THE FEASIBILITY OF USING HEPATOCYTES TO INVESTIGATE HORMONE INDUCED CHROMATIN CHANGES

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CERTIFICATE OF SUPERVISOR

In terms of paragraph five of "General Regulations for the Degree of M.Sc." I, as supervisor of the candidate, E.A. Venter, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.

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SCOPE OF THIS THESIS

Alterations at the chromatin level induced by hormonal induction have been reported widely in the literature "in vivo" as well as "in vitro" investigations, but still remain controversial.

To characterise such alterations, the suitability of the induction of tyrosine aminotransferase by glucocorticoids, was investigated. TAT as a marker enzyme for glucocorticoid induction in liver has been well described. However, the events at the chromatin level which contribute to the 2 hour delay in eliciting of the biological response (Izawa et al., 1982), still remain to be answered.

In order to conduct the "in vitro" investigation on identical cell preparations, hepatocytes with a high viability (Seglen, 1976) were chosen as a model system.

The above mentioned study is outlined in 2 major parts in this thesis:

1. The isolation and purification of hepatocytes and their nuclei, the development of culture conditions and the determination of the metabolic activity of the cells are

discussed.

2. The second part encompasses the study of triamcinolone acetonide uptake by the cells, the development of the TAT assay, induced TAT synthesis and the post-translational acetylation and phosphorylation of histones.

SUMMARY

The feasibility of using hepatocytes in culture, to study hormonal induced alterations at the chromatin level, was investigated.

Hepatocytes were isolated using a modified version of the physiological collagenase perfusion method of Seglen (1976). A compact perfusion system, which allowed continuous flow of the perfusate during the excision of the liver from the carcass and during changing over between perfusion buffers, was designed. A contamination free cell preparation with a viability in the excess of 80%, was obtained. Further biochemical analysis necessitated the liberation of the nuclei, hence different ways of breaking the cells were investigated.

Short term monolayer and suspension cell cultures were established. Protein and RNA synthesis were determined on the cells in suspension culture to assess their metabolic integrity.

Glucocorticoid induction was studied by determining the sequence and duration of events upon uptake of the glucocorticoid by the cell, until eliciting of tyrosine aminotransferase (TAT)

synthesis. A TAT assay, sensitive enough to determine the basal level of TAT present in 1×10^6 hepatocytes, was established. Radio-active labels were used to study the post-translational acetylation and phosphorylation of histones during induction.

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ABBREVIATIONS

Arg	=	arginine
ATP	=	adenosine 5' – triphosphate
BSA	=	bovine serum albumin
DEAE	=	diethylaminoethyl
DMEM	=	Dulbecco's Modification of Eagles Medium
DNA	=	deoxyribonucleic acid
DNase	=	deoxyribonuclease
DNPH	=	dinitrophenylhydrazone
EDTA	=	ethylenediamine tetra-acetate
EGTA	=	ethylenebis (oxyethylenenitrilo) - N,N,N',N'-tetra-acetic acid
FCS	=	fetal calf serum
GFC	=	glass fibre cellulose
HPLC	=	high pressure liquid chromotography
HTC	=	hepatoma cells
MEOH	=	methanol
MTV	=	mammary tumour virus
N.I.P.-40	=	Nonidet P-40
OPA	=	O – phthaldehyde
PPO	=	2,5 diphenyl oxazole
RNA	=	ribonucleic acid
SDS	=	sodium dodecyl sulphate
SRC	=	steroid receptor complex
TAA	=	triamcinolone acetonide
TAT	=	tyrosine aminotransferase
TCA	=	trichloro – acetic acid
TEMED	=	N,N,N',N' – Tetramethylenediamine
Tris	=	tris-(hydroxymethyl)-amino methane

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MATERIALS AND METHODS

PART 1

REVIEW ON HEPATOCYTES

1.1 INTRODUCTION

The liver, representing 4% of the total body weight of the rat (Jeejeebhoy et al., 1975), is with exception of the skin the largest organ in the body and has diverse functions. It serves first of all as the primary receiving depot, the chemical processing plant and the distribution centre for almost everything that enters the body through the walls of the alimentary tract. The fact that the liver occupies a central position in body metabolism, and its size, softness and relative homogeneity have made it a favourite organ for biochemical investigation.

Although many biochemical studies have been performed on the liver "in vivo" (Yuwiler et al., 1970; Grossman and Boctor, 1972; Chong-Cheng and Oliver, 1972; Izawa et al., 1982; Åström and Von der Decken, 1983; Grote et al., 1983), the isolated perfused rat liver has been used for the study of intact liver functions under controlled conditions (Seglen 1972 a).

Although this experimental system is excellent for many purposes, it has several shortcomings:

1. It is difficult to obtain many identical samples from a non-homogeneous organ like the liver, containing up to 40% non-parenchymal cells (Daoust, 1958).
2. The viability of an isolated liver can be maintained only for a limited period of time, hence long term studies are excluded.
3. Only one experimental treatment can be tested at a time, making experiments involving more than one parameter impossible.

Numerous attempts have been made to overcome these problems by the isolation and purification of intact parenchymal rat liver cells.

1.2 STRUCTURE OF THE LIVER

For a better understanding of the strategy behind all the liver cell isolation methods attempted over the years, a closer look at the liver is warranted.

The main structural component of the liver is the hepatocyte. The hepatocytes (epithelial cells) radially dispersed, are

grouped in plates which are interconnected in such a way as to form structural units called liver lobules. These plates are directed from the periphery of the lobule to its centre and anostomose freely, forming a complex labyrinth. The space left between these plates is filled by sinusoid capillaries (liver sinusoids). These capillaries are composed of a discontinuous layer of cells encompassing endothelial and Kupffer cells.

The spaces of Disse, between the liver cells and the wall of the sinusoid are usually filled by microvilli of the hepatocytes, reticular fibres and extracellular fluid. The sinusoid's form is maintained by a surrounding sheath of reticular fibres. Type III collagen and fibronectin have been identified in reticular fibres (Rubin et al., 1978).

1.2.1 Liver Blood Supply

In addition to the arterial supply, which oxygenate the liver cells, the portal vein brings in the bulk of blood charged with products of the reabsorption process from the intestinal tract.

The portal vein branches repeatedly and sends small venules, the portal venules, to the portal canals (interlobular branches). The latter penetrate into the lobules and empty their contents into

the liver sinusoids. These sinusoids run radially and converge in the centre of the lobule to form the centrolobular vein. The central vein progresses along the lobule, it receives more and more sinusoids and gradually increase in diameter to form larger veins. The latter veins form the sublobular veins, which gradually converge and fuse, forming the 2 or more large hepatic veins that end in the upper vena cava.

1.2.2 Hepatocytes

The cells are polyhedral, with 6 or more faces and have a diameter of 20 — 30 µm. The cell surface is covered by glycocalyx and the surface of the cell facing the space of Disse presents many microvilli. The cell presents one or two rounded nuclei with one or two typical nucleoli (Junqueira, 1977).

The cell membrane is approximately 50Å in thickness and is separated by a space of 50Å from the cell membrane of the adjacent cell (Coman, 1954). The liver cells are held together by tight (Heath and Wissig, 1966) and gap junctions (Revel and Karnovsky, 1967). These border the bile canaliculi, desmosomes, and the interdigitation of cytoplasmic extensions of adjacent cells (Fawcett, 1955).

Calcium is known to play a role in cellular adhesion. A molecular band of calcium between opposed cells, presumably by linkage of the calcium to the carboxyl groups of the proteins and to the phosphate groups of lipids was suggested (Coman, 1954). Desmosomes are .complex disk-shaped structures formed by the juxtaposition of 2 electron-dense regions (attachment plaques) present near the cell membranes of 2 neighbouring cells but within the cytoplasm of each cell (Junqueira, 1977). Studies by Amsterdam and Jamieson (1974) suggest that the attachment plaque is identical with a calcium-dependent adhesion factor.

1.3 ISOLATION OF HEPATOCYTES

1.3.1 Mechanical Dispersion

The methods, which were used in the first attempts to isolate single cells from liver, were essentially mechanical ones. Chopping and manipulating of slices, homogenization with a loosely fitting Potter-Elvehjem homogenizer, forcing the liver through cheese-cloth or steel screens, shaking with glass beads, and other mechanical procedures were used to obtain single isolated liver cells (for review see Schreiber and Schreiber, 1973). The cells obtained by such techniques were uniformly

damaged.

1.3.2 The Use of Chelators

In an attempt to reduce the damage caused to the cells by the harsh mechanical treatment required to disintegrate the liver, chelators were employed to lyse the intercellular matrix, followed by gentle mechanical treatment (mechanical shaking). Although the removal of calcium by divalent metal chelators, citrate (Anderson, 1953); EDTA (Coman, 1954; Leeson and Kalant, 1961); EGTA (Seglen, 1972b) or by the perfusion with a calcium-free buffer (Seglen, 1973a) may improve the separability of the liver cells to some extent, the mechanical force required for complete separation of the cells still results in damage to virtually all cells, less than 1% of the cells remain intact. The improvement in separability of the liver cells can be ascribed to the disruption of desmosomes by the removal of calcium, but tight and gap junctions remain intact (Schreiber and Schreiber, 1973; Seglen, 1973a). Friedmann and Epstein (1967) showed that the cells prepared with citrate have only limited value for the "in vitro" study of the regulation of hepatic protein synthesis.

1.3.3 Enzymatic Treatment

This approach entails the promotion of cell separation by enzymatic hydrolysis of the intercellular matrix by proteolytic enzymes, such as trypsin, chymotrypsin, papain, pepsin, pronåse and lysozyme. Disagreement exists as to the effectiveness of these enzymes in promoting cell dispersion. Major damage to cell membranes have been observed by all of them (Longmuir and Ap Rees, 1956; Easty and Mutolo, 1960; Laws and Stickland, 1961, Pisano et al., 1968). Trypsin however, was successfully employed for the separation of single cells from avian liver (Montalvo and Conrad, 1972) and Kupffer cells from rat liver, (Van Berkel et al., 1972). Pronase, while very effective in digesting most of the liver parenchyma and leaving the non-parenchymal cells intact, has been employed by many investigators for the preparation of pure non-parenchymal (Kupffer) cells (Mills and Zucher-Franklin, 1969; Van Wyk et al., 1971).

Great progress was made when the use of enzymes more appropriate for the lysis of the intercellular matrix, such as collagenase and hyaluronidase were introduced for the preparation of single cell suspensions from adult liver. Howard et al. (1967), (1968) described an enzymatic technique in which isolated cells were prepared by shaking liver slices in a buffered medium containing

hyaluronidase and collagenase. Cells isolated by them in this manner, appeared to have intact cell membranes (when studied by electron microscopy), respired in the presence of calcium and were not stained by trypan blue, but the percentage yield was only 5% of the original tissue. Dissociation of liver cells by Berry and Friend (1969), using a simplified version of the perfusion system devised by Mortimore (1961),which made the liver tissue uniformly accessible to the actions of 0,05% collagenase and 0,10% hyaluronidase, resulted in the conversion of about 50% of the liver into isolated, parenchymal cells. The effectiveness of hyaluronidase in cell dispersion has been questioned by many workers (Ingebretsen and Wagle, 1972; Clark et al., 1973; Veneziale and Lohmar,1973) and its presence has been found to be unnecessary; in fact was inhibitory to dispersion (Seglen,1973b). It also had the effect of enhancing the degradation of hepatic glycogen (Wagle, 1974).

1.3.4 Calcium and the Two-Step Procedure

The poor results obtained by Berry and Friend (1969),can be ascribed to their failing to recognize the dual role Ca^{++} plays in liver dispersion involving collagenase. Collagenase is a Ca^{++} - requiring enzyme (Seifter and Harper,1970), thus the inclusion of Ca^{++} during perfusion with collagenase enhances enzymatic

activity and accelerates dispersion. They had used the Ca^{++}, Mg^{++} — free Hanks solution, which had been found to be most suitable by Howard et al.(1967), (1968) in their study.

The partial success they achieved with their enzymatic dispersion of the liver could probably be ascribed to the inefficient removal of endogenous Ca^{++} during the pre-perfusion step.

A systematic, quantitative study of the various parameters of collagenase perfusion was undertaken in Seglen's laboratory, (Seglen, 1972b, 1973a,b). By modifying the collagenase perfusion technique it became possible to convert the whole liver to a suspension of intact cells, i.e. both the initial yield and the cellular viability approach 100% under ideal circumstances.

It was shown by Seglen (1973a) that Mg^{++} inhibits liver dispersion, probably by competition with Ca^{++} and should be omitted from the enzyme medium. The presence of K^{+} during pre-perfusion was found to be advantageous (reduce cellular adhesion) and cells isolated by the two-step technique have been reported to retain K^{+} better and to have a higher K^{+} uptake capacity than cells prepared without Ca^{++} addition (Barnabei et al., 1974), this has also been shown to be the case with cells prepared from liver slices incubated with collagenase in the

presence of Ca^{++} (Howard et al., 1973).

Crude collagenase has been used in concentrations ranging from 0,01% - 0,08%, but 0,05% was found to be optimal with 50 mℓ of perfusate. The optimal concentration may possibly depend on the total volume of the recirculating collagenase solution, collagenase batch (specific activity of collagenase) and the size of the liver (collagen content increases with age, Harkness, 1958).

1.3.4.1 Perfusion Flow Rate, Volume and Duration

Since the introduction of the liver perfusion step to be executed in the physiological direction with a medium close to the natural one, many theories have been put forward concerning the role the flow rate, volume and the duration of perfusion step, play in the quality of the cell preparation obtained.

When first performed (Berry and Friend,1969), a perfusion flow rate of 50 — 60 mℓ/minute with oxygen saturated buffers was maintained. A flow rate of 40 - 50 mℓ/minute is adequate for maximal oxygenation of a 8 - 10 g liver (300 g rat) (Zahlten and Stratman, 1974; Seglen,1976). Since the liver can tolerate hypoxia (up to 30 minutes) (Seglen,1976), using a non-

recirculating perfusion technique, the flow rate could be lowered to 5 mℓ/minute (Capuzzi et al., 1971, 1974). However, a low perfusion rate has two major disadvantages, namely:

1. A big volume of expensive collagenase buffer is required.
2. It is difficult to achieve a uniform perfusion of the tissue at such a low flow rate (Seglen,1976).

The latter problem might be solved by inclusion of erythrocytes in the buffer (Quistorff et al., 1973) or albumin (Schreiber and Schreiber, 1972; Michalopoulus and Pitot,1975; Holler and Breuer,1974), resulting in an increase in perfusate viscosity, bringing about a higher perfusion pressure (Schimassek, 1968).

A high perfusion rate was favoured by Seglen (1973a). He demonstrated that the blood is removed from the hepatic veins more efficiently if the perfusion rate is raised to 50 — 60 mℓ/minute during the first minute of perfusion. This view is contradicted by workers who maintain that a high flow rate right at the beginning might cause a build up in pressure, because of the resistance offered by the viscosity of the blood, resulting in the bursting of the capillaries. The perfusion is commenced at a flow rate of 5 — 30 mℓ/minute, which is gradually raised over a 10 minute period to 50 — 60 mℓ/minute (Seglen, 1972a, 1976; Cameron-Clarke et al., 1983).

Time allowed for the removal of blood and Ca^{++} ranged from 5 — 15 minutes and the volume of perfusate from 300 — 400 mℓ. The collagenase perfusion was performed with 80 — 200 mℓ recirculating buffer and disintegration of the liver was achieved in 7 - 30 minutes, depending on the enzyme concentration used.

1.3.4.2 pH Maintenance and Oxygenation

Various factors play a role in determining the optimum pH of the perfusate. The pH value of the portal blood in the rat ranges from 7,2 — 7,4 during feeding, respiration is also strongly pH dependent. However, no pH optimum in the physiological pH range 7 — 8 could be detected (Seglen, 1972a). Although the liver can tolerate a pH change of 7,1 — 7,5 (Gerschenson ,1974a), the optimum pH for collagenase activity is 7,5, hence the pH should not be allowed to drop below 7,3 (Seglen, 1976).

pH Maintenance in the perfusate can be accomplished by 4 ways:

1. The constant addition of NaOH by means of a pH stat/titrator. (Seglen, 1972 a,b).

2. The use of the CO_2/bicarbonate system (Berry and Friend, 1969; Berg,1972; Zahlten and Stratman,1974; Jeejeebhoy et al.,1975; Groothuis,1981; Crisp,1982). This buffer system

requires the continuous gassing with 5% CO_2 and has the advantage that it combines perfusate oxygenation (95% O_2, 5% CO_2) with a very high buffering capacity. A disadvantage of this system is the bubble formation that takes place during gassing of the perfusate, resulting in microembolies (Staib and Scholz,1968). This problem was solved by means of an "oxygen lung" (bubbling oxygen saturated water into the medium (Zahlten and Stratman, 1974).

3. Organic buffers e.g. Hepes (Howard, 1973; Seglen, 1972a, 1973a, 1976). Hepes is expensive and a high concentration (0,1 M) is required in order to achieve the same buffer capacity as with $NaHCO_3$. However, it is easier to maintain a pH close to 7,4 (pKa of 7,3 at 37°C) and oxygenation is achieved with 100% O_2 resulting in less bubble formation than with 5% CO_2.

4. The perfusion is commenced, employing a $NaHCO_3$ buffer in the presence of a 5% CO_2 gas phase. Due to carbon dioxide production by the liver, there is a rise in CO_2 pressure resulting in the decrease of the pH to 7,1. At this point the gas phase is changed to 100% O_2 and the optimal pH thereafter maintained by varying the percentage of the CO_2 in the gas phase (Berry and Friend, 1969).

1.3.4.3 Characteristics of Freshly Isolated Hepatocytes

Evidence presented showed that hepatic sinusoids distend and parenchymal cells appear as naked chords, maintaining contiguity with adjacent cells at their pericanalicular junctions, while the perfusion is still in progress. Desmosomes invaginate, cellular interdigitation separate and free surfaces of the hepatic cells bulge, but neither enzymes or EDTA could cleave tight or gap junctions. Separation of the latter is achieved by mechanical tearing after disruption of other adhesive elements, resulting in defects in the membrane.

The disappearance of tight junctions and desmosomes and the appearance of uniform villi all over the cell surface were described by Seglen (1976), Drochmans et al.(1978) and Wanson et al.(1979), although the latter sometimes found hemidesmosomes at the cell surface. Freshly isolated hepatocytes retain their intracellular polarity, but not their membrane specialization (Groothuis, 1981).

The discrepancies in the above-mentioned observations can probably be ascribed to small differences in isolation procedures, like temperature and in the length of time of

perfusion.

In conclusion it can be said that during the pre-perfusion step, the removal of Ca^{++} is achieved resulting in the separation of interdigitation and desmosomes, thereby weakening the intercellular links. During the collagenase perfusion step, the collagen fibres and reticulin fibres are digested, causing the collapse of the connective tissue support of the cells. In the absence of this support, a big strain is put on the tight and gap junctions. Although it has been reported that these junctions can only be broken by mechanical tearing, the amount of mechanical force required depends largely on the degree of Ca^{++} removal and connective tissue digestion achieved.

1.3.4.4 Purification of Parenchymal Cells

The initial cell suspension obtained after collagenase perfusion contains intact parenchymal cells, variable numbers of non-parenchymal and damaged cells, some cell clumps, pieces of connective and vascular tissue and subcellular debris (Seglen, 1976). The first step in the purification process (Berry and Friend ,1969) was to shake the cell suspension at 37°C for 15 minutes (37°C for 7 minutes, Groothuis,1981) in order to break up cell clumps and digest isolated nuclei and damaged cells. The

pre-incubation period presumably also served to allow freshly isolated intact parenchymal cells to assume a more rounded shape during incubation and Kupffer cells to bind to aggregates of cells and debris for which they have a particular affinity. Many Kupffer cells can then be removed selectively by filtration through filters with diminishing pore sizes (250 µm, 100 µm, 61 µm mesh openings).

