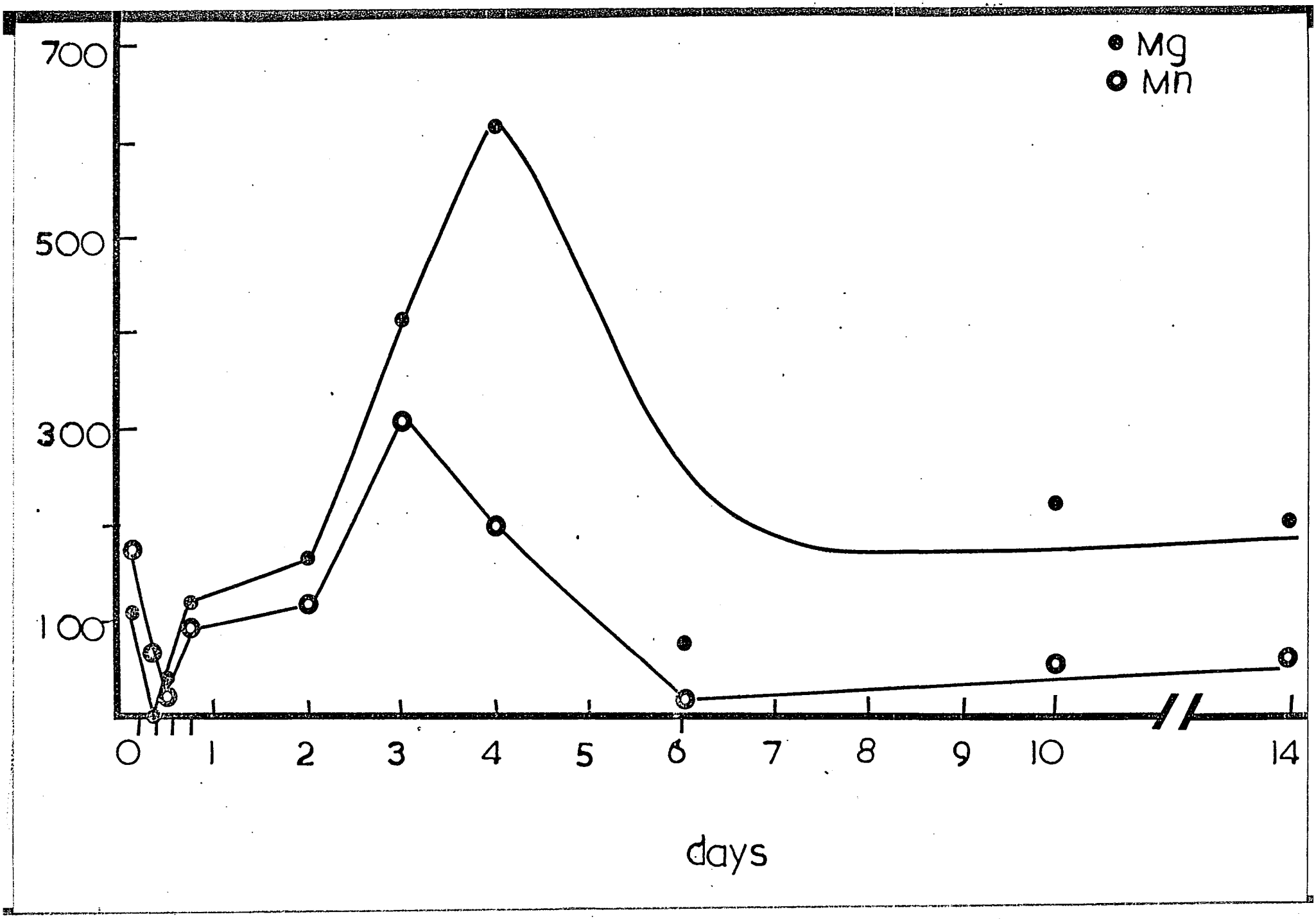
Percentage Changes over Time in the RNA Polymerase Activity following Aortic Banding

---

The changes in enzyme activity are expressed as mean values above control, in percentage. (Ordinate), abscissa shows the days after operation.

● represents System I (Mg^{++}-activated)

⊙ represents System II (Mn^{++}-activated)

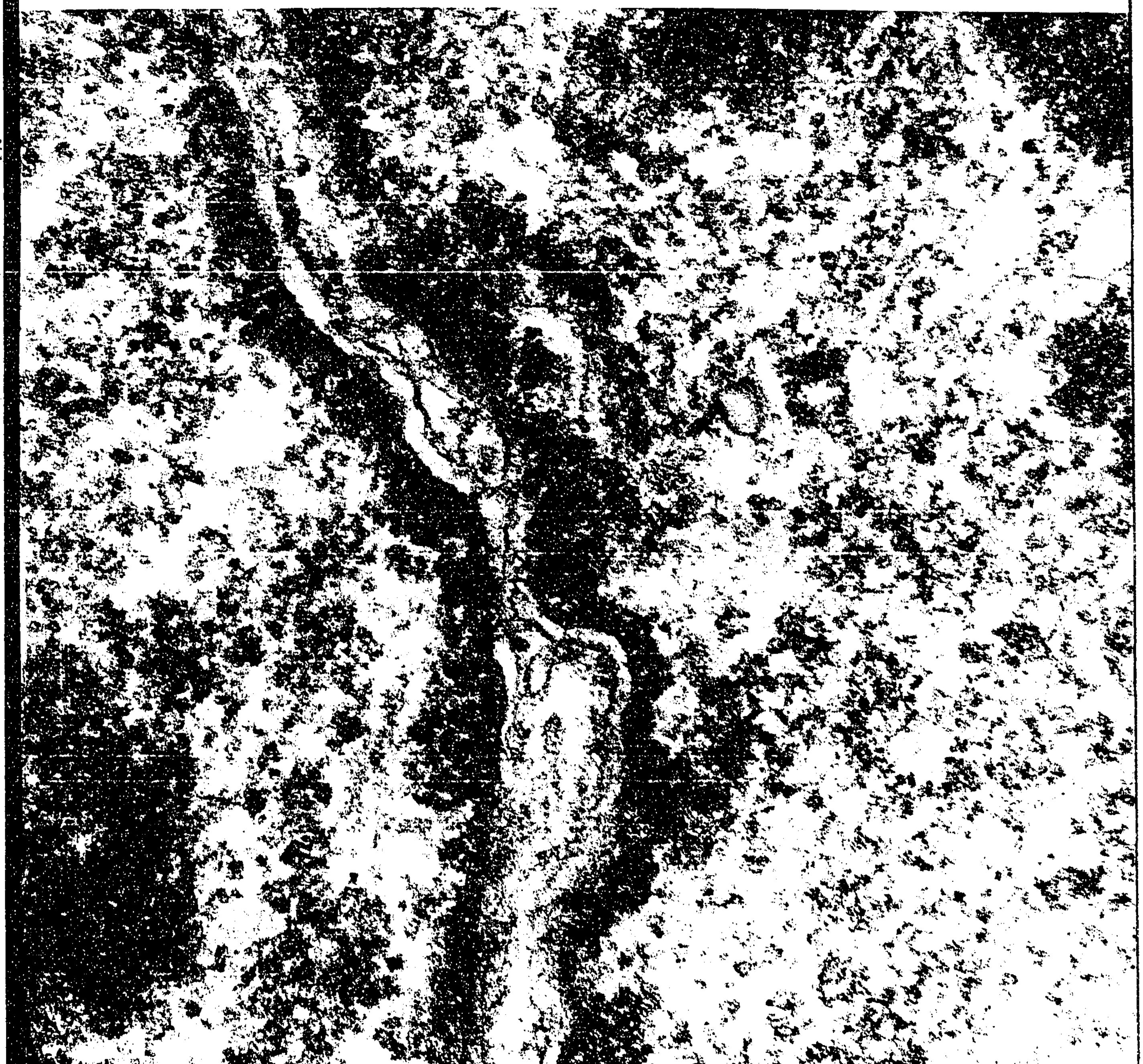

700
500
300
100
Mg
Mn
0
1
2
3
4
5
6
7
8
9
10
14
days

Electronmicrographs of the Nuclear Preparation

The final nuclear pellet obtained after the isolation procedure is shown at different magnifications. Electronmicrographs were prepared from normal heart muscle nuclei (described on p. 19).

First picture: magnification is 1:53000. It shows the nuclear membrane of two nuclei.

Second picture: magnification is 1:28000. It shows the isolated nuclei and the mitochondrial contamination



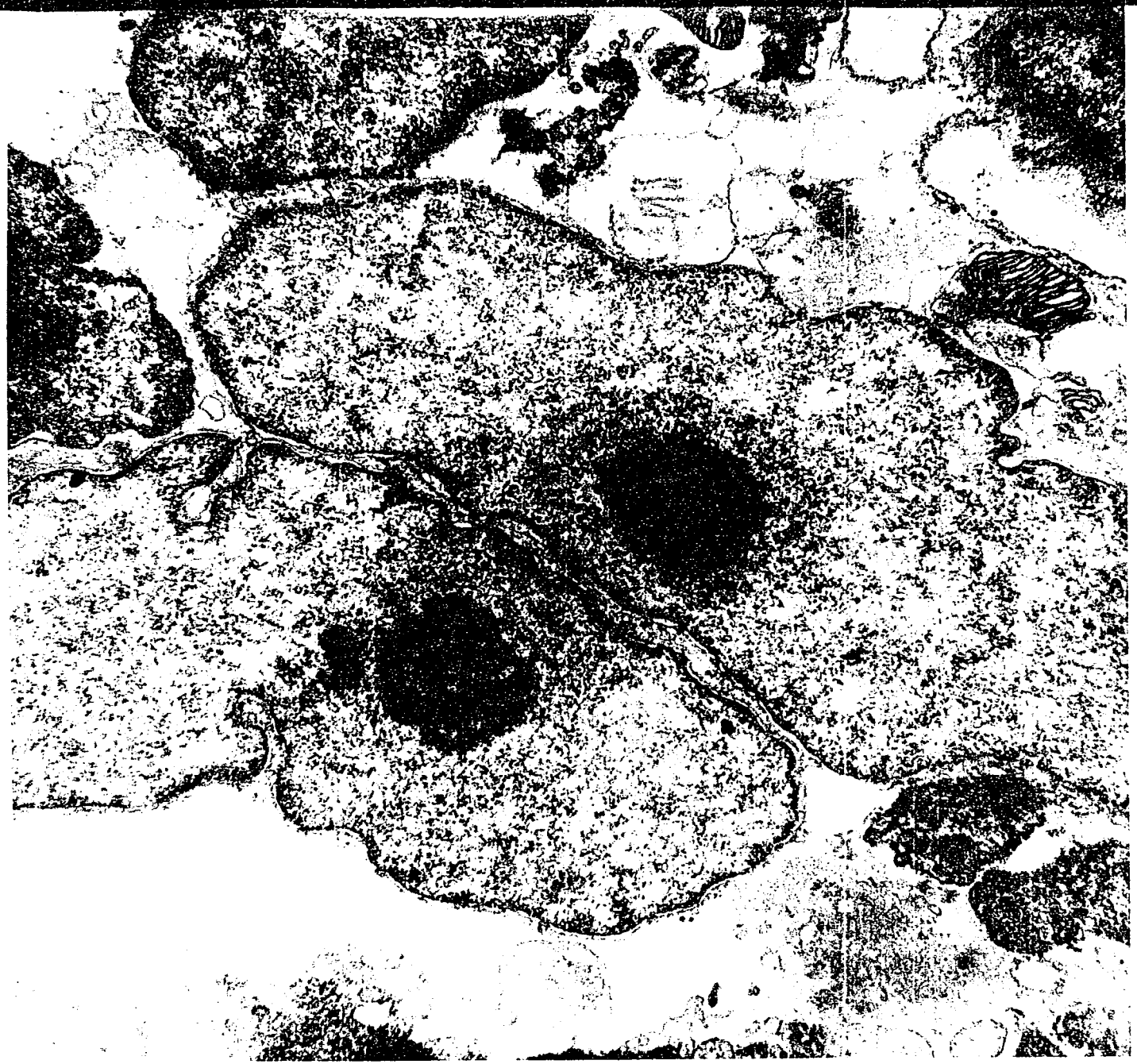


Table I

RNA Polymerase Activity of Normal Heart Muscle Nuclei in the Presence of Mg^{++} and Mn^{++} - $(NH_4)_2SO_4$

The activity is expressed as μμmole UTP-H^3 incorporated into RNA (a) per DNA of the nuclear preparation in mg; (b) of one left ventricle; (c) of one g of left ventricle.

