THE EFFECTS OF TRIAC ON DEVELOPING AND GROWING RAT HEART MUSCLE AND ITS MODIFICATION BY CERTAIN THERAPEUTIC AGENTS: WITH REFERENCE TO A POSSIBLE PATHOGENESIS OF HYPERTROPHIC CARDIOMYOPATHY

THESIS

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ABSTRACT

Hypertrophic cardiomyopathy is a heart muscle disease of unknown cause. This thesis describes a study, using the rat, which explores clinical evidence that thyroid hormones, especially their analogues, may play an important role in the pathogenesis of the disease.

Triiodothyroacetic acid, triac, has been administered to pregnant rats and has produced, in the young offspring, cardiac hypertrophy and, at the ultrastructural level, an increase in the extent of myofibrillar disarray. Both of these pathological features are seen in hypertrophic cardiomyopathy in man.

A series of experiments was designed to define the hitherto unknown site of action of triac. This compound was administered concurrently with selected antagonist drugs with each or all of the following activities; beta adrenergic blockade, membrane stabilisation and sympathomimetic activity. Oxprenolol, dl and d propranolol, and procainamide prevented the disarray but not the hypertrophy, and timolol had no effect. The results suggested that the most important property involved in the prevention of the action of triac was that of membrane stabilisation. A low dose of the calcium antagonist verapamil reduced both the triac induced disarray and hypertrophy, suggesting that triac may act by increasing intracellular calcium. This suggestion was supported by an

experiment using the calcium ionophore A23187. This compound did not affect the action of triac, but, when administered alone, produced hypertrophy and disarray in the hearts of the offspring.

An attempt was made to produce pathological features of hypertrophic cardiomyopathy, at the histological and macroscopic levels, by attempting to enhance the actions of triac so far delineated. For this purpose a beta 1 agonist, prenalterol, was used together with triac. This combination was administered to the offspring of treated rats until they were 3 months old. Severe cardiac hypertrophy was produced but not disarray.

Additional experiments showed that thyroxine produced the same effects as triac but that a much higher dose was required. Triac was also given during different stages of pregnancy and was shown to be most effective during the early stage.

These studies have shown the hitherto unknown site of action of triac on developing rat myocardium. They have also demonstrated how the influence of chemical and biological agents may be explored in the search for possible contributory factors in the pathogenesis of hypertrophic cardiomyopathy.

The preparation and analysis of tissue samples was carried out at:-

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The parts of the experiments involving the use of animals were carried out at:-

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GLOSSARY OF TERMS

Triac - Triiodothyroacetic acid

Tetrac - Tetraiodothyroacetic acid

T3 - Triiodothyronine

T4 - Thyroxine

TSH - Thyroid stimulating hormone

In tables the following abbreviations have been used:

Oxpren - Oxprenolol

Prop - Propranolol

Tim - Timolol

Verap - Verapamil

Proc - Procainamide

INTRODUCTION

The purpose of the experiments carried out in this thesis was to study a possible cause of hypertrophic cardiomyopathy. An increase in the knowledge of this condition is important as it can be subclinical and often results in sudden death, especially in the young.

Hypertrophic cardiomyopathy has been classified as one of three forms of cardiomyopathy which have been collectively defined as "heart muscle diseases of unknown cause" (WHO/ISFC 1980). The two other forms are dilated and restrictive cardiomyopathies and these will be briefly described. Hypertrophic cardiomyopathy appears to be an uncommon condition, as many patients are symptomless, but since it was first fully described in 1958 by Teare it has been the subject of a vast amount of literature and discussion. Part of the fascination of the subject is that there are almost as many viewpoints on the various aspects of the condition as there are people writing about them. From the pathological point of view there are several major characteristics of which gross cardiac hypertrophy with disproportionate septal thickening, disarray of the myocardial fibres and disarray of the myofibrils rank highly. None of these are pathognomic and all can be found in other conditions. Much of the literature has debated the importance of these features and some authors have even found themselves disagreeing with their previous work.

As the definition points out, aetiology remains unknown even though many workers have explored many ideas and put forward many proposals. One of these ideas comes from the clinical observation that covert hyperthyroidism has been associated with the condition in some patients. This finding prompted some initial experiments by Olsen, Symons and Hawkey to try and produce the condition in laboratory animals using a thyroid hormone analogue, triiodothyroacetic acid, or triac. They found that when this compound was administered to pregnant rats the hearts of the newborn offspring showed changes, at the ultrastructural level, similar to those observed in the clinical condition. These changes were the production of cardiac hypertrophy and disarray of the myofibrils. Could triac therefore be implicated as a possible endogenous cause of hypertrophic cardiomyopathy? Excess levels have been found in the circulation with no signs of overt hyperthyroidism being present (Symons et al 1971).

This initial experiment has prompted two lines of research which have been followed in this thesis. The first was to ensure that this action of triac could be repeated and to assess its mechanism of action. Hawkey et al (1981) had found that the beta adrenergic blocking agent propranolol, could prevent the disarray but not the hypertrophy. This finding has led to the use of other similar drugs with differing properties which could be utilised as pharmacological tools to investigate the mechanism of action of triac.

The other line of research was to attempt to improve the animal model and produce other features of hypertrophic cardiomyopathy seen at the histological and gross levels.

Thus this thesis brings together the subjects of pathology, endocrinology and pharmacology in the investigation of a possible cause of hypertrophic cardiomyopathy.

PART 1

BACKGROUND

1. CARDIOMYOPATHIES

1.1 Definition and Classification

The name cardiomyopathy means any abnormal condition or disease of the heart muscle (Greek: mys - muscle, pathos - suffering). The literal meaning, however, would include all heart muscle diseases and so to reduce confusion, myocardial lesions produced by ischaemic heart disease, hypertensive cardiovascular disease, valvular heart disease and congenital heart disease are excluded, even though the presenting clinical and haemodynamic findings may be indistinguishable.

The term was introduced by Brigden in 1957 who adapted it to uncommon myocardial diseases of non coronary origin . He emphasised that aetiology should be indicated whenever possible. Since then there has been much confusion concerning classification and definition but the recent WHO/ISFC task force (1980), based on the work of the Hammersmith group, in particular Goodwin, has decided upon "Heart muscle diseases of unknown cause" as the definition. (Goodwin et al 1961, Goodwin, 1975 and Oakley, 1978).

Previously cardiomyopathies were classified into "Primary" and "Secondary" groups but the current term refers only to the group originally known as "Primary". The term secondary

has now been replaced by "Specific Heart Muscle Disease" (Goodwin & Oakley, 1972; Goodwin, 1973) in which metabolic (including thyrotoxicosis), general systemic diseases, heredo-familial neuromuscular disorders, sensitivity and toxic reaction, and myocarditis are included (Olsen, 1972; Goodwin, 1975; Olsen, 1978a).

The task force classified the cardiomyopathies into three groups:

1) Dilated cardiomyopathy
2) Hypertrophic cardiomyopathy
3) Restrictive cardiomyopathy

These classifications have been based upon haemodynamic and structural changes.

It is pertinent here to describe briefly the dilated and restrictive cardiomyopathies to show the differences, but the major part of this thesis deals with hypertrophic cardiomyopathy.

1.2 Dilated Cardiomyopathy

This condition, also known as congestive cardiomyopathy, is recognised by dilatation of the ventricular cavities. It is invariably accompanied by hypertrophy which may be masked if the dilatation is severe. Systolic ventricular function is impaired as the muscle has a poor contractile force, demonstrated by a low cardiac output and stroke volume, reduced ejection fraction and increased residual volume. Congestive heart failure may or may not supervene. Patients often present with disturbances of ventricular or atrial rhythm and death may occur at any stage.

The endocardium is usually thickened and in 60% of cases a thrombus may be superimposed (Olsen, 1973a). No organic abnormalities are found in the heart, or elsewhere in the body, which could have resulted in heart failure, but some functional changes, such as thickening of the valve leaflets due to existing insufficiency, may be found. Coronary arteries are invariably normal (Gare et al, 1972).

The myocardial fibres are predominantly regularly arranged, with the very occasional focus of irregularity, and have normal diameters. Nuclear changes, however, and also features seen at the ultrastructural level, do show hypertrophy to be present, the discrepancy being due to the attenuation of the fibres, (Olsen, 1972, 1973a, 1975a).

There is an increase in fibrous tissue which may take the form of an increase in the interstitial collagen component or replacement of myocardial fibres.

Histochemical analysis of fresh endomyocardial tissue obtained by bioptome has shown an increase, normal amount or a decrease in substances such as glycogen and succinic dehydrogenase (Olsen, 1978b).

Features of alcoholic heart disease and heart failure following pregnancy are indistinguishable from dilated cardiomyopathy and it could be possible that these may be contributory factors to the disease. In a series of 74 patients Kristensson (1969) noted heavy alcoholic intake in 16, a history of previous infection in 14, while the onset of the illness was in pregnancy or puerperium in 7.

1.3 Restrictive Cardiomyopathy

Under this heading both endomyocardial fibrosis (EMF) and Löfflers endocarditis parietalis fibroplastica (Löffler's endocarditis, 1936) are included. Both diseases are histologically identical and clinically, virtually indistinguishable (Bell et al 1976) and it has been suggested that they both belong to the same disease spectrum (Brockington and Olsen 1973).

Pathologically, a thickened endocardium is arranged in characteristic layers. In the above retrospective study, three major morphological stages were identified: necrotic, thrombotic and fibrotic, each stage being dependent on length of history.

More recently an association with eosinophilia containing a significant number of degranulated eosinophils have been noted (Olsen and Spry 1979). It was suggested that if 1.5 X 10^9 per litre of eosinophils are degranulated then endomyocardial heart disease will be associated, irrespective of the cause of eosinophilia (infective, malignancy, idiopathic or possibly genetic) or geographic distribution.

1.4 Before describing hypertrophic cardiomyopathy in detail it is pertinent to mention that the term "unclassified cardiomyopathy" covers a few cases which do not fit readily into any group. This includes some with minor abnormalities in which progression to overt cardiomyopathy may or may not occur. This has been referred to as latent cardiomyopathy.

2. Hypertrophic cardiomyopathy

This condition is usually characterised by a disproportionate, or asymmetric, hypertrophy of the interventricular septum. This is not a pathognomic sign and several features in combination contribute to the diagnosis of hypertrophic cardiomyopathy (see 2.5.4). The left ventricular volume is either normal or reduced and systolic gradients are common. Obstruction of the left ventricular outflow tract may not be present but the essential haemodynamic abnormality is failure of diastolic compliance (Oakley, 1972).

2.1 Historical Review

The first descriptions of this condition were by two nineteenth century French pathologists, Liouville (1869) and Hallopeau (1869), followed by the German pathologist Schmincke (1907). Their findings were compatible with asymmetric hypertrophy of the heart. Hudson (1970) had previously credited Dittrich (1849) and Lauenstein (1875) with the first descriptions but the cases described by these authors were later found to be cases of fibrous subvalvular aortic stenosis.

It remained, however, for Brock in 1957 and Teare in 1958 to draw attention to the condition. In nine cases of sudden

death, Teare described a marked hypertrophy and thickening of the interventricular septum at post-mortem examination. In five of these cases, the hypertrophic process also involved the anterior wall of the left ventricle but the posterior left ventricular wall appeared not to be involved. The process was asymmetric. Microscopic examination of the hypertrophied areas of the myocardium revealed myocardial fibre disarray and significant amounts of interstitial fibrosis.

Since this report a vast amount of literature on the subject has appeared dealing with all aspects of the condition, for example Goodwin et al 1960, Goodwin 1970, 1980, Braunwald et al 1964, Van Noorden et al 1971, Olsen 1971, 1973ab, 1975abc, 1980 and Davies et al 1974. Many titles have been used when describing the condition and these have been excellently reviewed by Baandrup (1979). In cases where obstruction is present it is often called hypertrophic obstructive cardiomyopathy or H.O.C.M. (Cohen et al 1964) and in American literature the term idiopathic hypertrophic subaortic stenosis or I.H.S.S. (Braunwald et al 1960, Boiteau & Allenstein 1961), appears frequently. Hypertrophic cardiomyopathy was introduced by Goodwin in 1970 and remains the appropriate term.

2.2 Distribution and Occurrence

Owing to the fact that diagnosis is difficult and many patients do not present symptoms, the true distribution and occurrence is not known. The condition has been described world wide in all races but has been found to be rare amongst African negroes (Lewis et al 1973, Beck 1978). For an unexplained reason Braunwald et al (1964) found many patients to be Canadian, or could trace their history there.

It is not a common disease but for the above reasons its occurrence may be more frequent than is generally known. Reports and reviews in the literature are generally from large referral centres or multicentre projects and they quote the number of patients they have seen in the period of study. For example Shah et al (1973) reported on 190 patients from centres in England and U.S.A. In a survey of sudden deaths from a regional centre in London, Davies (1981) found 76 patients with hypertrophic cardiomyopathy. (In 48 of these cases the condition had been undiagnosed in life). In a clinical survey of 2800 patients undergoing cardiac catheterisation in Chicago, 1% had hypertrophic cardiomyopathy with obstruction (Falicov & Resnekov 1977). By contrast, Robinson in 1976 did not record a single case of asymmetric septal hypertrophy in 2790 autopsies in the Veterans Administration Hospital, Lake City, concluding that the condition is rare in the elderly.

Reports on sex ratio vary. Goodwin (1970) found an equal sex ratio in 91 patients whereas Braunwald et al (1964) reported a male preponderance of 1.5 : 1. In his report on sudden death Davies (1981) found 85% of those with hypertrophic cardiomyopathy undiagnosed in life were men but only 62% were men from the group previously diagnosed or symptomatic.

2.3 Clinical Recognition

Clincal aspects have been reviewed in many papers, including Goodwin et al (1961), Braunwald et al (1964), Goodwin (1970, 1974), Oakley (1974) and Shah (1979). Although this thesis concerns the pathology of the position, it is relevant here to briefly describe the clinical aspects.

Symptoms

When present, clinical symptoms are generally those of left ventricular outflow obstruction, namely dyspnoea, syncope, angina and palpitation. Sudden death is a common feature and congestive heart failure occasional. In patients without obstruction dyspnoea is the most common presenting symptom.

Effort dyspnoea and paroxysmal nocturnal dyspnoea represent evidence of pulmonary congestion.

Syncope and dizziness are common and are generally effort related (Sundararajan 1980) but the frequency of episodes is highly variable.

Angina of effort simulating symptomatic coronary artery disease is frequent, although chest pain episodes may be prolonged and may occur spontaneously at rest. As expected, nitroglycerine does not usually provide relief. In the presence of large patent coronary arteries, the cause of the ischaemia is probably related to increased myocardial tension and increased muscle mass, with oxygen requirements outstripping delivery.

Palpitations may represent awareness of forcible heart beat but it is more common that atrial and ventricular arrhythmias are responsible. Tachyarrhythmias are poorly tolerated and may be the cause of the many cases of sudden death (Goodwin & Krikler 1976). Patients who are in atrial fibrillation with grossly raised venous pressures and a low cardiac output might be expected to die suddenly, probably from ventricular fibrillation. Sudden death, however, does not only occur in advanced cases, but also in younger, often asymptomatic, patients. It may well be the first manifestation of the disease (Maron et al 1978c). In a series of 220 patients with hypertrophic cardiomyopathy, followed for a mean of 6 years, 27 patients died suddenly, most being under 40 years of age and asymptomatic before

death (McKenna et al 1979). Arrhythmia could still be responsible, though, as continuous electrocardiographic monitoring of patients outside hospital has shown that asymptomatic arrhythmia is surprisingly common in patients with hypertrophic cardiomyopathy (Savage et al 1979, McKenna et al 1980).

Signs

These are summarised by the following 5 hallmarks:-

1) 'Jerky' arterial pulse.
2) Palpable atrial beat.
3) Sustained left ventricular impulse.
4) A systolic murmur heart at the lower left sternal border.
   (This is the characteristic murmur of left ventricular outflow tract obstruction).
5) Mitral regurgitation may or may not be present.

Pressure gradients in systole are present in 70-80% of cases (Goodwin 1975) but they may be variable and transient (Braunwald et al 1962).

Echocardiography has proved to be the most useful non-invasive technique for aiding the diagnosis of hypertrophic cardiomyopathy. Goodwin (1975) has noted 6 important

features, none of which are diagnostic in isolation but when all are present in the same recording diagnosis is virtually certain.

Nuclear medicine imaging using 201 Thallium has also been used to diagnose the condition.

2.4 Natural History

The natural history of hypertrophic cardiomyopathy has been the subject of many papers including Braunwald et al 1964, Goodwin 1970, 1974, Swann et al 1971, Oakley 1971, Shah et al 1974, Hardarson et al 1973.

It is determined by the degree of stiffness of the left ventricle and the filling problems associated with it. The clinical course is related to the left ventricular end diastolic pressure, particularly on effort, (Oakley 1971). Most patients appear to have some degree of obstruction to left ventricular outflow tract, either persistently or on provocation, at some time during their lifetime, but others, in whom evidence of outflow tract obstruction disappears, may develop severe heart failure, often associated with atrial fibrillation and emboli. This course of the disease does not appear to occur in more than 10% of patients (Swann et al 1971). Most patients in the above study followed a stable course, but a minority tended either to deteriorate

progressively or to apparently improve symptomatically. Sudden death is a well recognised complication and is the most common cause of death (Hardarson et al 1973). It is most common in young males with a family history of the disease or when the left ventricular end diastolic pressure is particularly high and there is a short history of symptoms of increasing severity. In infants with hypertrophic cardiomyopathy, Maron et al (1982) found sudden death (2 out of 20 patients) less common than death due to progressive cardiac failure. Eleven infants had congestive heart failure and nine of these died within the first year of life. Swann et al (1971) found that symptoms and their deterioration were related to left ventricular end diastolic pressure and not to the left ventricular systolic gradient, suggesting the latter is a less important feature. The average age of onset of symptoms was 28 years and the average duration of symptoms before death was 9 years. In an average follow up of 5.2 years Shah et al (1974) found 18% of patients died, i.e. a rate of 3.5% per year.

2.5 Pathological Findings

An excellent review of macroscopic, microscopic and electron microscopic findings has recently been published by Olsen (1980). As an indication of the volume of published work on the subject in recent years he quotes over 80 references, which is extensive but still not complete. The following

descriptions can therefore only be a brief summary and the thesis itself will concentrate on electron microscopic findings, particularly disarray of the myofibrils.

2.5.1 Macroscopic Appearances

The heart is firm to the touch and biatrial and biventricular hypertrophy is usually present. The most striking feature, however, is the often extreme disproportionate hypertrophy of the interventricular septum, extending from the region of the apex to just below the aortic valve, (See Fig. 1). Teare (1958), in his original descriptions emphasised the hypertrophy of the upper portion of the septum adjacent to the anterior leaflet of the mitral valve. The hypertrophied septum, and in some instances the hypertrophied anterior wall of the left ventricle, bulge into, and impinge upon, the lumen of the left ventricular outflow tract (Paré et al 1961). The obstruction, when present, is usually at its peak in the subaortic region, but Falicov & Resnekov (1977) have shown it may also be found in the mid ventricular area. The asymmetric hypertrophy of the interventricular septum is best seen on cross section and the ratio of thickness of the septum to the thickness of the free wall of the left ventricle is calculated. Menges et al (1961) found that in cases of hypertrophic cardiomyopathy the ratio always exceeded 1.3 whereas the mean for normal hearts was 0.95 and for hypertrophied hearts, 0.98.

Fibrous tissue is often seen in the interventricular septum when the ventricles have been transected, giving it a 'watered milk' appearance (Olsen 1972). In patients with



Fig. 1. Illustration of macroscopic appearance of hypertrophic cardiomyopathy in man, showing disproportionate thickening of the interventricular septum.
(Courtesy of Dr E.G.J. Olsen, National Heart Hospital).
x 0.7

obstruction, abnormal positioning of the anterior mitral leaflet has occasionally been described (Björk et al, 1961, Björk 1964). The anterior mitral leaflet and, to a lesser extent, the posterior leaflet are usually thickened in patients with mitral regurgitation, (Olsen 1973b). A characteristic endocardial fibrous thickening in the outflow tract is often seen opposite the anterior mitral leaflet, the "mirror image of the leaflet" (Davies et al 1974).

Cases of hypertrophic cardiomyopathy without obstruction have been demonstrated by Olsen (1971), Davies et al (1974) and Roberts & Ferrans (1975) and these have generally shown varying degrees of concentric hypertrophy. They are often without any of the diagnostically helpful information mentioned above.

Coronary arteries are normal in the condition, although coexistence of coronary artery disease and hypertrophic cardiomyopathy has been reported by Gulotta et al (1972) and Moïse et al (1981).

Asymmetrical apical hypertrophy, in which giant T wave inversion is characteristic, has been studied using echocardiography and ultrasonic techniques by Sakamoto et al (1976). The giant forms were not observed in hypertrophic cardiomyopathy and a possible connection between the two conditions was not established.

2.5.2 Light Microscopy Appearances

The muscle most frequently involved is the deep bulbospiral muscle with thinning or absence of the overlying superficial fasciculi (Olsen 1971). See Fig. 2.

Severe hypertrophy of the muscle fibres is often present with mean diameters noted as 25-35μm (norm = 12μm) (Pearse 1964, Van Noorden et al 1971). Individual fibres measuring up to 100μm have been observed (Olsen 1973a). The abnormal fibres are typically interrupted by fibrous tissue. Serial sections of these areas have shown that fibrous tissue arises within the myocardial fibres and spreads towards the surface, replacing a variable amount of muscle tissue. This results in the observed shortening of the fibre and its termination in collagen tissue (Olsen 1971). The nuclei are large and "bizarre shaped" and frequently surrounded by a clear zone, the perinuclear halo.

Disarray of the myocardial fibres was noted by Teare (1958) and this finding has been confirmed by many workers, including Goodwin et al (1960), Menges et al (1961), Braunwald et al (1964), Olsen (1972, 1973b, 1978b, 1979), Roberts (1973), Davies et al (1974), and Maron & Roberts (1979), (see Fig. 3). It is the most characteristic, histological feature of hypertrophic cardiomyopathy but, like asymmetric septal hypertrophy, cannot be considered

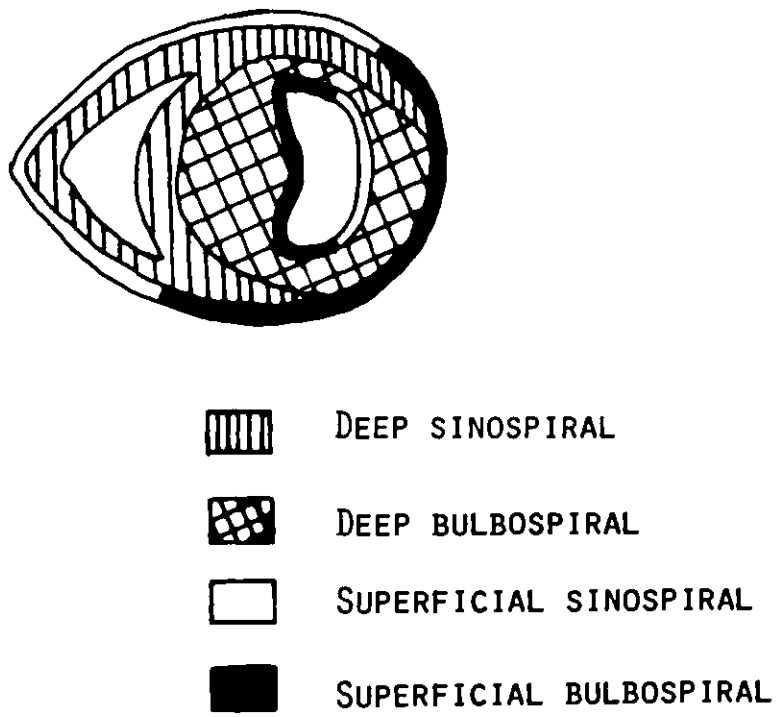


Fig. 2. Distribution of muscle bundles in a cross section of a human heart with extreme hypertrophy of the interventricular septum. (After Olsen 1971, based on Wartman and Saunders 1950).



Fig. 3. Photomicrograph from a clinical case of hypertrophic cardiomyopathy. Short, grossly hypertrophied myofibres are in total disarray.
Haematoxylin and eosin. x 500

pathognomic of the condition (see 2.5.4). An additional feature may be the presence of whorls, in which muscle fibres are oriented in a concentric ring-like arrangement (Van Noorden et al 1971). In cases with obstruction these changes are generally confined to the thickened septum but in cases without obstruction, foci of disarray are irregularly distributed throughout the ventricular walls (Olsen 1971, Maron et al 1974). An exception to this was noted in two children with clinical evidence of obstruction but at post mortem the distribution of disarray was that normally associated with non obstruction (Edwards et al 1977). The appearance of the muscle is essentially the same whether from the apex or from the outflow tract (Cohen et al 1964, McCallister & Brown, 1967, Van Noorden et al 1971).

Other microscopic changes reported have been those of the conduction system, particularly the sino-atrial and atrio-ventricular nodes (Ferris 1973, James & Marshall 1975). These changes are mild and have been described either as atrophy or hypertrophy. Clefts or channels, sometimes lined by endothelial cells, have been observed not only in the myocardium (Teare 1958, Van Noorden et al 1971) but also in the conducting system (James & Marshall 1975). Their significance is not yet fully understood as they have not been found consistently and are not specific for hypertrophic cardiomyopathy (Lev et al 1966, James & Puech 1974). Ferris (1973), McReynolds & Roberts (1975) and James

& Marshall (1975) have observed medial thickening and cellular intimal proliferation of intramural arteries and arterioles. However the narrowing of the lumen is usually mild and rarely exceeds 50%.

Extensive histochemical analysis has been undertaken (Van Noorden et al 1971, Van Noorden and Pearse 1971) in which a large number of enzymes were investigated. The increase in glycogen levels, particularly in the perinuclear areas, was helpful in distinguishing hypertrophic cardiomyopathy (HOCM) from myocardial hypertrophy due to other causes. However, a great deal of overlap was observed between HOCM and non HOCM cases when assessing the degrees of mitochondriosis (by dehydrogenases) and increased lysosomal enzyme activity (by acid phosphatase and non specific esterases). The assessment was semiquantitative with points 0-3 awarded for each parameter, producing a "histochemical HOCM index".

2.5.3 Electron Microscope Appearance

Electron microscope investigations have been carried out by many workers including Pearse (1964), Van Noorden et al (1971), Olsen (1971, 1978), Sekiguchi (1974) and Ferrans et al (1972, 1973). A large number of abnormal features have been identified and the following description is adapted from Ferrans et al (1973).

They carried out extensive evaluation on left ventricular outflow tract tissue from 14 patients with hypertrophic obstructive cardiomyopathy and described 5 distinctive features. They listed a further 8 abnormalities which were considered non-specific features of general hypertrophy and cellular degeneration. Controversy still exists in the literature, however, as to whether any of the features are specific for hypertrophic cardiomyopathy or just reflect hypertrophy (see 2.5.4). The five distinctive features were:-

1) Abnormal arrangement and shape of muscle cells which were wide, and often shorter than normal. Many bundles of cells were severely disorganised with muscle cells running in several directions instead of parallel. Some cells were normally shaped and arranged, whereas others had irregular or stellate shapes. These had several branches extending in different directions, so forming intercellular junctions with several adjacent cells.

2) Abnormal arrangement of myofibrils which often coursed in several directions within one given cell. This disarray could be such that immediately adjacent myofibrils were orientated perpendicular to each other (for example see Fig. 4). The extent of the abnormality varied. In some cells the arrangement was normal whereas in others total disarray was present, especially in the branched, stellate-shaped cells.

3) Abnormal arrangement of myofilaments was observed in some areas of myofibrillar disarray. Here myofilaments originating from one Z band were inserted into the Z band of an adjacent myofibril forming a cross-woven arrangement. Occasional actual branching of myofibrils was also seen.

4) Abnormal intercellular junctions, which were unusually convoluted and more extensive than in normal cardiac muscle. There were frequent large areas of side to side apposition of muscle cells instead of, or as well as, end to end apposition.

5) Frequent Z band abnormalities which consisted of irregular widening of the Z band, spreading of Z band material towards the centre of the sarcomere, attachment of Z bands to the sarcolemma and T tubules, and the presence of increased amounts of Z band like material at points of attachment of myofibrils to intercellular junctions.

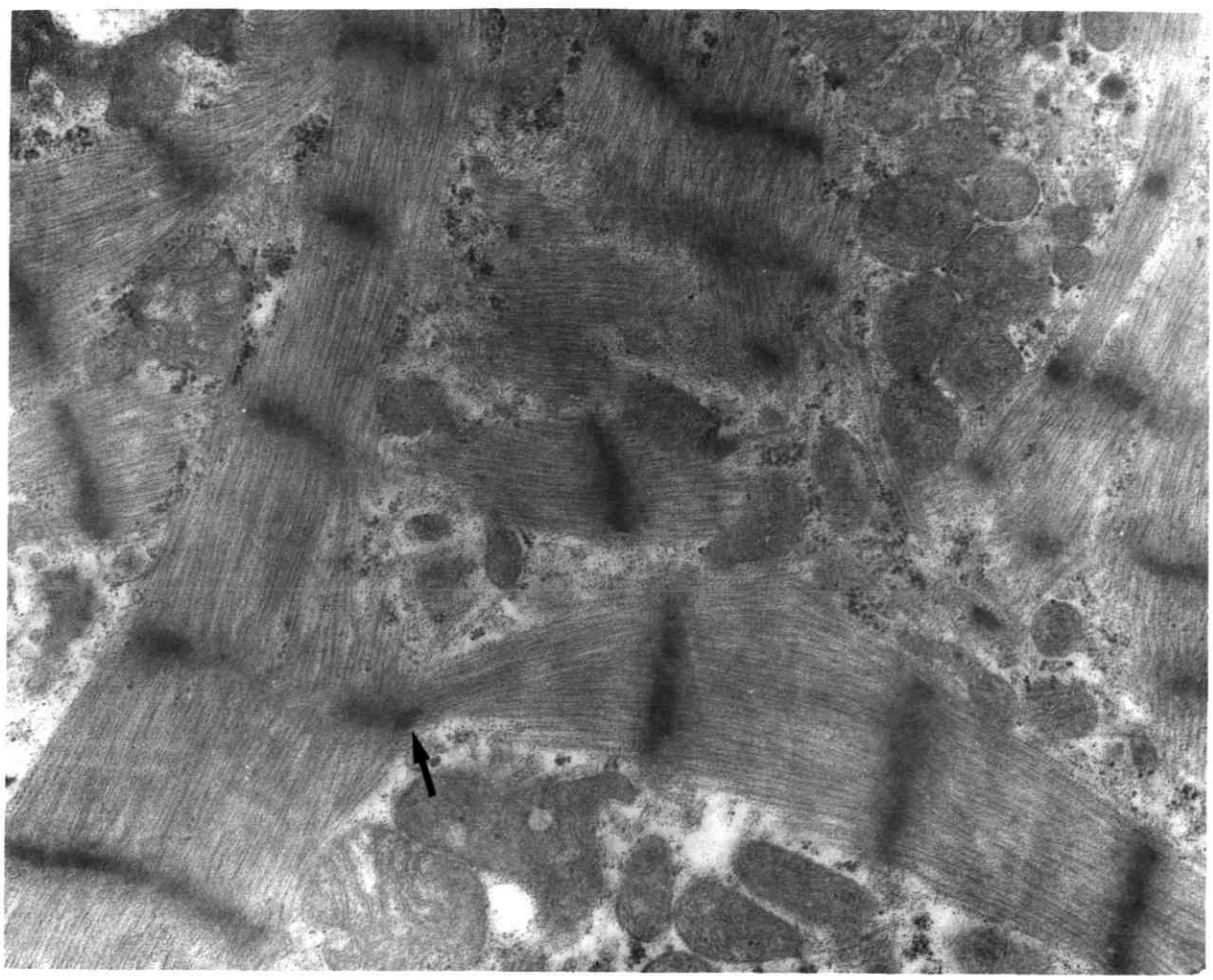


Fig. 4. Electronmicrograph from a clinical case of hypertrophic cardiomyopathy, showing abnormal arrangement of myofibrils. Two sarcomeres at right angles to each other share the same z band (⟶) x 26,000

The extent of the features varied within different areas of a given biopsy but all were present in each patient. The other features related to simple hypertrophy, and degeneration of the muscle cells, were:-

1) Increase in size of nuclei and Golgi complexes.
2) Marked irregularity in the contour of the nuclear membranes (nuclear crenation).
3) Increased numbers of mitochondria and glycogen particles, particularly at the nuclear poles. As the nuclear size was increased the myofibrillar free perinuclear areas were much larger than normal (the 'perinuclear halo') and filled with large masses of mitochondria (mitochondriosis) and glycogen granules.
4) Alterations in the mitochondria, consisting of swelling, disruption of cristae and formation of intramitochondrial concentric lamellae (myelin figures).
5) Increased numbers of free and membrane bound ribosomes, lipofusion granules and lysosomes.
6) Dilatation of T tubules was present in 9 out of 14 patients, the others being normal. This dilation was frequently accompanied by developing collagen in their lumina, which appears as coarse fibrils instead of fine filamentous lining. These fibrils were also found in the vicinity of the sarcolemma at the cell surfaces and in adjacent areas of interstitium. They characterise interstitial fibrosis.

7) The structure of the sarcoplasmic reticulum was normal in most areas, occasional dilatation being seen.
8) Lipid droplets were rarely observed in the muscle cells.

2.5.4 Discussion

The preceeding descriptions of hypertrophic cardiomyopathy at the various levels of investigation have been straightforward accounts of features present. However, certain of the abnormalities have also been found in other, unrelated conditions, and indeed, in some normal individuals. The following is a brief review of these findings and some comments as to whether an accurate diagnosis of the condition can be made.

A wide spectrum of hypertrophic cardiomyopathy exists, with at one end typical asymmetric hypertrophy of the septum with abnormal fibres aggregated in one area, while at the other end no macroscopic changes are evident but foci of similar abnormal fibres are scattered throughout the myocardium (Olsen, 1971). Obstruction is no longer regarded as an essential feature of the condition and is incidental. It is the powerful contraction of the greatly hypertrophied left ventricle, discharging virtually all of its contents in the first half of systole, with its narrowed and distorted cavity, that creates pressure gradients within and not obstruction as was originally suggested (Criley et al,

1965, Murgo et al 1980). Clinical diagnosis may be difficult in the absence of an outflow tract gradient, as there are usually no murmurs and the condition may mimic idiopathic pulmonary hypertension (Goodwin, 1975). Gradients are most marked in patients with asymmetric septal hypertrophy but they have been recorded in cases without this feature (Criley et al, 1965, Raizner et al, 1977).

The greater problem with asymmetric septal hypertrophy (ASH) is not that in some cases of hypertrophic cardiomyopathy it is not present, but that it is present in some other conditions. Echocardiography has been the major method of determining septal to free wall ratio during life and Henry et al (1973a) found a ratio of 1.03 in normal individuals, in patients with fixed left ventricular obstruction, and in patients suffering from miscellaneous cardiovascular diseases. By contrast the ratio in patients with hypertrophic cardiomyopathy exceeded 1.3 in every case and so ASH was considered pathognomic of hypertrophic cardiomyopathy. Since that report, however, ASH has been found in other situations, for example in normal children (Larter et al 1976), in athletes (Roeske et al 1976, Menapace et al 1977), in normal ageing people (Gerstenblith et al 1977) and in patients with congenital and acquired heart disease (Maron et al 1975, 1978d, 1979, Abbasi et al 1976, Raizner et al 1977, Henning et al 1978). More significant than these findings, though, was the septal thickening found in developing hearts (Bulkley et al 1977a, Maron et al 1978a). Nearly all embryos and young foetuses

were found to have disproportionate septal thickening and, although it disappears with age, it was still observed in 25% of live newborn infants without any congenital heart disease, decreasing to 12% by 2 weeks.

Fig. 5 is adapted from Maron & Epstein (1980) and shows the occurrence of asymmetric septal hypertrophy and septal disorganisation (to be discussed). All figures are approximate.

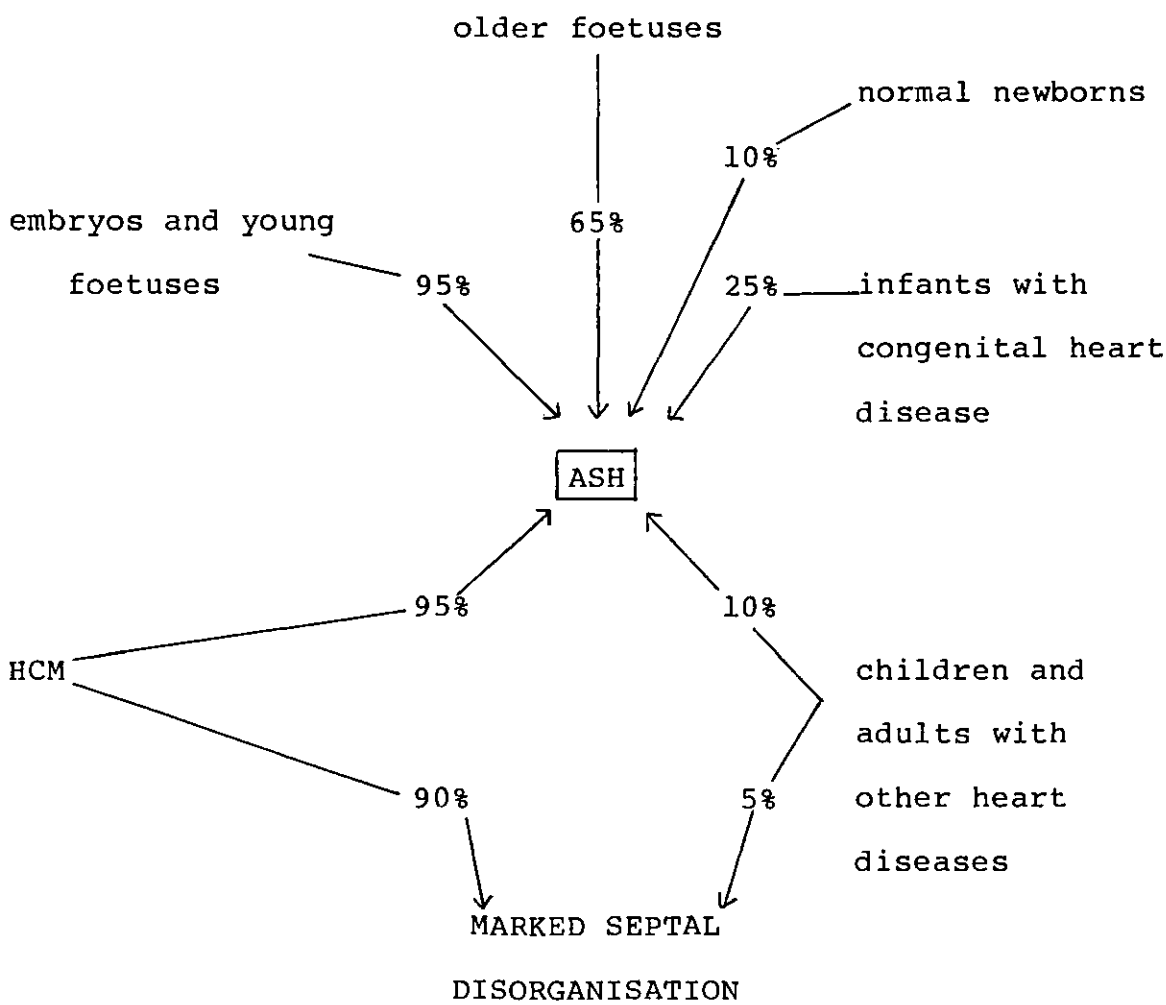


Fig. 5. HCM = hypertrophic cardiomyopathy
ASH = asymmetric septal hypertrophy

The disproportionate septal thickening in infants with congenital heart disease is associated with right ventricular abnormalities and only rarely accompanies abnormalities of left heart development (Bulkley et al 1977a). It usually disappears by age 2, primarily because continued thickening of the posterior left ventricular wall exceeds that of the ventricular septum (Larter et al 1976).

Some investigators have suggested that a septal to free wall ratio of 1.5 or 1.6 instead of 1.3 would be more specific in order to diagnose hypertrophic cardiomyopathy (Abbasi et al 1973; Criley, et al 1975) but this increase in specificity would be at the expense of decreasing sensitivity (Maron & Epstein 1980). Approximately 15% of patients with hypertrophic cardiomyopathy have a ratio in the borderline area of 1.3 or 1.4 and 5% have a normal ratio of less than 1.3.

In arriving at their conclusions Bulkley et al (1977a) suggested that significant asymmetric hypertrophy in the adult heart and in the 'proper clinical setting' can be a valuable diagnostic clue to the presence of hypertrophic cardiomyopathy. Olsen (1980) concluded that minor degrees of asymmetric septal thickening are non specific, and severe degrees in combination with right ventricular congenital anomalies, are also unreliable to associate with hypertrophic cardiomyopathy. Absolute asymmetric septal

hypertrophy, even when associated with left side cardiac anomalies, is more suggestive of cardiomyopathy but in any event caution must be exercised and confirmatory histologic evidence should always be sought.