1.3.4.4.1 Temperature

Further purification procedures have been performed at different temperatures. Earlier workers, acting on reports that liver slices take up water, Ca^{++} and Na^{+} while losing K^{+} and Mg^{++} at low temperatures (Heckmann and Parsons,1959; Von Rossum, 1969) carried out all the manipulations at temperatures ranging from 21°C - 37°C (Jeejeebhoy et al., 1975; Gerschenson et al., 1974a). On the contrary, Seglen (1973b) reported that low temperatures do not seem to be harmful to the cells and present workers do all the purification at low temperatures (0°C - 4°C), to minimize aggregation and to slow down the metabolism of the cells and so reduce the requirement for oxygen (Howard et al., 1973).

1.3.4.4.2 Differential Centrifugation

The harvesting of cells from the suspension is achieved through very low speed centrifugation (20 g — 50 g)for 35 seconds — 5 minutes (Howard, 1968; Gerschenson, 1974a; Jeejeebhoy and Phillips,1976; Groothuis ,1981). At the same time enriched parenchymal cell suspensions are obtained, since the much smaller non-parenchymal cells and the much lighter damaged parenchymal cells, are discarded with the supernatant. The initial centrifugation is followed by three to four washes. Jeejeebhoy et al.,(1975) found that centrifugation increased the number of cells stained by trypan blue and achieved very good results by permitting the cells to settle by gravity alone. Although the viability is good, it was found to be a very time consuming process (20 minutes per wash) (Cameron-Clarke et al., 1983).

1.3.4.4.3 Gradient and Cushion Techniques

Isopycinic gradient centrifugation can be applied in the separation of small quantities of cells. Partial separation of parenchymal and non-parenchymal rat liver cells, was obtained with Metrizamide but an iso-osmotic 30% Metrizamide cushion is claimed to be more useful for the separation of intact and damaged cells (Munthe-Kaas and Seglen, 1974).

Better separation was achieved with Percoll gradients: with viable hepatocytes banding around 1,08 — 1,09 g/mℓ and Kupffer cells around 1,05 g/mℓ (Pertoft et al., 1977). Weigand (1974) used a gradient of 7 cushions of iso-osmolar Ficoll (except the cushion with the highest density of 1,133 Ficoll) to separate the different liver cells. A small contamination of non-parenchymal cells was noticed in most of the fractions.

1.4 HEPATOCYTE CULTURES

1.4.1 The Establishment and Maintenance of Hepatocytes in Primary Culture

The lack of ability of adult mammalian hepatocytes to proliferate actively "in vitro", is not surprising, considering their longevity "in vivo" (several hundred days, Grisham,1973). In rats and mice, hepatocytes exhibit minimal mitotic activity of 1 mitosis per 1 to 2 x 10^4 hepatocytes. It has been suggested that the cells may survive for the life of the animal (Bucher, 1967).

All existing methods for establishing hepatocytes in primary culture, with the aim of studying liver specific functions, must be viewed with great caution.

"In vivo" hepatocytes can undergo multiplication as a result of some hormonal or metabolic imbalance or by a two-thirds hepatectomy (McGowan et al., 1984). In rats partial hepatectomy, results in initiation of DNA synthesis within 18 hours, followed by mitosis 8 hours later (Garrard et al., 1976). Parenchymal cells obtained from fully regenerated liver (4 days after hepatectomy), retained the subcellular structural elements, characteristic for normally functioning hepatocytes for several days (Chapman et al., 1973).

Work presented by Bissell et al., 1973 and Bonney et al., 1974 demonstrated that these cells retained several major metabolic functions, e.g. albumin synthesis, glycogen synthesis, gluconeogenesis and inducibility of tyrosine aminotransferase by hydrocortisone or dexamethasone. However, the metabolic usefulness of actively regenerating cells, exhibiting changes in important metabolic functions of hepatocytes, are limited to liver regeneration studies (Huerta-Bahena, 1983).

In studies involving non- or slow growing differentiated fetal hepatocytes, evidence was presented that surviving cells retain liver-specific urea cycle functions, albumin synthesis and DNA synthesis in glucose deficient medium or medium supplemented with insulin. Such responses can also be elicited if the cells are

maintained in media containing serum from hepatectomized rats. Such cells can be used for regeneration studies (Leffert and Paul, 1972; Leffert, 1974).

Several workers have presented evidence of alterations in metabolic characteristics of hepatocytes, the fetalization of several glycolytic enzymes (Walker, 1972; Leffert,1978; Sirica, 1978) and the loss of certain adult characteristics, including glycogen synthesis (Walker,1977) and enzymes from the urea cycle (Lin and Snodgrass, 1975) have been observed. In fetal hepatocytes, adult characteristics, ranging from development of gluconeogenesis (Coufalik and Monder, 1976) to synthesis of adult serum protein (Grieninger and Granick, 1975) came to the fore.

Cells have been cultured in various media (Leffert and Paul, 1972; Bissell et al., 1973; Bonney et al., 1974; Michalopoulus and Pitot,1975; Seglen,1976; Wanson et al., 1977; Siraca et al., 1979; Rojkind et al., 1980; Cameron-Clark et al., 1984), ranging from L-15 (Bissell et al., 1973) to DMEM buffered with Hepes (Wanson et al., 1977).

Whenever a high concentration of sodium bicarbonate was employed as a buffer, the maintenance of a 95% air, 5% Co_2 atmosphere was

required, otherwise the cells were cultured in the presence of air only.

Most workers in the field include fetal calf serum in the culture medium but great controversy still prevails as to what role, if any, it plays in promoting cell attachment to different substrata. As is known, fibroblasts secrete their own fibronectin (Grinnell and Feld,1979) and thus do not require exogenous fibronectin to facilitate their attachment to substrata "in vitro". However, contradicting reports have been received pertaining the presence (Vos et al., 1979; Rubin et al., 1981) or absence (Linder et al., 1975; Stenman and Vaheri, 1978) of endogenous fibronectin in hepatocytes. Dexamethason treated hepatocytes produce a well defined extracellular matrix of fribonectin (Marceau et al., 1980). An increase from 2 to 50% in the attachment efficiency of hepatocytes on a plastic substratum, was obtained in the presence of 25% FCS (Bonney et al., 1974).

Gjessing and Seglen (1980) showed that the percentage of hepatocytes attaching to naked polystyrene in the absence of fetal calf serum, is dependent on the cell concentration in the medium. This phenomenon, they ascribed to the degree of saturation of the negative charges in the polystyrene, determined

by the amount of protein present in the incubation medium at different cell concentrations. Taking all these observations into account, the possibility of a low fibronectin content in hepatocytes can not be excluded.

The survival of cultured hepatocytes have been extended from a few days to approximately 3 weeks and 5 months, respectively, with the introduction of collagen (Michalopoulus and Pitot,1975; Siraca et al., 1979) and biomatrix (extracted from rat liver, Rojkind et al., 1980), as culture substrata. Attachment efficiencies ranging from 60% in the case of collagen to more than 70% in the case of biomatrix were obtained. The inferior results obtained with the rat tail collagen can be ascribed to the absence of collagen Type III and fibronectin, two major components of reticulin fibres (Rojkind et al., 1980). Reticulin fibres are found in close contact with hepatocytes in the liver.

In order to retain tissue architecture and to preserve the differentiation state of the cells, it normally becomes necessary to resort to organ or tissue fragment culture with all its complications (Jacoby et al., 1979; Wolff and Wolff, 1976). Thus the advantages a biomatrix substratum present for the culture of the differentiated cells can be fully appreciated.

1.4.2 Short-Term Suspension Cultures of Hepatocytes

Although suspension cultures of hepatocytes have a limited lifetime of 2 - 7 hours (Berg et al., 1972; Seglen,1976), they also hold many advantages over serial cell cultures. Apart from the practical advantages of suspension cultures e.g. easy sampling, it has been shown that suspensions of freshly isolated hepatocytes are more likely to retain specific organ functions, e.g. serum albumin synthesis (East et al., 1973), which makes them more suitable for certain metabolic studies.

Several containers, usually immersed in a shaking waterbath to prevent aggregation, have been employed in suspension culture studies. Incubation in tubes (Garrison and Haynes, 1973; Seglen, 1976) is very convenient for further biochemical analysis but the tendency of the cells to adhere to the tube wall at the air-liquid interface, resulting in the slow deterioration of the cells, limits the incubation time to two hours. For longer term incubations (7 hours), wider vessels e.g. Erlenmeyer and cell culture flasks (Nilsson et al., 1973) and spinner flasks (Jeejeebhoy et al., 1975) gassed with 95% O_2 and 5% CO_2 are preferred. A shallow depth of incubation medium to improve oxygenation (Zahlten and Stratman,1974) with gentle reciprocating shaking, normally provides the best results.

Hanks buffer (Berry and Friend,1969, Nilsson et al., 1973) and Krebs buffer (Johnson et al., 1972; Zahlten and Stratman,1974; Berg and Morland,1975) have been used as the sole incubation medium. However, better results have been obtained in the presence of macromolecules such as defatted bovine albumin for which a drop in disintegration of cells has been reported (Berg and Morland, 1975). In the presence of gelatin, increased metabolic activity has been observed (Zahlten and Stratman, 1974). Improved results have been obtained when the salt buffers were replaced by Eagle's medium (Nilsson et al., 1973 ; Grant et al., 1974). Jeejeebhoy et al. (1975) achieved success using either Ham's F10 or Waymouth's MB 752/1 medium supplemented with inactivated horse serum (Jeejeebhoy, 1975).

The practical advantages of the isolated liver cell suspensions, their faithful reproduction of normal liver function (Ontko,1972; Schreiber and Schreiber,1972 ; Ingebretsen et al., 1972b) and their hormonal responsiveness (Berg and Morland,1975) combine to make this system very attractive for studying the control of liver metabolism.

PART 2

EXPERIMENTAL RESULTS

ISOLATION AND CHARACTERIZATION OF HEPATOCYTES FROM RAT LIVER

2.1 INTRODUCTION

The methodology for the isolation of hepatocytes from rat liver was based on a modification of the method of Howard (1967), who introduced collagenase as a liver dispersing agent using liver slices. This method was extended by Berry and Friend (1969), when they combined the techniques of physiological liver perfusion and collagenase digestion to make the tissue uniformly accessible to the action of collagenase and thus ensuring a high yield of intact liver cells. Seglen (1972 a,b) introduced an improved modification of the physiological collagenase perfusion method, enabling them to convert the whole liver to a suspension of intact and metabolically viable cells.

The improved technique of Seglen (1976)(Fig. 2.1) satisfied the requirements for this study but prevailing conditions necessitated further modifications.

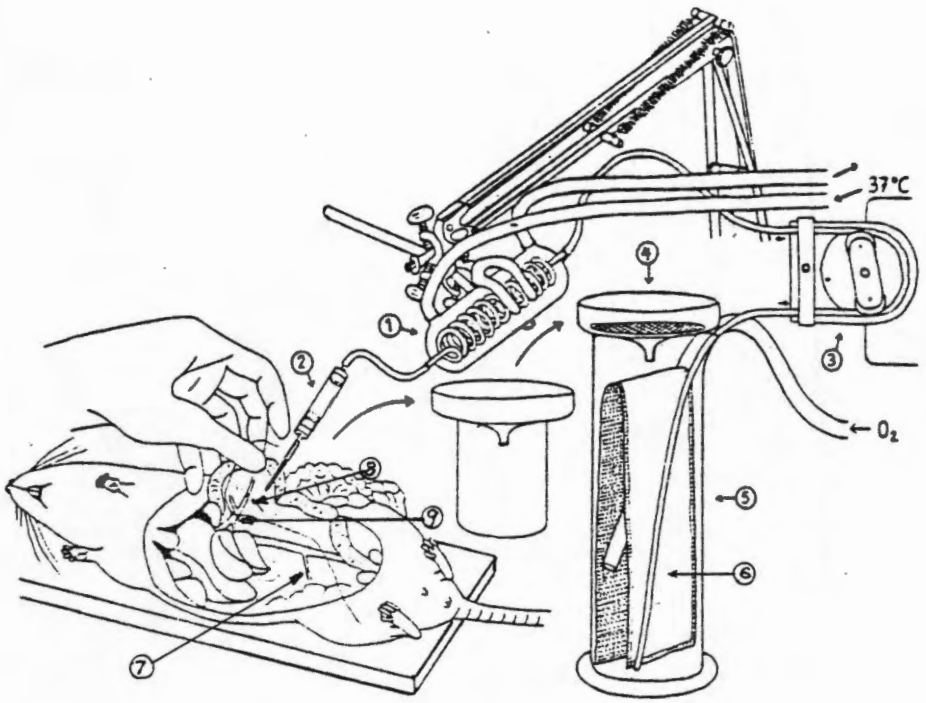


FIGURE 2.1 Experimental arrangement for isolation and perfusion of the rat liver (Seglen, 1976).

1. Water-jacketed (37°C) coiled tube fixed onto flexible lamp holder.
2. Filter and bubble trap.
3. Pump
4. Liver dish
5. Oxygenator cylinder and buffer reservoir
6. Oxygenator net
7. Site for heparin injection in the vena iliolumbalis dextra.
8. Site for insertion of the portal cannula
9. Loose ligature around vena porta.

(Fig.2.1 Cont.)

FIGURE 2.1 (Cont.)

Operative Technique used by Seglen (1976)

The rat was anaesthetized with ether and the abdomen opened. After having injected heparin into the vena iliolumbalis a loose ligature was placed around the vena porta. A deep cut was made with fine scissors in the lower vena cava (for perfusate efflux) followed by a rapid half-way cut through the vena porta. The perfusate flow was started at a rate of 20 - 30 mℓ/minute and the portal cannula rapidly inserted into the vein. After securing the ligature, the upper vena cava was cut and the perfusate flow increased to 50 mℓ/ minute. The first 2 — 3 minutes of pre-perfusion was performed in situ.

While the pre-perfusion process was still in progress, the liver was cut loose from the carcass and transferred to the liver dish supported on a beaker. When most of the Ca^{++}-free perfusion buffer in the oxygenator reservoir had been used, the flow was stopped momentarily and the remaining buffer rapidly poured onto the oxygenator net. The perfusate flow was re-established at 50 mℓ/minute and the liver dish placed on top of the oxygenator cylinder. The collagenase perfusion was continued for 7 — 10 minutes until the perfusion was naturally terminated by the

rupture of the vena porta.

2.2 DEVELOPMENT OF THE PERFUSION TECHNIQUE

Initially ether was employed as an anaesthetic, but problems encountered with maintaining light anaesthesia during the operation and inhalation of ether by laboratory personnel resulted in the adoption of Sagatal (phentobarbitone sodium) as an anaesthetic. 0,375 mℓ (60 mg/mℓ) were administered intraperitoneally per 200 g body mass and anaesthesia was achieved within 5 — 10 minutes after the injection.

0,5 mℓ of Pularin (heparin sodium solution, 1000 U/mℓ were injected into the lower vena cava, and a membrane pump (Gorman-Rupp) with a maximum flow rate of 18 mℓ/minute (the only pump available at the time) was used for the subsequent perfusion.

The following problems were encountered when the perfusion was performed according to the method of Seglen:

1. Bleeding occurred during and after administering the heparin solution into the vena cava.

2. Cutting a hole in the portal vein and inserting the nylon cannula without loss of too much blood, proved to be difficult.

3. Removing the liver, without damaging the liver itself or piercing holes in the intestines, was a cumbersome process.

4. The cannula tended to slip out of the portal vein during the transfer of the liver from the rat carcass to the liver dish.

5. Twisting of the liver lobes occurred during the transfer of the liver to the liver dish.

6. Interruption of perfusate flow while replacing the Ca^{++}—free buffer in the reservoir with collagenase buffer.

7. It was difficult to achieve efficient removal of blood due to the low flow rate used. The low flow rate also resulted in the exclusion of parts of the liver from collagenase digestion. In an attempt to achieve a more uniform perfusion, the penetration of the perfusate to all parts of the liver was improved by intermittent short pulses of rapid flow (suggested by Seglen,1976). This was achieved by

connecting up a 50 mℓ syringe to the perfusion system via a two-way tap. The short pulses lasted for 1 minute at a time (50 mℓ/minute) and the pump was only used to maintain flow, while the syringe was being filled. Although an improvement in the uniform perfusion of the liver was observed, this method was found to be very cumbersome and impractical.

8. Usually after 12 minutes perfusion, the vena porta ruptured due to collagenase digestion. This resulted in premature termination of perfusion with the result that total disintegration of the liver was never achieved.

9. On an average only 10% viable cells were found with a low total yield of cells per gram liver tissue.

To overcome these problems the following modifications were implemented.

1. The Gorman-Rupp membrane pump was replaced with a modified CFG membrane pump with a maximum flow rate of 50 mℓ/minute. To smooth out the pulsating flow a glass tube damping device was fitted (Fig.2.2.2).

Fig.2.2 PUMP MODIFICATIONS

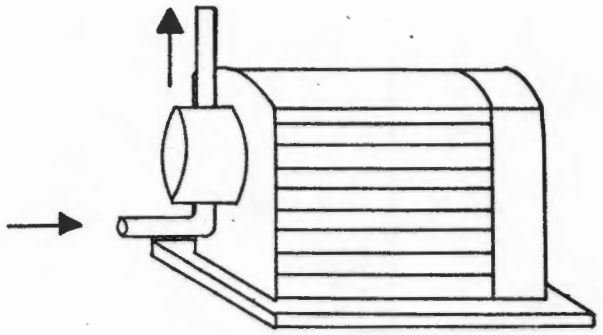


Fig.2.2.1 Pump.

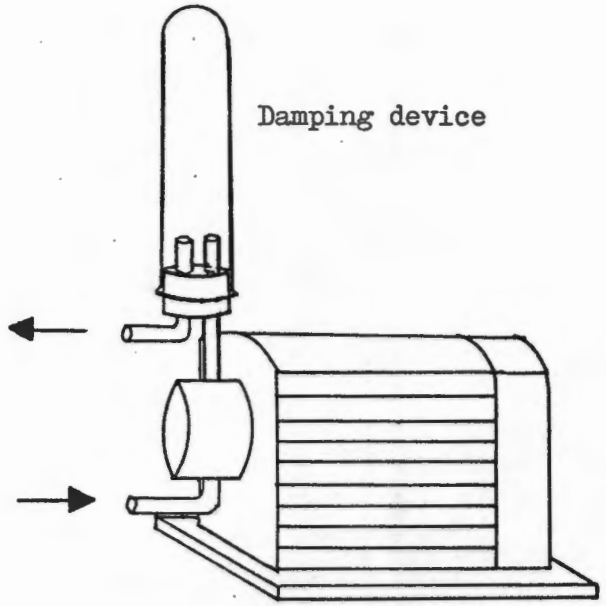


Fig.2.2.2 Pump fitted with simple glass tube damping device.

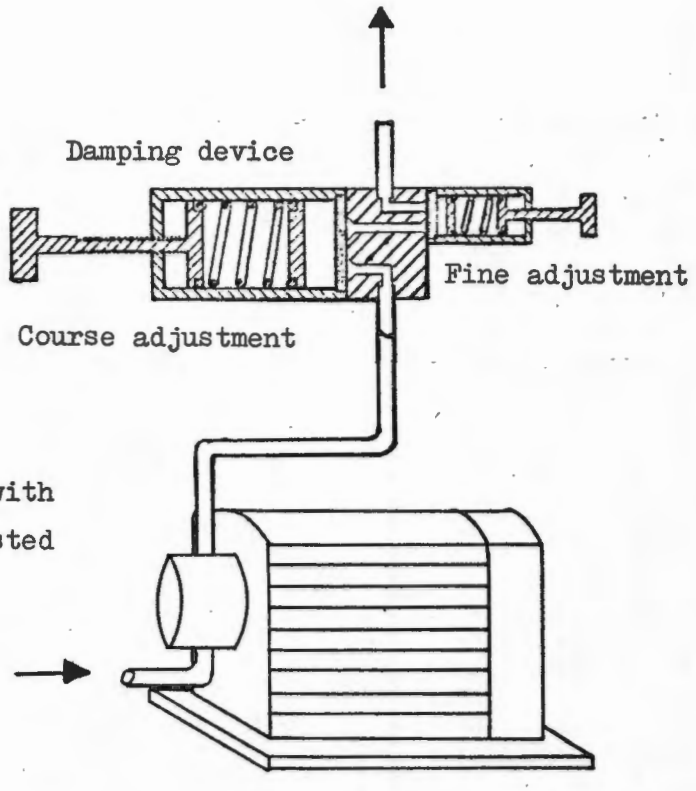


Fig.2.2.3 Pump fitted with custom built spring adjusted damping device.