TABLE I

Incub. Time \ Sample No		1	2	3	4	Mean + SE
5	a		180		203	192 + 11
	b		4,3		6,2	5,2 + 0,9
	c		6,2		10,7	8,5 + 2
15	a	243	280	325	324	268 + 22,3
	b	15,3	6,7	15,1	9,9	11,7 + 2,1
	c	20,4		26,5	17	21,3 + 2,9
30	a		302			302
	b		14			14
	c		24,5			24,5
5	a	430		219	858	502 + 188
	b	7,7		10	26,3	14,7 + 5,8
	c	11,5		17,9	45	24,8 + 9,8
10	a	1110	635	678	1225	912 + 149
	b	19,9	26	31,5	37,5	28,7 + 3,7
	c	29,9	39	55	64	47 + 7,7

Table II

Chemical Analysis of Nuclear Preparations obtained from Control Animals

Rows 1 - 13 show the results obtained from groups of normal rats and Rows 14 - 22 are those from sham-operated rats.

The verticle columns 1, 3 and 5 show the total protein, total RNA and total DNA in the nuclear suspension of each group of 12 pooled left ventricles.

Columns 2, 4 and 6 are protein, RNA and DNA expressed per 0.1 ml of nuclear suspension.

Columns 9 and 11 indicate the nuclear DNA and RNA contents in one left ventricle.

Columns 1, 12 and 13 show the nuclear DNA, RNA and protein concentrations expressed as μg/g left ventricle.

No.	1 Prot.	2 Prot.	3 RNA	4 RNA	5 DNA	6 RNA	7 DNA Prot.	8 DNA RNA	9 DNA heart	DNA left V	10 DNA g	11 RNA heart	12 RNA g	13 Prot. g	
1	12h. 10g.	2720	170	74	4,6	349,6	21,9	0,1285	4,72	29		35	6,2	7,4	272	August 22, 1969
2	12h. 8g.	2240	140	52,6	3,3	214,6	13,4	0,0958	4,08	18		27	4,4	6,6	280	August 26, 1969
3	12h. 8,3g.	2470	190	73	5,6	285,7	22	0,1157	3,9	24		34	6,0	8,8	298	September 1, 1969
4	12h. 9g.	2940	150	84,6	4,2	359	18	0,1221	4,24	30		40	7,0	9,4	327	September 3, 1969
5		1650	120	55,5	4	219	15,6	0,1327	3,94							September 5, 1960
6	12h. 9g.	3330	240	43,5	3,1	757	54	0,2273	17,4	63		84	3,6	4,8	370	September 14, 1969
7	12h. 8,4g.	3870	280	53	3,8	653	46,6	0,169	12,3	54		78	4,4	6,3	461	September 16, 1969
8a)	6h. 9,4g.	2400	290	32	4,1	293	36,6	0,1220	9,2	49		73	5,3	8	600	September 18, 1969
8b)	6h. 4g.	1900	240	29	3,6	266	33	0,1400	9,2	44		67	4,8	7,3	475	September 18, 1969
9	12h. 8g.	2400	175	72	5,2	493	35	0,2054	6,8	41		62	6,0	9	300	September 22, 1969
10	14lv 8g.	3400	210	59	3,7	651	41	0,1915	11.	W/lv 0,57	47	81	4,2	7,4	425	September 24, 1969
11	12lv 7g.	3760	250	55	3,7	368	24,5	0,0977	6,7	0,58	31	53	4,6	7,8	537	October 29, 1960
12	12 7,9g.	1300	80	16	1	260	16,2	0,200		0,66						February 13, 1970
13	14 6,6g.	2900	180	87	5,4	337	21,1	0,1162	3,4	0,47	24	51	6,2	13	439	February 15, 1970
14	12 4h.6,7g.	5060	340	90	6,0	426	28,4	0,0842	4,7	0,56	35,5	64	7,5	13,3	755	

4g.																
9 12h. 8g.	2400	175	72	5,2	493	35	0,2054	6,8	41		62	6,0	9	300	September 22, 1969	
10 14lv 8g.	3400	210	59	3,7	651	41	0,1915	11.	W/lv 0,57	47	81	4,2	7,4	425	September 24, 1969	
11 12lv 7g.	3760	250	55	3,7	368	24,5	0,0977	6,7	0,58	31	53	4,6	7,8	537	October 29, 1960	
12 12 7,9g.	1300	80	16	1	260	16,2	0,200		0,66						February 13, 1970	
13 14 6,6g.	2900	180	87	5,4	337	21,1	0,1162	3,4	0,47	24	51	6,2	13	439	February 15, 1970	
14 12 4h.6,7g.	5060	340	90	6,0	426	28,4	0,0842	4,7	0,56	35,5	64	7,5	13,3	755		
15 12 3h. 6g.	2300	150	66	4,4	380	25,3	0,1650	5,75		31,6	63	5,5	11	383	October 10, 1969	
16 12 3h.6,2g.	3880	260	66	4,4	478,5	32	0,1233	7,3	0,52	39,9	77	5,5	11	626	November 12, 1969	
17 11 12h.6,5g	2900	190	57	3,8	315	21	0,1084	5,5	0,59	28,6	48	5,2	8,7	446		
18 12 1d. 8,4g	3870	280	53	3,8	653	46,6	0,169	12,3		54,4	77,7	4,4	6,3	461		
19 12 1d. 7g.	3900	260	82	5,4	617	41	0,158	7,5	0,58	51,4	88	6,8	11,7	557		
20 12 2d. 8g.	3000	200	67	4,5	458	31	0,152	6,8	0,67	38	57	5,6	8,4	375		
21 12 3d. 8g.	3750	250	65,6	4,4	515	34	0,137	7,8	0,67	43	64	5,5	8,2	469		
22 6 3d. 4g.	1900	240	29	3,6	266	33	0,1400	9,2		44,3	66,5	9,8	7,3	475		
Mean Values		222±18				30,4±2,5	0,1417± 0,0098	7,3± 0,73	0,58	39±2,7	65,9± 3,68	5,5±0,28	9,5±0,69	496±31,4		13
		SD±65				SD±8,9	SD±0,035	SD±2,54		SD±9,4	SD±12,7	SD±0,98	SD±2,4	SD±108,7		12

Table III

Chemical Analysis of Nuclear Preparation obtained from Hypertrophied Left Ventricles

For further information see legend to Table II.

Time after operation	1	2	3	4	5	6	7 DNA	8 DNA	9 DNA	10 DNA	11 RNA	12 RNA	13 Prot.	Total W	μμ mole UTP/RNA	
	Ptot.	Prot.	RNA	RNA	DNA	DNA	Prot.	RNA	lv	g	lv	g	g	num. of ventr.	Mg	Mn
4 h.	12 6,2	3500	230	79	5,3	392	26	0,1119	4,9	33	63	6,6	13	565	0,52	6	15,7
	12 6,5	4600	340	60	9,5	446	33	0,097	7,4	37	67	5,0	9,2	708	0,54	7,4	10,3
	12 6,8	3310	200	37	2,5	337	22,5	0,102	9,1	28	50			487	0,57		
8 h.	12 6,2	3700	240	74	4,9	475	32	0,1283	6,4	40	76,6	6,2	12	597	0,52	1	5,4
	12 6,8	4000	270	71	4,8	596	40	0,149	8,3	50	88	5,9	10,5	588	0,56	2,7	7,1
2 h.	12 6,2	3400	230	62	4,1	290	19	0,0853	4,7	24	47	5,2	10	548	0,52	5,3	10,1
7 h.	12 6,7	2700	180	50	3,3	251	16,7	0,093	5,02	21	37	4,2	7,5	403	0,56	8,9	18,2
2 d.	12 6,6	3540	240	84	5,6	392	26	0,1106	4,7	33	59	7	13	536	0,55	8,5	15
	9 5,2	5200	430	98	8,2	464	39	0,089	4,7	52	89	11	19	1000	0,58	2	5,1
	12 7,9	4020	250	81	5,0	391	24,4	0,097	4,8	33	49				0,66	10,4	17,6
3 d.	12 7	3400	230	110	7,3	606	40	0,1782	5,5	51	87	9,2	15,6	486	0,58	1	3,6
	11 7	6200	420	168	11,2	612	41	0,099	3,6	56	87	15,3	24	886	0,64	2,7	6
	12 8,2	2100	140	37	2,5	200	13,3	0,095	5,4						0,68	17,6	
4 d.	12 7,5	3760	250	104	7,0	448	30	0,1191	4,3	37	60	8,7	14	501	0,63	6,9	10,4

	12															
	6,8	4000	270	71	4,8	596	40	0,149	8,3	50	88	5,9	10,5	588	0,56	2,7	7,1
12 h.	12																
	6,2	3400	230	62	4,1	290	19	0,0853	4,7	24	47	5,2	10	548	0,52	5,3	10,1
17 h.	12																
	6,7	2700	180	50	3,3	251	16,7	0,093	5,02	21	37	4,2	7,5	403	0,56	8,9	18,2
2 d.	12																
	6,6	3540	240	84	5,6	392	26	0,1106	4,7	33	59	7	13	536	0,55	8,5	15
	9																
	5,2	5200	430	98	8,2	464	39	0,089	4,7	52	89	11	19	1000	0,58	2	5,1
	12																
	7,9	4020	250	81	5,0	391	24,4	0,097	4,8	33	49				0,66	10,4	17,6
3 d.	12																
	7	3400	230	110	7,3	606	40	0,1782	5,5	51	87	9,2	15,6	486	0,58	1	3,6
	11																
	7	6200	420	168	11,2	612	41	0,099	3,6	56	87	15,3	24	886	0,64	2,7	6
	12																
	8,2	2100	140	37	2,5	200	13,3	0,095	5,4						0,68	17,6	
4 d.	12																
	7,5	3760	250	104	7,0	448	30	0,1191	4,3	37	60	8,7	14	501	0,63	6,9	10,4
	12																
	8,8	5200	320	107	6,7	468	29,3	0,09	4,4	39	53	8,9	12	591	0,73	9,9	13,5
6 d.	12																
	8	4800	320	140	9,3	707	47	0,1473	5,0	58,9	88	11,7	17,5	600	0,67	2,3	5
10 d.	10																
	7,2	3980	270	96	6,4	468	31	0,1175	4,9	46,8	65	9,6	13,3	553	0,72	4,2	6,7
11 d.	10																
	9	3500	220	64	4	308	19,2	0,088	4,8	30,8				389	0,90	10,8	18,0
14 d.	6																
	4,2	1200	120	42	4,2	129	12,9	0,1075	3,07	21,5	30,7	7,0	10	286		2,4	4,3

Table IV

Time Course of Changes in Nuclear RNA Polymerase Activity following Aortic Banding

Each number represents the mean value of 2-3 experiments. The activity is expressed as on table I.

