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TITLE OF THESIS....Tubercidin Triphosphate.....
Its Synthesis and Reactions.
with Myosin and Actomyosin.

UNIVERSITY.....Alberta.....

DEGREE FOR WHICH THESIS WAS PRESENTED..Ph.D.....

YEAR THIS DEGREE GRANTED.....1972.....

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THE UNIVERSITY OF ALBERTA

TUBERCIDIN TRIPHOSPHATE
ITS SYNTHESIS AND REACTIONS WITH MYOSIN AND ACTOMYOSIN

by

© JOHANN PHILIPP BORGER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

SPRING, 1972

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Tubercidin Triphosphate: Its Synthesis and Reactions with Myosin and Actomyosin" submitted by Johann Philipp Borger in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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21st. March. 1972.....
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ABSTRACT

The triphosphate ester of Tubercidin (TuTP) was chemically synthesized for the purpose of studying its reactions with myosin and actomyosin. TuTP, an analog of ATP, has a -CH group in place of N-7 of the adenine ring. The rate of TuTP hydrolysis was the same as that of ATP hydrolysis in the 10^{-7}-10^{-3}M range, whereas the increment of optical density of myosin at the 290 mμ region caused by TuTP was twice as much as that caused by ATP. Actomyosin hydrolyzed TuTP about 4 times (Vmax) as fast as it did ATP, while the Km values were of the same order of magnitude for both substrates. The rate of superprecipitation with TuTP was 50% of that with ATP at the nucleotide concentrations between 3 and 100 μM. A similar effect was observed with glycerol-extracted rabbit psoas fibers. Thus ATP is 800% more efficient as an energy source than its TuTP analog for muscle contraction. At 0.5 M KCl, actomyosin is dissociated by the addition of TuTP showing a second-order rate constant of $1.3 \times 10^{6} M^{-1} sec^{-1}$, which is 60% of that observed with ATP. The reassociation of actin and myosin took place at the similar rate regardless of the type of nucleotide used for the dissociation. Substitution of TuDP for ADP in F-actin did not affect the rate of superprecipitation or enzymic activity.

The results indicate that the hydrolytic site of myosin is modified by actin such that the enzymatic activity becomes more sensitive to the base structure of substrates, and suggest that the actin-myosin interaction is influenced by not only the terminal pyrophosphate group, but the base structure of nucleotides which affects the myosin conformation. The results are discussed in relation to the current understanding of the actomyosin-nucleotide interaction mechanism.

This work,

for reasons too many to enumerate here,

is dedicated

To my wife Doreen Delphine

and my children

Leslie Catherine

Linda May

Joshua Walter

Janine Elizabeth

Ivan Henry

John Paul

Michael Andrew

and to Jerome Fred and to Ronald John Borger,

whose lives on this earth, although short,

affected my own significantly.

ACKNOWLEDGMENTS

To Dr. T. Nihei for his guidance and critical evaluation throughout this study. His obvious patience over the past six years is deeply appreciated and will always be remembered.

To Dr. S. Masamune for providing guidance and laboratory space for the chemical syntheses.

To Mr. Glen Bigam and Mr. Dave Gifford for their experienced help in preparing and analyzing nmr spectra.

To Mr. Brian Read for drawing chemical structures.

To Mr. Gordon Blinston for providing assistance in measuring tension development in muscle fibre.

To Mrs. Cheryl Filipenko for providing excellent technical assistance and drawing figures for the manuscript.

To Mrs. Wendy Pysar for correcting and typing the manuscript.

To the Muscular Dystrophy Association of Canada and the Medical Research Council of Canada for financial assistance.

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LIST OF ABBREVIATIONS

A	F-actin
ADP	adenosine 5'-diphosphate
ADP-F-actin	F-actin with bound ADP
ADP-actomyosin	protein complex made up of ADP-F-actin and myosin
AM	actomyosin
ATP	adenosine 5'-triphosphate
ATP-G-actin	G-actin with bound ATP
F-actin	fibrous actin (polymer of G-actin monomer units)
G-actin	globular actin
HMM	heavy meromyosin
HMM-NDP	heavy meromyosin with bound NDP
HMM-NTP	heavy meromyosin with bound NTP
LMM	light meromyosin
M	myosin
myosin-NDP	myosin with bound NDP
myosin-NTP	myosin with bound NTP
NDP	ADP and/or TuDP
NTP	ATP and/or TuTP
Pi	inorganic phosphate
PP	inorganic pyrophosphate
Tris-HCl	2-amino-2-hydroxymethyl-1,3-propandiol-HCl
Tu	tubercidin
TuDP	tubercidin 5'-diphosphate
TuDP-F-actin	F-actin with bound TuDP
TuDP-actomyosin	protein complex made up of TuDP-F-actin and myosin
TuTP	tubercidin 5'-triphosphate
TuTP-G-actin	G-actin with bound TuTP

PART I

THE REACTIONS OF TUBERCIDIN TRIPHOSPHATE WITH MYOSIN AND ACTOMYOSIN

The mechanism of muscular contraction is one of the oldest and perhaps most intensely studied problems in all science. Yet an understanding of the mechano-chemical energy conversion in muscle has remained decidely elusive.

In addition to heart and skeletal muscle, the contractile systems are an integral part of the numerous tissues and organs that constitute life. In recent years an increasing number of new and specific muscle disorders has been described and neuromuscular diseases have become more familiar to the medical sciences. Thus research that further elucidates the mechanism of contractile action would enhance (an understanding for) early treatment of the many muscle diseases recognized to date.

Although there are a variety of contractile systems which provide mechanical energy for the proper functioning of living tissue, the basic mechanism is apparently similar. The fundamental contractile units are actin and myosin. Actin can be isolated as a globular unit of molecular weight 46,000 (G-actin) or as a fibrous polymer of the G-actin monomers (F-actin). The molecular weight values of myosin from skeletal muscle are on the order of 500,000. The energy for the contraction process is derived in some way from the enzymatic splitting of adenosine 5'-triphosphate (ATP) by myosin (Equation 1).

$$ATP^{-4} + H_2O \longrightarrow ADP^{-3} + H^{+} + Pi^{-2} \qquad (1)$$

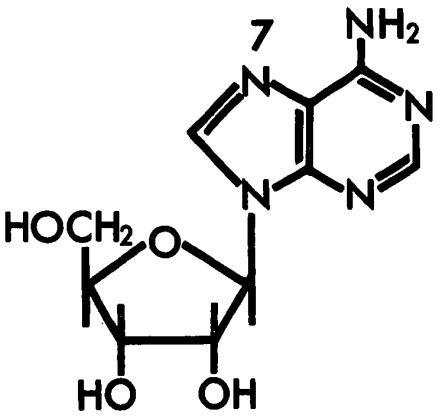
An <u>in vitro</u> complex of F-actin and myosin (actomyosin) may be prepared and used as a simple model to study the mechano-chemical energy conversion of the living systems.

Although it has been shown by Davies and his co-workers (Cain and Davies, 1962; Cain et al., 1962; Infante and Davies, 1962; Infante

et al., 1965) that the hydrolysis of pyrophosphate linkage in the ATP molecule is essential for muscle to generate mechanical work, the role played by the adenosine moiety in the contractile process is yet to be studied systematically. In the past, several investigators have reported observed effects of modified nucleosides on enzymatic and contractile functions using various muscle models. As will be cited below, most of the results were inconclusive in that the observations were made under limited conditions and the experimentalists were unable to describe the specific parameters which were directly affected by the modified nucleoside structure. Therefore, this study was conceived to examine as many aspects of the reactions between actin and myosin as possible using the 5'-triphosphate ester of tubercidin (II) which differs from ATP in having a -CH group in place of a nitrogen atom at the 7 position of the adenine ring. An attempt was also made to chemically synthesize the 5'-triphosphate ester of 1-(β-D-ribofuranosyl)indole (III) whose aromatic ring has chemical properties widely different from ATP.

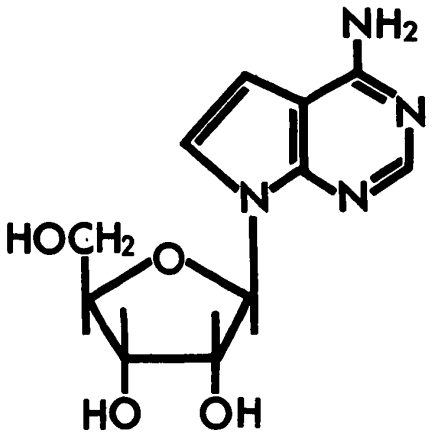
Previous Work on the Effects of Tubercidin (II)

Tubercidin is known to inhibit the growth of tumor cells (Anzai et al., 1957). Metabolism of tubercidin in mouse fibroblasts was reported by Acs, Reich, and Mori (1964). Nishimura et al. (1966) described the ability of tubercidin 5'-triphosphate to serve as a substrate for RNA polymerase obtained from E.coli. Ikehara and Ohtsuka (1965) studied trinucleotides containing tubercidin 5'-monophosphate indicating its ability to mediate binding of tRNA to ribosomes, and Uretsky et al. (1968) investigated the action of tubercidin on several of the enzymatic steps involved in polypeptide synthesis in extracts of mammalian



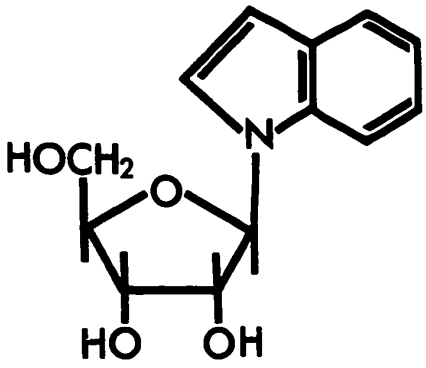
I

Adenosine



II

Tubercidin



III

cells. Both of the latter investigators (Ikehara and Ohtsuka, 1965; Uretsky et al., 1968) concluded that tubercidin is less capable in forming a base pair with uracil than adenosine, and they support the assumption that hydrogen bonding in the Watson-Crick model is an essential step in the codon-anticodon interaction (additional evidence cited by Ikehara and Fukui, 1969). The effects on biological activity by replacing one nitrogen atom at the 7 position of adenosine (I) by a carbon atom had thus been exemplified. Selecting an analog of ATP with such a minor structural alteration in the base would seemingly allow a precise focus on a specific step of a complicated process.

An investigation of the interactions between actomyosin systems and natural analogs of adenosine 5'-triphosphate, such as inosine 5'-triphosphate, uridine 5'-triphosphate, guanosine 5'-triphosphate, and cytidine 5'-triphosphate, has already been carried out by a number of investigators (Berkvist and Deutsch, 1954; Portzehl, 1954; Blum, 1955; Raney, 1955; Hasselbach, 1956; Keilley, 1956). These investigators threw some light on the role of purine and pyrimidine bases in the contraction of muscle models.

Tonomura and Ikehara and their collaborators (Ikehara et al., 1961; Azuma et al., 1962; Ikehara et al., 1964; Ikehara et al., 1965; Tonomura et al., 1967) synthesized various analogs of ATP and investigated their reactions with actomyosin systems. In their final paper (Tonomura et al., 1967) they summarized all their experimental results on ATP analogs and those of other investigators.

More recently Murphy and Morales (1970) employed 6-mercapto-9-β-D-ribofuranosylpurine 5'-triphosphate (SH-TP) as a chromophoric probe and as an affinity label of the ATPase sites of myosin. SH-TP was shown

to be a substrate for myosin and caused contraction of both myofibrils and glycerinated muscle fibers.

In the broad survey and cursory study of many nucleotides by Tonomura and Ikehara and co-workers, tubercidin 5'-triphosphate was included. Myosin B, directly extracted from muscle and myofibrils from glycerol extracted muscle fiber, were employed for their investigations. They observed that the rate of hydrolysis of TuTP by myosin B was greater than that of ATP at low ionic strength in the presence of Mg^{2+} and that this ATP analog did not induce myofibrillar contraction.

The Contractile Proteins: Actin and Myosin

Myosin - The morphological features of myosin have been extensively studied by making use of proteolytic enzymes. By treating myosin with trypsin under catalytic conditions two major fragments are obtained (Gergely, 1950, 1953; Perry, 1951; Mihalyi and Szent-Gyorgyi, 1953).

The low molecular weight component is called light meromyosin (LMM) and has a molecular weight of approximately 130,000 (Gergely et al., 1958; Szent-Gyorgyi et al., 1960). Electron microscopy has shown that LMM has a rod-like appearance 800 angstroms long and 20 angstroms in diameter (Huxley, 1963; Zobel and Carlson, 1963). Szent-Gyorgyi (1953) has shown that this fragment has solubility properties similar to intact myosin and fails to bind actin or exhibit ATPase activity. It comprises the major portion of the myosin thick filaments (Huxley 1963), has a high helical content as shown by optical rotatory measurements, and preliminary wide-angle X-ray diffraction data indicate a coiled-coil α-helical conformation (Szent-Gyorgyi, 1960).

The high molecular weight component obtained by tryptic digestion

is called heavy meromyosin (HMM) and has a molecular weight of approximately 340,000 (Gergely et al., 1958; Lowey and Holtzer, 1959). Electron microscopy was employed to show that HMM has a rod-like portion about 600 angstroms long and 20 angstroms in diameter that terminates in a globular portion approximately 200 angstroms by 50 angstroms (Huxley, 1963; Zobel and Carlson, 1963). Szent-Gyorgyi (1953) has shown that this fragment is soluble at low ionic strength and hydrolyzes ATP and binds actin in a manner similar to myosin. Huxley's studies (1963) indicate that HMM forms the cross-bridges between actin and the myosin thick filament in the myofibrils.

Mueller and Perry (1961, 1962) further digested HMM with trypsin and obtained a smaller unit (subfragment-1) which retained the actin-binding and ATPase properties. Kominz et al. (1965) obtained the same fragment by the proteolytic hydrolysis of native myosin by papain. Subfragment-1 has a molecular weight in the range 110,000-120,000 (Jones and Perry, 1966; Nihei and Kay, 1968). Stoichiometric studies by Stracher and Dreizen (1966) and Nauss and Gergely (1967) indicate that one HMM contains two particles of subfragment-1.

Thus it is now accepted that myosin is a duplex, Y-shaped molecule with two "heads" each containing one actin binding site and one hydrolytic site (Figure 1). The hydrolytic activity is activated by Ca^{2+} and inhibited by Mg^{2+}.

Actin - The globular actin (G-actin) monomer belongs to the class of spherical proteins with axial ratios between 1 and 4 (Cohen, 1966). An electrophoretically homogeneous preparation has a molecular weight of 46,000 and is constructed from a single, covalently linked polypeptide

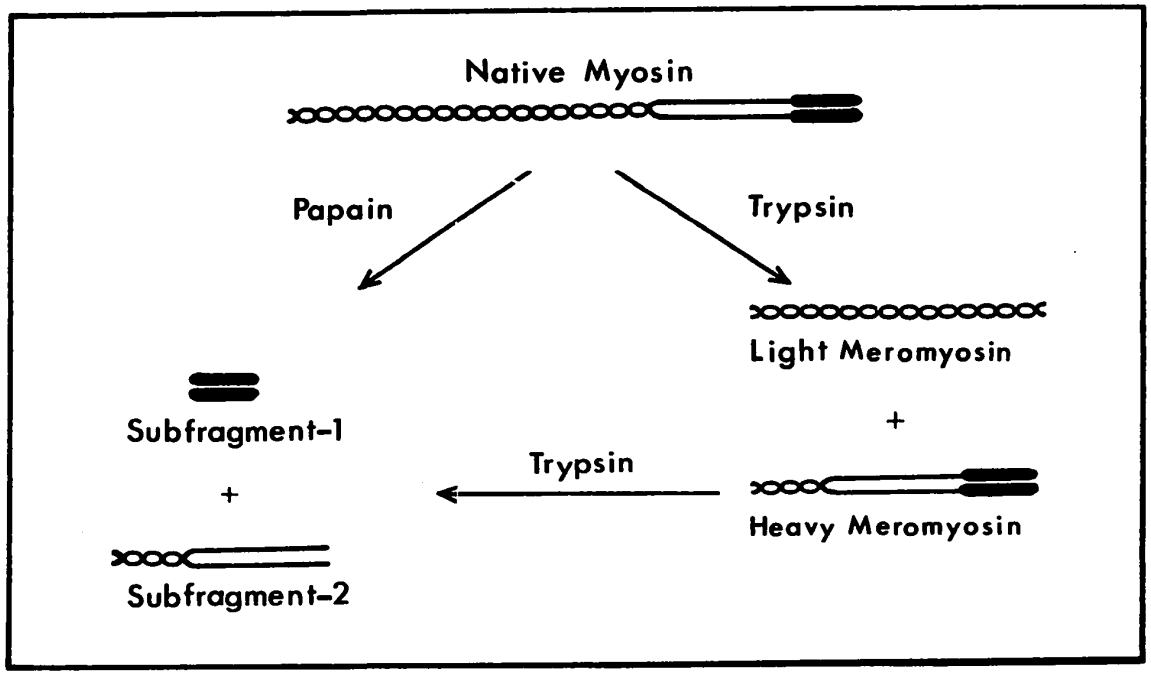


Fig.I. Rabbit skeletal myosin and its proteolytic fragments. The native myosin molecule is comprised of an axial core of two heavy polypeptide chains which terminate in a globular head. Each head contains one actin binding site and one hydrolytic site.

chain (Rees and Young, 1967). G-actin contains one mole of firmly bound nucleotide (usually ATP) and one mole Ca^{2+} per mole of protein (Gergely, 1964). Studies demonstrating exchangeability of the bound ATP and Ca^{2+} with nucleotide and Ca^{2+} in the solvent have been published (Martonosi et al., 1960; Barany et al., 1962). Upon addition of neutral salts to a solution of ATP-G-actin, the in vitro polymerization to fibrous protein (F-actin) occurs (Straub, 1942) with concomitant dephosphorylation of the bound ATP (Straub and Feuer, 1950; Laki, Bowen, and Clark, 1950).

$$n\ \text{ATP-G-Actin} \xrightarrow{0.1M\ KCl} \text{ADP-F-Actin} + n\text{Pi} \qquad (2)$$

Ultrastructural analysis of F-actin by X-ray diffraction (Selby and Bear, 1956), electron microscopy (Hanson and Lowey, 1963), and physico-chemical analysis (Oosawa and Kasai, 1962) shows that F-actin is a helical polymer of G-actin monomers. The ADP molecule and Ca^{2+} become non-exchangeable in the resulting F-actin polymer under normal experimental conditions (Barany et al., 1962; Martonosi et al., 1960).

Rapid homogenization of F-actin in a buffered salt-free solvent yields ADP-bound G-actin (Grubhofer and Weber, 1961; Hayashi and Tsuboi, 1960). The removal of bound nucleotide from ATP-G-actin or ADP-G-actin is always accompanied by loss of polymerizability. One exception has been reported by Kasai, Nakano and Oosawa (1965) who treated G-actin with EDTA-Dowex in sucrose, thereby removing nucleotides and divalent cations, yet retaining the polymerizability.

Actomyosin - An in vitro complex of F-actin and myosin (actomyosin) is formed at different actin-myosin ratios. Upon association with actin the enzymatic activity of myosin becomes activated by Mg^{2+} as well as

Ca^{2+}, whereas that of myosin alone is activated by Ca^{2+} only, (Banga, 1943; Banga and Szent-Gyorgyi, 1943).

At high ionic strength this protein complex is readily dissociated by Mg^{2+} and ATP or pyrophosphate. In a low ionic strength medium (<0.1) actomyosin forms a gelatinous, hydrated suspension which is viewed as a network of actin and myosin filaments (Huxley, 1963; Eisenberg and Moos, 1967). Upon addition of Mg^{2+} and ATP to a dilute suspension of actomyosin, a striking physical change occurs whereby the translucent gel becomes dense and opaque. The syneresis of this actomyosin complex is called superprecipitation and was first reported by Szent-Gyorgyi (1941, 1942). Studies on the processes of superprecipitation were extended by Weber (1958), who prepared the actomyosin thread which undergoes the contraction cycle and serves as a simple model for studying muscle action. It has been proposed that the actomyosin gel expels the trapped water during the superprecipitation process (Szent-Gyorgyi, 1951).

The stoichiometric ratio of myosin to actin monomers in actomyosin has received considerable attention. Huxley's (1963) electron microscopic studies suggest that one heavy meromyosin molecule binds one actin monomer. Young (1967) employed transport measurements to show that each monomer unit in the polymerized actin can bind one particle of HMM and one unit of subfragment-1.

Eisenberg et al. (1968a, 1968b) have shown that the Mg^{2+}-ATPase of HMM and subfragment-1 is activated by F-actin as in the case of the parent myosin molecule, yet no superprecipitation occurs even at low ionic strength (Eisenberg and Moos, 1966; Moos and Eisenberg, 1967).

Actin and Myosin in Muscle Fibers

Since muscle cells are generally long and thread-like; they are

called fibers. These fibers contain not only a contractile apparatus, but also other structures which control and fuel the contractile activity. By storing a strip of rabbit psoas (muscle connecting the thighbone and spinal column) in 50% glycerol at 0^{0} for several days, the more soluble activators and co-factors are extracted thereby leaving behind the intact structural material. When a small bundle of fibrils is removed from this preparation and suspended in buffered ionic medium containing Mg^{2+}, the fibers will contract and develop a measureable tension upon the addition of ATP, the structural unit making up the muscle fibers is shown in Figure 2.

The myofibril of vertebrate striated muscle contains an interlocking array of thin (actin) and thick (myosin) filaments from which cross-bridges extend at periodic intervals (Figures 3-4). These general features were derived by Hanson and Huxley (1953, 1954, 1955, 1960, 1969) employing electron microscopy and low-angle X-ray diffraction studies of relaxed and contracted muscle. The thin filament is comprised of a double-helical arrangement of two actin chains and each chain is made up of actin monomers which are about 55 angstroms in length (Oosawa and Kasai, 1962; Hanson and Lowy, 1963). The thick filament is composed of myosin molecules, with their rod-like LMM portions making up the filament axis in a staggered array and their enzymatically active heads protruding as cross-bridges at radial distances of approximately 60-130 angstroms from the filament axis (Huxley, 1960; Huxley and Brown, 1967). According to the Huxley model (1969), the cross-bridges interact in a repetitive manner with actin sites as the thick filaments slide past the thin filaments. The physico-chemical

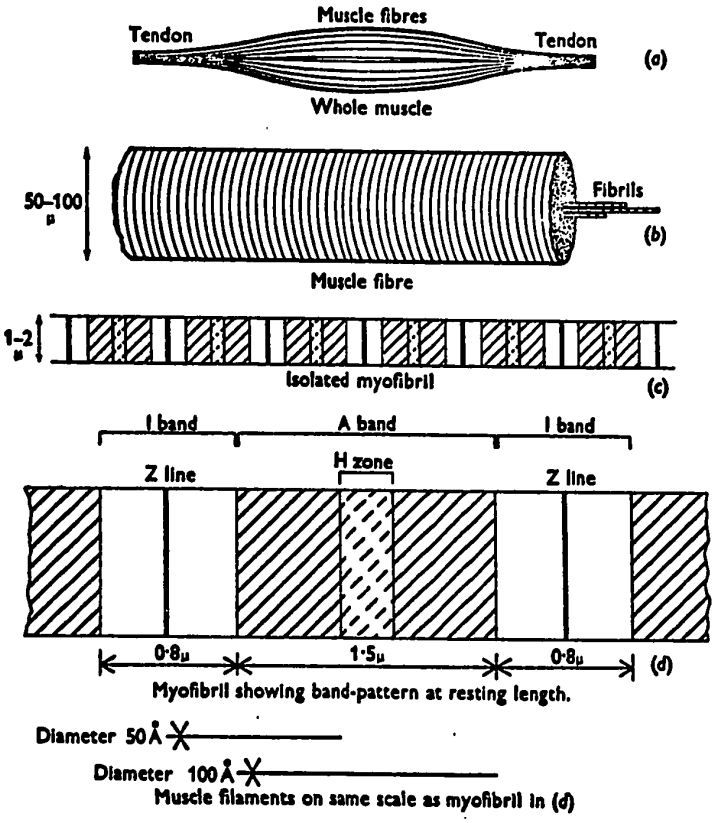


Fig. 2. The structure of muscle at various levels of organization; dimensions shown are for rabbit psoas (From Huxley, 1956).

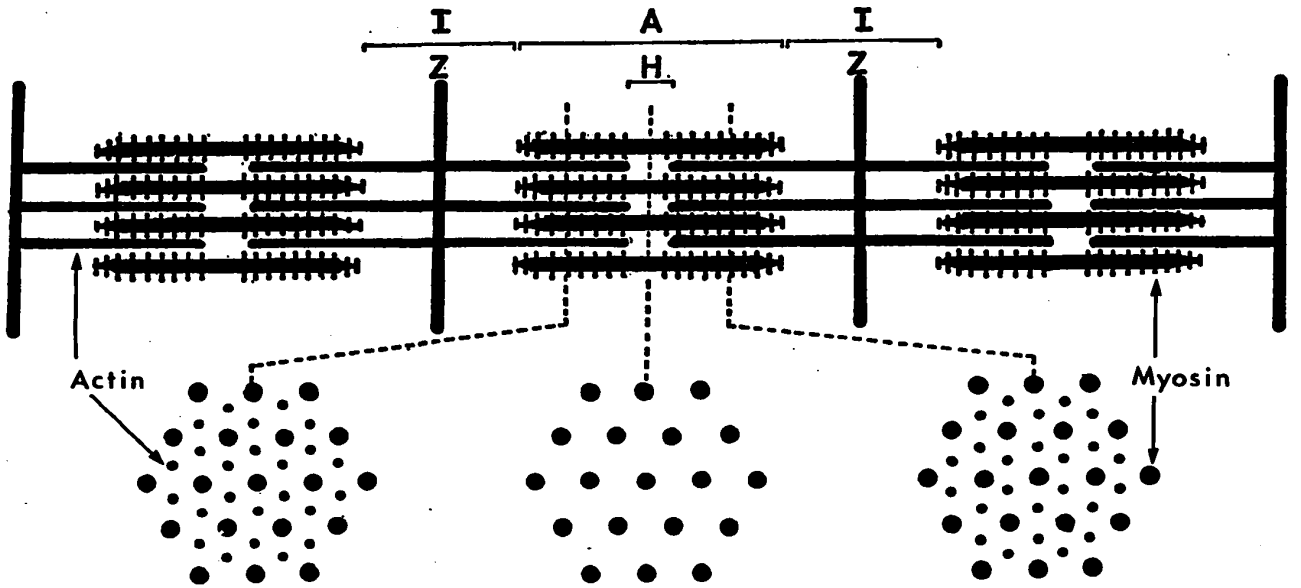


Fig.3. Diagrammatic representation of the structure of striated muscle, showing overlapping arrays of actin- and myosin-containing filaments, the latter with projecting cross-bridges on them. For convenience of representation, the structure is drawn with considerable longitudinal foreshortening; with filament diameters and side-spacings as shown, the filament lengths should be about five times the lengths shown (From Huxley, 1969).

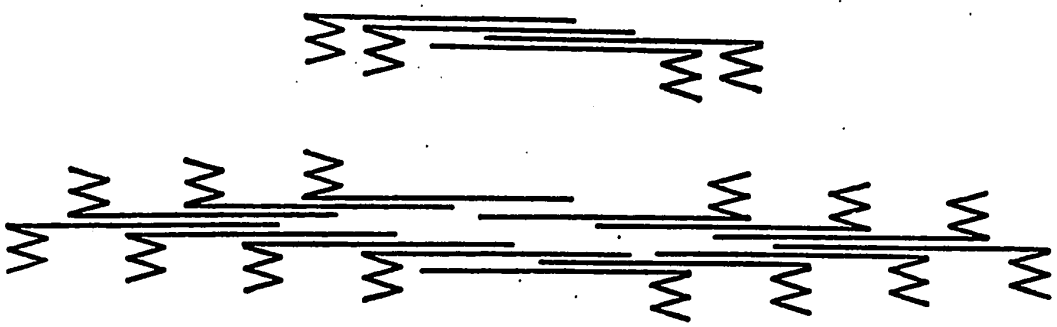


Fig. 4. Diagrammatic representation of the mode of aggregation of myosin molecules to form thick filaments whose structural polarity reverses at the midpoint. The light-meromyosin parts of the myosin molecules form the backbone of the filaments, and the globular ends of the heavy-meromyosin components form the projecting cross-bridges. Since these will be oriented in opposite senses in the two halves of the A-bands, they could generate sliding forces which are always directed toward the center of the bands. (From Huxley, 1969).

interaction of HMM cross-bridges with actin provides the necessary force during active contraction.

In our study of the contractile systems the analog TuTP together with ATP were employed as tools to further investigate myosin, actin, actomyosin and finally muscle fibers with the anticipation that a contribution would be made toward elucidation of a difficult and not well understood biochemical problem.

METHODS AND MATERIALS

1. The Preparation of Myosin

Myosin was prepared from the back and leg muscles of albino rabbits by the procedure of Tonomura et al. (1966). After the excision of fat deposits and connective tissue, the muscle was homogenized in 3 volumes of a 0.3M KCl solution containing 0.2mM ATP and 0.02M histidine (pH 6.8), in the stainless steel vessel of the Sorvall Omni-mixer (Sorvall Inc., Norwalk, Conn., U.S.A.). The homogenate, after standing for 10 minutes, was centrifuged for 20 minutes at 6,000 rev/min in the GSA rotor of a Sorvall RC2-B centrifuge. The supernatant was filtered through 4 layers of clean gauze into a chilled graduate cylinder. The volume of filtrate was estimated, and this was added to 9 volumes of double distilled water at $4^{o}C$ in a large conical container with a clamped opening at the bottom. The precipitated crude myosin sedimented in approximately 45 minutes. The sedimentation was sometimes prolonged and seemed to depend upon fat content and the proportion of leg muscle used. The precipitate was collected from the bottom and centrifuged at 6,000 rev/min for 20 minutes. The packed precipitate was resuspended in a buffer solution (0.5M KCl, 0.02M histidine, pH 6.8). Then it was diluted with double distilled water to suspend the protein in 0.28M KCl. This was stirred for 2 hours to ensure breaking up of lumps and dissolving of the myosin. Insoluble material was removed by centrifugation at 100,000 x g for 60 minutes in the Beckman Preparative Ultracentrifuge Model L3-50 using the Rotor Type 30. The supernatant was passed through 4 layers of gauze and myosin was precipitated at 0.05M KCl. The precipitate was again collected by

centrifuging at 6,000 rev/min for 20 minutes. The dissolution-precipitation cycle was repeated except that the second dissolution was at 0.25M KCl. The myosin prepared showed no turbidity change under the conditions for superprecipitation and no Mg^{2+} activations of the ATPase activity, both indicating the absence of actomyosin contamination. The final myosin precipitate was resuspended in a small volume of 0.5M KCl, 0.02M histidine (pH 6.8), and dialyzed against a large volume of the same. Before use this myosin solution was centrifuged at 100,000 x g for 2-3 hours to remove denatured material. The stock myosin solution usually contained 30-50 mg/ml. The average activity of 0.5 μmole Pi $min^{-1}mg^{-1}$ agrees with that of other workers (Lowey and Lueck, 1969). The enzymatic activity of myosin was stable for at least two weeks.

The ultraviolet absorption characteristics of myosin preparations were tested with a Cary 16 spectrophotometer. The absorbancy ratio of 280 mμ to 260 mμ was observed to be 1.70±0.05. The amount of nucleotide contaminant in myosin was estimated by treating proteins with 1 N perchloric acid at 90^0 for 15 minutes. The results indicated that less than 0.1 mole equivalents of nucleosides were present in 100,000 g myosin.

Some preparations were stored in 50% glycerol for 1-3 months before use. Pre-cooled glycerol was added slowly with stirring to a measured volume of myosin solution, the presence of air bubbles being avoided. The container was subsequently sealed and stored at -25^0C.

For the removal of glycerol, the myosin-glycerol mixture was equilibrated to 0^0C, and dialyzed against 0.5M KCl, 0.02M histidine (pH 7.4). Care was taken to immerse only the lower 50% of the dialysis bag in the buffer to create an outward hydrostatic pressure opposing the

inward osmotic pressure which may otherwise burst the bag. It was confirmed that glycerol storage did not adversely affect the ATPase activity.

2. Extraction and Purification of Actin

The acetone powder of muscle (Szent-Gyorgyi, 1951) was usually prepared from ground (not homogenized) back and leg muscle after the myosin extraction. The muscle was first suspended with constant stirring in 5 volumes of a 0.4% $NaHCO_3$ solution at room temperature for 30 minutes. The fluid was expressed through 4 layers of gauze and the residue suspended in one volume of a solution of chilled 0.01M $NaHCO_3$ and 0.01M Na_2CO_3 for 10 minutes. Then the suspension was diluted with 10 volumes of distilled water at room temperature and immediately strained through gauze. Three volumes of cold acetone were mixed with the residue at a temperature below 25°C. After stirring for 10 minutes, the acetone was squeezed out and this procedure repeated. The final residue was left to dry at room temperature and the dry fiber was stored at -25°C in a sealed container.