2. The common perfusate reservoir (oxygenator) was replaced by two reservoirs and the switching over from the Ca^{++}-free buffer to the collagenase buffer was simplified by a two-way tap.

3. The bubble trap was redesigned and replaced by a glass tube fitted with a side inlet and a fritted glass filter (Fig.2.3.2).

4. Heparin was injected into one of the tail veins of the rat prior to opening up the abdomen.

5. Making an incision in the wall of the vena porta and the insertion of the cannula, were accomplished in one step by replacing the nylon cannula with a medican T intravenous cannula.

6. Instead of being cut, the upper vena cava was cannulated and the blood and Ca^{++}-free perfusate efflux were directed to a beaker via a tube connected to the cannula. On commencement of the collagenase buffer perfusion, a few seconds were allowed for the Ca^{++}-free buffer in the tubing and the bubble trap to be flushed out, before placing the outlet of the tube in the collagenase reservoir. The liver was not

Fig.2.3 BUBBLE TRAP MODIFICATIONS

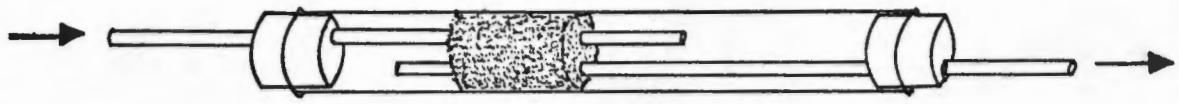


Fig.2.3.1 Seglen combined bubble trap and filter unit.

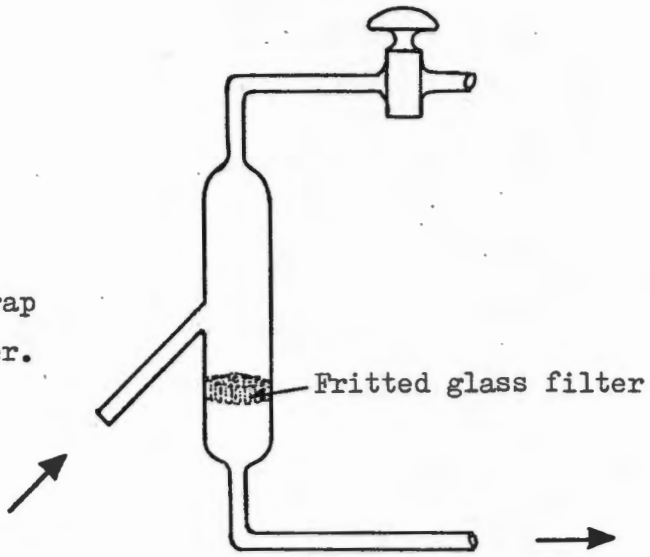


Fig.2.3.2 Glass tube bubble trap fitted with fritted glass filter.

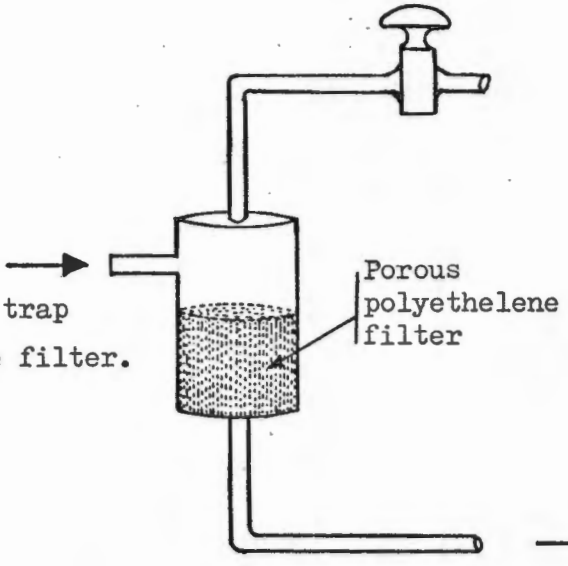


Fig.2.3.3 Custom built bubble trap fitted with porous polyethelene filter.

removed from the abdomen but perfused in situ.

7. A light source (100 watt bulb) served the dual purpose of providing adequate illumination during the operation, as well as keeping the outer surface of the liver warm, to avoid blood vessel constriction while the perfusion process was in progress.

8. While the perfusion was in progress, the liver capsule was kept wet with Ca^{++} — free buffer or collagenase buffer, respectively.

With those modifications the average percentage viability increased from 10% to 70% in the final cell suspension. This improvement could probably be ascribed to the following:

1. The increased flow speed (36 mℓ/minute) brought about a higher pressure in the liver during the perfusion process making the liver lobules in the periphery as well as in the centre of the liver accessible to the action of collagenase.

2. Although the natural intra-abdominal topology had been disturbed by placing the rat on its back and by the dis-

placement of the liver lobes to expose the vena porta, the liver lobes were returned to their natural position once the perfusion was in progress. Thus the liver lobes were not twisted. A better flow could be maintained than in the case of the excised liver.

3. The inclusion of a two-way tap in the perfusion system assured a constant, uninterrupted flow to the liver and subsequently a constant pressure was maintained in the liver during the changing over from the Ca^{++}-free buffer to the collagenase buffer.

4. The new bubble trap was more efficient in removing air bubbles from the pressurized solution. Air bubbles entering the liver under pressure expand and thus occlude the venules inside the liver. This causes a build up of fluid in that area of the liver, which then ruptures.

5. The administering of the heparin to one of the tail veins eliminated the risk of internal bleeding and the subsequent drop of blood-pressure in the liver and vena porta.

6. The efficiency of enzyme digestion was increased by

maintaining the liver at a constant temperature of approximately 37°C, the optimum temperature for collagenase action.

7. The vena porta remained intact for the whole duration of the perfusion, hence the collagenase perfusion could be extended till the liver started to disintegrate after 15 — 20 minutes and no further mechanical treatment was required to liberate the cells. This can be ascribed to the absence of any strain on the vena porta caused by stretching, twisting and the removal of its supportive tissue, during the excision of the liver from the rat.

In spite of these improvements this method, however, still had five disadvantages.

1. A large volume of expensive collagenase solution (300 — 400 mℓ) was required due to the increase in dead volume by the tubing leading from the upper vena cava to the collagenase reservoir and the loss of buffer incurred during the final stages of the perfusion when the buffer oozed out of the liver through ruptures which started appearing in the liver capsule.

2. There was a high risk of bacterial contamination from rat

hair, the intestines and blood present in the cavities of the carcass.

3. On occassions, up to 10 minutes were spent to ascertain that the heparin was injected into the tail vein and not into the tendons surrounding the veins. It is important to check that blood can be drawn up into the syringe prior to injection.

4. Small air bubbles still passed through the bubble trap.

5. The pump pulsations were still not completely smoothed out.

The first problem was solved by using a desiccator lid of suitable size covered with a piece of wire mesh as support for the rat during the operation (Fig.2.4). The lid acting as a collecting funnel was suspended with its outlet above a 1 ℓ beaker (blood reservoir) in a waterbath (Figs. 2.5 & 2.6). The operation was performed in the same way as before except that the upper vena cava was cut, allowing the efflux to collect initially in the chest cavity and then to overflow into the desiccator lid and into the beaker. Before commencement of the collagenase perfusion step, the desiccator lid and the carcass of the rat were cleaned by squirting on Ca^{++}-free buffer with a Pasteur

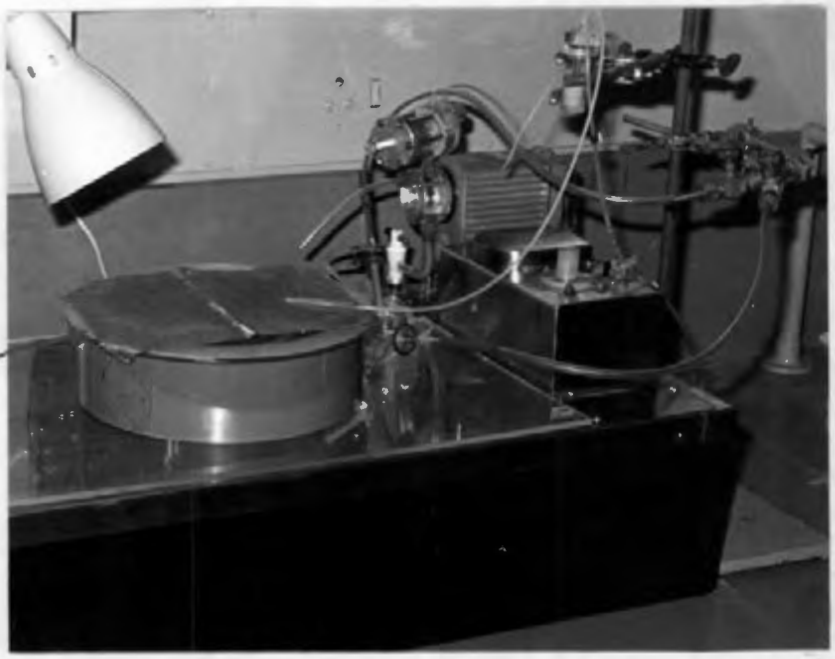


Fig. 2.4 Perfusion system showing the desiccator lid with the wire mesh cover.

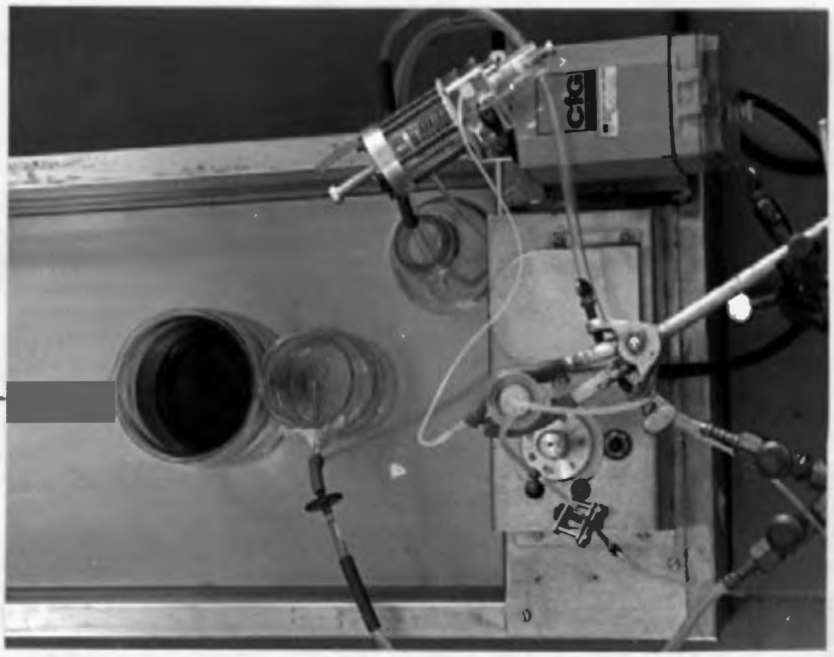


Fig. 2.5 Perfusion system showing blood reservoir.

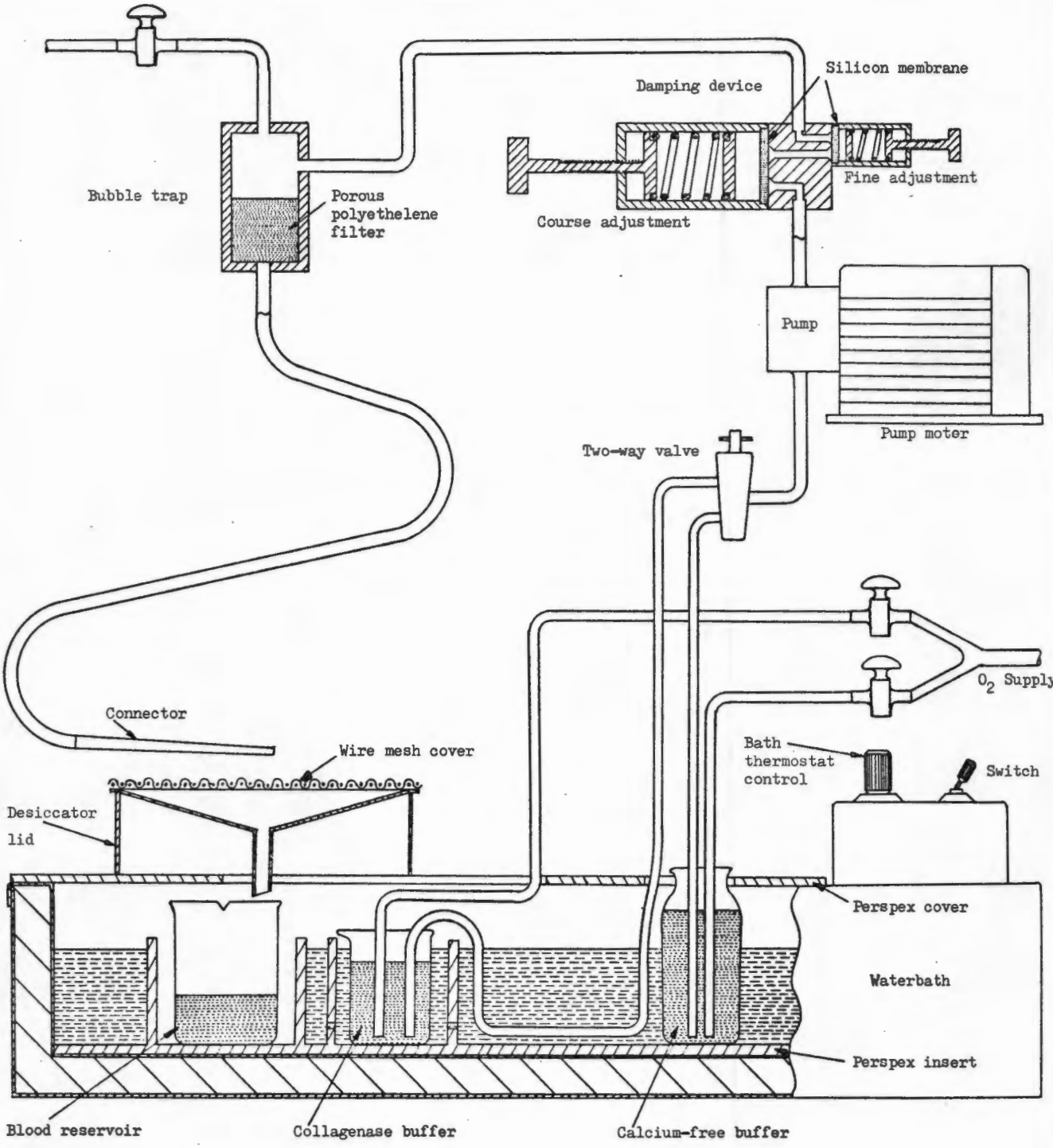


FIGURE 2.6 DIAGRAMMATIC OUTLINE OF PERFUSION SYSTEM.

pipette to remove all loose hair and blood which might contaminate the recirculating collagenase buffer (100 mℓ). After changing over to the collagenase buffer, the lid was positioned with the outlet right above the collagenase reservoir and thus 100% recirculation of the buffer could be achieved. Occasionally, blood contaminating the recirculating collagenase buffer reduced the efficiency of the collagenase buffer.

The time consuming process of trying to locate one of the tail veins was eliminated by injecting the heparin into the spleen. The damping device was redesigned with adjustable spring operated dampers and silicon membrane filters (Fig.2.2.3). The bubble trap was also redesigned (Fig.2.3.3).

2.3 "IN SITU" PERFUSION OF RAT LIVER

The rat had free access to food and water (Seglen,1976) up till being anaesthetized. The rat was then wiped down with 70% ethanol and placed on its back on the wire mesh on the desiccator lid with its legs taped to the wire mesh support in order to secure the body in a fixed position during the operation (Fig.2.7).

The abdomen was opened with a U-shaped transverse incision and

Fig. 2.7 Rat secured on wire mesh support.

Fig. 2.8 The abdomen was opened with a U-shaped transverse incision and the walls of the abdomen were cut away.

both the walls of the abdomen were totally cut away to facilitate the displacement of the intestines and to prevent the collection of the blood and buffers inside the carcass (Fig.2.8). 0,5 mℓ of heparin sodium solution (5000 U/mℓ) were injected into the spleen (Fig.2.9) before displacing the intestines to the left side of the body on a piece of perforated plastic in order to expose the lower part of the vena porta and the lower vena cava. The whole vena porta was exposed by carefully lifting the liver lobes up with a coarse point forceps and supporting them against the diaphragm.

Loose ligatures were placed around the vena porta, the artery coming from the stomach, the bile duct (Seglen,1972a) and the duodenum (Fig.2.10).

The connector at the end of the tube coming from the pump was placed on the desiccator lid and the perfusate flow started at 8 mℓ/minute. The artery coming from the stomach, the bile duct and the vena porta at the most distal ligature were tied off, followed by the immediate insertion of the catheter at a site just proximal to the knot (to prevent blood from leaking out at the end of the portal vein). The needle was slid down the vena porta just far enough for the point of the plastic cannula to be safely inside the vein, before carefully pulling out the needle.

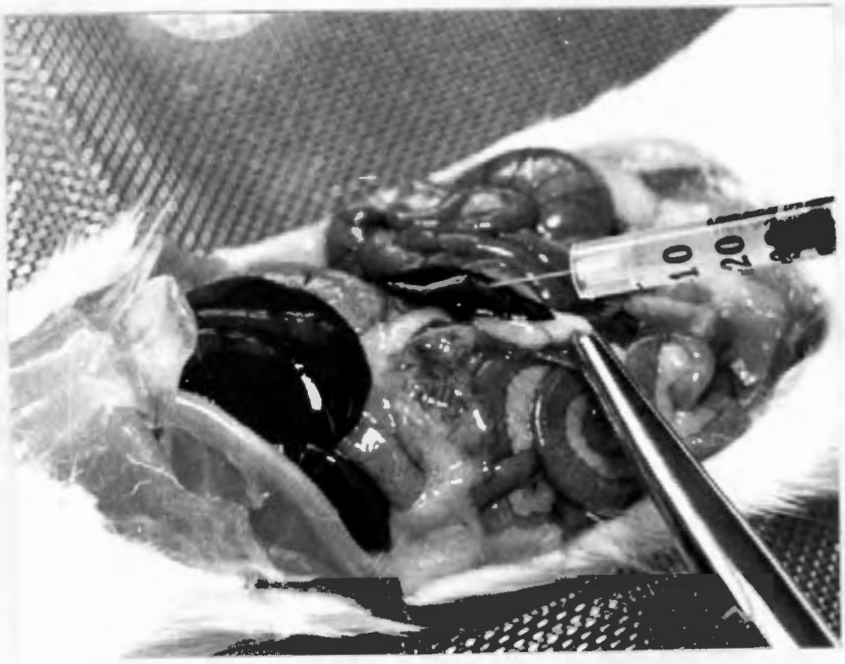


Fig. 2.9 Heparin solution was injected into the spleen.

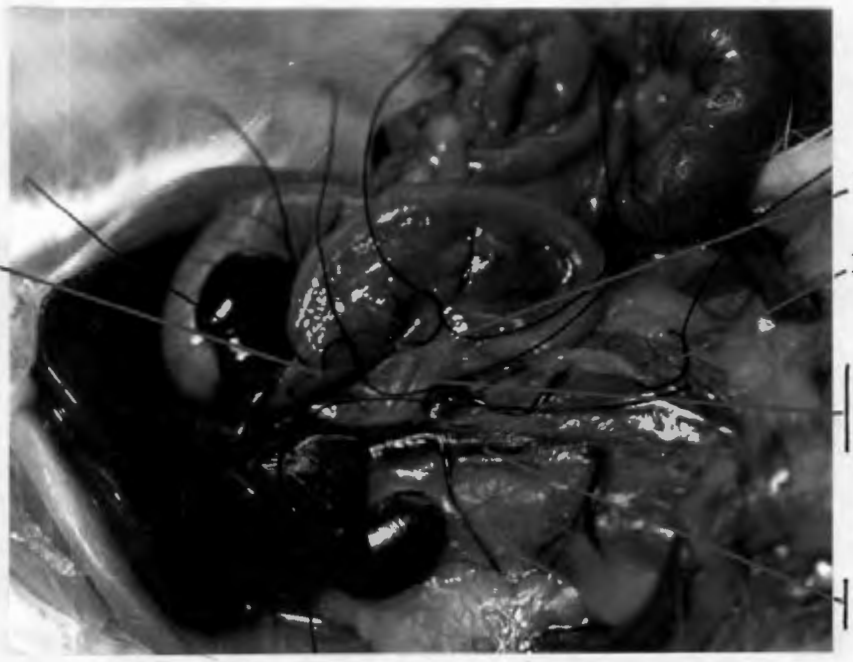


Fig. 2.10 The intestines were removed to the left side of the carcase and loose ligatures were placed around the vena porta, the artery coming from the stomach, the bile duct and the duodenum.