Myocardial fibre disarray is another hallmark of hypertrophic cardiomyopathy which was originally considered pathognomic of the condition (Ferrans et al 1972, Maron et al 1974). Since then, however, this concept has been questioned, as disarray has also been found in congenital heart disease, including semilunar valve atresia with intact ventricular septae and tetralogy of Fallot (Bulkley et al 1977a). It has also been found in some acquired heart conditions such as systemic hypertension, cor pulmonale, and coronary heart disease (Van der Bel-Kahn 1977, Maron et al 1978b). Small foci of disarray have been found at the anterior and posterior junctions of the interventricular septum with the ventricular free walls in normal hearts (Bulkley et al 1977a), normal infants (Maron et al 1978a), and in infants who have died suddenly without evidence of structural cardiac disease (Maron & Fisher 1977). These were very small areas and no abnormal amounts of disarray were found in any developing foetuses or infants, despite the frequent presence of disproportionate septal thickening (Bulkley et al 1977a). Small foci were also found at the borders of myocardial infarcts in humans (Bulkley et al 1977a) and in experimental animals (Dušek et al 1971).

It therefore seems likely that absolute presence of disarray is not enough to diagnose hypertrophic cardiomyopathy, but extensive presence may be more suggestive of the condition. Quantitative analysis to determine areas of myocardium occupied by fibres in disarray has therefore been carried out. Van der Bel-Kahn (1977) studied patients with congenital heart disease, systemic hypertension, coronary heart disease and patients with cor pulmonale and found fibre disarray present to a mild degree in all cases. The extent of the disarray varied in each individual but usually involved less than 11% of the myocardium and never more than 20%. The one case of hypertrophic cardiomyopathy showed more than 50% of the left ventricular myocardium, both septal and free wall, to be involved.

A more extensive quantitative analysis of fibre disorganisation was carried out on 54 patients with hypertrophic cardiomyopathy and 144 control patients (patients with other heart diseases or with normal hearts) by Maron and Roberts (1979). Marked septal disorganisation was defined as involving 5% or more of the relevant areas of tissue section and this was found to be a highly sensitive (89% of cases of hypertrophic cardiomyopathy showed at least this level of disarray) and specific (only 7% of control cases showed more than this) histologic marker for the condition. Septal disorganisation was actually present in 26% of the control cases but, other than the 7% above, always involved less than 5% of the myocardium. In the cases of hypertrophic cardiomyopathy, often much more than

5% was involved. In fifty per cent of patients over 25% of the tissue was involved, and in twenty-five per cent over 50% was involved. They also found that marked septal disorganization linked patients within the broad clinical and pathological spectrum of the disease; that is, disorganization was present, and did not differ appreciably in extent in those patients with or without symptoms before death, with asymmetric or symmetric ventricular wall thickening, or with or without obstruction to left ventricular outflow. In addition the presence or extent of septal disorganization was not related to absolute heart weight and hence could not be considered simply a manifestation of marked cardiac hypertrophy (Maron & Epstein, 1980).

In a less extensive study St. John Sutton et al (1980) agreed that disarray was qualitatively non specific, but quantitatively hypertrophic cardiomyopathy could be distinguished from normals and patients with congestive cardiomyopathy. There was no significant variability from sampling sites, whether from the left ventricular free wall or septum. They suggested that fibre disarray probably represents an exaggeration of a non-specific common pathway for many diverse pathophysiological processes.

By contrast Becker and Caruso (1982) suggest that the significance of disarray in hypertrophic cardiomyopathy in the clinical setting almost vanishes after concluding that disarray is a natural phenomenum in normal hearts. In many

such hearts examined they found that the plane of section of a piece of tissue affected the histology in the majority of cases. A change in the orientation of the tissue could turn "normality" into "disarray".

Van Noorden et al (1971) had previously used other features as well as disarray when attempting to diagnose hypertrophic cardiomyopathy and the histologic HOCM index was devised. This is a semi-quantitative analysis awarding points 0 - 3 for the following changes:-

1) Short runs of fibres interrupted by connective tissue.
2) Large bizarre nuclei.
3) Fibrosis.
4) Degenerating muscle with disappearing myofibrils and perinuclear spaces.
5) Disorganized "whorling" muscle.

In a longitudinal section the maximum points that could be obtained was 15 and this was expressed as 100%. Tissue was obtained at surgery and was compared with tissue from similar sites, also obtained at surgery, from cases of tetralogy of Fallot, subaortic stenosis, ventricular septal defects, mitral valve disease and congestive cardiomyopathy. In just over half the cases of hypertrophic cardiomyopathy an index of over 65% was obtained, permitting a diagnosis of the condition. However in the remaining cases overlaps between those clinically diagnosed as suffering from hypertrophic cardiomyopathy and tissue for comparison

existed. They concluded that although a diagnosis could be made on histologic criteria alone in several cases, the biopsy could only provide circumstantial confirmation of the clinical opinion. They found no justification for taking needle biopsy as a diagnostic procedure in a case of hypertrophic cardiomyopathy.

It seems, therefore, that marked disarray of the myocardium is present in cases of hypertrophic cardiomyopathy and this is easily visible in the large areas which can be viewed from the samples taken at necropsy (Maron & Roberts 1979). However these areas of disarray could easily be missed when diagnosis is being made from a small biopsy sample and so, as Olsen (1980a) concluded, the other accompanying changes, used by Van Noorden et al (1971), are essential for verification. The presence of glycogen pools, observed by histochemical analysis, increases the diagnostic reliability.

As with the macroscopic and light microscopic findings, there is also significant overlap between features of hypertrophic cardiomyopathy and control cases, of other heart diseases, at the electronmicroscopic level. This was concluded by Van Noorden et al (1971) when analysing seven electronmicroscopic features and has been confirmed by Snijder et al (1971), Olsen (1975a), Sekiguchi et al (1972/73), and Dingemans & Becker (1977) when comparing hypertrophic cardiomyopathy with other conditions resulting in hypertrophy.

Disarray of myofibrils and myofilaments are the most characteristic observations and, as with fibre disarray, were thought to be unique to hypertrophic cardiomyopathy, (Ferrans et al 1972, Roberts & Ferrans 1974, 1975). Other reports from the same institute found the abnormalities not to be specific and said they were commonly found in other forms of myocardial hypertrophy (Maron et al 1973, 1974). Most papers, however, from the groups agree that a pronounced quantitative difference exists. In patients with hypertrophic cardiomyopathy the abnormalities were extensive and widespread while myocardium from cases of congenital heart disease with right ventricular outflow tract obstruction showed only minimal and highly focal disorganisation (Jones et al 1975). A separate study, with only 2 patients, however, found more intracellular disarray in the case of cardiac hypertrophy with rheumatic valve disease than in the case with hypertrophic cardiomyopathy (Dingemans & Becker, 1977). Some minor disorganisation of myofibrils in congestive cardiomyopathy was found by Ferrans et al (1973) and Sekiguchi et al (1972/73) but a year later Sekiguchi (1974) did not observe any abnormal architecture in the myocardium from cases of hypertrophic cardiomyopathy. He concluded that this could be due to a different site of sampling, a conclusion also reached by Olsen (1975a). In any section observed under the electron microscope the area viewed must necessarily be very small and, as many cells in cases of hypertrophic cardiomyopathy exhibit normal myofibrillar arrangement, areas of disarray could easily be missed. Ferrans et al (1972) found that the myocardial

cells exhibiting the most intracellular disarray were found in areas where the fibres themselves were also in disarray. This finding was not, however, endorsed by Dingemans & Becker (1977).

In a semiquantitative study of biopsies from cases of ordinary hypertrophy, congestive cardiomyopathy, and hypertrophic cardiomyopathy Baandrup et al (1981) found crossover of sarcomeres more frequent in cases where hypertrophic cardiomyopathy was diagnosed.

It appears that intracellular disarray is a normal feature of developing or immature cells. A disorderly arrangement of myofibrils is characteristic of chick embryonic cardiac muscle, in which myofibrils in early stages of formation have myofilaments that radiate in several directions from a single Z band. Some myofibrils are seen stretching the whole length of the cells, sometimes in different directions, but most are only a few sarcomeres in length and seem unattached to the sarcolemma (Manasek 1970). Disorientation of myofibrils has been observed in regenerating cardiac muscle cells at the periphery of areas of infarction (Wilcken et al 1970, Dušek et al 1971) and also in primative hearts such as those in crustaceans (Howse et al 1971). It has also been observed in salamander hearts grown in organ culture for three months or longer at which time the pronounced changes of dedifferentiation also developed (Millhouse et al 1971). These observations may be significant in the etiology and development of hypertrophic cardiomyopathy (see section 2.6).

In analysing reports on the electronmicroscrope findings Olsen (1980a) concluded that features of hypertrophic cardiomyopathy can be extensively mimicked by other cardiac conditions resulting in hypertrophy but if intra- and intercellular disarray is extensive then the changes are suggestive but not pathognomic of hypertrophic cardiomyopathy.

From this discussion it can be concluded that for each pathological change noted, either it does not apply for all cases of hypertrophic cardiomyopathy or other conditions may also exhibit it. Therefore when making a diagnosis, no single change can be regarded as pathognomic but the spectrum of changes at all levels of investigation must be considered before arriving at a conclusion. It would seem that, if gross asymmetric hypertrophy, and extensive cellular and myofibrillar disarray were present, then hypertrophic cardiomyopathy would be the most likely diagnosis. If a small biopsy sample was the only tissue available then looking for fibre disarray under the light microscope would be the first choice of investigation (Van Noorden et al 1971).

2.6 Aetiological suggestions of hypertrophic cardiomyopathy

By definition the aetiology of hypertrophic cardiomyopathy is unknown. There are many theories as to possible causes of the disease, the only known fact being that there is a genetic basis behind some patients. A high familial occurrence and autosomal dominant traits with almost complete penetrance, have been reported (Brent et al 1960, Henry et al 1973b). Braunwald et al (1964) and Emanuel (1971) found that familial incidence accounted for approximately 30% of patients. Clark et al (1973), however, using echocardiography, detected familial occurrence of asymmetric septal hypertrophy in 28 of 30 families. Matsumori et al 1979 investigated the HL-A antigens, the human major histocompatibility complex, in patients with hypertrophic cardiomyopathy and found they could be involved in patients with familial occurrence but not in those without.

Whether or not the condition is genetic or acquired, the underlying biochemical or mechanical actions causing it remain unknown. The following are some suggestions of aetiology already made.

Earlier this century Schmincke (1907) had proposed that an embryonic growth disturbance could be involved in the production of the disease. It is highly likely, in the light of more recent work, that this may be the case, although no mechanism of action was proposed.

Teare (1958) in his original description of the condition, and later Cleland (1963) regarded the septal hypertrophy as a muscle hamartoma. Lannigen (1965) considered the case he described as an active degenerative process superimposed on the hypertrophied muscle fibres. He proposed that the abnormality was a primary metabolic disorder of the cardiac muscle fibres. Meerschwam and Hootsmans (1971) suggested that in an appreciable number of patients with HOCM, a generalised myopathy accompanying skeletal muscle changes with early and serious cardiac manifestations should be considered as a possible aetiological factor. Later Darsee et al (1979) found no primary skeletal muscle involvement, but neuropathic features were present.

Systemic hypertension preceding the development of obstructive cardiomyopathy has been suggested (Alday et al 1972) but the association with hypertension is rare. James (1977) included hypertrophic cardiomyopathy among other conditions which could involve small vessel disease in its genesis. This, however, has been questioned as small vessel disease was only found in 8% of patients with hypertophic cardiomyopathy (Olsen - personal observation).

Bulkley et al (1977) have suggested that the myocardial fibre disarray may result from altered wall stresses related to isometric systolic contraction. In the normally functioning heart ventricular contraction has two phases.

The first occupies approximately 10% of systole and is a period of isometric or isovolumic contraction preceding the opening of the aortic valve. After this is the period of ventricular ejection as the fibres shorten and the ventricular volume decreases. This phase occupies approximately 80 to 90% of systole and ends with aortic valve closure and the beginning of isovolumic relaxation. In patients with hypertrophic cardiomyopathy a third phase is added to ventricular systole, one of middle and late systolic isometric contraction which may occupy over half of systole.

Here the ventricle is nearly empty and high pressures are generated inside, thus altering the contraction-induced linear stresses which are probably necessary for proper muscle fibre allignment. As evidence the authors demonstrated that myocardial fibre disarray may also be seen in infants with aortic or pulmonary atresia with intact ventricular septa, both conditions in which the left or right ventricle, respectively, is believed to undergo isometric contraction. They believed the isometric contraction resulted from cavity obliteration of the left ventricle. This however does not occur in all cases of hypertrophic cardiomyopathy and is not an essential feature for its diagnosis (Adelman et al 1969). The same authors (Hutchins and Bulkley, 1978) describe a second type of isometric contraction, which could cause septal disarray and

hypertrophy, resulting from a septum which is catenoid in shape (convex to the left from apex to base, concave to the left in the horizontal plane) with net zero curvature at the left septal surface. They suggested that such a shape would lead to little or no myocardial shortening, i.e. isometric contraction. Wigle & Silver (1978), however, pointed out that they do not propose how the septum becomes catenoid in the first place and that the normal septum may be sigmoid shaped in infants (Wolska & Kobuszewska-Faryna 1974) and adults (Goor et al 1969). A sigmoid shaped septum is convex to the left from apex to base, not dissimilar to the catenoid shape. Wigle (1977) has proposed another possible cause for the isometric contraction from studies of patients with outflow tract gradients in excess of 100 mmHg, in whom the ejection fraction varied between 50-70% with no cavity obliteration. During the last 30% of systole the ventricular volume was unchanged, in spite of up to 40ml of blood remaining in the ventricle. Thus in these hearts studied, isometric contraction occurred at end systole in the absence of cavity obliteration.

An argument against isometric contraction causing fibre disarray comes from the quantitative analysis by Maron and Roberts (1979). They found that the infants with aortic or pulmonary atresia (evidence used by Bulkley et al 1977) only had small foci of disarray, similar in extent to that seen in normal foetuses and infants and infants with other congenital heart malformations.

Perloff (1981) believed that the asymmetric septal hypertrophy found in hypertrophic cardiomyopathy is a non regression of that found in developing foetuses. He proposed the opposite from the above theories, in that isometric contraction is inherent in malaligned cells and that this stimulates hypertrophy, interferes with regression and promotes progression of disproportionate septal thickness. Whether malaligned cells cause isometric contraction or isometric contraction causes malalignment of cells is still debatable, but what primary action causes either of these?

In 1964 Pearse concluded that sympathetic innervation and noradrenaline content were increased in the fresh septal myocardium of patients with hypertrophic cardiomyopathy. Doubt, however, was cast on these findings by Van Noorden et al (1971) who found that the autofluorescence emitted by fibrous connective tissue was very similar in colour and brightness to that emitted by noradrenaline. Cases of hypertrophic cardiomyopathy had tended to fluoresce more strongly than the controls as they contained more connective tissue.

The possible relationships with catecholamines was more recently raised by Goodwin (1974). He stated: "If the

catecholamine hypothesis has any validity, it is likely that the relationship is a complex one that exerts its effect on the growing myocardium, rather than a simple matter of excess amounts of catecholamine acting on the heart over a prolonged period later in life".

When reviewing cardiac development and the role of circulating and intracardiac catecholamines, Perloff (1981) proposed the following hypothesis: "A genetic fault results in disturbance of the delicate interaction between immature supersensitive myocardial adrenergic receptor sites and extracardiac catecholamines during cardiac embryogenesis and early foetal development. The interactions of excessive receptor site sensitivity, excessive circulating neurotransmitter, or abnormalities in number or locations of receptor sites, causes heightened adrenergic stimulation of the early developing myocardium, resulting in abnormal cellular disarray. Antagonistic (isometric) contraction of the malaligned cells interferes with the regression of foetal and embryonic disproportionate septal thickness, since isometric contraction is a powerful stimulus to hypertrophy". This theory, if it is valid, may enhance understanding of the pathenogenesis of the disease, i.e. the extensive cellular disarray and failure of regression of the disproportionate septal thickness, but does not aid the

understanding of the progression of the disease. One important unanswered question is, are patients who do not have evidence of hypertrophic cardiomyopathy until late in life, actually born with the pathological features of the condition?

It is possible that for some patients an endocrine factor could be involved in the pathogenesis and, perhaps, progression of the condition. There is a recognised association of cardiac hypertrophy with hyperthyroidism (Sandler and Wilson 1959 a, b, Cohen et al 1966) and some patients have been reported with hyperthyroidism and hypertrophic cardiomyopathy (Symons et al 1974, Bell et al 1978). The frequency of patients with both conditions may be far more than realised as the level of hyperthyroidism was relatively low, such that clinically overt hyperthyroidism was not present. The significance of this and experimental work carried out will be dealt with in chapter 5.

2.7 Treatment of hypertrophic cardiomyopathy

Although this thesis concerns mainly pathological aspects of the condition, many of the drugs used in the experiments are used clinically for treatment and so it is pertinent here to mention some forms of treatment used.

2.7.1 Drug therapy is the most common form of treatment, whereby alleviation of left ventricular inflow restriction (Cohen et al 1964) and of the slow filling of the left ventricle (Stewart et al 1968) are the important aims, rather than relief of obstruction.

Early interest in the use of beta adrenergic blocking agents for therapy of hypertrophic cardiomyopathy, came from the suggestion of Pearse et al (1964) that there was excess catecholamine activity in the myocardium of patients with the condition. The previous section has explained how this was later found to be unsubstantiated (Van Noorden et al 1971) although interest in catecholamines has remained alive (Goodwin 1974).

There does appear to be an abnormal response to sympathetic stimulation in hypertrophic cardiomyopathy since there is a rise in left ventricular end diastolic pressure after isoprenaline. This is in contrast with the fall or lack of change seen in both the normal and other disorders of the left ventricle (Webb-Peploe 1971).

Beta blockade by propranolol and practolol reduces myocardial oxygen consumption during exercise by reducing heart rate and contractile state by blocking catecholamine stimulation. Practolol is a cardioselective beta blocking agent and appears to be more effective than propranolol in reducing left ventricular end diastolic pressure but less effective in reducing myocardial oxygen consumption (Kristinsson 1971). Both drugs relieve symptoms, especially angina, but the primary cardiomyopathic process is not altered. The long term effect of beta blockade, at dose levels causing a complete block, is to improve symptoms by improving parameters of left ventricular filling (Roudaut et al 1980) but left ventricular distensibility is not improved with acute administration of propranolol (Speiser and Krayenbuehl 1981).

It was originally hoped that beta blockade would also be antiarrhythmic in action but this only appears to be the case when used in combination with other antiarrhythmic drugs (Canedo et al 1981). Despite the administration of these drugs some patients return to their severely symptomatic states and some die (Swan et al 1971, Adelman et al 1972).

A recent alternative to beta blocking therapy has been the use of calcium antagonist drugs. The heart in hypertrophic cardiomyopathy is in a hypercontractile state and the underlying factor responsible for this is still uncertain. However, it is known that increases in myocardial cell calcium content increase myocardial contractility (Nayler 1967).

Studies on the hereditory cardiomyopathy of Syrian hamsters (Bajusz and Lossnitzer 1968, Abelman et al 1971, Lossnitzer et al 1975) have shown that myocardial calcium uptake and content are increased and that the metabolic and anatomic abnormalities can be prevented by the administration of verapamil. This drug blocks inward calcium transport across cell membranes (Kohlhardt et al 1972) through the so called slow channel (Cranefield et al 1974). Human hypertrophic cardiomyopathy and the cardiomyopathy of the Syrian hamster are not the same condition but it was thought that the myocardial abnormalities leading to symptoms and death may be from a similar mechanism.

Kaltenbach et al (1979) treated 22 patients for an average of 15 months with verapamil. Symptoms disappeared or considerably lessened in two thirds of the patients who had been symptomatic on beta blockers, although it took several weeks before subjective improvement was noticed.

Electrocardiograms were improved and they also reported that heart size was significantly decreased. If this effect is true, then verapamil will be an improvement over beta blockade, but it was only observed when chest x-rays were taken in the supine position. Conventional x-rays showed no change. A reduction in coronary artery diameter of about 16% was also noted and this was attributed to be a consequence of a decrease in ventricular muscle mass.

Rosing et al (1979a) studied the effects of intravenous infusion of verapamil into patients with hypertrophic cardiomyopathy and showed that the basal and provoked left ventricular outflow tract gradients were diminished in a dose related manner. Cardiac output was maintained, indicating that the fall in gradient resulted from an actual increase in the effective orifice size of the left ventricular outflow tract. It was pointed out, however, that care would have to be taken as some patients with obstruction may respond by a reduction in systemic resistance, and thereby arterial pressure, which may increase left ventricular outflow tract obstruction (Wigle et al 1965). Oral verapamil also improved exercise capacity and symptomatic status although there was no correlation between this and reduction in left ventricular outflow tract obstruction with intravenous verapamil (Rosing et al 1979b).

Verapamil has been shown to decrease myocardial contractility in vitro at high concentrations (Nayler and Szeto 1972) and experimentally in vivo (Singh et al 1978, Newman et al 1977). Other studies, however, have not shown this (Atterhög and Ekelund 1975, Seabra-Gomes et al 1976, Smith et al 1976). If the contractile state is decreased it is not known whether this is due to decreasing normal calcium transport or to correcting a basic abnormality involving excessive uptake of calcium by the myocardium. Any agent that produces a decrease in contractility of the heart should, nonspecifically, diminish the degree of left ventricular outflow tract obstruction in patients with hypertrophic cardiomyopathy (Harrison et al 1964). This is believed to be the major mechanism by which propranolol reduces the outflow gradient in some patients.

More recently it has been suggested that the antiarrhythmic agent amioderone is superior to verapamil in the treatment of hypertrophic cardiomyopathy (McKenna et al 1981). These authors found that amioderone abolished ventricular tachycardia in 10 out of 13 patients and significantly reduced the number of extra systoles. Goodwin (1982) suggested that amioderone improved severe cardiac pain, possibly because of its beta blocking action.

2.7.2 Surgery

At one time surgery was advocated because of the obstructive element, but subsequent clinical experience showed that this was coincidental and that the basic haemodynamic abnormality was that of failure of diastolic compliance. Many authorities have reduced the number of patients subjected to surgery but, if obstruction is severe, then it is considered. As left ventricular outflow obstruction was the first abnormality to be recognised, it was early alieviated by surgical incision of the hypertrophied septal muscle (Bentall et al 1965). Symptoms are improved by this treatment, mainly through the reduction in outflow gradient. Morrow et al (1971) performed left ventriculomyotomy and myectomy on 43 severely symptomatic patients. Four of these died at or shortly after the operation (9% morbidity) and two later died of unrelated causes. An average follow up of 51 months showed 22 patients with no symptoms, the others only mildly symptomatic. Gradients were abolished, the raised left ventricular end diastolic pressure lowered, and mitral regurgitation abolished in most patients. Later studies have shown similar results but still with a 5-10% operative risk (Morrow et al 1975, Maron et al 1978b).

Although these operations have been successful in the majority of patients, it has been noted that spontaneous loss of outflow tract obstruction during the natural cause of the disorder has been associated with distinct deterioration (Oakley 1971).

3. Cardiac hypertrophy in animals

3.1 Hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy can occur naturally in animals and there have been reports on both cats (Tilley et al 1977, Liu et al 1981) and dogs (Liu et al 1979). In the latter report on ten dogs showing characteristic features of hypertrophic cardiomyopathy, only two exhibited extensive cellular disarray.

In the cardiomyopathic Syrian Golden hamster, which was previously mentioned in context with calcium antagonist drugs, the condition shows autosomal recessive transmission and also features skeletal muscle lesions. The animals are unhealthy and the cardiac hypertrophy is associated with failure rather than increased contractility (Lossnitzer and Bajuz 1973). It is therefore an unsuitable model for the study of hypertrophic cardiomyopathy.

3.2 Experimental production of cardiac hypertrophy

Cardiac hypertrophy has been produced using a variety of methods in several species, mainly dogs, rabbits, rats and mice. As the changes produced in the rats in this thesis include cardiac hypertrophy, it is relevant to briefly review other experimental models. None, however, have attempted to show, nor shown, changes mimicking those seen in hypertrophic cardiomyopathy in man.

Several methods involve stenosing part of the aorta in order to increase the afterload on the left ventricle, which compensates by hypertrophy.

Severe pressure overload was produced in rats by stenosing the abdominal aorta (Dowell et al 1976) and this produced an increase in left ventricular muscle mass of 75%. This compared with only a 10% increase produced by moderate pressure overload caused by desoxycorticosterone acetate and salt loading. Stenosing the abdominal aorta in rats (Novi 1968) and the ascending aorta (Wollenberger and Schulze 1961, Meerson et al 1964) in dogs and rabbits respectively have also been carried out. Malik et al (1974) considered banding of the suprarenal abdominal aorta a better experimental model than stenosing part of the thoracic aorta as less surgery is involved, less recovery time and less variability in the production of hypertrophy. They produced a 26% increase in left ventricular weight to body weight ratio of rats in 6 days using this method, which remained constant. Right ventricular hypertrophy has been produced by banding of the pulmonary artery in dogs (Laks et al 1974).

Prolonged physical exercise in rats such as swimming (Arcos et al 1968, Bozner and Meessen 1969) produced hypertrophy but the treadmill exercise used by Dowell et al (1976) over a period of 80-90 days, did not increase absolute left ventricular mass over the controls. A relative left ventricular enlargement, however, was established as the left ventricular mass increased at approximately the normal rate while simultaneously reducing the overall growth rate of the animal.

Lack of oxygen supply by simulating high altitude (Turek et al 1972) or the production of anaemia (Neffgen and Korecky 1972) also produced hypertrophy in rats. Anaemia was induced by a low iron diet and it was found that if it was induced during early post weaning life then the cardiac enlargement was by hyperplasia of the cardiac muscle and non muscle cells. If it was induced in adult rats then it was by hypertrophy of the muscle cells and hyperplasia of the non muscle cells.

The introduction of an aorto-caval fistula (Taylor et al 1974) and the production of renal hypertension (McCallister and Brown 1965) have also been used.

Hormonal production of hypertrophy has involved the use of thyroid hormones, e.g. thyroxine (McCallister and Page 1973) and analogues e.g. triac (Symons et al 1975, Olsen et al 1977) and catecholamines e.g. noradrenaline (Laks and Morady 1976) and isoprenaline (Taylor et al 1977). These studies will be dealt with in the following chapters.

3.3 Microscopic changes

In the electron microscopic examination of the hypertrophied heart, much interest has centred on mitochondrial appearance. Olsen (1980b) has reviewed these changes. Soon after the initial experimental procedures swelling and alteration of the normal pattern of cristae are observed (cristolysis) which is subsequently followed by normal

appearances. Later the number of mitochondria is increased with much greater variation in size and shape as well as cristolysis. The increased mass of mitochondria takes place without qualitative or quantitative signs of insufficiency (Laguens et al 1966, Laguens and Gómez-Dumm 1967, Arcos et al 1968).

When quantitative morphological analysis has been carried out, differing results from using different methods and different animals have been found. For example in the dog (Wollenberger and Schulze 1961), a normal relationship of mitochondria/myofibrils (0.5) was still present after the acute phase of stenosing the ascending aorta. This decreased to 0.4 after 6 months and 0.28 after 11-29 months. On the other hand, in rabbits an increase in mitochondria was observed in the initial stage (Meerson et al 1964). Onishi and Zittel (1968) and Onishi et al (1969) observed that in progressive hypertrophy, after stenosing the ascending aorta of dogs, the proportion of mitochondria to myofilaments may decrease in waves, recovering to normal values between the waves.

By stenosing the aortic conus in rabbits and dogs, or the subdiaphragmatic part of the abdominal aorta in the rat, Meerson (1965, 1969) described 3 stages of hypertrophy.

1. The first brief period (5-10 days) is the damage stage of isometric hyperfunction. Aerobic metabolism was disturbed, with mobilisation of glycogen and a severe decrease in high energy phosphates. "Cloudy swelling"

and fatty degeneration of muscle fibres was observed with a rapid increase in heart weight of 50-80%.

2. The second stage of relatively stable hyperfunction lasts 4-10 months in rats and rabbits where the weight of the heart increased 190-200% of normal. The degenerative changes from the first stage disappeared but myocardial fibrosis occured.

3. The third stage is of gradual exhaustion and progressive cardiosclerosis. High energy phosphates and noradrenaline concentrations fell and dilatation became evident. Atrophy and vacuolar degeneration and breakdown of muscle fibres were noted. Foci of fibrous replacement and fatty degeneration were also observed.

By histochemical analysis of enzyme changes Van Noorden and Pearse (1971) suggested that the hearts they studied from cases of hypertrophic cardiomyopathy were from the second stage of relatively stable hyperfunction and hypertrophy. Similar enzyme changes from this stage of hypertrophy were observed in rats by Vihert and Pozdyunina (1969).

In these studies of compensatory hypertrophy due to overload, systolic tension is initially increased and an increased expenditure of high energy phosphate occurs. In hypertrophy due to damage such as occurs in cardiomyopathy, ischaemia or myocarditis, a decrease in systolic tension and resynthesis and high-energy-phosphate utilisation efficiency takes place (Meerson 1976).

4. The Thyroid

As triac, the acetic acid metabolite of triiodothyronine, has been used in the experiments described in this thesis, it is pertinent here to review briefly thyroid function per se and the effects on the heart.

The function of the thyroid gland is to synthesise, store and secrete thyroxine (T4) and triiodothyronine (T3). This is controlled by pituitary thyroid stimulating hormone (TSH, thyrotropin).

The majority of circulating hormone is T4 (normal range for humans 60-150 nM/l) with only 1-2% as T3 (normal range 1.0-2.6 nM/l). All but a tiny fraction of this is bound to specific plasma proteins with only 0.05% of T4 (20-50 pM/l) and 0.5% of T3 (6-11 pM/l) existing free. It is the free moiety which is capable of entering cells and affecting the metabolic processes.

The major binding proteins are thyroxine-binding globulin (TBG), thyroxine binding prealbumin (TBPA), and albumin which respectively carry 60, 30 and 10% approximately of total circulating T4. The role of the binding proteins appears to be to act as a buffer in the extrathyroidal metabolism of the thyroid hormones, modulating their transfer to intracellular sites and preventing their loss in urine. TBG behaves as a stable store while fluctuation in

the level of TBPA which can occur allows for variation in the concentration of free hormone and thus of supply to tissues commensurate with the need of the moment.

Thyroidal production of T3 only accounts for 20% of total T3, the majority being derived from peripheral deiodination of T4. Approximately one third of the T4 pool is converted to T3 each day (Braverman et al 1970, Pittman et al 1971, Surks et al 1973). The iodothyronine deiodinases are found in the kidney, liver, heart and fibroblasts.

On a molar basis T3 is 3-4 times as potent as T4 and it has been proposed that peripheral generation of T3 may play a central role in the mediation of the biological activity of thyroid hormone. From this some investigators have concluded that T4 does not have intrinsic hormonal activity and may be considered a prohormone (Oppenheimer et al 1972a, Ingbar and Braverman 1975). There is some evidence, however, that T4 can have its own direct action as, not uncommonly, serum T3 concentration can be normal in patients with proven hypothyroidism associated with low T4 and high TSH (Chopra et al 1973). Also some cretins from endemic areas of iodine deficiency, with unquestionable evidence of hypothyroidism, may present with normal or even high serum T3 but low serum T4 (Refetoff et al 1976).

The protein bound hormone is in reversible equilibrium with the minute amount of free hormone, which in turn is in equilibrium with the intracellular hormone pool. Because T4 has a higher affinity than T3 for the serum hormone binding

proteins and T3 has a slightly higher affinity than T4 for the intracellular binding proteins, 60% of the extrathyroid T4 pool is located in the intracellular compartment as compared with 90% of the T3 pool. These binding proteins are present in the cytoplasm of most tissues, some having a passive role similar to that of the serum binding proteins and others, such as mitochondrial proteins may be specific, acting as receptors (Sterling and Milch 1975). Other specific binding sites are ribosomes and the nuclei, 10% of intracellular T3 being bound to the latter. A saturable nuclear iodothyronine-binding acidic protein associated with chromatin has been extracted from a variety of tissues and has a capacity of 7000 to 10000 molecules of T3 per cell. This nuclear T3 binding protein has 4-10 times greater affinity for lT3 than lT4. However dT3 binds with near equal affinity as the naturally occuring l isomer and triiodothyroacetic acid (triac) has even greater affinity (Oppenheimer et al 1972a, b, Samuels and Tsai 1973, DeGroot et al 1974, DeGroot and Torresani 1975, Docter et al 1976). It is likely that interaction with these nuclear proteins promotes DNA transcription into messenger and ribosomal RNA's facilitating protein synthesis.

The main pathways for the breakdown of the thyroid hormones are by:-

a) deiodination, accompanied by degradation of the alanine side chain to pyruvic, lactic or acetic acid derivatives and bought about by mitochondrial enzymes in liver and kidney. The acetic acid derivative of T4 is tetraiodothyroacetic acid (tetrac) and that of T3 is triac, the latter being the major compound under study in this thesis.

b) conjugation of the phenolic hydroxyl group mainly in the liver but also in the kidney and leading to the formation of glucuronide or sulphate esters which are excreted largely in the bile. Here some unaltered hormone is also found.

c) excretion in urine either as intact hormone due to filtration of free plasma T4 and T3, or as metabolites.

5. Thyroid and the Heart

Historical review

The possible relationship between altered thyroid gland function and the heart was first noted in 1785 by Caleb Hillier Parry, his notes being published posthumously in 1825. He described 8 patients in whom changes in heart rate and function were related to changes in the size of the thyroid gland. The actual concept of thyrocardiac disease was introduced by Levine and Sturgis (1924) who described atrial arrhythmias, congestive cardiac failure and cardiac enlargement in hyperthyroidism, occurring even in the absence of evidence of overt thyroid disorder. Symons et al (1978) found 5% of clinically euthyroid patients with paroxysmal or permanent atrial arrhythmias had underlying hyperthyroidism, as disclosed by routine laboratory screening.

The clinical effects of thyrotoxicosis on the heart include increased rate, stroke volume, cardiac output and pulse pressure and decreased circulation time (Kontos et al 1965). The velocity of contraction is shortened, myocardial oxygen consumption is increased and cardiac blood flow is greatly augmented (Rowe et al 1956). Sandler and Wilson (1959a) showed that the cardiac dysfunction could occur in an otherwise normal heart. Among 462 patients with thyrotoxicosis, 150 had evidence of cardiac dysfunction, 43% of which having no evidence of underlying organic heart disease to explain the fibrillation, congestive failure or cardiomegaly. When the thyrotoxicosis was controlled, 40% had complete clearance of symptoms.

Much information on the effects of thyroid hormone comes from the experimental production of thyrotoxicosis in animals, and care has to be taken when relating these results to the clinical situation in man, as the dosage employed may be exerting effects at the pharmacological level rather than the physiological level (Bernal and Refetoff 1977). Some of the effects of thyroid hormones are biphasic, being stimulatory at low doses and inhibitory at high doses (Hoch 1974, Vonderhaar 1975).

Of major interest to this thesis is the cardiac hypertrophy which accompanies hyperthyroidism. In 1920 Hewitt found that administration of fresh thyroid to rats caused hypertrophy of the heart and other organs. Since then hypertrophy has been produced in many other animal experiments (e.g. Shelley et al 1942, Sandler and Wilson 1959b, Beznak 1962 and Cohen et al 1966).

Lynn Skelton et al (1972) have shown that the cardiac hypertrophy induced in cats by long term thyroid hormone administration is completely reversible within 12 months once the hormone is stopped. In the dog, as noted above, right ventricular thickness is increased by chronic thyroxine administration, and, when congestive heart failure occurs, left ventricular wall thickness is also increased.

In thyrotoxic animals myofibrillar degeneration, oedema, focal necrosis, cellular infiltration and fibrosis have been described. Electron microscope studies (Poche 1957, Zaimis et al 1965, 1969) have demonstrated swelling of the

longitudinal system and sarcoplasmic reticulum, mitochondrial pleomorphism with granulation, oedema and disintegration of mitochondria (many with fewer christae). The most striking feature noted by Zaimis et al (1969) was mitochondrial pleomorphism. They found many of the mitochondria were either enlarged or enfolded or both. The enfoldings were of varying depth and often multiple. Sometimes neighbouring mitochondria appeared fused in long slender forms equal in length to several sarcomers. These abnormalities were found in mitochondria among the myofibrils and in subsarcolemmal areas, rather than in the perinuclear zone. Mitochondrial number was increased by 25% in the cat and unchanged in the guinea pig, although mitochondrial volume in relation to the total sarcoplasm was increased by approximately 20% in both species. Associated with the changes was an increase in excitability of the heart and an increase in mitochondrial respiration and phosphorylation. These changes occurred before the appearance of tachycardia and hypertrophy.

In rats injected with thyroxine, Page and McCallister (1973) noted an increase in the volume of myofibrils but this was in the same proportion as the increase in cell volume. The same occurred for mitochondrial volume but the christae were more densely packed. This was in contrast to hypertrophy caused by an aortic ring where myofibrillar volume increased at the expense of the mitochondria.

Reith and Fuchs (1973) also found increased density of christae in thyroxine treated rats and noted invaginations and tip like structures. They interpreted these as an expression of biogenesis.

In contrast Susin and Herdson (1967) noted no histological change in the hearts of rats after 5 daily doses of thyroxine, although necrosis later became evident. Nayler et al (1971) found no change in mitochondrial morphology in electron micrographs of perfused fixed heart muscle from the thyrotoxic dog.

In the development of hypertrophy, protein levels in the heart are increased (Michels et al 1963) and Fagg Sanford et al (1978) found that this was due predominantly to an increase in synthesis with only a minor contribution from decreased degradation. In a further investigation Carter et al (1980) found that hyperthyroidism reducted protein breakdown in the sarcoplasm of cardiac muscle without altering degradation in the myofibrillar fraction. This is in contrast to the effects hyperthyroidism has on skeletal muscle where there was increased protein breakdown in both sarcoplasmic and myofibrillar fractions.

5.1 Mechanism of action of thyroid hormones on the heart

There have been many proposals concerning how thyroid hormones exert there actions and these have been summarised and discussed in several reviews, including those by DeGroot (1972) and Symons (1979).

Some of the effects may be a result of the hypermetabolic actions of thyroxine requiring an increase in cardiac output to compensate for increased oxygen consumption in other tissues. This, however, does not account for the profound changes occurring in hyperthyroidism (DeGroot and Leonard 1970). When asprin was administered in quantities to raise the metabolic rate to levels comparable to that seen in thyrotoxicosis, it did not substantially change the cardiac output (Kroetz et al 1962).

Merillon et al (1981) did include a peripheral thyroid action as part of a complex interaction of mechanisms causing a hyperkinetic circulatory state, but considered it was due to a reduction of systemic arterial resistance and an increase in circulating blood volume.

The earliest action of thyroid hormone is thought to be an acceleration of the basal respiratory rate of mitochondria (Hoch 1967, Cohen 1974). The previous section describes some of the early changes observed in mitochondria structure and Zaimis et al (1969) used this as evidence for the hypothesis that the thyroid hormone acts on energy yielding

pathways, resulting in the uncoupling of oxidative phosphorylation. Challoner (1968) had also suggested this but other studies have indicated that coupled oxidative phosphorylation is typical of mitochondria derived from muscle of humans and animals with thyrotoxicosis (Stocker et al 1968, Tarjan 1971).

Myosin structural alterations

Recent biochemical evidence has suggested that the augmentation of contractile performance of the heart in thyrotoxicosis has its basis in the enzymatic properties of the myosin. Bárány (1967) suggested that the velocity of a muscle's contraction is related to the ATPase activity of its myosin and an increase in this activity has since been reported in the hearts of thyrotoxic animals by Thyrum et al (1970), Yazaki and Raben (1975), Katagiri et al (1975) and Banerjee et al (1976). More recently it has been suggested that under thyrotoxic conditions a new species of myosin is synthesised. Flink and Morkin proposed this in 1977 and later (Flink et al 1979) further characterised the difference from normal myosin. Morkin (1979) has reviewed the effects of thyroid hormone on myosin ATPase. In hypertrophy induced by mechanical pressure the new myosin isoenzyme produced is, by contrast, lower in ATPase activity (Mercadier et al 1981).

Smitherman et al (1979) pointed out that the increased contractility of the changed myosin can, under severe hyperthyroidism, lead to toxic changes that can damage the heart tissue or render it more susceptible to injury and thereby eventually reduce the contractility. Piatnek-Leunissen and Olsen (1967) had previously produced cardiac failure in dogs receiving thyroxine.