IONS	UNITS	4 h.	8 h.	12 h.	17 h.	1 d.	2 d.	3 d.	4 d.	6 d.	10 d.	11 d.	14 d.
Mg	a)	703	153	638	2399		864	926	712	275	333		372
5	b)	23,6	6,85	15,4			28,8	25,6	25,6	16	15,6		8
	c)	43,5	12,5	30			45,5	39	38,9	24	21,6		11
	a)	1187	250	1133	1774		1998	1384	1924	459	866		790
15	b)	39	11,4	27,3	37		65	37,4	73,7	27	46,4		17
	c)	72,5	20,5	53	66		108	57	107,5	41	56		24
	a)	1050	216	1426			2318		1795	454			
30	b)	36	8,5	34,4			75,6		67	26,8			
	c)	68,5	16,6	66,6			137		107	40			
Mn	a)	1763	506	1721	1582		2212	911	2140	531	724		778
5	b)	58,4	23,3	41,6	33		72	33,4	81,7	31,3	33,9		16,7
	c)	108,5	42	80	59		119	52	120,5	47	47		29
	a)	2363	847	2150	3633		3434	3748	2751	1001	1384		1431
10	b)	76	38	52	76		112	94,6	105	59	64,7		30,8
	c)	141,5	70	101	136		185,5	143	154,5	89	90		44

Table V

Time Course of Changes in Nuclear RNA Polymerase Activity following Aortic Constriction

The results are expressed as absolute changes over values obtained from controls.
For further information see Table I.

IONS	UNITS	4 h.	8 h.	12 h.	17 h.	1 d.	2 d.	3 d.	4 d.	6 d.	10 d.	11 d.	14 d.
Mg^{++}	a)	484	-200	220	1981		516	734	520	83	141	228	180
5	b)	15,9	-4,35	3,4			15,2	20,4	20,4	10,8	10,2	13,5	2,8
	c)	29,5	-9,5	10	27,4		25,5	30,5	30,4	15,5	13,1	10,9	2,5
	a)	620	-20	333	974		1244	1116	1656	191	598	546	522
15	b)	19	2,8	4	14		36,1	25,7	61,6	15,3	28,7	28	5,3
	c)	36,5	3,5	14,4	27,4		65	35,7	86,2	19,7	34,7		2,7
	a)		-55	464					1493	152			
30	b)		-0,1	7,7					53	12,8			
	c)		-0,4	20,6					82,5	15,5			
Mn^{++}	a)	1360	4	1287	1187		1017,5	409	1638	29	222		276
5	b)	44,4	7,3	29	20,5		26,5	18,7	67	16,6	19,2		2
	c)	83	10	59	38		51,5	24,2	95,7	22,2	22,2		-0,8
	a)	1486	337	392	1875		1848	2836	1839	300	472		519
10	b)	45	22	1,7	25,7		51,5	65,9	76,3	30,3	36		2,1
	c)	85,8	37,7	16	51		95,5	96	107,5	42	43		-5

Reference	Tissue	Isolation Medium	Fraction	Protein	Protein	RNA/DNA
Schneider and Petermann (15)	Mouse spleen	0.88 M sucrose + Ca^{++}	N	0.29	0.013	0.045
Gurr et al. (30)	Rat liver	1 percent citric acid	N	0.28		
		2.2 M sucrose + Mg^{++} + Na^{+} + glycerol P	N	0.12		
Chauveau et al. (7.8)	Rat liver	2.2 M sucrose	H	0.019	0.047	2.5
			N	0.21	0.034	0.16
Hogeboom et al. (2)	Rat liver	0.34 M sucrose + Ca^{++}	H	0.016	0.042	2.5
			N	0.12	0.051	0.38
Hymer and Kuff (42)	Rat liver	0.25 M sucrose + Mg^{++} + Triton-X	H			3.3
			N	0.29	0.063	0.22
Maggio et al. (1)	Guinea pig liver	2.2 M sucrose + Ca^{++}	N	0.31	0.065	0.21
Kay et al. (43)	Rabbit thymus	0.05 M citric acid	N	0.51	0.047	0.080
		non-aqueous solvent	N	0.23	0.057	0.21
Allfrey et al. (6)	Calf liver	Non-aqueous solvent	N	0.18		
	Horse liver (fasted)	Non-aqueous solvent	N	0.22		
	Calf heart	Non-aqueous solvent	N	0.24		
Siebert (5)	Rat liver	Non-aqueous solvent	H	0.015	0.053	3.5
			N	0.14	0.045	0.32
	Ox thymus	Non-aqueous solvent	H	0.016	0.022	1.4
			N	0.19	0.049	0.25
Edelman et al.	Rat skeletal muscle	2.15 M sucrose + K^{++} Mg^{++}, ATP	H	0.002	0.004	1.7
			N a	0.17	0.064	.30
			b	0.29	0.029	0.10
This study	Rat heart muscle	2.4 M sucrose + Mg^{++}	H	0.0024		
			N	0.1417	0.019	0.137
Ferguson et al.	Guinea pig heart muscle	0,32 M sucrose 10 mM $CaCl_2$	N			0.85

Table VII

Summary of Changes in Heart Weight and RNA Content in Experimental Cardiac Hypertrophy

Reference	Mode of producing hypertrophy	Animal	Duration	% Change in heart wt.	RNA control values (μg/mg wet wt)	% Change in left ventricular RNA	
						Concentration (μg/mg wet wt)	Total
Nowy and Frings (11)	constrict ascending aorta	mature rabbit	6 mths.	+ 51%	3.22*	+ 6%	+ 60%
Rossi and Mor (22)	constrict ascending aorta	mature rabbit	8 da 4 mo	not given	2.2*	+ 46% - 10%	not given
Meerson and Ramenskaya (14)	constrict ascending aorta	mature rabbit	7 da 50 da	+ 75% + 50%	not given	+ 22% + 5%	+ 110% + 57%
Gluck et al (18)	constrict ascending aorta	puppy	8 da	not given	5.5*	+ 545%	not given
Kleitke and Sydow (23)	constrict ascending aorta	mature dog	1-1/2-2 yr.	+ 48%	0.7	-11 %	+ 31%
Grimm et al (20)	constrict abdominal aorta	mature rat	5-7 mth	+ 100%	4.09	- 20%	+ 55%
Hoyle et al (13) (13)	diet-induced anemia	mature rat	120 da	+ 51%	2.63	0	+ 50%
Korecky and French (21)	diet-induced anemia	mature rat	120 da	+ 100%	2.00	0	+ 100%
Nair et al	constrict ascending aorta	mature rat	1-12 da 4-8 wk	+ 30% + 40%	2.2	+ 30% - 12%	+ 65% + 12%
Beznak (1954)	constrict abdominal aorta	mature rat	1-14 da	+ 33%			+ 44%

* Recalculated

DISCUSSION

I. CRITICISM ON METHODOLOGY

1. Isolation of Heart Muscle Nuclei

The difficulties of the isolation of striated muscle nuclei are formidable owing to the abundance and infiltrative nature of the surrounding connective tissue and a relatively large amount of myofibrils (Edelman et al, 1965). Furthermore, the peripheral location of the nuclei could lead to their damage or ultimate destruction during homogenization.

Three main types of isolation procedures have been described.

(a) Isolation of nuclei in non aqueous solvents after complete drying of the tissue. The advantages of this method are that the water soluble material is not diminished and enzymatic reactions are stopped immediately. The chemical constituents are preserved very closely to that of the original tissue, although the fractionation after the lyophilization cannot easily be accomplished and the nuclear membrane is mechanically damaged. This method is recommended for analytical purposes.

(b) The preparation of nuclei in dilute acids. In general, citric acid is employed. There is little destruction of nuclei, but the alteration of pH affects the nuclear membrane permeability and certain nuclear proteins may be denatured. It is obvious that this method is not suitable for enzymatic studies.

(c) Preparation of nuclei in sucrose solution. The advantages of this technique are described in the "Methods" section. Nuclei obtained in this way are the least damaged in their structure.

There is no doubt that for enzymatic investigations, as in this study, the third method has to be employed.

Table VI summarizes the chemical analysis of nuclei obtained by various isolation techniques. It shows that the DNA/protein; RNA/protein and RNA/DNA ratios in this study are comparable to those obtained with other tissues by other methods.

One of the first reports on a method for the preparation of relatively pure heart muscle nuclei was the one published by Ferguson et al (1965). They studied the biochemistry of cardiac muscle nuclei in an in vitro preparation. However, sucrose containing $CaCl_2$ was used for isolation. The objection can be raised that $CaCl_2$ inhibits the RNA polymerase activity and the "Omni-mixer" used for homogenization can damage the nuclear membrane by its sharp blades.

All the present studies on RNA polymerase are based on the work of Weiss (1960). He prepared the so called aggregate-enzyme from rat liver tissue. The method of preparation partly appears in the work of his followers. The homogenization was carried out in 0.25 molar sucrose containing 0.001M $MgCl_2$ with the aid of a loose fitting stainless steel homogenizer. Widnell and Tata (1964a) modified Weiss's technique according to the principle that by increasing the concentration of sucrose it is possible to get rid of the mitochondrial contamination. The crude nuclear fraction was purified by resuspension in 2.2 molar sucrose and by centrifugation at high speed. While Weiss and Widnell used liver tissue, Nair et al (1968) applied their isolation method for the first time to heart muscle.

As already mentioned, the method adapted in this study is based on Nair et al's work with minor modifications. These are as follows: both the homogenizing and heavy density sucrose medium contained tris-HCl, and the pH was adjusted to 7.6 in order to preserve physiological conditions. The filtration step was omitted, because no difference in the purity of the preparation could be detected with and without filtration. The dilution of the homogenate was determined according to the weight of tissue (1:10, w/v) instead of adding a constant volume. This step is very critical in that a little change in the preparation causes a difference in the purification and moreover the weight of tissue varied with the stage of hypertrophy (see "Methods" section). The volume of 2.4 M sucrose was 6 times the initial wet weight (g) of tissue, in contrast to Nair et al's original specification which was 3.8 times the tissue weight. This modification resulted in a decreased purity of the nuclear preparation but it was a necessary compromise for obtaining a larger amount of material. Nevertheless, the first nuclear preparation showed the RNA/DNA ratio of 0.14 which is smaller than the value (0.22) obtained by Widnell and Tata (1964a).

2. Determination of RNA Polymerase Activity

RNA polymerase has not been isolated from mammalian tissue, although experiments are in progress with chromatin-free RNA polymerase (Liao et al, 1968). Therefore, in most studies, including this one, nuclear preparations had to be used as the source of enzyme.