The catheter was then slowly slid down the whole length of the vena porta until the point was 2 — 3 mm beyond the most proximal ligature, before securing both ligatures with double knots (Fig.2.11).

By that time the blood normally started to drip out at the other end of the catheter provided that the heart was still beating strongly. The catheter was connected to the pump via the connector as soon as the blood appeared at that end of the catheter and great care was exercised not to trap an air bubble in the process (Fig.2.12). The lower vena cava was immediately cut to provide an outlet for the efflux, before the increased pressure inside the liver could cause any damage. The liver lobes were returned to their natural position and the perfusion rate raised to 26 mℓ/minute to ensure better removal of blood. The diaphragm was cut to expose the upper vena cava and oesophagus in the chest cavity (Fig.2.13). The upper vena cava was lifted up with a pair of tweezers and cut to allow the Ca^{++}-free buffer to follow the blood's normal pathway through the liver, followed by an immediate raise in flow rate to 36 mℓ/minute. The removal of blood was aided by gently moving the liver lobes with the fingers without exerting any pressure on them. At the same time the liver was being kept wet by the occassional squirt of warm Ca^{++}- free buffer. Under ideal

Fig.2.11 The catheter was inserted into the vena porta and secured in position by pulling the loose ligatures tight.

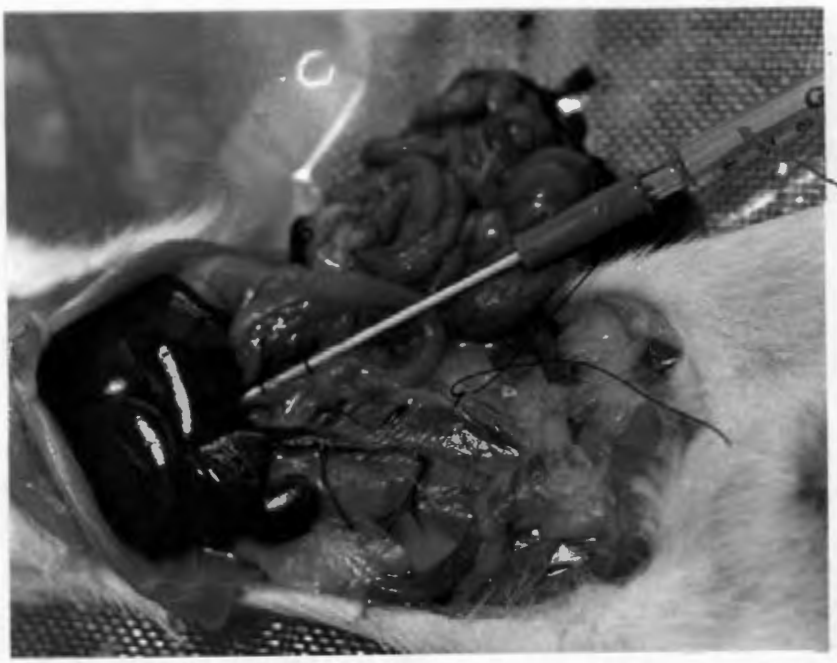


Fig.2.12 The catheter was connected to the pump via a connector.

Fig. 2.13 The diaphragm was cut to expose the chest cavity. The upper vena cava was cut and the alimentary tract tied off at the oesophagus and the duodenum.

Fig. 2.14 The liver and intestines were removed from the carcass by first lifting the liver with a forceps gripping a piece of diaphragm still attached to the preparation.

conditions, the whole liver turned light tan within the first minute of perfusion.

Once the perfusion process was well under way, the alimentary tract was tied off at the oesophagus and the duodenum.

The outside of the catheter is very smooth in order for it to be able to slide down the inside of a vein without offering any resistance, therefore, the catheter can easily slip out of the vein. To prevent this the loose ends of the most distal knot around the vena porta were used to tie a third knot around the catheter, gripping onto the piece of medical tubing that was slipped over the catheter for that purpose, thus securing the rear end of the catheter protruding from the vena porta to the outside of the vein.

While the perfusion was still in progress, the liver and intestines were carefully removed from the carcass by first lifting the liver with a pair of forceps gripping a piece of diaphragm still attached to the organ preparation (Fig.2.14) without stretching the latter. The alimentary tract, the vasculary and biliary supply and the peritoneum attaching the organ preparation to the abdominal wall as well as the other connective tissue securing the intestines to the carcass were

cut. The alimentary tract, and the vasculary and biliary supply were cut distally to the ligatures. The excised liver and intestines were transferred to the perforated piece of plastic positioned next to the rat, by carefully allowing the preparation to slide down the side of the carcass onto the plastic (Fig.2.15). The carcass was removed from the desiccator lid and Ca^{++}- free buffer was squirted onto the liver and the intestines to remove all traces of blood or hair present on the outside.

The desiccator lid was replaced by a small freeze drying flask lid with a wire mesh support. The organ preparation was transferred to the smaller lid on the piece of plastic (Fig.2.16). The plastic was carefully pulled from under it and although the liver was still more or less in its natural position the cannulated vena porta and liver were arranged in such a way as to put the least strain on them. The tied off ends of the alimentary tract were moved out of the pathway of the efflux to reduce bacterial contamination. The liver dish is just big enough to accommodate the preparation, thus the piece of catheter and the tube connected to it sticking over the side of the dish, were supported on a wooden block wrapped in tinfoil, the same height as the dish (Fig.2.17)

While the pre-perfusion was still in progress, 30 mℓ of

Fig. 2.15 The excised liver and intestines were transferred to the perforated piece of plastic positioned next to the rat.

Fig. 2.16 The organ preparation was transferred to the smaller lid on a piece of plastic.

Fig. 2.17 The cannulated vena porta and liver were arranged in such a way as to put the least strain on them and the tied off ends of the alimentary tract were moved out of the pathway of the efflux.

oxygenated collagenase buffer were transferred from the collagenase reservoir with the aid of a sterile pipette to a container with 35 mg of collagenase. The collagenase powder was dissolved and filtered through a 0,8 µm prefilter and a 0,22 µm filter (millipore, millex range) attached to the end of a syringe, directly back into the collagenase reservoir. The filtration served the dual purpose of sterilization and the removal of all undissolved particles which might clog up the small venules. Before the enzyme containing solution was added back to the rest of the buffer in the container, oxygenation was stopped to prevent denaturation of the enzyme due to air bubble formation. Pre-perfusion of the liver lasted for approximately 15 minutes and 600 – 700 mℓ of Ca^{++}–free buffer were used to wash the blood and Ca^{++} out as well as keeping the liver capsule wet. At this stage the liver had a uniform light tan colour and no swelling had occurred.

Collagenase perfusion was commenced by switching over to the collagenase reservoir by means of the two-way tap. A few seconds were allowed for the Ca^{++}–free perfusion buffer in the tubing and the filter unit to be flushed out, before sliding the liver dish along the slit in the perspex lid of the waterbath until the outlet of the dish was positioned right above the collagenase reservoir. The lamp was positioned approximately 8 cm above the

liver to keep it warm and small volumes of collagenase buffer (minus the enzyme) were squirted over the liver to prevent drying out of the liver capsule. As the collagenase perfusion progressed, the liver started to swell. The increase in size of the liver could be ascribed to the expansion of the extracellular space when the collagenaceous intercellular cement was dissolved and the cells moved apart. The rate of swelling, therefore, provided a good criterion of liver dispersion (Seglen, 1976). Ruptures started to appear in the liver capsule after 15 — 20 minutes of perfusion. The perfusion was continued for another 5 — 10 minutes until the liver started to disintegrate, before the perfusion was terminated. It is important to reach this stage because the amount of mechanical force required to disintegrate the liver is directly related to the percentage viable cells obtained (Berry and Friend, 1969).

The disintegrating liver was cut loose from the intestines and transferred to a beaker containing 50 mℓ of ice-cold pre-oxygenated washing buffer. It had been shown that at low temperature aggregation was minimized and that the cells became metabolically dormant and hence less sensitive to various types of stress (Seglen,1976). The liver cells were liberated from the connective-vascular tissue by holding the liver in the portal region with a pair of forceps and gently shaking it in the

absence of any other mechanical force and all that remained suspended from the forceps was a white gelatinous mass of connective and vascular tissue.

2.4 PURIFICATION OF PARENCHYMAL LIVER CELLS (HEPATOCYTES)

2.4.1 Filtration

The first step in obtaining a cell preparation consisting of mainly viable hepatocytes was achieved by filtering the suspension consisting of pieces of connective tissue, cell clumps, non-parenchymal cells and viable parenchymal cells through a coarse (250 µm mesh openings) nylon filter (Nytal, Switzerland). By this process connective tissue debris and big cell clumps were removed. The two other time consuming filtration steps making use of finer (100 µm and 61 µm mesh openings) nylon filters were omitted seeing that the only purpose they served as to remove smaller cell clumps. Depending on the perfusion conditions, the initial cell suspension could consist of only single cells with sometimes a few cell clumps present (removed by the coarse filter) or a very clumpy preparation (Fig.2.18) in which case all the cells would stay behind on the filters resulting in a very low cell

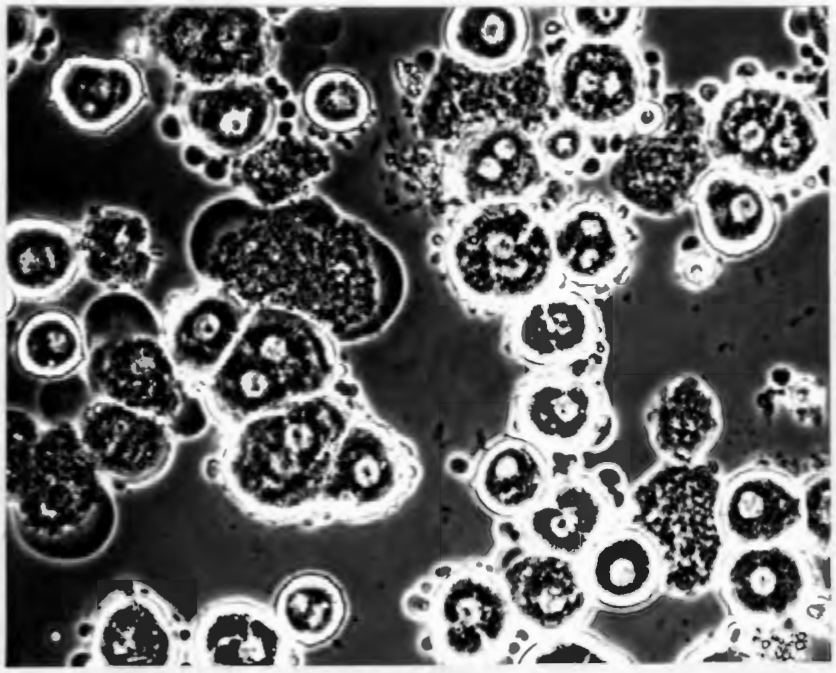


Fig. 2.18 Initial cell suspension.

yield. Normally a clumpy initial cell suspension was already an indication of a very low percentage viability and the cell preparation was not considered suitable for any metabolic studies.

2.4.2 Differential Centrifugation

Total and partial separation of the intact parenchymal cells from non-parenchymal and damaged parenchymal cells, respectively, were achieved by submitting the initial cell suspension to centrifugation at 7,5 g for 45 seconds, followed by three washes in pre-oxygenated washing buffer. The final spin was performed at 50 g for 45 seconds. The centrifugation was performed in a Hettich Rotunda benchtop centrifuge using pre-cooled Sm 34 tubes. After every spin the supernatant was carefully siphoned off and the cells were resuspended in a small volume of washing buffer by gentle agitation of the tube. The volume was then brought to 30 mℓ before the next spin.

2.5 RESULTS AND DISCUSSION

2.5.1 Nervousness of Rats

In a few experiments the rats were transferred from their

holding cages, housing a group of rats of the same age and sex, to individual cages one day before they were sacrificed. It was observed that they were very nervous the next morning and also used to put up a struggle when picked up to administer the anaesthetic. That normally resulted in a bad perfusion which could probably be ascribed to vasoconstriction in the liver brought on by the secretion of catecholamines. Similar observations have been made by Seglen (1976). Rats are gregarious animals probably becoming frightened when they are isolated and moved to a strange environment. Thus in all subsequent experiments the rats were kept in groups until the time of the experiment.

2.5.2 Dosage of Anaesthetic

Although good results were obtained by administering 22,5 mg of phentobarbitone sodium per 200 g body mass, a few rats died on the operating table before commencement of perfusion and occassionally the intravenous cannulation of the portal vein was foiled due to vascular collapse caused by a too deep anaesthesia (Seglen,1976).

2.5.3 Perfusion Flow Rate and Direction

In an attempt to save time, the oesophagus and upper vena cava were tied off with one ligature and the efflux allowed to escape via the lower vena cava. This, however, resulted in the swelling up of the liver capsule and subsequent rupture after approximately 10 minutes of collagenase perfusion. Traces of blood were noticed in the recirculating collagenase buffer, which served as an indication of inefficient removal of blood and Ca^{++} in the perfusion configuration. Only the outer cells could be liberated by shaking the liver in washing buffer. The remainder of the liver had to be mechanically disintegrated, resulting in a clumpy cell preparation with a very low viability (Table 2.1).

When the liver had been perfused in the opposite direction to the normal blood flow, the efflux was hindered by the valves in the lower vena cava. The resistance offered by the valves caused a drop in the rate of efflux resulting in a pressure build up in the liver and the subsequent rupturing of the liver capsule. During the pre-perfusion step, the slow flow resulted in the inefficient removal of Ca^{++} and blood. Although the increase in pressure inside the liver made the whole liver accessible to collagenase digestion, the enzymatic perfusion step was terminated prematurely by the rupturing of the liver capsule.

TABLE 2.1

	TYING OFF UPPER VENA CAVA			CUTTING UPPER VENA CAVA
Exp.	1	2	3	4
Viability (%)	0	40	64	78
No. of Viable Cells ($\times 10^6$)	0	1,62	26	113
Total No. of Cells ($\times 10^6$)	0	4,2	42	144
Collagenase Conc. (% (m/V))	0,035	0,035	0,035	0,035
Ca^{++}-Free Buffer				
Volume (mℓ)	350	350	450	700
Time (min)	15	15	15	15
Collagenase Buffer				
Perfusion Time (min)	30	30	30	30
Blood present	Yes	Yes	Yes	No
Liver blanched immediately after commencing pre-perfusion	No	No	No	Yes
State of dis-integration of liver at end of perfusion	not perfect	not perfect	not perfect	Perfect
Dispersion of liver	mechani-cal	mechani-cal	mechani-cal	Enzymatic
Degree of Cell Aggregation	Cells clumped	Cells clumped	Cells clumped	Less clumping
Rupture of liver Capsule (approx. min. after start of experiment.)	10	10	30	30

The resulting cell preparation was clumpy and had a low viability, probably due to intact intercellular links as a result of inefficient Ca^{++} removal and partial digestion of the connective tissue network.

2.5.4. Oxygenation of Buffers

Although the perfused liver can tolerate anoxia for up to 30 minutes (Seglen ,1976), the buffers used in this study were saturated with 100% medical oxygen because of the longer perfusion time involved (45 minutes in total). Oxygenation of the collagenase buffer was stopped on addition of the enzyme, in order to prevent foaming resulting in denaturation of the enzyme.

2.5.5. Quality of Collagenase

In this study, quite a few problems were experienced with the varying effectiveness in liver dispersion exhibited by different batches of collagenase. The quality of the collagenase used is a very important factor in the successful preparation of hepatocytes (Howard et al., 1973; Seglen,1976; Cameron-Clarke, 1983). Although the suppliers of collagenase (Sigma) guarantee that their Type IV collagenase will release liver cells with a viability in the excess of 70% when the liver perfusion is

performed according to the method of Seglen (1976), they do not comment on the perfusion time required, the degree of clumpiness of the cell preparation or the percentage yield obtained.

Initially, collagenase Type IV (Sigma) with a specific activity of 260 U/mg was used at a concentration of 0,05%. This resulted in the total disintegration of the liver in about 15 — 20 minutes. No mechanical force was required to release the cells after digestion of the liver and the cell preparation consisted of single cells only.

On reordering collagenase, samples from several batches were tried out, which proved not to be of the same quality as the first batch (Table 2.2). Very clumpy cell preparations were obtained which resulted in a large loss of viable cells during the purification process. Problems were also experienced in dispensing the cells evenly, judged by a variation obtained in the DNA determinations.

In an attempt to solve these problems, the concentrations and perfusion times were varied. In the end, the best results were achieved by lowering the collagenase concentration to 0,035% and extending the collagenase perfusion time to 30 minutes while using collagenase with a specific activity of 230 U/mg. Though

TABLE 2.2

COLLAGENASE

		COMPANY				
		Sigma				Worthington
		Type IV			Type V	
Collagenase (Units/Mg DW)		260	160	230	500	157
Clostripain (Units/Mg Dw)		0,26	0,29	0,32	0,42	-
Caseinase (Units/Mg DW)		178	94	62	241	-
Tryptic Activity (Units/Mg DW)		0,04	0,03	0,05	0,015	-
Initial cell suspension	Viability (%)	80±6	65±17	76±3	73±9	74±4
	No. of viable cells (x 10^6)	191±37	161±67	158±62	186±19	182±19
	Total No. of cells (x 10^6)	239±43	243±38	208±74	222±43	232±35
Final cell suspension	Viability (%)	83±3,83	83±6	77±5	84±0,7	85±3
	No. of viable cells (x 10^6)	101±23	90±17	83±31	158±59	65±5
	Total No. of cells (x 10^6)	123±34	111±25	107±39	128±16	75±6
% Increase in viability		3	18	1	11	11
		Average 8± 8				
% Yield	Viable cells	53	56	53	85	36
		Average 62±16				
	Total No. of cells	52	46	51	58	32
		Average 52±5				

The above data on enzyme activities were taken from the suppliers' specifications.

this led to an improvement, a cell preparation of the same quality as achieved with the first batch of collagenase could never be repeated.

In an attempt to find an explanation for these variations amongst the different batches, the protease content of the collagenase preparations supplied by the Sigma company was scrutinized, but no correlation could be demonstrated between the quality of the collagenase preparation and the protease content.