Comparison with catecholamines

The cardiovascular manifestations of hyperthyroidism are very similar to those of excess catecholamines, so much so that it was originally thought that noradrenaline was the predominant mediator of thyrotoxic changes (Brewster et al 1956). However, many studies reported before 1960 suggesting that both the chronotropic and inotropic responses to exogenous catecholamines were potentiated by hyperthyroidism have been criticised for not subjecting data to statistical analysis and only using one dose of noradrenaline (Levey 1971, Blonde and Lynn Skelton 1978).

The above articles are both excellent reviews on the interactions of thyroid hormones and catecholamines and outline the suggestions for the mechanism of action of thyroid hormones. They review the studies that have been designed to overcome the criticisms mentioned above. These investigations, however, failed to document an enhanced sensitivity of the cardiovascular system in hyperthyroidism to exogenous catecholamines both in animals and man.

Hypersensitivity to beta adrenergically mediated chronotropic agents has been produced by tiiodothyronine on isolated foetal mouse hearts in organ culture (Wildenthal 1972).

Williams et al (1977) found an increase in beta adrenergic binding sites in hyperthyroid rats, suggesting that thyroid hormones can regulate the number of beta receptors. In atria from hypothyroid rats Kunos et al (1974) found a decrease in sensitivity to beta receptor stimulation and a corresponding increase to alpha receptor stimulation. This could be reversed by the administration of thyroxine or tiiodothyonine. This experiment, however, did not distinguish whether there are reciprocal changes in sensitivity or availability of two independent pools of receptors or to a single type of adrenoceptor that is quantitatively altered by changes in thyroid state. Since then McConnaughy et al (1979) have shown a decrease in alpha receptors and an increase in beta receptors in hyperthyroid rats when compared with controls. The ratio of beta to alpha receptors was doubled, but in hypothyroid rats both alpha and beta receptors decreased in number and the ratio remained the same as that in euthyroid rats.

Another explanation of heightened sympathoadrenal activity in hyperthyroidism discussed by Blonde and Lynn Skelton is that sympathetic neuronal activity or adrenal medullary secretion of catecholamines might be increased. However, they point out that plasma (Coulombe et al 1976a, Stoffer et al 1973) and urinary (Wiswell et al 1963) noradrenaline

levels are either low or normal in hyperthyroidism and plasma (Coulombe et al 1976b) and urinary (Wiswell et al 1973) adrenaline levels are unaltered. Wurtman et al (1963) suggested a third possibility based on their findings that although there was no increase in the enzymes catabolising noradrenaline, a greater amount of injected noradrenaline and adrenaline was delivered to the heart in hyperthyroidism. There was also a decrease in catecholamine inactivation thus resulting in increased myocardial levels. This would have been a suitable explanation had other experiments by other workers shown enhanced cardiac responses to given doses of catecholamines under thyrotoxic conditions.

It seems most likely that thyroid hormones have their own direct inotropic and chronotropic actions on the heart and that adrenergic and thyroxine mediated effects are additive. The reason that beta adrenergic blocking agents can reduce heart rate in thyrotoxic patients, and hypothyroid patients, is probably by inhibiting the normal adrenergic contribution to the heart rate (McDevitt et al 1968). Also it was found that the enhancement of papillary muscle contractility of hyperthyroid cats was unaffected by reserpine pretreatment to deplete the catecholamine stores (Buccino et al 1967). It has been proposed that this direct action of thyroxine activates the adenyl cyclase - cyclic AMP system and levey and Epstein (1969) suggested that there are two separate systems for thyroid hormones and catecholamines.

Calcium availability

An alternative explanation for some of the cardiac effects of excess thryoxine could lie in an increased availability of intracellular calcium which would enhance myocardial contactility. In heart muscle from hyperthyroid dogs and rabbits, microsomes enriched with sarcoplasmic reticulum were found to accumulate and exchange calcium at increased rates (Nayler et al 1971, Suko 1973). The importance of calcium will be dealt with later in an experimental chapter (8.1).

Reverse triiodothyronine

A more recent theory concerning the action of propranolol in reducing the effects of thyrotoxicosis, stems from the finding that it lowers T3 concentrations (Roszhowska et al 1974). This is associated with a rise in reverse triiodothyronine (rT3) concentrations (Verhoeven et al 1977). Thus propranolol may divert T4 deiodination from the B ring (T3 production) to the A ring (rT3 production), in the periphery rather than the thyroid gland (Feely et al 1979).

5.2 Hyperthyroidism and hypertrophic cardiomyopathy

The previous chapter has outlined the effects that thyroid hormones have on the heart, including the production of cardiac hypertrophy. Hypertrophic cardiomyopathy is not considered to be just an extreme form of cardiac hypertrophy but is an idiopathic entity in itself. There have been many suggestions, though, concerning its aetiology, including an involvement of thyroid hormones in some cases.

Symons et al (1974) reported three cases of hypertrophic cardiomyopathy where hyperthyroidism was also diagnosed. The combination of the two conditions led to a complex clinical picture as many signs and symptoms are common to both. They suggested the long continued high output circulatory state in clinically undetected hyperthyroidism may prove to be a stimulus for unrestrained muscle hypertrophy.

In 1978 Bell et al investigated 31 patients with hypertrophic cardiomyopathy for thyroid abnormalities and found that 4 (13%) had a history of thyroid disease. An explanation considered for the apparent association was the effects of hyperthyroidism on normal or, more probably, abnormal myocardium.

Symons (1979) suggested that, as with other cardiac conditions such as arrhythmia, a long period of hidden or occult hyperthyroidism could be responsible for the cardiomyopathy. He also suggested that cardiomyopathy could

be allied to a metabolic disorder when commenting on the work of Resnekov and Falicov (1977) who described patients with hyperthyroidism and lactate producing angina pectoris but with normal coronary arteries.

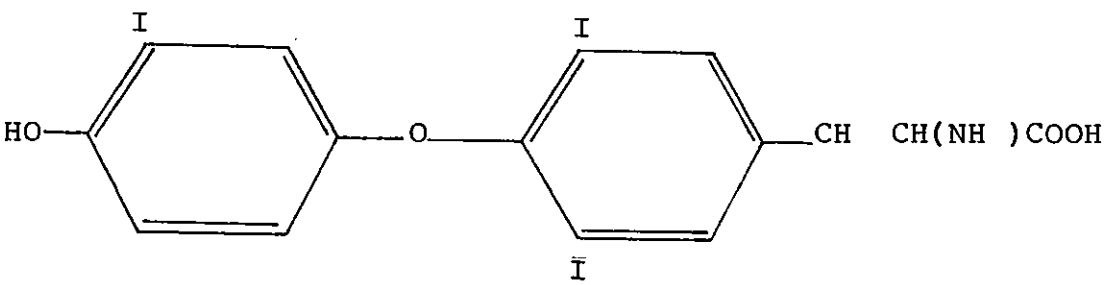
5.3 Triac and the heart

Triac, Triiodothyroacetic acid, is the acetic acid analogue of T3, see Fig. 6, and in comparison has very low hormonal potency (Money et al 1960, Westerfield et al 1965). It is bound as avidly to nuclear binding sites, which are the receptors involved in the initiation of hormone action, as T3 but duration of occupation is shorter. This can be attributed to a more rapid fractional metabolism of triac (Goslings et al 1976). Clinically, when administered to myxoedematous patients, it lowers serum cholesterol and reduces body weight but has only 1/6th the effect of T4 on stimulating overall body oxygen consumption (Lerman and Pitt Rivers 1956).

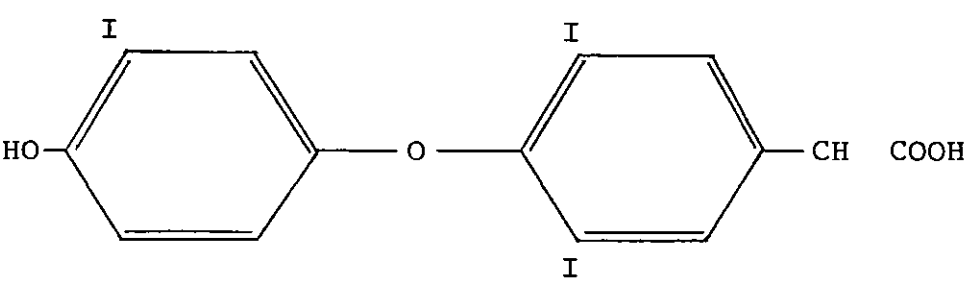
Interest in the cardiac actions of triac was stimulated by an unexplained finding of Barker and Lewis (1956). They measured cellular respiration of several tissues excised from thyroidectomised rats injected with T4, tetrac or triac. Oxygen consumption levels were higher in heart tissue slices than in other tissues for all three compounds. Triac, however, was over twice as active as T4 in stimulating the heart muscle. Tetrac was less active than T4.

Fig. 6 Structure of T3 and Triac

3 5 3' Triiodothyronine (T3)



3 5 3' Triiodothyroacetic acid (Triac)



In 1971 Symons et al reported the presence of circulating triac and tetrac in a euthyroid patient with heart disease who later became hyperthyroid. This finding along with the subsequently described association of hypertrophic cardiomyopathy with long standing hyperthyroidism led to a series of experiments with triac. The first, Symons et al (1975), was to use doses of triac and T4 which simulated longstanding covert hyperthyroidism and inject young adult rats daily for a period of one year. Doses used were T4, 0.13 mg/kg/day and triac, 0.18 mg/kg/day. There were no differences in weight gain between treated and control groups and when the hearts were examined after 15 and 33 weeks there were no changes observed either macro or microscopically. The hearts of T4 treated rats at 52 weeks, however, showed signs of mild hypertrophy but major changes were noted in triac treated rats. Histological examination showed definite nuclear changes of hypertrophy in the form of pyknosis and blunting and increases of glycogen and succinic dehydrogenase were also found.

At electron microscopic examination, increased numbers of mitochondria, which were variable in size and shape, were noted. In some, crystolysis was observed. However, no changes in myofibrils were noted in this experiment.

A further experiment was designed to investigate the effects of triac during a vulnerable phase of cardiac development. Olsen et al (1977) administered triac to pregnant rats and, whilst finding no change in the hearts of the mothers, found total disarray at the ultrastructural level in the hearts of

the 2 day old offspring whose mothers received triac at 60 µg/day. Also the numbers of mitochondria were increased and nuclear membranes showed more pronounced infolding, suggesting hypertrophy was present. No changes were found at the histological level other than an increase in the fibre diameters. Hypertrophic cardiomyopathy itself was therefore not produced but the ultrastructural findings showed great similarity to those seen in the condition in man. The authors suggested that the experiment provided a valuable model in which the effects of other compounds on the developing heart could be tested. The report, however, was a preliminary communication and there was no quantitation of data.

The next experiment in the series was to administer the beta adrenergic blocking agent propranolol with triac during pregnancy (Hawkey et al 1981). In this experiment the production of intracellular disarray was prevented by propranolol but hypertrophy was unaffected. The results were quantified in this study but only one dose of propranolol, 0.5 mg/day, was used. In addition, rats were killed at various times after birth, up to 490 days. Disarray was most prominent at 2 and 6 days, some was present at 14 days but by 28 days all the myofibrils were regularly arranged. Hypertrophy persisted for 56 days.

These results prompted the series of experiments which are described in this thesis. Beta adrenergic blocking agents have additional properties which were utilised in these experiments in order to attempt to elucidate the mechanism of the action of triac. Table 1 lists these properties and the drugs used, not all of them beta blockers. Further details of each drug and reasons for use are given in each individual experiment.

In addition some preliminary experiments were carried out to investigate placental transfer of triac and to produce an adult animal model of hypertrophic cardiomyopathy. Both experiments are described, and further work is suggested.

Table 1

Properties of drugs used to investigate the mechanism of action of triac on developing rat heart muscle

	B.B	M.S	P.A.	Ca Antag.	Ca. Influx
dl Oxprenolol	+	+	+	-	-
dl Propranolol	+	+	-	-	-
d Propranolol	-	+	-	-	-
dl Timolol	+	-	-	-	-
Procainamide	-	+	-	-	-
Verapamil	-	+	-	+	-
A 23187	-	-	-	-	+
Prenalterol	-	-	+ *	-	-

B.B. = Beta Blockade.

M.S. = Membrane stabilisation

P.A. = Partial Agonist Activity

* Prenalterol is a pure beta 1 agonist

Ca Antag = Calcium Antagonist

Ca Influx = Calcium ionophore, opening of calcium channels

PART 2

EXPERIMENTAL WORK

6. Experimental Methods

6.1 Introduction

The experiments described in the first part of this section all follow essentially the same methodology, which was based on that used by Olsen et al (1977) and Hawkey et al (1981). In this chapter the method will be described and any minor modifications will be mentioned in each chapter concerned.

Each experiment had two sets of controls, rats receiving buffer only as negative controls and rats receiving triac alone at 60 μg per day, acting as positive controls. In addition these served to verify the work of the above authors and to satisfy the present author that triac produces abnormalities which can be modified with selected drugs. The collective results of these will be discussed in this chapter. In addition, three doses of test drugs with potentially protective or additive effects were given with triac in each experiment. Also the highest dose of these was given alone in order to assess whether or not they would affect the structure of the developing myocardium.

6.2 Materials and methods

Triac (diethanolamine salt of triiodothyroacetic acid, Sigma Ltd.) was dissolved in glycine buffer, pH 10, at a concentration of 100 µg per ml. The formula for making up 50 ml of solution was as follows:-

Triac	5 mg.
Powdered sodium hydroxide	74 mg.
Sodium chloride	184 mg.
Glycine	237 mg.
Water for injection	to 50 ml.

The triac was first dissolved with the sodium hydroxide in 1 or 2 ml of water and then the other ingredients were added and made up to volume. The strength of the solution was checked by ultra-violet sprectrophotometry. The solution was sterilised by filtration, in a laminar flow cabinet, and stored in sterile 20 ml multidose vials in the dark at 4°C.

Glycine buffer was made up as above and stored under the same conditions.

Rats Virgin female Wistar rats weighing approximately 250 g were used and were mated with males of the same strain. They were caged individually in grid bottomed cages and all received standard rat pellets (diet 86, E. Dixon and Son Ltd.) and tap water ad libitum throughout the experiment.

Procedure

For a week before the start of the experiment the male rats were kept in close proximity, but in separate cages, to the females, so that oestrous could be stimulated if necessary.

On day 1, one male was introduced to each female and the injections to the females began. The weight of each female was recorded daily and the subsequent presence of vaginal plugs in the cage trays was an indication of mating. The males were removed on day 9.

All injections were intramuscular into the rear thigh and they were given 5 days per week for three weeks. Parturition took place during the 3 to 6 days after the final injection. The offspring received no further treatment. Gestation periods and litter sizes were noted and the percentage of live births calculated.

At 2 and 7 days after birth four offspring from each group were randomly selected, weighed and then killed by decapitation. They were pinned, in the supine position, to a cork board. An incision was made down the mid line of the chest wall which was then reflected. Using a fine pair of scissors, a cut was made just above the heart to sever the major blood vessels and the heart was carefully removed. Whilst still contracting it was weighed and then sectioned just below the atrioventricular groove.

The lower half was finely chopped into pieces of approximately 1 mm^3 under glutaraldehyde/formaldehyde fixative. These were processed for electron microscope examination.

The upper half was placed in buffered formalin fixative and processed for histological examination. Samples of other major organs, including liver, lung, kidney and spleen were also removed and stored in formalin for future examination.

6.3 Processing of Tissue Samples for Electron Microscopy

Fixation was carried out in a glutaraldehyde/formaldehyde mixture, which allows the rapid penetration and primary fixation by formaldehyde to be stabilised by the bifunctional activity of glutaraldehyde.

The preparation of the solution was:-

a) Paraformaldehyde (B.D.H.) 8g, was added to 80 ml of boiling deionised water and stirred. A few drops of 10 M NaOH were necessary to ensure that it fully dissolved. This was made up to 100 mls.
b) Glutaraldehyde 25% (Taab Iabs) 20 mls.
c) O.2 M phosphate buffer pH 7.2 80 mls.

This was made up each day as it was used.

The period of fixation was approximately 1.5 hours and was carried out at 34°C. The tissue pieces were then placed in 0.08 M phosphate buffer and stored at 4°C for no more than one week.

The rest of the procedure was as follows:-

1) Post - fixation in 1% osmium tetroxide in 0.08 M phosphate buffer..... 1 hour at 4°C.
2) 70% ethanol ..... 15 mins "
3) 90% ethanol ..... 20 mins "
4) Absolute ethanol ..... 2 x 30 mins at room temp.
5) Propylene oxide ..... 10 mins "
6) 50/50 Resin/propylene oxide. 2 - 4 hours "
7) Resin ..... Overnight at 37°C
8) Resin ..... 36 hours at 60°C.

The tissue blocks were polymerised in the resin in gelatine capsules, oo size.

The resin used was an Epon mixture, made up as follows:-

Stock A	Epon 812	124 ml
	DDSA	200 ml
Stock B	Epon 812	200 ml
	MNA	178 ml

These were stored at 4°C.

The working solution was:-

A ..... 60 ml

B ..... 40 ml

+ BDMA ..... 1.5 ml.

Thorough mixing was ensured at all times.

DDSA = Dodecenyl succinic anhydride

MNA = Methyl nadic anhydride

BDMA = Benzyl dimethyl amine

In an earlier experiment this method of fixation and processing was compared with a routine method using only glutaraldehyde in sodium cacodylate at 4^{o}C. Storage between fixation and processing was in a cacodylate/sucrose solution. Preservation of the myocardium was much improved when the method fully described was used and so this was repeated in all the other experiments.

Fixation at 34^{o}C gives an increased speed of penetration of the tissue and causes a much more uniform quality of tissue preservation. However, for osmium tetroxide post fixation, cold is necessary. Bullock (1979) has excellently reviewed the parameters affecting the quality of fixation of the myocardium for ultrastructural studies.

6.4 Histological examination

The upper halves of the hearts were orientated in wax blocks such that whole transverse sections could be cut. This was carried out on an *MSE base sledge microtome at a thickness of 5 μm. The sections were stained with haematoxylin and eosin and examined under a Projectina microscope. Here a screen instead of eyepieces made possible the measurement of fibre diameters. These were measured across the point of a nucleus in fibres which were in longitudinal section. This is the widest part of the fibre, which tapers towards the ends, thus reducing potential errors (Baandrup 1979). Two rats from each group were examined at 2 and 7 days and the diameters of at least 100 cells from each measured.

* MSE Scientific Instruments Limited.

Statistical analysis

The test of Comparison of means was used when comparing diameters of different groups.

6.5 Electronmicroscopic examination

Eight blocks of tissue from each rat heart were processed and were available for examination. This number were required to ensure that cells in longitudinal section could be found in at least one block.

Semi thin sections of 1 μm thickness were firstly cut on an *LKB mark 3 ultramicrotome and stained with toluidine blue. Under light microscopy a block showing cells in longitudinal section was chosen and 50 nm sections cut on the same ultramicrotome. They were picked up on 200 mesh copper/rhubidium grids and stained with uranyl acetate (a saturated solution in 50:50 water:ethanol) and lead citrate (Veneble and Coggeshall 1965).

A coding system ensured that the origin of each tissue section was unknown when examination took place. For this a Phillips 301G electron microscope was used. Five randomly chosen squares of cells in longitudinal section from each grid were examined in close detail.

Fig. 7 on page 107 illustrates cells in longitudinal section from a control rat. Myofibrils are pedominently regularly arranged.

* LKB Instruments Limited.

Abnormalities which were quantified were those of myofibrillar disarray and hypertrophy.

A score for disarray was the total number of foci of disarray and crossover of myofibrils and myofilaments observed in the five grid squares. Examples are given in Figs. 8, 9 and 10.

When scoring, care was taken to exclude areas where apparent disarray was due to changes of orientation of fibres, for example Fig. 11. Disarray was also commonly noted in all groups in areas close to intercalated discs, for example Fig. 12, and these were also excluded from the score.

The degree of hypertrophy was assessed by counting the number of mitochondria separating 2 adjacent parallel sarcomeres.

Score	
0	1-2 mitochondria per 2 sarcomeres (normal) See Fig. 13.
1	2-3 mitochondria per 2 sarcomeres See Fig. 14.
2	3-4 mitochondria per 2 sarcomeres See Fig. 15.
3	4-5 mitochondria per 2 sarcomeres.

Additional evidence for hypertrophy was provided by crenation of nuclear membranes (Fig. 16), areas of accumulated mitochondria away from the nucleus, especially when a variety of size and shape was noted (Fig. 15) and the appearance of excess glycogen (Fig. 9).

Statistical analysis

Where appropriate, the Wilcoxon rank sum test (Wilcoxon 1945) was used to compare scores from different groups (see page 117).

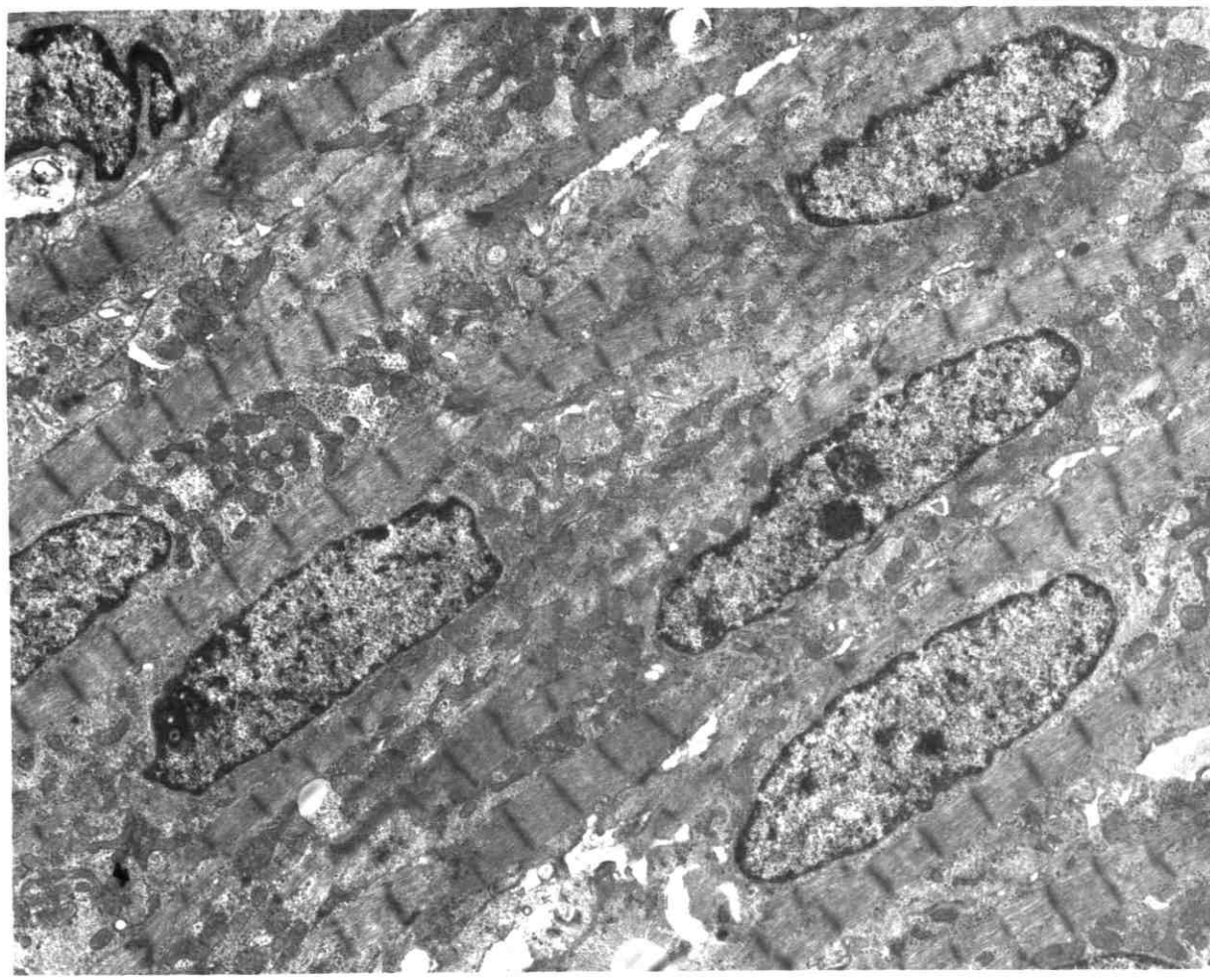


Fig. 7. Electronmicrograph illustrating regular arrangement of myofibrils within longitudinally sectioned myofibres. There is some chromatin clumping. (Control rat - 2 days). x 7,200

(All electronmicrographs are from sections stained with uranyl acetate and lead citrate).

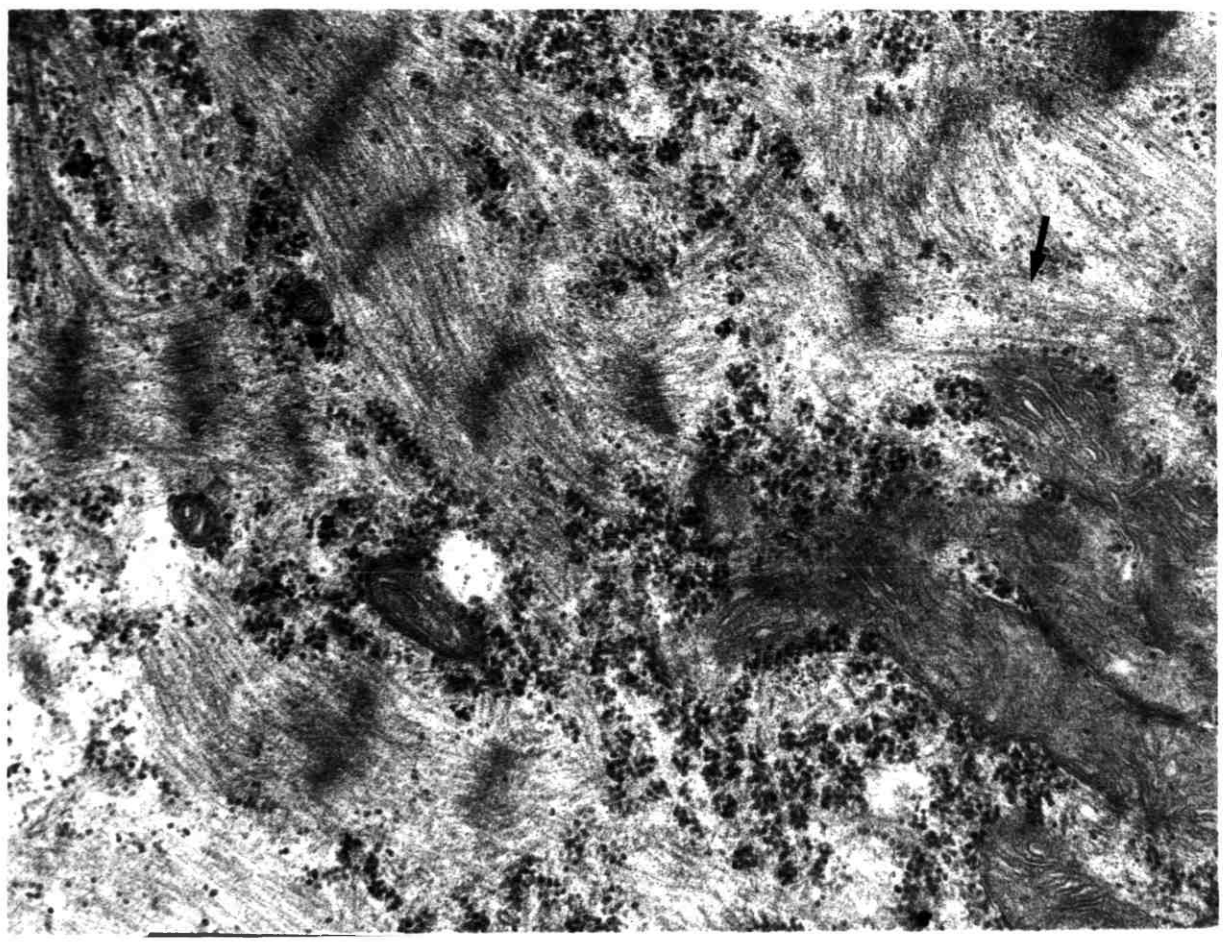


Fig. 8. Electronmicrograph illustrating abnormal arrangement of myofibrils. They run in all directions and appear to cross over each other. Disorganization of myofilaments is also shown (➡). (2 day offspring from triac treated rat). x 44,000

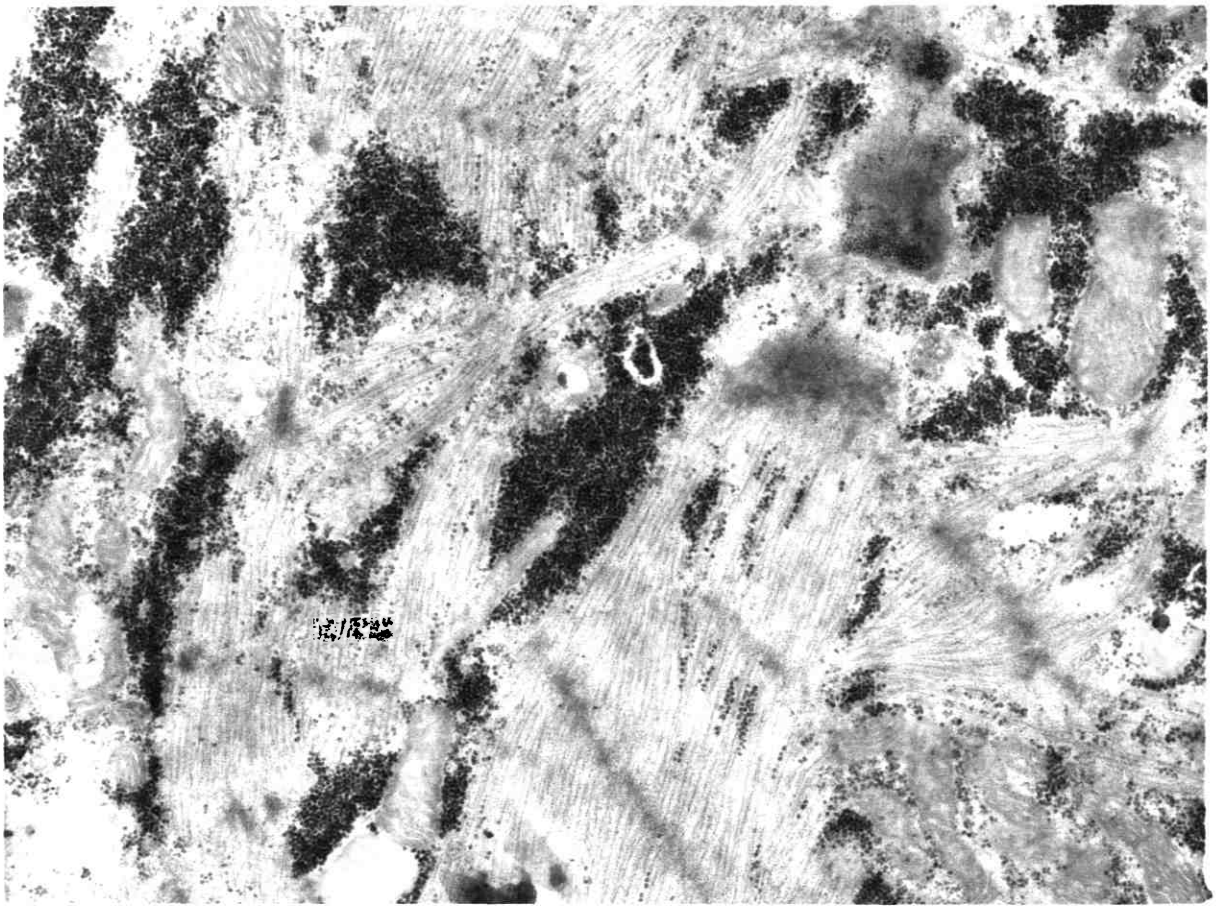


Fig. 9. Electronmicrograph illustrating myofilaments crossing between adjacent myofibrils. There is also an increase in glycogen content and diffusion of z band material. (7 day offspring from a triac treated rat).

x 26,000

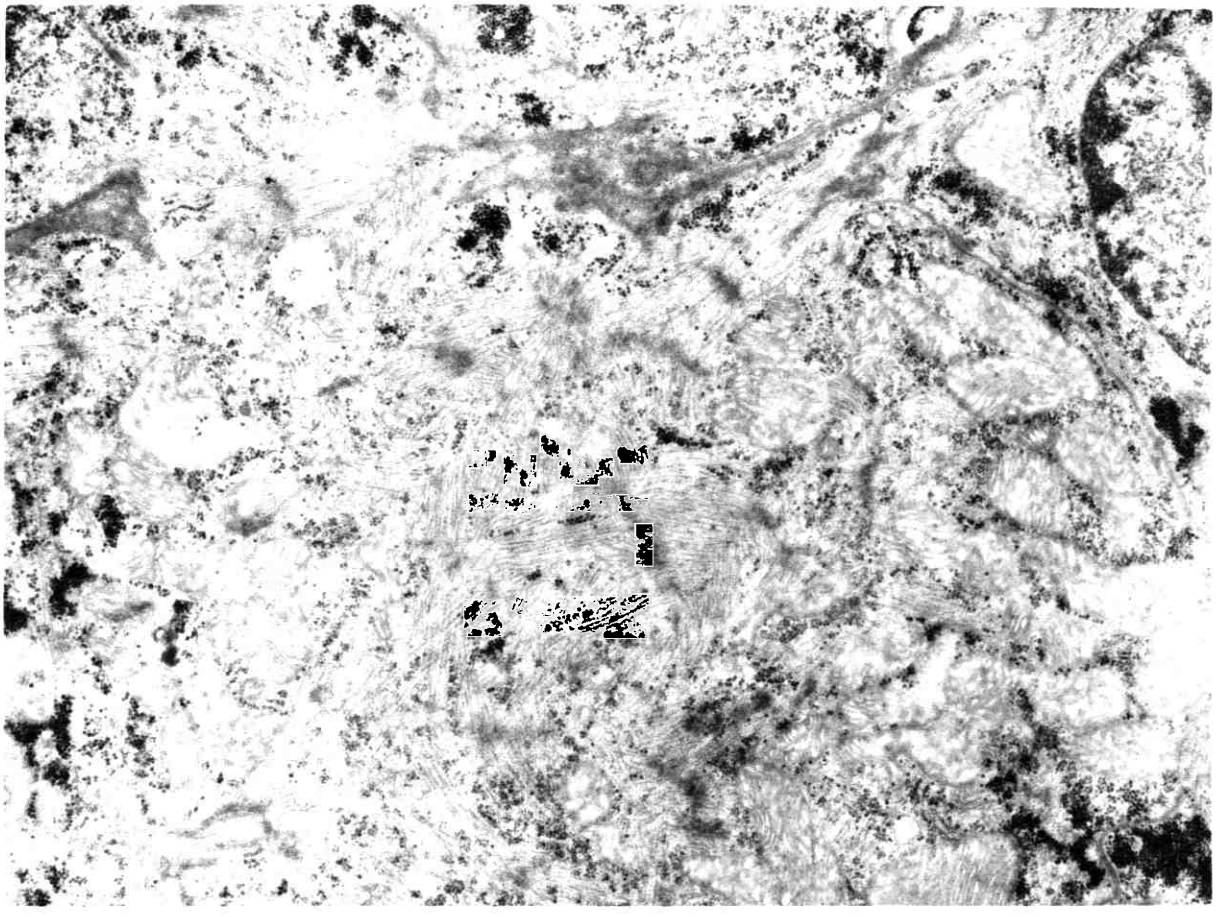


Fig. 10. Electronmicrograph illustrating myofilaments forming a "criss-cross" pattern. Myofibrils appear to be no longer than one sarcomere in length. (2 day offspring from triac treated rat).

x 14,500

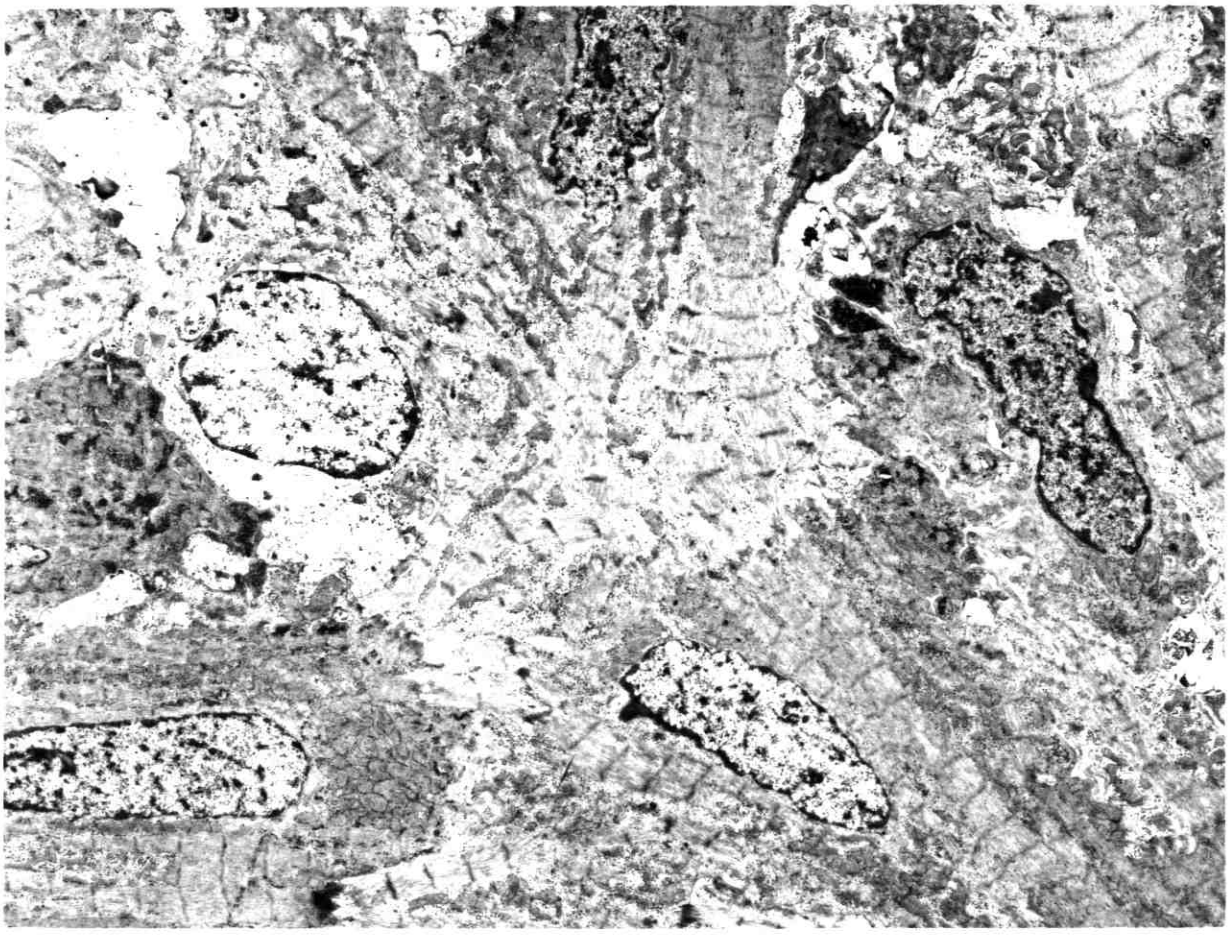


Fig. 11. Electronmicrograph illustrating a cell at a point where the fibres change direction. The disarray within that cell is not scored. (2 day offspring from triac treated rat). x 4,300