The extraction of actin from the fiber was done with 20 volumes of 0.2mM ATP, 3mM cysteine (pH 8.0), hereafter called the "depolymerizing solution", for 20 minutes at 0°C in order to minimize the tropomyosin contamination (Drabikowski and Gergely, 1962). The first phase of actin purification used the polymerization-depolymerization cycle of Mommaerts (1951). The extracted actin was polymerized with 0.1M KCl, 1mM $MgCl_2$, overnight at 4°C or at room temperature for 1 hour, and then centrifuged at 100,000 x g for 3 hours in the Beckman Preparative Ultracentrifuge Model L3-50 using the Rotor Type 30. The sides of the centrifuge tubes and the pellets were rinsed with cold water to float out fat and remove excess salt. Then the pellet was suspended in

a small volume of the depolymerizing solution (25 ml if 10 g of fiber was originally extracted), and homogenized in a Teflon-glass homogenizer. This suspension was dialyzed overnight against 20 times its volume of depolymerizing solution, followed by centrifugation at 100,000 x g. The resulting G-actin was aspirated from the central portion of the solution to avoid fat. This depolymerization-polymerization cycle was repeated

The second phase of actin purification involved a partial polymerization step to further eliminate tropomyosin contamination (Martonosi, 1962; Laki et al., 1962). The F-actin was depolymerized as before, except that it was suspended in a smaller volume of depolymerizing solution (6 ml for 10 g of originally extracted fiber) so as to maintain the actin concentration above the critical value (Oosawa et al., 1959) for polymerization (2.5 mg/ml seemed to be ideal). This G-actin was polymerized overnight by dialysis against 0.7mM $MgCl_2$, 0.4mM ATP, 3mM cysteine (pH 8.0). The partially polymerized actin was centrifuged at 70,000 x g for 3 hours. Under these conditions, the tropomyosin-contaminated actin was difficult to polymerize, and remained in the supernatant. The pure F-actin sedimented, forming a soft pellet. This polymerization cycle was also repeated. Finally the actin was polymerized with 0.1M KCl, 1mM $MgCl_2$ and dialyzed against 0.1M KCl to remove free Mg^{2+}.

3. Preparation of Heavy Meromyosin

Heavy meromyosin was prepared according to the methods of McManns and Muller (1966). Two ml of myosin (12 mg/ml) in 0.5M KCl, 20mM Tris-HCl (pH 7.4), were digested for 4½ min with 50 λ trypsin (10 mg/ml). The reaction was stopped by the addition of 50 λ trypsin inhibitor (20 mg/ml). The digestion mixture was dialyzed overnight against 0.05 M KCl, 2mM Tris-HCl (pH 6.9). The precipitated protein was removed by centrifugation at 37,000 x g for 30 min.

4. Estimation of Protein Concentration

The concentrations of actin, myosin, and heavy meromyosin were routinely determined by the biuret method as standardized with human albumin (Dade Reagents Inc., Miami, Fla., U.S.A.), and calibrated against a micro-Dumas nitrogen determination (Coleman nitrogen analyzer, Model 29), assuming 16.5% nitrogen in myosin (Kominz et al., 1954) and actin (Rees and Young, 1967). Actin and papain subfragment were also determined at 289 mμ using an extinction coefficient of 1149 cm^2/g (Estes and Moos, 1969) and 770 cm^2/g (Young et al., 1965), respectively.

5. Glycerinated Muscle Fibers

Glycerinated muscle fibers were prepared by extracting rabbit psoas fiber bundles in 50% glycerol at -25°C for more than two months (Szent-Gyorgyi, 1951). All fibers at rest length were tied to wooden medical applicators with coarse cotton strings before removal from the animal. Muscle fiber bundles used in these experiements had a resting cross section of approximately 0.12 mm^2 as calculated from their estimated diameters observed through a microscope with an attached eyepiece grid. The length of the fibers was in the range 1.5-2.0 cm. Before testing, a fiber bundle was allowed to equilibrate with the bathing solution (0.05M KCl, 20mM Tris-HCl, pH 7.4, 1mM $MgCl_2$ 10μM $CaCl_2$ at 22°C) for 15 min. The system was held and adjusted by a micro-manipulator to 10 mg tension. The concentrated nucleotide solution was added with mixing by mild air injection. Measurement of tension development by the fibers was performed by means of an isometric (force) transducer Type 2ST02, a 3C66 Tecktronix carrier amplifier with a Type 564 storage oscilloscope, and a Hewlett Packard Model 320 dual channel DC amplifier-recorder.

6. Superprecipitation

In a typical experiment, actomyosin (A:M = 1:2, W/W) was suspended

in 0.05M KCl, 20mM Tris-HCl (pH 7.4), 1.0mM $MgCl_2$, 10 µM $CaCl_2$ at 23°C. Twelve ml of the protein suspension (0.015-0.03 mg/ml) were placed in the sample cell (2.0 cm light path) of a Zeiss PMQ II spectrophotometer. Throughout the measurement of turbidity, the protein suspension was stirred by a rotating magnetic bar at the bottom of the cell. ATP and TuTP were added to the suspension with an Eppendorf pipette. The change in turbidity at 545 mµ was recorded continuously on a Beckman Photovolt linear-log recorder connected to the spectroscope.

7. Enzymatic Assays

A. Molybdovanadate Method for Inorganic Phosphate (Lecocq and Inesi, 1966)

The reaction was initiated by adding nucleoside triphosphate (ATP or TuTP) to a constantly stirred mixture containing myosin in 0.1 KCl, 20mM Tris-HCl (pH 7.4), 15mM $CaCl_2$ at 25°C. Aliquots (1 ml each) of the reaction mixture were taken at intervals and added to 2 ml of the molybdovanadate reagent. The mixture was centrifuged to remove precipitated proteins, and the absorbance at 400 mµ was determined after 10 minutes. A suppression of the colour was encountered when a high protein concentration was used. This difficulty was circumvented by adding 0.05 µmole of K_2HPO_4/ml of reaction mixture.

B. Raabe Method for Inorganic Phosphate (Raabe, 1955)

A description is given in the EM-test (E. Merck, Darmstadt, Germany; in the U.S.A., Brinkmann Instruments, Westbury, N.Y.). An aliquot (1 ml each) was added to the molybdate reagent, and the resulting phosphomolybdate was reduced to colloidal molybdenum blue. After fifteen minutes a sulfite-carbonate reagent was added

to solubilize the protein, and after a further fifteen minutes the blue color was read at 750 mμ.

C. Enzymatic Activity of Myosin in the Presence of Mg^{2+} at Low Ionic Strength

The enzymatic activity was determined in the presence of 5mM Mg^{2+} and 0.05M KCl using the auxiliary enzyme system, pyruvate kinase and lactic dehydrogenase. The decrease of optical density at 340 mμ as DPNH oxidation follows the hydrolysis of ATP (or TuTP), was measured using a Cary 16 spectrophotometer which allows for detection of an optical density change of 0.001 with an error of 10%. In this system it was possible to assay the NTPase with NTP concentrations from 10^{-7}M to 10^{-3}M, without changing the myosin concentration.

D. Enzymatic Activity of Actomyosin

Phosphate determinations were made according to the method of Lymn and Taylor (1970). The enzyme reaction was stopped by adding HCl to a final concentration of 0.4N and the combined mixture was added to 10 mg of Norit A. The mixture was agitated intermittently for 5 min (Vortex mixer) to facilitate absorption of nucleotides and was kept in an ice bath to reduce metal ion catalyzed hydrolysis at the low pH. The suspension was filtered and washed twice with 1.5 ml of 0.1N perchloric acid employing a Millipore filter (0.45 μ pore size). To the filtrate, whose volume was approximately 8 ml, was added 0.4 ml of freshly mixed molybdate-triethylamine solution (two parts of 10% by weight sodium molybdate to one part 0.2M triethylamine) to precipitate the phosphate. The solution was filtered on a Millipore filter and washed three times with 2-ml aliquots of the wash solution (2.5% ammonium molybdate, 0.025M triethylamine,

0.2N perchloric acid plus sufficient phosphate to saturate the solution, ca. 5×10^{-5} M).

The filter disc was placed in a vial with a 10 ml solution of Packard pre-mix "M" (91% PPO and 9% Dimethyl POPOP) in scintanalyzed toluene and counted in Beckman LS-100 Liquid Scintillation System.

8. Spectra

The ultraviolet and uv difference spectra were measured with a Cary 16 spectrophotometer. Dry nitrogen gas was passed through the instrument for the purpose of an improved response particularly in the shorter wavelength region. For the actin spectra 1-mm cells were utilized, and for the myosin and heavy meromyosin difference spectra 1-cm cells were used. Base lines were determined with double distilled water in the reference and sample cells. Spectra were obtained at several different slit widths to ensure reproducibility and minimal contribution of stray light. An extinction coefficient of 15.4×10^3 at 259 mμ and 11.2×10^3 at 271 mμ was used for ATP and TuTP respectively.

9. Chemicals

ATP was purchased from the Sigma Chemical Co., and used without further treatment. γ-^{32}P-ATP was ordered from Amersham/Searle corporation in Toronto, and ^{32}P-phosphoric acid was purchased from New England Nuclear. TuTP and γ-^{32}P-TuTP were chemically synthesized as described in Part II of thesis. The remaining chemicals were of reagent grade.

RESULTS

Hydrolysis of TuTP and ATP by Myosin in the Presence of Ca^{2+}

All nucleoside triphosphates tested to date have been found to be dephosphorylated by the enzymatic action of myosin. The simplest organotriphosphate, methyltriphosphate (Lecocq, 1968), and indeed tripolyphosphate (Friess and Morales, 1955) are hydrolyzed by myosin. The myosin inactivator 6-mercapto-9-β-D-ribofuranosyl 5'-triphosphate (SH-TP) is also a substrate as demonstrated by Murphy and Morales (1970).

The 5'-triphosphate of the potent antibiotic tubercidin (Acs et al., 1964) is readily hydrolyzed by myosin. At relatively high concentrations of TuTP the rate of hydrolysis by myosin in the presence of Ca^{2+} was found to be the same as that of ATP under the conditions tested (Figure 5). Thus no inhibitory effect on the enzymatic activity of myosin could be detected in a reaction medium conducive to high myosin ATPase activity.

It has been shown that the metal ions Ca^{2+} and Mg^{2+} act differently as modifiers of myosin and actomyosin. The hydrolysis of nucleotide by myosin is activated by Ca^{2+}, whereas in the presence of Mg^{2+} the enzymic activity of myosin is suppressed below the level that is detected in a monovalent salt solution (Bailey, 1942). When myosin is associated with F-actin, then the Mg^{2+} bound to the protein becomes an activator (Perry, 1951). Jacobson et al. (1971) used beef cardiac actomyosin, and Sugden (1971) employed rabbit skeletal actomyosin to demonstrate that the Mg^{2+}-activated (1mM Mg^{2+}) actomyosin superprecipitates at a considerably higher rate than the corresponding Ca^{2+}-activated protein (10mM Ca^{2+}) at low ATP concentrations. It has been shown by Lymn and Taylor (1970) that Ca^{2+} alone precipitates actomyosin, which appears to

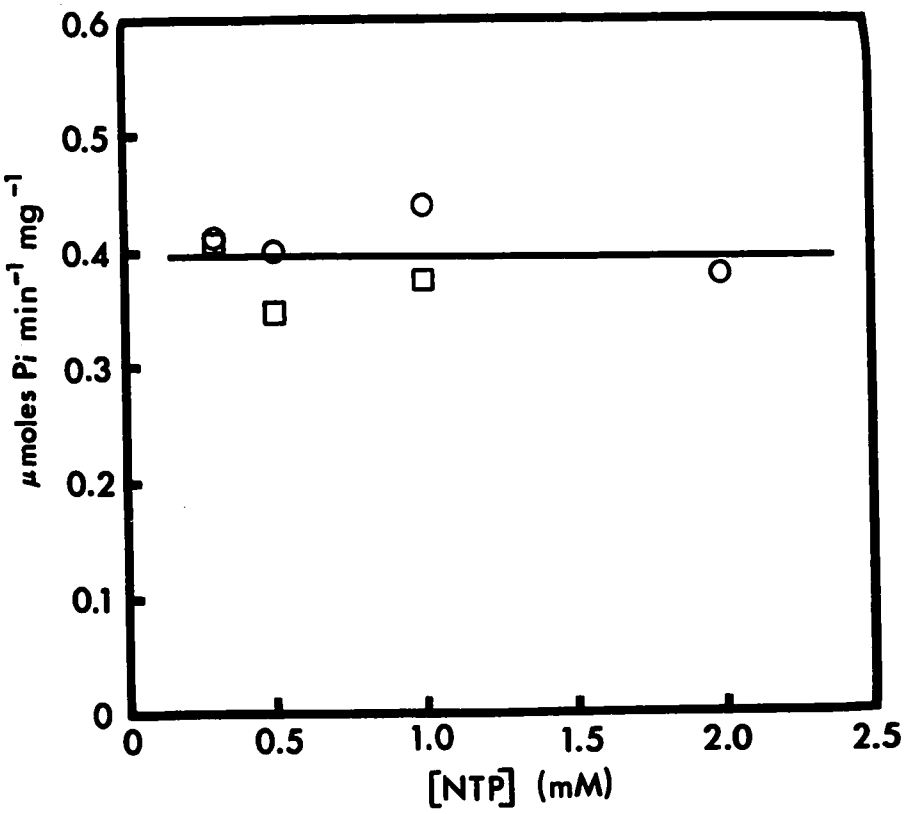


Fig.3: Hydrolysis of TuTP by myosin at high nucleotide concentrations. At zero time nucleotide was added to a myosin suspension (0.1mg/ml) in 0.1 M KCl, 15 mM $CaCl_2$, and 0.02 M Tris-HCl (pH 7.4) at 25°C. (O), ATP; (□), TuTP.

indicate the characteristic, but perhaps non-physiological feature of the Ca^{2+}-actomyosin complex.

Hydrolysis of TuTP and ATP by Myosin at Low Ionic Strength in the Presence of Mg^{2+}

Figure 6 shows the variation of the rate of hydrolysis of TuTP and ATP by myosin at 0.05M KCl in the presence of 5mM Mg^{2+} (pH 7.4, 20^{o}). This ionic condition was chosen so that the results could be compared with those observed in the actomyosin system. Concentrations were varied from 10^{-7} to 5 x 10^{-3}M at constant myosin concentrations. The same results were obtained with two different preparations of myosin, one of which had been stored in 50% glycerol at -20^{o}C for several months. It is apparent that the enzymic activity of myosin is also identical for the substrates ATP and TuTP at low ionic strength in the presence of Mg^{2+}. In addition to the above observation the data of Figure 6 support the hypothesis that the two active sites of the myosin molecule interact under certain conditions. In the lower range of nucleotide concentration (10^{-7} to 10^{-5}M) the activity increased rapidly and the data fit the Michaelis equation. The value of the Michaelis constant (Km) was estimated to be 7 x 10^{-7}M, which is similar to that reported by Nanninga and Mommaerts (1960). However, the remainder of the data at nucleotide concentrations above 10^{-5}M shows more complex features of this enzyme and would not give a straight Lineweaver-Burk plot. Lymn and Taylor (1970) suggested that, since two active sites are present in the myosin molecule, it is possible that these sites may interfere with each other in their reaction with ATP. They further proposed that the range of reported Km values for myosin ATPase of 10^{-4}M (Nihei and Tonomura, 1959; Kiely and Martonosi, 1968) to 3 x 10^{-8}M (Lymn and Taylor, 1970) could be a reflection of the site interaction.

When a small molecule interacts with a macromolecule which has two or more equivalent but not independent binding sites, a graphic presentation of the binding data may not reveal the binding parameters in a manner expected for a model with a single site interacting with a single reactant. Rather, a conjugate of parameters is shown (Klotz and Hunston, 1971). It may be that the Km values reported for the myosin ATPase are at variance from the real figures, unless the site interaction has been taken into account. Since most of the previous work concerning the kinetic parameters of myosin ATPase reactions has been carried out in a substrate concentration range of less than twenty-fold, the feature demonstrated in Figure 6 could not be demonstrated. The experiments with subfragment-1, however, showed a set of data which fit the Michaelis formulae. The ATP concentration range used, was the same as that for myosin ATPase, and a Michaelis constant of 10^{-6}M was obtained. It is, therefore, possible to assume that the two active sites in myosin are enzymatically identical but influence each other upon binding with the substrate. Consequently, a simple model of the myosin ATPase at steady state (Appendix I) can be used to calculate the activity-substrate concentration profile drawn as a solid curve in Figure 6, assuming the apparent Km values 10^{-7}M and 10^{-5}M for the first and second binding with the active sites.

Superprecipitation of ADP-Actomyosin by ATP and TuTP

Upon addition of ATP to an actomyosin suspension at low KCl concentration (0.05M), the contractile proteins become dense and opaque, and the turbidity increase can be followed by measuring the optical density change (Levy and Fleisher, 1965).

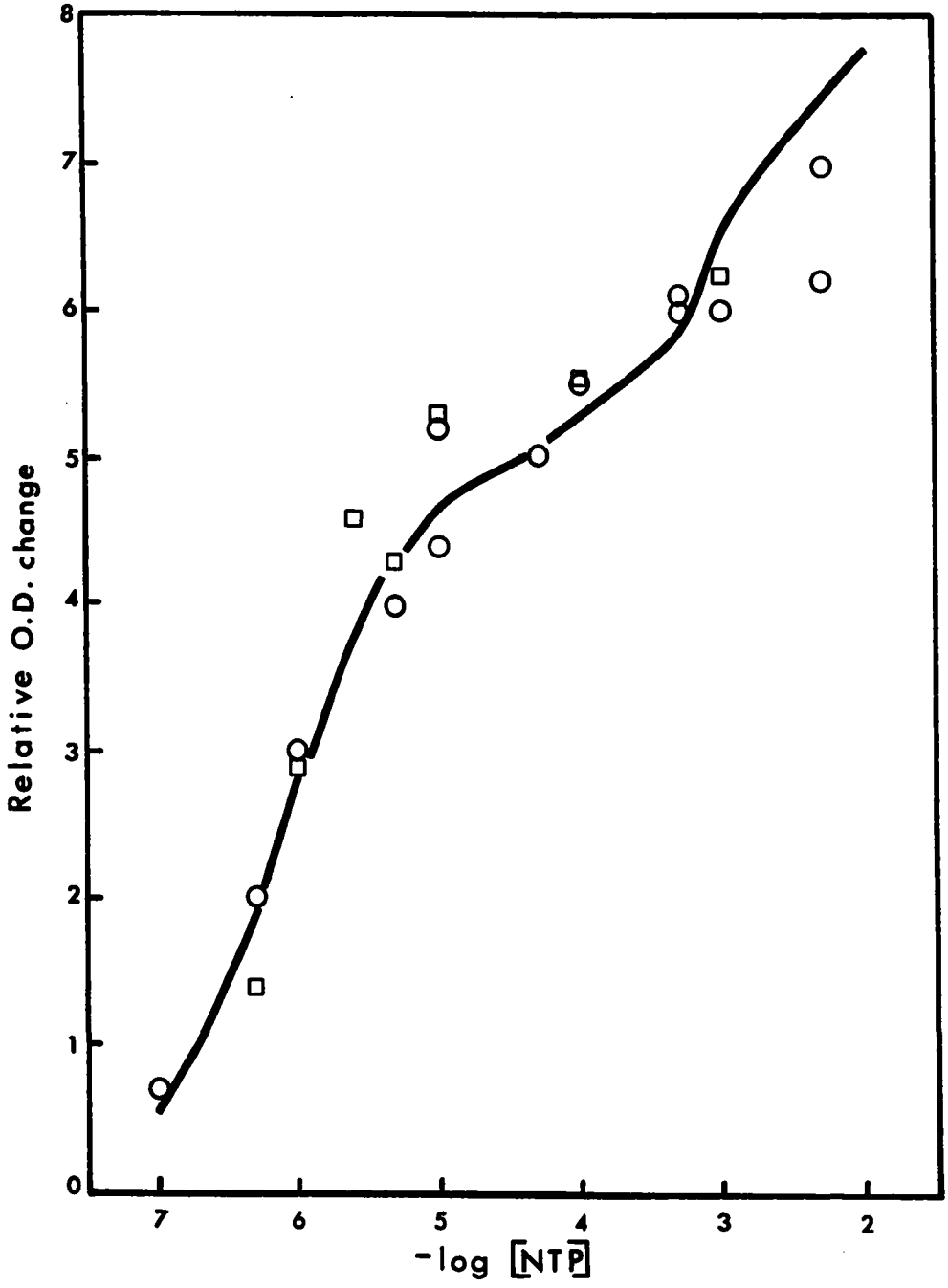


Fig.6: Variation of the myosin NTPase vs the substrate concentration. The assay medium: 0.5M KCl, 0.02M Tris-HCl (pH 7.4), 5mM $MgCl_2$, 5mM PEP, 2mM DPNH, 100 mg/ml each of PK and LDH. Temp.20 ± 1°C. The myosin concentration was 0.29-0.26 mg/ml. (O), ATP; (□), TuTP.

Figure 7 shows the differences in rates of superprecipitation induced by ATP and TuTP. Clearly TuTP is less effective in the superprecipitation process than is ATP, although myosin alone hydrolyzed TuTP and ATP at the same rates (Figures 5 and 6). In the experiments to obtain data for Figure 7, the F-actin in actomyosin contains bound ADP (ADP-F-actin).

Although the role of the ADP attached to F-actin in the process of muscle contraction or superprecipitation is not clear, it is known that the nucleotide bound to F-actin will exchange with free ATP in the reaction medium, and the rate of exchange is considerably enhanced in superprecipitated actomyosin (Szent-Gyorgyi and Prior, 1966; Moos et al., 1967). Moos et al. (1967) demonstrated an exchange of nucleotide in superprecipitated actomyosin at levels of 20 to 30 percent after 10 minutes of reaction time. The turbidity change during superprecipitation in our studies was complete in the order of seconds. Hence, it is unlikely that any sizable extent of the exchange occurred during the turbidity change measurement. However, since nucleotide exchange has been demonstrated and some investigators (Oosawa et al., 1961; Laki, 1969; West, 1970a and b), have suggested that the actin-bound nucleotide and actin conformational changes have roles in the steps leading to muscle contraction, it was considered necessary to determine whether TuDP or TuTP replaced the ADP in F-actin, thereby reducing the rate of superprecipitation. For this reason, the F-actin containing TuDP (TuDP-F-actin) was prepared using TuTP in the processes of extraction and depolymerization of actin.

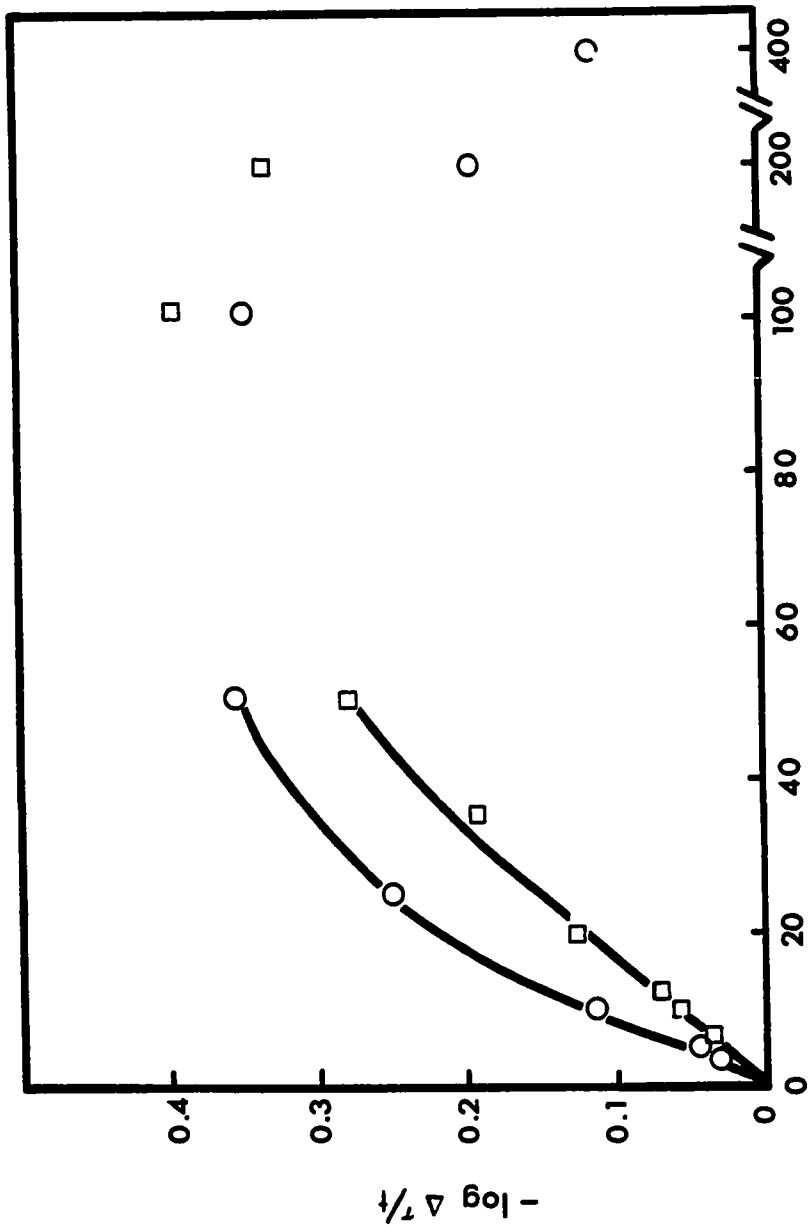


Fig. 7. Rate of turbidity change of ADP-actomyosin vs nucleotide concentration [NTP]. Actomyosin suspension was prepared just prior to the experiment from ADP-F-actin and myosin (0.01 mg/ml actin : 0.02 mg/ml myosin) in 20mM Tris-HCl (pH 7.4), 50mM KCl, 1.0mM $MgCl_2$ and 10μM $CaCl_2$ at 23°C. (○), ATP; (□), TuTP.

Superprecipitation of TuDP-Actomyosin by ATP and TuTP

F-actin was homogenized in the presence of TuTP and dialyzed against the same to obtain the globular TuTP-G-actin. By polymerization and depolymerization in the presence of TuTP, TuDP-F-actin was obtained. TuDP-actomyosin was prepared by mixing TuDP-F-actin and myosin and superprecipitated by ATP or TuTP. As shown in Figure 8, ADP-actomyosin and TuDP-actomyosin behaved identically when they underwent turbidity change upon addition of either ATP or TuTP. In the figure, the rate of turbidity change is expressed by the reciprocal of $t_{\frac{1}{2}}$, which represents the time required to give 50% of total turbidity change. The values of $t_{\frac{1}{2}}$ vary with the concentration of ATP when the other ion concentrations are constant, but are independent of protein concentration (<0.05 mg/ml) at fixed ATP concentration (Nihei and Yamamoto, 1969).

The change in turbidity can be brought about by the variation of such particle parameters as size, shape, and density. If the two actin preparations are different in effecting the change of individual parameters involved in turbidity, it is possible that the net results would still show the same rate of turbidity change with respect to nucleotide concentration. It appears likely, however, that the F-actin-bound nucleotide is not a dominant factor affecting the rates of superprecipitation.

By employing the Lineweaver-Burk graphical method, it has been shown that both actomyosin preparations follow Michaelis-Menten kinetics (Fig.9). The values of the maximum velocity, Vmax, and of Km calculated from the plots in Fig. 9, are listed in Table I. Apparently, the Km's for both actomyosin preparations are about the same for the TuTP and

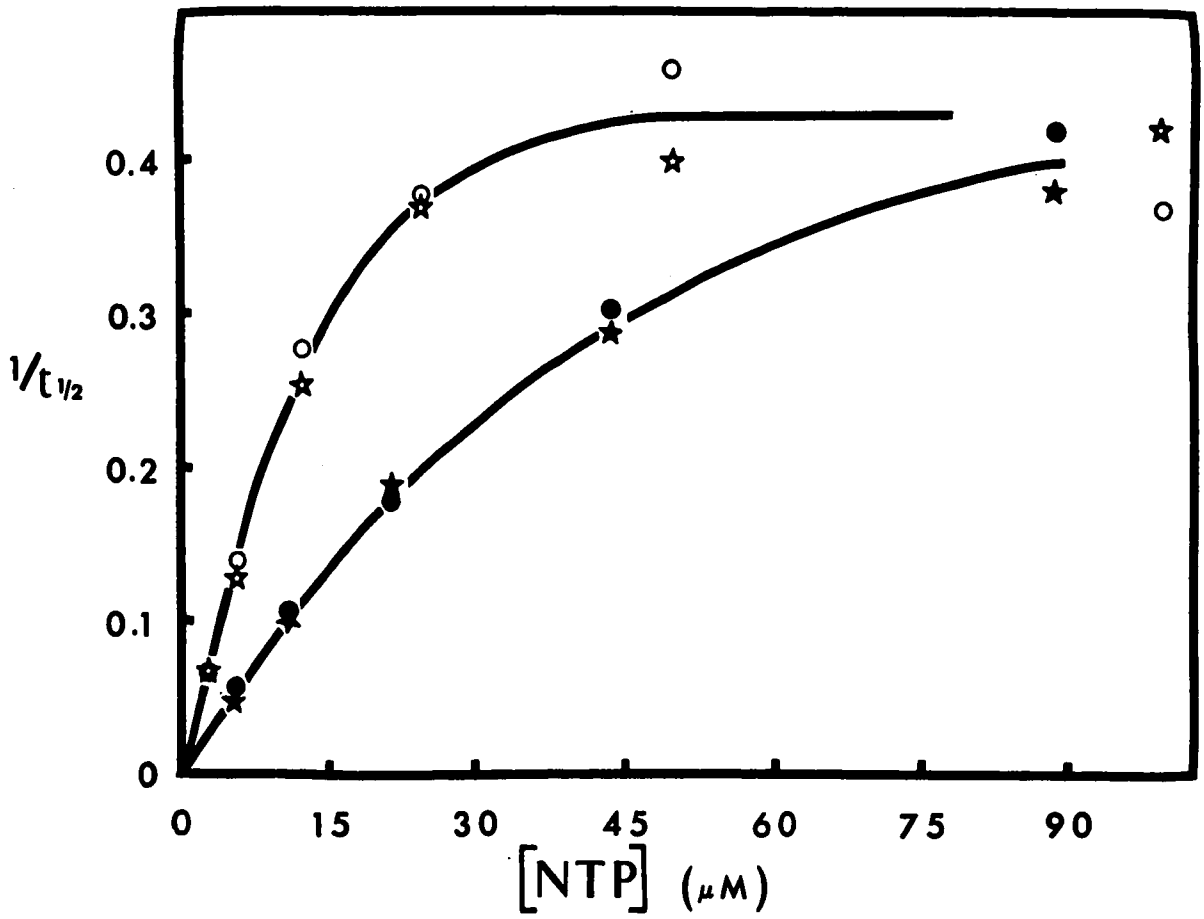


Fig.8. Rate of turbidity change of ADP-actomyosin (○, ●) and TuDP-actomyosin (☆, ★) vs nucleotide concentration (ATP: ○, ☆; TuTP: ★, ●). Actin and myosin were mixed just prior to the experiment in a weight ratio of 1:2. The reaction vessel had a volume of 10 ml containing a final protein concentration of 0.02 mg/ml in 20 mM Tris-HCl (pH 7.4), 50 mM KCl, 1.0 mM $MgCl_2$ and 10 μM $CaCl_2$ at 25°C.

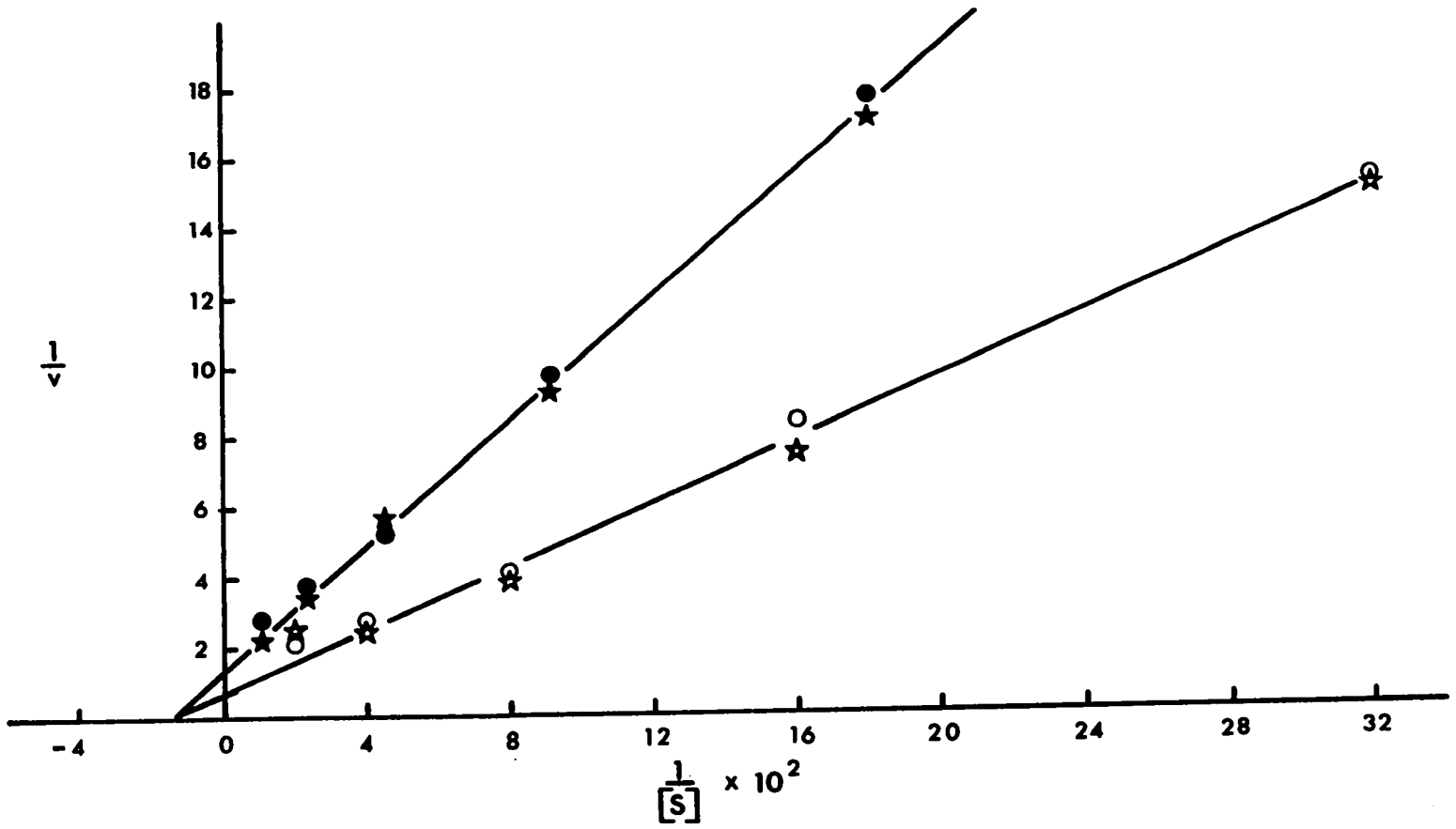


Fig.9. Lineweaver-Burk plots of rates of superprecipitation as a function of nucleotide concentration for ADP-actomyosin (○, ●) and TuDP-actomyosin (☆, ★). ATP : ○, ☆; TuTP : ●, ★. Conditions are the same as for Fig.8.