2.5.6. Viability Test

To assess the viability of the cell preparation, the cells were studied under a light microscope. The viable cells could be distinguished from the damaged cells by their more or less rounded appearance (Fig.2.19) and well defined plasma membrane (Howard,1968). To confirm these results routinely, a trypan blue exclusion test was performed on the final cell as well as the initial cell suspensions (Fig.2.20). Although it is known that viable cells exclude trypan blue (Howard et al., 1973), which serves as a clear indication of the structural intactness of the plasma membrane it does not reveal any intracellular damage (La Brecque et al., 1973). However, the trypan blue exclusion test serves as a simple test to assess the viability of the cell

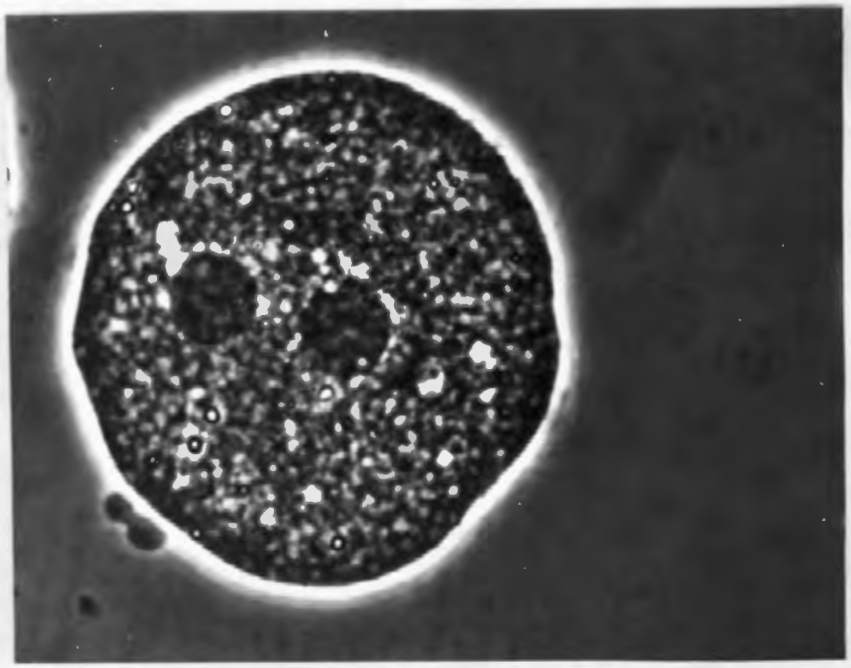


Fig. 2.19 Hepatocyte.

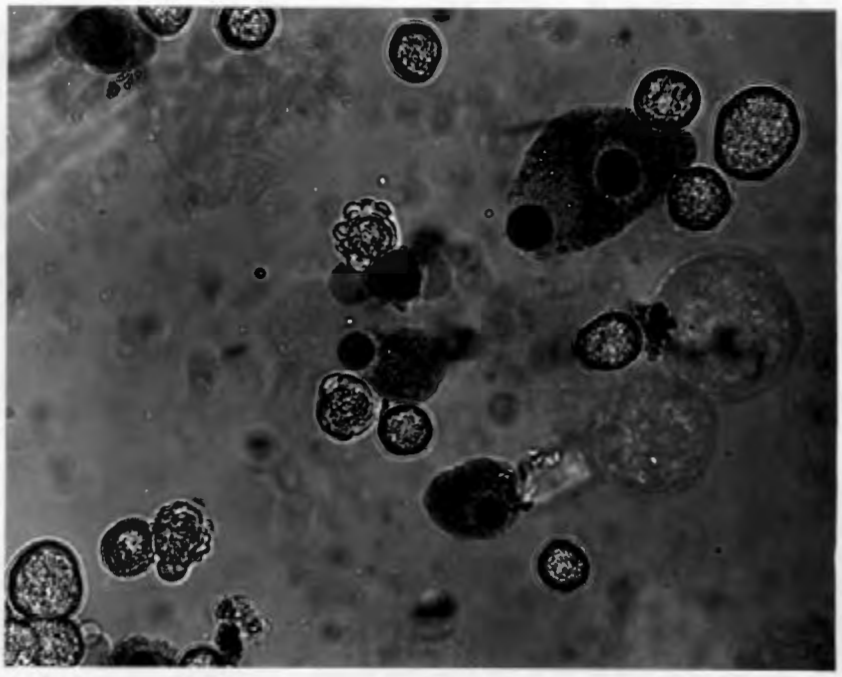


Fig. 2.20 Initial Cell Suspension Stained With Trypan Blue.

preparation before it is subjected to a more stringent test ascertaining the metabolic integrity of the cells.

In this study, trypan blue from only one batch was used, thereby eliminating any problems experienced with batch differences (Schreiber and Schreiber,1973). Dye concentrations ranging from 0,067% — 0,45% have been used in literature (Howard and Pesch, 1968; Jeejeebhoy et al.,1975; Seglen,1976). A study was conducted to compare the efficiency of different dye concentrations (Table 2.3.). All the cells could be clearly distinguished in the presence of 0,13% trypan blue, hence this concentration was used for all future tests.

Table 2.3

TRYPAN BLUE EXTENSION TEST		
Cell Preparations	Concentration of Trypan Blue Solution (% m/v)	
	0,13	0,2
1	73	74
2	84	81
3	62	59

Results are expressed in percentage cells excluding Trypan Blue

2.5.7 Initial and Final Cell Suspensions

After purification of the initial cell suspension by means of differential centrifugation, 52% of the cells were recovered in agreement with the literature (Van der Werwe,1980 ; Cameron-Clarke et al., 1983). On average the viability of the cell suspension was improved by 8%, resulting in the loss of 38% of the viable cells.
Normally non-parenchymal cells were totally absent from the final cell suspension.

2.6 DEVELOPMENT OF "IN VITRO"CULTURE CONDITIONS

Although it is known to be difficult to establish hepatocytes in primary culture, it was attempted in order to achieve a culture enriched in viable hepatocytes. It has been reported in the literature that dead hepatocytes do not adhere to a plastic substrate (Cameron-Clarke et al., 1983).

The percentage of viable hepatocytes attached was determined by the additon of a trypan blue solution to the culture flask, thus making it possible to distinguish between the different forms attached viable and dead cells take on. After a microscopic

investigation the cells were liberated and a cell count was performed (Table 2.4).

2.6.1 Medium

It was decided to use Dulbecco's Modification of Eagles Medium, acting on reports of improved metabolic activity in hepatocyte cultures observed in the presence of this more complete medium (Seglen, 1976; Wanson et al., 1977; Cameron-Clarke et al., 1983).

2.6.2. Buffers

Seglen (1976) used a mixture of organic buffers (Hepes, Tes and Tricine) with a high buffering capacity. Replacing a phosphate or sodium bicarbonate buffer with zwitterionic buffers in a culture medium, has many advantages. It is easier to maintain a physiological pH, they do not penetrate biological membranes and the initial alkalization in sparce cultures is minimized (Brimble, 1981). A CO_2 atmosphere is not required, which makes this culture system much more convenient.

Several buffers and buffer combinations were investigated (Tables 2.5&2.6). Problems were experienced in maintaining the pH, when high concentrations of sodium bicarbonate were used. In the

TABLE 2.4

Dislodging of Cells						
Methods used	Saline		Trysin - EDTA Solution		Trypsinization	
Viability test performed before (B) or after (A) dislodging the cells	B	A	B	A	B	A
Viability of the attached cells (%)	50	0,08	49	21	27	0
No. of viable cells attached (x 10^6)	5,1	0,006	4,46	1,6	2,37	0
Attachment efficiency of viable cells (%)	68	0,08	59	21	32	0
Total No. of cells attached (x 10^6)	10,22	6,92	8,74	7,6	8,78	5,2

TABLE 2.5

Buffer Combinations 2 Components								
Hepes		Combined with:						
		$NaHCO_3$				Tes		
mM		4	10	24	44	20	25	30
		*Buffer Capacities of Component Buffers						
		1,48	3,69	8,86	16,24	10,35	12,94	15,53
		Buffer Capacity of Combination						
10	4,42				20,6			
20	8,84	10,32	12,53	17,7				
25	11,05							
30	13,26							28,79
40	17,68							
60	26,57							

TABLE 2.6

Buffer Combinations 3 Components								
Tes		Combined with:					Tricine	
		Hepes						
mM		20	25	30	40	60		mM
		*Buffer Capacities of Component Buffers						
		8,84	11,05	13,52	17,68	26,52		
		Buffer Capacity of Combination						
20	10,35	24,19					5	20
25	12,94		30,24				6,25	25

* Buffer Capacity (mM $H^+/1\ell$)

absence of a CO_2 incubator, the Co_2 atmosphere was maintained by flushing the tissue culture flasks with 95% O_2 and 5% CO_2, screwing the caps on tightly. In order to maintain the pH in the range 7,2 – 7,4, a buffering system with a buffer capacity in the excess of 17 mM H^+/ℓ was required. This requirement could be met by several combinations of buffers or high concentrations of one buffer, Hepes. The buffer capacity provided by 60 mM Hepes was adequate for maintaining the pH, but proved toxic for hepatocytes (Eagle,1971). In an attempt to solve this problem, the concentration of the buffer was halved and the volume of the incubation medium doubled, but this increased volume made handling of the tissue culture flasks difficult. Adequate covering of the desired pH range was achieved by employing a combination of zwitterionic buffers i.e. 25 mM Hepes, 25 mM Tes and 25 mM Tricine, with pKa values ranging from 7,16 to 7,79 at 37°C.

2.6.3 Oxygenation

The next problem to be solved was whether the cells should be cultured in the presence of an air atmosphere (Bissell et al., 1973; Bonney et al., 1974; Sirica et al., 1979) or an oxygen enriched atmosphere.

No difference in viability was observed between cultures maintained in oxygen saturated or unsaturated culture medium in the presence of 100% oxygen atmosphere (Table 2.7). However, sharp drops in cell viability occurred when cells were incubated in O_2 unsaturated medium under an air atmosphere. This can probably be ascribed to the decrease in oxygen solubility with the increase in the temperature of a fluid. The lag period, before equilibrium is established between the air atmosphere and the incubation medium oxygen content, is longer due to the lower penetration potential of oxygen at a lower partial pressure. Another factor reducing the rate of oxygen transfer from air to liquid is the layer formed by proteinous material present in the medium at the air-liquid interface due to surface activities (McLimans et al., 1968).

2.6.4 Fetal Calf Serum (FCS)

When the effect of FCS was studied, it was observed that in the absence of serum, the cells were weakly attached and no spreading occurred. More confluent cultures of flattened cells were obtained in the presence of 5% and 10% FCS than in the presence of 2,5% FCS. This observation was in agreement with Bissell et al.(1973), who found that parenchymal cells from adult rat liver required 10% fetal calf serum for monolayer formation.

TABLE 2.7

Oxygenation of Media

Tissue Culture Bottles	Corning			Falcon		
Presaturation of the medium with O_2	Yes	No		Yes	No	
Atmosphere in Bottle	100% O_2	100% O_2	Air	100% O_2	100% O_2	Air
% Viability of attached cells	55	51	25	55	53	24
No. of viable cells attached (x 10^6)	4,96	4,45	0,67	5,23	4,23	0,97
% Attachment of viable initial incubated cells	66	60	9	70	56	13
Total No. of cells attached (x 10^6)	9,62 86	8,81 78	2,67 30	9,5 85	7,9 71	4,05 45

Although some workers include higher concentrations of FCS in the incubation medium (25%, Bonney et al., 1974), the concentration of FCS was not raised above 10%, due to the detrimental effects a too high concentration of serum might have on the cells (Barnes and Sato, 1980).

2.6.5. Osmolality

The osmolalities of all the perfusion buffers and incubation media were determined (Table 2.8). The osmolalities of the collagenase buffer and incubation media exceeded the normal iso-osmotic value of 283 mOsm for rat plasma (Seglen, 1972a), by far. The high osmolalities are due to replacing the buffer of the commercial medium with a different buffer system.

Comparative studies employing the medium with the high osmolality and media with osmolalities closer to the physiological value (medium A and diluted commercial media), revealed no improvement in survival and attachment efficiency of the cells.

2.6.6. Antibiotics

Initially 100 IU penicillin / mℓ and 100 µg streptomycin /mℓ (Bissell et al., 1973 ; Sirica et al., 1979) were included in

TABLE 2.8

Osmolalities (m Osmol/Kg H_2O) of Media and Buffers

Buffer:	Osmolality (m Osmol/Kg H_2O)
Ca^{2+} free buffer pH 7,4 at 37°C	295
Collagenase buffer (without collagenase) pH 7,4 at 37°C	320
Washing buffer pH 7,4 at 4°C	295
Dulbecco's modified Eagles Medium (without buffer)	270
DMEM, 44 mM $NaHCO_3$ pH 7,4 at 37°C	345
DMEM, 30 mM Hepes, 30 mM Tes pH 7,4 at 37°C	350
DMEM, 25 mM Hepes, 25 mM Tes, 25 mM Tricine pH 7,4 at 37°C	370
DMEM, 25 mM Hepes, 25 mM Tes, 25 mM Tricine 10% FCS pH 7,4 at 37°C	365
DMEM, 25 mM Hepes, 25 mM Tes, 25 mM Tricine 10% FCS O_2 pH 7,4 at 37°C	370
DMEM, 60 mM Hepes pH 7,4 at 37°C	355
DMEM, 25 mM Hepes, 25 mM Tes, 25 mM Tricine 4 mM $NaHCO_3$ pH 7,4 at 37°C	390
Medium A pH 7,4 at 37°C	300
Diluted Commercial Medium Dilution 1 pH 7,4 at 37°C	296
Dilution 2 pH 7,4 at 37°C	305

all the perfusion buffers and the incubation medium. However, these antibiotics did not suppress the bacterial infection and to establish the main source of contamination, sterility checks were performed on all the perfusion buffers before and after completion of perfusion. The main source of contamination was identified as the recirculating collagenase buffer.

The disk method as well as preparing agar plates containing varying amounts of antibiotics were used to test the sensitivity of the bacteria to a range of antibiotics. No bacterial growth was observed in the presence of tetracycline (>15 µg/mℓ), ampicillin (> 500 µg/mℓ) and gentamicin (> 30 µg/mℓ).

Thereupon, gentamicin was routinely included in the collagenase and washing buffers as well as the incubation medium at 35 µg/mℓ, a non-toxic concentration to the cells (Jeejeebhoy et al.,1975). Bacterial growth in the presence of 35 µg/mℓ gentamicin was observed in the overnight incubation medium of cells isolated from a preparation which may have been contaminated due to a rupture of the intestines during isolation of the cells. The concentration of gentamicin in the relevant buffers was raised to 100 µg/mℓ. This together with the extracorporal collagenase perfusion, resulted in routinely sterile cell suspensions.

2.6.7 Cell Density

Hepatocytes have been seeded at densities ranging from 107 000 – 700 000 cells/cm^2 growth area (Wanson et al., 1977, Siraca et al., 1979).

In this study the cells were seeded at 100 000 viable hepatocytes /cm^2 growth area in a tissue culture flask (Table 2.9). Fifteen mℓ incubation medium was the smallest volume that could be used in the 270 mℓ tissue culture flask, covering the whole growth area of the flask.

2.6.8 Culture Conditions

The hepatocytes, at a cell density of 100 000 viable cells /cm^2 growth area, were cultured in 15 mℓ DMEM buffered with 25 mM Hepes, Tes, Tricine, pH 7,4 at 37°C. 10% FCS (heat-inactivated) and 100 µg/mℓ gentamicin were added. The medium was pre-heated to 37°C, pre-oxygenated (100% O_2) and the culture flask filled with 100% O_2, before screwing the cap on tightly.

2.6.9 Discussion

Under the above mentioned conditions, the cells attached to the

TABLE 2.9

Dimensions of Culture Vessels

	Tissue Culture Flask	Scintil-lation Vial	Tissue Culture Tube
Total Volume (mℓ) (Vessel)	270	21	16
Volume Medium (mℓ)	15	2	2
Total No. of cells (x 10^6)	7,5	0,5-1	0,5-1
No. of cells per mℓ medium (x 10^6)	0,5	0,25-0,5	0,25-0,5
No. of cells per mℓ airspace (x 10^3)	28	24-48	31-63

plastic substratum, but they were all still in the rounded form. After 20 hours all the viable cells had flattened out and formed colonies. The dead cells were also attached to the substratum, sometimes on top of the monolayer formed by the viable ones, but they were still in the rounded form. Neither the viable or dead cells could be dislodged by gentle agitation of the tissue culture flask. All the cells floating in the medium were dead, as determined by the trypan blue exclusion test. It could be argued that the cells died after they had attached, however, in view of the observation in Table 2.4 that the total number of cells attached exceeds the number of viable cells inoculated, proves that at least some of the attached dead cells were already dead before they attached.

After 24 hours the attachment efficiency was 68% (viable cells) which compares favourably with values obtained in literature (Bonney et al., 1974). After 48 hours hardly any of the attached cells were still viable and all of the cells in the medium were dead. Cells were beginning to detach. After 72 hours all the cells were dead and the remaining attached cells had all taken on a rounded form.

2.6.10 Suspension Culture

After having studied the advantages and disadvantages of establishing a monolayer culture, it was decided that a suspension culture may be more advantageous for the short term metabolic studies planned. Smaller numbers of cells are required for suspension cultures, thus more experiments can be performed on one cell preparation, eliminating the variation introduced by individual differences. To set up a suspension culture is also less time consuming.

Initially siliconized scintillation vials were used (Zahlten and Stratman, 1974) (Table 2.9). Cell aggregation was prevented by rolling the vials on a roller machine at 60 rpm (Schreiber and Schreiber, 1973). At the same time in this incubation mode a shallow depth and large surface were provided, which allowed adequate oxygenation of the cells.

Evidence was presented (Jeejeebhoy et al., 1975) that spinning as opposed to shaking and the inclusion of serum in the incubation medium, prolongs the lifetime of cells in suspension culture. Rolling as well as spinning are more gentle ways of keeping the cells in suspension in comparison with vigorous shaking.

After initial successful experiments the scintillation vials were replaced with plastic disposable, presterilized and siliconized tissue culture tubes (Johnson et al., 1972). This arrangement allowed immediate centrifugation of the suspension at the end of the experiment. By adopting this method,cell suspension cultures were kept well oxygenated and cells exposed continuously to new medium, factors which have been shown to prolong the lifetime of cells in suspension in the spinning mode.

2.7 METABOLIC INTEGRITY OF THE ISOLATED HEPATOCYTES

2.7.1 Introduction

Before the hepatocytes could be used in any study of liver specific functions, it had to be ascertained whether the cells carried out basic metabolic reactions.

An average drop of 10% in cell viability as determined by the trypan blue exclusion test, was observed during the first 5 hours of incubation (Table 2.10).

TABLE 2.10

Cell Viability during 5 hour Incubation Period							
	Cell Preparation						Av.
Viability of freshly isolayed hepatocytes (%)	84	74	84	82	87	69	80 ± 8 *
Viability of hepatocytes after 5 hour incubation period (%)	67	60	78	72	80	60	70 ± 9

*Standard Deviation

Protein and RNA synthesis were studied at short intervals over 5 hours and then after a further 16 hours to determine the life span of the suspension.

The results required that in all future metabolic experiments metabolic parameters had to be expressed in terms of viable hepatocytes present at the end of the experiment.

All metabolic studies were preceded by a pre-incubation period of 30 minutes, allowing time for the cells to reach equilibrium with the incubation medium. In this way a sigmoid shape curve of

metabolic activities was not observed (Schreiber and Schreiber, 1973).

2.7.2. RNA and Protein Synthesis

A nearly linear relationship between RNA synthesis and incubation time was obtained for the whole duration of the study (Fig.2.21).

The more pronounced decrease in protein synthesis as compared with RNA synthesis, after 5 hours of incubation, can be understood in terms of the amplification taking place during translation, i.e. metabolic damage to the amplifying system has more drastic results than damage to the RNA synthesis. The protein could also have been degraded by proteases during the long incubation period.

Both RNA and protein synthesis could still be observed after leaving the cells in the cold overnight, however at a considerable reduced rate (Figs.2.22&2.23).