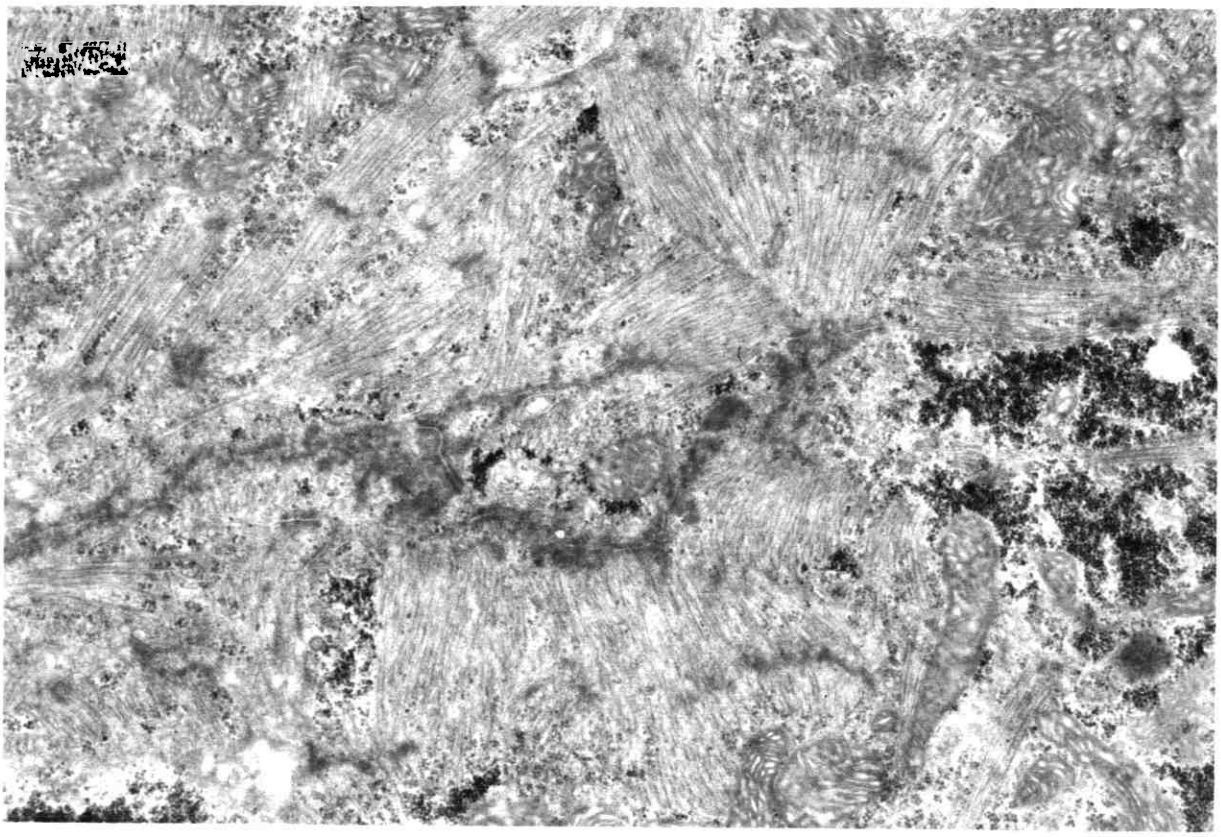


Fig. 12. Electronmicrograph illustrating disarray of myofibrils around an intercalated disc. This was not included in the score for disarray. (2 day offspring from triac treated rat).

x 19,000

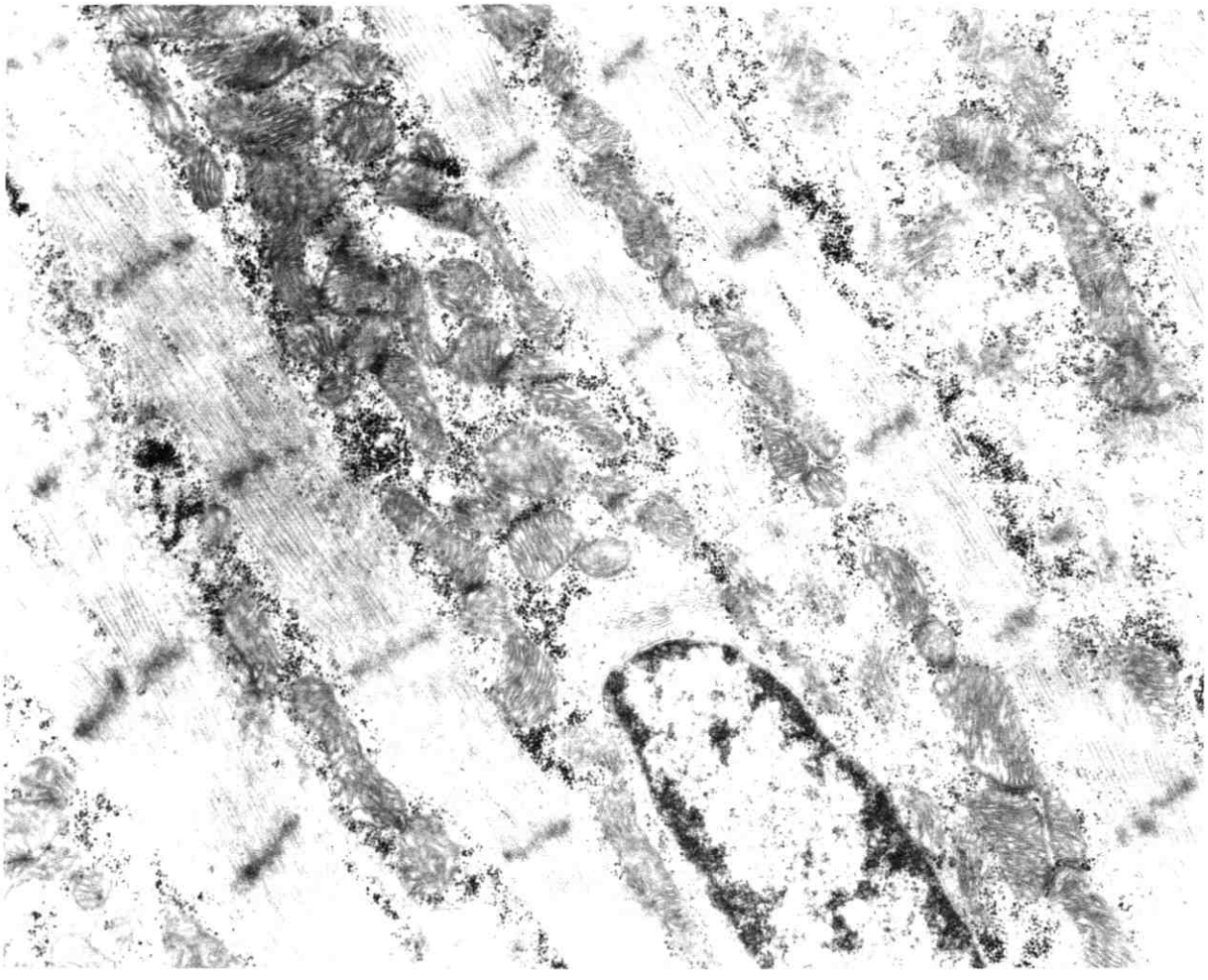


Fig. 13. Electronmicrograph illustrating separation of adjacent sarcomeres by one or two mitochondria. Score for hypertrophy = 0. (7 day offspring from a control rat). x 19,000

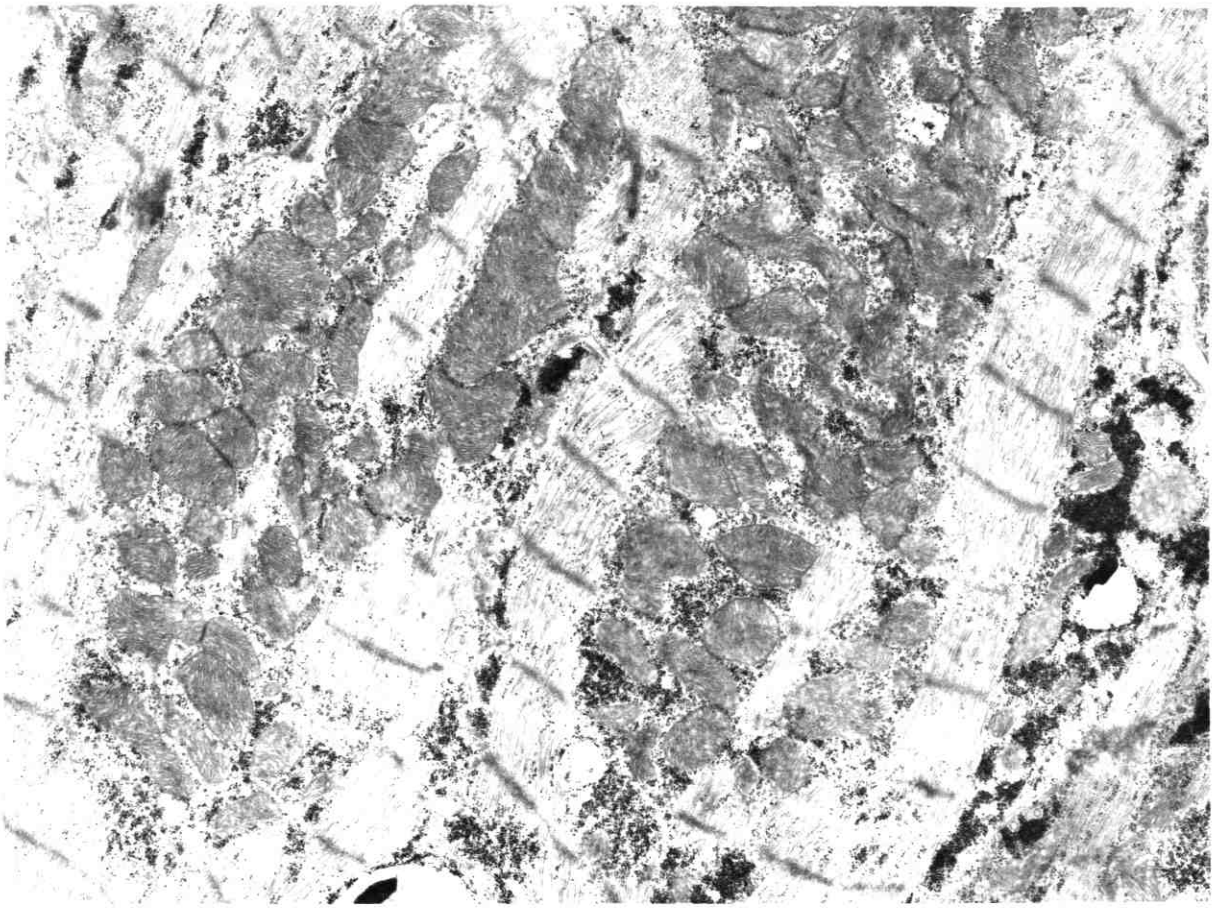


Fig. 14. Electronmicrograph illustrating separation of adjacent sarcomeres by two or three mitochondria. Score for hypertrophy = 1. (7 day offspring from a triac + propranolol treated rat).

x 28,000

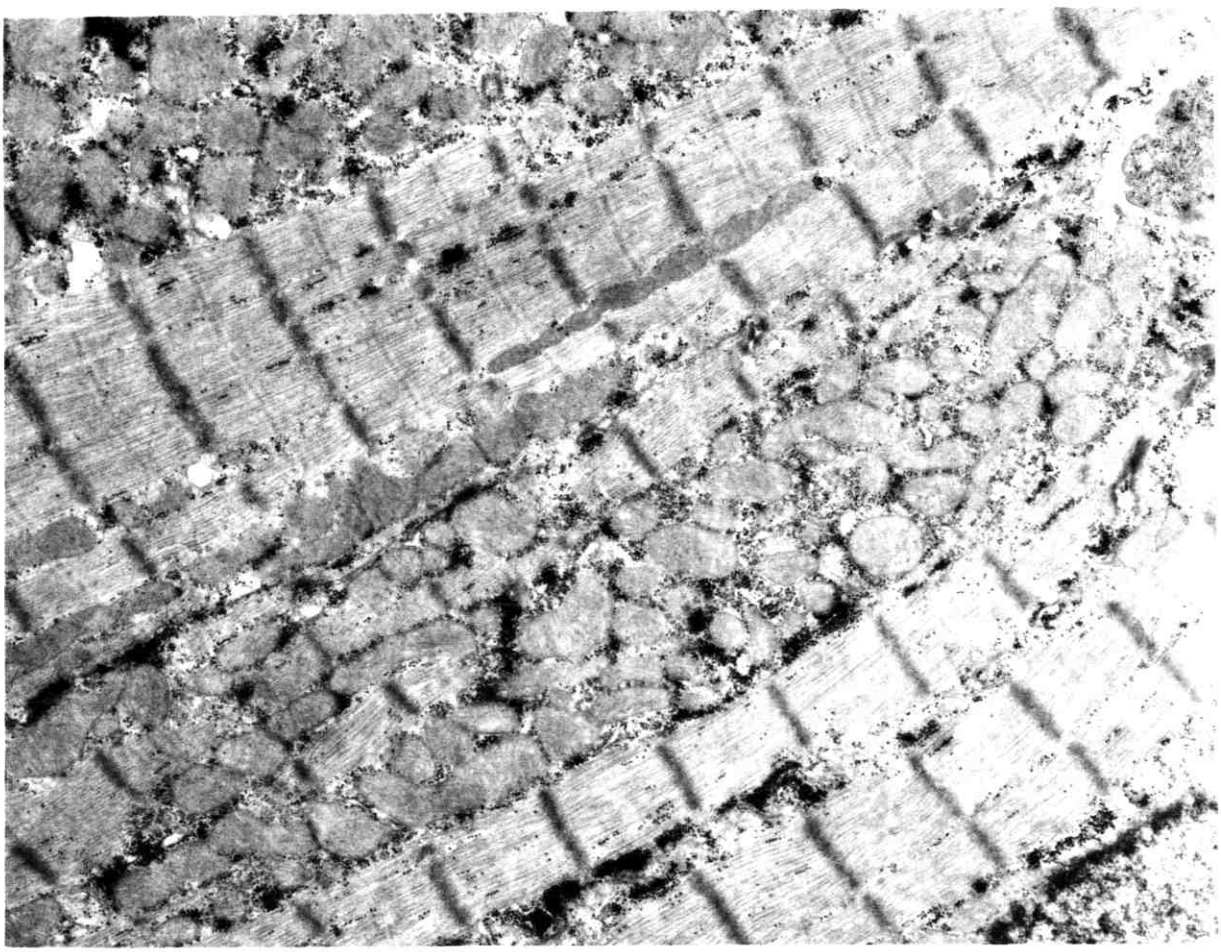


Fig. 15. Electronmicrograph illustrating separation of adjacent sarcomeres by three to four mitochondria. Score for hypertrophy = 2. (7 day offspring from a triac treated rat). x 15,000

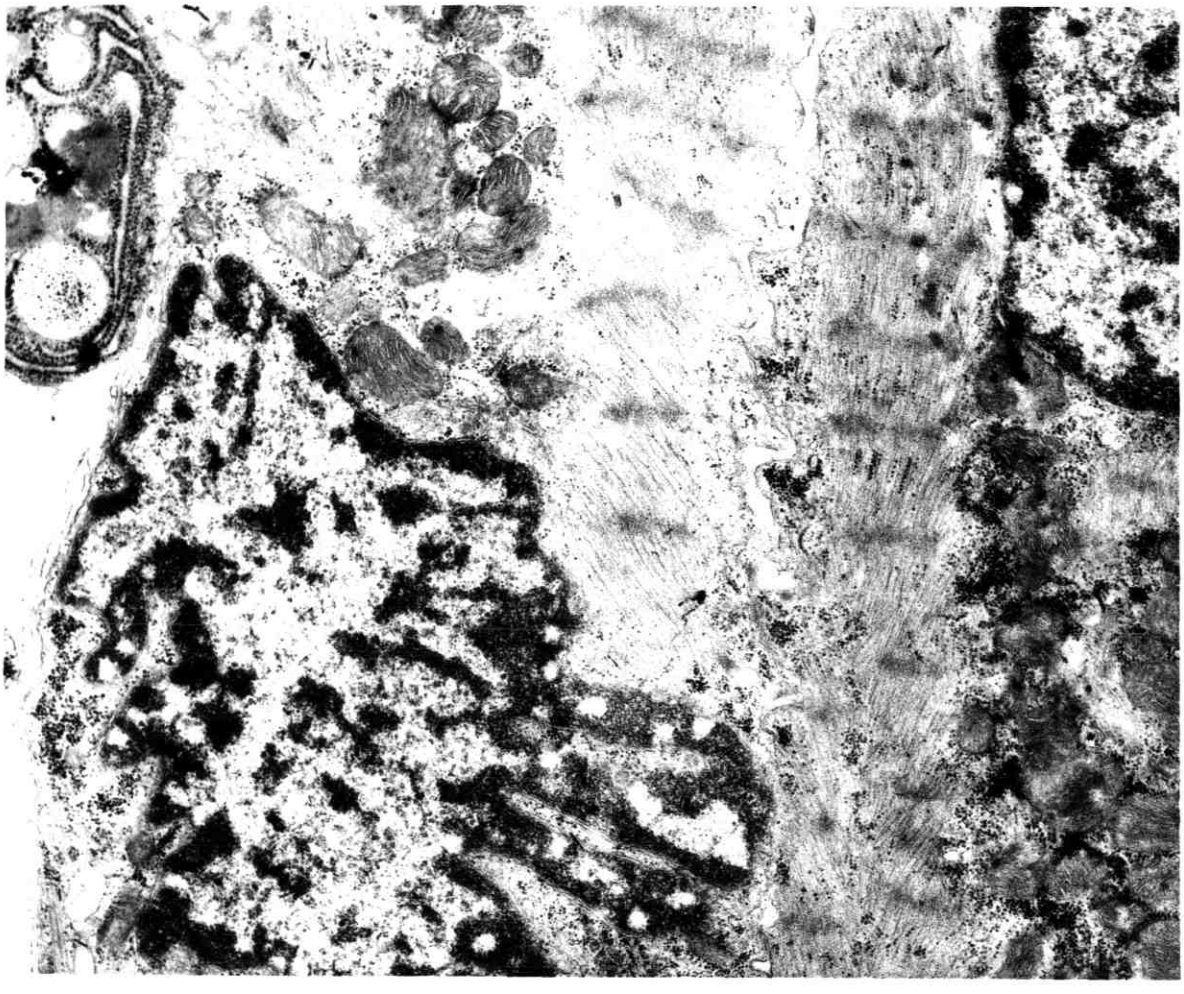


Fig. 16. Electronmicrograph illustrating severe crenation of the nuclear membrane. This is a feature associated with hypertrophy. (2 day offspring from a triac treated rat). x 19,600

6.5.1 Results

The results presented in tables 2 and 3 (page 119) are those of the electronmicroscope analysis of the buffer and triac alone treated groups from all the experiments.

The difference between the negative control and the triac treated groups was quantitative rather than qualitative. There were some foci of disarray observed in the control groups but in rats from triac treated mothers the disarray was much more widespread. The amount of disarray was greater at 2 days than at 7 days. Distribution was, however, in patches and occasionally a high score in a control rat and a low score in a triac treated rat was noted. Cells in which disarray was severe could be adjacent to normal cells. Observation of 5 grid squares under the elctronmicroscope however was considered sufficient to separate the amount of disarray present in normal neonatal rats from that caused by triac.

Statistical analysis of individual experiments is, unfortunately, difficult, with only four at each age to compare in each group. The Wilcoxon rank sum test (Wilcoxon 1945) on these results requires no overlap of numbers for there to be a significant difference between groups at the

5% level. However, significant difference at this level was observed in the groups marked *. In other individual experiments a trend is observed.

When all the data are analysed together using Wilcoxon's rank sum test, then there is a high level of significant difference between the two groups ($p < 0.05$ at 2 and < 0.01 at 7 days).

The same applies when analysing the results for hypertrophy. In individual experiments trends were noted. Scores in the buffer treated control groups were either 0 or 1 whereas in the triac treated groups they were all 1 or more. When all the data are analysed together then there is a significant difference between the two groups at both 2 and 7 days ($p < 0.05$).

Table 2

Results - Em Scores for disarray and crossover

Group	2 Day	7 Day
Buffer 0.6 ml per day	7 2 6 2 * 0 0 1 1 * 2 3 3 2 1 0 1 2 0 n = 17, Range 0-7 mean = 2.0 ± 2.0	* 3 0 0 1 0 2 0 1 * 0 0 0 1 2 1 2 1 n = 16, Range 0-3 mean = 1.0 ± 1.0
Triac 60 µg per day	9 20 5 21 * 9 11 15 10 * 10 9 15 11 8 7 9 n = 15, Range 5-21 mean = 11.5 ± 4.5	* 13 14 9 4 4 13 7 2 * 7 6 15 14 8 4 n = 14, Range 2-15 mean = 8.5 ± 4.5

All scores are for individual rats

Each horizontal line represents rats from the same experiment.

n = total number of rats

* $p < 0.5$

Table 3

Results - Em Scores for hypertrophy

Group	2 Day	7 Day
Buffer 0.6 ml per day	1 0 0 1 1 0 0 1 0 1 0 1 1 1 0 0 0 n = 17, Range 0-1 mean = 0.5 ± 0.5	1 1 0 0 1 1 0 1 0 0 1 0 0 1 0 0 n = 16, Range 0-1 mean = 0.4 ± 0.5
Triac 60 µg per day	2 1 1 2 2 2 1 1 2 1 3 2 2 2 1 n = 15, Range 1-3 mean = 1.7 ± 0.6	1 1 1 2 3 1 1 1 1 1 2 1 1 1 n = 14, Range 1-3 mean = 1.3 ± 0.6

Each score for hypertrophy corresponds to the score for disarray in the same position in the previous table.

6.5.2 Discussion

The combined results confirm the findings of Olsen et al (1977) and Hawkey et al (1981), that triac, when administered to pregnant rats, produces in the myocardium of the young offspring, hypertrophy and myofibrillar disarray. As this was shown consistently in all experiments it was considered that use of the model to investigate the mechanism of action of triac by its combination with selected compounds, was justified.

Four rats were sampled from each group at both 2 and 7 days and they were taken, where possible, from at least 2 of the litters in each group. The sampling was a compromise reached to ensure that sufficient samples could be analysed from several doses of drugs combined with triac. One block from each rat was analysed but often, when cutting them, only one out of the eight processed would contain cells in longitudinal section which were acceptable to be analysed under the electron microscope.

Assessment of the degree of hypertrophy by counting the number of mitochondria separating adjacent myofibrils was considered suitable for these experiments. Hawkey et al (1981) used the same method and in most cases hypertrophy

was confirmed by measurement of cell diameters at the histological level. The use of an image analyser was investigated. This used electron micrographs and measured the areas occupied by the various cellular components. However, for the purpose of these experiments, this time consuming procedure did not result in improvement in the data.

Disarray at histological level

The phenomenon of disarray, and the recent proposal from Becker and Caruso (1982) that it may be an artefact of orientation of the sections cut, requires discussion. They found that of all tissue sections examined histologically from five normal human hearts, 44% showed a "deviation from normality" and 33% showed "myocardial fibre disarray". In 87% of the blocks studied the histology changed when the orientation of the block changed. This is not surprising because, as they point out, "fibres are not simply cylindrical units but they bifurcate and connect with adjacent fibres to form a complex three-dimensional network (Bloom and Fawcett, 1975)". Parallel alignment of fibres cannot be regarded as the only form of normality. However, when arrangement of fibres, such as that observed in Fig. 3 on page 38 is widespread, this type of disarray must surely be regarded as abnormal. Some of the types of patterns of arrangement described in their paper were observed in the

rat hearts, but these were regarded as part of the normal interweaving of bundles of fibres. Thus it is agreed that care has to be taken when making a diagnosis from tissue sections. The authors point out that this work was only on normal hearts and they do not deny that myocardial disarray may be more extensively present in hearts from patients with hypertrophic cardiomyopathy. Olsen (1982) has recently reviewed this work.

Disarray at ultrastructural level

The logical progression from this work on fibre disarray must be that the same applies when looking at fibrillar disarray. This was realised early on in the experimental work, and if sections observed were not longitudinal then far more disarray was apparently present. Myofibrils could often appear in short runs and in varying directions but these were not scored unless orientation abnormalities could be discounted. The experiments described in this thesis have shown that abnormal arrangements of myofibrils were present in some control rats, as has previously been shown in developing hearts (Manasek, 1970), but again the difference between controls and rats from triac treated mothers is quantitative rather than qualitative. Scores from individual rats vary, a few control rats have even given higher scores than rats from triac treated mothers, but, taking all the experiments into account, the level of disarray is significantly greater in the offspring of triac treated rats.

The actions of triac with selected antagonist and agonist compounds on developing rat heart muscle.

7. The effects of beta adrenergic blocking and membrane stabilising agents on triac induced myocardial abnormalities in the developing rat

7.1 Oxprenolol

7.1.1 Introduction

Chapter 5.3 has described the effects that triac has on developing heart muscle and that concurrent administration of dl propranolol prevents the triac induced disarray but not the hypertrophy.

The mechanism of the action of triac, however, remains unknown. It could involve direct or indirect stimulation of beta receptors, or have a direct action of its own. Oxprenolol, like propranolol, possesses beta blocking and membrane stabilising properties and also possesses a partial agonist action. In this experiment oxprenolol was given with triac to the pregnant rats to assess the importance of this third property.

7.1.2 Methods and materials

Table 4 shows the injections given to each group of 5 rats, in this experiment.

Oxprenolol was supplied by Ciba-Geigy Ltd. and made up to concentrations of 3.3, 6.7 and 13.3 mg/ml in glycine buffer at pH 10.

Triac was made up to a concentration of 200 µg/ml.

In this experiment all injections were 0.6 ml and triac and oxprenolol were mixed in the syringe immediately before each injection.

Table 4

Triac with oxprenolol

DRUGS AND DOSES

Group	Treatment	Dose
1	Buffer (control)	0.6 ml
2	Triac	60 µg
3	Triac + Oxprenolol	60 µg 1 mg
4	Triac + Oxprenolol	60 µg 2 mg
5	Triac + Oxprenolol	60 µg 4 mg
6	Oxprenolol	4 mg

7.1.3 Results

No behavioural changes or abnormal clinical signs were noted in any of the pregnant rats. Weight gains and food intake were similar in all groups. Table 5 (page 135) gives details of the pregnancies and shows that litter sizes and gestational periods were similar in all groups. The slight reduction in percentage live birth rate in group 2, triac alone, was due to one rat giving birth to several which were found dead.

7.1.3.1 Macroscopic findings

Table 6 shows the results of the heart weights expressed as a percentage of total body weight, for each group. No significant differences between any of the groups were found.

7.1.3.2 Light microscopic findings

Table 7 shows the results of measurements of cell diameters. All groups receiving triac, with or without oxprenolol, showed a significant increase in diameters compared with the controls at both 2 and 7 days - see Figs. 17 and 18. Oxprenolol did not influence the triac induced hypertrophy, even at the highest dose - see Fig. 19, nor did it have any effect when given on its own.

Very occasional areas of cells in disarray were found in some sections - see Fig. 20, but these were not associated with a particular group.

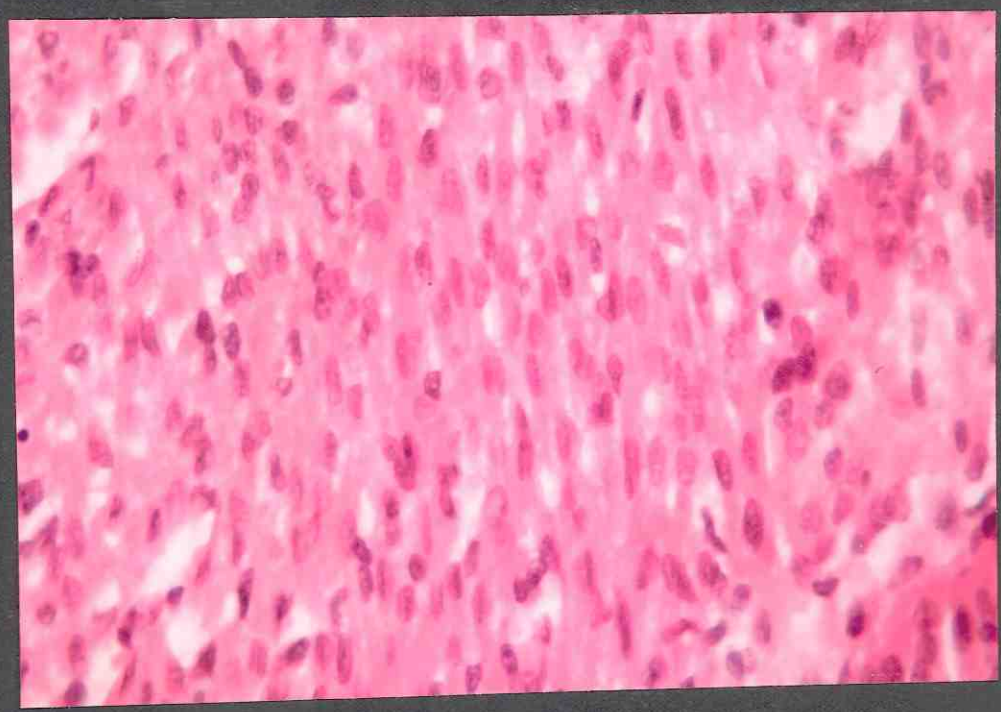


Fig. 17 Photomicrograph illustrating normal myocardium (2 day control rat).
Haematoxylin and eosin. x 500

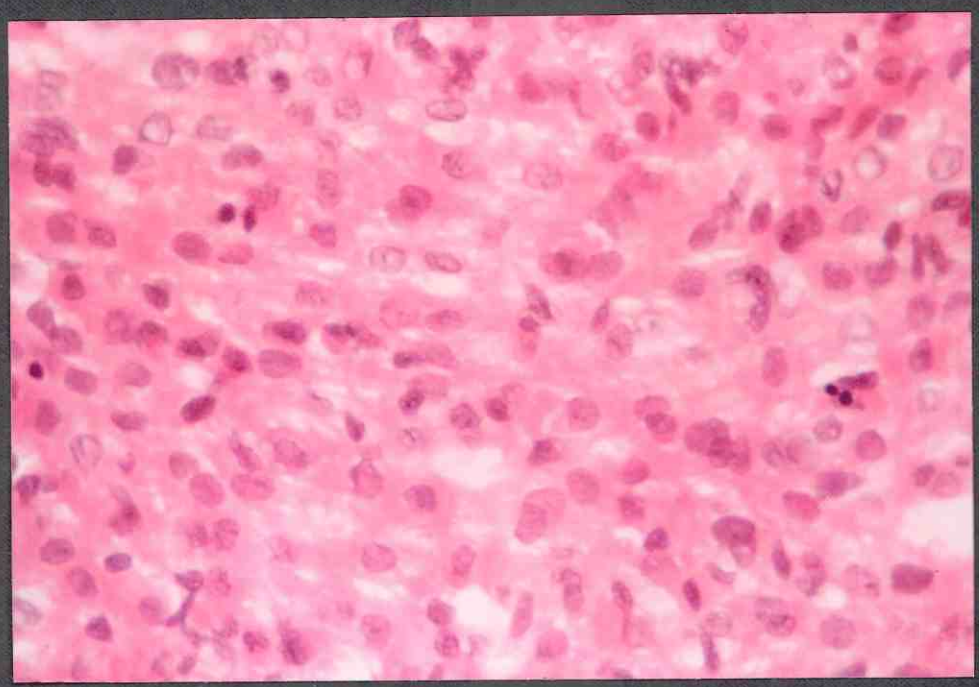


Fig. 18 Photomicrograph illustrating hypertrophied fibres with enlarged nuclei. (2 day offspring from a triac treated rat). Compare with Fig. 17. Haematoxylin and eosin. x 500

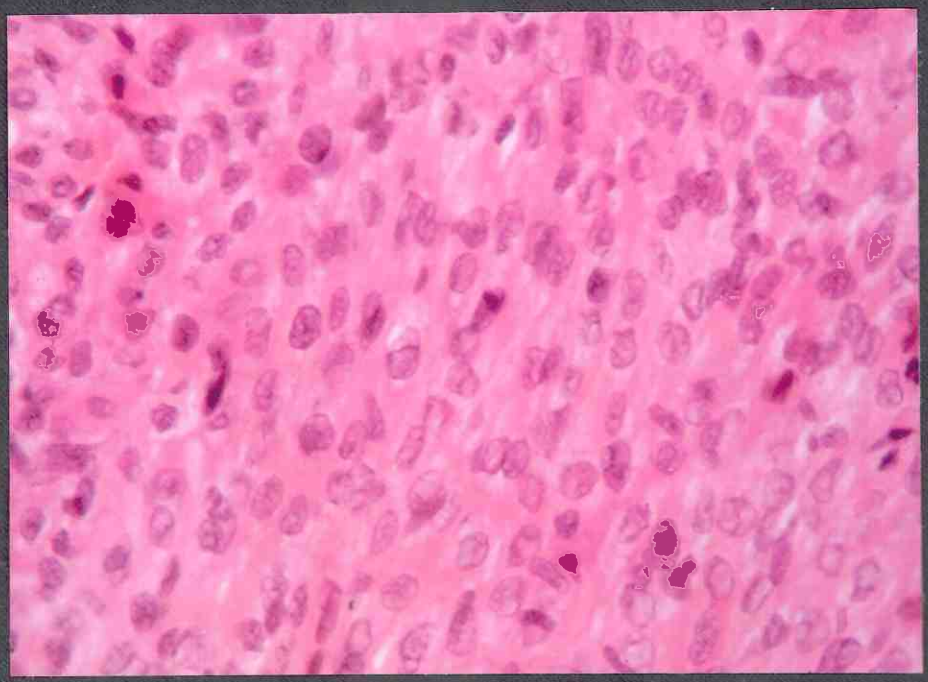


Fig. 19. Photomicrograph illustrating hypertrophied fibres. (2 day offspring from a triac + oxprenolol (4mg) treated rat). Comparison with Figs. 17 and 18 show that oxprenolol did not affect triac induced hypertrophy.
Haematoxylin and eosin x 500

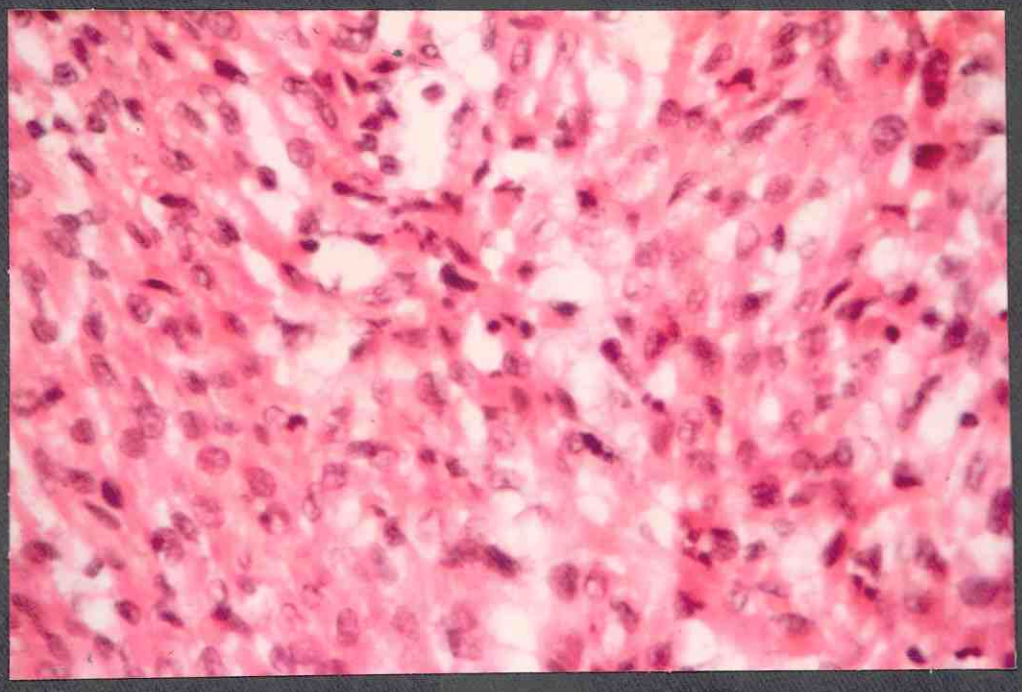


Fig. 20. Photomicrograph illustrating disarray of myofibrils. (2 day offspring from triac treated mother). Haematoxylin and eosin. x 500

7.1.3.3 Electron microscopic findings

Myofibrillar disarray (see table 8, page 138) was present as a few foci in the control group but regular arrangement of myofibrils predominated, as seen in the example in Fig. 7 (page 107). The number of foci, however, was greatly increased in the offspring of rats which received triac alone, and in most cases the severity was greater at 2 days than at 7 days. When 1 and 2 mg oxprenolol were given with triac the number of foci was slightly reduced but when 4 mg was given, scores were very similar to the control group. When oxprenolol was given alone at 4 mg all rats except one at 7 days showed levels of disarray similar to control rats.

Very occasionally cells similar to that seen in Fig. 21 were found. They were not associated with any particular group. In these cells myofilaments were observed in total disarray, with no formation into myofibrils. They were assumed to be developing rather than degenerating cells and so were not taken into account when scoring.

Scores for hypertrophy reflect a trend rather than distinct scores for each group (see table 9). In the control group half the rats showed scores for mild hypertrophy, the remainder all scoring 0. Rats from triac treated mothers all showed scores of at least 1, three having scores of 2. When oxprenolol was administered with triac, mild and moderate hypertrophy was still present in all groups. Scores from rats from oxprenolol (4 mg) alone treated mothers were similar to control rats.

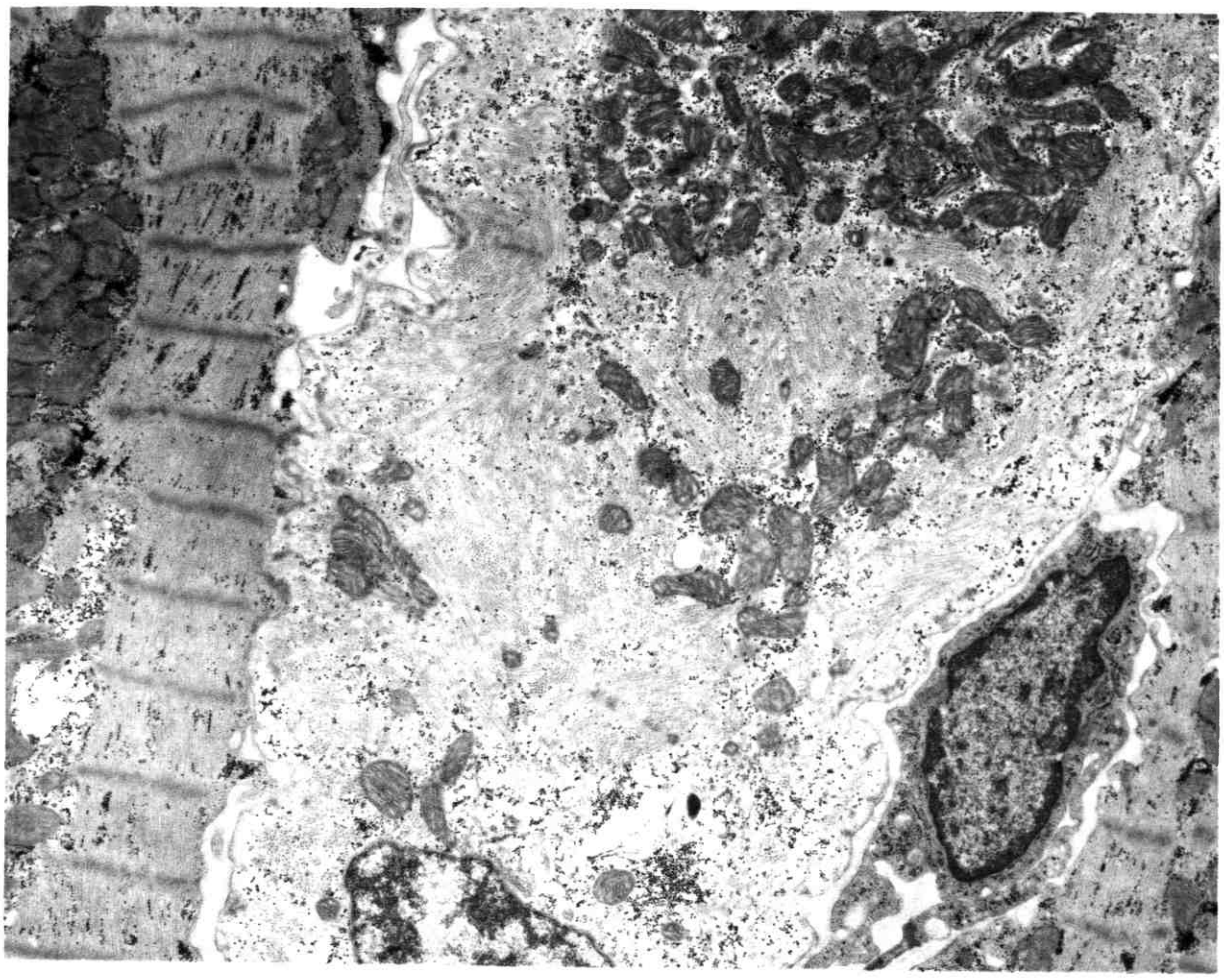


Fig. 21. Electronmicrograph illustrating myofilaments running in all directions in a developing cell. (2 day offspring from a triac + oxprenolol (4mg) treated rat). This type of disarray was not included in the score. x 11,800

Table 5

Details of Pregnancies

Group	No. of Pregnancies	Av. Litter Size	% Live Births	Gestation Time-Days
1	4/5	12	90	22
2	4/5	11	67	22-23
3	5/5	13	87	22
4	4/5	10	92	21-23
5	4/5	12	94	21-22
6	4/5	11	84	21-22

Table 6

Triac with Oxprenolol

Heart weight - expressed as a percentage of total body weight

GROUP	2 DAY RATS	7 DAY RATS
1. Control	0.612 ± 0.093	0.748 ± 0.064
2. Triac 60 µg	0.622 ± 0.049	0.719 ± 0.042
3. Triac 60 µg + Oxpren 1 mg	0.653 ± 0.057	0.709 ± 0.043
4. Triac 60 µg + Oxpren 2 mg	0.598 ± 0.057	0.655 ± 0.062
5. Triac 60 µg + Oxpren 4 mg	0.622 ± 0.041	0.699 ± 0.089
6. Oxpren 4 mg	0.626 ± 0.047	0.711 ± 0.041

Oxpren = Oxprenolol

Table 7: Triac with Oxprenolol

Results: Cell diameters - histological assessment

2 DAY RATS

Group	n	mean cell diameter µm	S.D.	p1	p2
1 Control	200	6.94	1.09		
2 Triac 60 µg	200	8.84	1.27	< 0.001	
3 Triac 60 µg + oxpren 1 mg	200	9.19	1.24	< 0.001	NS
4 Triac 60 µg + oxpren 2 mg	200	9.02	1.37	< 0.001	NS
5 Triac 60 µg + oxpren 4 mg	200	8.82	1.41	< 0.001	NS
6 Oxpren 4 mg	200	7.13	1.04	NS	<0.001

7 DAY RATS

Group	n	mean cell diameter µm	S.D.	p1	p2
1 Control	200	8.19	1.15		
2 Triac 60 µg	200	9.68	1.44	< 0.001	
3 Triac 60 µg + oxpren 1 mg	200	9.64	1.44	< 0.001	NS
4 Triac 60 µg + oxpren 2 mg	200	9.49	1.19	< 0.001	NS
5 Triac 60 µg + oxpren 4 mg	200	9.46	1.45	< 0.001	NS
6 Oxpren 4 mg	200	8.27	1.30	NS	<0.001

p1: compared with Group 1

p2: compared with Group 2

NS: not significant

n: No. of cells measured

Table 8

Triac with Oxprenolol

Results: E.M. Score for disarray

GROUP	2 DAY RATS				7 DAY RATS			
1 Control buffer	7	2	6	2	3	0	0	1
2 Triac 60 µg	9	20	5	21	13	14	9	4
3 Triac 60 µg + Oxpren 1 mg	26	1	19	2	25	8	3	0
4 Triac 60 µg + Oxpren 2 mg	11	5	8	15	5	7	7	3
5 Triac 60 µg + Oxpren 4 mg	1	5	1	6	7	3	2	4
6 Oxpren 4 mg	0	4	4	0	9	2	2	2

All scores are for individual rats.