TABLE I

Maximum velocity and Michaelis constants derived from Lineweaver-Burk plots of rates of superprecipitation as a function of nucleotide concentration for ADP-actomyosin and TuDP-actomyosin.

Nucleotide	Vmax Sec^{-1}	Km µM
ATP	1.6 ± 0.1	71 ± 5
TuTP	0.8 ± 0.1	83 ± 5

the ATP-induced superprecipitation. On the other hand, the Vmax for TuTP is about one-half that of the natural nucleotide, and again no differences are observed between the two types of actomyosin (Table I).

Tension Development by Muscle Fibers Induced by ATP and TuTP

Although the sliding filament model of muscular contraction has gained wide acceptance in recent years, the molecular mechanism which actually produces the force that causes the actin filament to slide past the myosin filament is still unknown. The glycerol-extracted muscle fibers have been widely employed as a contractile model (reviewed by Hasselbach and Weber, 1955). The ATP-induced contraction of these glycerol-treated muscles is considered to be an excellent system in which the conversion of chemical potential into mechanical work takes place (Hotta and Bowen, 1970). Some investigators hold that the superprecipitation system may not in the true sense serve as a contractile model (Hayashi, 1966). Consequently, glycerin-extracted skeletal muscle fibers of rabbit psoas were tested for tension development induced by ATP and TuTP.

In Figure 10, the recorded time course of tension development is reproduced. In the experiments, the identical glycerol-treated muscle fibers were suspended in a medium identical to that used for the superprecipitation experiments. The fibers were fastened between two hooks to maintain the length of fiber as near the resting state as experimentally feasible. Upon addition of ATP, tension development was expressed by the voltage transmitted from the transducer. As seen from the curves in Figure 10, the addition of ATP at a final concentration of 0.6mM caused the fiber to develop tension immediately. On the other

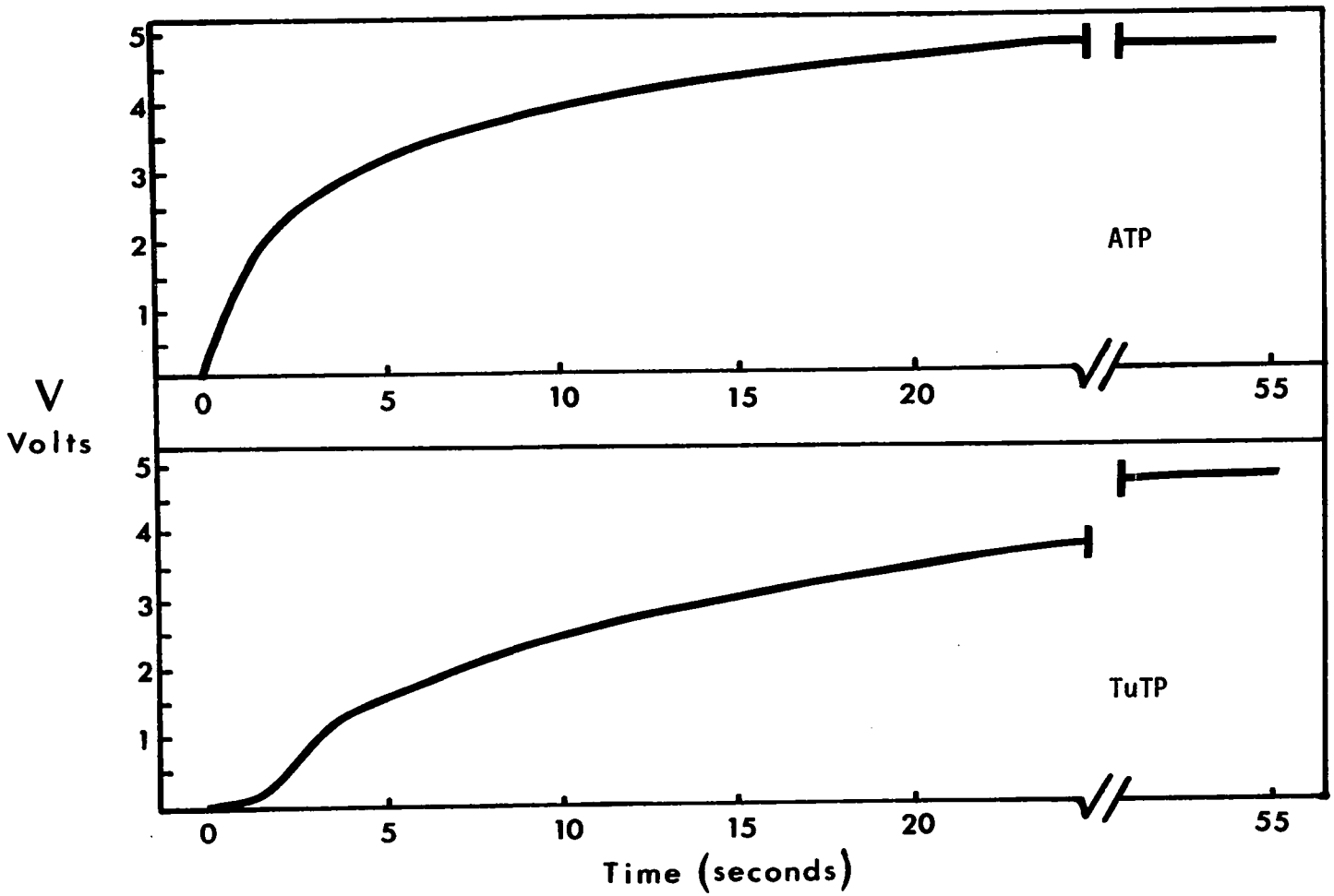


Fig.10. Tension development by glycerol-extracted rabbit psoas induced by ATP and TuTP in 20mM Tris-HCl (pH 7.4), 50mM KCl, 1.0mM $MgCl_2$ and 10μM $CaCl_2$ at 22°C. Initial nucleotide concentration was 0.6mM. Fiber diameter was about 350μ.

hand, the addition of the same amount of TuTP as that of ATP, effected the fiber to contract slowly after a certain lag period. It was noted that the final level of developed tension was approximately the same with the addition of ATP and TuTP. To compare the rates of tension development more quantitatively, the increment of voltage per second was calculated at intervals of 0.5 seconds, and plotted as shown in Figure 11. Figure 12 shows the results of the experiments with 0.3mM nucleoside triphosphate.

Although the experimental data can be treated according to the equation of Hill (1938), the interpretation of constants involved is not clearly understood. For this reason, the observations described in this section are used as supporting evidence of the experimental results obtained with the actomyosin system.

Enzymic Activity of ADP-Actomyosin and TuDP-Actomyosin during the Superprecipitation Process

Since Weber and Portzehl (1954) have shown that the actomyosin type of ATPase activity is necessary for the contraction of glycerinated muscle fibers and that the force of contraction is proportional to the rate of ATP splitting, it is imperative that the superprecipitation of both preparations of actomyosin be correlated with the rate of hydrolysis of the nucleotides ATP and TuTP. In many experiments the superprecipitation is complete within 10 seconds. The rate of hydrolysis is normally measured by determining the amount of inorganic phosphate formed. The classical method of colorimetric determination of phosphate concentration is not sufficiently sensitive. As a result γ-^{32}P-nucleotide was employed.

By plotting the rate of hydrolysis of γ-^{32}P-ATP versus nucleotide

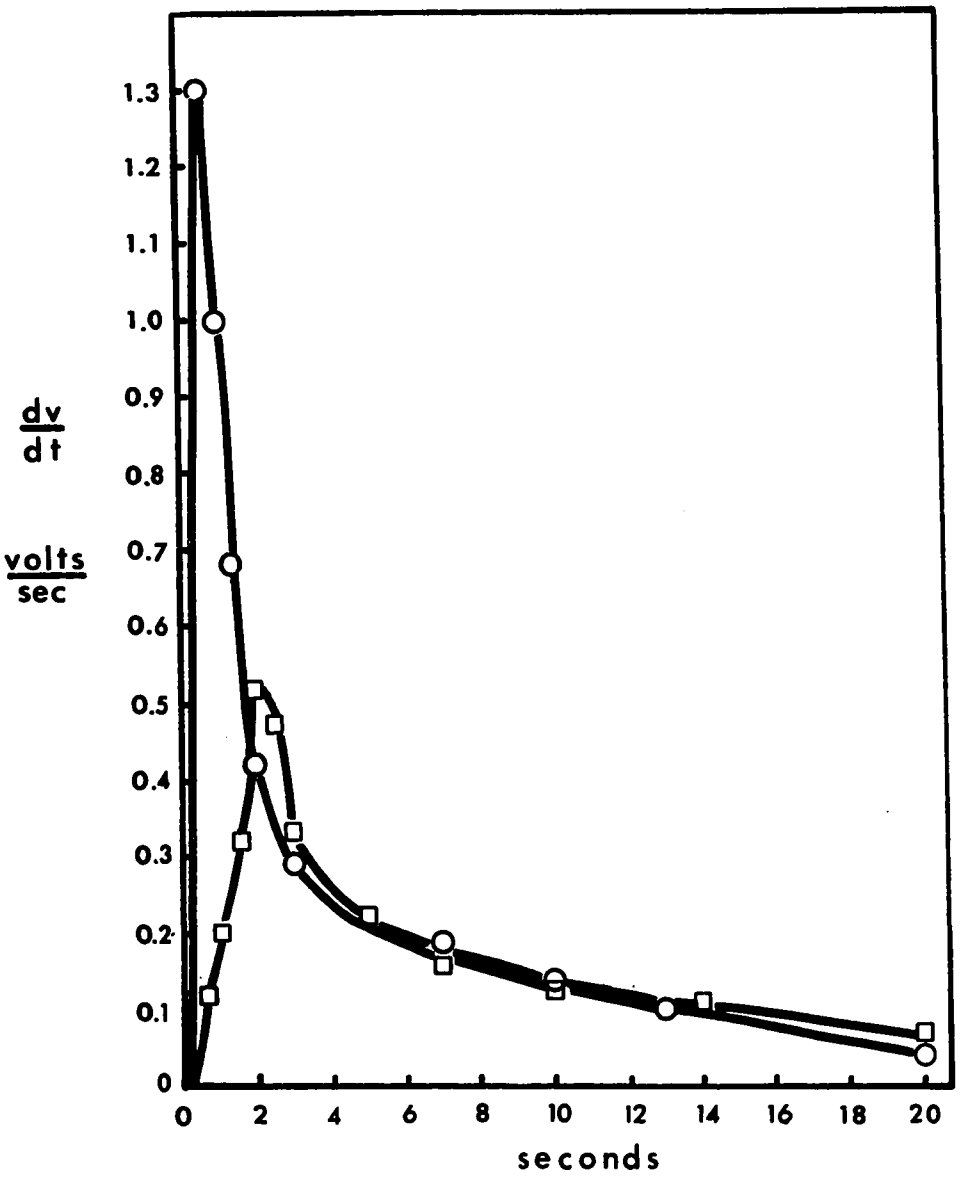


Fig.11. Rate of tension development as a function of time. Conditions are the same as for Fig.10. (○), 0.6mM ATP; (□) 0.6mM TuTP.

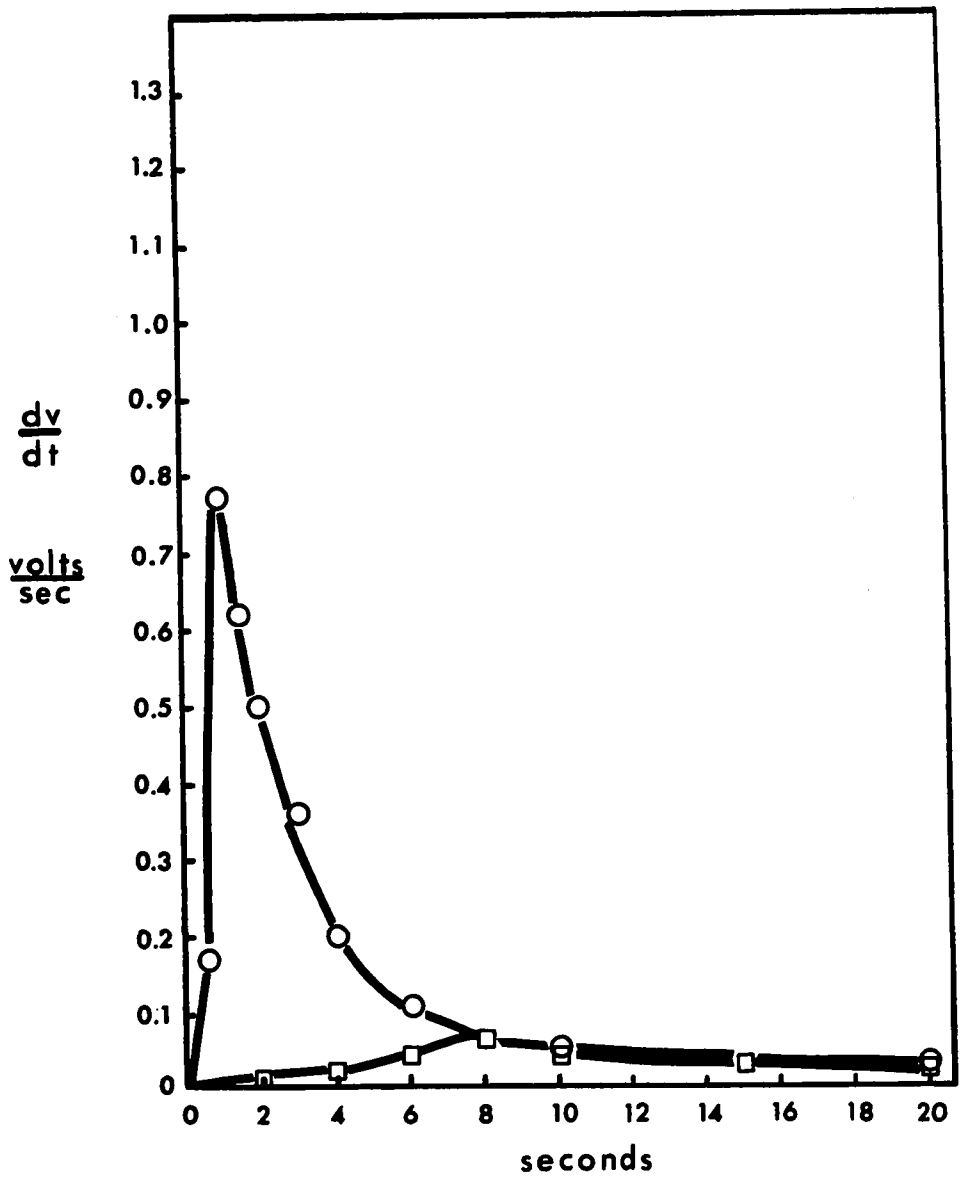


Fig.12. Rate of tension development as a function of time. Conditions are the same as for Fig.10. (○), 0.3mM ATP; (□), 0.3mM TuTP.

concentration (Fig.13), it seems that ATP is hydrolyzed more rapidly by ADP-actomyosin than by the TuDP-actomyosin system. Similarly, under identical conditions, ADP-actomyosin appeared to hydrolyze γ-^{32}P-TuTP at a somewhat higher rate. However, utilizing the Lineweaver-Burk plots for rates of nucleotide hydrolysis (Figure 14) and therefrom calculating Vmax and Km, the values of these parameters for the two types of actomyosin were found to fall in the ranges listed in Table II. It is thus reasonable to assume that there is no significant difference in the hydrolytic behaviour of the two actomyosins. Again, it is apparent that the Km value for TuTP hydrolysis is about the same as that for ATP hydrolysis but the Vmax value for TuTP is about 4 times as great as that of ATP (Table II).

If the efficiency of a nucleotide in superprecipitation is defined as the ratio of the rate of turbidity change to the rate of hydrolysis, then the natural energy source, ATP, is about 800% more efficient than its analog TuTP in the process.

Association, Dissociation and Reassociation Studies of the Actomyosin Complex

It has been known for some time that the actomyosin complex in high ionic strength medium will dissociate into the component proteins, F-actin and myosin, upon the addition of ATP (Szent-Gyorgyi, 1951; Spicer, 1952; Gergely, 1956). Maruyama and Gergely (1962) observed the dissociation (so-called "clearing") by ATP of an actomyosin dispersion at physiological ionic strength. The "clear phase" is followed by precipitation and superprecipitation as the substrate, ATP, is depleted. The duration of the "cleared" state ("clearing time") diminished with decreasing ionic strength (Eisenberg and Moos, 1967) and finally superprecipitation appears to occur without dissociation at low (0.05M)

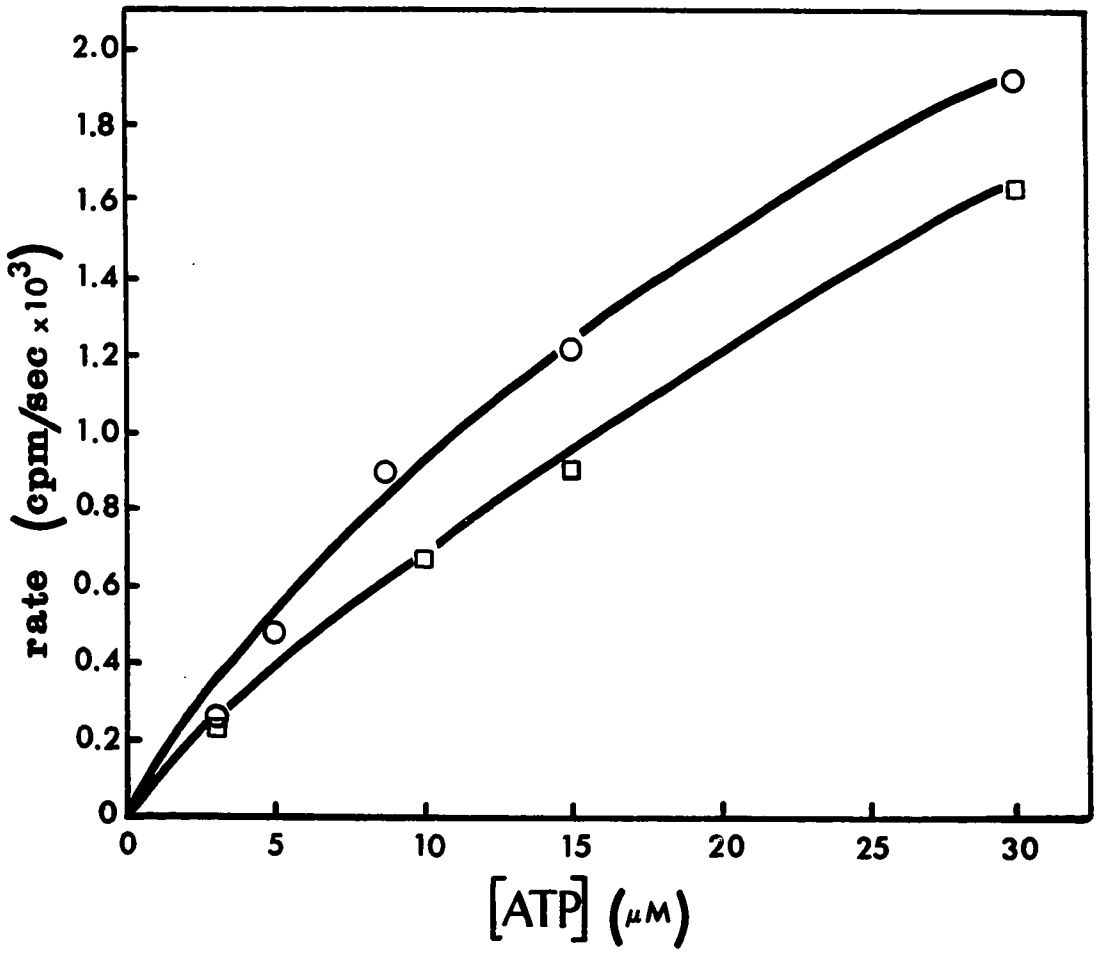


Fig.13. Rate of hydrolysis of γ-^{32}P-ATP by actomyosin as a function of nucleotide concentration. Actomyosin suspensions were prepared just prior to the experiment (0.005 mg/ml actin : 0.01 mg/ml myosin) in 20mM Tris-HCl (pH 7.4), 50mM KCl, 1.0mM $MgCl_2$ and 10 μM $CaCl_2$ at 23°C. (○), ADP-actomyosin; (□), TuDP-actomyosin.

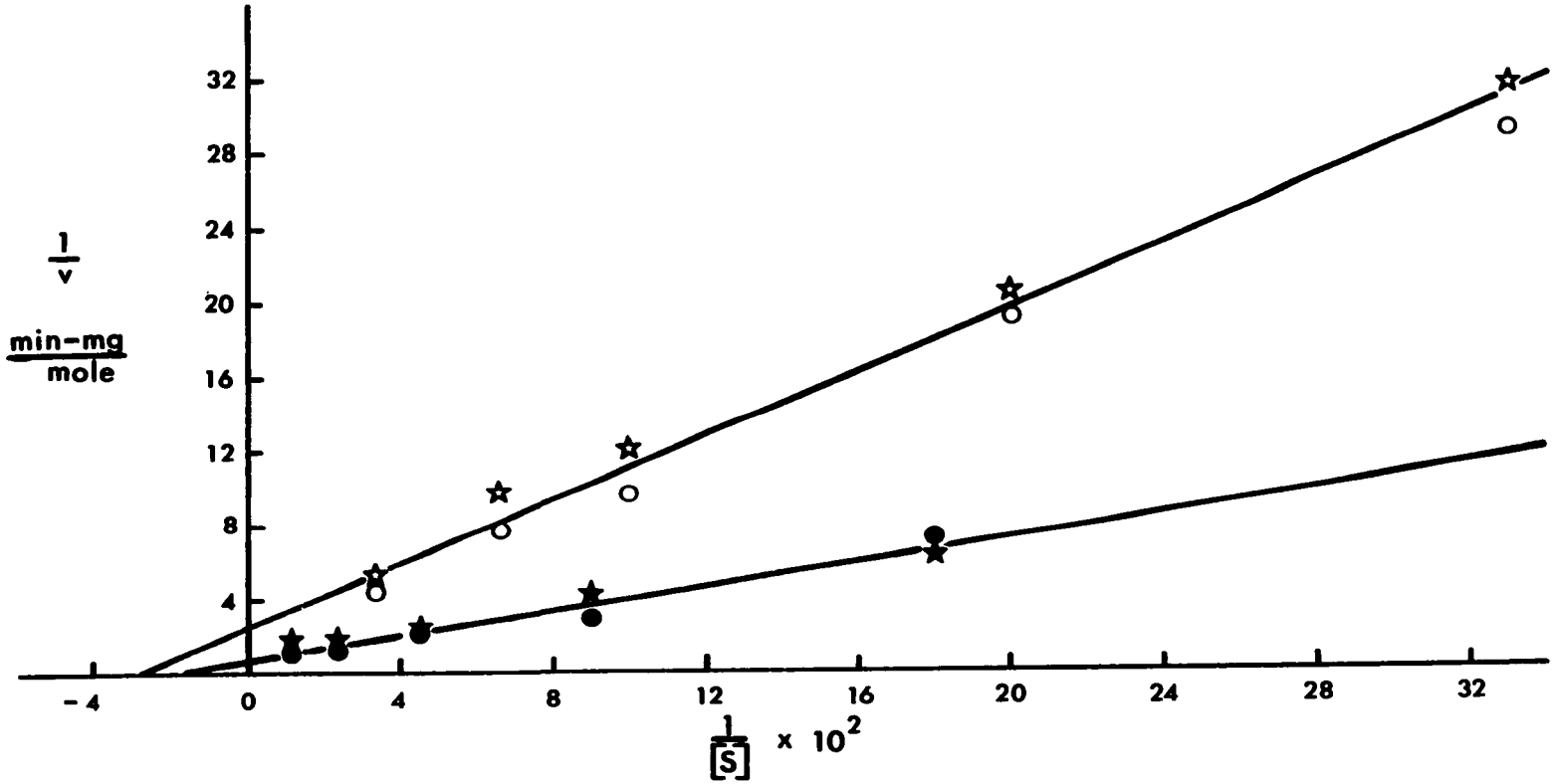


Fig.14. Lineweaver-Burk plots of NTPase activity as a function of nucleotide concentration for ADP-actomyosin (○,●) and TuDP-actomyosin (☆,★). γ-^{32}P-ATP : ○,☆; γ-^{32}P-TuTP : ●,★. Conditions are the same as for Figure 13.

TABLE II

Maximum velocity and Michaelis Constants derived from Lineweaver-Burk plots of rates of hydrolysis as a function of nucleotide concentration for ADP-actomyosin and TuDP-actomyosin.

Nucleotide	Vmax μmoles/min-mg.	Km μM
ATP	0.4 ± 0.1	40 ± 10
TuTP	1.5 ± 0.3	32 ± 10

KCl concentrations (Maturaga and Noda, 1966; Nihei, 1967). Even after superprecipitation (turbidity change) is complete, ATP hydrolysis continues at an elevated rate. Nihei and Yamamoto (1969) have employed electron microscopy and observed morphological changes in the superprecipitated proteins up to 30 minutes after ATP addition. It is therefore inferred that, at least in solution, ATP serves both as substrate for myosin and actomyosin and as regulator of the interaction between F-actin and myosin.

Many workers have studied the dissociation of actomyosin and Finlayson et al., (1969) have studied the kinetics of the association-dissociation process. All association-dissociation and reassociation studies were carried out at 0.5M KCl in the presence of 5mM Mg^{2+}.

Association - The rate of formation of the actomyosin complex (AM) upon addition of myosin (M) to a suspension of F-actin (A), may be measured by the increase in optical density which is proportional to the turbidity change. If the interaction of actin and myosin can be expressed in terms of the following equation;

$$A + M \underset{k_d}{\overset{k_a}{\rightleftharpoons}} A - M \qquad (3)$$

the association step is a second order reaction. The rate constant, k_a, is related to the concentrations of free myosin, (Mo) and (M), at zero time and after a period of reaction time, t, respectively, in the case where (Mo) is identical to the initial concent ation of free actin, (Ao). The relationship is written in Equation 4 as shown by Frost and Pearson (1961).

$$k_a t = \frac{1}{(M)} - \frac{1}{(Mo)} \qquad (4)$$

Figure 15 shows a typical recorder tracing of the association reaction of actin and myosin. In the figure, the dissociation phenomenon is also shown, which is caused by the addition of ATP at the time indicated by the arrow.

If the amount of actin in the reaction mixture is adjusted to let all the molecules of myosin combine with actin, the values of (Mo) and (M) can be represented by the maximal optical density change, ΔODmax, and ΔODmax-ΔOD, respectively, where ΔOD, the optical density change at time t, is proportional to (AM) = (Mo) - (M), hence (M) = (Mo) - (AM) = ΔOD max - ΔOD. Thus according to Equation 4, a plot of $(\Delta OD_{max} - \Delta OD)^{-1}$ versus t should be linear with a positive slope equal to k_a. It is shown in Figure 16 that the data fit the equation and that ADP-actin and TuDP-actin exhibit the same rate constant ($1.1 \pm 0.2 \times 10^5$ $M^{-1}Sec^{-1}$) in combining with myosin to form the actomyosin complex.

Dissociation - Another important step in the mechanism of muscle contraction involves the dissociation of actin and myosin as the cross-bridges interact with the actin filament. Again, a 0.5M KCl solution is used to measure the rate of dissociation of actomyosin, because at a lower ionic strength, the dissociation step is not clearly separated from the reassociation reaction.

As observed in the data for Figure 15, addition of ATP dissociates the actomyosin complex and the reaction may be followed by measuring the optical density change. In their studies of dissociation, Finlayson et al. (1969) found that the rate constant λd (Equation 5) depended on pH, temperature, metal ion, and substrate concentration.

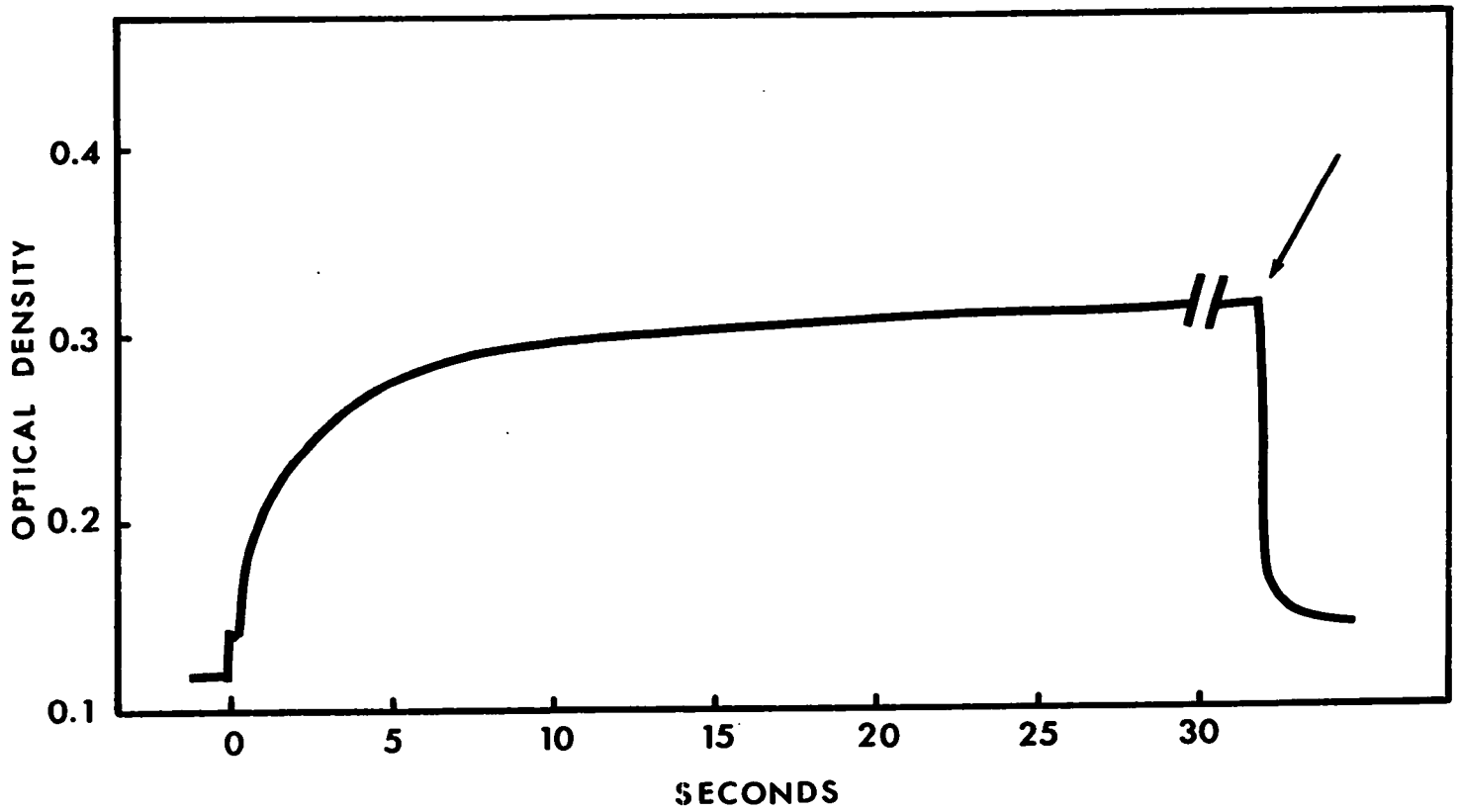


Fig.15. Turbidity change in the formation of the actomyosin complex upon addition of myosin to a suspension of F-actin. The reaction vessel had a volume of 7 ml. containing a final protein concentration of 1 mg/ml myosin and 0.3 mg/ml actin in 20mM Tris-HCl (pH 7.4), 0.5M KCl, 5.0mM $MgCl_2$ at $23^{o}C$. The arrow indicates the addition of 0.1mM ATP.