Weiss (1960) investigated the requirements for the proper functioning of this enzyme under various experimental conditions. The enzyme action was defined as catalysis of the synthesis of ribonucleoside triphosphates into the interpolynucleotide linkage of RNA. The enzyme activity was measured as the rate of incorporation of a labelled nucleotide into RNA. He showed that the activity was dependent on the presence of all four ribonucleoside triphosphates and DNA. The experimental evidence was (i) omission of any one of the four ribonucleoside triphosphates resulted in a marked reduction of incorporation, and (ii) preincubation with extremely small quantities of desoxyribonuclease completed inactivated the system. The radioactive product was identified in two ways: (i) by the addition of ribonuclease, which altered the properties of the product, and (ii) by hydrolysis, which degraded the product into the four kinds of mononucleotides (Weiss, 1960).

In a series of experiments, cation requirement of the enzyme action has been explored. Tata and Widnell (1966), later Nair et al (1968), and others found two incubation solutions for the optimal enzyme activity, one containing Mg^{++} and the other containing Mn^{++} and $(NH_4)_2SO_4$. It was shown that in different ionic milieu some characteristics of the enzyme, such as for example pH optimum, also differ. The problem of Mg^{++}, Mn^{++} and $(NH_4)_2SO_4$ activated reactions are discussed in a later section.

In order to prove in my experiments that true RNA synthesis did in fact occur, a few experiments were done based on Weiss's work. The omission of any ribonucleotide, the addition of ribonuclease or deoxyribonuclease to the incubation medium caused marked decrease in the enzyme activity, i.e. the final product and the requirements for

the enzyme action both indicated that synthesis of ribonucleotide triphosphates into RNA took place. It was shown that the apparent enzyme activity is higher at 37° (see "Methods," p.). However, Maul and Hamilton (1967) found that the incubation lasting from 15 min to 1 hour at 30° resulted in a high level of incorporation. As stated in "Methods" the above two results can be harmonized when one assumes that the ribonuclease activity can be suppressed at a lower temperature, whereas the polymerase activity reaches its highest after longer incubation at such a temperature.

In this work the determination of RNA polymerase was carried out by a modification of the method described by Nair et al (1967). In this modified method, the incubation time for the Mn^{++} plus $(NH_4)SO_4$ activated reaction was 10 minutes instead of 45 minutes. It was found that after 15 minutes a definite decline in the rate of reaction occurred. This could be one reason for differences between the work of Nair and this work (as discussed elsewhere).

3. Method for Production of Cardiac Hypertrophy

A general discussion is presented in the "Introduction". The most widely used methods for inducing cardiac hypertrophy can be listed as: constriction of the aorta or pulmonary artery; partial ligation of the renal artery after unilateral nephrectomy; treatment with thyroid hormone and related compounds; treatment with sympathomimetic drugs; production of anemia; creation of nutritional deficiency states; exposure to hypoxic conditions; production of myocardial ischemia; and exposure to stressful circumstances (Norman, 1962).

The basic functional alterations leading to cardiomegaly induced by the various methods just enumerated were classified by Friedberg and Schmal in 1937 as follows:

(a) increased outflow resistance;

(b) increased venous return to heart;

(c) disturbances leading to weakness of the heart muscle.

The methods described above can be placed into one of these classes according to the way in which they affect the circulation. In addition, each category could be subdivided based on its variation, for example according to the relative rapidity of changing heart size produced.

Coarctation of the aorta was adopted in this study. This is equivalent to stenosis in man, which may be a consequence of rheumatic fever, syphilis or arteriosclerosis (Beznak, 1954). Although no definite correlation has been established between the increased heart weight and blood pressure (Beznak, 1954), there is little doubt that this type of hypertrophy is due to hypertension.

The increased heart weight produced in Beznak's experiments was relatively greater in smaller hearts than in larger ones. This may be of importance in considering the relationship of the degree of differentiation and response of the heart to stress at various ages. In this study the animals were carefully chosen from the same range of body weight, and twelve left ventricles were pooled, whereby the difference among the operated groups was lessened.

II. MISCELLANEOUS FACTORS WHICH MAY INFLUENCE RESULTS

Before discussing the results obtained in this study, consideration should be given to secondary factors which may influence metabolic changes during hypertrophy.

One of the most important factors is the type of experimental method used to produce hypertrophy. The ratio between the parenchymal and connective tissue components of an organ changes according to the method used for inducing cardiomegaly. The rapidity of the growth of the muscular and collagenous components of the tissue mutually influences not only the mechanical properties of the organ but other functions such as diffusion of metabolites and the distribution of water and electrolytes as well (Bartošova et al, 1969). Thus variations in the relative proportion of collagen influence the speed and magnitude of the biochemical events during hypertrophy.

In addition to the method of producing hypertrophy the age of the experimental animal is another factor affecting the rapidity of the growth of the muscle and stroma (structural) proteins. This is one of the reasons why a greater percentage increase in cardiac weight was observed in younger rats following aortic narrowing (Beznak, 1954).

Another critical factor regulating the magnitude of hypertrophy is the size of the stimulus. Even using the same method, such as aortic constriction with utmost care, the increase in weight varied from 10 - 60% above control values (Nair et al, 1968). The reason for this scattering may be that a small difference in the degree of narrowing may result in a great difference in the degree of resultant hypertrophy.

Capers (1964) postulated that the degree of narrowing influences the degree of hypertrophy through the change in the maximal pressure loading of the left ventricle. He showed that the RNA concentration which is one of the most characteristic metabolic variables in the hypertrophying heart varies with the extent of pressure loading at each chamber of the heart. However, Posner and Fanburg (1968) could not demonstrate correlation between the extent of RNA labelling and the pressure time index.

Differences among studies on induced protein synthesis may be attributed to different types of tissue used. Until recently, the liver has been extensively used for technical reasons. However, the reaction to partial hepatectomy differs basically from the reaction to hemilateral nephrectomy, or aortic constriction, since liver tissue has a much greater mitotic ability in adults than do the other tissues. There are differences not only among different types of tissue, but also among the muscle tissues used, for example the neuro endocrine regulation is much more complex in cardiac muscle than in skeletal muscle. An interesting example of the role of the neuroendocrine system, especially on the heart, is that experimental hypertrophy could be produced solely by applying light or sound stress (Norman, 1962).

The last condition, which is not very well defined but which results in variations in experimental data, is the general state of the experimental animal. An example is that of Ilan and Taubert (1968) who observed a higher rate of RNA synthesis in the uterus during the preovulatory phase as compared to the postovulatory phase. Seasonal changes appear to influence protein and nucleic acid metabolism. Rats showed an unexpectedly high mortality after pinealectomy around the beginning of spring. At springtime, the rats become more sensitive to sedatives.

Many rats suffered from the subclinical stage of supperative disease of the lungs. Sections through small foci of inflammation showed accumulation of mucosa or mucopurulent plugs in the bronchi and adjacent parenchyma (G. Radford, personal communication). This illness can only be eliminated by breeding pneumonia-free animals. Although animals with clinical symptoms were never used for experiments, the exclusion of subclinical cases was not possible and this might have contributed to the reaction to aortic constriction. However, the results obtained by control animals were extremely reproducible.

In order to compare my data with data obtained from similar experiments by others, attention should be given to these secondary factors. For example, the method in this study is based on Nair et al's (1968) work, but the rats used in their study were of a different type (Sprague-Dawley), the body weight was smaller (200 - 220 g) and their operative method differed (aorta ascendens was constricted). These differences probably influenced the results which are somewhat dissimilar.

III. CHEMICAL CHANGES CAUSED BY EXPERIMENTAL HYPERTROPHY

The observed alterations in the chemical composition of each tissue resulting from induced hypertrophy depend upon the tissues used and the methods applied. It is not yet settled whether a new type of RNA or a new protein or both plays a role in the initial nuclear response. Although there is experimental evidence that a humoral factor, probably a new RNA, can induce protein synthesis in normal animals (Villee, 1970) and, according to a recent theory (Pirrotta and Ptashne, 1969) newly synthesized RNA polymerase initiates a series of changes, the basic composition of RNA or amino acid sequences of protein have been shown not to differ from those existing in normal conditions.

In this study, DNA, RNA and protein determinations were carried out in the nuclear preparation of normal and hypertrophied left ventricles from 4 hours to two weeks following aortic constriction.

RNA Synthesis

RNA has been measured in hypertrophied hearts in numerous studies (Nair et al, 1967; Beznak et al, 1969; Meerson, 1966; Fanburg et al, 1968, etc.) and the total content has invariably been found to increase over control values. In several of the acute experiments, RNA concentration increased very early after the application of the hypertrophy producing stimulus. The concentration of RNA usually returned to normal levels after a period of time, reflecting a later increase in other tissue components.

The values reported by several workers for changes in RNA and heart weight in experimental cardiac hypertrophy are summarized in Table VII. The differences in published values of RNA content of hypertrophied hearts probably reflect differences in the degree of hypertrophy and in the time interval between measurements, application of the stimulus, and by different methods on different species.

In Nair et al's (1968) experiments, the maximal increase in RNA concentration was 30% and in total ventricular RNA, 65%, two days after the production of acute supravalvular aortic stenosis, whereas Beznak (1969) found a 72% increase in the total left ventricular RNA during 4 weeks of observation.

Some workers suggest that the stimulation of RNA synthesis occurs selectively during hypertrophy. For example, Moroz (1967) obtained data demonstrating that ribosomal RNA synthesis increases to a great extent. Similarly, Nair et al (1968) observed an increase in RNA polymerase activity, the product of which is believed to be ribosomal RNA. In contrast, Schreiber (1968) emphasized the importance of m RNA formation.

However, the distribution of labeling in various types of RNA (28S, 18S and 4S) which were separated by sucrose density-gradient centrifugation remain unchanged in heart hypertrophy (Fanburg, 1968; Koide and Rabinowitz, 1969).

The changes in RNA synthesis demonstrated in this study in nuclear preparation confirms some of the experimental findings in the literature.

Maximal increase in RNA concentration (100%) and RNA content(100%) - Fig. 6, 7 - are comparable with data on total RNA (see Table VII), but the concentration in this study increased much more than was observed by Nair et al (1968) (30%). This discrepancy is likely due to the fact that the RNA concentration in nuclear preparation, and not that of heart muscle, was measured in this study.

Protein Synthesis

In vivo measurements of the incorporation of radioactive precursor into protein have been used in several studies to determine changes in the rate of protein synthesis in hypertrophy (Posner and Fanburg, 1968; Gudbjarnason et al, 1964). These studies demonstrated that labelling of heart protein increased within a day or two after aortic constriction. However, the rate of protein degradation and analysis of the type of protein synthesized are yet to be investigated.

Because only a very small percentage of protein synthesis occurs in the nucleus, the detectable change in protein synthesis should be much less than that in RNA synthesis. In this study, however, the protein concentration in the nuclear suspension, obtained from hypertrophying left ventricles, showed a 50% increase on the second day after aortic constriction compared to the protein concentration found in control nuclear preparation.