Each score = the number of points of disarray and crossover in 5 squares of a 200 mesh grid.

Table 9

Triac with Oxprenolol

Results: E.M. Score for hypertrophy

GROUP	2 DAY RATS	7 DAY RATS
1 Control buffer	1 0 0 1	1 1 0 0
2 Triac 60 µg	2 1 1 2	1 1 1 2
3 Triac 60 µg + Oxpren 1 mg	1 1 2 1	1 0 1 1
4 Triac 60 µg + Oxpren 2 mg	1 0 2 1	2 1 1 2
5 Triac 60 µg + Oxpren 4 mg	1 1 3 1	1 1 1 1
6 Oxpren 4 mg	0 0 0 0	1 0 1 0

All scores are for individual rats.

Each score is based on the number of mitochondria per 2 parallel sarcomeres.

Conclusion

This experiment has shown that triac induced disarray of myofibrils was prevented by the highest dose of oxprenolol. Hypertrophy, however, was not significantly affected by oxprenolol.

This result has prompted the next experiment, which was to assess the other property common to both oxprenolol and propranolol, that of membrane stabilising activity.

The two experiments are discussed together in section 7.2.4 (page 152).

7.2 dl Propranolol and d propranolol

7.2.1 Introduction

The racaemic mixture of dl propranolol, when administered throughout pregnancy to rats, is known to prevent triac induced myofibrillar disarray, but not the triac induced cardiac hypertrophy in the newborn rat (Hawkey et al 1981). In the above paper only one dose, 0.5 mg/day, of propranolol was used.

A feature of both propranolol and oxprenolol is that they both possess membrane stabilising activity, a local anaesthetic like action, and so an experiment to investigate the influence of this property of the drugs was designed. The dexro isomer of propranolol has equivalent membrane stabilising activity to the racaemic mixture but has only one hundredth the potency as a beta blocking agent (Barrett and Cullum 1968).

The experiment described compares the actions of the same doses of both dl propranolol and d propranolol on the triac induced cardiac abnormality.

7.2.2 Materials and methods

Table 10 shows the injections given to each group.

dl Propranolol (Inderal, ICI Ltd.) was used as supplied by the manufacturer for injection. This was in glass ampoules containing 2 ml of sterile solution at a concentration of 1 mg per ml. Citric acid was present as a buffering and stabilising agent. Storage was in the dark at room temperature.

d Propranolol was supplied as the powder by ICI Ltd. It was made up to a concentration of 1 mg per ml in water for injection in 20 ml sealed multidose vials. Citric acid (at 14 mg per 50 mg d propranolol) was added as above. Storage was in the dark at $4^{o}C$.

Table 10

Triac with d and dl Propranolol

GROUP	TREATMENT	DOSE
1	Buffer	0.6 ml
2	Triac	60 µg
3	Triac + dl Propranolol	60 µg 0.25 mg
4	Triac + dl Propranolol	60 µg 0.5 mg
5	Triac + dl Propranolol	60 µg 1 mg
6	Triac + d Propranolol	60 µg 0.25 mg
7	Triac + d Propranolol	60 µg 0.5 mg
8	Triac + d Propranolol	60 µg 1 mg
9	dl Propranolol	1 mg
10	d Propranolol	1 mg

7.2.3 Results

There were no abnormal findings during pregnancy (table 11). A high rate of pregnancy was achieved for each group, the litter sizes were all normal and so were the gestation periods. The percentage live birth rate was high for all groups although the rate for group 2, those receiving triac alone, was significantly lower than the other groups. (The value falls lower than 2 standard deviations away from the mean).

7.2.3.1 Macroscopic findings

Table 12 shows the results of heart weights, expressed as a percentage of total body weights, for each group. No significant difference between any of the groups was found.

7.2.3.2 Light microscopic findings

Table 13 shows the results of measurements of cell diameters. All rats whose mothers received triac, with or without d or dl propranolol, had significantly greater mean cell diameters than either the controls or those with d or dl propranolol alone. At 2 days none of the groups receiving triac with d or dl propranolol differed significantly from those receiving triac alone. The mean cell diameter for the group receiving d propranolol alone was smaller than the controls.

At 7 days, in the groups receiving triac with 0.5 and 1 mg of either d or dl propranolol, the mean cell diameters fell between those of the control and triac alone treated groups suggesting that, although the triac induced hypertrophy was not prevented, it was slightly reduced.

Also, at 7 days, the mean cell diameter for the group receiving dl propranolol alone was higher than the controls.

7.2.3.3 Electron microscopic findings

Results for disarray and crossover are given in table 14. A few foci of disarray were seen in the control group and in the groups receiving d and dl propranolol alone. However in the group receiving triac alone (group 2) much higher scores were seen, with the exception of one 7 day rat with a score of 0. The number of foci of disarray was reduced by dl propranolol at 0.25 mg (group 3) but 0.5-1 mg was required to return to approximately control values. d Propranolol also reduced the amount of disarray and crossover but not by as much as dl propranolol. 1 mg did not totally abolish the disarray but the scores were very similar to those from the 1 mg d propranolol alone treated group (group 10).

Results for hypertrophy are given in table 15. Using the scoring criteria previously described, some control rats showed hypertrophy to a mild degree (score 1). However all the rats from the triac alone treated group (group 2) showed at least this level and some (3 out of 8) gave scores of more severe hypertrophy (score 2). dl Propranolol, at the lower doses of 0.25 and 0.5 mg, had no effect on the triac induced hypertrophy but 1 mg at 2 days did show some reduction. d Propranolol had no effect on the hypertrophy at any of the doses used.

Table 11

Triac with d and dl Propranolol

Details of Pregnancies

Group	No. of pregnancies	Average litter size	% live births	Gestation time-days
1	3/3	9	100	21 - 22
2	2/3	7.5	79	22
3	2/3	10.5	95	22 - 23
4	3/3	11	100	22
5	3/3	8	100	21 - 24
6	3/3	10	97	21 - 22
7	3/3	8.5	94	22
8	2/3	8.5	94	22
9	2/3	12	100	21 - 22
10	3/3	9	94	22 - 24

Table 12

Triac with dl propranolol and d propranolol

Heart weights expressed as a percentage of total body weight.

GROUP	2 DAY RATS	7 DAY RATS
1. Control	0.717 ± 0.047	0.819 ± 0.071
2. Triac 60 µg	0.619 ± 0.089	0.636 ± 0.045
3. Triac 60 µg + dl Prop 0.25 mg	0.633 ± 0.038	0.730 ± 0.064
4. Triac 60 µg + dl Prop 0.5 mg	0.665 ± 0.023	0.706 ± 0.112
5. Triac 60 µg + dl Prop 1 mg	0.579 ± 0.085	0.686 ± 0.062
6. Triac 60 µg + d Prop 0.25 mg	0.488 ± 0.036	0.640 ± 0.054
7. Triac 60 µg + d Prop 0.5 mg	0.620 ± 0.091	0.540 ± 0.027
8. Triac 60 µg + d Prop 1 mg	0.771 ± 0.055	0.720 ± 0.181
9. dl Prop 1 mg	0.666 ± 0.079	0.784 ± 0.023
10. d Prop 1 mg	0.625 ± 0.031	0.672 ± 0.021

Prop = Propranolol

Table 13a

Triac with dl and d Propranolol

Results: Cell diameters - histological assessment

2 DAY RATS

Group	n	mean cell diameter µm	S.D.	p1	p2
1 Control	200	8.49	1.26		
2 Triac 60 µg	200	9.48	1.12	<0.001	
3 Triac 60 µg + dl Prop 0.25 mg	200	9.51	1.28	<0.001	NS
4 Triac 60 µg + dl Prop 0.5 mg	200	9.26	1.25	<0.001	NS
5 Triac 60 µg + dl Prop 1 mg	200	9.31	1.32	<0.001	NS
6 Triac 60 µg + d Prop 0.25 mg	200	9.36	1.25	<0.001	NS
7 Triac 60 µg + d Prop 0.5 mg	200	9.34	1.25	<0.001	NS
8 Triac 60 µg + d Prop 1 mg	200	9.28	1.37	<0.001	NS
9 dl Prop 1 mg	200	8.37	1.10	NS	<0.001
10 d Prop 1 mg	200	7.94	1.11	<0.001	<0.001

p1: compared with Group 1

p2: compared with Group 2

NS: not significant

n: No. of cells measured

Table 13b

Triac with dl and d Propranolol

Results: Cell diameters - histological assessment

7 DAY RATS

Group	n	mean cell diameter μm	S.D.	p1	p2
1 Control	200	7.19	1.01		
2 Triac 60 μg	200	8.43	1.25	< 0.001	
3 Triac 60 μg + dl Prop 0.25 mg	200	8.42	1.16	< 0.001	NS
4 Triac 60 μg + dl Prop 0.5 mg	200	7.68	1.08	<0.001	<0.001
5 Triac 60 μg + dl Prop 1 mg	200	7.82	0.98	< 0.001	<0.001
6 Triac 60 μg + d Prop 0.25 mg	200	8.15	1.15	< 0.001	< 0.05
7 Triac 60 μg + d Prop 0.5 mg	200	8.01	1.08	< 0.001	<0.001
8 Triac 60 μg + d Prop 1 mg	200	8.01	0.98	< 0.001	<0.001
9 dl Prop 1 mg	200	7.70	1.10	< 0.001	<0.001
10 d Prop 1 mg	200	7.26	1.05	NS	<0.001

p1: compared with Group 1

p2: compared with Group 2

NS: not significant

n: No. of cells measured

Table 14

Triac with dl and d propranolol

Results: E.M. score for disarray

GROUP	2 DAY RATS				7 DAY RATS			
1) Control	0	0	1	1	0	2	0	1
2) Triac 60 µg	9	11	15	10	4	13	7	2
3) Triac 60 µg + dl Prop 0.25 mg	1	9	3	3	3	4	0	5
4) Triac 60 µg + dl Prop 0.5 mg	2	0	2	1	0	0	0	1
5) Triac 60 µg + dl Prop 1 mg	0	0	0	2	7	3	0	0
6) Triac 60 µg + d Prop 0.25 mg	8	4	3	10	6	5	1	0
7) Triac 60 µg + d Prop 0.5 mg	4	2	6	1	2	5	1	0
8) Triac 60 µg + d Prop 1 mg	3	3	2	0	2	2	0	0
9) dl Prop 1 mg	3	0	0	0	2	3	0	1
10) d Prop 1 mg	3	1	1	4	0	0	0	2

Table 15

Triac with dl and d propranolol

Results: E.M. score for hypertrophy

GROUP	2 DAY RATS				7 DAY RATS			
1) Control	1	0	0	1	1	1	0	1
2) Triac 60 µg	2	2	1	1	3	1	1	1
3) Triac 60 µg + dl Prop 0.25 mg	2	1	0	1	2	2	1	1
4) Triac 60 µg + dl Prop 0.5 mg	1	1	1	2	1	0	3	0
5) Triac 60 µg + dl Prop 1 mg	0	0	1	1	1	1	2	1
6) Triac 60 µg + d Prop 0.25 mg	1	1	2	1	1	1	1	1
7) Triac 60 µg + d Prop 0.5 mg	1	2	2	1	1	1	2	1
8) Triac 60 µg + d Prop 1 mg	2	1	2	2	1	1	2	0
9) dl Prop 1 mg	0	0	0	1	1	1	0	0
10) d Prop 1 mg	1	0	1	1	1	0	0	1

7.2.4 Discussion

Although the findings are along similar lines to those which have been previously reported by Olsen *et al* (1977) and Hawkey *et al* (1981), the experiments were carried out in order to confirm that the results could be reproduced. The results also provided a base line for subsequent experiments in this thesis.

New information provided by the results of these experiments is that dl oxprenolol, despite its additional sympathomimetic activity, has the same effect as dl propranolol, although a higher dose is required. The same results were also noted with d propranolol, which has membrane stabilising but minimal beta blocking activity, when it was administered with triac.

Of the many theories concerning the cause of hypertrophic cardiomyopathy, two are considered here. These are the association with thryoid hormones (Symons *et al* 1974) and the possibility that catecholamines may be involved (Goodwin 1974). The latter suggestion was that, "The relationship with catecholamines is a complex one and the effect is probably exerted on the growing myocardium rather than a simple matter of excess amounts of catecholamine acting on the heart over a prolonged period later in life". This concept would also probably be applicable if thyroid hormones are involved, as prolonged administration of both triac and thyroxine to adult rats produced cardiac

hypertrophy but no alteration in cardiac structure (Symons et al 1975). In contrast the brief administration of triac during pregnancy does affect the proper alignment of the myofibrils as well as produce cardiac hypertrophy in the offspring.

Beta blockade by dl propranolol is a recognised treatment for patients with hypertrophic cardiomyopathy (Cohen and Braunwald 1967) but is only effective in relieving symptoms for some patients and does not cure the condition. At concentrations needed to block the beta receptors, propranolol also exerts a direct negative inotropic effect and this cardiac depressive action can lead to congestive failure in long-term treatment. Oxprenolol, on the other hand, with its additional sympathomimetic or partial agonist action, exerts a positive inotropic action which proveds a safeguard against cardiac insufficiency. Approximately 10 times the dose of oxprenolol, compared with propranolol, is needed to diminish the contractile force by the same amount. In clinical studies on the symptomatic treatment of moderate thyrotoxicosis, Gibberd and Staffurth (1973) found oxprenolol no better than a placebo, and concluded that intrinsic sympathomimetic activity countered the beneficial actions of beta blockade. The results in the first experiment suggest that the sympathomimetic property could have an influence. Over 8 times the dose of oxprenolol compared with propranolol was required to produce the same effect, when, in terms of beta blockade, the difference in potency is only double.

In order to assess the importance of the other property common to both oxprenolol and propranolol, the membrane stabilising activity, d propranolol was used. This action is a powerful one as both isomers of propranolol are twice as potent in reducing isolated nerve trunk spike amplitude as the local anaesthetic procain (Barrett and Cullum 1968). Oxprenolol is 1.4 times as active as procain.

At the doses used d propranolol reduced the level of triac induced disarray and crossover, though not completely preventing it. The results indicate that some of the action of triac is via direct or indirect beta receptor stimulation but that most is via a direct action which can be blocked by membrane stabilisation. This finding is in part contrast to Perloff's (1981) suggestion that the stimulus for cellular disorganisation may be an adrenergic stimulus that accompanies hyperthyroidism, rather than a primary effect of the thyroid hormone or circulatory analogue. In 1967 Howitt and Rowlands found that the heart rate and circulatory output from hyperthyroid patients were reduced by d propranolol, but not by as much as by the racaemic mixture.

An experiment using a beta blocking agent with no membrane stabilising activity is therefore suggested from these results. Comparison with a pure membrane stabilising agent, not related to beta blocking agents, would also help clarify the degree of involvement of beta receptors.

7.3 Timolol and procainamide

7.3.1 Introduction

The previous experiments have shown that beta adrenergic blocking agents can prevent triac induced diarray but not cardiac hypertrophy. The finding that d propranolol was also effective in reducing disarray suggests the possibility that the membrane stabilising property exhibited by both propranolol and oxprenolol is responsible for their protective effects.

In order to substantiate this theory, two further drugs, timolol and procainamide, were tested. Timolol is a beta adrenergic blocking agent which, like dl propranolol and oxprenolol, is non selective. It has, however, minimal agonist activity, and no membrane stabilising activity. Procainamide belongs to the quinidine like group of drugs and is a pure membrane stabilising compound. It is used clinically as an antiarrhythmic agent.

7.3.2 Methods and materials

Table 16 shows the injections given to each group.

Timolol maleate - was supplied as the powder by Merck, Sharp and Dohme Development laboratories (batch 691). It was made up to a concentration of 0.2 mg/ml in water for injections and stored in the dark at $4^{o}C$.

Procainamide HCl (Pronestyl, Squibb) was diluted from the commercially available 10% solution to make a concentration of 2 mg/ml in 0.9% sodium chloride. Storage was as above.

Table 16

Triac with timolol and procainamide

Drugs and Doses

Group	Treatment	Dose
1	Buffer (Control)	0.6 ml
2	Triac	60 µg
3	Triac + Timolol	60 µg 0.05 mg
4	Triac + Timolol	60 µg 0.1 mg
5	Triac + Timolol	60 µg 0.2 mg
6	Timolol	0.2 mg
7	Triac + Procainamide	60 µg 1.25 mg
8	Triac + Procainamide	60 µg 2.5 mg
9	Triac + Procainamide	60 µg 5.0 mg
10	Procainamide	5.0 mg

7.3.3 Results

No abnormalities were found during pregnancy in any of the groups, although the lowest pregnancy rate was in the triac alone treated group (see table 17). Two rats, one receiving triac + timolol (0.1 mg) and the other receiving triac + procainamide (2.5 mg) had longer than normal periods of gestation. As this was an unusual finding and the times were 3 to 4 days longer than normal, it could have meant that a plug was found but fertilisation did not take place until mating at the next cycle.

Lower percentage live birth rates were found in the groups receiving triac with procainamide and one rat in group 9 had all her litter die by 48 hours. This may have been due to lack of milk production as none of the offspring appeared to have been fed.

7.3.3.1 Macroscopic findings (see table 18)

No pattern of change was observed between groups when comparing heart weights as a percentage of total body weights.

7.3.3.2 Light microscopic findings

Results for cell diameters are given in table 19. The cell diameters for the control groups, buffer and antagonist alone, at both 2 and 7 days, did not differ significantly from each other (except the procainamide alone treated group at 7 days which had slightly smaller diameters). Triac increased the cell diameters and this hypertrophy was not altered by timolol at either 2 and 7 days. When procainamide was given, on the other hand, the cell diameters were reduced compared with triac alone, but, in most cases, were still higher than the controls.

7.3.3.3 Electron microscopic findings

Results for disarray and crossover are given in table 20. A few foci of disarray were seen in the control groups, with two rats in the timolol alone treated group (day 2) showing higher scores than normal for these groups. Consistently high scores were seen in the triac alone treated group and also in the triac plus timolol treated groups. Two rats in the groups receiving higher doses of timolol showed scores slightly lower than for triac alone but overall the results indicate that timolol, at the doses used, did not prevent triac induced disarray.

By contrast, when procainamide was given with triac the scores were all lower than for triac alone, but not as low as control levels. There did not appear to be any dose relationship.

Scores for hypertrophy are given in table 21. Positive scores were seen in some of the control rats but higher scores were consistently seen in the triac alone treated group. When timolol was given with triac, scores for hypertrophy were observed in virtually all rats, but fewer scores of 2, and none of 3, were observed. When procainamide was given with triac some scores for hypertrophy were noted, but several scores of zero were also recorded.

Table 17

Triac with timolol and procainamide

Details of Pregnancies

Group	No. of pregnancies	Average litter size	% live births	Gestation time-days
1	2/3	9	100	23 - 24
2	1/3	12	92	22
3	3/3	10.3	100	20 - 22
4	3/3	8.3	100	21 - 26
5	2/3	9.5	100	20 - 22
6	2/3	11.5	100	22
7	2/3	9.5	83	22
8	3/3	9	96	21 - 25
9	3/3	11.7	97[1]	22
10	3/3	10	100	21 - 22

[1] 97% were born live but by 48 hours, in one litter, all the siblings (12) were dead.

Table 18

Triac with timolol and procainamide

Heart weights expressed as a percentage of total body weight

Group	2 day rats	7 day rats
1	0.605 ± 0.044	0.640 ± 0.041
2	0.603 ± 0.051	0.719 ± 0.022
3	0.668 ± 0.058	0.696 ± 0.017
4	0.593 ± 0.067	0.629 ± 0.104
5	0.583 ± 0.036	0.602 ± 0.029
6	0.624 ± 0.063	0.638 ± 0.055
7	0.651 ± 0.041	0.635 ± 0.054
8	0.553 ± 0.014	0.709 ± 0.063
9	0.604 ± 0.073	0.660 ± 0.061
10	0.652 ± 0.050	0.687 ± 0.007

Table 19a

Triac with timolol and procainamide

Results: Cell diameters - histological assessment

2 DAY RATS

Group	n	mean cell diameter μm	S.D.	p1	p2
1 Control	200	8.00	1.22		
2 Triac 60 μg	200	9.99	1.57	< 0.001	
3 Triac 60 μg + Tim 0.05 mg	200	9.93	1.65	< 0.001	NS
4 Triac 60 μg + Tim 0.1 mg	200	10.09	1.51	< 0.001	NS
5 Triac 60 μg + Tim 0.2 mg	200	9.60	1.63	< 0.001	NS
6 Tim 0.2 mg	200	8.20	1.41	NS	< 0.001
7 Triac 60 μg + Proc 1.25 mg	200	8.56	1.38	< 0.001	< 0.001
8 Triac 60 μg + Proc 2.5 mg	200	8.85	1.52	< 0.001	< 0.001
9 Triac 60 μg + Proc 5.0 mg	200	8.85	1.44	< 0.001	< 0.001
10 Proc 5.0 mg	200	8.15	1.35	NS	< 0.001

Tim = Timolol

Proc = Procainamide

p1: Compared with group 1

p2: Compared with group 2

NS: Not significant

n: No. of cells measured

Table 19b

Triac with timolol and procainamide

Results: Cell diameters - histological assessment

7 DAY RATS

Group	n	mean cell diameter µm	S.D.	p1	p2
1 Control	200	8.69	1.26		
2 Triac 60 µg	200	9.98	1.63	< 0.001	
3 Triac 60 µg + Tim 0.05 mg	200	10.52	1.60	< 0.001	< 0.005
4 Triac 60 µg + Tim 0.1 mg	200	9.85	1.55	< 0.001	NS
5 Triac 60 µg + Tim 0.2 mg	200	9.68	1.49	< 0.001	NS
6 Tim 0.2 mg	200	8.50	1.20	NS	< 0.001
7 Triac 60 µg + Proc. 1.25 mg	200	8.92	1.37	NS	< 0.001
8 Triac 60 µg + Proc 2.5 mg	200	9.22	1.36	< 0.001	< 0.001
9 Triac 60 µg + Proc 5.0 mg	200	9.17	1.31	< 0.001	< 0.001
10 Proc 5.0 mg	200	8.25	1.31	< 0.001	< 0.001

p1: Compared with group 1

p2: Compared with group 2

NS: Not significant

n: No. of cells measured

Table 20

Triac with timolol and procainamide

Results: E.M. score for disarray and crossover

GROUP	2 DAY RATS				7 DAY RATS			
1. Control	2	3	3	2	0	0	0	1
2. Triac 60 µg	10	9	15	11	7	6	15	14
3. Triac 60 µg + Tim 0.05 mg	10	13	14	14	12	6	8	7
4. Triac 60 µg + Tim 0.1 mg	8	6	15	8	9	3	6	8
5. Triac 60 µg + Tim 0.2 mg	9	15	3	15	6	8	7	2
6. Tim 0.2 mg	0	6	7	2	0	0	0	0
7. Triac 60 µg + Proc 1.25 mg	5	2	1	2	7	3	2	5
8. Triac 60 µg + Proc 2.5 mg	3	8	3	1	5	2	1	4
9. Triac 60 µg + Proc 5.0 mg	4	1	3	1	4	5	0	4
10. Proc 5.0 mg	3	1	2	0	1	2	1	0

Table 21

Triac with timolol and procainamide

Results: E.M. score for hypertrophy

GROUP	2 DAY RATS				7 DAY RATS			
1. Control	0	1	0	1	0	0	1	0
2. Triac 60 µg	2	1	3	2	1	1	2	1
3. Triac 60 µg + Tim 0.05 mg	2	1	1	1	1	2	1	1
4. Triac 60 µg + Tim 0.1 mg	1	1	1	1	1	2	1	2
5. Triac 60 µg + Tim 0.2 mg	1	1	2	2	0	2	1	1
6. Tim 0.2 mg	0	0	1	0	0	0	0	0
7. Triac 60 µg + Proc 1.25 mg	2	0	0	0	2	1	1	1
8. Triac 60 µg + Proc 2.5 mg	0	2	1	0	1	0	1	1
9. Triac 60 µg + Proc 5.0 mg	0	2	1	0	1	1	0	1
10. Proc 5.0 mg	0	0	0	0	1	0	1	0

7.3.4 Discussion

The results show that timolol, a pure beta adrenergic blocking agent with no membrane stabilising activity (Hall et al 1975) did not prevent triac-induced myocardial damage. Procainamide, a pure membrane stabilising drug reduced both disarray and hypertrophy, though, at the doses used, not to control levels.

It would thus appear that beta blockade does not prevent the action of triac, which is therefore likely to have a direct action on the cell membrane which can be prevented by the action of membrane stabilising drugs.

The doses of timolol were 5 times less than those of propranolol so that the equivalent beta blocking dose was used (Scriabine et al 1973). Table 20 hints that a higher dose might have produced a significant reduction in disarray. This does not rule out membrane stabilising action as, although Hall et al (1975) found no such action, Scriabine et al (1973) did find some which was approximately 5 times less than that of propranolol. Alternatively there could be a limited involvement of beta receptors, which would also account for the difference between the results of d and dl propranolol.

The doses of procainamide were approximately 5 times more than those of propranolol as clinically effective doses for controlling arrhythmias are of a similar ratio (Martindale Pharmacopea). Quinidine itself was not used because of lack of availability.

Electrophysiological cycle of the heart

In order to propose a theory of how triac may act it is pertinent here to briefly review the electrophysiological cycle of the heart.

From this point of view there are two types of cell in the heart. The first type is found in the sino atrial and atrioventricular nodes and the conducting system and these cells are able to discharge automatically. Under normal conditions the sinoatrial node controls the rate of contraction of the heart. The second type is the muscle cell, which does not have the capacity to discharge spontaneously, but when an impulse reaches it through the conducting system the potential rapidly reverses, i.e. it depolarises and the cell contracts. The phases of contraction are:-

0- rapid depolarisation of the cell membrane which is associated with the inflow of sodium ions.

1- a short initial period of rapid repolarisation, which leads to

2- a period of more gradual repolarisation in which there is a movement of calcium ions into the cell.

3- is a second period of repolarisation during which potassium ions move out of the cell until

4- which is the fully repolarised state during which potassium moves in and sodium out of the cell again to enable the next cycle to begin. During this phase the pacemaker cells discharge slowly to reach a critical threshold at which rapid depolarisation begins.

The antiarrhythmic drugs have been classified according to their electrophysiological properties (Vaughan Williams 1970) and those with membrane stabilising activity were called class 1. They express a number of effects including reducing the rate of spontaneous depolarisation of pacemaker cells (phase 4) and increasing the threshold at which pacemaker cells discharge. Probably more relevant to the action of triac is that procainamide also reduces the rate of rapid depolarisation of cardiac cells (phase 0) and reduces the conduction velocity of these cells. The contribution of membrane stabilising drugs in the clinical treatment of arrhythmias is by reducing the automaticity of pacemaker cells and limiting the responsiveness to excitation of cardiac cells in general. At high doses the drugs affect nerves to produce local anaesthesia.

In the experimental model procainamide reduced not only the triac induced disarray, but also hypertrophy. Thus triac may be acting by increasing the excitability and contractility of the cardiac cells as they are developing.

Procainamide reduces the excitability of the heart by preventing the initial phase of depolarisation. This means that the second phase of calcium inflow will not follow. Calcium ions control the contraction of the myofibrils and as calcium antagonists have been successfully used in the treatment of hypertrophic cardiomyopathy they may show some effect on the experimental model.

7.4 Conclusion

Triac, when administered to pregnant rats, produced, in the young offspring, cardiac hypertrophy and increased intracellular disarray. The level of disarray was reduced by the concurrent administration of oxprenolol, dl and d propranolol, and procainamide, but not by timolol. It would thus appear that the most important property of these drugs in preventing the action of triac is that of membrane stabilisation. The increased dose of oxprenolol required to prevent the triac induced disarray suggests a minimal involvement of beta receptors.

8. The effects of compounds which alter intracellular calcium levels, on triac induced myocardial abnormalities

8.1 Verapamil

8.1.1 Introduction

Clinical treatment by drugs of hypertrophic cardiomyopathy has mostly involved the use of beta adrenergic blocking agents. These can be effective in the relief of symptoms but do not alter the disease process. More recently a second group of drugs, the calcium inhibitors, have been successfully used with reports suggesting superiority over beta blockers (Rosing et al 1979a,b).

Linder (1960) first observed that prenylamine, a newly developed coronary dilator, depressed cardiac performance in canine heart-lung preparations. Soon after this Haas and Hartfelder (1962) noted that verapamil, another phenylalkaline with coronary dilating properties, exerted negative inotropic effects on isolated cat and rabbit myocardium. As the effects of these drugs were opposite to those elicited by catecholamines, they were thought to be

adrenergic blocking agents (Melville and Benfey 1965, Haas and Busch 1967). However, Fleckenstein et al (1967) recognised a difference when they noted that the drugs depressed contractility without altering the height and contour of the monophasic action potential and concluded that the agents acted as uncouplers of excitation contraction coupling. The action of the drugs was attributed to an inhibition of the influx of calcium ions into myocardial cells. Fleckenstein (1970) later specified that these "calcium antagonists" acted by reversibly sealing specific calcium channels in the membrane of the mammalian myocardial cell.

As hypertrophic cardiomyopathy is associated with hypercontractility of the heart and increased cell calcium content can cause this (Nayler 1967), the calcium antagonists, notably verapamil, have been used to treat the condition (see section 2.7.1). It was therefore decided to use the neonatal rat model of triac induced cardiac hypertrophy and intracellular disarray to observe the influence of verapamil.

8.1.2 Materials and methods

Table 22 shows the injections given to each group.

Verapamil (Abbott Laboratories Ltd.) was supplied as verapamil hydrochloride powder and was made up to a concentration of 2.5 mg/ml in water for injections. Storage was in the dark at $4^{o}C$.

Table 22

Triac with verapamil

DRUGS AND DOSES

GROUP	TREATMENT	DOSE
1	Buffer	0.6 ml
2	Triac	60 µg
3	Triac + Verapamil	60 µg 1 mg
4	Triac + Verapamil	60 µg 2 mg
5	Triac + Verapamil	60 µg 4 mg
6	Verapamil	4 mg

8.1.3 Results

Table 23 (page 180) shows the details of the pregnancies. Several abnormalities were noted. The overall pregnancy rate was low, with only groups 1 (control) and 3 (triac + verapamil 1 mg) having more than one rat becoming pregnant. In group 2 (triac alone) only 1 became pregnant and in groups 4 and 5 (triac with 2 and 4 mg verapamil respectively) none became pregnant. In group 6 (verapamil 4 mg alone) two became pregnant but one died very close to term. At post mortem two fully grown foetuses were found to be present and there were no gross abnormalities observed in either the foetuses or the mother. The other rat in this group produced a relatively small litter and all the offspring were alive. In the other groups the rats which became pregnant all had litters of normal sizes and there were no deaths among the offspring.

8.1.3.1 Macroscopic findings

Table 24 shows the results of heart weights expressed as a percentage of total body weights for each group. The heart weights for group 2 (triac alone) are higher than the controls at 2 and 7 days but the significance levels is low ($0.01<p<0.5$). The varying weights in the other groups are

probably due to experimental variation in the removal of the hearts. The speed at which the hearts have to be removed, weighed, sectioned and fixed is such that the amount of great vessels removed with the heart each time may account for the variations in heart weights.

8.1.3.2 Light microscopic findings

The hearts of the two foetuses from the dead mother in group 6 showed signs of autolysis, but no other abnormalities were present. The heart of the dead mother also appeared normal.

Table 25 shows the results of measurements of cell diameters. At both 2 and 7 days cell diameters were increased when compared with the control group, and this hypertrophy was reduced, though not totally prevented, by verapamil. Unfortunately there are no data for groups 4 and 5. Group 6 was the highest dose of verapamil (4 mg), given alone. In this group the cells were hypertrophied and at 2 days the cell diameters were not significantly different from those of the triac alone treated group.

8.1.3.3 Electron microscopic findings

Results for disarray and crossover are given in table 26. As there was a low pregnancy rate in this experiment the number of hearts of offspring analysed were fewer than previous experiments.

Some foci of disarray were observed in some of the control rats at both 2 and 7 days but higher scores were seen in group 2 (triac alone). When verapamil, 1 mg, was given with triac, the amount of disarray was reduced to control levels. In group 6, verapamil alone at 4 mg, one 7 day rat received a score of 4 but control levels were noted in all other rats.

Results for hypertrophy are given in table 27.

In this experiment the triac induced hypertrophy was reduced when verapamil at 1 mg was administered. This is illustrated in Fig. 22. By contrast, when verapamil at 4 mg per day was given alone, hypertrophy was observed in all the rats (see Fig. 23).

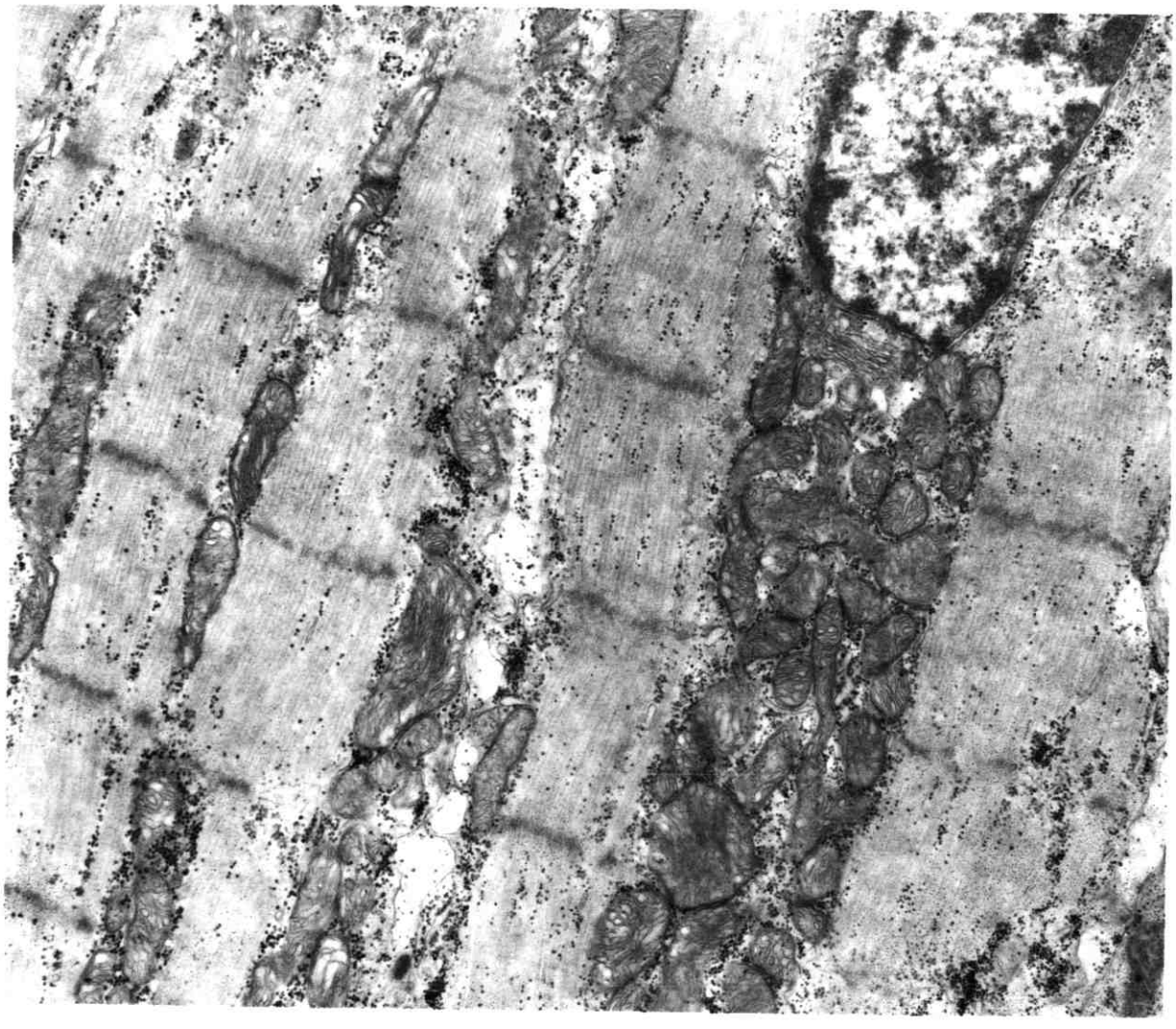


Fig. 22. Electronmicrograph illustrating normal myocardium (7 day offspring from triac + verapamil treated rat). Accumulation of mitochondria at the nuclear pole is a normal feature. x 19,600

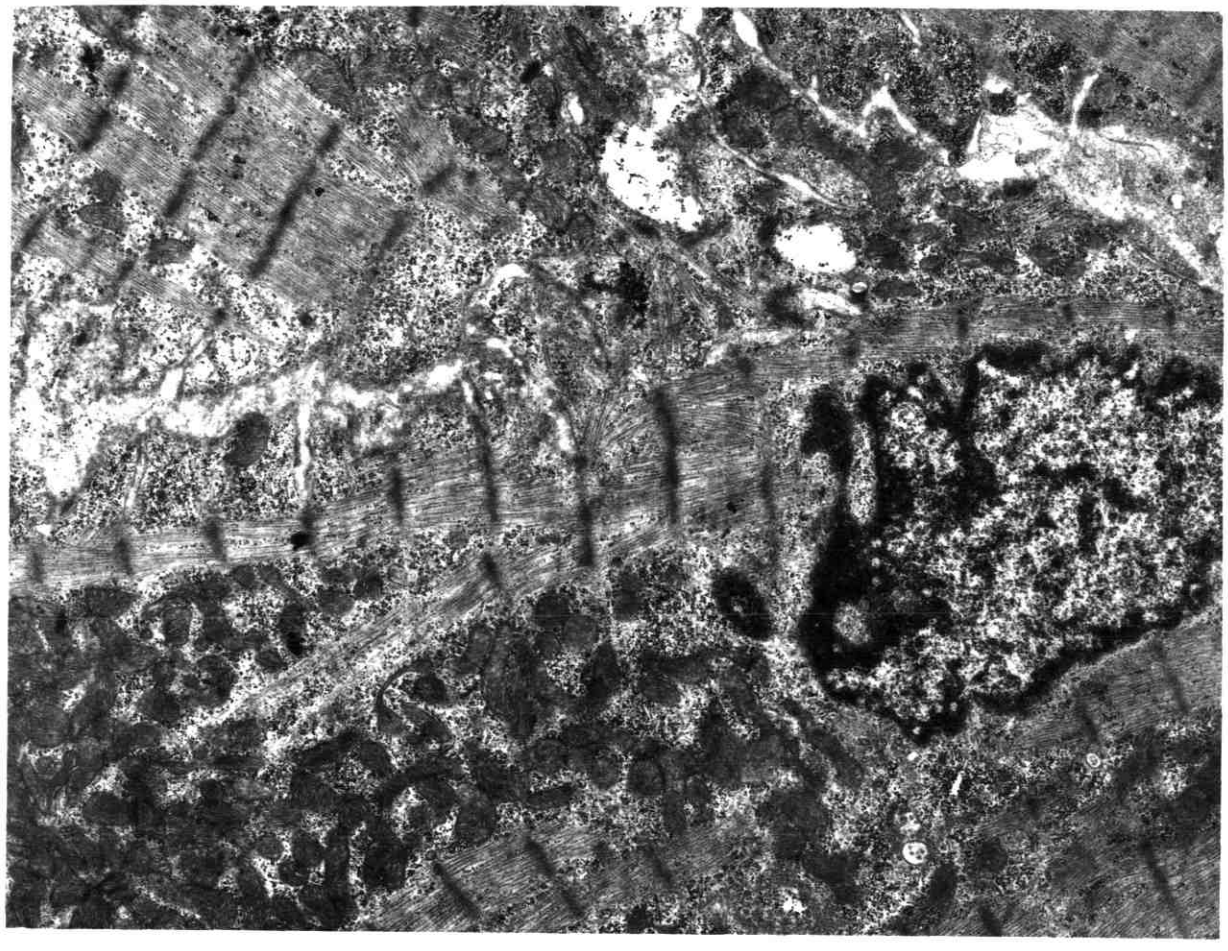


Fig. 23. Electronmicrograph illustrating verapamil induced hypertrophy. The nuclear membrane is crenated. (2 day offspring from verapmil (4mg) treated rat). x 11,800

Table 23

Triac with verapamil

Details of Pregnancies

Group	No. of pregnancies	Average litter size	% live births
1	2/3	11.5	100
2	1/3	9	100
3	3/3	10.7	100
4	0/3		
5	0/3		
6	1/3[1]	5	100

[1] 1 additional mother in group 6 became pregnant but died on approx. day 20. Two foetuses were present.