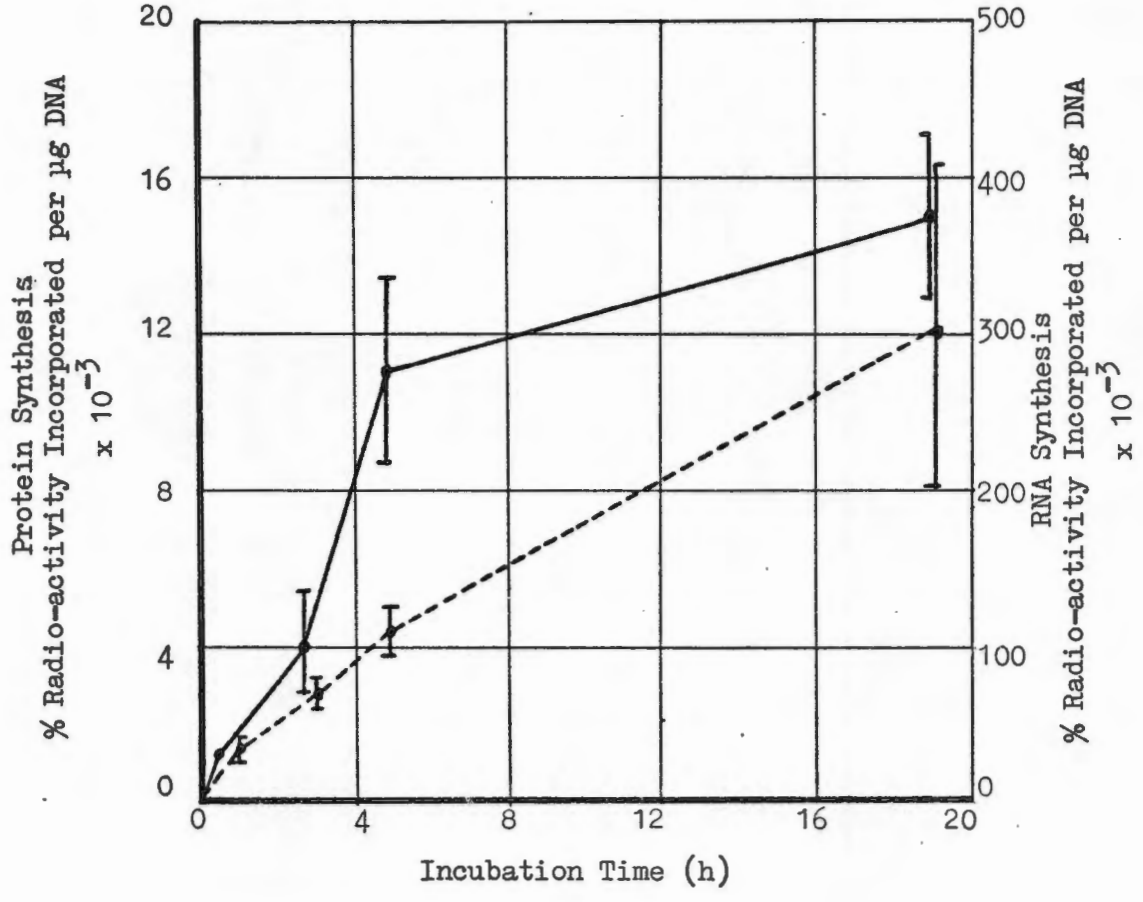


Fig. 2.21 Protein (————) and RNA (– – – –) synthesis in hepatocytes.

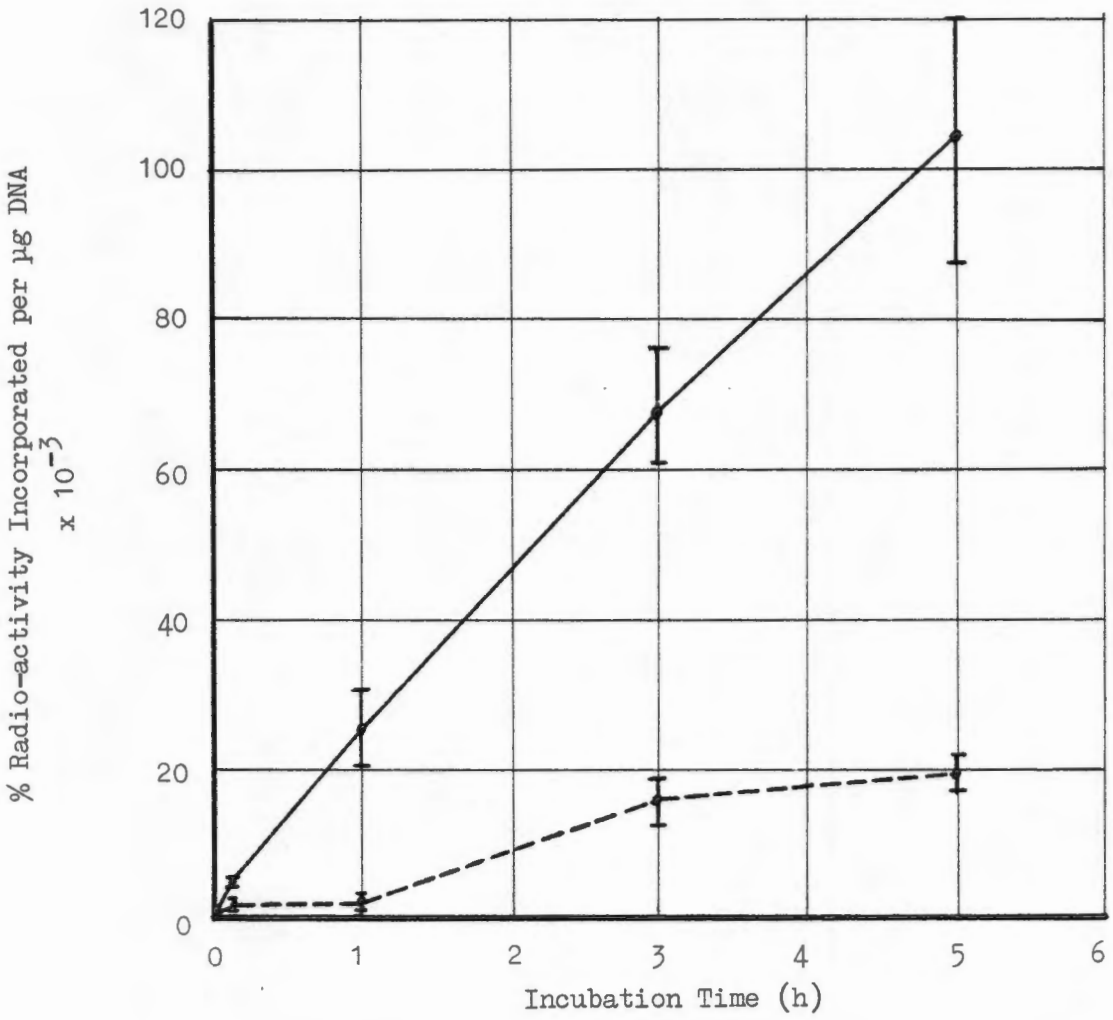


Fig. 2.22 RNA synthesis in freshly isolated hepatocytes (————) and hepatocytes left overnight in washing buffer at 4°C (- - -).

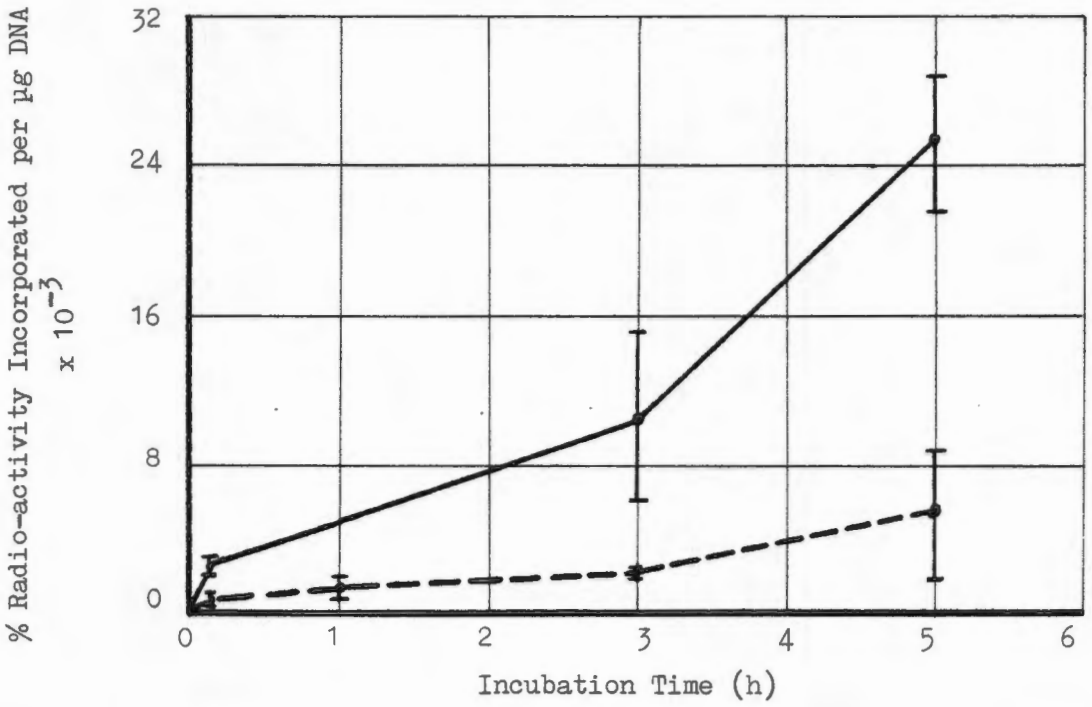


Fig. 2.23 Protein synthesis in freshly isolated hepatocytes (——) and hepatocytes left overnight in washing buffer at 4°C (- - -).

2.8 ISOLATION OF NUCLEI

2.8.1 INTRODUCTION

For analytical purposes, the breaking of the isolated hepatocytes by mechanical force was required. The choice of methods to liberate the contents of the intact hepatocytes was determined by the specific requirements of a chosen analytical method.

For the tyrosine aminotransferase assay, only breaking of the plasma membrane was required to release the enzyme present in the cytoplasm. However, the studies involving triamcinolone acetonide uptake into the nuclei and the phosphorylation and acetylation of nuclear proteins, especially histones, required a nuclei isolation method yielding nuclei with intact nuclear membranes free of cytoplasmic components.

In order to find methods to satisfy all the above mentioned requirements the following were investigated: homogenization involving different types of micro-tissue grinders, swelling buffers, detergents and sonication.

2.8.2 Sonication

Cells (1×10^6) suspended in 1 mℓ sonication buffer (isolation buffer – TAT extraction) in a Sm 24 centrifuge tube, suspended in ice-water were sonicated for time periods of 30 and 60 seconds. The sonicator was a Biosonik III with a probe which was operated at 30% of the maximum output. After 30 seconds 21% of the total number of cells and 18% of the total number of nuclei were still intact. On extension of the sonication period to 60 seconds, 3,5% of the cells were found still to be intact, but no intact nuclei could be distinguished.

Inclusion of 0,1% of the nonionic detergent Nonidet P-40 in the sonication buffer, resulted in the total disintegration of all the cells and nuclei, following 15 seconds of sonication.

2.8.3 Homogenization

Nuclei can be prepared on a large scale from a rat liver, according to the modified method of Blobel and Potter (1966). A nuclei preparation with only a few intact cells still present can easily be obtained by resuspending the minced liver in 10 volumes of 0,25 M sucrose buffer in a 50 mℓ homogenization vessel followed by 3 strokes (1 stroke comprises one-up-and-down movement of the plunger)at top speed in a

Potter Elvehjem homogenizer. The nuclei obtained are relatively free from cytoplasmic contamination. On inclusion of 0,1% Triton-X- 100 in the homogenization buffer, hardly any cytoplasm is left clinging to the nuclei as observed under phase contrast. After sedimenting the two homogenates (prepared in the absence or presence of detergent) through 2,3 M sucrose, no difference could be observed in the quality of the nuclear preparation. All the nuclei were cytoplasm free.

In order to achieve the same results, but on a microscale and effecting liberation of nuclei from a liver cell suspension instead of intact tissue, different micro-tissue grinders were employed. In an attempt to compensate for the smaller shearing force, due to the shorter length of the stroke, the number of strokes were increased. After 20 strokes in the following 1 mℓ micro-tissue grinders, Dounce Type A (tight fit), Dounce Type B (loose fit), Tenbroeck (all glass) and a 2 mℓ Potter Elvehjem vessel with a Teflon pestle at top speed (1200 rpm), 80% of the cells were still intact.

By extending the duration of homogenization, the results achieved with the small vessels improved dramatically. Employing a tapered 1 mℓ mortar with teflon pestle operated at top speed, proved to be the most successful. It appears that the pestles in

the other vessels did not fit tightly enough. It was found that the homogenization time could be reduced by about half, by the inclusion of 0,1% Triton-X-100 (Method 3) (6.3.2.3) in the homogenization buffer or replacing the homogenization buffer with a hypotonic 'swelling' buffer (Method 2)(6.3.2.2)(Tables 2.11 and 2.12).

Inspection under a light microscope revealed that the nuclei liberated according to Methods 1 (6.3.2.1) and 2, still had pieces of cytoplasm clinging to them. Nuclei prepared in the presence of 0,1% Triton- X-100 were cytoplasm free.

Although the use of either a hypotonic swelling buffer or the inclusion of the detergent facilitated the liberation of the nuclei, these procedures can result in the damage of the nuclear membrane (Holtzman and Smith, 1966). Electron microscopy however, shows the selective disappearance of the outer nuclear membrane by Triton-X-100. Subsequently it has been shown that the detergent disrupts the nuclear membrane mainly by the extraction of the phospholipid which ultimately leads to collapse of the double membrane structure (Jackson, 1976; Richardson and Agutter, 1980).

Since the structural integrity of the nuclear membrane was a

TABLE 2.11

Comparison of Homogenization Buffers			
Number of Strokes*	Percentage Nuclei Liberated		
	Method 1	Method 2	Method 3
25	83	100	100
60	100		

TABLE 2.12

The Effect of Hypotonic Buffer		
Percentage of Nuclei Liberated		
Time exposed (min.)	10	180
Number of strokes *		
5		32
10		67
15		74
20		85
25	100	100

* A stroke comprises one downwards and one upwards movement of the rotating pestle.

prerequisite for the planned biochemical analysis, Method 1 was adopted. The long homogenization process required a careful control of the temperature in the homogenizing vessel suspended in a large volume of ice-water.

In four tapered 1 mℓ mortar homogenizing vessels, small individual differences in the sizes of the vessels and pestles necessitated the calibration of the different sets to achieve reliable results.

TABLE 2.13

Calibration of Homogenizing Sets

Number of Strokes	Percentage Nuclei Liberated			
	Homogenizing Sets			
	1	2	3	4
25			90	100
30			97	
35	80			
45		72		
55		81		
60	100			
70		100		

Due to wear and tear of the teflon pestle, the number of strokes required to liberate a certain number of nuclei changed quickly necessitating frequent recalibration. Therefore only one homogenizing vessel was used per experiment, wherever possible, or if more than one was used, only recalibrated sets were used (Table 2.13).

2.8.4 Comparative Study between Isolation of Nuclei from Intact Tissue and Isolated Cells

In an attempt to gain more information on the role various homogenization conditions play in the effective liberation of nuclei, a comparative study was conducted employing intact liver tissue and isolated hepatocytes.

As can be gathered from Tables 2.14 and 2.15 the speed at which the teflon pestle is moved up and down plays a very important role in the effective liberation of nuclei from cells or intact tissues. This effect is much more pronounced in the case of the intact tissue, and this can probably be ascribed to the contribution of the slow up and down motion to the already existing potential shearing force present between adjacent cells in intact liver tissue. The absence of such potential shearing forces probably contributes to the large differences between

TABLE 2.14

Isolation of Nuclei from Intact Liver Tissue				
No. of Strokes	Percentage Nuclei Liberated			
	Homogenizing Conditions*			
	1	2	3	4
1	67	63	54	24
2	94	89	86	34
3	99	97	95	42
4				65
5				67
10				64
20				100

(7,87 mg wet weight liver tissue equivalent to 1 x 10^6 hepatocytes)
(Seglen, 1976)

TABLE 2.15

Isolation of Nuclei from Isolated Hepatocytes				
No. of Strokes	Percentage Nuclei Liberated			
	Homogenizing Conditions*			
	1	2	3	4
5	30	33	31	20
10	37	43	35	28
20	57	55	54	38
30	65	64	69	59
40	71	76	72	60
50	95	78	77	78
60	96	91	88	88
70		100	94	93
80				97

(1 x 10^6 cells were homogenized.)

To be continued

* Homogenization Conditions

The same micro-tissue grinder set (1 mℓ tapered mortar with teflon driven pestle) was used in the liberation of nuclei from a cell suspension or intact tissue.

1. Control

   Rotation speed of pestle : 1500 rpm

   Homogenization buffer volume : 1 mℓ

2. Rotation speed of pestle : 750 rpm

   Homogenization buffer volume : 1 mℓ

3. Rotation speed of pestle : 1500 rpm

   Homogenization buffer volume : 500 mℓ

4. Rotation speed of pestle : 1500 rpm

   Homogenization buffer volume : 1 mℓ

   Up and down movement of pestle speeded up (2 times as fast)

intact tissue and isolated cells. In addition there may well be differences in the expression of the cytoskeleton between cells in the context of tissues and floating cells. The longer stroke in larger homogenizing vessels appears not to make a major contribution to the shearing force, because the same results were obtained when varying the homogenization volume.

2.8.5 Purification of Nuclei

Seeing that pieces of cytoplasm were still clinging to the nuclei after the homogenization step the nuclei had to be subjected to a further purification step. Nuclei, free from cytoplasmic membrane were prepared (Fig.2.24) by spinning the homogenate through 2,3 M sucrose for 40 minutes at 49 500g (Blobel and Potter,1966). A 1 M sucrose solution resulted in contamination. A nuclear yield of approximately 20% and 45% were achieved, using nuclei (approximately 1 x 10^6)liberated by Methods 1 and 2, respectively.

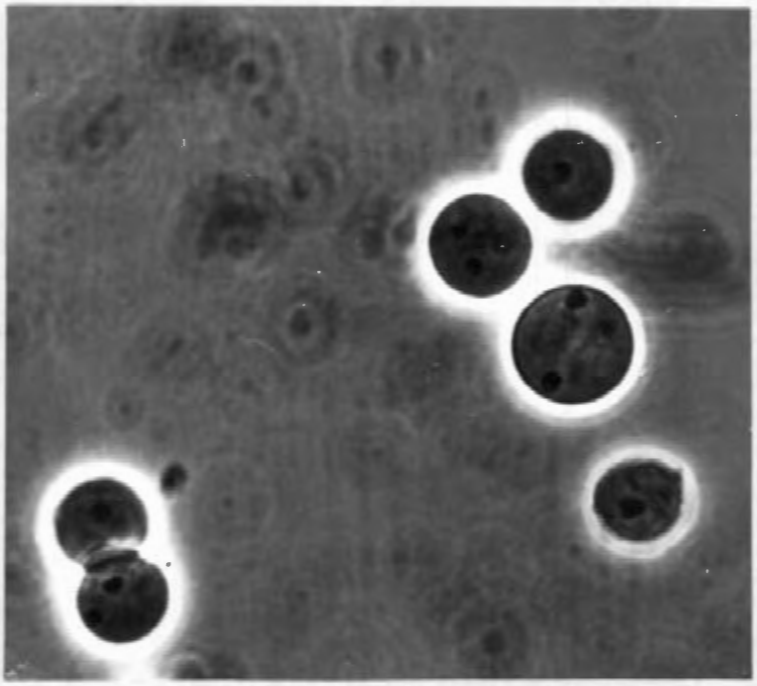


Fig. 2.24 Purified nuclei suspended in 0,25 M sucrose buffer.

PART 3

REVIEW ON GLUCOCORTICOIDS AND THEIR RECEPTORS

3.1 INTRODUCTION

Steroid hormones play an important role in development and physiological regulation in animals ranging from arthropods to primates (Yamamoto and Alberts,1976). The theory that various steroids function via a common molecular mechanism, in which the steroid acts as an allosteric effector molecule that binds tightly to its specific receptor protein in the cell cytoplasm, enjoys wide support. The binding event stabilizes or induces an "activated" state of the steroid receptor, increasing the affinity of the receptor for chromosomal sites in the cell nucleus. Once the interaction between the hormone receptor complex and the genome has taken place, the biological response characteristic of the particular hormone and target tissue is elicited (Fig.3.1).

Glucocorticoids, produced by the adrenal cortex, are a major class of steroid hormones and are important physiological regulators of gene expression (Feinberg et al.,1983).