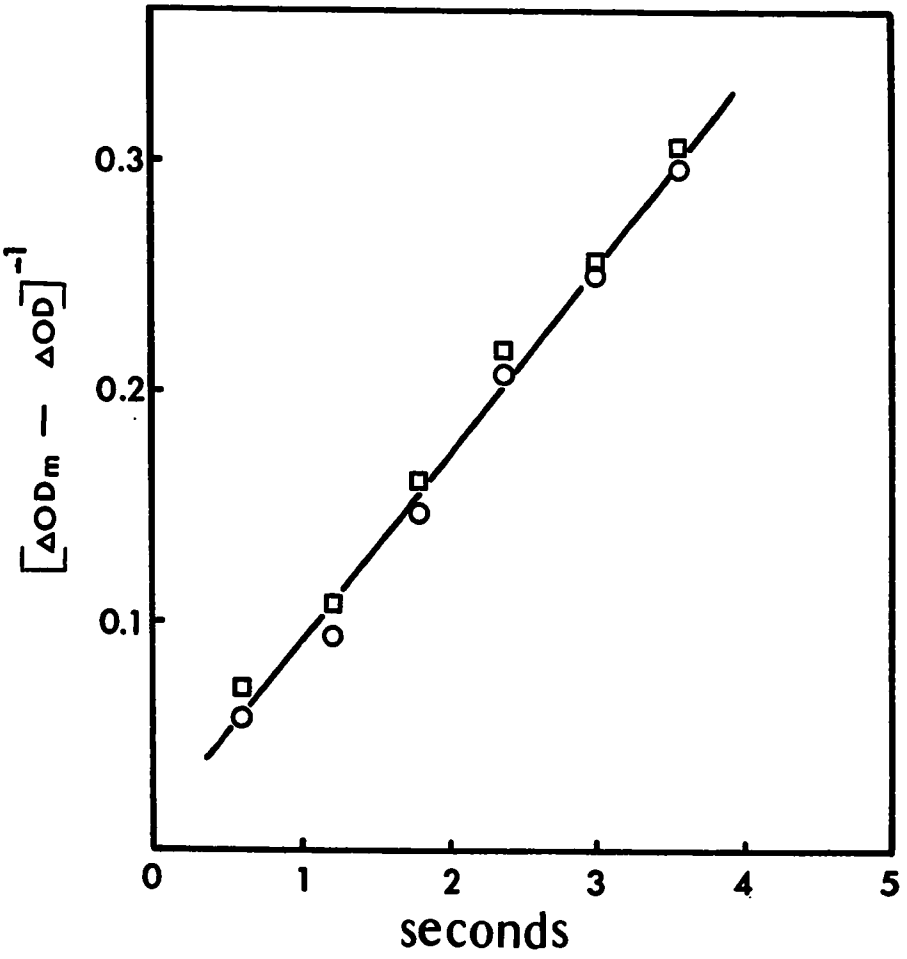


Fig.16. Plot of the reciprocal of the optical density difference in the reaction of F-actin and myosin. ΔOD_m is the total optical density change obtained at long times. Conditions are the same as for Figure 15. (○), ADP-actomyosin; (□), TuDP-actomyosin.

Since our primary purpose was to compare the substrate TuTP to ATP, all the variables except the nucleotide concentration were kept constant.

$$AM \underset{\lambda r}{\overset{\lambda d}{\rightleftharpoons}} A + M \qquad (5)$$

By plotting -log(ΔODmax-ΔOD) versus time, linear plots were obtained, which indicate that the dissociation of actomyosin is a first order reaction in terms of turbidity. The results showed that TuTP was less effective in the dissociation of the protein complex (Figure 17). The data in Figure 17 yielded the rate constants (λd) of 23sec^{-1} and 13 sec^{-1} for ATP and TuTP, respectively. Finlayson et al., (1969) found that λd was proportional to MgATP concentration and observed that it satisfied the relation

$$\lambda d = k(MgATP) + c \qquad (6)$$

where k has the typical second-order reaction unit and "c" is a constant. Theoretically "c" should equal zero because, in the absence of ATP, dissociation does not occur. The data of Finlayson et al., (1969) show a non-zero "c" which they interpreted to be not much greater than the experimental error.

In plotting λd versus nucleotide concentration in the range 10-50 μM it was found that "c" in Equation 6 was 9 sec^{-1} for TuTP and 19 sec^{-1} for ATP in their dissociation of actomyosin. Since the dissociation of the protein complex is extremely rapid at higher NTP concentrations (>10μM), the resolvable time by our method was limiting and subject to increasing error with increasing nucleotide concentration. Finlayson

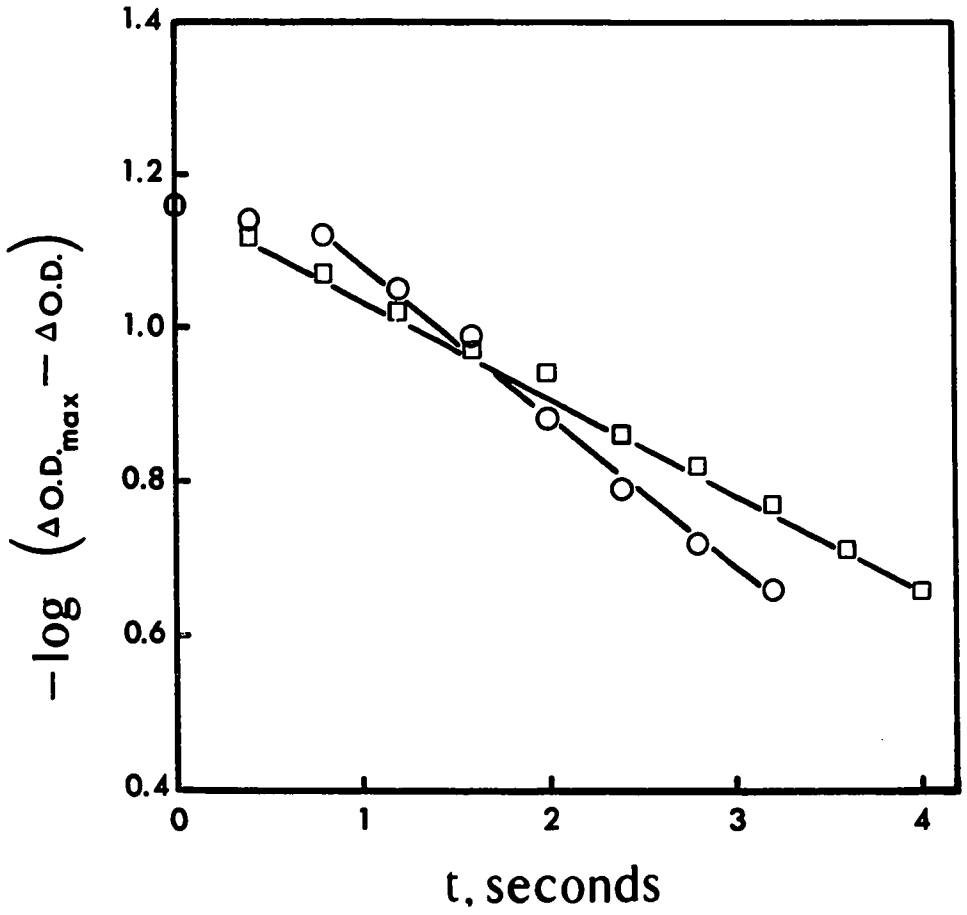


Fig.17. The negative log plot of the optical density change in the dissociation of the actomyosin complex by ATP (○) and TuTP (□). The reaction vessel had a volume of 7 ml. containing a final protein concentration of 0.29 mg/ml in 20mM Tris-HCl (pH 7.4), 0.5 M KCl, 5.0mM $MgCl_2$ at 23°C.

et al., used high ATP concentrations (100-1000μM) and employed a stopped-flow spectrophotometer which responded in the order of milli seconds.

If the value of "c" is not zero, the dissociation process is not a second-order reaction. If our λd data are made to fit Equation 6 then the second-order rate constant, k, is 1.3×10^6 $M^{-1}sec^{-1}$ for TuTP and 2.3×10^6 $M^{-1}sec^{-1}$ for ATP.

Reassociation - When the actomyosin complex is dissociated with low concentrations of nucleotide (10μM), the triphosphates are rapidly depleted and the actin and myosin proteins reassociate once again. However, the rates of reassociation are substantially reduced in comparison to those observed by mixing actin and myosin (Figure 18).

When an actomyosin preparation is allowed to reassociate after dissociation by ATP or TuTP, the subsequent rates of dissociation are increased by a factor of approximately 2 (Figure 19 and Table III). This effect may be due to the fact that the protein complex forms a more homogeneous suspension after the dissociation and reassociation cycles.

As the substrate concentration is varied from 50μM to 10μM the extent of the change in turbidity is reduced in the dissociation step. This observation may be the result of incomplete dissociation. On subsequent reassociations the regain in turbidity exceeded the former values (Figure 18). These results are in disagreement with those of Finlayson et al., (1969) and Tonomura and Watanabe (1953) who reported an average regain in turbidity of only 80% of the original drop brought about by substrate. No satisfactory explanation can be offered other

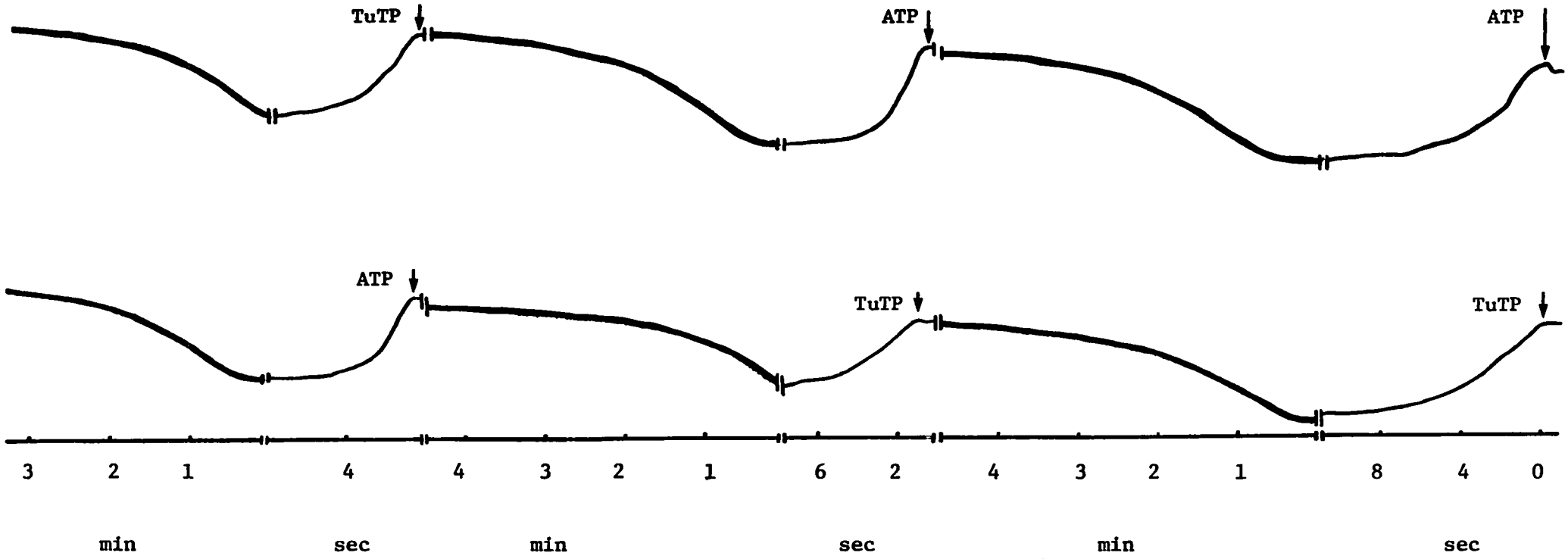


Fig.18. Dissociation and reassociation phenomena of actomyosin observed by following turbidity changes. Arrows show the initiation of dissociation upon addition of a pulse of 10μM ATP (↓) or TuTP (↓) to a 7 ml reaction vessel with 3.1 mg/ml actomyosin (A:M = 1:2, W/W) in 5mM Tris-HCl (pH 7.4), 0.5M KCl and 1mM Mg^{2+} at 23°C.

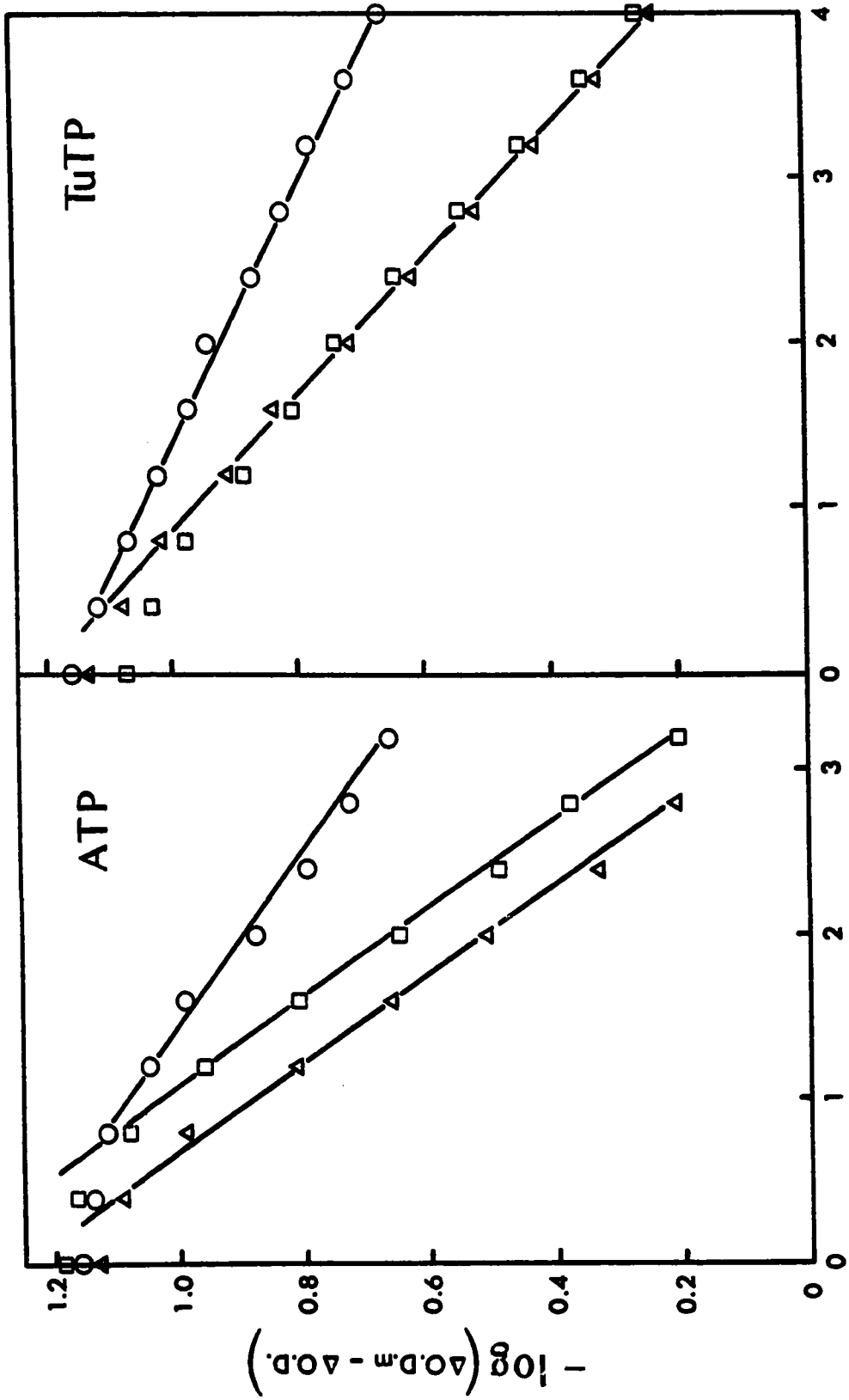


Fig.19. The negative log plot of the optical density change in the dissociation of the actomyosin complex by ATP and TuTP. conditions are the same as for Figure 17. (O), initial dissociation; (□), dissociation subsequent to a dissociation cycle by ATP; (Δ), dissociation subsequent to a dissociation cycle by TuTP.

Table III

Rate Constants for Dissociation of the Actomyosin Complex by 10μM NTP[a]

Substrate	Rate Constant λ_d (sec^{-1})
ATP	22.5
TuTP	13
ATP	43.5[b]
TuTP	29[b]
ATP	43.5[c]
TuTP	29[c]

a. All optical density measurements were made with protein concentration at 1.9 mg/ml (A:M = 1:4, W/W) in 20mM Tris-HCl (pH 7.4), 0.5 M KCl, 5mM $MgCl_2$, at 23°C.

b. Dissociation constant subsequent to one dissociation and reassociation.

c. Dissociation constant subsequent to two successive dissociation and reassociation steps.

than perhaps the differences in protein preparations and/or in the optical arrangement of instruments used.

At low nucleotide concentrations ($\sim$10μM) the maximum optical density change incurred by TuTP dissociation is approximately 80-85% of that for ATP. This might suggest that the extent of dissociation by TuTP is reduced in addition to the lower rate of dissociation as already observed in Figure 17.

Also, the reassociation step of F-actin and myosin appears to be a first-order reaction because the plot of the negative log of the optical density change versus time was linear and the rate constant, λr, did not depend on the actomyosin concentration (Figure 20). In the dissociation-reassociation sequences, TuTP was used to dissociate actomyosin subsequent to the dissociation by ATP. Similarly, ATP was used to dissociate the complex after it had been dissociated by TuTP and subsequently allowed to reassociate. The rate constant for reassociation after TuTP dissociation was found to be $5.7 \pm 0.5 \times 10^{-3} sec^{-1}$ which is not significantly different from that of the corresponding reassociation after ATP dissociation ($4.9 \pm 0.4 \times 10^{-3} sec^{-1}$).

Clearly, a different mechanism is in operation in the reassociation steps when compared to the initial association of independently prepared F-actin and myosin. If one assumes that the conformation of myosin is altered upon the binding of NTP or NDP with subsequent actin-activated hydrolysis of the substrate (vide infra, uv difference spectra in myosin), then it may be proposed that the reduced rate of reassociation of actin and myosin is due to the conformational change in myosin.

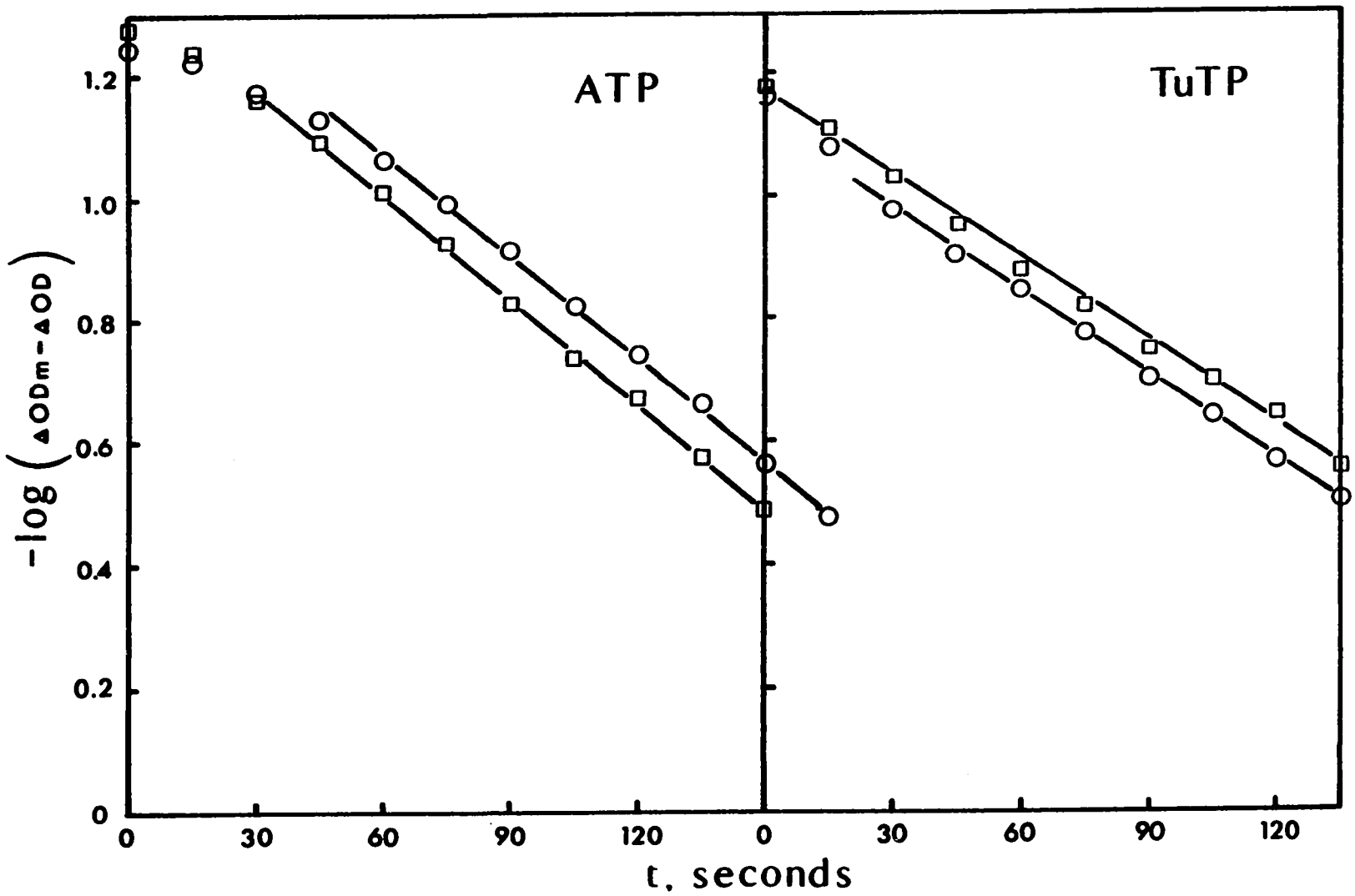


Fig. 20. The negative log plot of the optical density change in the reassociation of F-actin and myosin. Conditions are the same as for Figure 17. (□), reassociation after an initial pulse of NTP; (○), reassociation after a dissociation pulse by NTP subsequent to a completed dissociation-reassociation cycle.

Ultraviolet and Difference Spectroscopy of the G- and F-Actins with Bound Tubercidin and Adenine Nucleotides

The most generally accepted model for muscle contraction is the one proposed by Huxley (Huxley and Niedergerke, 1954; Huxley and Hanson, 1954; Huxley, 1969). In the proposed mechanism the actin and myosin filaments are linked by, and interact through, the so-called "cross-bridges" which are a permanent part of the myosin structure. The series of small displacements between actin and myosin results in movement (muscle contraction).

Ever since the discovery that the ATP bound to globular (G-) actin is transformed to ADP and inorganic phosphate during polymerization to fibrous (F-) actin (Straub and Feuer, 1950; Laki et al., 1950), the role of the actin-bound nucleotide has been rigorously investigated. However, agreement in the interpretation of results and understanding of the function of bound nucleotide have not been reached.

Barany et al. (1966) suggest that the bound ADP of F-actin is not involved in the physiologically important properties of F-actin, namely, the activation of the ATPase activity of myosin and superprecipitation with myosin in the presence of ATP. On the other hand, Hayashi (1966) found that ADP bound to actin is necessary for contraction of an actomyosin system to occur. Presently research is continuing with the view of determining the possible role of actin-bound nucleotide in the biochemical events leading to contraction (West, 1971).

Clearly then, it was also necessary to consider the effects, if any, of substituting tubercidin-bound actin for the naturally occurring molecules. As reported above, TuDP-actomyosin and ADP-actomyosin

superprecipitated at the same rates when TuTP or ATP was used as the energy source. ADP- and TuDP-F-actin associated with myosin at the same rate to form the actomyosin complex. Both actomyosin complexes dissociated at the same rates by the addition of either ATP or TuTP. There was no significant difference in rates of hydrolysis of NTP when TuDP-actomyosin was compared to ADP-actomyosin. Despite the above negative results, a study of the actin monomers and polymers employing the ultraviolet difference spectroscopy was pursued in view of the fact that the function of bound-nucleotide is still unclear.

Most of the work on ultraviolet light absorption by proteins has been carried out in the 250-320 $m\mu$ region where low intensity $\pi \rightarrow \pi^*$ transitions of the aromatic side chains can be detected. The peptide bond absorbs the light in the 200-240 $m\mu$ region due to the $n \rightarrow \pi^*$ transition of the carbonyl groups. Tryptophan is the strongest absorber of the aromatic amino acids. The molar extinction coefficient for N-acetyl-L-phenylalanine ethyl ester is 195 at 258.5 $m\mu$, 1340 at 274.5 $m\mu$ for N-acetyl-L-tyrosine ethyl ester, and 5550 at 282 $m\mu$ for N-acetyl-L-tryptophan ethyl ester (Beaven and Holiday, 1952; Wetlaufer, 1962). Rabbit skeletal actin is comprised of 13 phenylalanines, 17 tyrosines and 5 tryptophans per G-actin monomer (Adelstein and Kuehl, 1970; Elzinga, 1970).

The G-actin preparations for ultraviolet spectroscopic studies were extensively dialyzed against 0.2mM nucleotide and pH 7.4 tris buffer. The reference cell contained dialysate and the sample cell contained protein in dialysate medium. The absorption spectrum

of TuTP-G-actin in the 280 mμ region was identical to that of ATP-G-actin (Figure 21). When the free nucleotide concentration in the sample cell was adjusted so that the bound nucleotide plus free nucleotide in the sample cell was equal to the nucleotide concentration in the reference cell, then the two spectra differed considerably (Figure 22). The λmax for TuTP and ATP are 269 and 259 mμ respectively and the λmin are 242 and 227 mμ respectively (Figure 23).

Figures 21-23 suggest that either the $\Pi \rightarrow \Pi^*$ transition energies of the aromatic rings of the respective nucleotides become equal upon binding to G-actin or the bound nucleotides do not absorb in the 230-290 mμ region.

The absorbance spectra of the F-actins were also the same. The large F-actin polymers also gave rise to an increase in optical density due to Rayleigh light scattering (Leach and Scheraga, 1960; Higashi et al., 1963). Light scattering by F-actin could be reduced by two additional depolymerization-repolymerization cycles.

The ultraviolet difference spectra between G-actin in the reference cell and F-actin in the sample cell were obtained (Figure 24). After four polymerization-depolymerization cycles no significant difference in the scattering intensity between G-actin and F-actin was observed in the 800-350 mμ region. As a result these spectra could not be corrected for light scattering by extending a double logarithmic plot from the visible region into the ultraviolet (Leach and Scheraga, 1960). Below 300 mμ the TuDP-F-actin preparations showed a larger degree of light scattering. This difference in Rayleigh light scattering could be due to the larger F-actin polymers formed with TuTP-G-actin by slower nucleation and growth processes

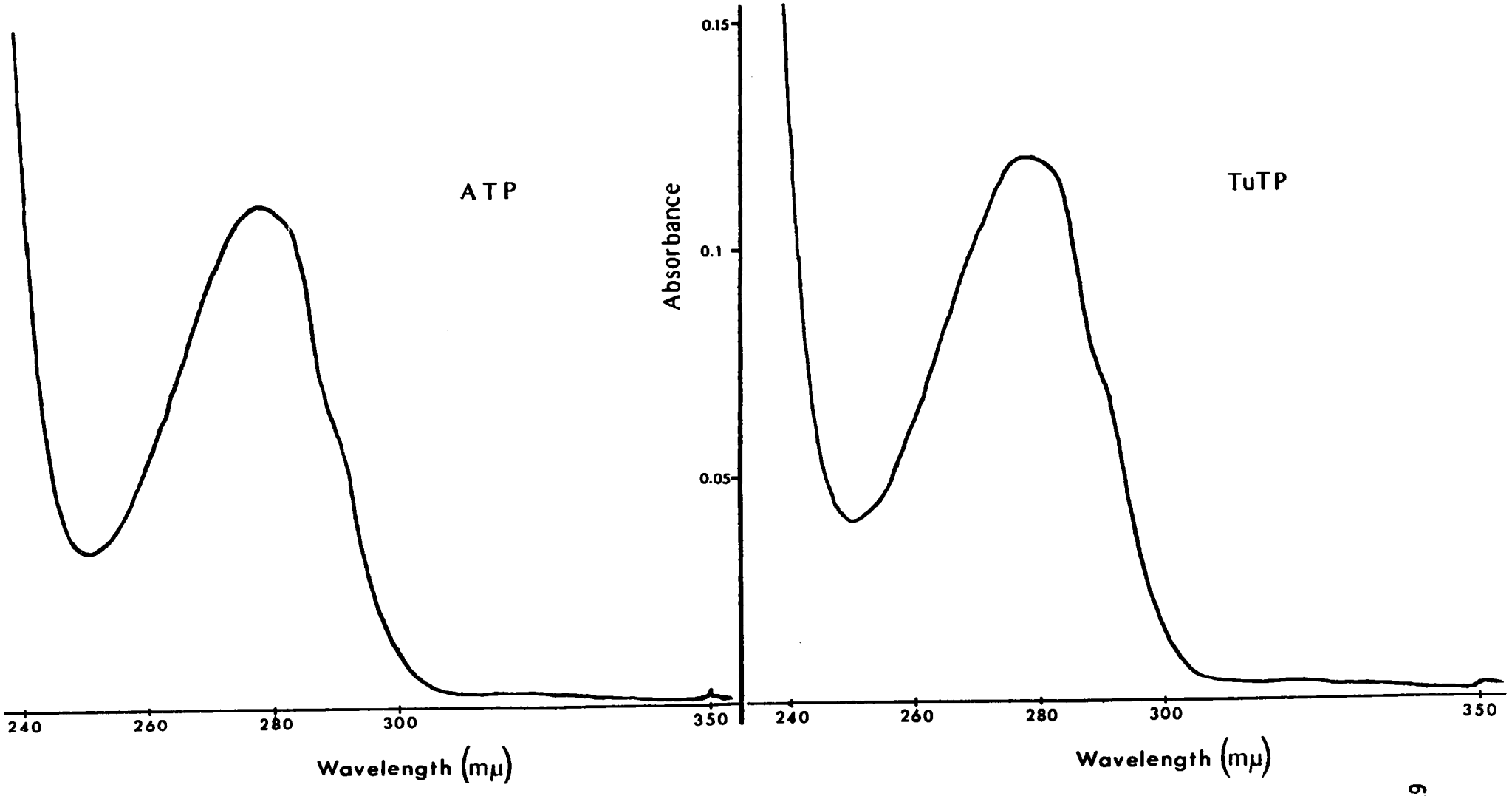


Fig.21. Ultraviolet spectra of ATP-G-actin (1.5 mg/ml) and TuTP-G-actin (1.5 mg/ml). Protein was dialyzed 3 days against three changes of 5mM Tris-HCl (pH 7.4) and 200 μM NTP. The reference cell contained dialysate. Absorbance changes were observed with 1-mm path length balanced cells.

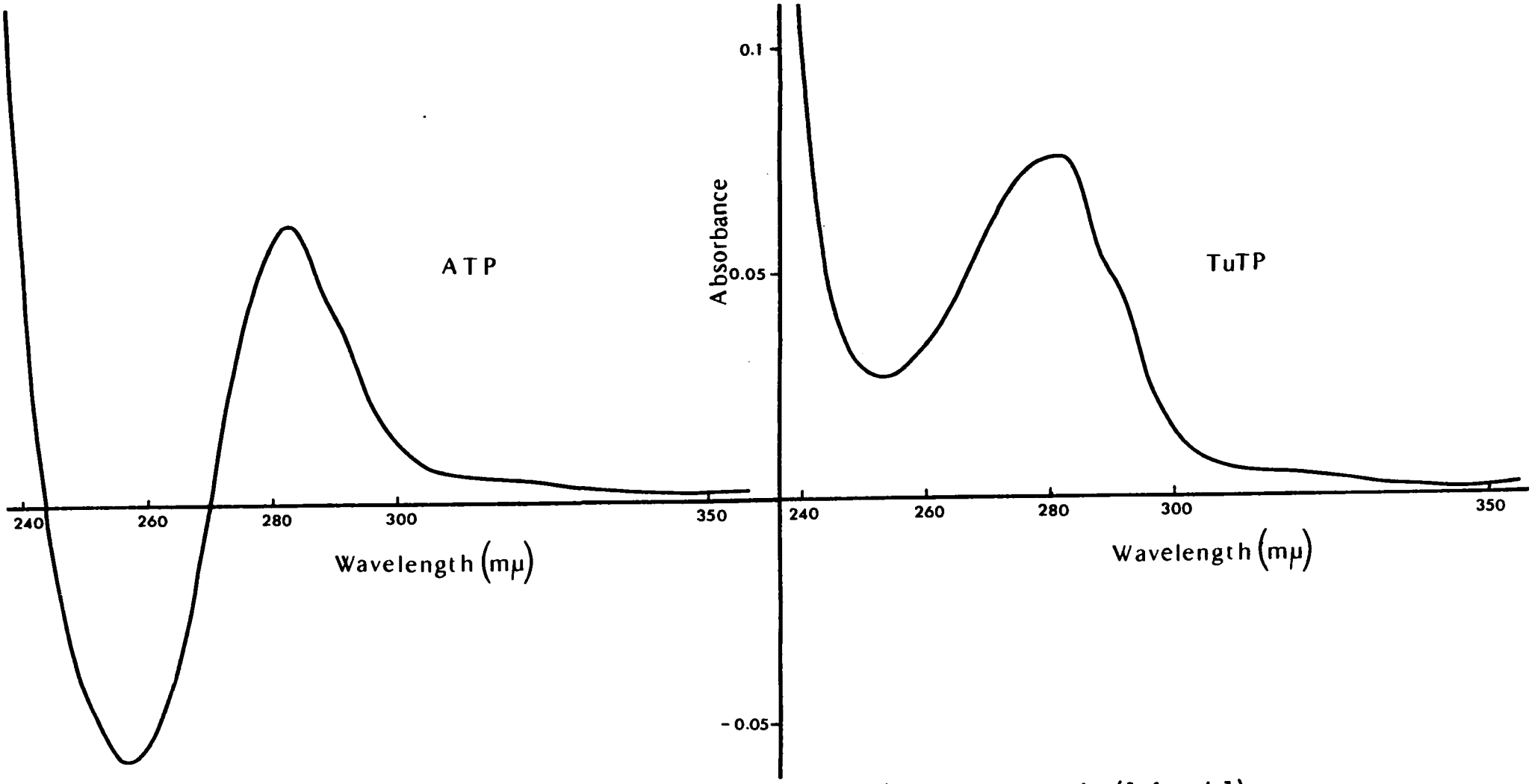


Fig.22. Ultraviolet spectra of ATP-G-actin (1.0 mg/ml) and TuTP-G-actin (1.4 mg/ml). Protein was dialyzed 3 days against three changes of 5mM Tris-HCl (pH 7.4) and 200 μM NTP. The reference cell contained dialysate and the sample cell had a free nucleotide plus a bound nucleotide concentration of 200 μM. Tris-HCl (pH 7.4) does not absorb above 210 mμ.