Table 24

Triac with verapamil

Heart weights expressed as a percentage of total body weight

Group	2 day rats	7 day rats
1. Control	0.601 ± 0.050 n = 5	0.508 ± 0.043 n = 4
2. Triac 60 µg	0.642 ± 0.040 n = 3	0.639 ± 0.077 n = 4
3. Triac 60 µg + verap 1 mg	0.558 ± 0.076 n = 6	0.652 ± 0.046 n = 4
6. Verap 4 mg	0.605 ± 0.028 n = 3	0.622 ± 0.012 n = 2

verap = verapamil

n = No. of hearts

Table 25

Triac with verapamil

Results: Cell Diameters - histological measurement

2 DAY RATS

Group	n	mean cell diameter μm	S.D.	p1	p2
1 Control	200	7.22	1.06		
2 Triac 60 μg	200	8.20	1.11	< 0.001	
3 Triac 60 μg + verap 1 mg	200	7.51	1.07	< 0.01	< 0.001
6 Verap 4 mg	200	8.07	1.22	< 0.001	N.S.

7 DAY RATS

Group	n	mean cell diameter μm	S.D.	p1	p2
1 Control	200	8.41	1.12		
2 Triac 60 μg	200	9.41	1.38	< 0.001	
3 Triac 60 μg + verap 1 mg	200	8.56	1.41	N.S.	< 0.001
6 Verap 4 mg	200	8.97	1.48	< 0.001	0.01

p1: compared with Group 1

p2: compared with Group 2

NS: Not significant

n: No. of cells measured

Table 26

Triac with verapamil

Results: E.M. score for disarray and crossover

Group	2 day rats	7 day rats
1. Control	1 0 1 2 0	2 1 2 1
2. Triac 60 µg	8 7 9	8 4
3. Triac 60 µg + verap 1 mg	3 3 2 2 1 1	1 1 1 0
6. Verap 4 mg	1 0 3	4 0

Table 27

Triac with verapamil

Results: E.M. score for hypertrophy

Group	2 day rats	7 day rats
1. Control	1 1 0 0 0	0 1 0 0
2. Triac 60 µg	2 2 1	1 1
3. Triac 60 µg + verap 1 mg	0 0 1 1 0 1	0 1 0 0
6. Verap 4 mg	1 2 2	2 1

8.1.4 Discussion

The calcium antagonist verapamil reduced both the triac induced disarray and cardiac hypertrophy when 1 mg per day was administered. The higher doses, however, apparently had damaging effects, with many rats not becoming pregnant and one dying. In the one rat receiving verapamil alone at 4 mg which gave birth, hypertrophy was observed in the myocardium of all the offspring.

The importance of calcium in cardiac contraction has long been recognised (Ringer 1883). The transition from resting to the active state is initiated by cell depolarisation, the transmembrane action potential, which is composed of an initial sharp spike followed by a prolonged plateau. The former is caused by the movement of sodium ions into the cell, whereas the latter results mostly from the inward movement of calcium (Tarr et al 1973). This elevation of intracellular calcium concentration removes the inhibitory influence of the troponin -tropomysin complex on the interaction between actin and myosin and thus contraction ensues (Ebashi and Endo 1968). Alternatively Fabiato & Fabiato (1975, 1978) have proposed a model in which a small amount of Ca^{2+} enters the cardiac cell and controls a graded release of Ca^{2+} from the sarcoplasmic reticulum, which supports contraction. Bers et al (1981) later suggested that the frog, neonatal rat and rabbit ventricles depend more directly on the entry of calcium ions from sarcolemmal sites for the control of tension development, whereas the adult rat ventricle and rabbit atrium depend to a greater extent on calcium released from the sarcoplasmic reticulum.

The sodium and calcium ions enter the cell through channels in the cell membrane, the former through the so-called fast channel and the latter through the slow channel. During excitation the free calcium concentration can double from the normal of 10^{-7}M and this leads directly or indirectly to contraction. A transport system in the sarcoplasmic reticulum with a high affinity for calcium ions reduces the calcium concentration again and therefore causes relaxation of the contractile proteins. The calcium ions are then stored in the sarcoplasmic reticulum but to avoid overload these ions are pumped out of the cell across the cell membrane by a transport system involving exchange of external sodium ions for internal calcium ions (Na-Ca exchange). Mechanisms proposed for inward calcium movement during action potential and outward calcium transport have been reviewed by Reuter (1974).

In the atrium of some species, such as the rat and frog, the slow current is carried by both calcium and sodium (Rougier et al 1969, Garnier et al 1969). The influx of sodium through the fast channel can be blocked by local anaesthetic drugs such as procainamide. This blockade may be the action by which both propranolol isomers reduce the triac induced disarray. Verapamil exerts its action by preventing the influx of calcium in the slow channel.

In very young hearts, such as the three day chick embryo there is no fast inward sodium channel, both ions entering via the slow channel, and verapamil blocks the slow inward flow of sodium as well as calcium (Shigenbu et al 1974). Bayer et al (1975a, b, c) have shown that verapamil consists of two isomers and the calcium antagonistic activity is bound to the l form. The d form mainly blocks the fast inward sodium channels, thus the racaemic mixture probably acts in the same way as propranolol as well as blocking the calcium inflow.

Anderson (1978) has reviewed the action of calcium and the antagonists on excitation-contraction coupling and Henry (1980) has described the comparative pharmacology of the calcium antagonists.

In the experiment described here a low dose, 1 mg, of verapamil was shown to reduce both the level of disarray and the hypertrophy caused by triac. It therefore provides an advantage over beta blockers which do not appear to affect the hypertrophy. Reduction of hypertrophy due to anoxia has also been shown in rats by Genoveses et al (1980) using verapamil. Clinically Kaltenbach et al (1979) found a reduction in heart size of patients with hypertrophic cardiomyopathy treated with verapamil.

It is not known why the high dose of verapamil (4 mg) alone produced hypertrophy in the offspring of the treated mothers but the findings may not be so unexpected in view of some electrophysiological findings. In doses above those blocking the slow responses, verapamil has been shown to cause a small depolarisation (Singh and Vaughan Williams 1972, Haeuster 1972). The mechanism for this is unknown but it may result from partial inhibition of Na^+K^+ pumping or from a decrease in the ratio of K^+ to Na^+ conductances (Shigenbu et al 1974).

In high doses, verapamil has toxic effects. It is not possible to ascertain whether or not verapamil was responsible for the fact that there were no births in groups 4 and 5. Data subsequently supplied by Abbott Laboratories Ltd. stated that an increased rate of foetal absorption occurred at an oral dose of 60 mg/kg in rats. The intramuscular doses used in this experiment were 8 and 16 mg/kg and this may have been approaching an equivalent oral dose to that above. In post mortems conducted at the end of the experiment, however, there was no evidence of pregnancies having been present.

In subacute toxicity tests intravenous doses of 0.2 to 10 mg/kg were tolerated well over 4 weeks. At 5.0 mg/kg the general condition was slightly disturbed. In acute toxicity tests the LD50 was 16 mg/kg for intravenous and 67 mg/kg for

intraperitoneal verapamil. Abbott Laboratories Ltd. did not supply the intramuscular dose level but it is possible that the 16 mg/kg dose of verapamil used could have been responsible for the death of the rat in group 6. Clinically, high doses of verapamil (80 x 80 mg tablets taken as an overdose) cause sinus bradycardia, a third degree AV block, asystole, depression of myocardial contractility and hypotension (Immonen et al 1981).

In a trial of long term treatment of hypertrophic cardiomyopathy by verapamil Rosing et al (1981) concluded that verapamil plays an important role for the treatment of the condition but patients must be carefully selected and followed up closely for the development of adverse haemodynamic and electrophysiological effects.

The conclusion from this experiment is that a low dose of verapamil is effective in reducing both the triac induced disarray and hypertrophy but a high dose has toxic effects, including the production of hypertrophy in the offspring. As calcium would thus appear to have major importance in the production of disarray and hypertrophy, a compound increasing levels of available calcium ions will be of interest if administered to pregnant rats.

8.2 Calcium ionophore A23187

8.2.1 Introduction

Previous experiments have shown how various drugs with differing properties protect the myocardium from the effects of triac. However, the damage caused by triac has so far only been observed at the ultrastructural level, with only hypertrophy diagnosable at the histological level. As part of a search to produce an animal model showing some of the other pathological features found in hypertrophic cardiomyopathy in man, for example disarray of the fibres and asymmetric septal hypertrophy, it was decided to find out if the damage caused by triac could be made worse. It is known that this cannot be done by simply raising the dose of triac as higher doses than 60 μg per day caused pregnancy problems and maternal deaths (Hawkey, unpublished data).

Experiments carried out so far have suggested that triac has a direct action on the cell membrane. The likely result of this is an increased entry of calcium ions into the cardiac cell which could be the main cause of the damage. An increase in the availability of calcium could perhaps worsen the effects of triac. In order to do this a calcium ionophore (A23187) was used. This compound is a divalent cation specific ionophore which facilitates the passage of calcium across cell membranes. Thus the level of intracellular calcium should be raised.

8.2.2 Materials and methods

Table 28 shows the injections given to each group.

A23187 Calcium ionophore was supplied as the free acid by Calbiochem-Behring Corp. 10 mg of powder was dissolved in 1 ml of ethanol with the aid of ultrasonic agitation to ensure complete dissolution. This stock solution was kept in the dark at room temperature. On each day of injections an aliquot of 50 µl was removed and added to 5 ml of rapidly mixing 5% dextrose. The resulting solution of 100 µg/ml was colloidal, giving a faintly opalescent appearance, and was used for the injections.

This experiment was carried out at the same time as triac with timolol and procainamide and therefore the results for buffer and triac alone treated rats are the same.

8.2.3 Results

Details of pregnancy are given in table 29 (page 196).

No abnormalities were found during pregnancy and although a low rate of pregnancy was found in the groups receiving triac and the low doses of A23187, all became pregnant in the high dose group and the A23187 alone group. The one rat which did become pregnant in group 3 (triac + A23187 -15 µg) had a larger than normal litter (17 babies) but by 48 hours these were all dead. Also one of the rats in group 6 (A23187 -60 µg alone) gave birth to 3 dead plus 8 which died within 24 hours. Periods of gestation were normal in these rats.

Table 28

Triac with A23187

Drugs and Doses

Group	Treatment	Dose
1	Buffer (Control)	0.6 ml
2	Triac	60 µg
3	Triac + A23187	60 µg 15 µg
4	Triac + A23187	60 µg 30 µg
5	Triac + A23187	60 µg 60 µg
6	A23187	60 µg

8.2.3.1 Macroscopic findings

Table 30 gives results for heart weights expressed as a percentage of total body weight. No pattern of difference was noted in any of the groups.

8.2.3.2 Light microscopic findings

Results of measurements of cell diameters are given in table 31. They show that A23187 did not alter the hypertrophy caused by triac, except in group 5 (triac + A23187 -60 μg) at 7 days, where cell diameters were greater than those of group 2 (triac alone). When A23187 was given alone, group 6, cell diameters were similar to those of group 2.

8.2.3.3 Electronmicroscopic findings

Results for disarray and crossover are given in table 32. At 2 days scores from the triac alone and triac + A23187 treated groups were very similar. When A23187 was given alone, the 2 day rats also showed high scores for disarray, for example see Fig. 24. However, at 7 days, scores were lower, with only one rat from the A23187 alone treated group showing disarray above control levels.

Results for hypertrophy are given in table 33. At 2 days, scores indicating the presence of hypertrophy were given in all rats whose mothers received triac and/or A23187. The highest score was in the triac alone treated group. At 7 days scores were less than at 2 days but hypertrophy was present in the triac and triac + A23187 treated groups. Only two rats gave positive scores in the A23187 alone treated group at 7 days.

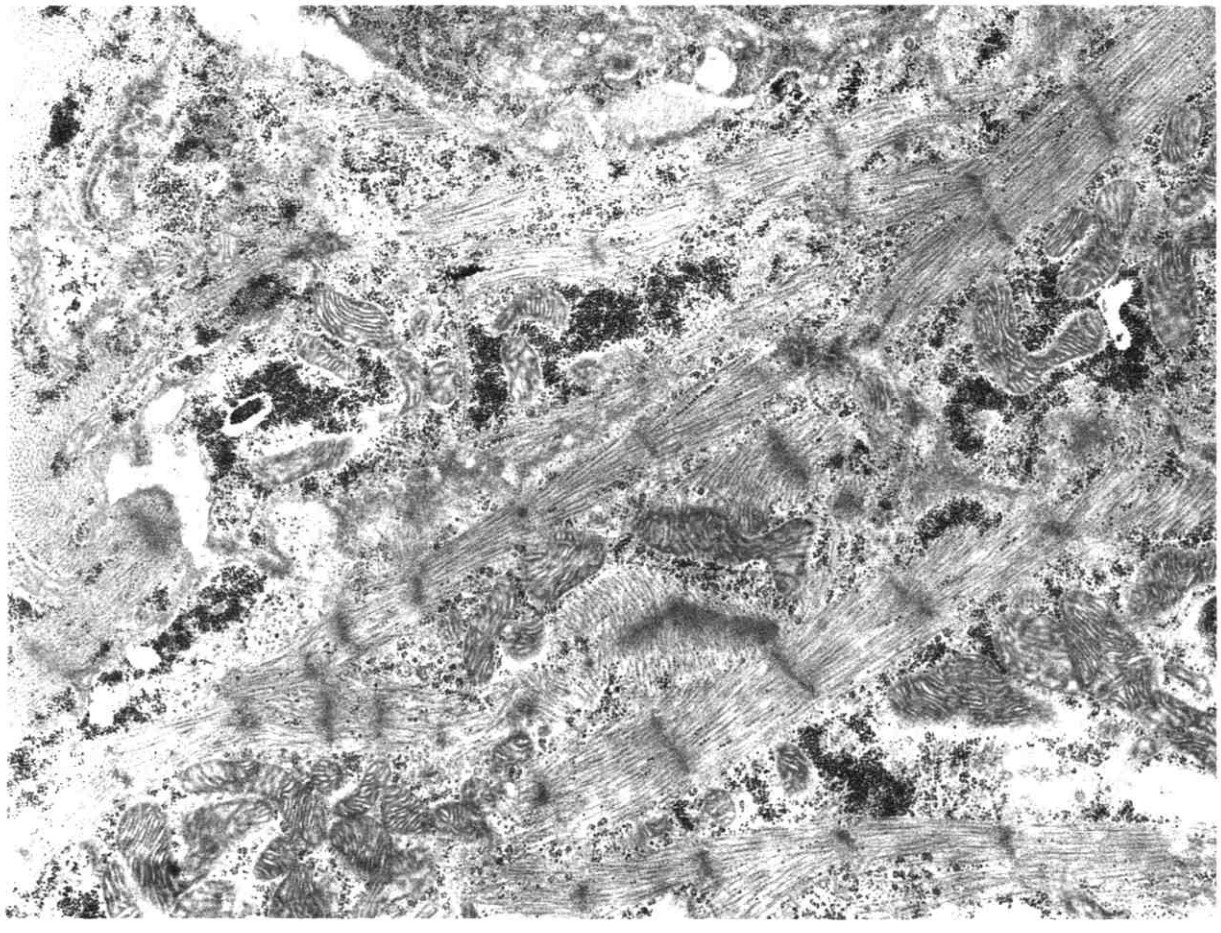


Fig. 24. Electronmicrograph illustrating disarray of myofibrils. (2 day offspring from A23187 (60μg) treated rat). x 14,500

Table 29

Triac with A23187

Details of Pregnancies

Group	No. of pregnancies	Average litter size	% live births	Gestation time-days
1	2/3	9	100	22
2	1/3	12	92	22
3	1/3	17	100[1]	22
4	1/3	9	100	22
5	3/3	7.7	85	22
6	3/3	10.3	94[2]	21-22

[1] All were born alive but by 48 hours all were found dead

[2] In one litter 8 out of 11 were born alive but by 24 hours these were dead.

Table 30

Triac with A23187

Heart weights expressed as a percentage of total body weight

Group	2 day rats	7 day rats
1. Control	0.605 ± 0.044	0.640 ± 0.041
2. Triac 60 µg	0.603 ± 0.051	0.719 ± 0.022
3. Triac 60 µg + A23187 15 µg	None pregnant	
4. Triac 60 µg + A23187 30 µg	0.603 ± 0.047	0.628 ± 0.079
5. Triac 60 µg + A23187 60 µg	0.624 ± 0.058	0.766 ± 0.177
6. A23187 60 µg	0.654 ± 0.022	0.691 ± 0.075

Table 31a

Triac with A23187

Results: Cell diameters - histological measurement

2 DAY RATS

Group	n	mean cell diameter µm	S.D.	p1	p2
1 Control	200	8.00	1.22		
2 Triac 60 µg	200	9.99	1.57	< 0.001	
3 Triac 60 µg + A23187 15 µg	None pregnant				
4 Triac 60 µg + A23187 30 µg	200	9.77	1.41	< 0.001	N.S.
5 Triac 60 µg + A23187 60 µg	200	10.34	1.61	< 0.001	N.S.
6 A23187 60 µg	200	9.53	1.76	< 0.001	N.S.

p1: Compared with group 1

p2: Compared with group 2

NS: Not significant

n: No. of cells measured

Table 31b

Triac with A23187

Results: Cell diameters - histological measurement

7 DAY RATS

Group	n	mean cell diameter µm	S.D.	p1	p2
1 Control	200	8.69	1.26		
2 Triac 60 µg	200	9.98	1.63	< 0.001	
3 Triac 60 µg + A23187 15 µg	None pregnant				
4 Triac 60 µg + A23187 30 µg	200	10.10	1.41	< 0.001	N.S.
5 Triac 60 µg + A23187 60 µg	200	10.51	1.55	< 0.001	< 0.001
6 A23187 60 µg	200	10.41	1.62	< 0.001	< 0.01

p1: Compared with group 1

p2: Compared with group 2

NS: Not significant

n: No. of cells measured

Table 32

Triac with A23187

Results: E.M. score for disarray and crossover

Group	2 day rats	7 day rats
1. Control	2 3 3 2	0 0 0 1
2. Triac 60 µg	10 9 15 11	7 6 15 14
3. Triac 60 µg + A23187 15 µg	None pregnant	
4. Triac 60 µg + A23187 30 µg	16 16 12 8	3 1 8 3
5. Triac 60 µg + A23187 60 µg	14 12 13 15	4 7 11 9
6. A23187 60 µg	11 16 13 12	1 3 8 1

Table 33

Triac with A23187

Results: E.M. score for hypertrophy

Group	2 day rats	7 day rats
1. Control	0 1 0 1	0 0 1 0
2. Triac 60 µg	2 1 3 2	1 1 2 1
3. Triac 60 µg + A23187 15 µg	None pregnant	
4. Triac 60 µg + A23187 30 µg	2 2 1 1	1 1 2 2
5. Triac 60 µg + A23187 60 µg	1 2 1 1	2 1 0 1
6. A23187 60 µg	2 1 1 1	1 0 1 0

8.2.4 Discussion

The results show that although the calcium ionophore A23187 did not actually worsen the effects of triac it did have an effect when given on its own, causing both disarray and hypertrophy. Results from electronmicroscopic and histological analysis showed closer agreement at 2 days than at 7 days. The finding that A23187 has its own effects is important as it has a much more specific action than is known for triac.

A23187 is a carboxylic acid antibiotic which is crystallised from broths of Streptomyces chartreusenis and it uncouples oxidative phosphorylation and inhibits ATPase of rat liver mitochondria (Reed and Lardy 1972). It has weak antimicrobial activity against gram positive organisms (Calbiochem literature on properties of A23187). Most publications on calcium ionophores have been on in-vitro usage, using them as biochemical tools to carry ions, but Pressman, who carried out many initial experiments with ionophores, has written an extensive review on their biological applications (Pressman 1976). He chose the name ionophore, meaning ion bearer, and in the review covers all types of ionophores.

In-vivo experiments have been carried out using the ionophore X537A on intact dogs and this was found to cause a) a 2-3 fold increase in myocardial contractility, b) a doubling of cardiac output, c) a modest rise in aortic pressure and heart rate, d) a drop in total peripheral resistance, and e) a decrease in coronary artery resistance leading to as much as a ten times increase in blood flow through the coronary arteries. However X537A also binds monovalent ions and catecholamines and so it was not sure how much calcium was involved. A23187, on the other hand, is specific for divalent ions and Holland et al (1975) found that on isolated guinea pigs left atria it increased the force of contraction and the rate of tension development. This was not mediated by the activation of beta receptors or by the release of endogenous catecholamines. They believed that the ionophore was acting by increasing the calcium influx across the myocardial cell membrane or by facilitating the release of calcium from the sarcoplasmic reticulum. This work was on an isolated preparation, not on the intact animal and Pressman (1976) believed that the results could be different in-vivo.

However, as A23187 is a specific divalent ionophore, it was decided to use it in the experiment described. The results add support to the theory that disarray could be caused by an increased influx of calcium into the cells of the developing heart muscle.

The doses of ionophore used were calculated from toxicity test information supplied by the manufacturer. The LD50 from intravenous injections into rats was found to be 2.25 to 3.00 mg/kg. The highest dose used was 60 µg, i.e. 240 µg/kg which was thought would be large enough to be effective on the foetus but small enough to not affect the mother.

8.3 Conclusion

Verapamil blocks the entry of calcium into the cells. The low dose of 1 mg prevented the triac induced disarray and also reduced the level of hypertrophy but the high dose of 4 mg had toxic effects.

A23187 is a calcium ionophore, which allows greater flow of calcium into the cell. This did not affect the actions of triac, but, when given alone, caused cardiac hypertrophy and intracellular disarray in the 2 day offspring.

The results suggest that production of disarray and hypertrophy is via an action which results in the increased availability of intracellular calcium ions.

9. Additional experiments

The following section describes some additional experiments where the methodology varied from that previously described.

These experiments were designed to:-

1) Ascertain the timing of the action of triac on the developing heart.

2) Attempt to produce an animal model of hypertrophic cardiomyopathy in the adult rat by using triac and prenalterol.

3) Assess how thyroxine affects the developing myocardium.

4) Exclude the possibility that triac induced changes might be due to low haemoglobin levels.

5) Assess whether or not triac crosses the placenta.

9.1 An assessment of the timing of the action of triac

9.1.1 Introduction

Each experiment carried out has confirmed the report of Olsen et al (1977) which showed that cardiac hypertrophy and myofibrillar disarray can be produced in the hearts of offspring of rats treated with triac throughout pregnancy. Protocols for the injections have meant starting the day the rats were paired with males and giving daily injections 5 days per week for 3 weeks. The triac-induced damage has been observed even when vaginal plugs did not appear until several days after the experiment had started, such that once the injections stopped after 3 weeks there was still some time until they gave birth. This posed the thought that triac may have its actions over a brief period of pregnancy and an experiment was carried out in order to find out at which stage triac acts.

9.1.2 Methods

The methods were as previously described on page 98. Triac and buffer were administered as shown in table 34. To ensure a greater amount of accuracy in dating the beginning of pregnancy, vaginal smears were taken each day and a Greenstein's stain (Greenstein 1964) was carried out to look for the presence of sperm.

The experiment with group 5, triac administered in the third week only, was carried out at a separate time from the other groups, hence 2 sets of controls are presented.

Table 34

Timing of Triac

Drugs and Doses

Group	Week	Administration	Dose
1	1 2 3	Buffer " "	0.6 ml " "
2	1 2 3	Triac " "	60 µg " "
3	1 2 3	Triac Buffer Buffer	60 µg 0.6 ml 0.6 ml
4	1 2 3	Buffer Triac Buffer	0.6 ml 60 µg 0.6 ml
5	1 2 3	Buffer Buffer Triac	0.6 ml 0.6 ml 60 µg

9.1.3 Results

9.1.3.1 Light microscopic findings

Measurements of cell diameters are given in table 35. They show that at 2 days cell diameters were increased above control values in all triac treated groups, though when it was given for short periods the diameters were not as great as when it was given for the full 3 weeks. In the 7 day rats hypertrophy was observed in all triac treated groups apart from group 4, i.e. mothers who received triac only in the second week.

9.1.3.2 Electron microscopic findings

Scores for disarray and crossover are given in table 36. They show that disarray can be caused by triac even when given in only the first or second weeks, but not as much was observed when compared to administration for the full three weeks. When triac was given only during the third week, one rat at 7 days showed disarray above control levels but no others did.

Scores for hypertrophy are given in table 37. Mild hypertrophy was observed in all triac treated groups, but scores of two were found only when triac was administered for all three weeks and during the second week only.

Table 35a

Timing of Triac

Results: Cell diameters - histological measurement

2 DAY RATS

Group	n	mean cell diameter µm	S.D.	p1	p2
1 Control	200	7.22	1.06		
2 Triac 60 µg 3 weeks	200	8.20	1.11	< 0.001	
3 Triac 60 µg 1st week only	200	7.91	1.32	< 0.001	< 0.05
4 Triac 60 µg 2nd week only	200	8.07	1.24	< 0.001	N.S.
1a Control	200	8.00	1.22		
2a Triac 60 µg 3 weeks	200	9.99	1.57	< 0.001	
5 Triac 60 µg 3rd week only	200	9.59	1.79	< 0.001	N.S.

p1: compared with group 1

p2: compared with group 2

NS: Not significant

n: No. of cells measured

Table 35b

Timing of Triac

Results: Cell diameters - histological measurement

7 DAY RATS

Group	n	mean cell diameter µm	S.D.	p1	p2
1 Control	200	8.41	1.12		
2 Triac 60 µg 3 weeks	200	9.41	1.38	< 0.001	
3 Triac 60 µg 1st week only	200	9.15	1.19	< 0.001	< 0.05
4 Triac 60 µg 2nd week only	200	8.58	1.26	N.S.	<0.001
1a Control	200	8.69	1.26		
2a Triac 60 µg 3 weeks	200	9.98	1.63	< 0.001	
5 Triac 60 µg 3rd week only	200	10.17	1.54	< 0.001	N.S.

p1: Compared with group 1

p2: Compared with group 2

NS: Not significant

n: No. of cells measured

Table 36

Timing of Triac

Results: E.M. score for disarray and crossover

Group	2 day rats	7 day rats
1 Control	1 0 1 2 0	2 1 2 1
2 Triac 60 µg 3 weeks	8 7 9	8 4
3 Triac 60 µg 1st week only	4 7 2 9	5 9 1 3
4 Triac 60 µg 2nd week only	9 12 1 3	4 2 7 1
1a Control	2 3 3 2	0 0 0 1
2a Triac 60 µg 3 weeks	10 9 15 11	7 6 15 14
5 Triac 60 µg 3rd week only	3 2 1 1	6 2 1 0

Table 37

Timing of Triac

Results: E.M. score for hypertrophy

Group	2 day rats	7 day rats
1 Control	1 1 0 0 0	0 1 0 0
2 Triac 60 µg 3 weeks	2 2 1	1 1
3 Triac 60 µg 1st week only	1 1 0 1	1 1 0 0
4 Triac 60 µg 2nd week only	2 2 0 1	0 1 1 1
1a Control	0 1 0 1	0 0 1 0
2a Triac 60 µg 3 weeks	2 1 3 2	1 1 2 1
5 Triac 60 µg 3rd week only	1 1 1 0	1 0 1 1

9.1.4 Discussion

The results suggest that when causing disarray, or preventing the proper alignment of myofibrils, triac may only be acting during the first two weeks of pregnancy. This could either be due to difficulties of placental transfer, see section 9.5, or that triac acts during the stages of embryonic heart development, which is early in pregnancy.

In the rat myofibrils appear, and the first contractions are observed by 9 days, at the three somite stage. Blood flow occurs at the eight somite stage, by 9.5 days, and the heart is fully formed at 16 days (Sissman 1970). Because of availability and ease of experimental manipulation, the chick has provided the greatest number of studies of the ultrastructure of cardiac development (e.g. Manasek 1968). In the chick, randomly organised thick and thin filaments are observed as early as between 30 and 36 hours of incubation. As development progresses the myofilaments become more abundant and more orientated and begin to resemble myofibrils. At 60 hours all developing heart muscle cells contain some myofibrils and the first cross striated myofibrils of the adult heart are seen at 72 hours. Subsequently the number of myofibrils per cell increases and the fibres line up parallel to one another so that the cross striations and Z lines become aligned in register. By the 15th day the muscle cells resemble those of the adult heart

(Zak 1973). Chicks hatch at 20-21 days. Challice and Virágh (1973) and Manasek (1974) have reviewed in much closer detail the histology and ultrastructure of developing heart muscle.

Thus the heart is formed very early in pregnancy and it is likely that triac is acting during the first 2 weeks in the rat. Previous experiments have suggested that triac destabilises the cell membranes, perhaps increasing the contractility of the myofibrils as they are formed. Forces within the cell may become altered (e.g. as suggested by Bulkley et al 1977) making more difficult the proper alignment of the myofibrils. The precise mechanism by which myofibrils become aligned is unknown (Manasek 1974).

Future experiments on the timing of the action of triac could involve administering triac for shorter periods at different times during the first two weeks of pregnancy. Higher doses could be used, which may be less damaging to the mothers than when given for the full 3 weeks.

9.2 The influence of prenalterol on triac induced abnormalities in the developing rat and the action of both compounds on the growing rat.

9.2.1 Introduction

The triac induced changes in the neonatal rat hearts at electronmicroscopic level are very similar to those which the author has observed in hypertrophic cardiomyopathy in man. However, these changes have been regarded as non specific and have been observed in other conditions resulting in hypertrophy of the myocardium (Van Noorden et al 1971). Disarray of the histological level has also been noted in other conditions (Bulkley et al 1977a) but if the changes are widespread then hypertrophic cardiomyopathy is the most likely diagnosis (Maron and Roberts 1979). If disarray could be produced in the rat at both the electron and light microscopic levels, together with disproportionate hypertrophy of the interventricular septum, then an animal model of hypertrophic cardiomyopathy will have been produced. It will only be then that one can be certain that an endogenous mechanism may be in operation in the pathogenesis of the condition.

The previous experiment showed that triac may have its principle action in causing disarray early in pregnancy. However, a continuation of the stimulus of triac throughout life until maturity was hoped may produce the three characteristic features. Triac is known to cause ordinary cardiac hypertrophy after long term administration to adult rats (Symons et al 1975).

Another of the many proposals of the aetology of hypertrophic cardiomyopathy has been the involvement of catecholamines, especially in early foetal development and cardiac embryogenesis (Goodwin 1974, Perloff 1981). Catecholamines have been detected in very early embryos (Ignarro and Shideman 1968) and the adrenal foetal myocardial receptor sites are supersensitive to circulating noradrenaline (Friedman 1973). An imbalance of adrenergic stimulation during development is a possible cause of hypertrophic cardiomyopathy and it was thought that an increase in catecholamines might potentiate the triac induced changes, both in developing and growing rats.

Pagano and Inchiosa (1977) concluded that isoprenaline induced cardiac hypertrophy was via stimulation of beta 1 receptors. An adrenergic agonist with only beta 1 actions, prenalterol, was therefore used in the experiment described here, administered alone and in combination with triac.

9.2.2 Methods

Eighteen virgin females Wistar rats, each weighing approximately 200g, were divided into 6 groups of 3 rats. They were fed as previously described and table 38 shows the intramuscular injections given to each group. Each female was injected 5 days per week for 7 weeks.

A male was placed in each cage of 3 females the day the injections started and was removed on day 8. During this time the males were swapped between groups daily to eliminate the consequences of one being infertile. On day 19 the females were separated and caged individually in preparation for giving birth.

Offspring were killed at 2 and 7 days and samples taken as previously described on page 98.

After the birth of the offspring in groups 1 to 4 the mothers still continued to receive injections until the siblings were 10 days old[1]. At this age the siblings were considered old enough to be injected themselves and were given intraperitoneal injections as shown in table 39. The offspring used were female, apart from 2 in group 3. They were weighed and injected daily, 5 days per week for 3 months.

[1] It is not known whether or not triac is secreted in the milk of lactating mothers but as injected triiodothyronine appears in milk and this causes a raised level in the serum of the sucklings (Koldovský 1980) it was not unreasonable to suggest that triac may behave in a similar manner.

The experimental rats were therefore under the influence of raised levels of triac from conception to maturity.

Table 38

Triac with Prenalterol

Drugs and Doses to pregnant females

Group	Treatment	Dose
1	Buffer	0.6 ml
2	Triac	60 µg
3	Triac + Prenalterol	60 µg 2 mg
4	Prenalterol	2 mg

Table 39

Triac with Prenalterol

Drugs and Doses to Offspring

Group	Treatment	Dose
1	Buffer	Same vol. as triac solution
2	Triac	0.4 mg/kg
3	Triac + Prenalterol	0.4 mg/kg 10 mg/kg
4	Prenalterol	10 mg/kg

9.2.2.1 Perfusion fixation

At 3 months the rats were killed by whole body perfusion fixation. The apparatus used - see Fig. 25, consisted of 2 jacketed reservoirs linked by jacketed tubing to a bubble trap and cannula.

The reservoirs were positioned 4 feet above the level of the bench such that a pressure of 100 mg mercury could be maintained. The left side reservoir contained Kreb's solution, see section 9.1.3 which was gassed with 5% carbon dioxide in oxygen. The right side reservoir contained the glutaraldehyde/formaldehyde fixative solution. The water jackets surrounding the reservoir and tubing were kept at a constant temperature by circulating water at 37^{o}C. When filling the system all bubbles were removed and any later appearing would be trapped. A sphygmomanometer gauge connected to the bubble trap monitored the pressure of the system. The tubing and bung in the bubble trap were made of silicon.

The rats were weighed and anaesthetised with 0.5 mg/kg Hypnorm. This consists of 0.315 mg/ml fentanyl citrate (a narcotic analgesic with morphine like properties) and 10 mg/ml fluanisone (a neuroleptic belonging to the group of butyropherones, which induces depression of the central nervous system) (Janssen Pharmaceutica).

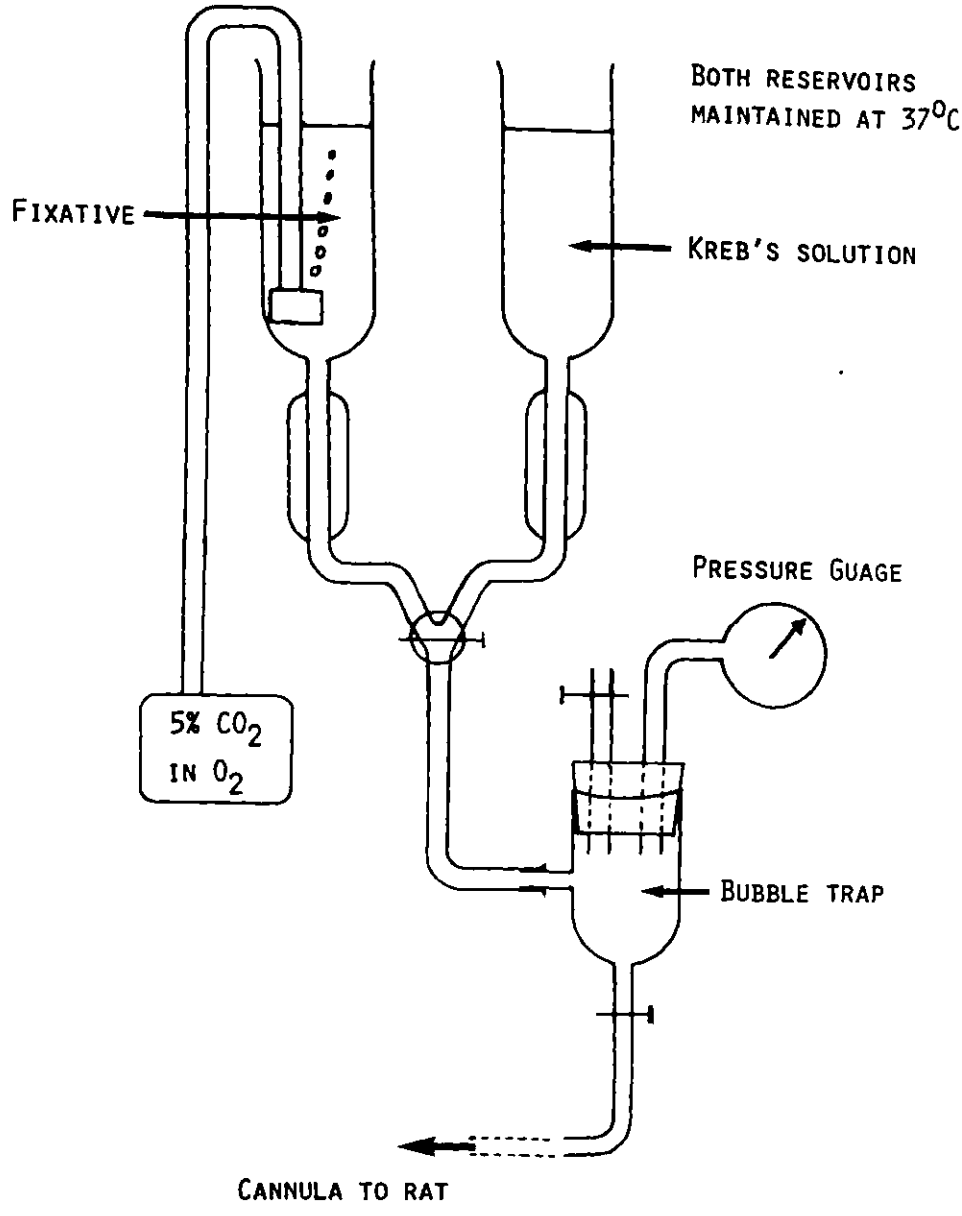


Fig. 25. Diagram of perfusion fixation apparatus.

The anaesthetised rat was pinned, in the supine position, to a cork board and the head tied back such that the anterior of the neck was fully exposed. A flap of skin was cut away and the exposed muscles on the left side were teased apart using a small pair of scissors. Care was taken not to sever any small blood vessels. An adjustable retractor kept the muscles apart and the left carotid artery was exposed. It was separated from the nerve and all the surrounding tissue was completely removed. Using a fine pair of jewellers forceps, two lengths of thread were passed under the artery and the upper piece tied. The artery was then clamped approximately 1 cm below this using a small artery clamp. A small cut was made across the top using a pair of fine bow scissors and the cannula inserted. It was secured using the lower piece of thread. The femoral artery in the right thigh was also dissected.