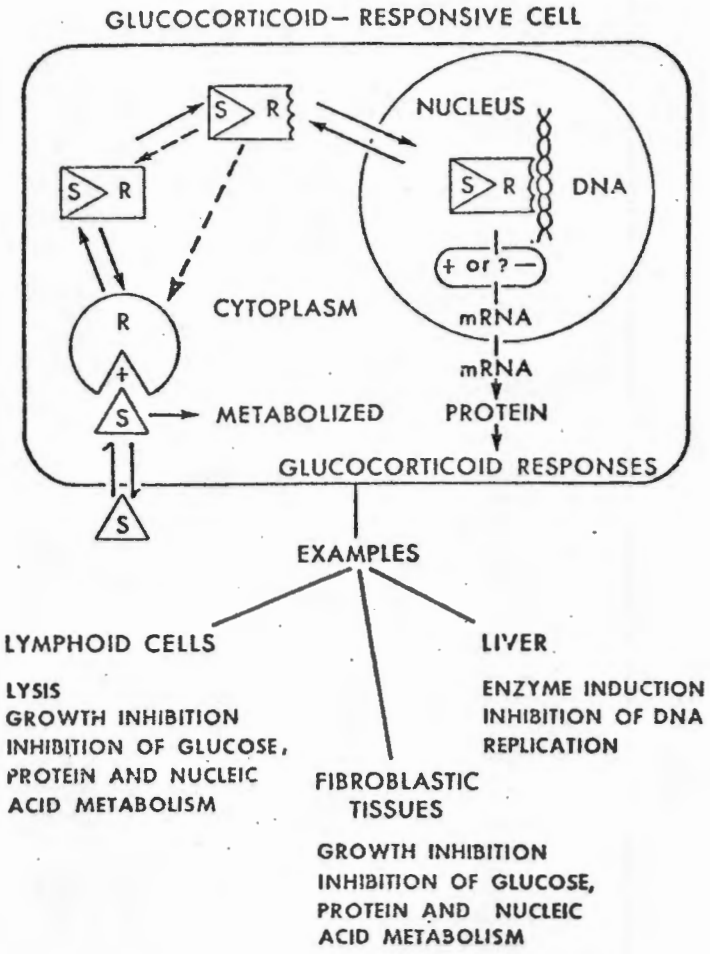
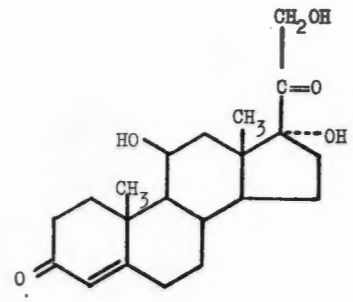


Fig. 3.1 Steps in glucocorticoid action (Baxter and Harris, 1975)
S = glucocorticoid
R = specific glucocorticoid receptor
Different shapes of R indicate different conformational states of the receptor. The dotted line indicates that it is not known how dissociation of the activated complex proceeds.

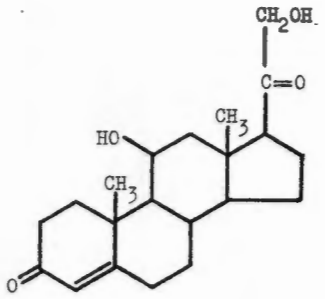
Birds, mice and rats secrete corticosterone, while cats, cows, sheep, monkeys and humans secrete predominantly cortisol. Approximately equal amounts of the two glucocorticoids are secreted by dogs (Ganong,1977)(Fig.3.2).

The natural glucocorticoids are often replaced by their synthetic analogues in biological studies. Evidence has been presented, showing that the 9 α- fluorinated glucocorticoids, triamcinolone acetonide and dexamethasone are biologically more active than the natural glucocorticoids (Munck and Brinck-Johnsen,1968; Samuels and Tomkins,1970; Agarwal, 1976; Nakanishi et al.,1977). Affinity for the receptor and glucocorticoid activity are highly sensitive to nature of the side chains of the latter and the surrounding substituents (Baxter and Rousseau,1979; Golikov et al., 1982).

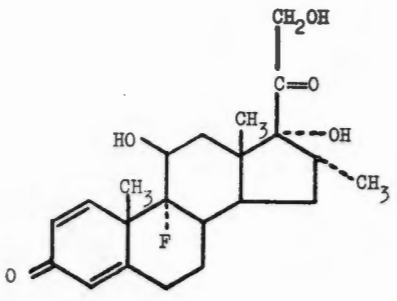
Competition studies showed that corticosterone and dexamethasone interact with the same binding site on the hormone receptor in the cytoplasm (Wrange,1976; Carlstedt-Duke et al., 1977). This observation was questioned when dexomethasone and cortisone complexes eluted under different conditions from a DEAE-52 cellulose column (Argawal, 1976). Schmid et al.(1976) demonstrated that dexamethasone receptors bind cortisol but to a



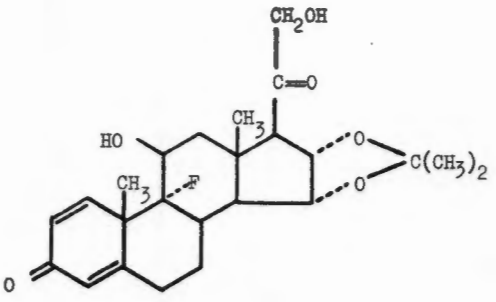
Cortisol



Corticosterone



Dexamethasone



Triamcinolone Acetonide

Fig. 3.2 Glucocorticoids and their Synthetic Analogues.

varying extent.

The uptake of the steroid hormone by target cells, has been studied under "in vivo "and "in vitro "conditions. Within 5 minutes after an intraperitoneal injection, 30%-40% of the glucocorticoid receptors in the rat liver were activated (Markovic et al., 1980). In isolated cells, there is a quick uptake of hormone during the first 3 - 4 minutes followed by a levelling off in the rate of uptake. A plateau is reached in about 10 — 30 minutes (Munck et al., 1972; Rao et al., 1976).

Most of the glucocorticoid receptor complexes in the cytoplasm are translocated to the nucleus within 7 — 10 minutes after addition of the hormone. Equilibrium is reached after 30 minutes (Baxter and Tomkins, 1970; Rousseau et al., 1973).

mRNA synthesis is initiated 15 minutes to 2 hours after the binding of the hormone receptor complex to the nuclear chromatin (Spelsberg et al., 1976; Grote et al., 1983).

The biological response is elicited approximately 2 hours after administration of the hormone (Izawa et al., 1982).

The first step in steroid hormone action, the rapid uptake of the

hormone by the target cells, has received little attention up till now. Different ways of penetration of the plasma membrane by the steroids have been suggested : passive diffusion (Turnell et al., 1974) and protein carriers (Milgrom et al., 1973).

When uptake of cortisol by isolated liver cells were studied, two saturable uptake systems were demonstrated. The high and low affinity systems probably mediate the transport of the glucocorticoid into the cells by protein carriers in the plasma membrane. At higher hormone concentrations, saturation is not achieved, simple diffusion becomes the major route of transport into the cell.

Uptake velocities increase with increasing temperature and a plateau is reached earlier at higher temperatures (Rao et al., 1976).

The classical 'two-step' model of steroid hormone intracellular interaction (Gorski et al., 1968; Jensen et al., 1968) was based on the studies of the uptake and binding of radio-active estrogens to receptors in reproductive tissue. Upon entering the target cell, the hormone binds to a receptor in the cytoplasm. The hormone receptor complex is then translocated to the nucleus by a temperature dependent activation process.

3.2 STEROID RECEPTORS

Several steroid receptors have been identified in the cytoplasm by different groups. Three types of binding sites for aldosterone in toad bladder and corticosterone in rat liver cytosol have been found (Snart 1967; Snart et al., 1970). The receptors in the rat liver cytosol had molecular weights of the order 50 000, 100 000 and 200 000. The first two sites were destroyed by mild heating.

Two other groups identified 3 rat liver cytoplasmic glucocorticoid binding proteins, i.e. A, B and G with the following molecular weights, 51 000, 64 000 and 200 000, respectively. At a higher ionic strength the G protein's molecular weight decreased to 66 000 (Koblinsky et al., 1972; Beato and Feigelson,1972). All these proteins are thermolabile, but the A and B proteins can resist heating for 10 minutes at 40°C. The A and B proteins are transcortin, steroid binding proteins, while the G protein is the only physiologically significant glucocorticoid receptor (Beato et al., 1973). The G

protein is also the only one of the above mentioned proteins, which binds 9 α— fluoro-glucocorticoid derivatives (Koblinsky et al., 1972).

Another group, Litwack et al. (1973) identified four binding proteins. Protein I (40 000 D) proved to be ligandin and protein IV (50 000 — 60 000 D) was identified as transcortin. Protein III (5 000 — 7 000 D) was a breakdown product. Protein II (67 000 D) is the glucocorticoid receptor in the liver.

Protein IB has been identified in male adrenalectomized rat liver cytosols (Litwack and Rosenfield,1975) and in kidney cortex and proximal colon. It is smaller than the traditional receptor and the pattern of elution from DEAE, relative affinities for binding of steroids, potency of binding to DNA and nuclei and preference of binding to homodeoxypolymers also differ (Mayer and Litwack, 1983). This receptor binds unmetabolized glucocorticoids (Litwack et al., 1978).

Following adrenalectomy, there is an increase in the number of unoccupied glucocorticoid receptors in the hepatocyte cytosol. This is followed by a subsequent decrease in the concentration of the nuclear glucocorticoid binding sites. This distribution of glucocorticoid binding sites is reversed within 30 minutes of an

intraperitoneal injection of cortisol to the adrenalectomized rat (Beato et al., 1974).

A lower concentration of glucocorticoid receptors in liver from female rats than male rats has been reported. Different responses were also shown by male and female rats to endocrine manipulation, e.g. adrenalectomy, hypophysectomy and ovariectomy. The different responses are probably due to associated changes in plasma corticosterone concentrations which are influenced by the ovary (Endres et al., 1979).

Another difference between male and female rats concerns the metabolism of glucocorticoids, e.g. cortisol. Cortisol is metabolized to produce a small amount of a non-anionic compound and three anionic derivatives in male rats. Adult female liver on the other hand forms only one anionic derivative (Singer and Litwack, 1971).

Specific receptors for glucocorticoids are present in fetal rat liver as early as the 18th day of gestation. There is a drop in the concentration of glucocorticoid receptors after the 20th day of gestation reaching undetectable levels shortly before and after birth. Glucocorticoids do not induce TAT synthesis in fetal liver, but induction is observed within 24 hours after

birth (Kalimi and Gupta, 1982). Fetal rat liver TAT gene is much less active than in adult liver (Granner et al., 1968). The concentration of the receptor again increases 1 — 2 hours after birth.

The induction of TAT can also be investigated in rat hepatoma tissue culture cells (HTC). Two classes of steroid responsive gene products have been identified. Class I proteins, representing less than 1% of the total proteins synthetized in HTC, are induced with induction ratios ranging from 3 — 10. TAT synthesis, representing 0,02% — 0,05% of total protein synthesis (Steinberg et al., 1975) is grouped in this class. HTC lack the liver specific enzyme tryptophan oxygenase. Class II proteins are infrequently induced or repressed with repression ratios never exceeding 3. The inducibility of a gene product may be lost and even reversed in different hepatoma cell lines (Ivarie and O'Farrell, 1978).

In the cytoplasm of hepatoma cells two forms of activated glucocorticoid receptor have been found . The labile form binds only to nuclei and the other form binds to DNA and nuclei (Simons, 1977).

Mature hepatocytes and hepatoma tissue culture cells are only

sensitive to steroid induction of TAT during the late G_1 and S phases of the cell cycle (Sellers and Granner,1974; Currie and Cidlowski, 1982).

Binding of the steroid to the receptor in the cytosol can take place at low temperature and low ionic strength. This interaction between the receptor and the steroid is a prerequisite for activation of the receptor (Milgrom et al.,1973). The binding site probably consists of a hydrophobic pocket with in addition polar groups that form hydrogen bonds. It has been suggested that the principal driving forces for interaction are the hydrophobic bonds. Hydrogen bonds confer specificity to the interactions (Munck et al., 1972).

Activation of the hormone receptor complex can be elicited by a number of factors. Activation of the steroid receptor complexes on an increase in temperature, have been observed by several groups (Munck et al., 1972; Baxter et al., 1973; Atger and Milgrom, 1976).

Activation is accelerated by an increase in ionic strength (Milgrom et al., 1973; Higgins et al., 1973; Kalimi et al., 1975; Bailly et al.,1978). An increase in pH from 7 to 8 resulted in a linear increase in activation (Bailly et al., 1978).

Activation can also be achieved by subjecting the hormone receptor complex to dialysis, a Sephadex G-25 column or ammonium sulphate precipitation, resulting in the removal of the low-molecular weight inhibitor (Cake et al., 1976; Bailly et al., 1977; Goidl et al., 1977; Sato et al., 1980; Leach et al.,1982). The low-molecular weight inhibitor may have a role in keeping glucocorticoid receptors in the untransformed steroid binding form (Leach et al., 1982).

The degree of phosphorylation of the receptor may play a role in the activation process. Munck et al. (1972) suggested that the phosphorylated receptor binds the glucocorticoid. During the activation process the receptor is dephosphorylated. Upon binding to the nuclear acceptor site, the hormone is released and the receptor recycled to the cytoplasm and rephosphorylated (Munck et al., 1972; Litwack et al., 1982). Another model based on the presence of a heat-stable factor, associated to the phosphorylated active receptor has been proposed. According to this model, after binding the steroid the receptor complex is then activated by dephosphorylation and dissociation of the low-molecular weight heat-stable factor. The heat-stable factor might act as an endogenous phosphatase inhibitor. A 3rd model suggests that the heat-stable factor, rather than the receptor protein is phosphorlyated. During activation, the release of

this phosphorylated factor or dephosphorylation of the factor itself may result in its dissociation from the receptor (Sando et al., 1979).

A role for pyridoxal phosphate as an "in vivo" regulator of further binding of activated steroid complexes, to nuclear acceptor sites, has been suggested. Pyridoxal phosphate combines with the activated form, preventing nuclear binding (Litweack and Cake 1977; DiSorbo et al., 1980; DiSorbo and Litwack, 1982). Pyridoxal phosphate binds to a site on the activated receptor which is inaccessible in the unactivated receptor (Cake et al., 1978). There is good evidence that the ε-amino group of lysine is involved in the reaction between receptor and pyridoxal phosphate (O'Brien and Cidlowski, 1981; Litwack and Cake, 1977). Evidence has been presented, that activation results in the change of the molecular size and the conformation of the receptor. Holbrook et al. (1983) observed a large reduction in size of the receptor. It was suggested by Carlstedt-Duke et al. (1979), that lysosomal proteases play a regulatory role in glucocorticoid receptor complex binding to nuclei. It was observed that lysomal proteases convert the glucocorticoid receptor complex to smaller forms, one of which lacks the capacity to bind DNA.

The different conformations of the receptor protein induced by corticosterone and dexamethasone binding can explain the qualitative differences between the biological response elicited by natural glucocorticoids and their synthetic analogues (Carlstedt-Duke et al., 1977).

Contradictory observations have been made concerning the change of the receptor protein surface charge during activation. No separation between activated and unactivated hormone receptor complexes, using diethylaminoethyl cellulose (DEAE-cellulose) (Beato and Feigelson, 1972) and DEAE-Sephadex (Litwack et al., 1973) could be obtained by earlier workers. However, the conditions employed for these chromatographic procedures, were such that activation was favoured. The single peak detected probably represents activated complexes (Schmidt and Litwack, 1982). Separation between the more acidic unactivated rat liver glucocorticoid receptor complex and the more basic activated glucocorticoid receptor complex has been obtained (Parchman and Litwack,1977; Parchman et al., 1977). In the DNA binding site, exclusively expressed in activated receptor complexes, Lys, Arg and His are involved (Litwack et al., 1982).

3.3 TRANSLOCATION OF ACTIVATED HORMONE RECEPTOR COMPLEX TO NUCLEUS

Evidence has been presented, showing that in cells incubated with steroid at 0°C, cytosol binding of the steroid occurs readily, but nuclear binding proceeds very slowly. On briefly warming the cells to 37°C the activated hormone receptor complex is translocated to the nucleus even if the cells are again chilled (Munck, et al., 1972; Bloom et al., 1980).

Recently workers started to question the validity of the classical two step model of steroid hormone action, i.e. that a cytoplasmic receptor interacts at the plasma membrane site with the steroid and after activation this charged complex translocates the steroid to the nucleus. This model is based on evidence obtained in homogenized cell preparations, and thus might represent an extraction artefact.

King and Greene (1984), making use of a immunocytochemical procedure encompassing monoclonal antibodies to estrogen, showed that specific staining was confined to the nucleus in target cells. Welshons et al. (1984) used cytochalasin B-induced enucleation to obtain cytoplast and nucleoplast fractions from receptor-containing GH3 cells derived from rat pituitary tumours.

They found that cytoplasts prepared from GH3 cells contain little estrogen binding activity and that most of the unfilled estrogen receptors are associated with the nuclear fraction.

Steroid receptor complexes could be associated with any of the following locations in the nucleus:

1. Nuclear envelope (Jackson and Chalkley, 1974; Lefevre, and Novosad 1980; Smith and Von Holt, 1981).
2. Ribonucleoproteins (Liao et al., 1973).
3. Histone proteins (Sluyser, 1969; Monder and Walker, 1970).
4. Basic non-histone proteins (Puca et al., 1974; Mainwaring et al.,1976).
5. Acidic non-histone proteins (King et al., 1969; Spelsberg et al., 1975).
6. DNA (Yamamoto and Alberts 1974; Andre and Rochefort, 1975).

Most nuclear bound dexamethasone was found to be located in extranucleolar chromatin (Beato et al., 1973). Of all the components of chromatin, non-histone proteins and DNA display the greatest acceptor activity for the steroid receptor complexes (SRC) (Steggles et al., 1971). The acceptor sites in the nuclear matrix, accounting for less than 1% of total nuclear DNA, seem to be at least in part responsible for specific receptor recognition (Buttyan et al., 1983). Nuclear envelope fragments,

isolated from purified chromatin, bind activated glucocorticoid receptor with the same equilibrium constant as nuclear envelopes (Smith and von Holt 1981).

It has been shown "in vitro" that the structural integrity of DNA is important for the efficient interaction with the SRC (Rousseau et al., 1975) and this binding can be competitively inhibited by basic proteins such as histones (Simons et al., 1976). It has been proposed that steroid intercalates between T-A base pairs. Intercalation of the steroid moiety causes helix destabilization, permitting increased transcription (Sluyser, 1983).

Payvar et al. (1981, 1983) showed that glucocorticoid receptor protein stimulates transcription within murine mammary tumour virus (MTV) DNA sequences "in vivo", and interacts selectively with MTV DNA "in vitro" Five regions of MTV DNA that were bound specifically by purified receptor were identified. One resides upstream of the transcription initiation site and the others are distributed within transcribed sequences between 4 and 8 Kb from the initiation site.

Histones are not acceptor molecules (Spelsberg et al., 1971, 1972) and cannot account for the tissue specificity of nuclear binding. A small amount of cortisol was observed bound to Arg-

rich histones. It has been suggested that the cortisol is probably bound to a non-histone protein associated with the Arg-rich histones (Stein et al., 1974).

High affinity interaction between the calf uterine estrogen receptor and a preparation of acid extracted basic chromosomal proteins, that are not histones, has been observed (Puca et al., 1974).

Non-histone proteins in the nucleus have been divided up into three classes based on differing affinities towards binding SRC. The two lower affinity classes, mask the high binding affinity (5 — 10 000 binding sites per cell nucleus) especially in the non-target tissues (Spelsberg et al., 1976).

It was observed that the affinity of the receptor for DNA appeared to be significantly lower than that of the receptor for nuclei and chromatin in the chick oviduct system (Buller and O'Malley, 1976). The binding affinity of the progesterone-receptor for a DNA-associated acidic protein fraction in the chick oviduct chromatin is only observed when it is complexed with DNA (Spelsberg, 1974). These observations suggest that the receptor binds either to DNA sites altered by chromosomal protein interaction or to protein sites modified by DNA interaction

(Thrall et al., 1978).

DNase is capable of digesting transcriptionally active regions in chromatin (Garel and Axel, 1976; Weintraub and Groudine, 1976). Chromatin regions to which estrogen, aldosterone and glucocorticoid receptors bind appear to be highly susceptible to digestion by DNase (Chamness et al., 1974; Marver et al., 1974; Higgins et al., 1973). Baxter et al. (1972) produced evidence demonstrating that chromatin DNA with bound receptor was more resistant to DNase digestion. From this observation it can be concluded that receptors are binding directly to exposed regions of DNA.