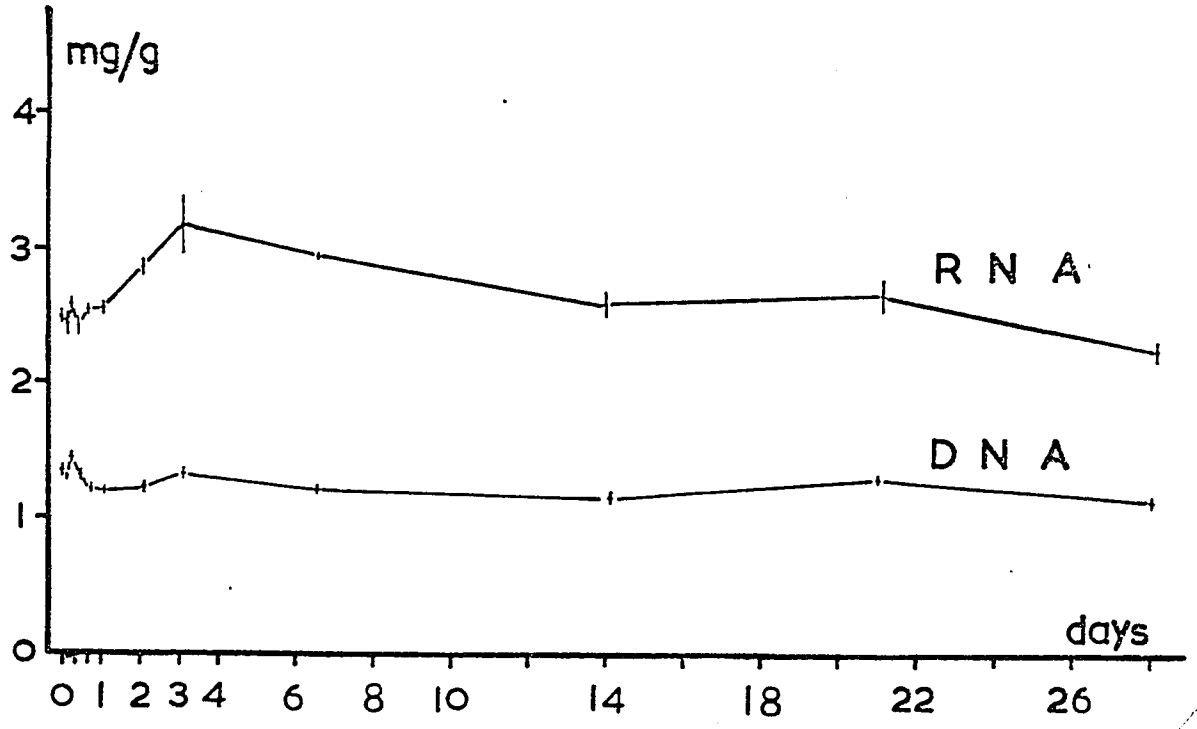
Relation of RNA to DNA in Hypertrophied Left Ventricle

The purity of the nuclear preparation could change when the nuclei of hypertrophying ventricle are isolated. Therefore, changes in DNA/RNA ratios obtained could either be due to true biochemical changes, or to alterations in the yield or purity of the

nuclear suspension. However, it is shown in this work that the ratios DNA/protein and DNA/RNA, which were used as an indicator of the purity in control experiments, are taken to indicate biochemical changes in DNA, RNA and protein metabolism. This assumption may not be correct, since it is possible that nuclei from muscle cells and connective tissue cells may behave differently during the isolation procedure. It can be considered that DNA content is proportional to the number of cells. Increase in the ratio without any change in DNA concentration is considered to be due to an enlargement, i.e. hypertrophy of cells (Matsumoto et al, 1965). When cardiac hypertrophy is induced in rats, the total quantity of DNA usually increases, whereas the concentration either remains the same or decreases (Grimm et al, 1966).

Beznak et al (1969) used the same method to induce hypertrophy. She found a delayed increase in DNA content in ventricular tissue as compared to that of RNA (see Figure below) and thus implied that those cells in which RNA synthesis is increased are not necessarily the cells in which DNA synthesis increased. In other words, the early RNA synthesis probably occurred in myocardial cells, while the delayed increase in DNA synthesis took place in non-muscular tissue.

Change in RNA and DNA Concentration in the Left Ventricle following Aortic Constriction of Rats

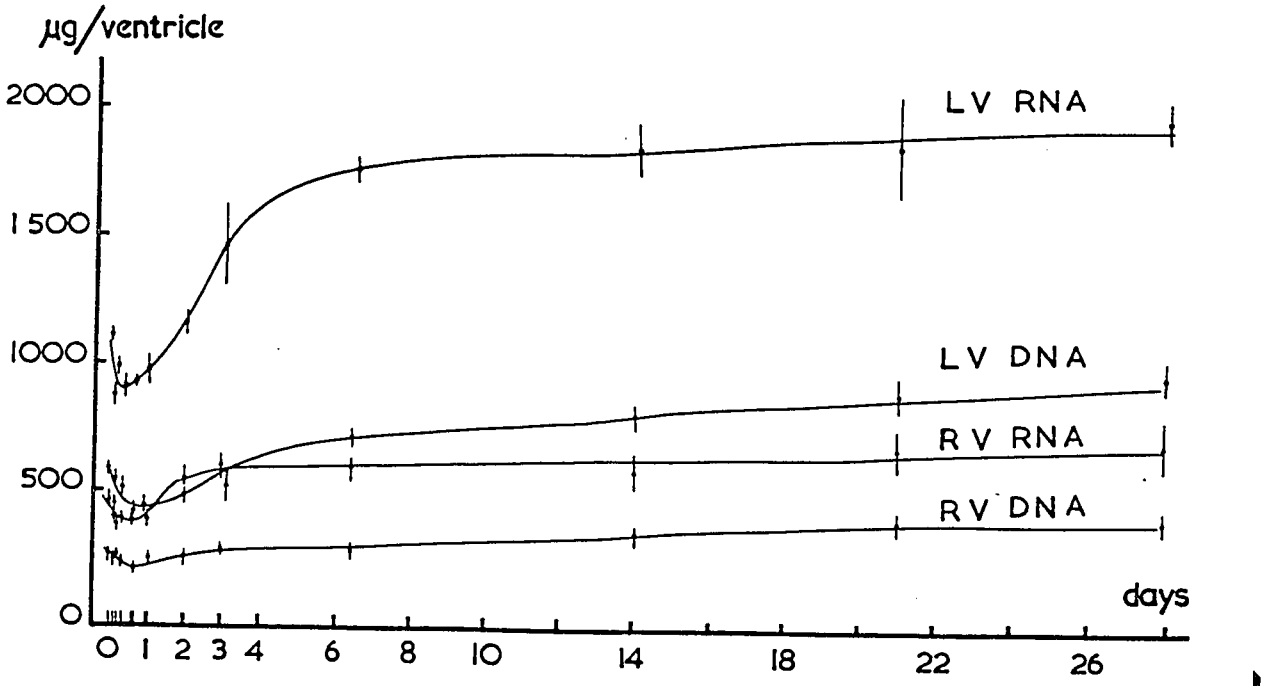


Nucleic acid contents, mg/g wet weight, are shown in the ordinate and the days after the operation in the abscissa. Number of experiments are average 8.

The figure indicates among other findings, the DNA concentration is quite stable.

The changes of the total ventricular nucleic acids after the constriction can be seen in the following figure, which is also taken from the work by Beznak et al (1969).

Change in Total RNA and DNA Contents of the Ventricles during the Development of Hypertrophy



Total contents of nucleic acids in left and right ventricles (LV and RV) are expressed in the ordinate as µg/ventricle.

The time after operation is indicated on the abscissa in days.

The figures show that DNA concentration decreased immediately after the operation, then returned towards normal values, while DNA content increased gradually around the 2nd to 4th day. In general, similar results were obtained using purified nuclei in this study; namely DNA concentration decreased immediately after the operation and only after the second day increased over the control value. DNA concentration decreased again around the 6th day, and 10 days after the operation it became comparable with the control values (Fig. 9).

The difference between the changes in DNA concentration and content is that the DNA content remains above the control value after a rapid increase, the latter corresponding to the mitotic activity of the connective tissue as a reaction to the stress. This can be explained as the induced hypertrophy being a kind of growth process: the hypertrophying heart reaches a certain weight which makes the heart able to compensate for the extra work and maintains this weight, although the mechanism by which the growth is levelling off is unknown.

The ratio DNA/RNA falls very early after constriction (Fig. 10), suggesting a few possibilities as follows:

(a) Experimental evidence is obtained in this study proving the <u>true increase in RNA synthesis</u>: the rate of incorporation of labelled nucleotide into RNA increased during the development of hypertrophy. The ratio remains under control values at least for the first two weeks following constriction, indicating that in spite of the increased mitotic activity of connective tissue cell at approximately the same time, relative increase in RNA synthesis also takes place.

(b) A different partition can take place between RNA in the nucleus and cytoplasm, depending on the rate of migration of RNA. Though knowledge about the distribution of the newly synthesized RNA is not well advanced, it has been proven that the nucleus maintains the normal concentration of RNA in the nucleus even if RNA synthesis is disturbed. Consequently, it is possible to suppose that in the case of increased RNA synthesis an increased migration of RNA to cytoplasm occurs. In other words, passage of RNA through the nuclear membrane appears to play a secondary role. The data showing an increased amount of RNA in the cytoplasm (Moroz, 1967) suggest that the increased nuclear RNA cannot be a result of decreased migration to the cytoplasm.

(c) The increased RNA concentration of some cell organelle, e.g. ribosomes, which is a constant contamination of the nuclear fraction, may add to the RNA concentration measured in the nuclear preparation. The electron microscopic picture did not indicate any ribosomal contamination and considering the constant results obtained from control animals, it is unlikely that the increased nuclear RNA obtained from narrowed animals could be due to ribosomal contamination.

(d) A change in the cell during hypertrophy alters the efficiency of the nuclear fractionation procedure. As mentioned previously, an unanswered question is whether the isolation procedure of nuclei of hypertrophied hearts is as good as that of the control.

The protein synthesis also increases out of proportion to the connective tissue proliferation (Fig. 9) as is indicated by the ratio of DNA over protein. It fell to 63% after a few hours of induced hypertrophy and did not reach the control ratio for at least two weeks.

These data support the view that hypertrophy occurs after aortic constriction in the muscle cell nuclei. The increased RNA and protein contents in relation to DNA of the nuclear preparation from the left ventricles are reflections of the nuclear hypertrophy in the enlarged muscle cell. In support of this, increased number of nucleoli in hypertrophied heart muscle was observed (Meerson et al, 1968).

IV. TIME COURSE OF INCREASED RNA AND PROTEIN SYNTHESIS

It is generally accepted that increased nucleic acid and protein synthesis plays a central role in the growth of any tissue. Therefore, an evaluation of pattern of increase over a period of time of nucleic acid and protein synthesis should help in clarifying processes translating a stimulus which induces cardiac hypertrophy to the actual increase in tissue mass (Fanburg, 1970).

However, caution should be exercised that the occurrence of one event after another may not necessarily reflect the physiological sequence. Further, the precedence in time of the first event does not imply necessarily that it is causal. Rather, both events could be the result of the initial stimulation or of some intermediary process.

Three major approaches have been used: (a) the rate of synthesis of RNA or protein which can be measured by in vivo labelling using one of their precursors; (b) the concentration of RNA or protein in the tissue, and (c) the RNA polymerase activity during the development of hypertrophy.

These methods can also be used to follow the sequence of biochemical changes in the presence of inhibitors of RNA or protein synthesis.