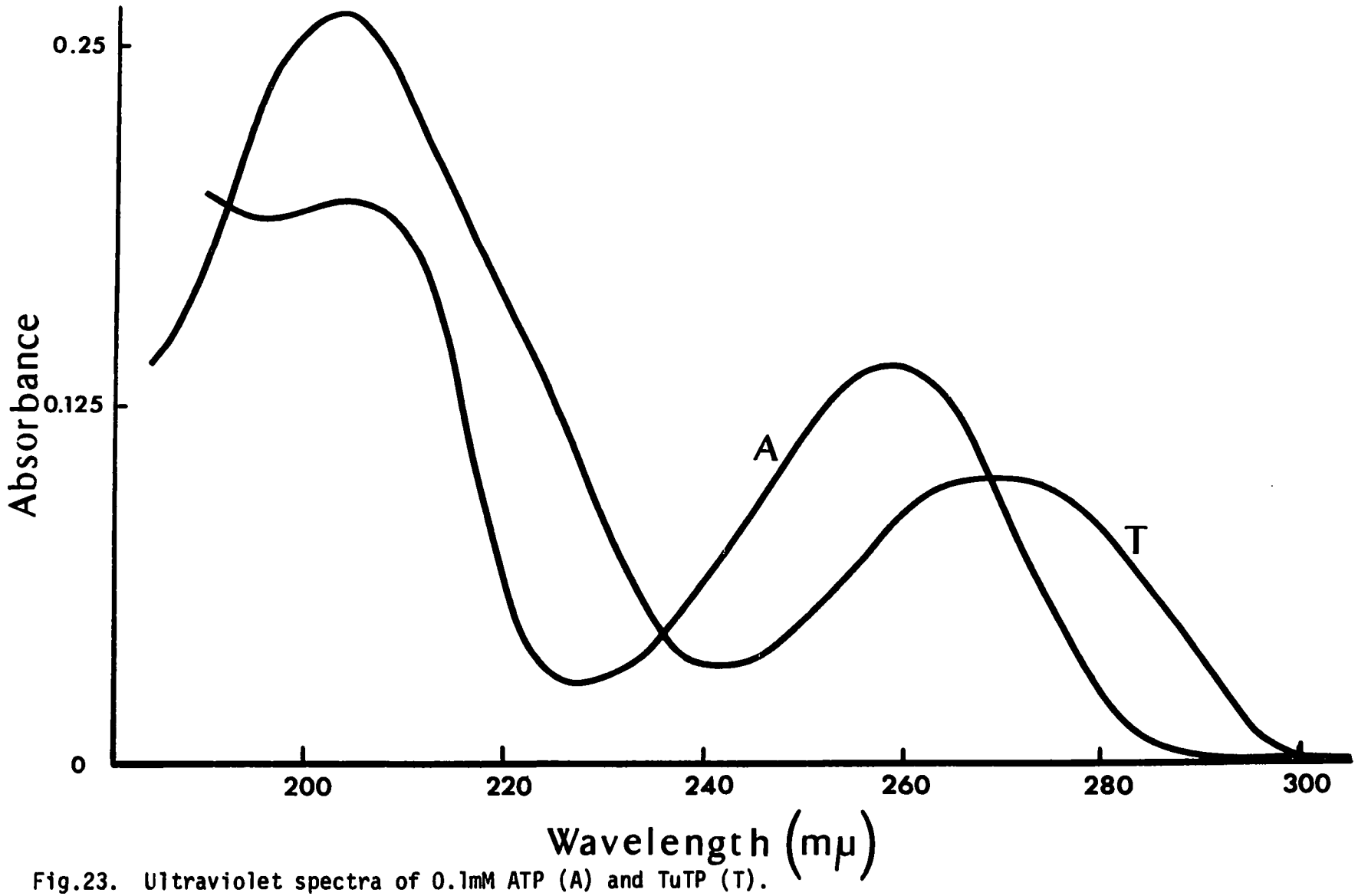


Fig.23. Ultraviolet spectra of 0.1mM ATP (A) and TuTP (T).

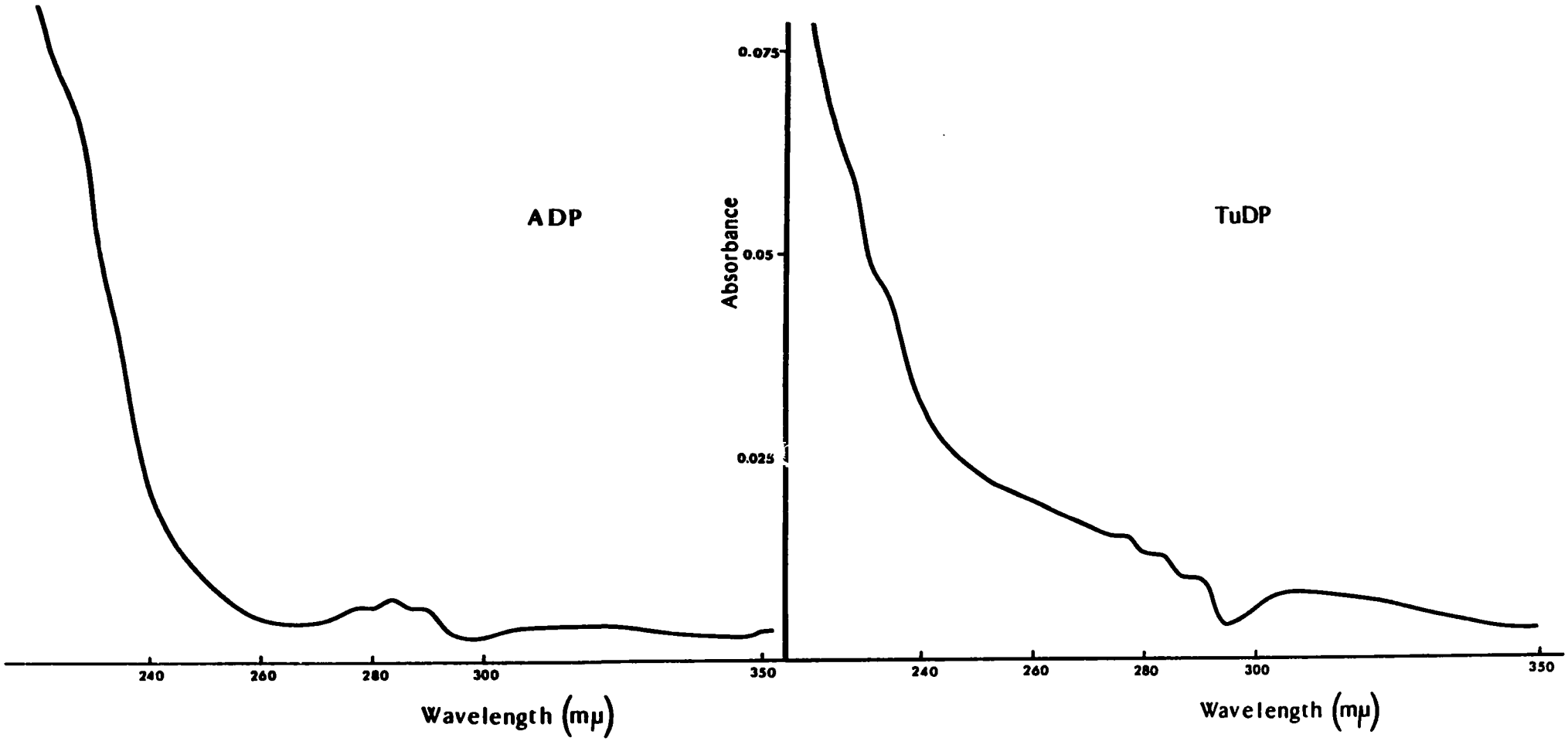


Fig.24. Difference spectra between NDP-F-actin and NTP-G-actin. All protein concentrations were 2.2 mg/ml. The reference cell contained NTP-G-actin in 5mM Tris-HCl (pH 7.4) and 0.2mM NTP. The sample cell contained NDP-F-actin in 5mM Tris-HCl (pH 7.4) and 0.2mM NTP together with the polymerizing ions 0.1M KCl and 1mM Mg^{2+}. 0.1M KCl and 1mM $MgCl_2$ do not absorb above 210 mμ. Absorbance changes were observed with 1-mm path length and no absorption occurred between 800 and 350 mμ.

(Kasai et al., 1962; Oosawa and Kasai, 1962; Oosawa et al., 1961). The co-operative interaction among monomers was shown by Oosawa (1961) to be essential for the formation of the F-actin polymer.

Solvent perturbation difference spectra of N-acetyl ethyl esters of trytophan and tyrosine centre around 292 and 287 mμ, whereas the corresponding phenylalanine ester accounts for a negligible absorptivity difference (Herskovits and Sorensen, 1968). Weber (1960) observed from polarization spectra that tyrosine has one near uv $\pi \rightarrow \pi^*$ electronic transition at 275 mμ and tryptophan has two, one at 272 and the other at 289 mμ. Since the actin monomer contains 17 tyrosines and 5 tryptophans, then both tyrosyl and tryptophyl residues contribute materially to the absorption and difference spectra. On this basis, the 289 mμ absorption peak of Figure 24 may tentatively be assigned to tryptophan and the 278 and 283 mμ peaks represent algebraic sums of tyrosine and tryptophan perturbation difference spectra.

The difference minimum of the spectrum of TuDP-F-actin (Figure 24) suggests that the tryptophyl shoulder at 294 mμ has undergone a blue shift. Blue shifts indicate a transfer of the chromophore to a more polar (not necessarily more aqueous) environment (Wetlaufer, 1962). Both spectra also showed some difference at 225 and 232 mμ which could be a reflection of the change of environment of the aromatic residues or a change in conformation of the peptide bond or both.

Ultraviolet Difference Spectra in Myosin and Heavymeromyosin Induced by ATP and TuTP

It has been shown that, upon binding with ATP or its analogs,

heavy meromyosin and subfragment-1 alter their light absorption characteristics in the ultraviolet region (Morita and Yagi, 1966; Morita, 1967; Morita, 1969; Morita and Shimizu, 1969). The spectral shift of these derivatives of myosin caused by ATP is quantitatively larger than that caused by ADP, and the difference spectrum between the ATP-protein system and the ADP-protein system disappears as ATP is hydrolyzed by the ATPase action. The maximal positive change in absorption occurs at 288 mμ indicating that some of the aromatic chromophores in myosin are involved in this phenomenon.

In this study, the difference spectra of myosin and heavy meromyosin caused by TuTP as well as by ATP were observed as shown in Figure 25 and Figure 26. In the experiments, the optical cells contained the protein-nucleotide mixtures of the same composition, except that the reference mixture had been made 20 to 30 minutes before the sample cell components were mixed so that the triphosphate in the former was completely converted to diphosphate prior to the measurement. It was demonstrated that myosin gives the identical spectral shift as observed with heavy meromyosin indicating the molar absorbance change at 288 mμ of 2200 cm^{-1}. Although the difficulty of using myosin for the spectrophotometry has been its tendency to aggregate resulting in a turbid suspension, it was delineated by the optical arrangement in the instrument. The Cary 16 spectrophotometer was employed which minimized the turbidity contributed in the transmitted beam. The experimental results showed clearly that the effect of TuTP on the spectra of myosin and heavy meromyosin is qualitatively similar to that of ATP, whereas the molar absorbancy change at 288 mμ with TuTP is 4200 cm^{-1}.

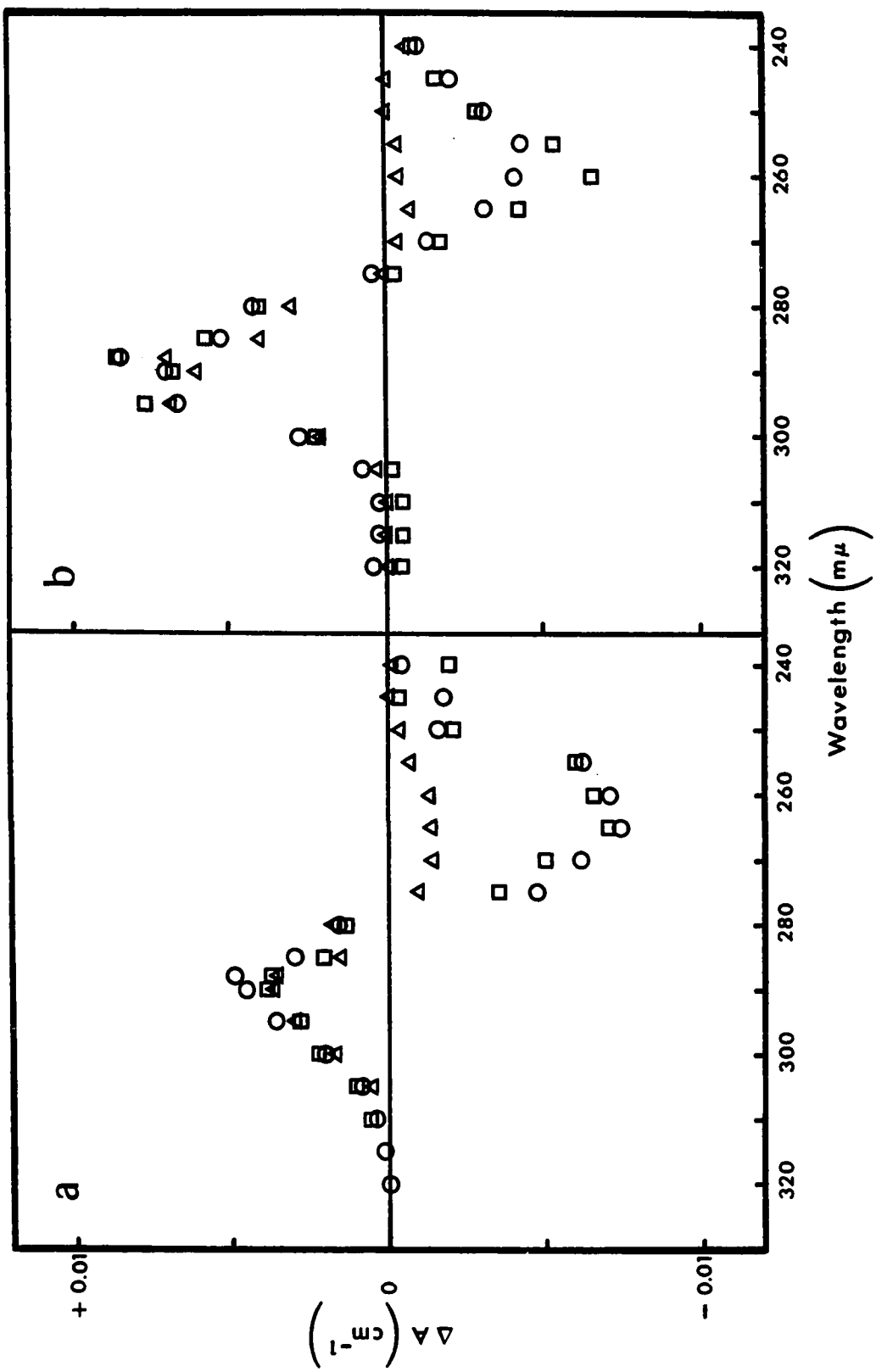


Fig.25. Ultraviolet absorption difference spectra between myosin-NTP and myosin-NDP. Experimental condition was 2 mg/ml myosin in 20mM Tris-HCl (pH 7.4), 0.5M KCl, 5mM $MgCl_2$ at 25°C. Spectra were obtained: (a) 90 sec (O), 365 sec (□), and 730 sec (Δ) after the addition of 90μM ATP; (b) 53 sec (O), 343 sec (□), and 722 sec (Δ) after the addition of 90μM TuTP.

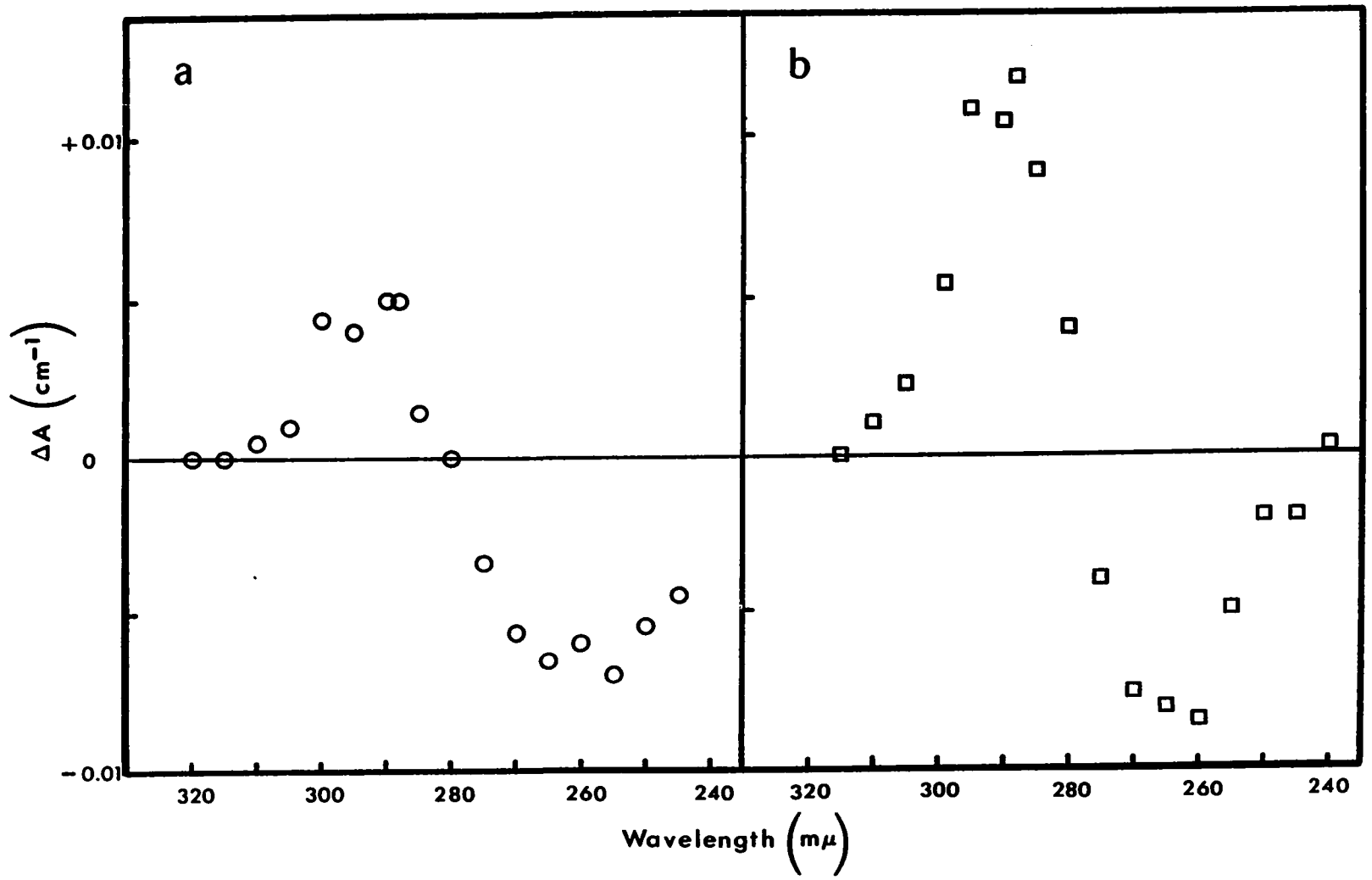


Fig.26. Ultraviolet absorption difference spectra between HMM-NTP and HMM-NDP. Experimental condition was 1.9 mg/ml heavy meromyosin in 20mM Tris-HCl (pH 7.4), 0.5M KCl, 5mM $MgCl_2$ at 25°C. Spectra were obtained immediately following the addition of (a) 90μM ATP (O); (b) 90μM TuTP (□).

In regard to the change in absorbancy below 280 mμ, no explanation has been offered by the previous workers. Obviously, the change in the lower wavelength region is larger and it disappears at the same time as the change at 288 mμ becomes zero. The observed changes in the shorter wavelength range were less reproducible probably because the slight change in size distribution of myosin aggregates occurs upon addition of ATP, and is detected at the near far ultra-violet region. As for the question whether the absorption spectrum of nucleotides alters during the interaction with proteins, it has been reported that the creatine phosphokinase reaction involves some change in the nucleotide conformation (Kagi et al., 1971). Since the size of the absorbance change is larger below 280 mμ than above 280 mμ, and it is observed with ADP as well as with ATP and TuTP, the spectral shift of nucleoside moiety is likely to be involved. For the detailed investigation on this aspect, the volumetric device was employed which gave a reproducibility of ± 0.001% for the transfer of 1 ml. and more importantly allowed the rapid mixing of solutions which contained myosin or its fragment without causing aggregate formation. The attempts with available facilities have so far been unsuccessful.

It seems quite significant that the effects of ATP and TuTP on the absorption spectrum of myosin are different, whereas these nucleotides are identical as substrates of the enzymatic action of myosin. This may reflect the difference between ATP and TuTP in the reactions with actomyosin and in the contractile process of glycerinated muscle fiber.

DISCUSSION

Two types of nuclear magnetic resonance measurements have led Glassman et al. (1971) to the conclusion that the paramagnetic metal ion (Co^{2+}, Mn^{2+}, and Ni^{2+})-ATP complex contains a water molecule that is simultaneously coordinated to the metal ion and hydrogen bonded to N-7 of the adenine ring. In the TuTP analog, in which N-7 of the adenine ring is replaced by a CH group, metal ion-adenine ring complexing does not occur. If a water-bridged structure exists for ATP complexes of Mn^{2+}, Co^{2+}, and Ni^{2+}, it seems likely that it could also exist for such diamagnetic ions as Mg^{2+} and/or Ca^{2+} but particularly for Mg^{2+} which has the largest charge density thereby making it a stronger Lewis acid. It would appear that the significant chemical difference between ATP and TuTP that would affect biological activity is the hydrogen bonding ability of N-7 and/or the formation of an outer sphere complex with metal ion through an inner sphere water molecule.

In the comparative studies of the behaviour of myosin and actomyosin in their interactions with ATP and TuTP it has been shown that the chemical structure of the nucleoside base has a significant role in the contractile process. The importance of aromatic base structure has been predicted for some time but direct experimental evidence has been lacking. Previous observations employing numerous ATP analogs led to the conclusions that the nucleoside moiety played a role in the binding of ATP to myosin or actomyosin.

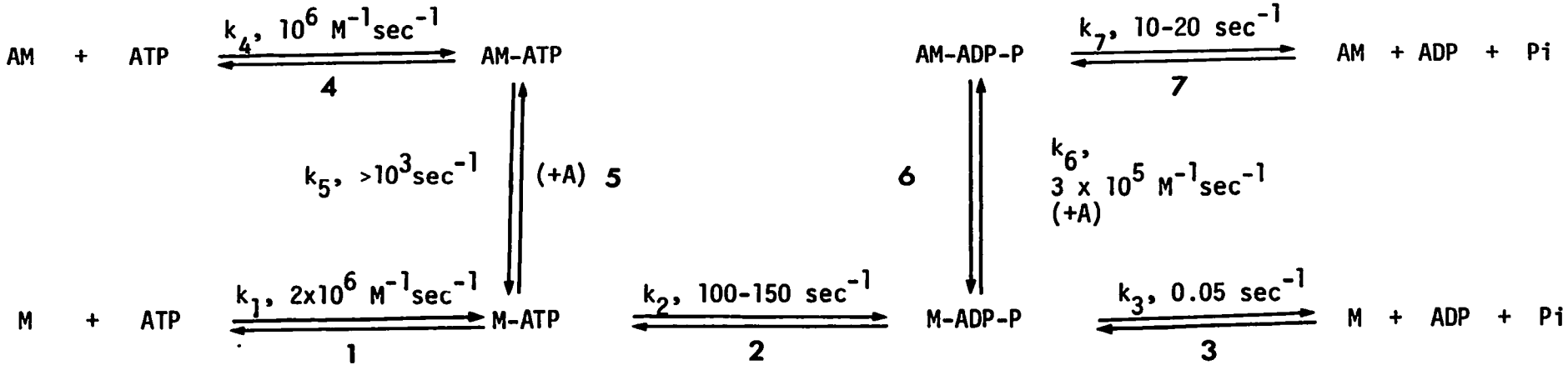
This study shows the catalytic activity of myosin in the hydrolysis of the pyrophosphate linkage to be identical for ATP and TuTP in a

wide range of substrate concentrations. It is most unlikely therefore, that the affinity of TuTP to myosin differs from that of ATP, despite the fact that the kinetic parameters of enzymatic action of myosin are likely to be more complex than hitherto anticipated.

On the other hand the experimental results showed that ATP and TuTP had considerably different effects in the enzymatic reaction and superprecipitation of actomyosin. To interpret these results it was necessary to relate them to a basic mechanism in the interaction of the actin-myosin-ATP system. For this reason, attempts were made to compare the effects of ATP and TuTP on the individual steps of actin-myosin interaction such as the association and dissociation reactions of actomyosin. Also, the role of the F-actin bound nucleotide in this system had to be made reasonably clear.

At the time the experimental part of this work was completed, Lymn and Taylor (1971) proposed a simple kinetic scheme which accounted for their results in a study of the transient and steady-state behaviour of actomyosin in the hydrolysis of ATP. In their scheme they included the results of their earlier studies and the work of others particularly that of Eisenberg and Moos (1968, 1970; Eisenberg et al., 1969). These workers used heavy meromyosin so that they could study the acto-HMM interaction at low ionic strength and Eisenberg and Moos also used subfragment-1.

According to Lymn-Taylor's mechanism, actomyosin ATPase action proceeds following the diagram shown in Figure 27. In the diagram, each step is designated by a number, and actomyosin, myosin and actin are represented by AM, M and A, respectively. Based on their experimental results, Lymn and Taylor described the Michaelis constant from the



A = F-actin M = Myosin AM = Actomyosin

Fig.27. Kinetic scheme of Lymn and Taylor (1971).

measurement of steady state rates in the presence of saturating amounts of actin for myosin to bind, as

$$Km = \frac{k_{-4} + k_7}{k_4} \qquad (1)$$

and the maximum rate of ATP hydrolysis, Vm, is

$$Vm = k_7 (AMo)$$

where AMo is the total concentration of actomyosin.

In the case of myosin ATPase, these authors (Lymn and Taylor, 1970) discussed that the Michaelis constant is either

$$Km = \frac{k_{-1}\, k_3}{k_1\, k_2} \qquad (2)$$

or

$$Km = \frac{k_3}{k_1} \qquad (3)$$

To arrive at this expression of Km, it is necessary to impose several assumptions regarding the relative values of rate constants. Without such assumptions, Km is written as

$$Km = \frac{k_{-1}\, k_{-2} + (k_{-1} + k_2)k_3}{(k_2 + k_{-2})k_1} \qquad (4)$$

$$Km = \frac{k_{-1}\, (k_{-2} + k_3) + k_2 k_3}{(k_2 + k_{-2})k_1} \qquad (5)$$

and the maximum rate of myosin ATPase is

$$Vm = \frac{k_2}{k_2 + k_{-2}} \cdot k_3 (Mo) \doteqdot k_3(Mo) \qquad (6)$$

where (Mo) is the total concentration of myosin in the reaction mixture. Since k_1, k_{-1} and k_{-2} are not known, though the latter two constants are to be very small, it is not warranted to ascertain the real meaning of K_m values for myosin ATPase. Moreover, these arguments are based on the assumption that two active sites of myosin are equal and independent. Further complications arise when one considers the indication (Figure 6) that the two sites are not independent. It appears reasonable however, to assume that all the rate constants involved in steps 1, 2, and 3 do not change when ATP, as a substrate, is replaced by TuTP, because of the similar activity observed for both substrates in the concentration range from 10^{-7} M to 10^{-3} M.

In the case of the actomyosin system, the Vm value for TuTP is at least 3 times as high as that for ATP, while the Km values for both substrates are of the same order of magnitude. If equation (1) is correct, then k_4 and/or k_{-4} should depend on the substrate. This may correspond to the result that the dissociation rate of actomyosin with TuTP is 57% of that with ATP.

It should be mentioned that with actomyosin, the ATPase activity-substrate concentration profile follows the simple Michaelis formulae. This is probably due to the fact (Nauss, Kitagawa and Gergely, 1969; Lymn and Taylor, 1971) that only one mole of ATP or pyrophosphate combine with a mole of myosin when actin is present. On the other hand, two moles of ADP (Lowey and Luck, 1969) and of pyrophosphate (Nauss et al., 1969) were found to bind with one mole of myosin or

heavy meromyosin. For the above reasons and in order to simplify the arguments, the actomyosin system can be regarded as a myosin complex with a single ATP binding site.

In considering the mechanism of superprecipitation, several characteristics of this phenomenon are to be taken into account:

(1) It is well known that superprecipitation requires the hydrolytic action of ATP or its analog to continue simultaneously. The hydrolysis of the pyrophosphate bond at the pre-steady state amounts to at least one mole of inorganic phosphate production per mole of myosin, which some workers (Tonomura et al., 1961; 1964a; 1964b) considered to energize myosin resulting in a contractile state. As shown by Taylor's group (Lymn and Taylor, 1970; 1971), the pre-steady state lasts less than 0.1 second and the reaction is a second-order reaction. On the other hand, if the turbidity change is used to detect the rate of superprecipitation, it is a first-order reaction in protein and is compatible with Michaelis kinetics. Furthermore, the rate constant of turbidity change is in the order of 10-20 sec^{-1} at the most, which is similar to that of the steady state rate constant of actomyosin ATPase.

(2) Although the turbidity change, under usual experimental conditions, reaches the final level in a period of seconds, the hydrolysis of ATP or its analog continues until the substrate level falls substantially. The data for Figure 13 illustrated that the rate of hydrolysis does not change significantly at the time of turbidity change completion. A slight decrease in the hydrolytic rate after the turbidity change was estimated to be due to the aggregate formation of actomyosin which

restricts the diffusion of substrate resulting in a slow enzyme-substrate complex formation (Endo, 1964), or it is because of strong myosin-myosin interaction preventing the actin activation of hydrolysis.

(3) From an electron microscopic study, it was shown that the appearance of actomyosin continues to change after the end of turbidity change (Nihei & Yamamoto, 1969). During the turbidity change, the arrow-head structure of actomyosin (Huxley, 1964) becomes less prominent. Eventually, if the supply of ATP is kept sufficient, superprecipitated actomyosin shows heavily clustered protein aggregates - mostly myosin and some actin threads left free (Ikemoto, Kitagawa and Gergely, 1966).

(4) Tokiwa and Morales (1970) found that 6-SH-ATP (SH-TP) binds covalently to the hydrolytic site of myosin, blocking the ATPase activity and that, if one of the two sites of myosin is bound with 6-SH-ATP, no superprecipitation ensues. This finding seems to contradict the above statement that only one site of myosin is available in actomyosin.

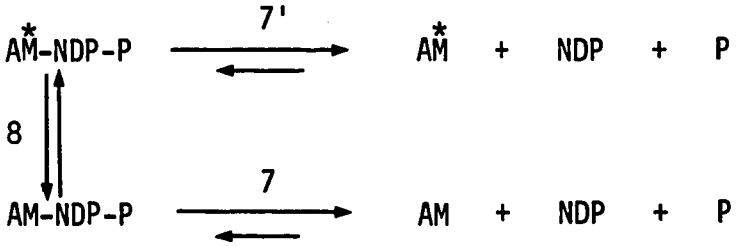
(5) The rate of reassociation of actin and myosin is slower than that of actin and myosin when these are mixed in the absence of a nucleotide (Figures 15 and 18) (Finlayson, Lymn, and Taylor, 1969). The reassociation rate constant at 0.6 ionic strength is $5 \times 10^{-3} - 10^{-2}$ sec^{-1}.

All the observations briefly noted above, with the exception of 6-SH-ATP effects, do not contradict the idea that superprecipitation is a physical process of actomyosin occurring during the steady state of nucleoside triphosphate hydrolysis which proceeds following

the steps ascribed to Lymn-Taylor's mechanism. It must be recognized, however, that superprecipitation involves at least two phases: one is the change in turbidity of actomyosin as observed in this study, and the other is the formation of heavy aggregates of proteins. The following part of the discussion deals with the turbidity change which shows fair parallelism with the isometric contraction of glycerol treated muscle in regard to the response toward ATP and TuTP (Figures 10 and 11). The turbidity change therefore, is associated with the conversion of the energetic state of actomyosin - most likely to be myosin, since myosin aggregates during the course of superprecipitation.

The main question to be answered in this study was, how does the energy transfer occur during the interaction between actomyosin and ATP or its analog? In other words, what intermediary step(s) is (are) the link between the hydrolytic process and the turbidity change? The answer is that it is apparently the intermediary stage between steps 6 and 7: it occurs in the complex of AM-ADP-P or AM-TuDP-P. The reasons are as follows: The Km values for the hydrolysis and the turbidity change are approximately the same for both ATP and TuTP, indicating that the rate constants participating to determine Km's are of the same order of magnitude. The kinetic parameters involved in myosin enzyme activity are also likely to be the same for both ATP and TuTP. Moreover, the size of the rate constants of the rate limiting steps of the hydrolytic process and the turbidity change, are between 10 and 20 sec^{-1}, which are less than one-tenth of the rate constants of other reaction steps in the

forward direction (toward the product release). In short, the steps up to the formation of the final intermediate, i.e., AM-ADP-P or AM-TuDP-P, in the hydrolytic process can proceed at about the same speed either with ATP or with TuTP. To explain why the Vm values in the actomyosin system are different, an additional step may be proposed:



In this equation the rate constants of steps 7 and 7' are the same for a given substrate. The experimental data (Table II) indicate that with TuTP, k_7 is 3-4 times larger than that with ATP. Step 8 forms actomyosin with an altered physical state and the rate of this step with TuTP is about one half of that with ATP. To continue the hydrolysis of triphosphates, AM as well as $\overset{*}{AM}$ should enter step 4 which dissociates actomyosin. It seems feasible that at step 4, $\overset{*}{M}$ forms aggregates but maintains the same enzymatic characteristics as does M. Although the myosin molecule, which is covalently bound to SH-TP at one of its two sites, can go through step 7 (Tokiwa and Morales, 1970), it may not proceed via step 8; thus no superprecipitation occurs. In this connection, it is of interest to further speculate that the difference in the ultraviolet spectral change of myosin with ATP and TuTP reflects the characteristics of AM-NDP-P which determine whether it can effectively undergo step 8. It seems evident that the structure of nucleoside base is not only important for the binding of triphosphate but determines the efficiency of energy transfer

in the actomyosin system.