The 3 way tap linking the reservoirs to the bubble trap was then opened such that the Kreb's solution could pass through the cannula. The small artery clamp was released and the femoral artery severed. This meant that blood could flow out of the body and be replaced by Kreb's solution at the same pressure with the heart still beating. When the flow from the femoral artery became clear the 3 way tap was

turned such that the fixative solution could pass through the system. This killed the rat and after approximately 5 minutes the whole body was perfusion fixed. The thorax was then opened and the heart removed and kept in fixative solution. A sample of lung, liver, spleen, kidney, trachea and skeletal muscle from the left thigh were also removed.

The heart was sectioned just below the atrioventricular groove and the upper half processed for histology. A sample of the interventricular septum was taken from the lower half, chopped into approximately 1 mm^3 pieces and processed for electronmicroscopy.

9.2.3 Materials

Glycine buffer - was prepared and stored as previously described (page 98).

Triac - was made up in concentrations of 50 and 100 µg/ml and stored as previously described.

Prenalterol - Astra Pharmaceuticals Ltd., was supplied as prenalterol hydrochloride powder and made up into concentrations of 1, 2 and 4 mg/ml in water for injections. It was stored in the dark at $4^{o}C$.

Kreb's solution

-	Sodium Chloride (NaCl)	0.77M	81.4 ml
-	Potassium Chloride (KCl)	0.77M	1.76 ml
-	Calcium Chloride (CaCl)	0.55M	2.4 ml
-	Potassium dihydrogen Phosphate (KH_2PO_4)	0.77M	0.8 ml
-	Magnesium Phosphate ($MgSO_4$)	0.77M	0.8 ml
-	Sodium bicarbonate ($NaHCO_3$)	0.154M	84.0 ml

These solutions were made up to 520 ml with distilled water and 1.05 g glucose was added.

The final solution was bubbled with 5% CO_2 in O_2 for ten minutes before it was used.

9.2.4 Results

Details of pregnancy are given in table 40. This experiment was carried out at the same time as the previous experiment with verapamil and so the control rats from the groups receiving buffer and triac alone were the same.

The pregnancy rate was good with at least 2 out of 3 becoming pregnant in all groups apart from group 2 which received triac alone. Here only one rat became pregnant and as some offspring were to be kept and injected throughout life, fewer were killed for analysis as neonates. In group 4, those receiving prenalterol alone, the litter sizes were smaller than average and so, as above, fewer were sampled as neonates.

One of the rats in group 3, receiving triac and prenalterol, gave birth to 12 offspring which were all alive, but by 24 hours after birth only 2 remained, the rest were presumed eaten by the mother. Occasionally mothers have been observed eating offspring, which she has probably killed, but ten is far more than normally noted. Rats which are actually born dead are generally left in the cage and not eaten.

Table 40

Triac with Prenalterol

Details of Pregnancy

Group	No. of pregnancies	Average litter size	% live births
1. Control	2/3	11.5	100%
2. Triac 60 µg	1/3	9	100%
3. Triac 60 µg + Prenalterol 2 mg	2/3	11	92% *
4. Prenalterol 2 mg	2/3	4.5	90%

* One mother in this group gave birth to 12 offspring, all alive, but after 24 hours 10 were missing, presumed eaten by the mother.

9.2.4.1 Macroscopic findings

See table 41 (page 237).

Results of heart weights appeared rather varied but, as in previous experiments, there were no significant differences between them. This applied to the 3 month rats as well.

Septal to free wall ratios were measured in the adult rats and most calculations were very close to unity in all groups. However, one rat in the group receiving triac with prenalterol showed a figure of 1.27. This approaches the value of 1.3, the suggested ratio indicative of hypertrophic cardiomyopathy in man (Henry et al 1973).

9.2.4.2 Light microscopic findings

Cell diameters are given in table 42. In the neonatal rats from triac treated mothers, cell diameters were significantly greater than in control rats. By giving prenalterol in combination with triac, cell diameters were greater than when triac was given alone, but levels of significance were low. When prenalterol was given alone, cell diameters were greater than controls at 2 days but not at 7 days.

Results from 3 month rats (table 43) show that cell diameters from triac treated rats were greater (by an average of 35%) than control rats. Fig. 26 shows cells from a control rat and is compared with Fig. 27 which shows the hypertrophied cells from a triac treated rat. Prenalterol did not worsen the level of hypertrophy caused by triac but on its own did produce a mild increase in cell diameters. These were on average 9% greater than the controls.

No extensive cellular disarray was noted in any of the rats.

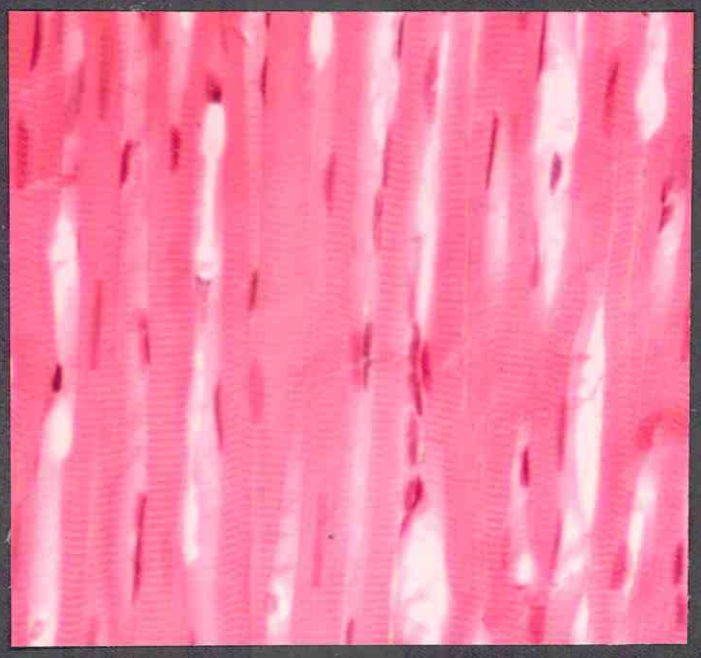


Fig. 26. Photomicrograph of normal myocardium (control rat at 3 months).
Haematoxylin and eosin x 500

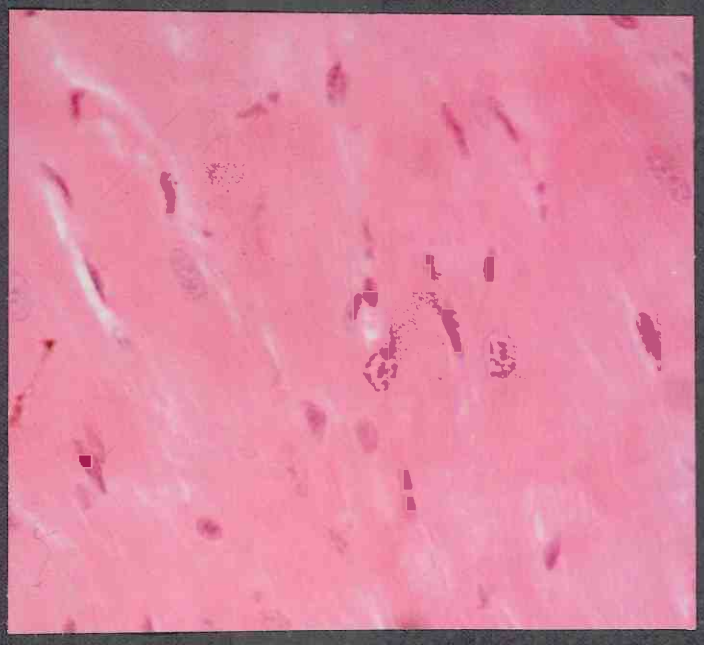


Fig. 27. Photomicrograph of hypertrophied myocardium (3 month triac treated rat).
Haematoxylin and eosin x 500

9.2.4.3 Electron microscopic findings

Results for disarray and crossover are given in table 44 (page 240). The level of disarray in the hearts of offspring of triac treated mothers was not altered by the concurrent administration of prenalterol. When prenalterol was given alone, disarray was no worse than in control rats at 2 days. It was only possible to kill one rat in this group at 7 days as others were needed for continued injections, but this only showed 3 foci of disarray.

Scores for hypertrophy (table 45) show that prenalterol did not affect that produced by triac, or produce any when given alone.

In the 3 month rats no foci of disarray or crossover were found in any rats in any of the groups. Additional samples were analysed from the left ventricular free wall in 2 rats from group 2 and 2 from group 3 but, again, no disarray was found.

Hypertrophy, however, was found in the triac and triac plus prenalterol treated groups. The scores for hypertrophy in table 46 give the mean score when observing the whole section under the microscope but often some individual groups of cells were hypertrophied to a much greater degree.

Fig. 28 is from a control rat where myofibrils are parallel and separated by one mitochondrion per 2 sarcomeres. This is compared with Fig. 29 which shows parallel sarcomeres from a triac treated rat which are separated by up to 10 mitochondria per 2 sarcomeres. In two rats from group 2 and two from group 3, widespread breakdown of cristae within the mitochondria (cristolysis) was noted. This is illustrated in Fig. 30 which also shows another common feature in these groups, that of elongated mitochondria. Some were up to 4 sarcomeres in length. In two rats, one from group 2 and one from group 3, severe cellular oedema was noted. This is illustrated in Fig. 31. Many myofibrils were in short runs.

In group 4, prenalterol alone, two rats appeared normal and two gave scores for mild hypertrophy as diagnosed by mitochondrial numbers.

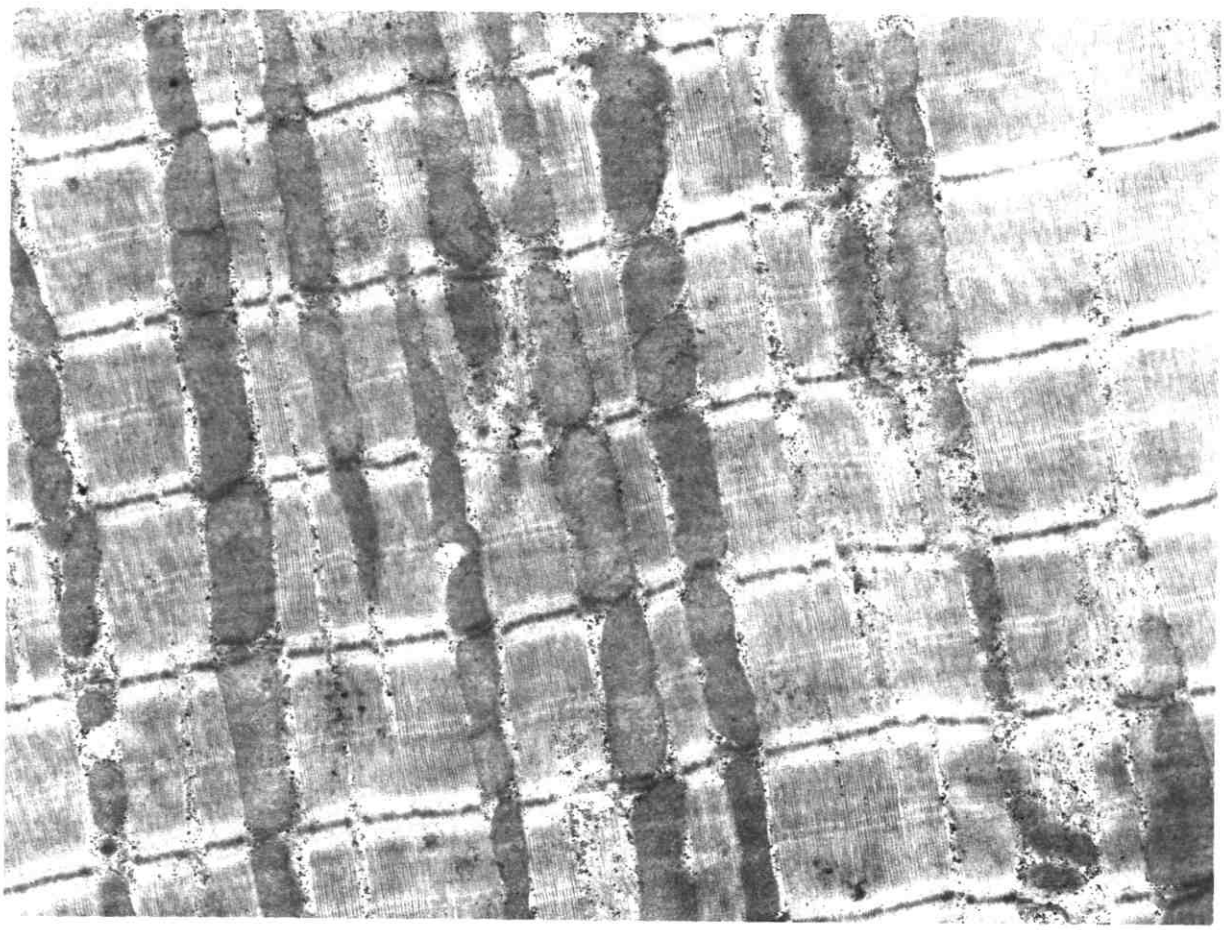


Fig. 28. Electronmicrograph of normal myocardium. Parallel sarcomeres are separated by one or two mitochondria. (control rat at 3 months).

x 18,600

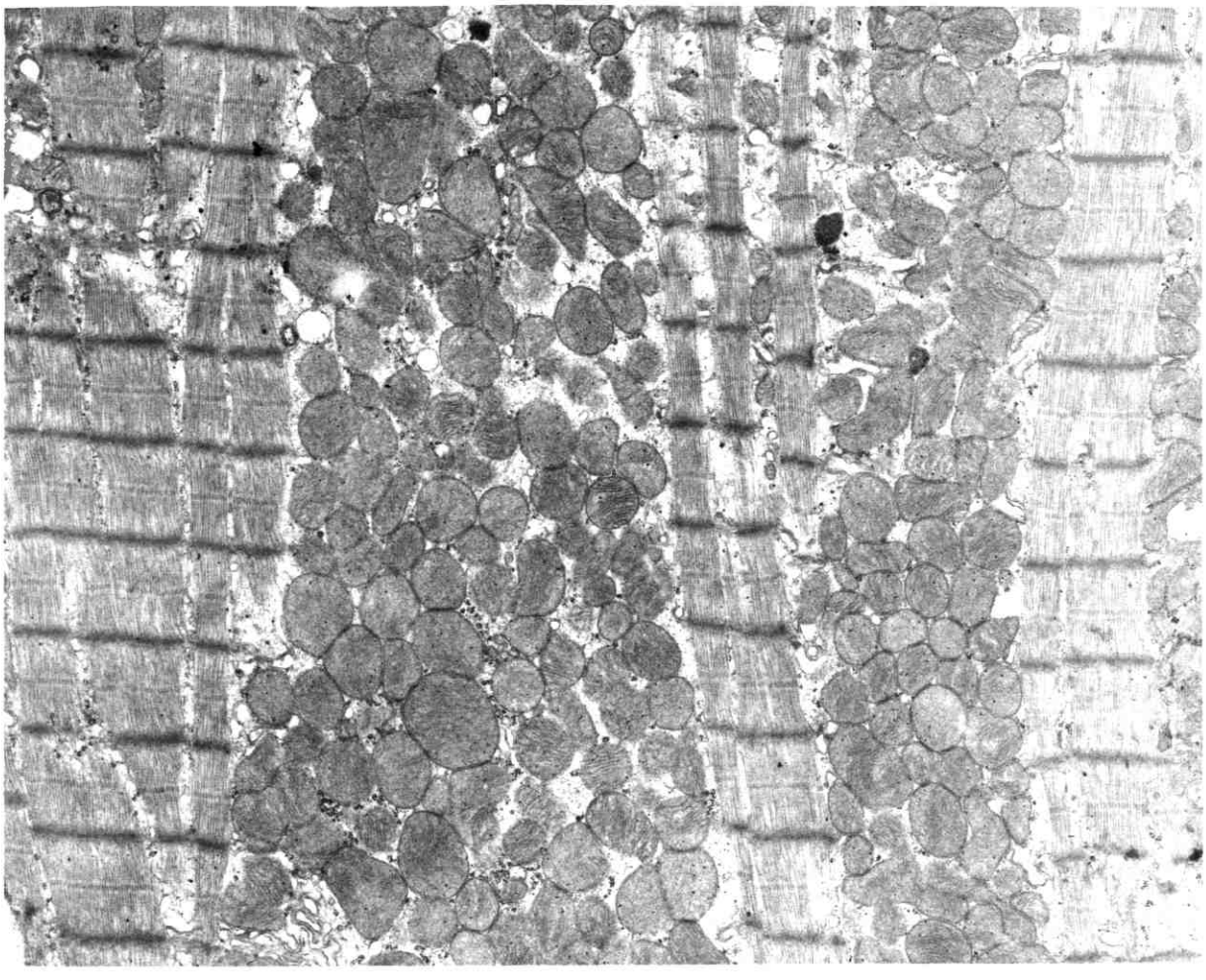


Fig. 29. Electronmicrograph of hypertrophied myocardium, with up to 12 mitochondria separating parallel sarcomeres. (triac treated rat at 3 months).

x 11,800

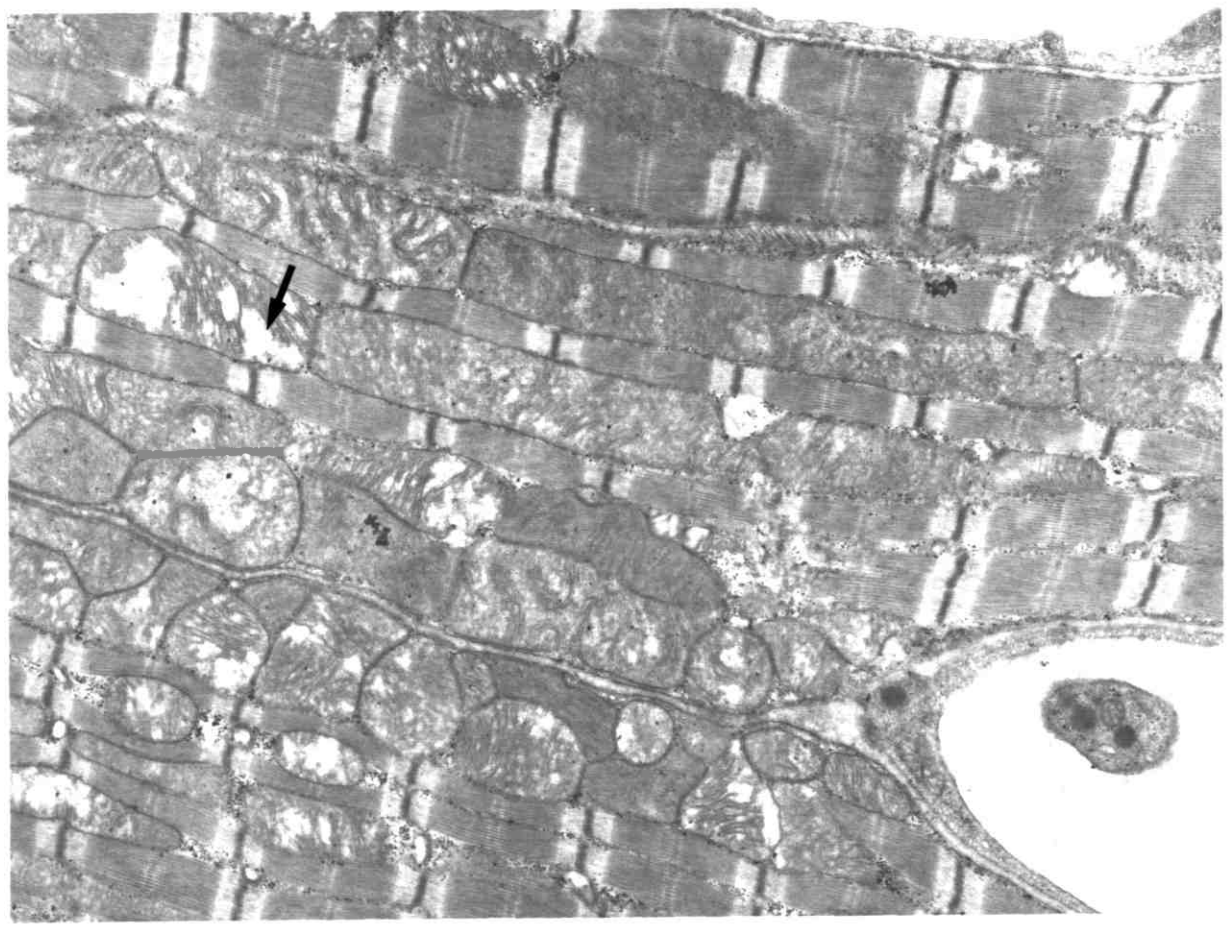


Fig. 30. Electronmicrograph illustrating breakdown of christae (➡) and mitochondria up to four sarcomeres in length. (triac treated rat at 3 months).

x 15,000

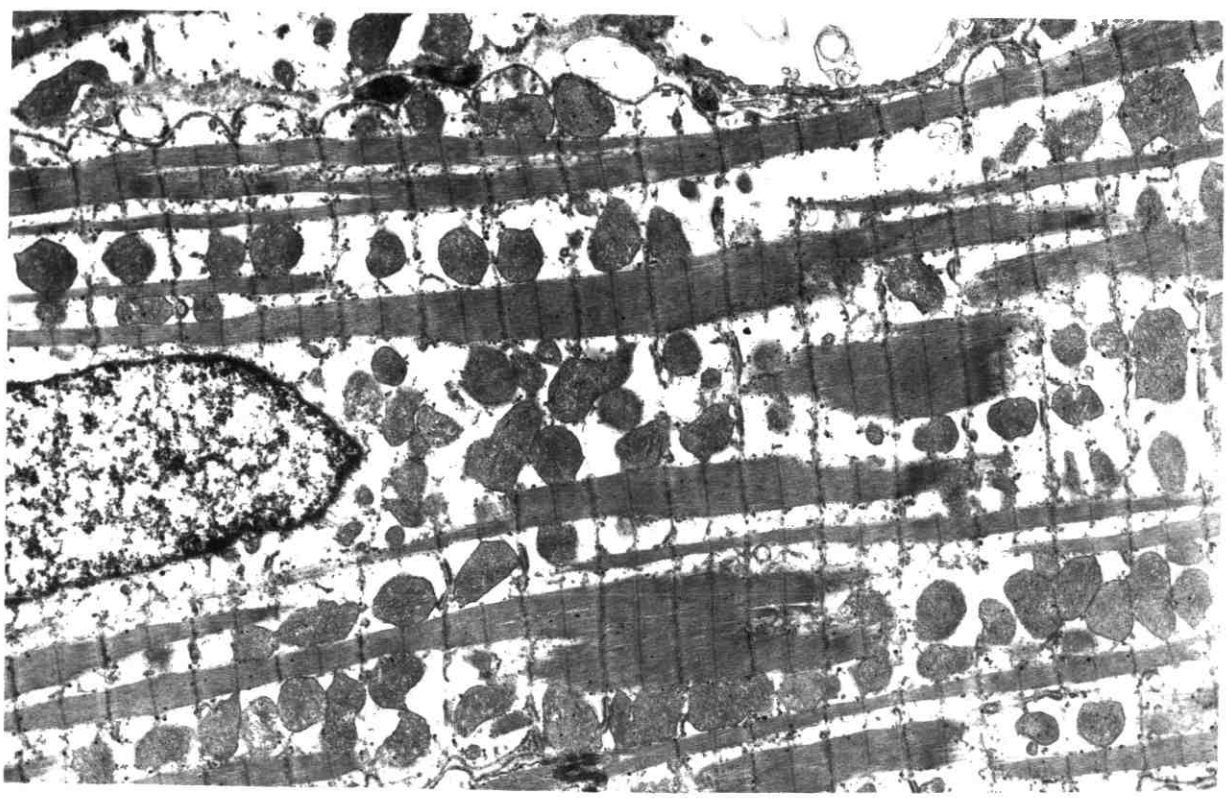


Fig. 31. Electronmicrograph illustrating severe cellular oedema and short runs of some myofibrils.
(triac treated rat at 3 months).

x 7,200

Table 41

Triac with Prenalterol

Heart weights expressed as a percentage of total body weight

Group	2 day rats	7 day rats
1 Control	0.601 ± 0.050 n = 6	0.508 ± 0.043 n = 4
2 Triac 60 µg	0.642 ± 0.040 n = 3	0.639 ± 0.077 n = 2
3 Triac 60 µg + Prenalterol 2mg	0.666 ± 0.039 n = 5	0.666 ± 0.153 n = 2
4 Prenalterol 2mg	0.695 ± 0.046 n = 4	0.694 n = 1

3 Month Rats

Group	% heart weight	n	Septal/Free Wall
1 Control	0.658 ± 0.107	5	0.99 ± 0.07
2 Triac 0.4 mg/kg	0.559 ± 0.094	4	0.97 ± 0.06
3 Triac 0.4 mg/kg + Prenalterol 10 mg/kg	0.519 ± 0.090	5	1.06 ± 0.14*
4 Prenalterol 10 mg/kg	0.666 ± 0.0743	4	0.98 ± 0.03

* One rat in this group had a ratio of 1.27.

n = No. of rats

Table 42

Triac with Prenalterol

Results: Cell Diameters - histological measurement

2 DAY RATS

Group	n	Mean cell diameter µm	S.D.	p1	p2
1 Control	200	7.22	1.06		
2 Triac 60 µg	200	8.20	1.11	< 0.001	
3 Triac 60 µg + Prenalterol 2mg	200	8.45	1.21	< 0.001	< 0.05
4 Prenalterol 2 mg	200	7.75	1.16	< 0.001	< 0.001

7 DAY RATS

Group	n	Mean cell diameter µm	S.D.	p1	p2
1 Control	200	8.41	1.12		
2 Triac 60 µg	200	9.41	1.38	< 0.001	
3 Triac 60 µg + Prenalterol 2mg	200	9.78	1.41	< 0.001	< 0.01
4 Prenalterol 2 mg	100	8.56	1.20	N.S.	< 0.001

p1: compared with group 1

p2: compared with group 2

NS: Not significant

n: No. of cells measured

Table 43

Triac with Prenalterol

Results: Cell Diameters - histological measurement

3 MONTH RATS

Group	n	Mean cell diameter um	S.D.	p1	p2
1 Control	500	11.59	1.96		
2 Triac 0.4 mg/ml	400	15.93	3.88	< 0.001	
3 Triac 0.4mg/ml + Prenalterol 10 mg/ml	500	15.29	2.83	< 0.001	< 0.01
4 Prenalterol 10 mg/ml	400	12.61	2.48	< 0.001	< 0.001

p1: compared with group 1

p2: compared with group 2

n: No. of cells measured

Table 44

Triac with Prenalterol

Results: E.M. score for disarray and crossover

Group	2 day rats	7 day rats
1 Control	1 0 1 2 0	2 1 2 1
2 Triac 60 µg	8 7 9	8 4
3 Triac 60 µg + Prenalterol 2 mg	6 7 7 6 3	8 2
4 Prenalterol 2 mg	4 2 1 1	3

Table 45

Triac with Prenalterol

Results: E.M. score for hypertrophy

Group	2 day rats	7 day rats
1 Control	1 1 0 0 0	0 1 0 0
2 Triac 60 µg	2 2 1	1 1
3 Triac 60 µg + Prenalterol 2 mg	2 1 2 1 1	1 2
4 Prenalterol 2 mg	1 0 0 0	0

Table 46

Triac with Prenalterol

Results: E.M. score for hypertrophy

Group	3 month rats				
1. Control	0	1	0	0	0
2. Triac 0.4 mg/ml	3	0	2	3	
3. Triac 0.4 mg/ml + Prenalterol 10 mg/ml	2	2	2	2	1
4. Prenalterol 10 mg/ml	0	1	1	0	

9.2.5 Discussion

An animal model showing the three major pathological features of hypertrophic cardiomyopathy has not been produced in this experiment. Triac produced myofibrillar disarray and hypertrophy in the neonatal hearts but continued administration of triac for three months did not retain the disarray. Hypertrophy was, however, maintained and worsened by the administration of triac to the growing rats. Other signs of myocardial damage at the ultrastructural level were also noted but these have been attributed to changes associated with general hypertrophy and are not specific in any way (Baandrup 1979). Prenalterol did not enhance the action of triac on either the developing or growing rats. When given alone it caused mild cardiac hypertrophy in the growing rats only.

Hypertrophic cardiomyopathy in man is associated with an elevated contractile state of the myocardium and prenalterol was chosen for this experiment because of its inotropic actions. It has been shown to be effective in patients with impaired left ventricular function, where it enhances the contractile state of the myocardium without altering heart rate. (Hutton et al 1980).

Carlsson et al (1972) showed that there were both beta 1 and beta 2 receptors in the heart, and later found (Carlsson et al 1977) that the relative distribution differed within the heart. Beta 1 receptors were found to be dominant in both the sinus node, controlling the rate of contraction, and in the myocardium, influencing the force of contraction, but the beta 1 : beta 2 concentration ratio was higher in the myocardium. A non selective beta receptor agonist, such as isoprenaline, acts on all the beta receptors and produces a greater chronotropic than inotropic response, whilst a beta 1 selective agonist, prenalterol, acts on more receptors in the myocardium than in the sinus node and so the relative inotropic to chronotropic response is increased. In dogs Manders et al (1980) found the inotropic stimulation of prenalterol to be greater in both magnitude and duration than the chronotropic action. It was hoped that an increase in the force of contraction with less effect on the rate, and in combination with triac, would produce changes in the myocardium of rats similar to those observed in hypertrophic cardiomyopathy in man. Another theoretical advantage of prenalterol over drugs such as isoprenaline and adrenaline was the longer duration of action. The foetus is very sensitive to catecholamines administered directly but the short half life of isoprenaline prevents any pharmacologically effective amounts crossing the placenta (van Petten 1975). Adrenaline also does not cross (Jackson and Egdahl 1962) but agonists with a longer half life, in

theory, have greater potential for crossing. Evidence for this came from van Petten and McCracken (1972) who were able to measure foetal chronotropic responses when isoxsuprine and buphenine were administered to pregnant ewes, but not from bamethan. Thus prenalterol should cross the placenta and subsequent company information (Astra Pharmaceuticals Ltd.) stated that it does so in rabbits.

Prenalterol is able to produce the same intensity of responses as isoprenaline although absolute doses required to do this are more (Carlsson et al 1977). However, as isoprenaline has been shown to produce ordinary hypertrophy at doses as low as 0.2 mg/kg (Pagano and Inchiosa 1977) and 0.3 mg/kg (Taylor et al 1977) over periods of 14 and 10 days respectively, then 10 mg/kg of prenalterol over 3 months to growing rats, in combination with triac, was considered a sufficient dose to produce a morphological effect.

Since this experiment was carried out, a paper by Tanaka (1981) appeared where adult rats were given isoprenaline at 5 mg/kg for 18 weeks, among other schedules, and disarray of myofibrils was produced. This occurred in cells described as non-destroyed degenerated type and he suggested that cardiomyopathy may be a disease of adaptation due to intense or excessive beta stimulation. However, there was no quantitation of results, no mention of disarray at the histological level, nor any mention of asymmetric septal

hypertrophy. Also the overall condition of the animals when sacrificed was not mentioned. If the disarray was widespread, his results would give support to the possibility that beta receptors may be involved in the aetiology of hypertrophic cardiomyopathy.

Triac produced hypertrophy, but not disarray, in otherwise healthy adult animals. The dose given was based on previous experiments. Symons et al (1975) showed that 0.18 mg/kg of triac per day over the period of one year produced ordinary hypertrophy, though it was not observed in rats sampled at 33 weeks. In a previous experiment in this series, not documented, triac at 0.18 and 0.25 mg/kg/day was administered to 10 day old rats, whose mothers received the standard dose of triac throughout pregnancy, for 3 months. No changes of disarray or hypertrophy were found at either of these dose levels and so a higher dose of 0.4 mg/kg was used for this experiment.

The period of 3 months was chosen as by this age the rats are fully grown and sexually mature. It is at this equivalent stage in humans that hypertrophic cardiomyopathy can manifest itself, often by sudden death.

The results have thus confirmed that triac increases the level of disarray in developing hearts and normal alignment returns with age (Hawkey et al 1981) with or without the presence of triac. Stimulation of beta receptors, within the limits of this experiment, did not affect the developing heart but did have minimal effect on the growing heart.

9.3 The effect of T4 on developing rat heart muscle

9.3.1 Introduction

The experiments described in this thesis have used the acetic acid metabolite of triiodothyronine, triiodothyroacetic acid (triac), to produce damage to the myocardium of the offspring of treated rats. Triac was originally chosen as it has overall weak metabolic actions (Lerman and Pitt-Rivers 1956), it is powerful in increasing oxygen consumption of heart muscle slices (Barker and Lewis 1956), and causes greater left ventricular hypertrophy (Symons et al 1975) than thyroxine.

To investigate whether production of disarray and hypertrophy in the young rat hearts is a specific action of triac, T4 has also been used. In an initial experiment, not presented here, three doses were used (60, 120 and 230 µg/day). Very little disarray was observed but some hypertrophy was present. This experiment was used to improve techniques and compare two methods of fixation.

The results presented here are from rats which were injected with a higher dose of T4 at the time when the experiment of triac with oxprenolol was being carried out, hence the controls are the same.

9.3.2 Materials and methods

T4 was dissolved in glycine buffer, pH 10, and stored in multidose vials in the dark at 4°C. The maximum concentration that could be achieved in solution was 230 µg/ml and so when the rats were injected 2 x 1 ml injections were given, one in each thigh.

Glycine buffer and triac were prepared as previously described on page 98. Other procedures were also as previously described.

9.3.3 Results

The details of pregnancies given in table 47 show that even at the high dose of 460 µg T4 per day there were no abnormalities. There were no clinical signs of thyrotoxicosis in the female rats being injected.

No pattern of change was seen in measurements of heart weights (table 48).

9.3.3.1 Light microscopic findings

Measurements of cell diameters in table 49 show that T4 produced almost as much hypertrophy as triac at 2 days but not as much at 7 days.

9.3.3.2 Electron microscopic findings

Scores for disarray and crossover are given in table 50. In 2 rats in the control group at 2 days up to 7 foci of disarray were observed. This is a high result when comparing control figures from other experiments. However scores from the T4 treated group showed that in all 4 rats, at least this figure was observed, with 2 rats giving a much higher result. At 7 days there were fewer foci present but a much greater distinction from controls could be seen.

Scores for hypertrophy, table 51, show that hypertrophy was present in all but one rat from the thyroxine treated group.

Table 47

Effects of Thyroxine

Details of pregnancies

Group	No. of pregnancies	Ave. litter size	% live births	Gestation period-days
1 Control	4/5	12	90	22
2 Triac 60 µg	4/5	11	67	22-23
3 T4 460 µg	2/2	13.5	87	21-22

Table 48

Heart weights expressed as a percentage of total body weight

Group	2 day rats	7 day rats
1 Control	0.612 ± 0.093	0.748 ± 0.064
2 Triac 60 µg	0.622 ± 0.049	0.719 ± 0.042
3 T4 460 µg	0.596 ± 0.045	0.792 ± 0.080

Table 49

Effects of thyroxine

Results: Cell diameters - histological measurement

2 DAY RATS

Group	n	mean cell diameter µm	S.D.	p1	p2
1 Control	200	6.94	1.09		
2 Triac 60 µg	200	8.84	1.27	< 0.001	
3 T4 460 µg	200	8.76	1.29	< 0.001	N.S.

7 DAY RATS

Group	n	mean cell diameter µm	S.D.	p1	p2
1 Control	200	8.19	1.15		
2 Triac 60 µg	200	9.68	1.44	< 0.001	
3 T4 460 µg	200	9.09	1.53	< 0.001	< 0.001

p1: compared with group 1

p2: compared with group 2

NS: Not significant

n: No. of cells measured

Table 50

Effects of thyroxine

Results: E.M. score for disarray

Group	2 day rats				7 day rats			
1 Control	7	2	6	2	3	0	0	1
2 Triac 60 µg	9	20	5	21	13	14	9	4
3 T4 460 µg	17	6	6	23	7	9	10	5

Table 51

Results: E.M. score for hypertrophy

Group	2 day rats				7 day rats			
1 Control	1	0	0	1	1	1	0	0
2 Triac 60 µg	2	1	1	2	1	1	1	2
3 T4 460 µg	2	1	0	1	1	1	1	1

9.3.4 Conclusion

The results from this experiment have shown that triac is not the only substance to produce disarray and hypertrophy in developing rat hearts. T4 produced the same effects, but a dose approximately 8 times that of triac was required.

It is likely that the site of action of T4 in causing these effects is the same as that proposed for triac, but it is not known whether the active molecule is T4 itself or if it becomes deiodinated to T3 and this is responsible. T3 will cross the placenta in greater amounts than T4, (see section 9.5.4). Alternatively, the molecule may be metabolised to tetrac or triac which may be acting on the developing heart. Further work with radioiodinated thyroid compounds will be required to identify the active molecule.

The dose of T4 which the pregnant rats received did not, surprisingly, affect their health. T4 has far greater effect on overall metabolism than triac and the dose administered was comparatively high. It has previously been noted that when greater amounts than 60 μg of triac were administered per day, there were pregnancy problems and several maternal deaths (Hawkey unpublished data).

9.4 Measurement of Haemoglobin levels

9.4.1 Introduction

Cardiac hypertrophy can be caused by many factors, including anaemia in developing animals (Poupa et al 1964). In order to exclude the possibility that triac may be acting by simply producing anaemia, which may then be the primary cause of the hypertrophy, haemoglobin levels were measured. The results are presented here so that the flow of the thesis was not interrupted earlier.

9.4.2 Method

During the experiment in which triac was administered with timolol, procainamide and A23187, blood from the neonatal rats was collected. This was carried out by placing a heparin coated capillary tube against the severed neck immediately after decapitation. This filled very quickly and did not delay the removal of the heart. Haemoglobin levels were measured as cyanmethaemoglobin on a haemoglobinometer (Coulter Electronics Ltd.).

9.4.3 Results

Results of measurements of haemoglobin levels are presented in table 52. The levels were very similar in all groups, though they were higher at 2 days than at 7 days. The offspring of rats which received triac alone had slightly lower levels compared with the other groups.

9.4.4 Conclusion

The results show that hypertrophy was produced in the offspring of triac treated rats without anaemia. Haemoglobin levels in the triac alone treated group were slightly lower than in other groups, but not severely low. It is unlikely that these differences in values are significant, as other groups, notably those receiving triac with either timolol or A23187, also showed hypertrophy and disarray with no difference in haemoglobin levels from the control rats.

Table 52

Results of measurements of Haemoglobin levels

Group	Hb at 2 days g/dl n = 4	Hb at 7 days g/dl n = 4
Control buffer	11.6 ± 0.8	9.4 ± 0.3
Triac 60 µg	9.1 ± 0.6	8.0 ± 0.5
Triac 60 µg +		
Timolol 0.05 mg	9.7 ± 1.7	9.9 ± 0.5
Timolol 0.1 mg	11.6 ± 0.8	9.0 ± 0.5
Timolol 0.2 mg	10.1 ± 0.8	9.7 ± 0.6
Timolol 0.2 mg	11.5 ± 1.4	9.5 ± 1.3
Triac 60 µg +		
Procainamide 1.25 mg	11.9 ± 1.4	8.1 ± 1.4
Procainamide 2.5 mg	11.5 ± 0.3	9.6 ± 0.4
Procainamide 5.0 mg	10.1 ± 1.4	9.3 ± 0.9
Procainamide 5.0 mg	10.6 ± 0.8	9.1 ± 0.9
Triac 60 µg +		
A23187 15 µg		
A23187 30 µg	10.4 ± 1.0	11.4 ± 1.8
A23187 60 µg	11.0 ± 1.4	10.4 ± 1.6
A23187 60 µg	10.8 ± 1.0	11.2 ± 0.9

9.5 Placental transfer of triac

9.5.1 Introduction

Although it is not relevant to the terms of reference of this thesis, it is important to know whether or not triac crosses the placenta. A pilot study was, therefore, undertaken to investigate this.

T4, T3 and TSH do not cross the placenta (Fisher 1975) but triac may behave differently. Hawkey et al (1981) suggested three possibilities, a) triac may cross the placenta, especially in high doses, b) some form of amniotic diffusion may take place, or c) the changes in the myocardium may be stimulated indirectly by triac.

In the following experiment an attempt was made to label and inject triac into a pregnant rat and measure the level of radioactivity in the foetus.

9.5.2 Materials and methods

9.5.2.1 Radiolabelling of triac with ^{125}I was carried out by the method of Burger and Ingbar (1974) using the modifications suggested for optimum efficiency for triac.

The following solutions were mixed in a plastic 1 ml reaction vial:-

Triac - 5×10^{-9} moles in 0.01 ml methanol
(taken from a stock of 0.3085 mg/ml)
+Na ^{125}I. 200μCi in 0.01 ml.
+0.1M Phosphate buffer - 0.05 ml to adjust pH to 6.2.

The reaction was started by adding to this 0.01 ml of Chloramine T (200 μg/ml in PO_4 buffer). The chloramine T was dissolved in buffer 15-60 minutes before use.