RNA Inhibition

Actinomycin D is known to inhibit RNA synthesis in both bacteria and mammalian tissues. Although it appears that the action of this antimetabolite is not specific, it is known that one of its actions is to block the DNA template so as to interfere directly with RNA synthesis. Actinomycin D has been used to study the hypertrophying heart after aortic constriction. Meerson et al (1965) and Zuhlke et al (1966) found that actinomycin D inhibited the incorporation of radioactive precursors into heart proteins in animals the aorta of which had been constricted. This dose of the drug had no effect on RNA labelling in normal hearts, suggesting that actinomycin D may interfere selectively with the RNA synthesis of hypertrophying heart and that this RNA synthesis is a prerequisite for the increased rate of protein synthesis.

Protein Inhibition

Puromycin is believed to inhibit protein synthesis at the level of translation. At a dose that inhibited protein synthesis, this antibiotic was found to inhibit increased RNA labelling in the hypertrophying rat heart but not in the control heart (Fanburg and Posner, 1968). The enhanced RNA labelling was not observed until four to eight hours after aortic constriction in the absence of puromycin. The administration of puromycin inhibited protein synthesis within the first four hours after aortic constriction, thereby depressing subsequent increase in RNA synthesis. These authors assumed that inhibition of RNA synthesis by the antibiotic results solely from its effects on protein synthesis.

A similar reaction to puromycin was observed using estrogen stimulation on rat uterus (Noteboom and Gorski, 1963; Gorski and Morgan, 1967). During the first two hours, i.e. before a general effect on protein synthesis occurred, puromycin was capable of blocking the effect of estrogen on RNA synthesis and on RNA polymerase activity. From these experiments, it can be concluded that protein synthesis precedes the increase in RNA synthesis in induced hypertrophy.

The data obtained from the inhibition of RNA and protein synthesis appear to be contradictory. This could be explained as follows: protein synthesis may initially be required for the increased RNA synthesis but this protein is very small in amount and cannot be detected by existing methods.

To study the early biochemical events following cardiac hypertrophy the rapid production of weight change, i.e. protein synthesis is most suitable and this was the main reason for the use of aortic constriction.

Although experiments using inhibitors of the protein synthesis provided evidence that a stimulation of protein synthesis is required for the increased RNA synthesis, the majority of experiments on metabolic changes during the development of hypertrophy deal directly with RNA synthesis. In contrast to the work concerned with the rate and type of RNA synthesized during hypertrophy, there have been only few experiments to observe the protein synthesis which appears to be a prerequisite for the changes in RNA synthesis.

In 1964, Meerson et al had provided data supporting the importance of early stimulation of protein synthesis. They found that when the contractile function of the heart increases, so does the intensity of protein synthesis as compared to the intensity of breakdown. According to Meerson et al's suggestion, the increased breakdown product affects the stimulation of RNA synthesis by derepressing the DNA template for transcription.

When RNA synthesis was measured by the in vivo labelling technique, an increase was observed within the first 4 hours after aortic constriction (Fanburg and Posner, 1968). Similar early incorporation was found in Beznak et al's experiments (1969). However, RNA polymerase activity in nuclei of the heart of narrowed rats showed the earliest detectable change only 12 hours after operation (Nair et al, 1968). The maximal increase was obtained on the second day (Nair et al, 1968; Beznak et al, 1969).

In this study it was possible to detect increased polymerase activity as early as 4 hours following aortic constriction (Fig. 14), the highest activity being found on the 3rd - 4th day. Such an early increase in the in vitro assay has not been reported previously, although there are studies showing the early increase in the in vivo labelling (Beznak et al, 1969; Fanburg and Posner, 1968, etc.). This is in agreement with Sobel and Kaufman's (1970) suggestion about the biphasic alteration in RNA metabolism. They postulate that the early increase in the enzyme activity was not detected but it has to exist (more will be mentioned in the following chapter).

The change in RNA concentration (Fig. 6) becomes obvious 8 hours after the operation, but in some experiments it was detected as early as four hours. The maximum level is reached on the third day. These data correspond to the observed changes in the polymerase activity in this study and in the cited works (Nair et al, 1968; Beznak et al, 1969; Sobel and Kaufman, 1970).

The time course study (Fig. 5) demonstrates an increase in nuclear protein synthesis. This result suggests that the size of nuclei is increased. It is also possible that the change may be a reflection of the changes in the protein concentration in the whole tissue. The sudden, 50% increase in the protein concentration on the second day following narrowing of the aorta is similar in course and magnitude to the increase found in ventricular protein in other studies. These results are also in harmony with Moroz's (1967) findings, i.e. the increased ability of microsomes to incorporate amino acid into protein in hypertrophied condition is due to the increased number of ribosomes.

The magnitude of hypertrophy, as expressed as the ratio of the weight of left ventricle to body weight, becomes greater after an approximately 24 hour lag period (Beznak et al, 1969), reaching its highest between 48 and 72 hours. Comparing this with the just discussed time course of biochemical changes it becomes evident that the alteration in RNA synthesis occurred before the major increase in the cardiac tissue mass. In other words, the nucleic acid synthesis responded to the stimulation preceding the increase in tissue mass.

Nucleic Acid and Protein Synthesis in Various Organs

Different organs show various patterns of nucleic acid and protein synthesis following an application of a stress.

For many years, liver regeneration after partial hepatectomy has served as a model for study of the regenerative growth process (Pogo et al, 1967; Bucher, 1967). As it appears later, the growth process which is the most akin to cardiac hypertrophy is that which occurs in kidney after unilateral nephrectomy. Some works indicated that this process is a true hypertrophy of nephrons (Halliburton and Thomson, 1965; Malt and Miller, 1967; Malt and Lemaitre, 1968). Thyroxin and growth hormone were found to stimulate growth of the liver (Korner, 1966, 1967, 1968; Tata and Widnell, 1966) and estrogen stimulated growth of the uterus (Hamilton et al, 1965, 1967, 1968; Gorski, 1964, 1965, 1967).

For comparison, the results obtained using kidney, uterus and skeletal muscle are briefly discussed.

Kidney : The synthesis of bulk renal RNA during compensatory growth of mouse kidney was studied for 4 weeks after unilateral nephrectomy (Malt and Lemaitre, 1968). The maximal rate of RNA synthesis was observed 2 days after nephrectomy and the plateau in compensatory growth was reached during the 9th week. The increase in weight was accounted for largely by increasing amounts of protein. Halliburton and Thomson (1965) found fully-developed increased RNA synthesis at the 24th hour following nephrectomy and the highest rate of growth occurred between the first and fourth day. Increases in the mitotic activity of the highly differentiated cells were found in the kidney in contrast to heart muscle tissue.

Uterus: It has been shown that the biological action of estrogen on the uterus of the ovariectomized rat in vivo began by synthesis of rapidly-labelled nuclear RNA (Hamilton et al, 1967) and an increased activity of DNA dependent RNA polymerase in isolated nuclei (Gorski and Morgan, 1967). Nuclear RNA synthesis in vivo was accelerated prior to an increased activity of the two polymerases. During the 3 week period following ovariectomy of the adult rat, it was found that the rate of synthesis of nuclear RNA was decreased by the in vivo labelling technique, followed by the decrease in the activities of polymerase in isolated nuclei (Hamilton et al, 1968). Changes in protein synthesis followed the change in RNA synthesis. It is possible that the increased polymerase activity during the early phase of hypertrophy is below the resolution of the analytical technique. Or perhaps, participation of other cellular components may be required for an early change in nuclei (Sobel and Kaufman, 1970). Thus, the increase in RNA synthesis observed in vivo appeared to precede the increased polymerase activity measured in the nuclear preparation.

Figure 16 gives a summary of the findings which have been obtained in relation to this problem using uterus tissue (Hamilton et al, 1968). The hormone induced stimulation of nuclear protein synthesis precedes an increase in RNA polymerase assayed in the Mg^{++} activated system. Both precede the increased concentration of cytoplasmic ribonucleoprotein and the C^{14}-leucine incorporation activity by polyribosomal preparations. Thus, the early protein synthesis responding to induced hypertrophy is that of nuclear ribosomal protein. In order to synthesize the ribosomal protein, the existence of a type of RNA is required for nuclear ribosome-like particles.

FIG. 16

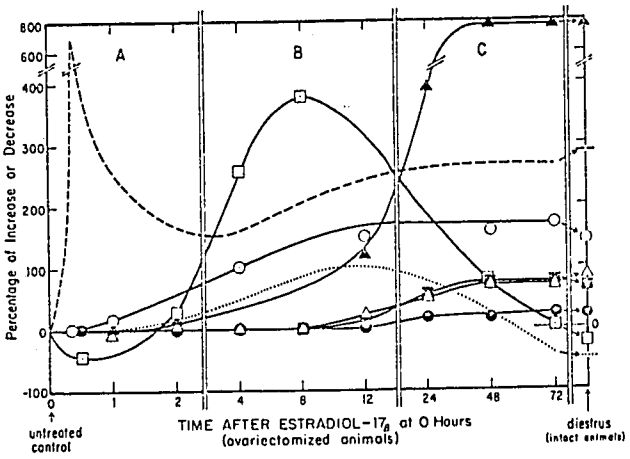


FIG. 1. Time course for the effects of administration to the ovariectomized rat of estradiol-17β on six of the uterine biochemical parameters listed in Tables 1 and 2. The data from the tables are expressed as percentage of increase or decrease in the values over or under the values observed for the untreated control animal. The symbols on the right ordinate indicate the biochemical parameters of the uterus of the intact animal in diestrus similarly expressed as percentages of the values for the untreated control.

O—O, Activity of the Mg^{2+}–activated RNA polymerase reaction; Δ—Δ, activity of the Mn^{2+}-$(NH_4)_2SO_4$-activated RNA polymerase reaction; □—□, specific activity of nuclear protein rapidly labeled *in vivo* with 1-H^3-methionine; ⊙—⊙, ratio of nuclear RNA/DNA; ⊡—⊡, ratio of nuclear protein/DNA (percentage values not shown prior to 8 hr, see Table 2); ▲—▲, concentration of cytoplasmic RNP; — specific activity of nuclear RNA rapidly labeled *in vivo* with H^3-uridine; ...., 1-C^{14}-leucine incorporation activity *in vitro* of cytoplasmic polyribosomal preparation. The last two parameters are taken from refs. 1 and 4.

Hamilton et al. (1968) Proc. Nat. Acad. Sc. U.S. 5a, 1265.

Skeletal muscle: Kaufman and Sobel (1970) studied the time course of certain biochemical events occurring in rat soleus muscle undergoing hypertrophy in response to tenotomy of the synergistic muscles. Their studies revealed that during the first 24 hours after the onset of the stimulus there is little change in the gross biochemical characteristics of the muscle; the quiescent period ends dramatically during the next 24 hours. By that time the following changes have almost reached their maxima; increase in tissue RNA; increase in microsomal and ribosomal RNA content, and increase in the rate of in vitro amino acid incorporation into protein.

V. CHANGES IN THE APPARENT ACTIVITY OF RNA POLYMERASE

1. Significance of a Change in RNA Polymerase Activity

The genetic information of m RNA is obtained through transcription of the DNA template by RNA polymerase. The basic function of the enzyme is to synthesize nucleotide triphosphates into RNA. The messenger RNA (m RNA) determines the sequence of amino acids in the protein and therefore the composition of m RNA varies according to the protein. Further, a different corresponding transfer RNA exists to carry each amino acid to the site of protein synthesis. The variabilities and specificities of proteins probably necessitate more than one polymerase for the synthesis of t RNA.