Despite the uncertainties concerning the rate constants, in particular k_4, k_6 and those in the reverse direction, the above explanation of the interaction between actomyosin and a nucleoside triphosphate makes a step forward by narrowing the scope of the problem to the understanding of the mechanism of energy transfer in muscle contraction. This work now poses several questions which should be answered in the near future: How does the structural change in myosin or actomyosin occurring during superprecipitation relate to the contraction of muscle fibers? If the proposed reaction step 8 does exist, how does the energy transfer proceed within the domain of the actomyosin complex?

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There seems no simple way to determine the kinetic parameters. At an extremely low range of ATP concentration, Equation (3) becomes

$$v = \frac{Mo\ k}{1 + \frac{K_1}{S} + \frac{k'}{k_1 K_2}} \qquad (4)$$

In this case, the Michaelis constant obtained from the double reciprocal plot is

$$K_1 \ / \ (1 + \frac{k'}{k_{-1} K_2})$$

If $k'/k_1 K_2 \ll 1$, the plot should yield the value of K_1. As it has been shown (Lymn and Taylor, 1970), K_1 is a complex of the rate constants of two intermediary steps, the value of K_1 may well depend on the condition for the assay of ATPase.

When the substrate concentration becomes large enough so that K_1/S approaches zero, and if $k \doteqdot k'$, Equation (3) can be written as:

$$v = \frac{Mo\ k\ (K_2 + S)}{K_2 + S + \frac{k'}{k_1}} \qquad (5)$$

APPENDIX

Klotz and Hunston (1971) have shown that when two or more sites of a macromomolecule bound with substrate interact with each other, the commonly used graphical methods for kinetic and binding parameters indicate the algebraic complexes of these parameters. If the two identical active sites of myosin interfere with each other upon binding NTP, then the myosin NTPase activity cannot be explained by a mechanism which involves a single type of active site. To describe the myosin NTPase in a simple model, two equations fulfill the minimum requirement:

$$M + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} MS \xrightarrow{k} P \qquad (1)$$

$$MS + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} MS_2 \xrightarrow{k'} P \qquad (2)$$

where k's are the rate constants of the steps shown, M the free myosin site, S the substrate, P the products, MS the first enzyme substrate complex and MS_2 the second enzyme substrate complex. With the treatment of these equations on the basis of steady state kinetics, one obtains the following rate equation.

$$v = \frac{Mo\ (k + k')\frac{S}{K_2}}{1 + \frac{K_1}{S} + \frac{S}{K_2} + \frac{k'}{k_1 K_2}} \qquad (3)$$

where v is the rate of ATP hydrolysis, Mo the total concentration of the active sites, and K's the apparent Michaelis constants.

$$K_1 = \frac{k_{-1} + k}{k_1} \qquad K_2 = \frac{k_{-2} + k'}{k_2}$$

PART II

CHEMICAL SYNTHESIS OF THE TUBERCIDIN NUCLEOTIDES AND THE INDOLE-INDOLINE NUCLEOSIDES

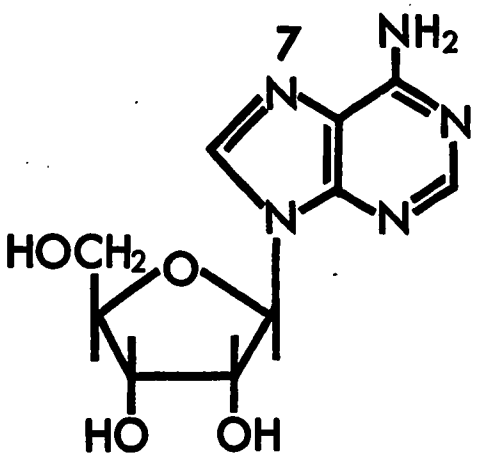
Introduction

The interactions between actomyosin systems and the 5'-triphosphorylated analogs of adenosine (I) have been extensively investigated by a number of workers (Berkvist and Deutsch, 1954; Portzehl, 1954; Blum, 1955; Raney, 1955; Hasselbach, 1956; Kielley, 1956; Ikehara et al., 1961; Azuma et al., 1962; Ikehara et al., 1964; Ikehara et al., 1965; Tonomura et al., 1967; Murphy and Morales, 1970). Despite the conclusions hypothesized by Tonomura et al., (1967) based on the accumulation of pertinent experimental results it seemed necessary to further investigate the effects of structural change in adenosine base on the contractile proteins in muscle. It was therefore proposed to synthesize the 5'-triphosphorylated esters of tubercidin (II, Tu) and 1-(β-D-ribofuranosyl)indole (III, Io) and to use them as tools in studying the actomyosin systems.

Tubercidin (7-deazaadenosine) is a moderately modified nucleoside and 1-(β-D-ribofuranosyl)indole (6-deamino-1,3,7-trideazaadenosine) is a more drastically modified analog with base aromaticity and lacking the necessary hydrogen bonding capability. The antibiotic tubercidin, isolated from Streptomyces tubercidicus, was phosphorylated directly. 1-(β-D-Ribofuranosyl)indole was synthesized by converting the indoline derivative into the corresponding substituted indole.

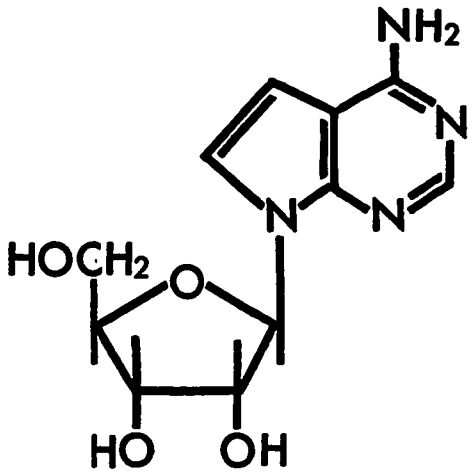
The Indoline-Indole Nucleosides

The indoline (IV, dihydroindole) ring system is a substituted aniline system. As such, N-acylation and N-alkylation reactions can be accomplished by standard methods appropriate for secondary amines (Sundberg, 1970; Preobrazhenskaya, 1967). When the foregoing steps are coupled



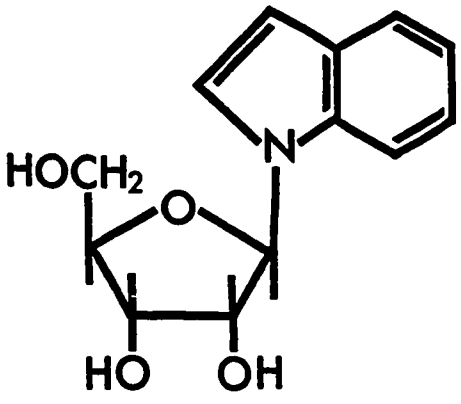
I

Adenosine

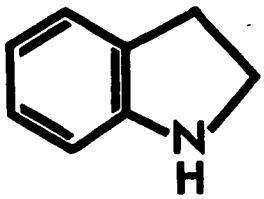


II

Tubercidin

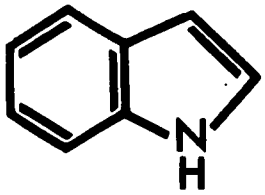


III



IV

INDOLINE



IVa

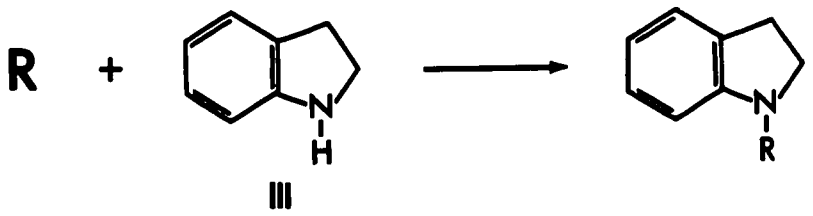
INDOLE

with an efficient dehydrogenation of the indoline to the corresponding indole (IVa), a useful synthetic approach to substituted indoles has been achieved. This method has largely been developed by Russian workers and a recent review summarizes the work in this area (Preobrazhenskaya, 1967).

The first synthesis of an indole nucleoside was reported by Suranov and Preobrazhenskaya (1961). The methods used for the synthesis of purine or pyrimidine nucleosides (Fox and Wempen, 1959; Montgomery and Thomas, 1962; Michelson, 1963; Fox, Watanabe, and Bloch, 1966) could not be applied to the synthesis of 1-glycosylindoles (i.e., XIX, XXI). The synthetic approach used by these workers involved reaction of 1,2,3,4,6-penta-O-acetyl-D-glucopyranoside (V) with 2 moles of indoline (IV). The intermediate, 1-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl)indoline (IX) was separated from 1-acetylindoline and oxidized with tetrachloro-p-benzoquinone (chloroanil) to produce the glycosyl indole X which was deblocked to give XI.

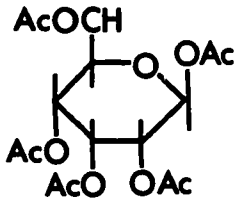
In a second publication Preobrazhenskaya and Suranov (1965) noted the superiority of 2,3-dichlor-5,6-dicyanobenzoquinone (DDQ) over chloroanil (90% yield vs. 30%) in the oxidation of the indoline nucleoside to the corresponding indole analog. Subsequently the Russian chemists (Preobrazhenskaya et al., 1967) and Walton, Holly and Jenkins (1968) reported the synthesis of 1-(β-D-ribofuranosyl) indole (XXI). The former workers refluxed 5-O-tritylribose (VI) with indoline in alcohol obtaining crystalline 9-(β-D-5'-O-trityl-ribofuranosyl)indoline (XII). The latter compound was acetylated to 1-(β-D-5'-O-trityl-2',3'-di-O-acetyl-ribofuranosyl)indoline (XIII).

Scheme I



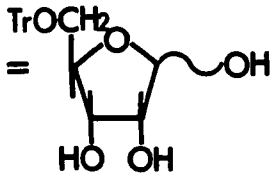
III

V. R =



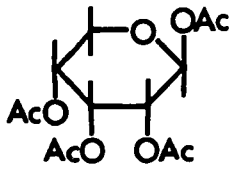
Ac = CH_3CO

VI. R =

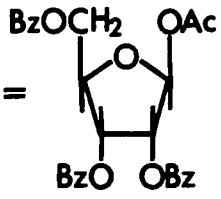


Tr = $(C_6H_5)_3C$

VII. R =

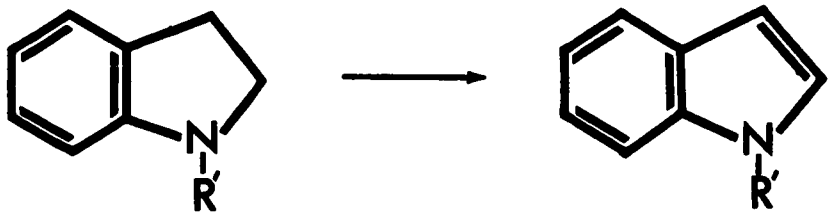


VIII. R =



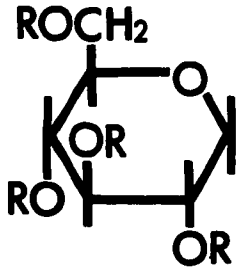
Bz = C_6H_5CO

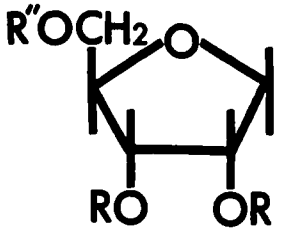
Scheme II

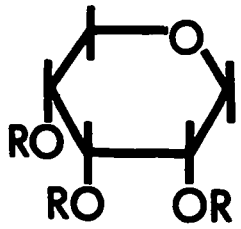


IX	R′ = A
XII	R′ = B
XIII	R′ = B_1
XV	R′ = C
XVI	R′ = B_2
XX	R′ = B_3

X	R′ = A
XI	R′ = A_1
XIV	R′ = B_1
XVII	R′ = C
XVIII	R′ = B_2
XIX	R′ = C_1
XXI	R′ = B_3







A, R = CH_3CO

A_1, R = H

B, R = H; R″ = $(C_6H_5)_3C$

B_1, R = CH_3CO; R″ = $(C_6H_5)_3C$

B_2, R = R″ = C_6H_5CO

B_3, R = R″ = H

C, R = CH_3CO

C_1, R = H

XIII was dehydrogenated in hot xylene using DDQ to yield the indole derivative XIV. The Merck chemists reacted indoline with 1,2,3,4-tetra-O-acetyl-β-D-ribopyranoside (VII) and 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranoside (VIII) producing the related 1-(β-D-ribosyl) indolines XV and XVI respectively which were subsequently dehydrogenated with DDQ to the corresponding protected indole nucleosides XVII and XVIII. The acyl blocking groups were removed with methanolic sodium methoxide yielding the desired 1-(β-D-ribopyroanosyl)indole (XIX) and 1-(β-D-ribofuranosyl)indole (XXI).

Nucleoside 5'-Mono-and Polyphosphates

Several excellent comprehensive texts dealing with the general chemistry of the nucleotides have appeared (Chargaff and Davidson, 1955; Khorana, 1961; Michelson, 1963; Brown, 1963; Ueda and Fox, 1967). Others have given special emphasis on details of procedures (Todd, 1957; Smith and Khorana, 1963; Moffatt, 1967; Zorbach and Tipson, 1968).

Numerous phosphorylating agents have been developed and employed for the purpose of converting nucleosides to the corresponding mononucleotides. Ueda and Fox (1967) have tabulated them and listed the specific nucleosides chemically phosphorylated together with pertinent references.

Recently Yoshikawa et al. (1967) have reported successful use of phosphoryl chloride which had earlier been found ineffective by Gulland (Gulland and Hobday, 1940; Barker and Gulland, 1942) for the preparation of 5'nucleotides from unprotected nucleosides due to low yields and lack of specificity of the reaction. Yoshikawa et al. (1967) found that unprotected nucleosides were readily phosphorylated

in good yields by use of the phosphoryl chloride reagent previously treated with a small amount of water in trialkyl phosphate.

Methods of synthesis of triphosphate compounds have been developed by many investigators, especially by Todd (Baddiley et al., 1949; Clark et al., 1957) and Khorana (Smith and Khorana, 1958) and their collaborators. Chemical methods, as opposed to enzyme-catalyzed phosphorylation (Canallakis, et al., 1960; Preiss et al., 1961), would appear to be more uniformly applicable. The method which has been most widely employed (Smith and Khorana, 1958), was also used for our purposes. It involves the condensation of an activated nucleotide derivative, e.g., a nucleoside 5'-phosphoromorpholidate (Moffatt and Khorana, 1961), with inorganic pyrophosphate (Moffatt, 1964) and offers greater versatility with a minimum of side reactions. Cramer and co-workers (Cramer et al., 1961; Schaller et al., 1961; Cramer and Neunhoeffer, 1962) have demonstrated that phosphorimidazolidates are useful intermediates for the synthesis of pyrophosphate bonds. Their procedure has been shown to offer the advantages of simplicity and adaptability to microscale synthesis but overall yields are not as good as those for the morpholidate method (Ott et al., 1963; Hoard and Ott, 1965).

The 5'-mono-, di-, and triphosphate derivatives of tubercidin were synthesized by using phosphoryl chloride and the morpholidate method. Although 1-(β-D-ribofuranosyl)indoline and 1-(β-D-ribofuranosyl)indole were prepared in good yields, their phosphate esters were obtained in trace amounts only under the conditions employed.

RESULTS AND DISCUSSION

Preparation of Indoline from Indole

Indoline (IV,2,3,-dihydroindole) was prepared according to the method of Kuhn and Butula (1968). Indole was hydrogenated by adding a hydrogen chloride solution of indole to a palladium catalyst on barium sulfate (Mozingo, 1955) in acetic acid (80-88% yields). Although satisfactory yields were obtained, other reactions of indoles are also acid catalyzed: namely, dimerization (Hodson and Smith, 1957), trimerization (Noland and Hammer, 1960), and attack by molecular oxygen (Hinman and Frost, 1961; Sundberg, 1970).

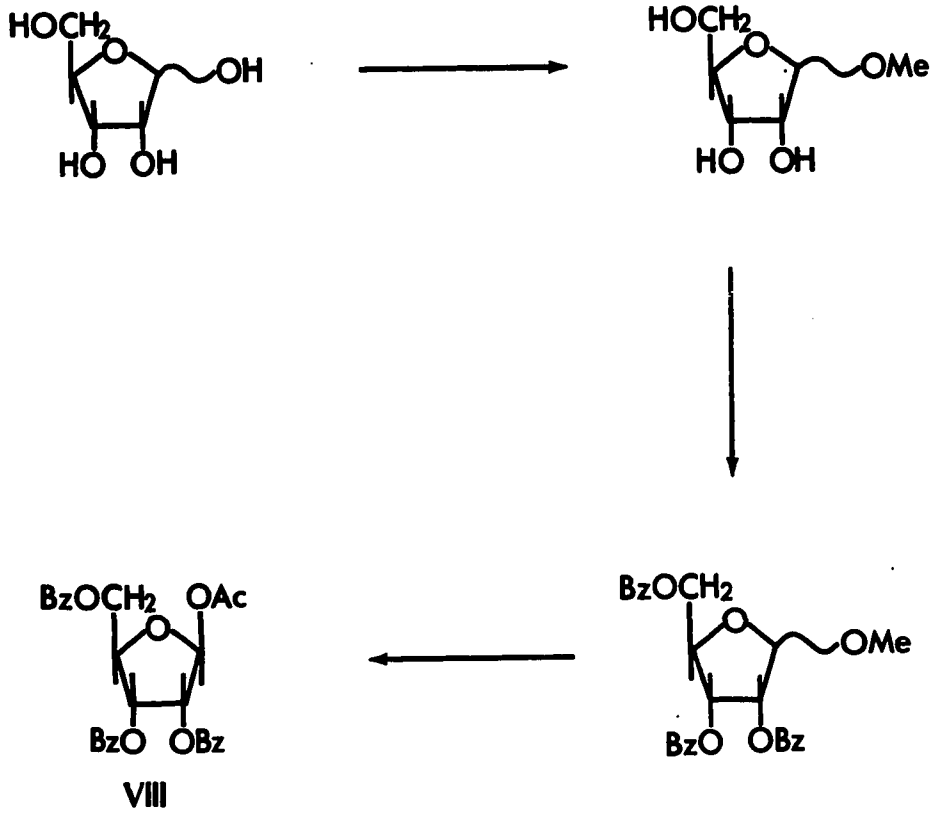
1-O-Acetyl-2,3,5-Tri-O-benzoyl-β-D-Ribofuranoside (VIII)

Compound VIII was first obtained from adenosine by Weygand and Wirth (1952) and later synthesized by Ness et al. (1954) from D-ribose. Kissman et al. (1955) developed some additional modifications to the Fletcher procedure making the method more attractive for large-scale preparations.

Recondo and Rinderknecht (1959) reported a new simple 3-step synthesis of acylated sugar (VIII). D-ribose was methylated to yield 1-O-methylribofuranoside and the latter benzoylated to give 1-O-methyl-2,3,5-tri-O-benzoyl-D-ribofuranoside. Their desired product was obtained directly and in good yield (56% overall) by acetylating with a mixture of acetic acid, acetic anhydride and sulfuric acid. (Scheme III.)

In our hands poor yields were obtained (15-35% overall) due to the accumulation of unwanted by-products. Other workers (Follman and Hogenkemp, 1970) have similarly encountered difficulty in obtain-

Scheme III



VIII

Ac = CH_3CO

Bz = C_6H_5CO

Fig. 1. Proton nmr spectrum (60 MHz) of indoline (IV) in $CDCl_3$. Chemical shifts are relative to tetramethylsilane (TMS, τ=10) as internal standard.

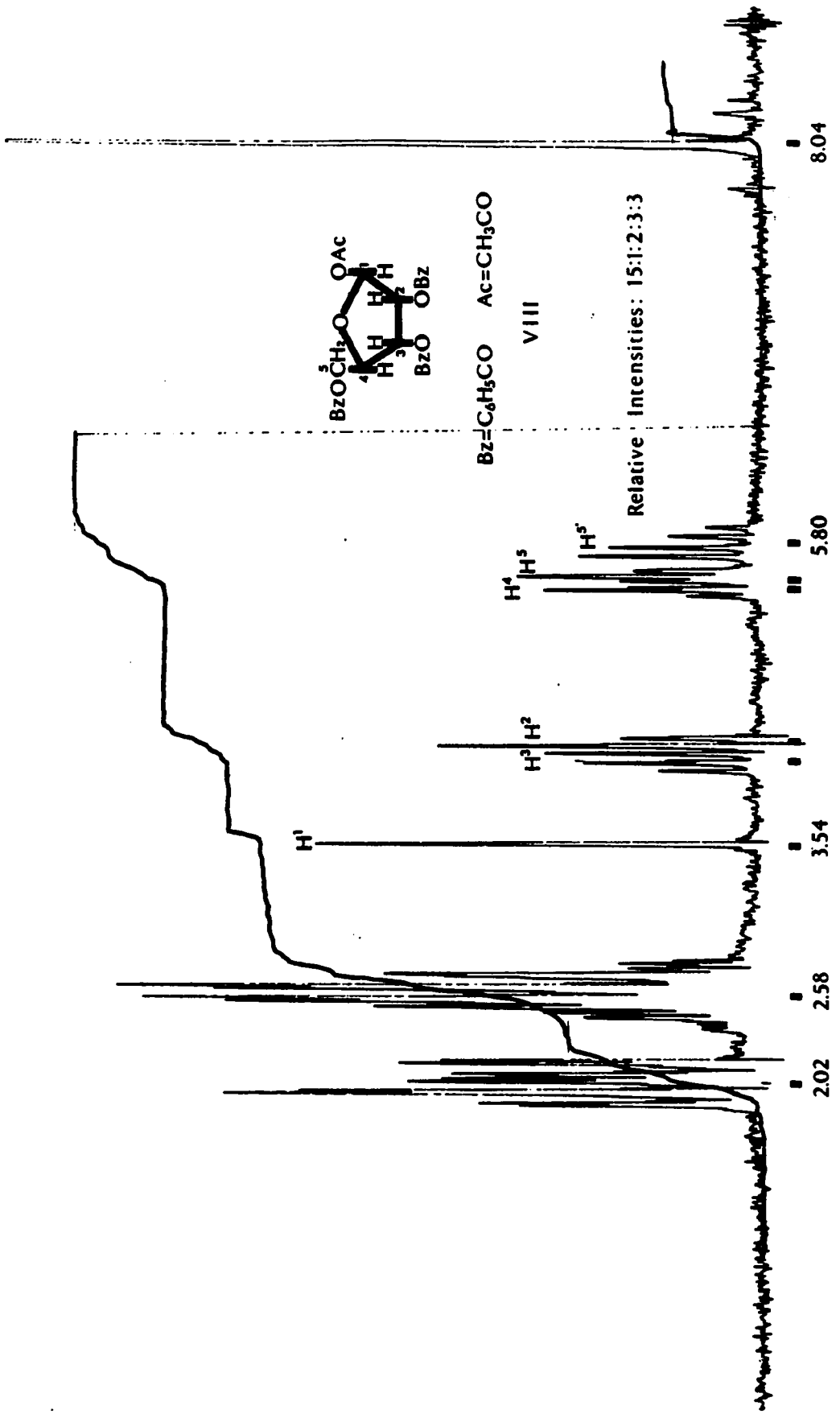
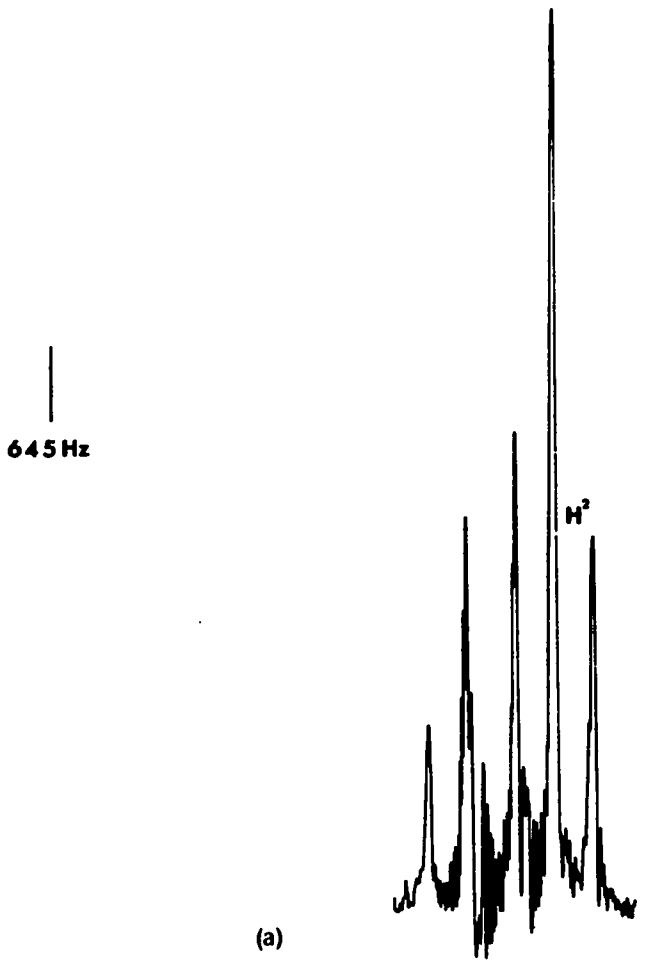


Fig. 2. Proton nmr spectrum (100 MHz) of 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranoside (VIII) in $CDCl_3$. Chemical shifts are relative to tetramethylsilane (TMS, τ=10) as internal standard.



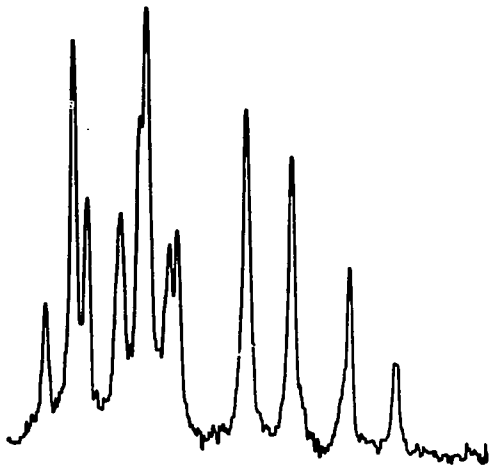


Fig. 2 (a) H^2 nuclei decoupled from H^1 nuclei.

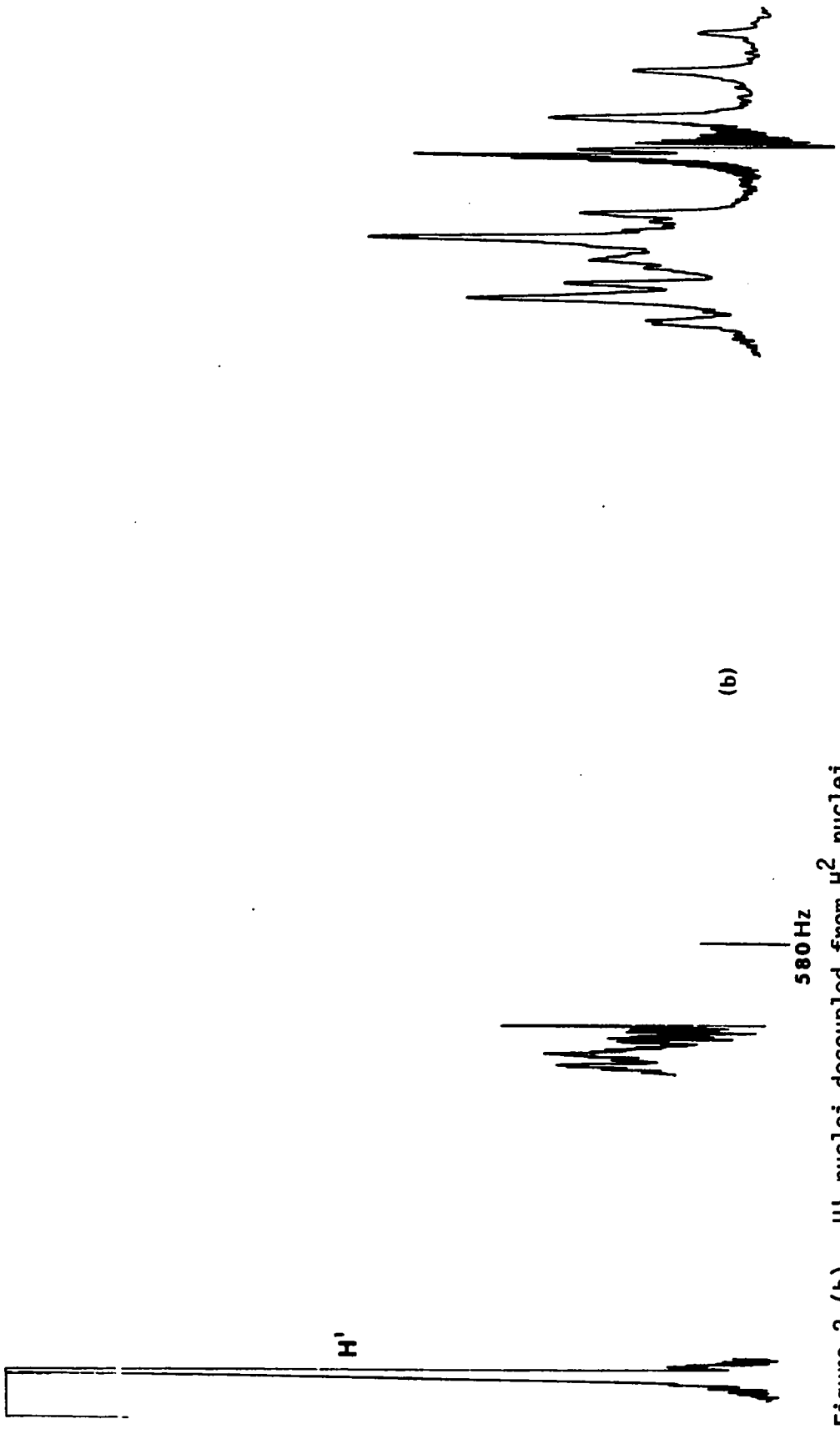
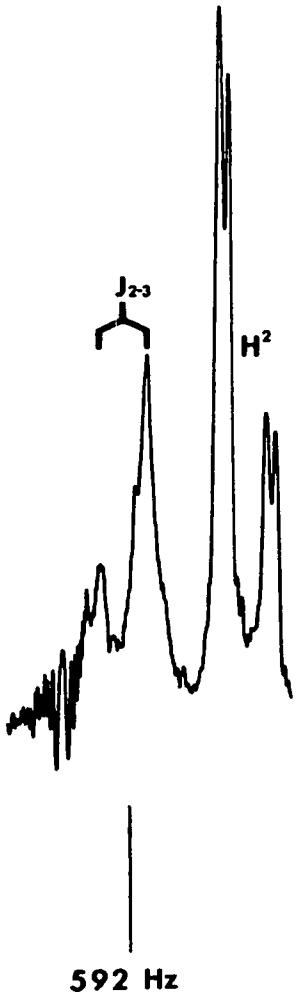


Figure 2 (b). H' nuclei decoupled from H^2 nuclei.



(c)

(d)

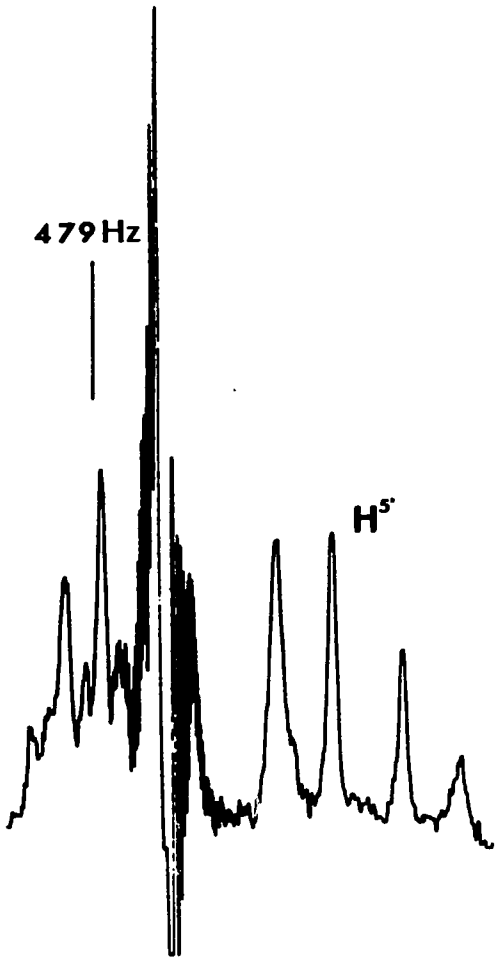


Fig. 2 (c). H^3 nuclei decoupled from H^4 nuclei.
Fig. 2 (d). H^4 nuclei decoupled from H^3 nuclei.