The reaction was stopped after 2 minutes by adding 0.02 ml of saturated sodium metabisulphite (Na_2SO_5) diluted 1 : 10.

Separation of the reaction mixture was by using a 2.5 ml plastic syringe as a column, filled to 1.5 ml with Sephadex LH20 in phosphate buffer. An even packing of the column was ensured.

The reaction mixture was added to the column which was then washed with:-

1 ml PO_4 buffer
4 ml distilled water
1.2 ml methanol : 2N NH_3 (99 : 1)
4 ml " " "

All free iodine should have been eluted in the first 3 washes and the radioiodinated compounds eluted in the final wash. Radioactivity (15 - 60 kev) of 1 µl alioquots from the reaction mixture and each wash was measured and the final wash was shown to contain 57% of the initial amount of radioactivity used.

A sample of the final wash was taken for paper chromatography (see below) and the rest dried under nitrogen and resuspended in sodium hydroxide and glycine buffer to be suitable for injection.

Paper chromatography using the method of Bellabarba et al (1968) was then carried out to test the labelling efficiency.

This was done by a descending system using 3MM Whatman paper. The solvent used was:-

Hexane	:	tertiary amyl alcohol	:	2N ammonia	
1	:	5	:	6	v/v/v

This mixture was thoroughly shaken and allowed to stand so that the two phases separated. The lower phase was used for saturating the jar and the upper used as the solvent. Spots from the final eluate in methanol/ammonia, the water eluate containing the free iodide and the final injected solution, were run alongside a spot of a higher concentration of unlabelled triac that could be identified colorimetrically. The system was allowed to run overnight and 14 x 1 inch strips were then cut from each chromatogram and the radioactivity from each counted. The samples were counted for 1 minute at 15-60 kev.

The spot of unlabelled triac was identified by spraying with 0.1% palladium chloride ($PdCl_2$), then 2% aminoantipyrine, and finally 2% potassium ferricyanide ($K_3\ Fe(CN)_6$).

9.5.2.2 Animals

At approximately 19 days of pregnancy one rat was injected intramuscularly with labelled triac and the other with the solution of free iodine. The rats were killed 18 hours later and the foetuses removed. The foetal hearts, livers and thyroids were removed and counted collectively. Samples of amniotic fluid and blood were also collected as were all the placentas. Organ samples from the mothers were also taken and counted.

9.5.3 Results

Chromatography Profiles of chromatograms; counts per minute of each strip are expressed as a percentage of the counts from the whole chromatogram, (see table 53).

Tissue samples - the counts of each sample are expressed as a percentage of the activity of the injection, (see table 54.

Table 53

Profile of chromatogram

a = Solution of injected labelled triac

b = Solution of injected free iodine

c = Solution of final wash from the labelling procedure.

a	b	c	
1.6	9.6	1.7	origin
13.3	15.6	1.4	
18.4	57.8	0.4	$Na^{125}I$
3.1	1.3	2.6	
0.5	0.8	4.2	
27.6	0.3	7.4	^{125}I Tetrac
0.5	4.0	3.7	
0.2	0.3	2.2	
7.3	0.2	3.0	
5.8	0.4	4.2	
16.8	3.6	29.1	^{125}I Triac
3.9	0.4	13.8	
0.3	1.1	3.0	
0.5	4.0	23.4	Solvent front

Table 54

Radioactive measurements of tissue samples

	1 ml ^{125}I triac	1 ml Na^{125}I
% of dose in:		
Maternal		
blood 2 ml	0.28	0.08
thyroid	0.1	0.42
heart	0.006	0.02
liver	1.7	0.63
spleen	0.07	0.03
Foetal		
blood 200 µl	0.06	0.01
hearts	0.002	0.002
livers	0.11	0.06
thyroids	0.16	0.06
Amniotic fluid 500 µl	0.08	0.11
Placentas	0.65	0.14
1 whole foetus	0.09	0.16

9.5.4 Discussion

Unfortunately very little can be concluded from these results. The experiment was a pilot study and clearly much future work is required to determine whether triac crosses the placenta.

The labelling procedure itself was efficient, with over 57% of the initial amount of ^{125}I used being recovered from the column and 44% of the activity from the last wash being located in the triac band. It is not known what the activity at the solvent front represents. The procedures of drying the solution under a stream of nitrogen and redissolving in an injectable medium altered the profile of the chromatogram. More radioactivity appeared in the region of the tetrac band (Bellabarba et al 1968) and over 30% became released as free iodide. In an earlier experiment the triac band was eluted from the paper but in the process even more iodine became free. Further research is needed to develop an efficient method of labelling triac which will remain as ^{125}I triac when dissolved in an injectable medium.

The levels of radioactivity in the foetuses were probably from the 30% free iodine in the injection solution, as distribution levels from the two rats were very similar. Iodine is known to be actively transported across the placenta (Logothetopoulos and Scott 1956). In the triac treated rat there was more radioactivity present in the

placentas. A significant amount of triac (or tetrac) would have been present in the injection as maternal thyroid levels were higher in the iodine treated rat and liver levels were higher in the triac treated rat.

Little seems to be known concerning the placental transfer of thyroid hormones or their metabolites, especially early in pregnancy, where triac may be exerting its actions, (see section 9.1). Fisher (1975) had concluded that thyroxine did not cross the placenta either at term or early in pregnancy. However in a later review, Burrow (1978) suggested that thyroid hormones do cross, but poorly. When Grumbach and Werner (1956) investigated placental transfer at term in humans they concluded that, although transport is slow, that of T3 is more rapid than that of T4, and they proposed that this difference could have been a property of the less intense binding of T3 to plasma proteins. The degree of binding of triac, if at all, is not known. Kearns and Hutson (1963) quantified the amount of transfer in humans just before delivery and found cord blood levels of T4 to be 7% of that of the mother. Levels of T3 were 25% of that of the mother, suggesting a higher rate of transfer of T3 than that found by Grumbach and Werner (1956). By contrast, Knobil and Josimovich (1958) suggested that, in the rat, transfer of T4 was better than T3. Geloso and Bernard (1967) concluded that there was limited transfer in the rat but it was not enought to compensate for an absence of foetal thyroid.

The paper which Fisher (1975) quotes to say that there is no transfer of thyroxine early in pregnancy is the work of Myant (1958) on humans. He injected women with ^{131}I T4 and T3 at stages varying between 11 and 25 weeks of pregnancy and then measured the levels of radioactivity in the aborted foetuses. He found that the foetal level of T4 never exceeded $1/10^{th}$ that of the mother, with T3 levels slightly higher. These levels are similar to those reported above in late pregnancy. Myant did not however identify the source of the foetal organic radioiodide and did not exclude the possibility that it could be from a metabolite of T3 or T4. In human pregnancy at 12 weeks the foetus is very nearly fully formed and is growing rather than developing. Also at this time the foetal thyroid gland is able to secrete its own T4 in response to foetal pituitary TSH, (Jost 1966). An article by Fisher and Dussault (1974) also states that T4 does not cross the placenta early in pregnancy but the reference quoted is that of Kearns and Hutson (1963) mentioned above, which is looking at transfer just before delivery.

It would thus appear that very little is known about placental transfer of thyroid hormones early in pregnancy. Experiments with rats have been late in pregnancy (e.g. Knobil and Josimovich 1958, Geloso and Bernard 1967) when the foetal thyroid is able to synthesise its own T4 (Legothetopoulos and Scott 1956). In the rat, maturation of

the hypothalamus, and therefore of the thyroid, is much slower than in humans. Rat foetal T4 is synthesised at approximately 18 days, i.e. at about 80% of pregnancy, whereas in humans it is produced at 12 weeks, i.e. at about 30% through pregnancy (Jost 1969). No studies of placental transfer of thyroid hormones, nor metabolites appear to have been carried out before these stages, by which times the hearts are fully formed and functioning. Triac may cross the placenta in significant amounts in the early stages of pregnancy and cause the abnormalities described. An experiment needs to be carried out whereby pure radioactive triac is administered from the beginning of pregnancy and the foetuses examined as early as possible for radioactive content, both quantitatively and qualitatively.

Placental transfer of other drugs used

There is also very little information concerning transfer of the other drugs used in the experiments. There is some published work but most information has come from the Companies selling them.

An important determinant of diffusion of drugs across biological membranes is the lipid solubility of the molecule. When this parameter was determined for beta blockers in vitro, Van Petten (1975) found good agreement between lipid solubility and the ability of the drug to

cross sheep placentas. Thus propranolol and oxprenolol crossed well but sotalol did not. For this reason timolol was chosen instead of sotalol as a beta blocker with no membrane stabilising activity and subsequent information from the Company (Merck, Sharpe and Dohme) has stated that it does cross in rats. Other Company information has stated that verapamil (Abbott) and procainamide (Squibb, Lima et al 1978) cross in humans and that prenalterol (Astra) crosses in rabbits. The calcium ionophore A23187 acts on cell membranes and so this may well cross, but no information has yet been found.

10. Final Conclusion & Discussion

The work for this thesis has been designed to investigate a possible pathogenetic mechanism for hypertrophic cardiomyopathy. In man, macro- and microscopic changes must be present in order to make a diagnosis of the condition, with, ideally, electronmicroscopic evidence to act as an adjunct. Some groups, notably Maron and co-workers, however consider electronmicroscopic changes sufficient to make a diagnosis. Experiments are presented in this thesis which have achieved changes at electronmicroscopic level which are similar to those seen in man.

The pathogenetic process must start somewhere and the earliest changes are obviously going to be seen at electronmicroscopic level. The possibility of a set of aetiological factors being located at the subcellular level has been investigated here by using a variety of therapeutic agents in order to assess whether they enhance or reduce triac induced damage. Two aetiological suggestions which have been important in leading to the investigations carried out have been the proposal that catecholamines may be involved (Goodwin 1974) and that there may be an abnormal enhancement of sensitivity or number of adrenergic receptors in developing hearts (Perloff 1981). By following this line, the experiments have defined the hitherto unknown site of action of triac and have led to a better understanding of the pathogenetic mechanism of disarray.

The starting point for the experiments came from the clinical usage of the beta adrenergic blocking and membrane stabilising agent dl propranolol in the treatment of patients with hypertrophic cardiomyopathy. This compound was used by Hawkey et al (1981) on the rat model and they found it prevented triac induced disarray but not hypertrophy. The same experiment was repeated for this thesis in order to ascertain the reproducibility of the previously published work and to test the efficacy of different batches of triac. Additional doses of dl propranolol were employed and the results confirmed that disarray could be prevented. Results from the group receiving the highest dose of dl propranolol suggested that hypertrophy may also be reduced.

As disarray could be prevented, could it be made worse? The possibility that partial agonist activity could affect the actions of triac was investigated by using oxprenolol. The results were similar to those found when dl propranolol was used, but approximately eight times the dose was required. This finding suggested that the partial agonist, or sympathomimetic, activity was having some effect. The difference in dose was more than could be accounted for purely in terms of differences in beta blocking and membrane stabilising potencies. If beta agonist properties were effecting a deleterious action it was therefore thought that a pure beta agonist, prenalterol, may have had an effect on the developing myocardium. However, at the dose given, this did not occur, nor were the effects of triac enhanced by concurrent administration of the compound.

To ascertain the extent that membrane stabilisation has played in the protective actions of dl propranolol and oxprenolol, d propranolol was administered with triac. This compound has no sympathomimetic properties, minimal beta blocking activity but equivalent membrane stabilising action to the racaemic mixture. At the highest dose of 1 mg, it prevented disarray but not hypertrophy. This result therefore suggested that prevention of triac induced disarray was primarily by membrane stabilisation and not by beta blockade.

In order to confirm these findings, two further drugs, timolol and procainamide, were tested on the animal model. Timolol is a pure beta adrenergic blocking agent with no membrane stabilising activity and procainamide is a pure membrane stabilising agent unrelated to beta blockers. Doses of the former compound were lower than those of propranolol but they were of equivalent beta blocking potency. It had no effect on the triac induced abnormalities. Procainamide, on the other hand, reduced the level of disarray and also showed some reduction in hypertrophy. Results from this experiment therefore supported the conclusion that membrane stabilisation is the major mechanism by which the action of triac can be prevented.

To further elucidate the action of triac in relation to hypertrophy, an investigation of possible interference with calcium channels was carried out. It is well known that clinical treatment of hypertrophic cardiomyopathy has had success with calcium antagonists (Rosing et al 1979a,b) and

some authors (Kaltenbach et al 1979) have further suggested that hypertrophy may be reduced. For this purpose verapamil was administered with triac. A low dose of 1 mg per day reduced the disarray and also significantly reduced the level of hypertrophy. Surprisingly though, when a high dose of 4 mg per day was administered alone, it induced hypertrophy and produced signs of damage to the myocardium. Thus triac may have a direct action on the myocardial cell membrane which facilitates the entry of calcium. The calcium ionophore A23187 acts specifically in this manner and this produced the same abnormalities as triac. The increase in intracellular calcium could possibly increase the contractility of the developing myofibrils and prevent their proper alignment, thus resulting in abnormal intracellular disarray. The aetiological suggestion of isometric systolic contraction (Bulkley et al 1977) may possibly be operative and the resulting actions could be the mechanism of progression of the condition.

From the evidence presented in this thesis it is reasonable to suggest that disarray begins in utero or early in life, but so far this experimental production of disarray has not persisted with age. An animal model of hypertrophic cardiomyopathy showing morphological features at all levels has yet to be produced. The attempt to do this by injecting triac and prenalterol throughout pregnancy and then to the offspring until adulthood, unfortunately produced results which did not fulfil the criteria required of an animal model of the condition. The hypertrophy produced was severe at both electron and light microscopic levels, but disarray was minimal. Dose levels and timing are, however, critical.

Symons et al 1975 produced severe hypertrophy in adult rats after 1 year of treatment with triac, but no changes at all were observed in a rat killed at 27 weeks. A preliminary experiment, not described in detail in this thesis, involved giving triac at a slightly lower dose of 0.25 mg/kg to offspring of treated mothers and, again, no changes were observed at any level. Long term experiments, of at least one year's duration, with different dose combinations of both thyroid and catecholamine related substances, may well lead to the production of morphological changes, at all levels, similar to those seen in hypertrophic cardiomyopathy. This would clearly be essential work to be undertaken in the future.

The pilot experiment on placental transfer of triac was included at the end of the thesis as, although the question is of fundamental importance, the work involved was not strictly within the perview of the project. The results were inconclusive and further experimental work is required. However, analysis of the literature does not exclude the possibility that triac may cross the placenta, especially early in pregnancy. The role of thyroid hormones during this period is unclear, but the experiment on the timing of the action of triac shows that production of disarray can be the result of administering triac during the first 2 weeks only.

This thesis has contributed to the knowledge of how thyroid hormones or their analogues may cause disarray and has elucidated their site of action, but several questions remain unanswered. Little is known of the aetiology of disarray observed in hypertrophic cardiomyopathy, probably because little is known of the mechanism responsible for the proper alignment of myocardial cells and their constituents. Perhaps manipulation with thyroid hormones and catecholamines may never produce a full blown picture of hypertrophic cardiomyopathy. An additional, as yet unidentified, factor such as a genetic predisposition, may be required. Hypertrophic cardiomyopathy is familial in many cases and a clarification of the genetic process is essential. Work on the HLA system, as proposed by Matsumori et al (1979), must be expanded and the genetic mechanism expressed at the molecular level. Further work on dogs (Liu et al 1979) and cats (Liu et al 1981) with the condition may well be helpful. In addition, it would be feasible to investigate the possibility that extra systolic isometric contraction may be involved during foetal development in these larger animals.

Although it is recognised that one cannot directly extrapolate the findings from experimental animals to man, the work in this thesis has clinical implications, not only in the drugs used, but also in the actions of the thyroid analogue. The association of hyperthyroidism and

hypertrophic cardiomyopathy is rare, but the hyperthyroidism is covert. The co-existance of the two condtions may, therefore, be more frequent than so far known. The development of sensitive assays for triac and tetrac is required such that levels of these metabolites can be measured along with other thyroid parameters in patients presenting with obscure heart disease.

The work presented in this thesis has contributed towards answering some fundamental questions. Further work already mentioned in this section must include investigation of placental transference of triac, long term experiments and more detailed analysis of timing of the action of triac. These investigations may well lead to the establishment of an experimental model of this fascinating disease.

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EFFECT OF TRIAC AND β-ADRENERGIC BLOCKING AGENTS ON THE MYOCARDIUM OF DEVELOPING RATS

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Effect of triac and β-adrenergic blocking agents on the myocardium of developing rats

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SUMMARY Triiodothyroacetic acid, triac, when given to rats during pregnancy, causes hypertrophy and intracellular disarray in the hearts of their offspring and concurrent treatment with dl propranolol can prevent the latter abnormality. Further experiments have been carried out to test the effect of dl oxprenolol and d propranolol on triac-induced myofibrillar disarray. Administration of dl oxprenolol at the same time as triac prevented disarray but a higher dose than that of dl propranolol was required to produce this effect. Disarray was also prevented by d propranolol. Selective consideration of the membrane stabilising, β blocking and agonist activities of dl oxprenolol and d and dl propranolol leads to the conclusion that although direct or indirect β stimulation by triac may play a small part in its disruptive effect on the developing myocardium, its main deleterious action can be blocked by membrane stabilisation.

Hypertrophic cardiomyopathy is characterised by extreme and bizarre cardiac hypertrophy and, by definition, is of unknown origin.[1] The association between hyperthyroidism and cardiac hypertrophy has long been recognised both in man[2] and in other animals,[3,4] and more recently hypertrophic cardiomyopathy has been reported in patients with mild long-standing hyperthyroidism.[5,6] Identification of triiodothyroacetic acid (triac) and tetraiodothyroacetic acid (tetrac), the acetic acid metabolites of triiodothyronine and thyroxine respectively, in the circulation of a patient with covert thyro-cardiac disease[7] suggested that a thyroid hormone imbalance could be an underlying cause of the disease in some cases. This possibility was strengthened by the finding that administration of triac to rats during pregnancy produced hypertrophy and myofibrillar disarray in the hearts of their newborn offpspring.[8] An understanding of the mechanism by which triac produces these effects should therefore help to elucidate the aetiology of hypertrophic cardiomyopathy.

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The starting point for the experiments described in this paper was the observation that dl propranolol, administered to pregnant rats at the same time as triac, protected the developing heart muscles of their offspring from myofibrillar disarray.[9] The actions of dl propranolol are β-adrenergic blockade and membrane stabilisation,[10] either or both of which could be responsible for these protective effects. In order to study their relative importance we have now used the rat model to investigate the effect on triac-induced myocardial damage of d propranolol, which has membrane stabilising activity but minimal β blocking action, and of oxprenolol, a β blocking and membrane stabilising drug with additional partial agonist action.

Materials and methods

The methods used were essentially those described by Hawkey *et al.*[9] The basic outline and important differences are described here.

Groups of virgin female Wistar rats were mated and given daily intramuscular injections of triac, alone or in combination with dl oxprenolol, dl propranolol or d propranolol, throughout pregnancy. Suitable controls were included. Tables 1 and 2 show the injections given to each group and the details of pregnancy.

TABLE 1 *Experimental groups and details of pregnancies — Triac with oxprenolol*

Group	*Treatment*	*No of pregnancies*	*Average litter size*	*% live births*	**Gestation period/days*
1	Glycine buffer	4/5	12	90	22
2	Triac 60 μg	4/5	11	67	22-23
3	Triac 60 μg + oxprenolol 1 mg	5/5	13	87	22
4	oxprenolol 2 mg	4/5	10	92	21-23
5	oxprenolol 4 mg	4/5	12	94	21-22
6	Oxprenolol 4 mg	4/5	11	84	21-22

*Gestation period — number of days between appearance of vaginal plug and delivery of litter.

TABLE 2 *Experimental groups and details of pregnancies — Triac with dl and d propranolol*

Group	*Treatment*	*No of pregnancies*	*Average litter size*	*% live births*	**Gestation period/days*
7	Glycine buffer	3/3	9	100	21-22
8	Triac 60 μg	2/3	7.5	79	22
9	Triac 60 μg + dl propranolol 0.25 mg	3/3	11	100	22
10	dl propranolol 0.5 mg	3/3	8	100	21-24
11	dl propranolol 1 mg	3/3	8	100	21-24
12	Triac 60 μg + d propranolol 0.25 mg	3/3	10	97	21-22
13	d propranolol 0.5 mg	3/3	8.5	94	22
14	d propranolol 1 mg	2/3	8.5	94	22
15	dl propranolol 1 mg	2/3	12	100	21-22
16	d propranolol 1 mg	3/3	9	94	22-24

*Gestation period as in table 1.

Triac (diethanolamine salt of triiodothyroacetic acid, Sigma Ltd) was dissolved in glycine buffer; dl propranolol (Inderal ICI Ltd) was used as supplied by the manufacturer for injection; d propranolol HCl was supplied as powder by ICI Ltd and was dissolved in water for injection with citric acid added at 14 mg per 50 mg d propranolol as a buffering and stabilising agent. Oxprenolol HCl was supplied by Ciba Geigy Ltd as powder and was also dissolved in glycine buffer.

All solutions other than dl propranolol were prepared in 20 ml sealed multidose vials and stored in the dark at 4°C.

After birth, the offspring received no further treatment. Four randomly selected individuals from each group were killed at 2 and 7 days after birth. The hearts were removed, weighed and transversely sectioned just below the atrioventricular groove. An entire cross section of the upper half was processed for histological examination and measurement of cell diameters. Two hundred cell diameters were measured from two rats from each group at 2 and 7 days.

Multiple areas of the lower half of the heart were processed for ultrastructural examination on a Philips 301 G electron-microscope. A coding system ensured that examination was carried out blind. The degree of *myofibrillar disruption* was assessed by counting the number of foci of disarray and cross over in 5 squares of longitudinally arranged cells on the 200 mesh grid.

Hypertrophy was evaluated by counting the number of mitochondria per two sarcomeres of parallely alligned myofibrils. Up to two mitochondria per two

sarcomeres was considered normal and scored 0. Up to three indicated mild hypertrophy and scored 1, up to four scored 2, etc.

Results

No behavioural changes or abnormal clinical signs were noted in any of the pregnant rats. Weight gains, food intake, litter sizes and gestation periods were similar in all groups. The percentage live birth rates for the groups receiving triac alone were less than for the other groups (table 2).

LIGHT MICROSCOPE FINDINGS

Cell diameter measurements are summarised in tables 3 and 4. To reduce the mass of numbers the figures for the lower doses of the drugs have been excluded. The fibre diameters of the offspring of rats receiving triac were significantly greater than their corresponding controls. Oxprenolol did not reduce the level of triac-induced hypertrophy, nor did it have any effect when given alone. With dl and d propranolol there was no reduction in hypertrophy at 2 days, although by 7 days the cell diameters were less than those for triac alone but still greater than the controls. Occasional

TABLE 3 *Cell diameters — triac with oxprenolol*

Group	*Mean cell diameter μm*	*SD*	*P1*	*P2*
2 days				
1 Control	6.94	1.09		
2 Triac 60 μg	8.84	1.27	<0.001	
5 Triac 60 μg + oxprenolol 4 mg	8.82	1.41	<0.001	NS
6 Oxprenolol 4 mg	7.13	1.04	NS	<0.001
7 days				
1 Control	8.19	1.15		
2 Triac 60 μg	9.68	1.44	<0.001	
5 Triac 60 μg + oxprenolol 4 mg	9.46	1.45	<0.001	NS
6 Oxprenolol 4 mg	8.27	1.30	NS	<0.001

P1: compared with group 1
P2: compared with group 2
NS No significant difference

TABLE 4 *Cell diameters — triac with d and dl propranolol*

Group	*Mean cell diameter μm*	*SD*	*P1*	*P2*
2 day rats				
7 Control	8.49	1.26		
8 Triac 60 μg	9.48	1.12	<0.001	
11 Triac 60 μg + dl prop 1 mg	9.31	1.32	<0.001	NS
14 Triac 60 μg + d prop 1 mg	9.28	1.37	<0.001	NS
15 dl prop 1 mg	8.37	1.10	NS	<0.001
16 d prop 1 mg	7.94	1.11	0.001	<0.001
7 day rats				
7 Control	7.19	1.01		
8 Triac 60 μg	8.43	1.25	<0.001	
11 Triac 60 μg + dl prop 1 mg	7.82	0.98	<0.001	<0.001
14 Triac 60 μg + d prop 1 mg	8.01	0.98	<0.001	<0.001
15 dl prop 1 mg	7.70	1.10	<0.001	<0.001
16 d prop 1 mg	7.26	1.05	NS	<0.001

prop=propranolol

TABLE 5 *EM scores for disarray and crossover — triac with oxprenolol*

Group	*2 day rats*				*7 day rats*			
1 Control buffer	7	2	6	2	3	0	0	1
2 Triac 60 μg	9	20	5	21	17	14	9	4
Triac 60 μg +								
3 oxprenolol 1 mg	26	1	19	2	25	8	3	0
4 oxprenolol 2 mg	11	5	8	15	5	7	7	3
5 oxprenolol 4 mg	1	5	1	6	7	3	2	4
6 oxprenolol 4 mg	0	4	4	0	9	2	2	2

areas of slight disarray of fibres were found in some sections but they were not associated with any particular group.

ELECTRON MICROSCOPE FINDINGS

Myofibrillar disarray (see tables 5 and 6) was present as a few occasional foci in the control groups, but in these, regular arrangement of myofibrils predominated (fig 1). The number of foci was greatly increased in offspring of rats which received triac alone and in most cases the severity was greater at 2 days than at 7 days. Fig 2 illustrates a focus of disarray

TABLE 6 *EM scores for disarray and crossover — triac with d and dl propranolol*

Group	*2 day rats*				*7 day rats*			
7 Control buffer	0	0	1	1	0	2	0	1
8 Triac 60 μg	9	11	15	10	4	13	7	2
Triac 60 μg +								
9 dl propranolol 0.25 mg	1	9	3	3	3	4	0	5
10 dl propranolol 0.5 mg	2	0	2	1	0	0	0	1
11 dl propranolol 1 mg	0	0	0	2	7	3	0	0
Triac 60 μg +								
12 d propranolol 0.25 mg	8	4	3	10	6	5	1	0
13 d propranolol 9.5 mg	4	2	6	1	2	5	1	0
14 d propranolol 1 mg	3	3	2	0	2	2	0	0
15 dl propranolol 1 mg	3	0	0	0	2	3	0	1
16 d propranolol 1 mg	3	1	1	4	0	0	0	2



FIG 1 *Electromicrograph of a 2 day rat from control group 1. Myofibrils are predominantly regularly arranged. Original magnification × 3600*

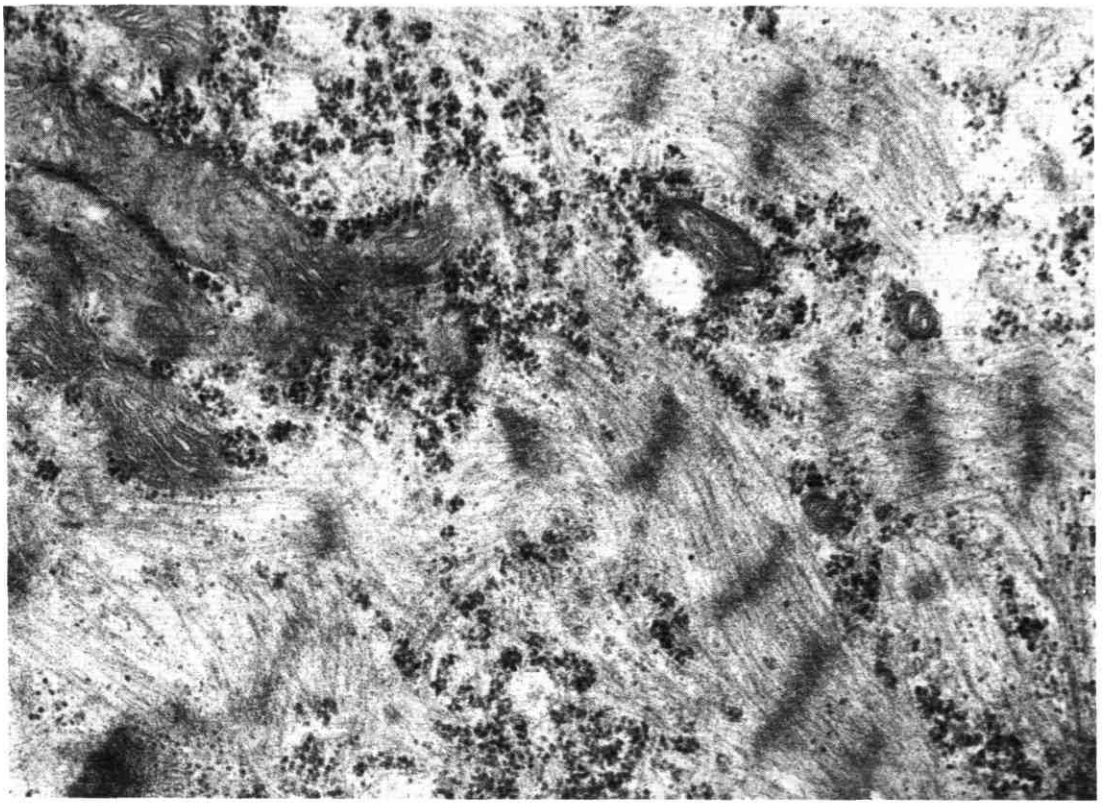


FIG 2 *Electromicrograph of a 2 day rat from triac treated mother, group 2. Severe disarray of myofibrils and myofilaments are noted. Original magnification × 22000*

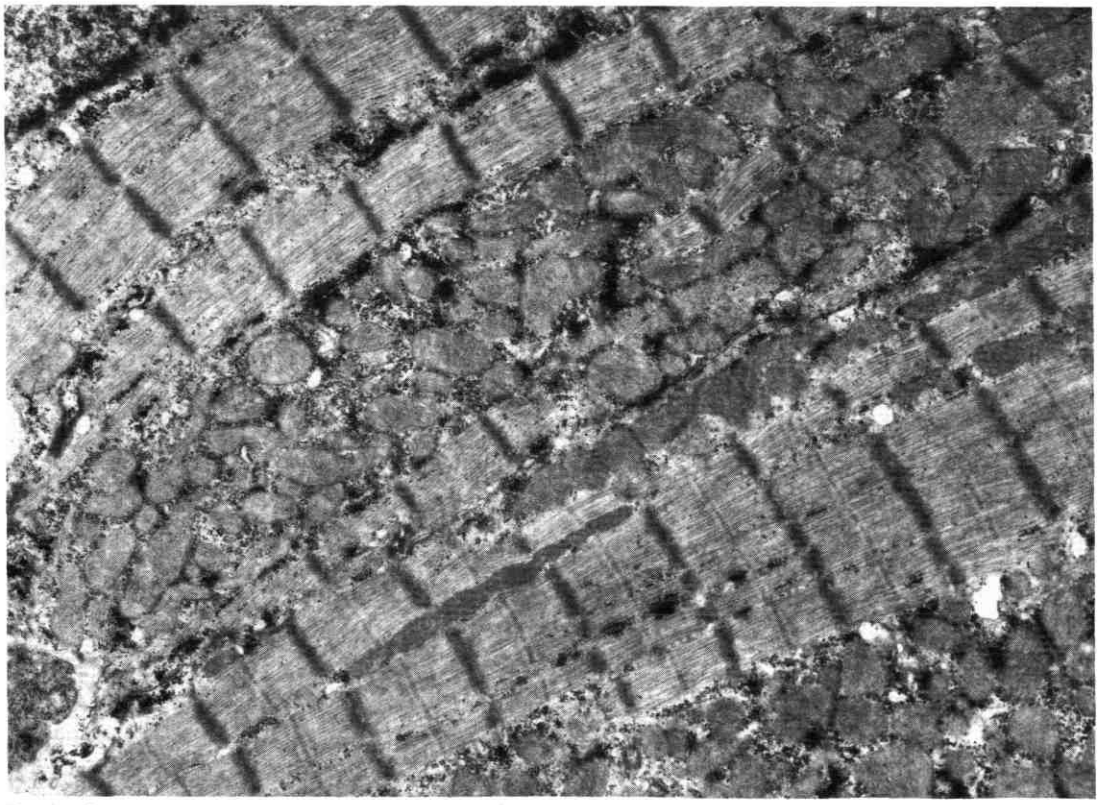


FIG 3 *Electromicrograph of a 7 day rat from group 10. Myofibrils are regularly arranged but mitochondrial numbers indicate mild hypertrophy is present. Original magnification × 15000*

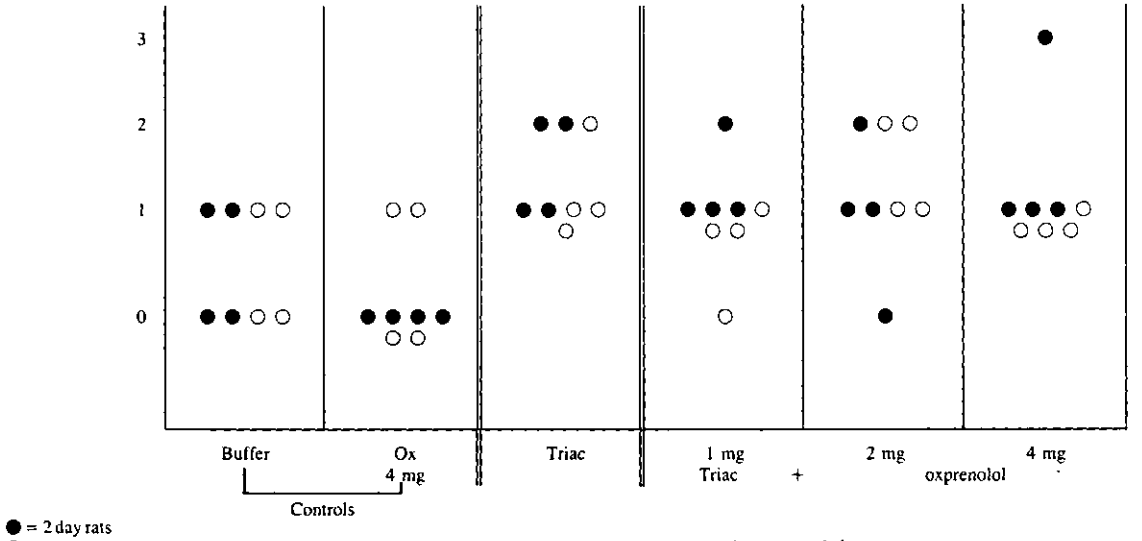


FIG 4 *EM scores for hypertrophy — triac with oxprenolol*

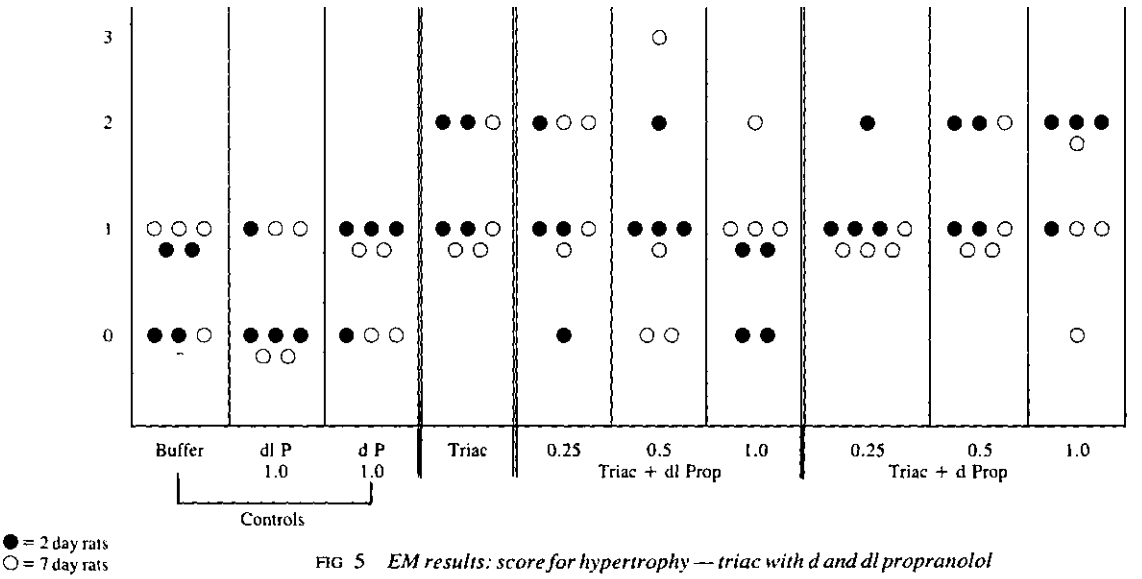


FIG 5 *EM results: score for hypertrophy — triac with d and dl propranolol*

from a rat in group 2. Oxprenolol, at 4 mg per day reduced the triac-induced disarray to levels similar to those of the controls. When 1 and 2 mg were given high scores for disarray were still noted. Disarray was reduced by dl propranolol at 0.25 mg and control levels were seen when 0.5 and 1 mg were given. The level of disarray was also reduced by d propranolol at the highest dose rate tested.

Hypertrophy. In approximately half of the control rats (buffer and antagonist alone) scores of 1 were found, the remainder being 0. However, when triac was given alone, all scores were 1 or 2 (see figs 3, 4 and 5). Despite some individual variation, the scores all remained at this level when either oxprenolol or d propranolol was given with triac. Some low scores for hypertrophy were observed in the groups receiving dl propranolol with triac.

Additional evidence of the presence of hypertrophy was provided by a general increase in the number of mitochondria in all parts of the cell and crenation of the nuclear membranes.

Discussion

In man, hypertrophic cardiomyopathy is characterised by often extreme cardiac hypertrophy, intra- and intercellular disarray and disproportionate septal thickening.[11–13] The experiments described in this paper confirm that two of these abnormal changes, cardiac hypertrophy and intracellular disarray, can be produced by exposure of rats to triac, or to its effects, during foetal development.[8] Hypertrophic cardiomyopathy itself has not been produced but the experiment provides a useful model for the study of disarray. The finding that triac-induced disarray can be prevented by concurrent administration of drugs with different actions provides new information which may help to clarify the mechanism producing the disease.

The possibility that excess catecholamine activity is responsible for pathological changes in the heart muscle has been proposed by Goodwin[14] who suggested that "the relationship with catecholamines is a complex one and the effect is probably exerted on the growing myocardium rather than a simple matter of excess amount of catecholamine activity on the heart over a prolonged period later in life". This concept would also probably be applicable if thyroid hormones or their metabolites are involved, as prolonged administration of both triac or thyroxine to adult rats produced concentric cardiac hypertrophy but no alteration in cardiac structure.[15] In contrast, the brief administration of triac during pregnancy affects the proper alignment of myofibrils as well as producing hypertrophy in the offspring.[8] Thyroxine can also produce this effect but much higher doses are required (unpublished data). The cause of the disarray could possibly be altered ventricular wall stresses resulting from an extra systolic isometric contraction of the foetal heart, as suggested by Bulkley *et al.*[16] An understanding of the precise mechanism of the action of triac is therefore important.

β blockade by dl propranolol is a recognised treatment for patients with hypertrophic cardiomyopathy[17] but is only effective in relieving symptoms for some patients and does not cure the condition. At concentrations needed to block the beta receptors, propranolol also exerts a direct negative inotropic effect and this cardiac depressant action can lead to congestive cardiac failure in long-term treatment. Oxprenolol, on the other hand, with its additional sympathomimetic or partial agonist action, exerts a positive inotropic action which provides a safeguard againt cardiac insufficiency. Approximately 10 times the dose of oxprenolol, compared with propranolol, is needed to diminish the contractile force by the same amount. In clinical studies on the symptomatic treatment of moderate thyrotoxicosis, Gibbard and Staffurth[18] found oxprenolol no better than a placebo, and concluded that intrinsic sympathomimetic activity countered the beneficial actions of β blockade. The results presented here suggest that the sympathomimetic property could have an influence. Over eight times the dose of oxprenolol compared with propranolol was required to produce the same effect, when, in terms of β blockade, the difference in potency is only double.

In order to assess the importance of the other property common to oxprenolol and propranolol, the membrane stabilising activity, d propranolol was used. This action is a powerful one as both isomers of propranolol are more than twice as potent in reducing isolated nerve trunk spike amplitiude as the local anaesthetic procaine.[10] Oxprenolol is 1.4 times as active as procaine. Clinically the action could be important as d propranolol reduced the heart rate and cardiac output of hyperthyroid patients, though not by the same extent as the racaemic mixture.[19]

At the doses used, d propranolol reduced the level of triac-induced disarray and crossover although not completely preventing it. The results therefore indicate that some of the action of triac is via direct or indirect β receptor stimulation but that most is via a direct action which can be blocked by membrane stabilisation. This finding is in part contrast to Perloff's[20] suggestion that the stimulus for cellular disorganisation may be an adrenergic stimulus that accompanies hyperthyroidism, rather than a primary effect of the thyroid hormone or circulatory analogue.

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