The question as to whether there are two polymerases, one synthesizing r RNA and the other m RNA or whether there is one kind of enzyme synthesizing two RNA's according to the different biochemical conditions has been dealt with by many investigators. Widnell and Tata in 1966 pointed out that the behaviour of the polymerase changes in different ionic milieu. Incubation conditions have been studied with regard to the presence and absence of ammonium sulphate, differences in the activation by Mg^{++} and Mn^{++}, the pH optima and the effects of NaF and cysteine. Although under all conditions, the reaction was DNA-dependent and dependent on the presence of all four nucleotide triphosphates, the products of the reaction in the presence and absence of the salt were found to have different base compositions, different nearest neighbour base frequencies for AMP and GMP and different sedimentation

characteristics in sucrose density gradients. Nair et al (1967) confirmed these results using rat heart muscle.

Next the question of localization of polymerase was dealt with. Pogo et al in 1967 investigated the effects of salt and specific divalent cations on ribonucleic acid synthesis in nuclei isolated from normal and regenerating liver. On the premise that carefully isolated nuclei preserve their natural organization and continue to synthesize the types of RNA characteristics of the original cell, the newly formed RNA was analyzed and the localization was followed by electron microscopic autoradiography.

It was found that the composition of RNA formed in the nucleus was influenced by a very low concentration of divalent cations which does not disrupt the nuclear structure. They showed that in the presence of Mg^{++}, in agreement with Widnell and Tata (1966), a ribosomal type of RNA was synthesized in the nucleolus, and in the presence of Mn^{++} and $(NH_4)_2SO_4$, a messenger type of RNA was formed. This occurred in the nuclear plasma associated with nuclear chromatin (extranucleolar region of the nucleus). The addition of Mn^{++} and $(NH_4)_2SO_4$ increased the grain densities over the extranucleolar region, indicating that the m RNA synthesis became predominant, while the nucleolar r RNA synthesis continued. It became evident from these experiments that the Mn-NH_4 medium increases the synthesis of informational RNA, although the mechanism of this effect has to be evaluated in more detail.

The measured RNA polymerase activity is an apparent activity representing the sum of polymerase and ribonuclease activity. The ribonuclease has a very special role in RNA production, because the actual RNA existing in the cytoplasm and synthesizing protein are the products of RNA broken by ribonuclease. Homoki et al (1967) pointed to

the inhibitory action of salt on nuclease activity of isolated rat liver nuclei. Heller and Kulka in 1968 found two ribonuclease activities in the developing chick pancreas, one requiring Mg^{++}, the other being a "neutral" polymerase.

Similarly, in this study, ribonuclease acitivity was shown to increase 50% in the Mg^{++} activated system, compared to that in the presence of ammonium sulphate. But the difference between the activities of the two systems is more than the effect on ribonuclease which can only partly explain the higher apparent activity of polymerase measured in the presence of the salt.

The selective activation of m RNA synthesis by salt requires further explanation. During an increased nucleic acid synthesis several authors observed no increase in the Mn^{++} and $(NH_4)_2SO_4$ activated system compared to the Mg^{++} activated reaction. For example, Pegg and Korner (1967) observed a stimulation of rat liver RNA polymerase activity in the Mg^{++} activated system following growth hormone administration but not in the presence of the ammonium salt. Similarly, Gorski (1964) observed a marked increase in uterine Mg^{++} activated polymerase activity within 1 hour after estrogen injection, but in the presence of $(NH_4)_2SO_4$ estrogen had no effect; the activity was equal to that of the control. Less increase in polymerase activity in $(NH_4)_2SO_4$ system in regenerating liver nuclei was reported by Bucher (1967).

With heart muscle, Nair et al's (1968) experiments indicate a 40% increase following aortic constriction in the Mg^{++} activated system, but only a 10% increase in the presence of the ammonium salt.

In contrast, O'Malley and McGuire (1969) observed a similar increase in both systems after estrogen stimulation using immature chick oviduct.

From these experiments the following hypothesis can be proposed: ammonium sulphate can nullify some inhibitory or controlling factors. An inducer of RNA synthesis cannot exert an effect on the stimulation of polymerase in the presence of salt, because the reaction is already de-repressed. Experiments in which some increaseswere detected suggest that the physiological effect on the repressor control mechanism by an inducer was stronger than the de-repressor effect of the salt.

Goldberg (1970) made another contribution to the present knowledge about the mechanism of the salt effect. He showed that using the purified enzyme, ammonium sulphate no longer stimulates the enzyme, making clear that the salt effects are not effects on the RNA polymerase enzyme but on the nuclear control elements. Travers and Burgess (1969) observed that the RNA polymerase contains an easily dissociated protein subunit required for proper initiation, called the sigma factor, and each class of promoters is recognized by a different sigma factor. Experimental evidence supports the idea that the so-called ammonium sulphate-activated enzyme is basically not a different enzyme from the nucleolar enzyme but is an enzyme with altered properties due to the presence of ammonium sulphate and Mn^{++}, which changes the enzyme's associations to the control elements of the nucleus in some unknown way. Therefore it is postulated from evidence quoted as well as from my study that there is a single enzyme, the apparent activity of which is greatly influenced by the presence of the salt.

Expressing in percentage the changes in enzyme activity following constrictinn (Fig. 15) except in the first few hours, the Mg^{++} activated system showed a greater change than was obtained in the experiments enumerated above (Pegg and Korner, 1967; Gorski and Morgan, 1967; Bucher, 1967; Nair et al, 1968). This suggests that the stronger stimulus

did overcome the effect of the salt resulting in the de-repression of the control inhibitory mechanism. In other words, the depression could not be reversed by the salt to the same extent as that produced by the induced hypertrophy.

In this study, the salt activated system showed an increase of 60% and thereafter was less stimulated than in the Mg^{++} activated system. If there was one common product, the activity in the two systems should have been always in the same relationship. Therefore it can be concluded that the products of the reactions, RNA, in the presence and absence of the salt, are different but their rate of synthesis is similar rather than the rate of synthesis of the one type of RNA varying according to the incubation condition.

How can one unite these experimental results suggesting the existence of one enzyme and two products ? Data obtained by autoradiography together with biochemical studies (Hulbert et al, 1969; Page et al, 1967) as well as the results showing the effect of salt, all suggest the validity of the following hypothesis, namely that most of the total polymerase of the nucleus is associated with non-nucleolar templates and is strongly repressed in its activity. In the case of de-repression by salt the nucleoplasmic DNA templates become readable for the polymerase, the rate of synthesis by the de-repressed reaction becomes dominant over the nucleolar reaction and therefore the amount of the new product exceeds that of the product synthesized in the absence of ammonium. Although the characteristic of the product is determined by the DNA template and not by various ionic milieu, both the quantity and quality of the product changes.

2. Changes in the Two Polymerase Systems during the Development of Hypertrophy

The Mg^{++} activated reaction forms r RNA and the product of the reaction in the presence of ammoninm sulphate and Mn^{++} is m RNA. As an initial biochemical event in response to a stress, particular significance lies in the sequence of changes in the two systems synthesizing RNA.

Previous experiments produced results of either (i) simultaneous change or (ii) non-simultaneous change of r RNA and m RNA as follows:

(i) Experiments using precursor labelling showed that the increased RNA synthesis during hypertrophy occurs to about the same extent in all species of RNA (Fanburg and Posner, 1968; Koide and Rabinowitz, 1969). The distribution of labelling in various types of RNA (28S, 18S, 4S) separated by sucrose density-gradient centrifugation was the same for hearts of animals with aortic constriction as for those of control animals and there was no selective increase in labelling for any one species of RNA.

Sobel and Kaufman (1970) observed that RNA polymerase activity of hypertrophying muscle nuclei was enhanced in both magnesium-dependent and ammonium sulphate-manganese dependent assay systems.

(ii) Some of the experiments could not reveal significant stimulation in the ammonium salt dependent reaction (Nair et al, 1968; Korner, 1967). Other authors found increased polymerase activity in the r RNA-producing system earlier than in the m RNA formation. For instance, Malt and Miller (1967) observed a reciprocal stimulation of m RNA

and r RNA production, i.e. the peak production of r RNA was on the 2nd day following unilateral nephrectomy when m RNA synthesis began to increase, reaching its peak activity on the 4th day. Others reported that the stimulation of r RNA synthesis precedes the increased m RNA synthesis, e.g. the results of Hamilton et al (1967) on the uterus after estrogen injection, Tata and Widnell (1966) on liver tissue after thyroid hormone administration, etc.

However, Schreiber et al (1969), by over-loading the perfused heart, observed reciprocal increases in the m RNA and r RNA synthesis in the opposite order to that just described. The use of the perfused preparation allows the study of very early changes which were not possible to detect in the operated animals.

The results of this study (Fig. 13-15) are in general in agreement with those findings in which selective stimulation of one kind of RNA occurs during hypertrophy.

However, at a very early time (4 hours) following constriction the activity in the Mn^{++}-$(NH_4)_2SO_4$ dependent system was stimulated 60% above that measured in the Mg^{++} dependent system. The early and greater increase in the NH_4-dependent reaction suggests an important role for the m RNA in the initial period of increased protein synthesis. This finding is supported by the experimental evidence presented by Schreiber et al (1969), who demonstrated a distinctive response in the m RNA producing reaction. However, Nair et al (1968) did not demonstrate any significant change in the Mn^{++} activated system. My observation showed that after the third day of operation, the smaller stimulation in the salt activated system becomes evident.

The delayed increase in DNA synthesis (Fig. 9) as compared to the RNA (Fig. 15) is of particular importance. One of the reasons is the fact that the enzyme activity was mainly expressed as incorporation per mg DNA, because there is no great fluctuation in DNA content in the muscle cell. However, from the third day, the increase in DNA content due to connective tissue hyperplasia, may have an important influence on our results. The early changes in polymerase may therefore reflect the actual changes in muscle cell nuclei more closely than those seen after the 3rd day. The activity of RNA polymerase in Fig. 13 - 15 is expressed as μμmole of labeled UTP incorporated into RNA per mg DNA. Since the DNA content did not change, the increased enzyme activity in the early hours represents a real increase. However, the highest level of activity (3rd - 4th day after narrowing) reflects the contribution of hyperplasia in the connective tissue and very likely the increase in the amount of RNA polymerase as well.

The time course of biochemical events during the process of hypertrophy has a particular importance in the investigation of the possible critical reaction causing stimulation in protein synthesis. Sobel and Kaufman (1970) demonstrated an increased synthesis of RNA measured by the in vivo labelling technique within 24 hours following an operation producing hypertrophy of skeletal muscle, while an increased activity of RNA polymerase of isolated nuclei was not found until 48 hours. A similar time course of events has been observed with heart muscle (Fanburg, 1970).

The biphasic alteration in RNA metabolism was also observed by Sobel and Kaufman (1970) on skeletal muscle undergoing hypertrophy, though in their experiments the early increase was detected only by the incorporation studies and not by polymerase assay. Sobel and

Kaufman proposed that the changes in the intracellular environment which led to an early increase in the in vivo activity of polymerase, cannot exert an effect on nuclei separated from other cellular components. Besides the differences between skeletal muscle and heart muscle, differences in the isolation technique may account for slight differences in the results between Sobel and Kaufman's work and this study.