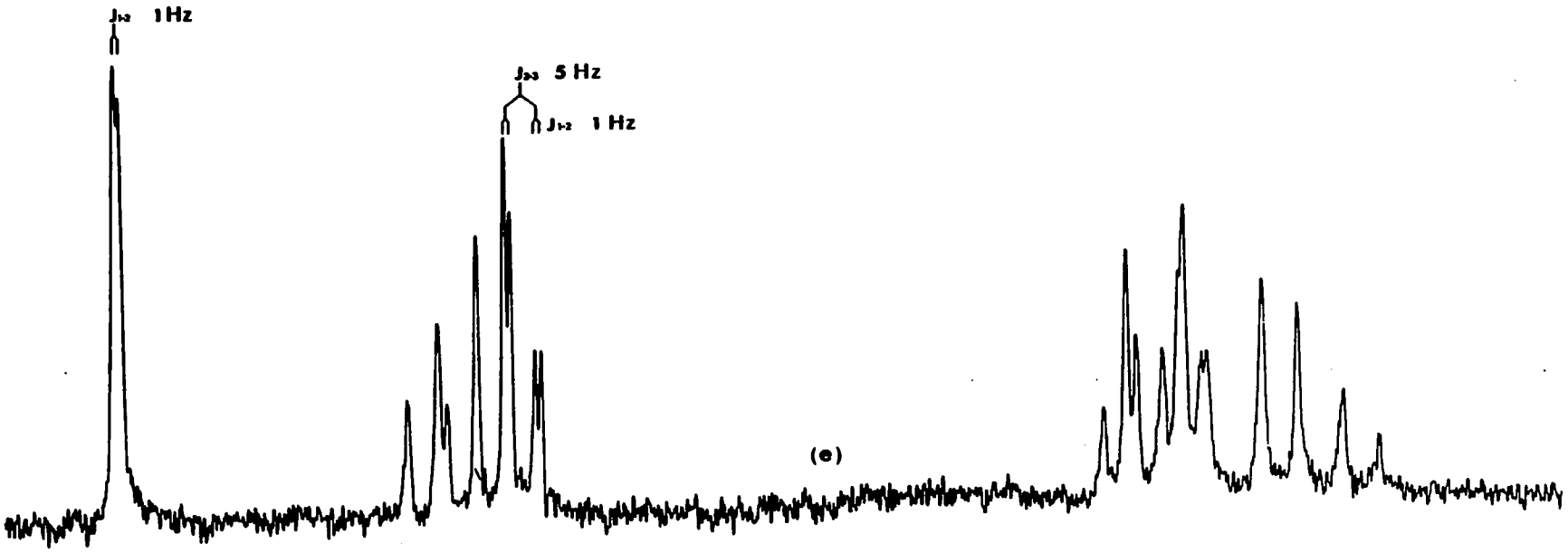


Fig. 2 (e). Proton nuclei of ribose in compound VIII with coupling constants shown.

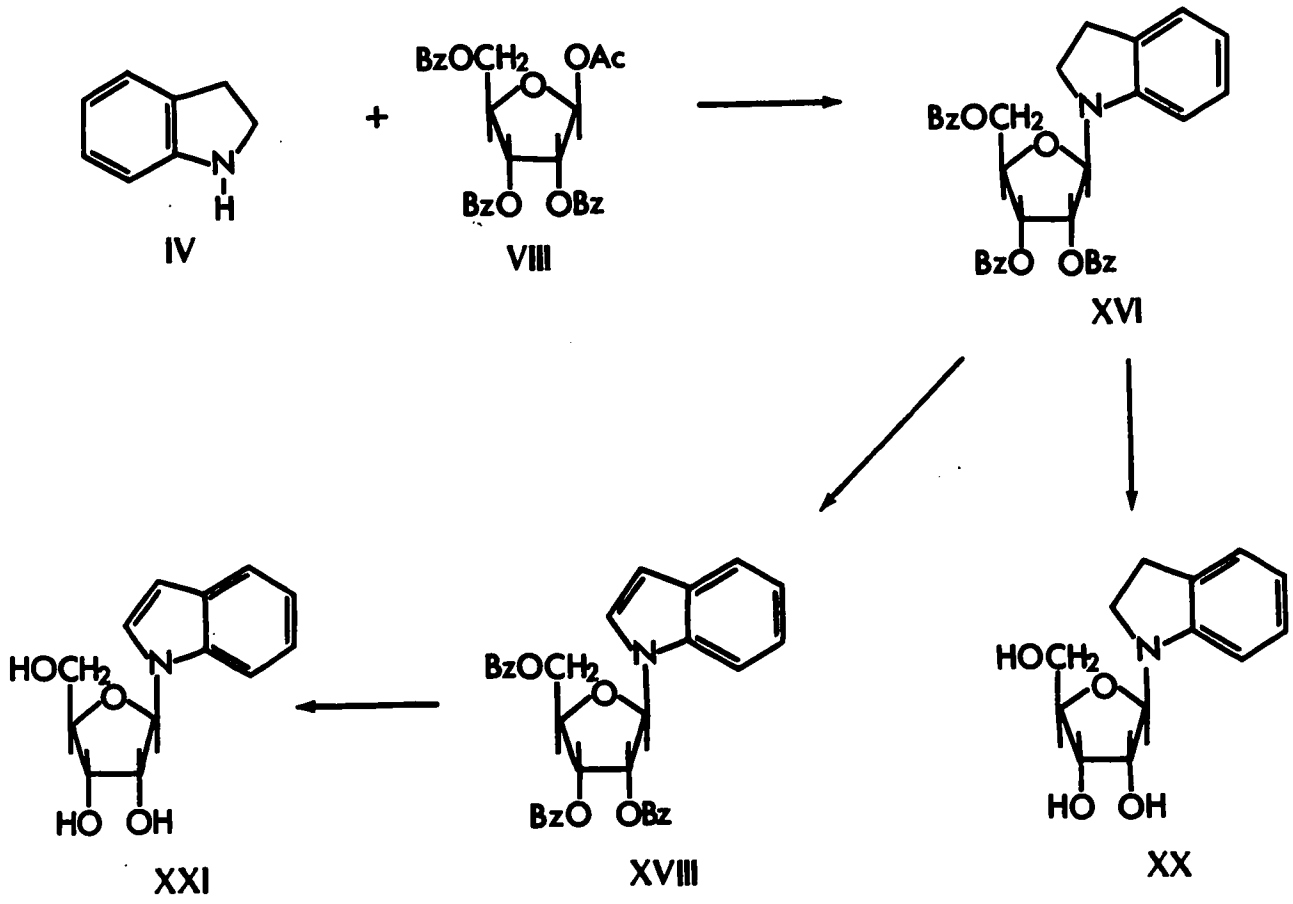
ing good yields by following the simple 3-step synthesis and reported a yield of 32%. Our synthesis of the desired product involved the incorporation of a few procedural alterations to the above methods (cf. Experimental section) designed particularly to purify the intermediate compounds (Scheme I) and thereby obtaining more respectable yields (68-72%). Figures 2 (a-e) verify the structure of the final product (1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranoside, VIII).

1-(β-D-Ribofuranosyl)indoline (XX) and 1-(β-D-Ribofuranosyl)indole (XXI)

Nucleoside XX was synthesized by a procedural modification of some of the steps developed by Preobrazhenskaya et al. (1965, 1967) and Walton, Holly and Jenkins (1968) using the protected precursor 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranoside (VIII). Acylated sugar VIII dissolved in ethanol-acetic acid was purged with argon followed by the addition of freshly distilled indoline. The reaction is permitted to go to completion under argon pressure. It is readily followed by TLC (silica gel GF_{254}), developed with benzene-ethyl acetate (19:1) and the spots identified by ultraviolet light absorption or iodine vapor. 1-(2,3,5-Tri-O-benzoyl-β-D-ribofuranosyl)indoline (XVI) was deacylated by dissolving in a mixture of dry, oxygen-free methanol and sodium methoxide which was refluxed to yield 9-(β-D-ribofuransyl)indoline (82%). Dehydrogenation of XVI in dry, refluxing, oxygen-free xylene with DDQ yielded the indole analog 1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)indole (XVIII) which was similarly deacylated to give 1-(β-D-ribofuranosyl)indole (yields of 86 and 78% respectively). Scheme IV.

The n m r spectra (Figure 3 and 4) of the indoline and indole

Scheme IV



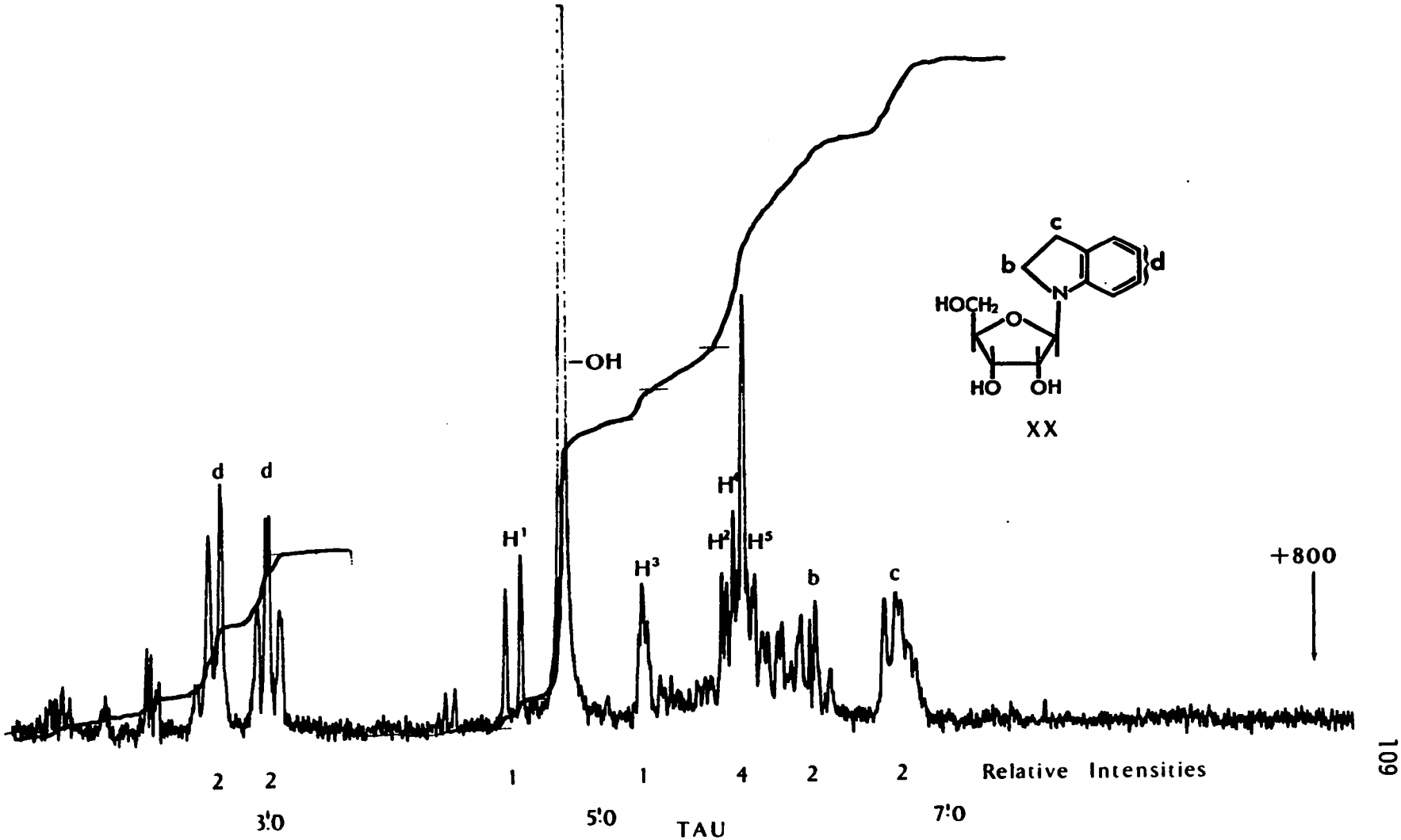


Fig. 3. Proton nmr spectrum (100 MHz) of 1-(β-D-ribofuranosyl)indoline (XX) in D_2O. Chemical shifts are relative to pyrazine as internal standard.

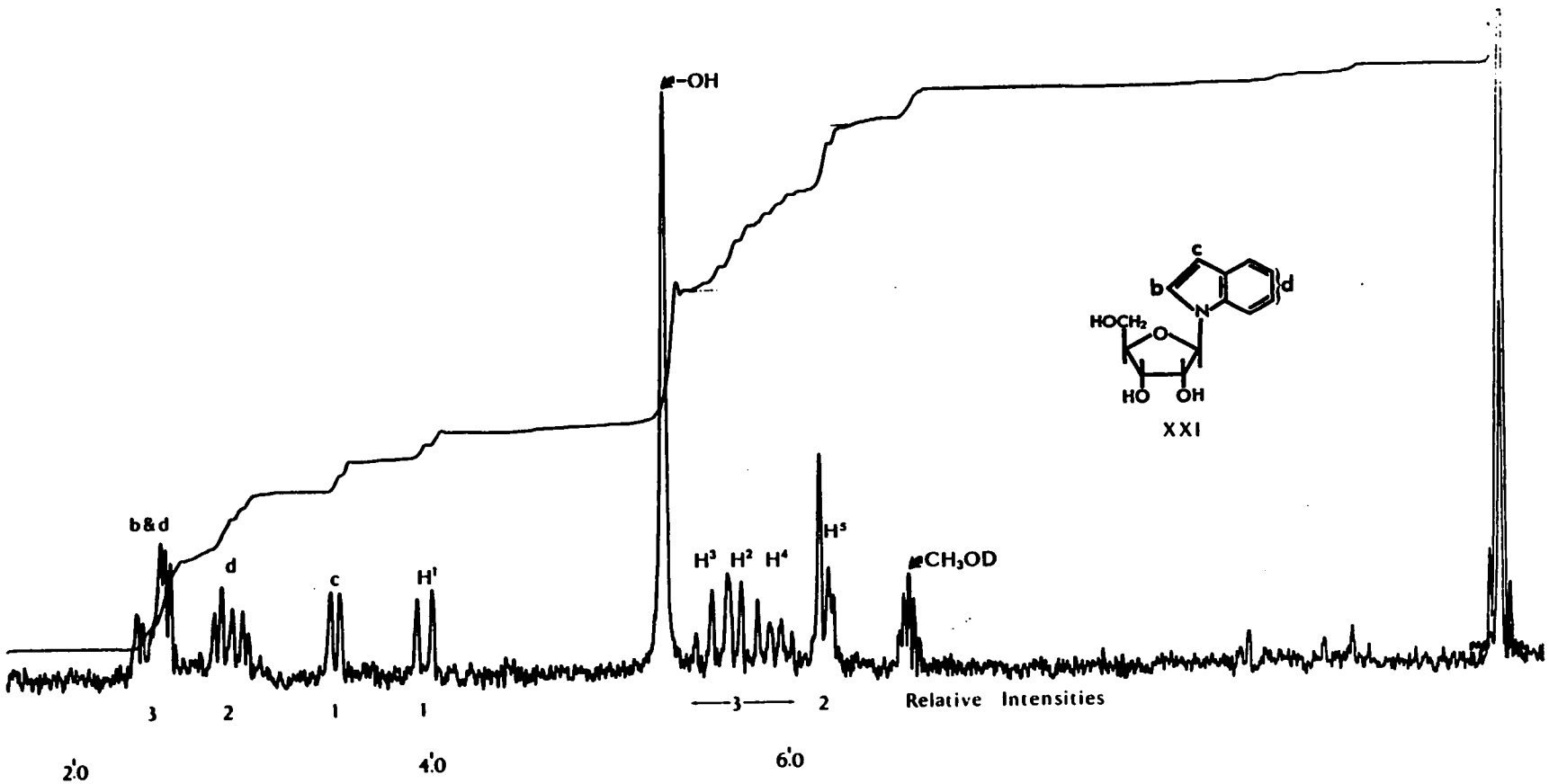


Fig. 4. Proton nmr spectrum (60 MHz) of 1-(β-D-ribofuranosyl)indole (XXI) in CD_3OD. Chemical shifts are relative to tetramethylsilane (TMS, τ=10) as internal standard.

nucleosides (XX and XXI) together with the spectra (Figures 5 and 6) of their respective benzoylated precursors (XVI and XVIII) analyzed in conjunction with the spectra of their starting material (Figures 1 and 2) verify the above assigned structures.

Preparation of the 5'-Monophosphate Nucleotides

Tubercidin (n m r spectra, Figures 7 and 8) was readily monophosphorylated at the primary alcoholic functional group by adding it to a solution containing two equivalents of phosphoryl chloride in triethyl phosphate previously treated with 1 equivalent of water. The secondary alcoholic functional groups were not esterified under these conditions since the presence of 2'-or 3'- phosphorylated by-products could not be detected. The reaction can be followed with TLC using silica gel and chloroform-methanol (85:15) as plate coating and developing solvent respectively or PEI-Cellulose F plates and 0.25M LiCl solution. When the reaction was complete the 5'-phosphorodichloridate and the excess phosphoryl chloride was hydrolyzed and neutralized by the addition of 6M ammonium hydroxide. The triethyl phosphate was removed from the aqueous solution by shaking with chloroform. The inorganic phosphate was precipitated and removed at pH 9 by the addition of 1M barium acetate. The addition of an equal volume of ethanol slowly precipitated the barium salt of tubercidin 5'-monophosphate in 82% yield. A slurry of this barium salt and an equivalent quantity of washed AGC-244 ion exchange resin (Dowex 50W-X8; H^+ form) was poured onto a column containing 3-4 equivalents of the same resin and the 5'-dihydrogenmonophosphate derivative was washed through the column.

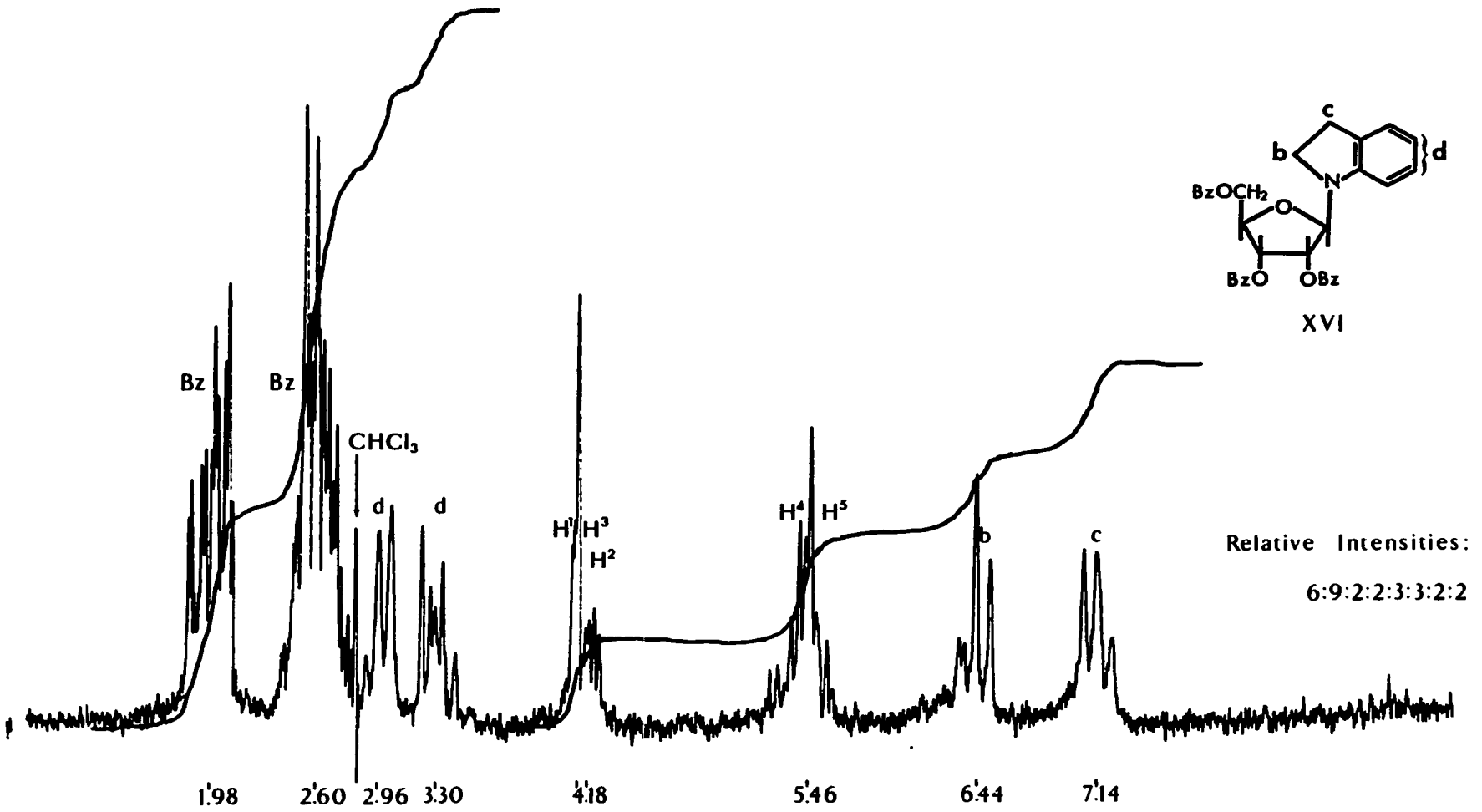


Fig. 5. Proton nmr spectrum 100(MHz) of 1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)indoline (XVI) in $CDCl_3$. Chemical shifts are relative to tetramethylsilane (TMS, τ=10) as internal standard.

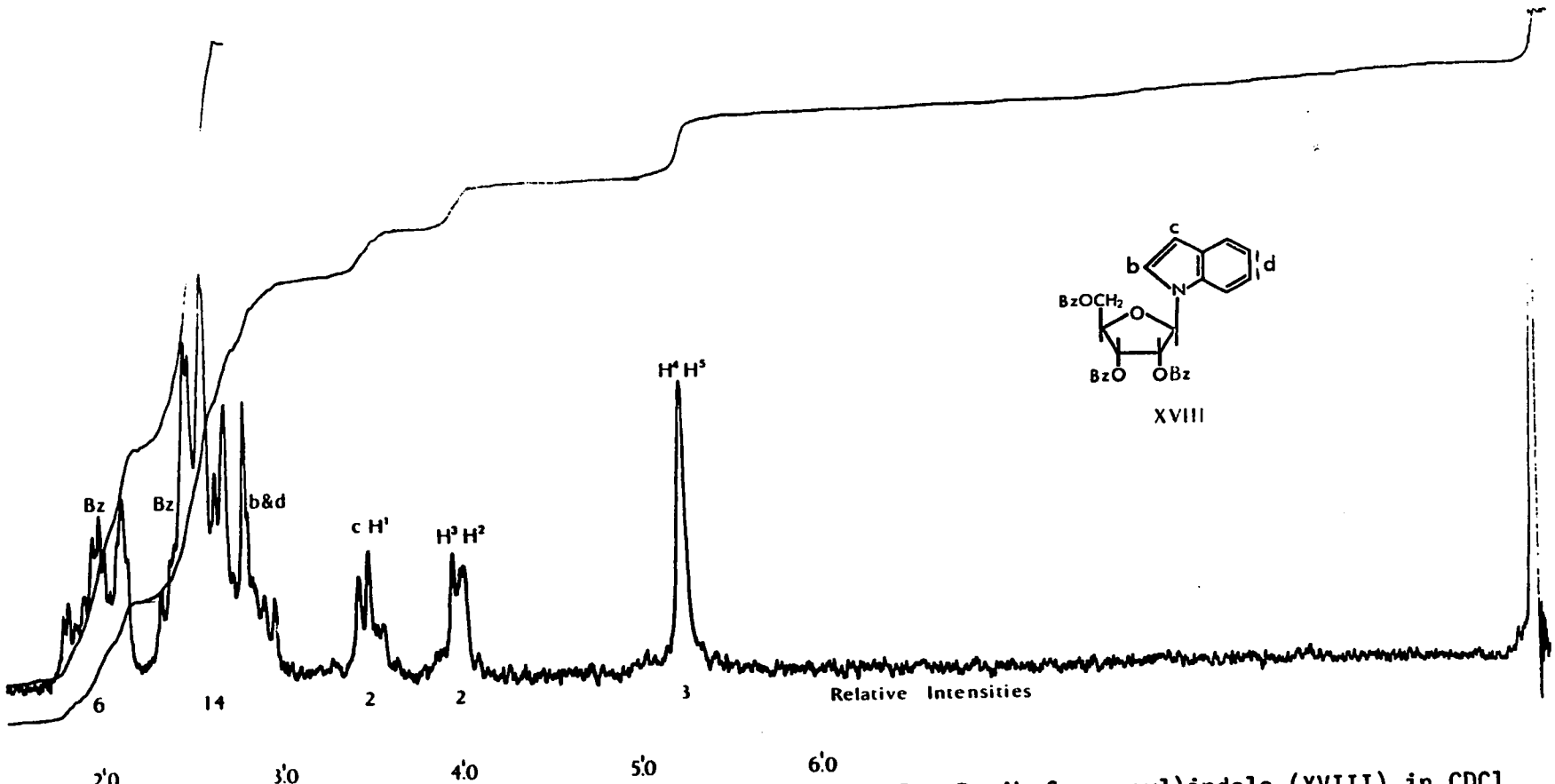
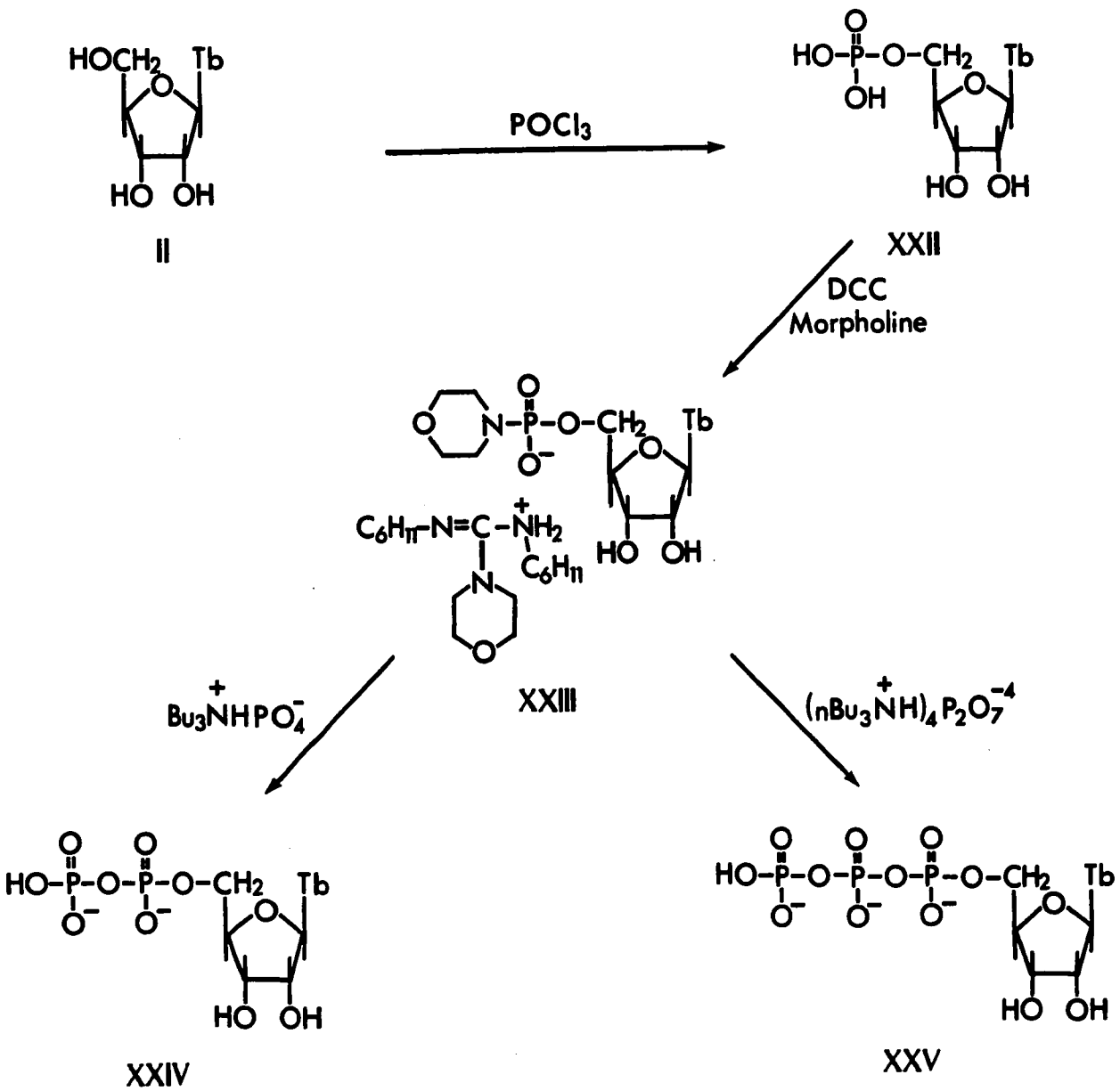


Fig. 6. Proton nmr spectrum (60 MHz) of 1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)indole (XVIII) in $CDCl_3$. Chemical shifts are relative to tetramethylsilane (TMS, τ=10) as internal standard.

Scheme V


HOCH2 Tb
HO OH
II
POCl3
HO-P-O-CH2 Tb
OH
HO OH
XXII
DCC
Morpholine
C6H11-N=C-NH2
C6H11
XXIII
Bu3NHPO4
(nBu3NH)4P2O7-4
HO-P-O-P-O-CH2 Tb
HO OH
XXIV
HO-P-O-P-O-P-O-CH2 Tb
HO OH
XXV

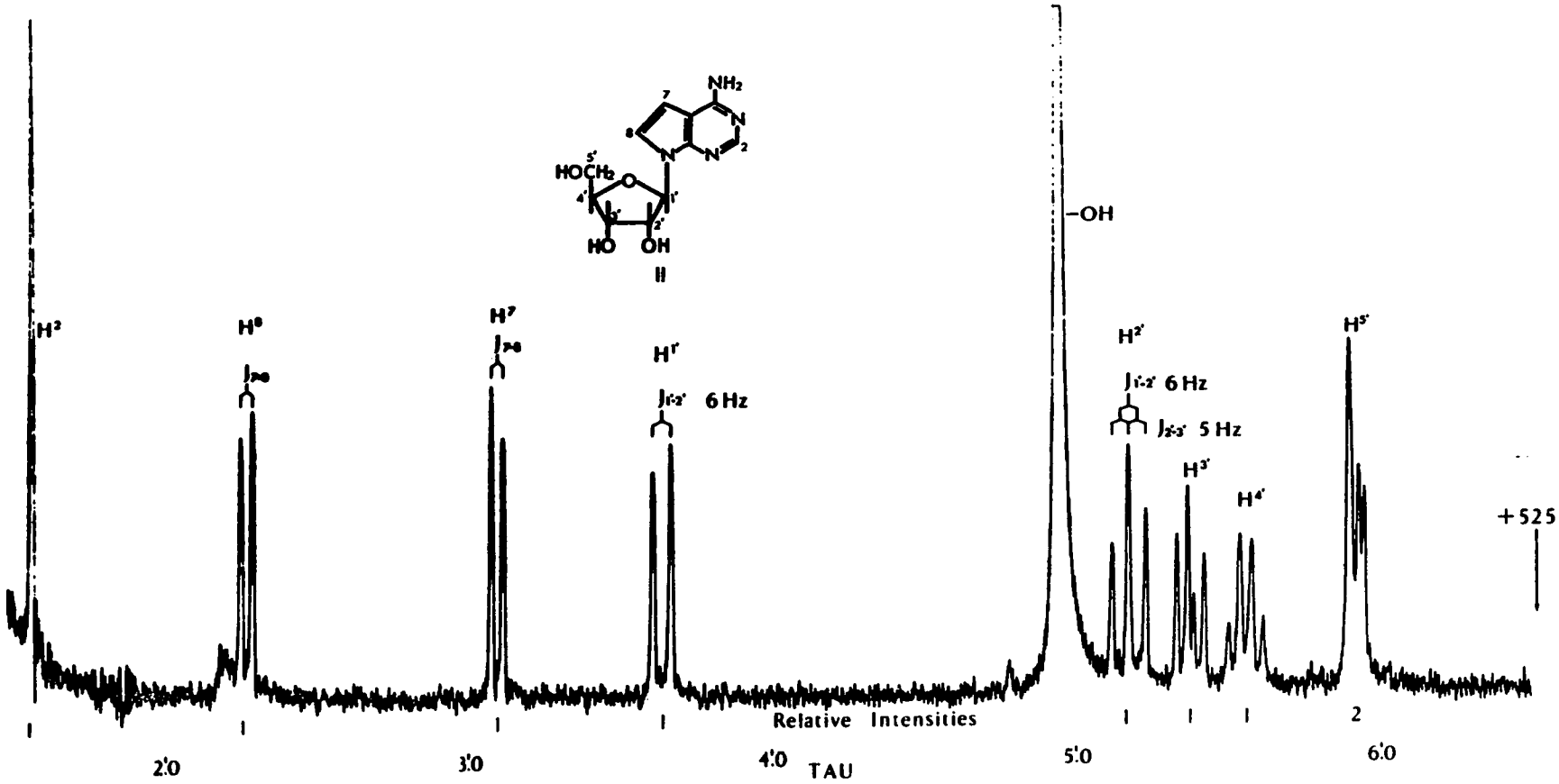


Fig. 7. Proton nmr spectrum (100MHz) of tubercidin (Tu, II) in a 36% solution of DCl in D_2O. Chemical shifts are relative to pyrazine as internal standard.