The results obtained by this study agree with the experimental work dealing with protein synthesis during the development of cardiac hypertrophy. Moroz (1967) demonstrated an increased content of ribosomal RNA in the microsomes, suggesting the presence of a greater number of ribosomes and D. Brodie (M.Sc. thesis) showed a 200% increase in the specific activity of microsomes to incorporate amino acid into protein on the second day following the same operation as was applied here. There was no increased detected before that time, suggesting that the increased microsomal activity corresponds to the second phase of increase in polymerase activity observed in this study. The microsomal activity was still high 10 days after the operation, in agreement with the findings of this work.

VI. FACTORS CONTROLLING THE APPARENT ACTIVITY OF RNA POLYMERASE DURING THE DEVELOPMENT OF HYPERTROPHY

The majority of experiments dealing with the mechanism of transcription were carried out by using bacterial polymerase because of the availability of the method to purify bacterial enzyme. The basic reaction of this enzyme that synthesizes RNA from nucleotide triphosphate does not differ from the mammalian. This is indicated by the fact that bacterial polymerase is able to synthesize RNA in mammalian preparation (Bannai and Terayama, 1967). The control mechanism, however, which influences the composition and amount of products, differs in the two reactions in their complexity.

The RNA polymerase of the bacteria does not respond to the same control mechanism as the enzyme in the mammalian tissue and transcribes much of the genome in an indiscriminate, uncontrolled manner. This was shown by the difference between the products synthesized by the endogenous bacterial enzymes in the mammalian system (Goldberg, 1970).

Repression of DNA template

The theory that the control mechanism works through repression rather than stimulation led to some very important discoveries. Possible repressors, histones were first discovered by Miescher 100 years ago. Histones are always associated with DNA in the chromosomes of organisms with an organized nucleus and they have not been found in any other place (Bonner, 1966).

Removal of histone from chromosomal DNA causes de-repression of genetic material previously repressed i.e. it increases

the template activity for RNA synthesis. An addition of histone to the polymerase enzyme aggregate on the other hand reduces the RNA synthesis by 50% (Bonner, 1966, Bannai and Terayama, 1967).

However, histones are not the only repressors. Protein removed from the DNA-protein complex with increasing concentration of salt solution contained lysine and arginine rich histones and also non-histone proteins (Georgiev, et al. 1966). $(NH_4)_2SO_4$ was able to de-repress the DNA template, resulting in an increased template activity. The type of RNA is uninfluenced by the repression, since the base sequences of RNA synthesized on DNA-protein complex and on free DNA appeared to be similar.

Nair et al. (1968) postulated that the stimulus increases the availability of active DNA template by removing some of the repressor elements during hypertrophy. According to their hypothesis, the high concentration of $(NH_4)_2SO_4$ unmasks the DNA template which permits functioning of all the existing polymerase, the activity of which could not further be increased by stimulation. The results, showing no increase in polymerase activity in the presence of salt during development of hypertrophy, could be explained in this way.

However, according to the experimental evidence obtained by Hulbert et al. (1969), the ribosomal cistrons (exclusively confined to the nucleoli) are almost entirely associated with RNA polymerase of nucleoli and the enzyme seems to be fully active rather than repressed by proteins or histones. Although the non-nucleolar templates are strongly repressed, the enzyme activity may be detected by an assay under conditions of high ionic strength. Since the nucleolar RNA synthesis is not repressed, it cannot be

increased by de-repression of the DNA template. Thus the increased availability of active DNA templates for nuclear r-RNA synthesis does not seem to be the reason for the observed increase in Mg^{++} activated polymerase (Nair et al. 1968, Fanburg and Posner, 1968, Fanburg, 1970, Sobel and Kaufman, 1970, Hamilton et al. 1968 and Schreiber et al. 1969, etc.).

Removal of inhibition of the enzyme

Considering the enhancement in polymerase activity of hypertrophying muscle nuclei in both Mg^{++} and Mn^{++} - $(NH_4)_2SO_4$ dependent assay systems (Sobel and Kaufman, 1970 and this study), one can assume that the mechanism of increased r-RNA and m-RNA synthesis is similar and is not solely influenced by changes in availability of DNA template. This assumption is in agreement with the fact that added exogenous DNA is ineffective in increasing activity of nuclei from hypertrophied or control tissue in either assay system. (Sobel and Kaufman, 1970, my results).

As Liao et al. (1968) has shown, an extract having RNA polymerase activity can be obtained in a soluble form from isolated nuclei in much larger amounts than would be predicted from the activity of intact nuclei. Therefore the enzyme appears to be present in excess in nuclei perhaps in a "reserve form". In agreement with this, Spelsberg et al. (1969) found that arginine-rich histones inhibited the DNA-dependent RNA synthesis most likely through interaction with the enzyme, while lysine-rich histones set on DNA templates.

In adaptation to such an acute stress as aortic constriction it is possible that a rapid increase in RNA synthesis is obtained

through the most efficient process, that is, through the removal of an inhibitor from the enzyme rather than through the new synthesis of polymerase. Very early increase in enzyme activity observed in this study is probably a result of such a mechanism. The de-repressor may be a kind of protein, the synthesis of which is necessary for the stimulation of RNA synthesis, as was shown by the protein inhibition experiments (Posner and Fanburg, 1968).

According to Meerson et al. (1964) who also supports this assumption, the increased contractile function leads to an elevated amount of protein breakdown product which acts as a de-repressor for RNA synthesis. Thompson and McCarthy (1968) found a cytoplasmic "stimulating factor" in the cytoplasm of 18 hour regenerating liver which caused an increase in RNA synthesis of normal mouse liver nuclei. This factor may be a de-repressor of polymerase.

Other factors

Another mechanism which may have a role in the regulation of RNA synthesis is ribonuclease activity. An attempt was made to assay the alteration of this enzyme activity during hypertrophy as follows: Ribonuclease activity in the nuclear preparation was determined at 0 time of the polymerase assay. No activity could be detected. The reason for the negative result may be that there was no ribonuclease activity present in the preparation at 0 time or that the activity could not be measured because it was relatively low compared to the synthetic activity of RNA polymerase. It could also be supposed that the condition of incubation (for example, ionic milieu) was not suitable for ribonuclease assay. Ribonuclease activity should have been studied under other conditions, since

this condition could be taken to be quite suitable for a polymerase assay, partly because it is not appropriate for ribonuclease activity.

However, in the control preparation after incubation in system I and II, a measurable ribonuclease activity was obtained. It would have been useful if an assay of the ribonuclease activity from constricted animals had been carried out after incubation in the Mg^{++} and Mn^{++} - $(NH_4)_2SO_4$ system.

Many other controlling factors have been described: increased utilization of the product, (Schreiber, 1968) increased precursor synthesis, availability of more ribosomal protein (Hamilton et al. 1968), alteration in the DNA-RNA polymerase linkage, (Novak and Doty, 1968) or in the timing mechanism initiating the transcription (Baker and Yanofsky, 1968). However, this study cannot provide a direct answer to these problems.

In conclusion, the time course of changes in RNA polymerase activity during the development of hypertrophy may be summarized as follows: RNA polymerase activity in the presence and absence of the ammonium salt shows roughly similar pattern over a 14-day observation period, i.e. in both systems a biphasic increase is demonstrated in the enzyme activity. However, the magnitude of increase in the enzyme activity in the Mg and Mn activated systems was not equal in the two phases.

The early phase is characterized 4 hours after aortic constriction. This very early increase in enzyme activity may be the result of a de-repression (de-inhibition) of the polymerase, since it is known that the enzyme functions below its maximal capacity. The de-repression may occur by the aid of a protein, the synthesis

of which therefore is required for the increased rate of RNA synthesis. Since the increased stimulation in m RNA synthesis occurred at this stage compared to the increase in r RNA synthesis, it is concluded that the mechanical stress caused a greater alteration in the extranucleolar regions which is responsible for m RNA synthesis. Thus, this extra stimulation may be also due to a de-repression of DNA templates in addition to the removal of an inhibitor from the enzyme.

In the late phase, the change of polymerase activity starts around the second day, reaching its maximum about 3-4th day following operation. In this phase much smaller stimulation was observed in the m RNA synthesis than in the r RNA synthesis. Other experimental data showing the greatest rate of hyperplasia in the connective tissue during this period suggest the role of RNA synthesis other than that by muscular tissue. The changes in the nuclear protein synthesis in this study and the time course of changes in protein synthesis in other work, both suggest that the new synthesis of the enzyme itself probably contributes in the second increase. Less stimulation observed in the presence than in the absence of $(NH_4)_2SO_4$ in this phase may be explained that the stimulating effect on the m RNA synthesis by the stress declined below the level which could be obtained by de-repression by the salt.

Investigations on changes in RNA-ase activity following constriction and on the type of RNA-s synthesized during the early and late phase are required to described more definitively the biochemical alterations in hypertrophying heart.

VII. CONCLUDING REMARKS

This chapter attempts to summarize the questions which arose during the course of this study. The importance of raising the right question was put forward in one of Beznak's lectures (1964). She stated that a possible reason why a research work does not provide a reasonable answer to the question may be that the question was not put well. Unfortunately, my study produced more questions than answers, but I only hope that some of these questions born out of this study will be starting points for elucidating the relationship between RNA and protein synthesis in the beginning of hypertrophy and the control mechanism of the action of RNA polymerase.

There are technical difficulties which have to be overcome. First of all, at the present time no method has been developed to separate the nuclei of muscle cell from the connective tissue nuclei. It is known that the reaction to increased workload (experimental hypertrophy) is different in the highly differentiated muscle cell than in the connective tissue cell. As a result, cellular hyperplasia takes place exclusively in interstitial cells in experimentally induced cardiac hypertrophy. Until the time when the two kinds of nuclei can be separated, we may provide only indirect evidence about RNA polymerase and RNA synthesis of various cell types.

The role played by the connective tissue should not be underestimated because of its participation in the growth process of heart tissue. Any alteration of the collagen content, which varies

depending upon the type of stimulus, is very likely associated with alterations in muscle mechanics (Fanburg, 1970). But it is still obscure how information is transmitted from the increased muscle work to heart growth. Also obscure is the mechanism which maintains the increased tissue mass after certain tissue growth.

Another problem concerning the investigation on RNA polymerase is that most of the work is done on crude systems and not with pure preparations. There is a drawback that the control mechanism cannot be investigated under these conditions, i.e. in a crude preparation the RNA polymerase is mixed with its control proteins. Research done on pure bacterial polymerase should be re-evaluated by using purified mammalian system. Simultaneously, the exact physiological milieu of the enzyme (ionic strength, etc.) has to be defined.

Research into the biochemical relationship among the cell organelles (mitochondria, nucleus, microsomes) has to be done, e.g. time course of their activities at different intervals following hypertrophy. The importance of mitochondria in particular has not received its share of attention (McCallister and Brown, 1969). Even in the nucleolus, the nucleolar r RNA synthesis was investigated separately from the nucleolar protein synthesis. Their changes after induced protein synthesis should concurrently be studied.

It is postulated in this thesis that the factor initiating cardiac growth in experimental hypertrophy is very likely a protein. Neither the origin and composition, nor the mode of action of this protein are known. The existence (synthesis) of this

protein is a fundamental requirement for the increased RNA synthesis at the initial stage (Posner and Fanburg, 1968). For this reason the mitochondrial and nucleolar protein synthesis are of particular interest since McCallister and Brown (1969) showed an early increase in mitochondrial protein synthesis.

Further research in this direction may greatly clarify the mechanism of the initiation of protein synthesis and its regulation under normal and hypertrophying conditions.

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