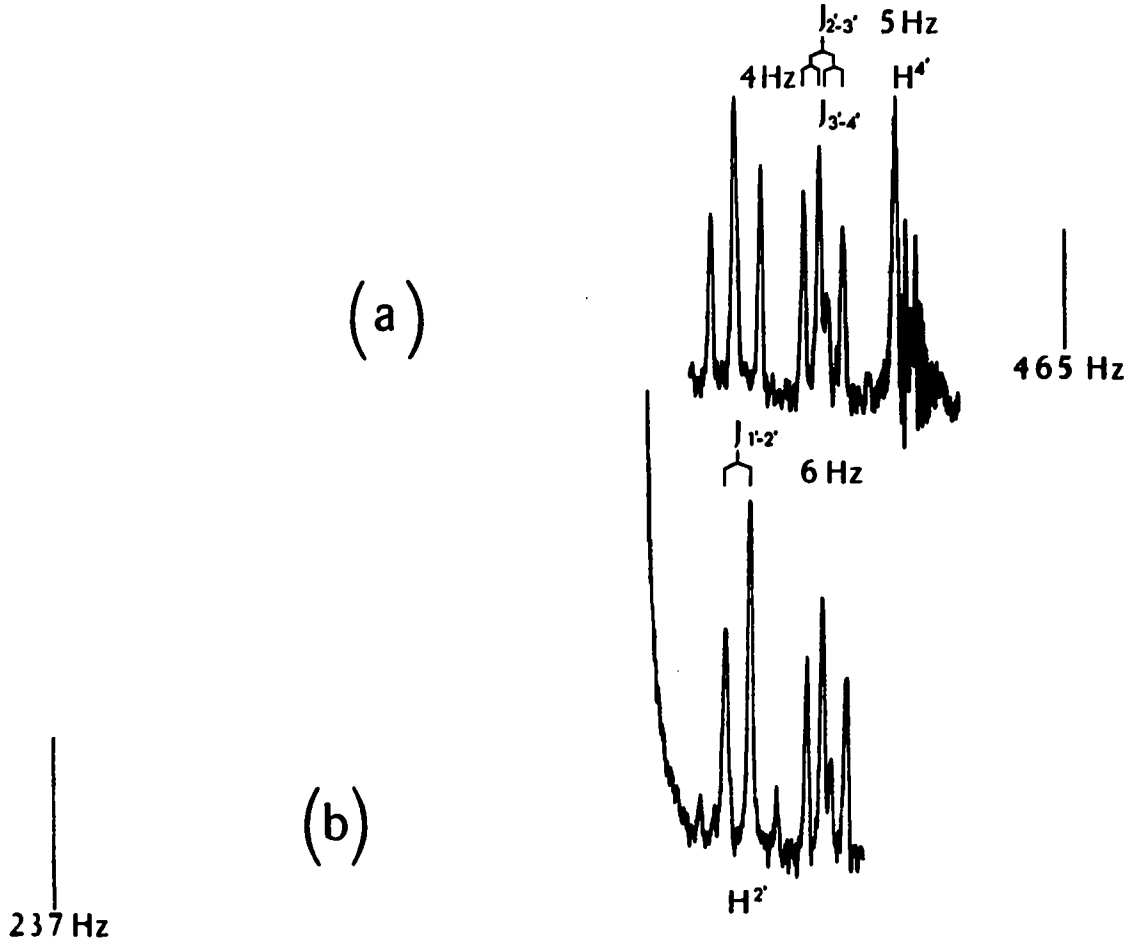


Fig. 8.(a) $H^{4'}$ nuclei of tubercidin (II) decoupled from $H^{5'}$ nuclei.
(b) $H^{2'}$ nuclei of tubercidin (II) decoupled from $H^{1'}$ nuclei.

1(β-D-ribofuranosyl)indoline (XX) nor the indole analog XXI could be phosphorylated effectively using the above described technique. The severe acidic environment (pH 1) yielded only trace amounts of the desired product (the indoline derivative is seemingly least stable).

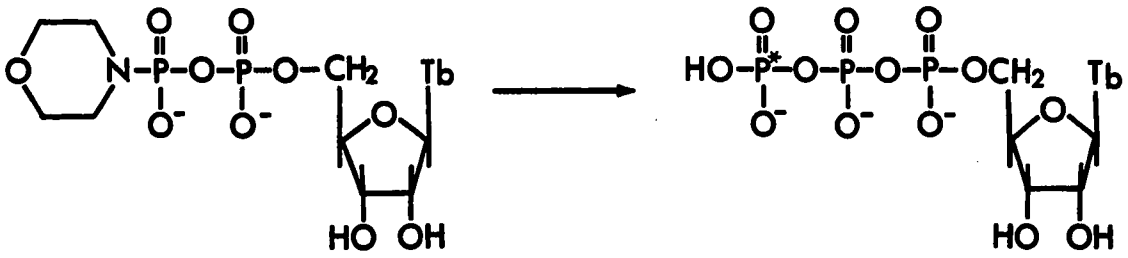
Tubercidin 5'-Di- and Triphosphates

Tubercidin 5'-monophosphate (XXII) is converted into tubercidin 5'-phosphoromorpholidate by reaction with morpholine and dicyclohexylcarbodiimide (DCC) (Moffatt and Khorana, 1961). This material was prepared and isolated as its 4-morpholine N,N'-dicyclohexylcarboxanidinium salt XXIII. The latter was condensed with tri-n-butylammonium phosphate and tri-n-butylammonium pyrophosphate in anhydrous dimethyl sulfoxide (Moffatt, 1964) yielding tubercidin 5'-diphosphate (XXIV) and the 5'-triphosphate derivative (XXV) respectively. Scheme V. Products were readily separated and purified on a column of DEAE-sephadex A-25.

γ-^{32}P-Tubercidin 5'-Triphosphate

The reaction of tubercidin 5'-diphosphate and morpholine in the presence of dicyclohexylcarbodiimide gave the terminally activated derivative P^1-(tubercidin-5') P^2-(4-morpholine)-pyrophosphate (TuDP-morpholidate, XXVI) in 76% yield. Condensation of XXVI with ^{32}P-labeled tri-n-butylammonium phosphate yielded γ-^{32}P-tubercidin 5'-triphosphate (XXVII). The crude product was chromatographed on a DEAE - Sephadex A-25 column and the fractions were followed by both radioactivity and ultraviolet absorption. Scheme VI.

Scheme VI



XXVI XXVII

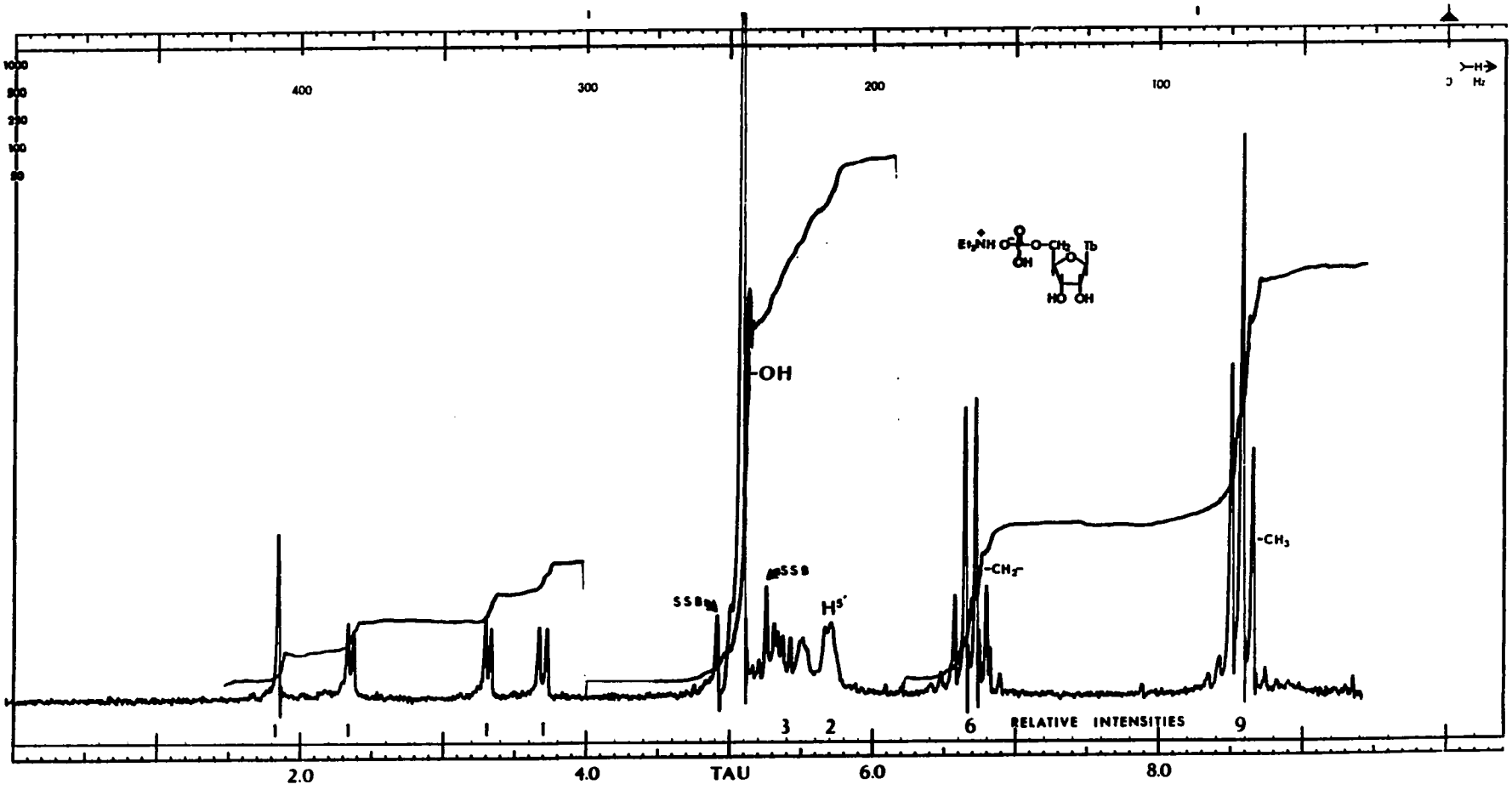


Fig. 9. Proton nmr spectrum (99.8 MHz) of the chromatographically pure triethylammonium salt of tubercidin 5'-monophosphate in D_2O. Chemical shifts are relative to tetramethylsilane as external standard.

EXPERIMENTAL SECTION

General. The experimentally determined melting points are reported uncorrected in degrees Celsius. Nuclear magnetic resonance spectra were obtained using a Varian A-60 or HA-100 spectrometer with approximately 20% solutions in deuterated solvents and 1% tetramethylsilane or pyrazine as reference. Descending paper chromatography on Whatman No. 1 paper and Whatman Chromedia DE 81 was carried out in the following solvent systems: solvent 1, 2-propanol-acetic acid-water, 3:1:1; solvent II, 2-propanol-ammonium hydroxide-water, 4:4:1. Thin layer chromatography on silica gel GF_{254} was developed in the following systems: solvent III, benzene-ethyl acetate, 19:1; solvent IV, chloroform-methanol, 85:15. Thin layer chromatography on pre-coated plates of PEI-cellulose F was carried out in the following solvent systems: solvent V, water; solvent VI, 0.25M lithium chloride; solvent VII, 0.85M LiCl and 1M formic acid pH2. Carbohydrates on chromatograms were detected with 0.5% sodium periodate and 0.5% benzidine. Benzoylated sugars, nucleosides and nucleotides were located by their absorption of ultraviolet light and/or their reaction with iodine vapor. Phosphorus-containing compounds were localized on chromatograms using the Hanes and Isherwood spray (1949) and developed according to Bandurski and Axelrod (1951).

^{32}P-containing compounds were separated by employing PEI- cellulose F TLC plates and DEAE-sephadex columns. An LKB fraction collector with a uv recorder was used for separating aromatic compounds.

All high b.p. solvent evaporations were done at 0.1-1 mm pressure using a rotary-type evaporator and a vacuum pump fitted with dry ice-acetone and liquid nitrogen traps. A Virtis freeze drying apparatus was used to remove large quantities of water from the water soluble compounds.

Preparation of Palladium Catalyst on Barium Sulfate

Palladium chloride (4.1g, 0.023 mole) was dissolved in a solution of 10 ml concentrated HCl and 40 ml water by heating on a steam bath (dissolution is complete in approximately 2 hours). Barium hydroxide (63 g of the octahydrate, 0.2 mole) was dissolved in 600 ml distilled water, placed in a 2 liter beaker, to which 60 ml 6N sulfuric acid was added all at once with vigorous stirring forming the necessary finely divided barium sulfate precipitate. The palladium chloride solution was added to this suspension followed by the addition of 4 ml 37% formaldehyde. Constant stirring was maintained as 30% sodium hydroxide was added until the suspension turned slightly alkaline. Stirring was continued for an additional five minutes and thereafter the catalyst was allowed to settle.

The supernatant was decanted and the catalyst was washed ten times with distilled water by allowing the catalyst to settle followed by decantation. The catalyst was finally collected on a medium-porosity sintered-glass funnel by suction filtration (avoiding breaking or channeling the cake) and was further washed with five 25-ml aliquots of distilled water. The cake was dried by placing the funnel and its contents in an 80 degree oven. The dried catalyst was ground to a powder using a mortar and pestle and finally stored in an air tight bottle. Yield: 43g.

Preparation of 2,3-Dihydroindole (Indoline, IV)

A mixture of 10g of palladium catalyst on barium sulfate and 250 ml acetic acid was hydrogenated using a Parr Instrument Company, Series 3910 Hydrogenation apparatus. After all reducing groups had been hydrogenated a solution of 11.7g (0.1 mole) indole in 100 ml 2N HCl was added to the catalyst-acetic acid mixture. The temperature was maintained at 55-65^{0} and the hydrogen pressure 4-8 p.s.i. When the uptake of hydrogen ceased (1-2 hour, approximately 0.1 mole) the catalyst was filtered off and the solvent removed in vacuo. The dark brown liquid was dissolved in 200 ml dilute aqueous NaOH and extracted with chloroform. The chloroform was removed and the desired colorless liquid was distilled off at 0.03 mm and 38^{0} (10.5g, 88% yield). The nmr spectrum (Figure 1, $CDCl_3$) showed bands at τ7.01 (2,t,H at C_3, $J_{2,3}$ = 7.0 Hz), τ6.49 (2,t,H at C_2, $J_{2,3}$ = 7.0 Hz), τ6.36 (1,s,H at N), and τ2.78-3.43 (4,m, aromatic H at C_4-C_7).

Preparation of 1-O-Acetyl-2,3,5,Tri-O-benzoyl-β-D-Ribofuranoside (VIII)

D-Ribose (75 g, 0.5 mole) was dissolved in 1600 ml anhydrous methanol (dried according to Lund and Bjerrum, 1931; Lund, 1934; 1952; and stored over Molecular Sieve Type 3A). To this stirred solution was added a fresh preparation of methanolic hydrogen chloride (6.6 g anhydrous HCl in 22 ml dry methanol). The reaction was continued (3-4 hr.) until the concentration of the reducing group reached a minimum (Levene et al., 1932, found that after 90 minutes 93% of the D-ribose was present as 1-O-methyl-D-ribofuranoside). Thereafter the reaction was quenched by the addition

of 150 ml pyridine which had previously been dried by distillation from barium oxide and stored over Molecular Sieve Type 4A. The solvents were removed in vacuo, the straw yellow oil taken up in 300 ml chloroform, and the solution was washed three times with 75-ml portions of water. The aqueous extracts were back-extracted with 75-ml chloroform and the organic phase was concentrated on the rotary evaporator.

The residual yellow oil was dissolved in 400 ml methylene chloride and 800 ml pyridine. The solution was cooled by setting in ice-water and benzoyl chloride (192 ml, 5 equivalents) was added slowly with vigorous stirring. The cold mixture was set in the refrigerator for 2-3 days. After benzoylation was complete the heterogeneous mixture was added to an equal volume of ice-water which was stirred and kept in an ice-bath. The resulting layers were separated, the aqueous layer extracted three times with 100-ml portions of chloroform, and the combined extracts and organic layer washed with 100-ml aliquots of saturated sodium bicarbonate and finally with 100 ml water. The aqueous washings were back extracted with 50-ml aliquots of chloroform, the combined organic phases dried over anhydrous sodium sulfate, filtered and evaporated in vacuo. Remaining traces of pyridine were removed from the reddish viscous oil by codistillation with toluene and benzene.

This oil was taken up in 150 ml acetic acid and 300 ml acetic anhydride and cooled in an ice bath. Concentrated sulfuric acid (45 ml) was added slowly with stirring. After addition was complete the mixture was allowed to reach room temperature, kept at room temperature for one hour, and then refrigerated. The following day the mixture was poured into 850 ml ice-water and 400 ml chloroform. After separation

of the two layers the aqueous phase was extracted five times with 75-ml aliquots of chloroform. The combined extracts and the organic phase were washed three times with 80 ml saturated sodium bicarbonate and the washings back extracted with chloroform. The combined organic extracts were dried over anhydrous sodium sulfate. The chloroform was removed and the yellow oil suspended in hot 98% ethanol. At room temperature crystals formed with a second crop precipitating after refrigeration of the filtrate yielding 180g (72% overall), m.p. 131-132°; reported (Recondo and Rinderknecht, 1959) 131-132°. The nmr spectrum (Figure 2 a-e, $CDCl_3$) showed bands at τ8.03 (3,s, H at CH_3CO), τ5.44 (1,q, H at C_5), τ5.29 (1,q, H at C_5), τ5.19 (1,q, H at C_4), τ4.21 (1,q, H at C_2, $J_{2,3}$ = 5.0 Hz and $J_{1,2}$ = 1.0 Hz), τ4.08 (1,q, H at C_3, $J_{2,3}$ = 5.0 Hz), τ3.54 (1,d, H at C_1, $J_{1,2}$ = 1.0 Hz), τ2.02 and 2.58 (15,m, aromatic H; 2:3).

Preparation of 1-(2,3,5-Tri-O-benzoyl-β-D-ribofuranosyl)indoline (XVI)

1-O-Acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranoside (25.2g, 0.05 mole) was dissolved in a mixture of 250 ml ethanol and 15 ml acetic acid. The solution was purged with argon for 1.5-2 hours. To the solution freshly distilled indoline (15.7g, 0.13 mole) was added dropwise. Refluxing was carried out under argon pressure for 12-16 hours. When the reaction was judged complete (followed by tlc on silica gel in benzene-ethyl acetate; 19:1) the solvent was removed in vacuo. The reddish oil was taken up in 300 ml chloroform, washed two times with saturated sodium bicarbonate and two times with distilled water. The chloroform was removed and the oil taken up in 100 ml benzene which in turn was removed with two final additions

of 25 ml dry benzene and removed in vacuo. A final yield of 40.4 g of reddish oil was obtained and chromatographed on a 900 g silica gel (28-200 mesh Grade 12) column (8.5 x 25 cm) using benzene-ethyl acetate (19:1) as the eluant. Most of the product (Rf 0.42; tlc, solvent III) had been washed through the column with 1.5-2 l of eluant. The solvent was removed from the combined fractions yielding 12.0 g (79%) straw yellow glassy amorphous material, 1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl) indoline (XVI). The nmr spectrum (Figure 5, $CDCl_3$) showed bands at τ7.14 (2,t, H at C_3, $J_{2,3}$ = 7.0 Hz), τ6.44(2,t, H at C_2, $J_{2,3}$ = 7.0 Hz), τ5.46 (3,m, H at $C_{4'}$ and $C_{5'}$), τ4.12 and 4.18 (3,m, H at $C_{1'}$, $C_{2'}$ and $C_{3'}$), τ2.96 and 3.30 (4,m, aromatic H at C_4-C_7; 2:2) and τ1.98 and 2.60 (15,m, aromatic benzoyl H; 2:3).

In addition to the desired product, starting material was recovered and 1-acetylindoline obtained.

Preparation of 1-(β-D-Ribofuranonsyl)indoline (XX)

Benzoylated nucleoside XVI (1.02 g, 1.8 m mole) in 15 ml dry methanol was dissolved upon warming and treated with 10 ml freshly prepared sodium methoxide in methanol (0.25 g sodium dissolved in 50 ml dry methanol). The mixture was refluxed under argon pressure and the reaction followed by tlc (silica gel, solvent IV). When debenzoylation was complete (30 min), the methanol was removed in vacuo and the adduct was taken up in 25 ml ether and 25 ml water. The aqueous layer was brought to neutral pH by bubbling CO_2 through the mixture. The organic layer was removed and washed three times with 7-8 ml water. The aqueous extracts were desalted on a column of Sephadex G-25. Water was removed with the

Virtis freeze dryer yielding 0.38 g (84%) of the desired product 1-(β-D-ribofuranosyl)indoline (XX) as a colorless amorphous glass. The nmr spectrum (Figure 3, D_2O) showed bands at τ6.73 (2,t, H at C_3), τ6.27 (2,t, H at C_2), τ5.7-6.1 (4,m, H at $C_{2'}$, $C_{4'}$, $C_{5'}$), τ5.29 (1,t, H at $C_{3'}$), τ4.69 (1,d, H at $C_{1'}$, $J_{1',2'}$ = 9 Hz), and τ2.7 and 3.0 (4,m aromatic H at C_4-C_7).

Oxidation of 1-(2,3,5-Tri-O-benzoyl-β-D-ribofuranosyl)indoline (XVI) to 1-(2,3,5-Tri-O-benzoyl-β-D-ribofuranosyl)indole (XVIII)

2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ; 3.08 g, 13.6 m mole) was dissolved in 100 ml dry xylene with several ml distilled off under vacuo at rt and the mixture deoxygenated with argon. Protected nucleoside XVI was dissolved in 150 ml dry xylene which had been previously deoxygenated with argon and was added to the DDQ mixture with a further distillation of 3 ml. The reaction medium was refluxed for 22 hr under argon pressure. When the reaction was judged complete, by following with tlc (silica gel, solvent III; Rf 0.44), the reddish mixture was filtered and the xylene was removed in vacuo yielding 7.8 g of dark red oil. The desired product 1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)indole was obtained as a light yellow glass by purification on a silicic acid column using chloroform as the eluant. The nmr spectrum (Figure 6, $CDCl_3$) showed bands at τ5.27 (3, H at $C_{4'}$ and $C_{5'}$), τ4.0 (2,m, H at $C_{2'}$ and $C_{3'}$), τ3.5 (2,m, H at $C_{1'}$ and C_3), τ2.75-3.0 (5,m, aromatic H at C_2 and C_{4-7}) and τ1.8-2.7 (15,m, aromatic benzoyl H; 2:3).

Preparation of 1-(β-D-Ribofuranosyl)indole (XXI)

The blocked precursor of XXI, 1-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl) indole, (2.61 g, 4.65 m mole) in 30 ml dry methanol was dissolved upon warming and treated with 10 ml freshly prepared sodium methoxide in

methanol (0.50 g sodium dissolved in 50 ml dry methanol). The mixture was refluxed under argon pressure and the reaction followed by tlc (silica gel, solvent IV; Rf 0.80). When deacylation was complete (30 min), the methanol was removed under vacuo and the adduct was taken up in 40 ml ether and an equal amount of water. CO_2 was bubbled through the mixture until pH was reduced to neutral range and the remaining liquid was washed 3-4 times with 10 ml CCl_4. The aqueous layer was continuously extracted with ether for 24 hr. and was removed yielding crude product XVIII (1.6 g) which was purified on a silicic acid column using ethyl acetate as eluant (0.19 g of crude XVIII was washed through a 1.75 x 5.5 cm column yielding 0.16 g, 74%, pure compound, mp 145°). The nmr spectrum (Figure 4, CD_3OD) showed bands at τ 6.23 (2,m, H at $C_{5'}$), τ5.94 (1,q, H at $C_{4'}$) τ 5.80 (1,q, H at $C_{2'}$), τ 5.63 (1,q, H at $C_{3'}$), τ3.95 (1,d, H at $C_{1'}$, $J_{1'-2'}$ = 9.0 Hz), τ 3.47 (1,d, aromatic H at C_3, $J_{2,3}$ = 6.0 Hz) and τ2.32-2.56 and 2.78-3.0 (5,m, aromatic H at C_2 and C_{4-7}).

Preparation of Tubercidin 5'-Monophosphate (XXII)

Triethylphosphate (dried by storing over CaH_2 for 48 hr and distilled under vacuo) was cooled to ice temperature. Phosphoryl chloride ($POCl_3$; 3.67 g, 20 m mole) was added to 25 ml of the $(EtO)_3PO$ followed by the addition of 0.18 g (10 m mole) water. After 10 min tubercidin (2.66 g, 10 m mole) was added as a powder and when dissolution was complete (40 min) the reaction mixture was stirred at 0° and followed by tlc. When the starting material (silica gel, solvent IV; Rf 0.18) was depleted (3-4 hr), water (20 ml) was added at ice temperature and neutralized by the addition of 6M NH_4OH. The mixture was washed 3 times with 20 ml and 3 times with

5 ml aliquots of chloroform to remove the organic phosphate. Water was removed by freeze drying yielding the salt of XII as a white solid. The vicinal diol and a phosphate were identified by the benzidine-periodate and Hanes-Isherwood sprays respectively at the same spot on Whatman Chromedia DE 81 ion-exchange cellulose paper using solvents I and/or II. The salt of XII was purified by dissolving in water and applying to a 1.5 x 30 cm DEAE-Sephadex A-25 column followed by a thorough wash with distilled water and subsequently eluted with a linear gradient of triethylammonium bicarbonate (2 liters from 0.05-0.5 M). Fractions of the ultraviolet peak were combined and water removed on the freeze drier. Residual bicarbonate was removed by 3-4 evaporations with 20 ml portions of methanol (2.96g, 82%). Phosphorous analysis and ultraviolet measurements showed one phosphate per aromatic ring. PEI-Cellulose F plates and 0.25 M LiCl and 1 M formate (pH 2) as developing solvent gave similar Rf values (TuMP 0.83; AMP 0.80). The nmr spectrum (Figure 9, D_2O) showed bands at τ8.58 (9,t, H at CH_3 of ethyl ammonium), τ6.69 (6,q, H at CH_2 of ethyl ammonium), τ 5.70 (2,m, H at $C_{5'}$), τ 5.52 (1,m, H at $C_{4'}$), τ5.28-5.44 (2,m, H at $C_{2'}$ and $C_{3'}$), τ 3.70 (1,d, H at $C_{1'}$), τ3.32 (1,d, H at C_7, $J_{7,8}$ = 4.0 Hz), τ2.36 (1,d, H at C_8, $J_{7,8}$ = 4.0 Hz), and τ1.84 (1,s, H at C_2).

The dihydrogen form of TuMP was also obtained. Barium chloride 10 m mole was added to an aqueous solution of 5.0 m mole of the crude ammonium salt of TuMP at pH 9. The barium phosphate was removed by filtration and an equal volume of ethanol was added to the filtrate. Ba TuMP crystals formed overnight in the refrigerator, were removed by centrifugation and added to an equivalent of washed AGC-244 ion exchange resin (Dowex 50W-X8; H^+ form). The resin was poured onto a 1.5 x 6 cm

column of additional Dowex-50W-X8. The tubercidin 5'-dihydrogen monophosphate was washed through the column with 0.1 N HCl and concentrated by freeze drying (4.1 m mole by uv absorption, 84%).

Preparation of Tubercidin 5'-Phosphoromorpholidate

The triethylammonium salt of tubercidin 5'-monophosphate or the dihydrogen form of the same (1.0 m mole) was added to a refluxing solution (30 ml) of 50% aqueous tert-butanol which contained 0.3 ml (3.5 m mole) of freshly distilled morpholine. Dicyclohexylcarbodiimide (DCC), 0.72g (3.5 m mole), in 15 ml tert-butanol was added dropwise over a 1.5-2 hr. period to the mononucleotide solution. The reaction was followed by tlc (PEI-cellulose F plates, solvent VII) and when the starting material (Rf 0.76) was depleted and replaced by the slower moving morpholidate refluxing was discontinued. When the reaction mixture had cooled the 4-morpholine N,N'-dicyclohexylcarboxamidine was removed by filtration and the additional base filtered off following removal of the tert-butanol from the reaction mixture. The aqueous solution was washed three times with 10 ml ether and the desired product was obtained as the 4-morpholine N,N'-dicyclohexylcarboxamidine salt of tubercidin 5'-phosphoromorpholidate as a glass (0.69 g, 95%) by removing water on the freeze drier.

Preparation of Tributylammonium Phosphate and Pyrophosphate

Dowex 50W-X8 (H^+ form) was converted to the pyridinium form by passing pyridine through a 1.5 x 30 cm column of the resin. An aqueous solution of sodium phosphate (3 m mole, 0.49 g) or sodium pyrophosphate (2.5 m mole, 1.1 g) was washed through the column. The effluent and water wash was concentrated to a viscous yellow liquid and taken up in 45 ml pyridine and 4 (10 m mole, 2.5 ml) equivalents of tri-n-butylamine

plates and 0.85 M LiCl and 1 M HCOOH (pH 2) as developing solvent gave an Rf value of 0.56 similar to ADP (0.52). The yield was 0.62 m mole (86%) as measured by uv absorption where E max was taken as 1.12×10^4 at a λmax_2 of 271 nm.

Preparation of Tubercidin 5'-Triphosphate(XXV)

The experimental procedure in the preparation of XXV was basically the same as that for the synthesis of TuDP except that tri-n-butylammonium pyrophosphate was used to form the triphosphate ester. The major peak (76%) was the desired TuTP with the minor peaks TuMP-morpholidate (4%) and TuMP (4-5%) also present. Phosphorus analysis and ultraviolet measurements showed a base to phosphate ratio of 1.0:2.88 and a labile phosphate to total phosphate ratio of 1.89:3.0.

Before use the triethylammonium salt of TuTP (0.6 m mole) was dissolved in methanol (8 ml) and precipitated as its sodium salt by the addition of 0.25 ml of 1 M sodium iodide in acetone and 60 ml acetone. The white flocculent precipitate was removed by centrifugation and washed with purified acetone (25 ml) an additional 2-3 times and dried in vacuo. A 1.0mM solution of the product was hydrolysed by myosin to form Pi and TuDP at a rate of 0.4 μ moles/min-mg protein in 0.1 M KCl 15mM $CaCl_2$, and 20mM Tris-HCl (pH 7.4) at 25°C.

Preparation of γ-^{32}P-5'-Triphosphate (XXVII)

The triethylammonium salt of tubercidin diphosphate (0.2 m mole) was added to a refluxing solution 15 ml of 50% aqueous tert-butanol which contained 0.1 ml (1.2 m mole) of freshly distilled morpholine. DCC (0.15 g, 0.7 m mole) in 10 ml tert-butanol was added dropwise over a 1.5-2 hr period to the TuDP solution. The reaction was followed by tlc

(3 equivalents upon preparation of the orthophosphate). The mixture was shaken until homogeneous and the pyridine was removed with two subsequent additions and evaporations of 20 ml pyridine. The tri-n-butyl ammonium phosphate and pyrophosphate compounds were further dried by three 20 ml additions and evaporations of each of dry benzene and rigorously anhydrous dimethyl sulfoxide (distilled over CaH_2 and stored over type 4A molecular sieve).

Preparation of Tubercidin 5'-Diphosphate (XXIV)

The crude tubercidin 5'-phosphoromorpholidate (0.72 mmole) was dried by three evaporations of 10 ml each of dry pyridine and benzene and finally with three evaporations with anhydrous dimethyl sulfoxide (DMSO). The dried compound was dissolved in 15 ml DMSO and added to a 15 ml solution of anhydrous tri-n-butylammonium phosphate (3.0 mmole) in DMSO. The viscous solution was sealed in a 100 ml r.b. flask and vibrated in a 35-40^{o} atmosphere for 48 hr. Water (40 ml) was added to the reaction mixture and the phosphates were precipitated with $BaCl_2$. The solids were filtered off and added to 50 ml 0.5N sulfuric acid with stirring overnight in the refrigerator. The barium sulfate was filtered off, the filtrate was neutralized with 4 N NaOH and added to a 1.5 x 30 cm DEAE-Sephadex A-25 column. After a water wash the column was eluted with a linear gradient of 0.05-0.5 M triethylammonium bicarbonate (3 l). The main peak fractions were combined and the product was obtained by freeze drying followed by three evaporations with 30 ml aliquots of methanol. Phosphorus analysis and ultraviolet measurements showed two phosphates per aromatic ring. PEI-Cellulose F

(PEI-Cellulose F plates, solvent VII) and when the starting material (Rf 0.56) was depleted and replaced by the slower moving morpholidate refluxing was stopped. The reaction mixture was allowed to cool and the 4-morpholine N, N'-dicyclohexylcarboxamidine was removed by glass wool filtration. After the tert-butanol was removed under vacuo the additional base was filtered off. The filtrate was washed 3 times with 5 ml ether and the 4-morpholine N, N'-dicyclohexylcarboxamidine salt of tubercidin 5'-diphosphoromorpholidate was obtained by removing water on the freeze drier. The product was further dried by three evaporations of 5 ml each of pyridine and benzene and finally with 3 evaporations with anhydrous DMSO. At the same time 0.8 ml of a 1 M solution of phosphoric acid (0.8 m mole) was mixed with 1.0 ml of 10 mc/ml ^{32}P-phosphoric acid in 0.02 N HCl. Tri-n-butylamine(0.16 ml, 0.8 m mole) was added to the radioactive material and the solution was evaporated to dryness (Virtis) followed by repeated evaporations of dry pyridine, benzene and finally anhydrous DMSO.

The radioactive phosphate was taken up in 2 x 5 ml anhydrous DMSO and transferred to the dried TuDP-morpholidate in 15 ml anhydrous DMSO. The viscous solution was sealed in a 50 ml r.b. flask and vibrated in a 35-40^{o} atmosphere for 48 hr. Water (50 ml) was added to the reaction vessel and the mixture was applied to a 1.5 x 30 cm DEAE-Sephadex A-25 column (HCO_3^- form). A linear gradient of triethylammonium bicarbonate was used (0.05 to 0.5M) after a water washing. Fractions were followed by ultraviolet absorption and the radioactivity measured in aliquots. The uv absorbing radioactive fractions were pooled and the water removed on the Virtis freeze drier. The product was taken up in 20 ml of methanol

which was evaporated (repeated 3 times) and converted to the sodium salt by adding NaI in acetone and precipitating. The yield was 86 mg (67%) with a specific activity of 8.8 μc/μmole. A 0.1 mM solution was hydrolyzed by myosin in 0.1 M KCl, 15mM $CaCl_2$, 20mM Tris-HCl at 25^{o} for 10 min and yielded TuDP free of radioactivity. Thus all the label was in the γ-position.

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