Contrary to the above mentioned observations, Beatriz and Baxter (1976) found that most of the receptors for thyroid and glucocorticoid hormones are located in the "inactive" chromatin fraction. They claim that this observation is due to the superior chromatin fractionation technique employed as opposed to other mechanical chromatin fractionation techniques used by earlier workers which did not yield good separation of the sequences that are mostly transcribed "in vivo".

Steroid merely facilitates the expression of the gene. The basal level of the marker enzyme, tyrosine aminotransferase is

synthetized in the absence of the inducer. Two different mechanisms might control the same gene (Granner et al., 1968). The extent of receptor depletion (Yamamoto and Alberts, 1976) is closely dependent and is well correlated with the biological potency of the steroids administered (Izawa et al., 1982). The biological effect of different classes of steroids can be related to their capacity to promote nuclear binding of the receptor (Rousseau et al., 1973). Target cells lacking any steroid receptor (Bardin and Bullock, 1974) or having defective receptors which fail to translocate to the nucleus (Gehring and Tomkins, 1974) show no response to the receptor steroid hormones either in transcriptional alterations or in the later biological responses. Recently a contradictory observation has been made by Grote et al. (1983), who found no correlation between induction of the biological response and saturation "in vivo" of any of the cytoplasmic binding components, but a good correlation was observed for the dose dependences of enzyme induction and stimulation of RNA polymerase B.

Five to ten times more hormone is required to obtain maximal protein synthesis over the amount required to obtain maximal response in the polymerase activity. A sustained nuclear binding of a steroid is required for a large range of hormone actions on target cells.

3.4 ACETYLATION AND PHOSPHORYLATION OF NUCLEAR PROTEINS AND STEROID HORMONE ACTION

3.4.1 A Model for Gene Activation by Steroids

It has been shown that active regions of the eucaryotic genome contain DNA in a more open and accessible conformation (Gottesfeld et al., 1974).

With reference to the correlation between histone acetylation and genetic activation discovered by Allfrey (1971) the following model for eucaryotic gene activation was proposed. Steroid receptors interact with the genome at specific and non-specific loci, inducing a local structural alteration. This alteration is then chemically conducted a short distance along the chromosome. Specific DNA sequences that bind the receptor with relatively high affinity give rise to clustered receptor binding which is required for formation of a large enough patch, including both a specific promoter and its structural gene.

The chromosomal protein change e.g. histone acetylation is spread for a limited distance along the DNA helix to neighbouring nucleosomes by e.g. endogenous histone acetylases (Yamamoto and

Alberts, 1976).

Studies involving estrogen receptor (Schwartz et al., 1975) and androgen receptor (Davis and Griffiths 1973, 1974) effects on chick oviduct and rat prostate respectively showed enhanced RNA polymerase activity.

Estradiol treatment resulted in increased acetylation of histones H3, H2A and H2B in rat uterus (Libby 1972). In the kidney an increase in histone H4 acetylation was observed upon aldosterone treatment (Libby 1973). Induction of TAT by dexamethasone was delayed in artificially hypoacetylated HTC cells (Pleslko et al., 1983). An increase in liver histone acetylation was observed upon induction of TAT by hydrocortisone (De Villiers Graaff and von Holt, 1973).

An increase in acetylation and phosphorylation of chromosomal proteins was demonstrated prior to RNA polymerase activity and RNA synthesis in rat kidney and heart, following aldosterone administration. There was no apparent stimulatory effect of aldosterone on liver histone acetylation indicating some degree of organ specificity (Liew et al., 1973).

Hydrocortisone leading to an increase in TAT activity, did not

cause a detectable increase in histone phosphorylation in liver (Langan 1969; De Villiers Graaff and Von Holt 1973). In contrast, Murthy et al. (1970) reported an increase in phosphorylation of Lys-rich and to a lesser extent that of Arg-rich histones of rat liver in the presence of hydrocortisone. An increase in phosphorylation of histone H2A in L cells were observed in the presence of dexamethasone (Prentice et al., 1978).

Rapid histone acetylation and enzyme induction of glucocorticoids are inhibited to the same extent by sodium butyrate. The slow form of histone acetylation was unaffected in the sodium butyrate concentration range studied. Therefore it can be concluded that a correlation exists between rapid histone acetylation and induction of TAT by glucocorticoids (Plesko et al., 1983).

Enzyme induction by glucocorticoids is selectively inhibited at the transcriptional level by sodium butyrate, a deacetylase inhibitor (Tichonicky et al., 1981; Plesko et al., 1983). Half-maximal inhibition of the enzyme occurred at 0,9 mM butyrate in hepatoma tissue culture cells. The inhibition of TAT synthesis is reversed more rapidly upon removal of sodium butyrate in dexamethasone pretreated cells than in control cells. It can thus be concluded that part of the induction process occurs in

the presence of sodium butyrate.

Sodium butyrate is active inside the cell within a few minutes after its addition to the medium (Cousens et al., 1979). Direct interaction between sodium butyrate and histone deacetylase results in inhibition of the enzyme without inhibiting histone acetyl transferases (Vidali et al., 1978). Hyperacetylation is also caused by homologous short fatty acids proportionate to hexanoate and by the following di-dicarboxylic acids, succinic acid to C12 dicarboxylic acid.

Apart from causing hyperacetylation of histones, sodium butyrate also has various other effects on mammalian cells in culture. Butyrate treatment leads to an alteration in the structure of chromatin as shown by modification of chromatin accessibility to micrococcal nuclease and DNAse I (Mathis et al., 1978). It was also observed that butyrate leads to the cessation of DNA synthesis and cell division by blocking the cell cycle in the early G1 phase (Ginsberg et al., 1973; Baxter and Harris, 1975; Rubenstein et al., 1979).

All the above mentioned observations just once again underline the role histone acetylation plays in transcription.

3.5 TYROSINE AMINOTRANSFERASE AND GLUCOCORTICOIDS

In rats tyrosine aminotransferase (TAT) activity occurs primarily in the liver. Smaller amounts are also present in the kidney, heart (Lin and Knox, 1958), in the adrenals and brain (Wurtman and Larin, 1968). Hepatoma tissue culture cells derived from a rat hepatoma (Thompson et al., 1966) retained the TAT activity.

Tyrosine aminotransferase is the marker enzyme for glucocorticoid induction. Dexamethasone at a optimum concentration of 10^{-5} M elicits a 3 – 4 times increase in TAT synthesis during the first 8 – 9 hours of induction. Induction is preceded by an initial lag period of 2 hours in rat liver cells "in vitro" (Gerschenson et al., 1974b).

3.5.1. Synthesis of Tyrosine Aminotransferase and Isozymes

TAT polypeptides are completed on the polyribosomes in the absence of the cofactor, pyridoxal phosphate. The release of TAT from hepatic polysomes specifically requires adenosine 3',– 5' – cyclic monophosphate (cAMP) and a release factor which can be extracted from liver microsomes. The concentration of cAMP is

controlled by hormones (Chong-Cheng et al., 1972).

The half-life of TAT in the presence of the inducer is approximately 2 hours in hepatocytes (Ernest and Feigelson,1979) and hepatoma cells (Wicks et al., 1978; Culpepper et al., 1983). Numerous forms or isozymes of rat hepatic tyrosine amino-transferase have been identified. Four forms of which three are regulated by hormones were partially purified and characterized (Iwasaki et al., 1971, 1973). These three forms of TAT are all induced by corticosteroids and by glucagon. Forms II and III are also induced by adenosine 3′ 5′— monophosphate and form IV by insulin. A heat-sensitive mitochrondrial form has also been characterised (Inoue et al., 1971).

Form II is converted to forms III and IV by a heat labile, pH and temperature dependent, non-dialyzable component present in the lysozyme and mitochondrial fractions (Rodriguez et al., 1976). Form I is heat sensitive at 65^{o}C and its heat resistance is increased during the induction phase (Gerschenson et al., 1974b). A minor more cathodal form, differing from the major anodal form only in charge but not in molecular weight has also been identified. This form can utilize oxalacetate in place of ketoglutarate in the transamination of tyrosine and can transaminate aspartate as well as tyrosine. Immunological tests

proved this enzyme to be a "pseudo-isozyme" of TAT and this form probably represents aspartate aminotransferase (Spencer et al., 1974).

3.5.2 The TAT Reaction and the Isolation of the Enzyme

Some aspects of the reaction of the enzyme and its isolation are considered here, since they are relevant to the methodology employed in this investigation.

TAT catalyses the first and rate limiting reaction in the pathway by which tyrosine is finally degraded to acetoacetate and fumarate. α -Ketoglutarate is probably the only keto acid which accepts the amino group of tyrosine. The transamination of a number of aromatic amino acids including phenylalanine and tryptophan are also catalyzed by TAT, but at much slower rates. The 5'-phosphates of pyridoxal and pyridoxamine are the active cofactors. The pH optimum for activity is between 7,5 – 7,6.

Phosphate ions inhibit the TAT reaction competively with the 5'-phosphates of pyridoxal and pyridoxamine. Hydroxylated derivatives of tyrosine (e.g. dopamine and norepinephrine) and other aromatic compounds (e.g. p-hydroxyphenylacetic acid) also

inhibit the reaction. The enzyme is sensitive to sulfhydryl inactivation and there is some evidence that heavy metals cause loss of activity (for review of the properties of the enzyme see Granner and Tomkins, 1970).

Several methods of purification of this enzyme from rat liver have been described (Hayashi et al., 1967; Granner et al., 1968; Valeriote, 1969). The enzyme is stable to freezing and thawing at all stages of purification if the protein concentration is at least 1 mg/mℓ. The protein concentration for the heat treatment step should at least be between 5 and 10 mg/mℓ (Granner and Tomkins, 1970). Pyridoxal phosphate is a prerequisite for TAT stabilization. A 95% pure enzyme can be attained after having subjected a crude extract to the following steps: heat treatment, ammonium sulphate precipitation, dialysis, DEAE-cellulose column, Sephadex G-100 column and a sucrose gradient. The purification factor was 2500 (Valeriote et al., 1969).

PART 4

EXPERIMENTAL RESULTS

GLUCOCORTICOID HORMONE ACTION

4.1 INTRODUCTION

The events occurring in the cytoplasm during glucocorticoid induction have been well characterised. However, controversy still prevails as to how the biological response is elicited at the chromatin level.

This problem was approached by determining the sequence and duration of events upon uptake of the glucocorticoid by the cell until the eliciting of the biological response for which the TAT activity is an important indicator.

4.2 TYROSINE AMINOTRANSFERASE ASSAY

Methodology to extract and measure TAT activity had to be

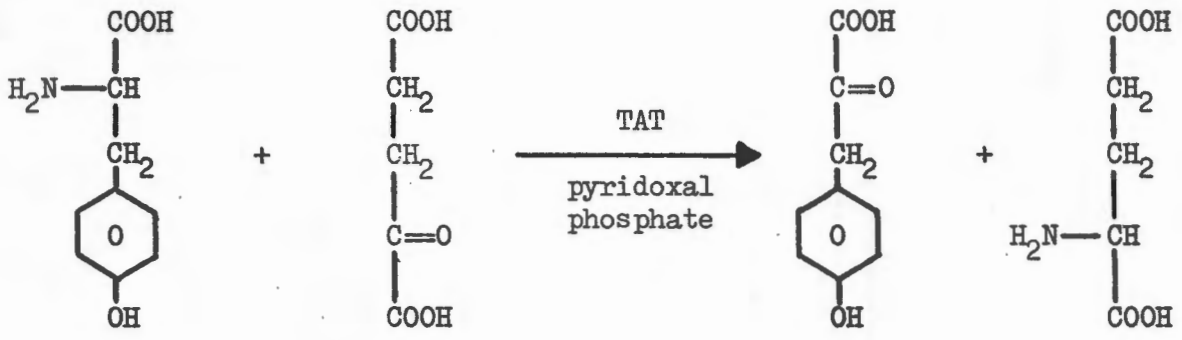
developed. This was based on the methods developed by Diamondstone (1966), Spencer and Gelehrter (1974).

First the sensitivity had to be increased. This was done by using ^{3}H— labelled tyrosine which allowed to replace the spectrophotometric assay by a radio-active determination of the p-hydroxyphenyl-pyruvate dinitrophenylhydrazone (Fig.4.1).

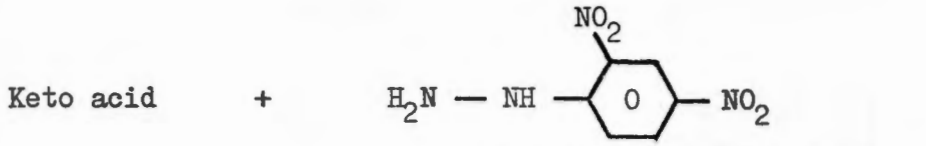
On adopting this method (Method 1) (6.5.4.1) for hepatocytes it was, however, found that the initial sample of 1 x 10^{6} cells became too diluted, which in turn made the enzyme more susceptible to inactivation. It had been observed that the enzyme activity was best preserved at approximately 10 mg protein/mℓ.

To overcome this difficulty to the cells to be lysed, a solution of buffered serum albumin was added (Method 2)(6.5.4.2) as the basal protein in 1 x 10^{6} hepatocytes amounts only to 1,79 mg (Seglen,1976). It is a further requirement for the stability of the enzyme that there should be excess of pyridoxal phosphate. This, however, interfered with the protein determination. Therefore, all enzyme activities are expressed in terms of DNA only.

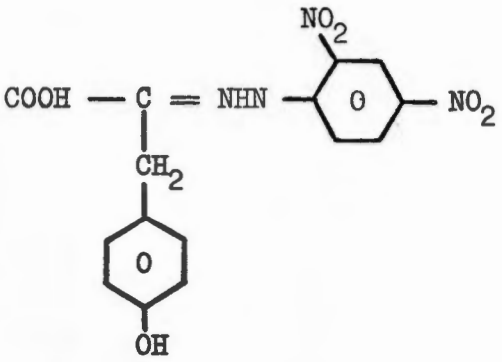
Different concentrations of the detergent used for cell lysis



Tyrosine — α - ketoglutaric acid — p-hydroxyphenyl pyruvic acid (Keto acid) — glutamic acid



2,4 dinitrophenylhydrazone



p-hydroxyphenylpyruvate
2,4 dinitrophenylhydrazone

Fig. 4.1 Tyrosine Aminotransferase Assay.

were explored (Table 4.1).

Table 4.1

The Effect of Nonidet P-40 on TAT Activity	
Non-Idet P-40 (%)	TAT Activity* nUnits/mg : prot.
0,1	0,42
1	0,23
10	0,174

* One unit of TAT activity is the amount of enzyme catalysing the formation of 1 µmol of p-hydroxyphenylpyruvate.

Concentrations higher than the minimal necessary to lyse the cells appeared to inhibit the enzyme. The latter method was also compared with two other mechanical methods to break the cells (Table 4.2).

Table 4.2

The Effect of Different Ways of Breaking the Cells on TAT Activity	
Method	TAT Activity per mg protein
0,1% (v/v) Nonidet P-40	0,369
Homogenization	0,342
Sonication	0,340

The storage of the crude enzyme extract until the assay could be performed also posed a problem. Although the enzyme could be stored at – 20°C (Granner and Tomkins,1970),the total protein concentration had to be in the excess of 1 mg/mℓ to protect the enzyme against inactivation (Table 4.3).

Table 4.3

Freezing of Enzyme	
Total Protein Concentration of Crude Extract (mg/mℓ)	TAT Activity per mg protein
> 1	0,384
< 1	0,038

4.3 UPTAKE OF [6,7 H (N)] TRIAMCINOLONE ACETONIDE BY THE CELL.

Uptake of TAA by the cell was studied at 4°C and 37°C to investigate the effect of temperature on the efficiency and duration of its transfer from the medium to the nucleus (Fig.4.2).

Maximal uptake was attained within 10 minutes. The slower uptake of the steroid at 4°C is probably due to a reduction in permeability of the plasma membrane at low temperatures (Munck and Wira,1970). Another contributing factor may be the

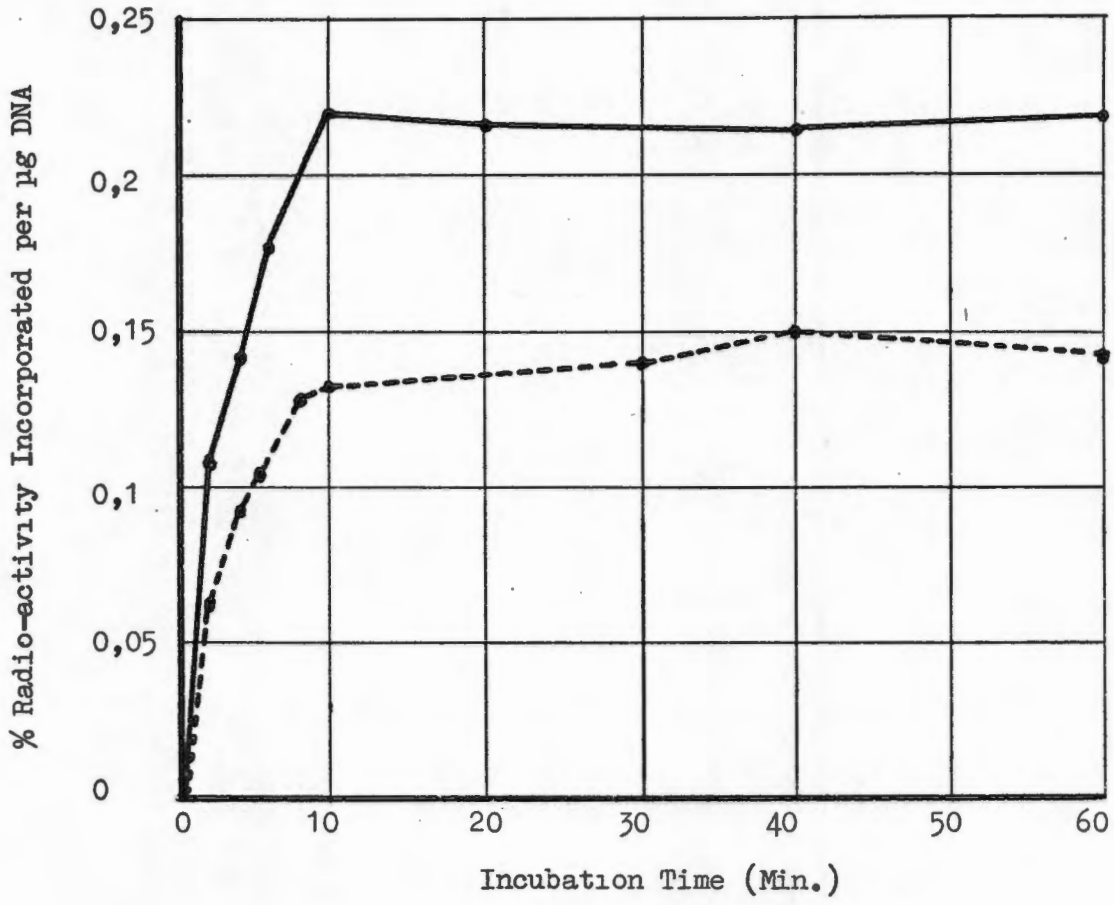


Fig. 4.2 Uptake of 6,7 ^{3}H (N) triamcinolone acetonide by hepatocytes at 4°C (- - - -) and 37°C (————).

inhibition of activation of the hormone receptor complex and subsequent nuclear translocation of the complex at low temperatures. This in turn will lead to a depletion of free receptor for uptake of new radio-activity.

4.4 RNA SYNTHESIS

Total RNA synthesis was studied in the absence and presence of the inducer, but no change in the RNA synthesis pattern was observed (Fig.4.3). These results corresponded with results obtained "in vivo". Considering that the basal rate of TAT synthesis is 0,005% — 0,010% of total protein synthesis in liver (Ernest and Feigelson, 1979) and that amplication takes place during translation, the increase in mRNA representing TAT would not increase total RNA synthesis of the cells significantly.

4.5 TYROSINE AMINOTRANSFERASE SYNTHESIS

TAT synthesis, induced by triamcinolone acetonide was studied at one hour intervals over six hours (Fig.4.4). An increase in TAT activity was only observed after a lag period of 2 hours and after six hours of incubation there was an approximate 3 fold

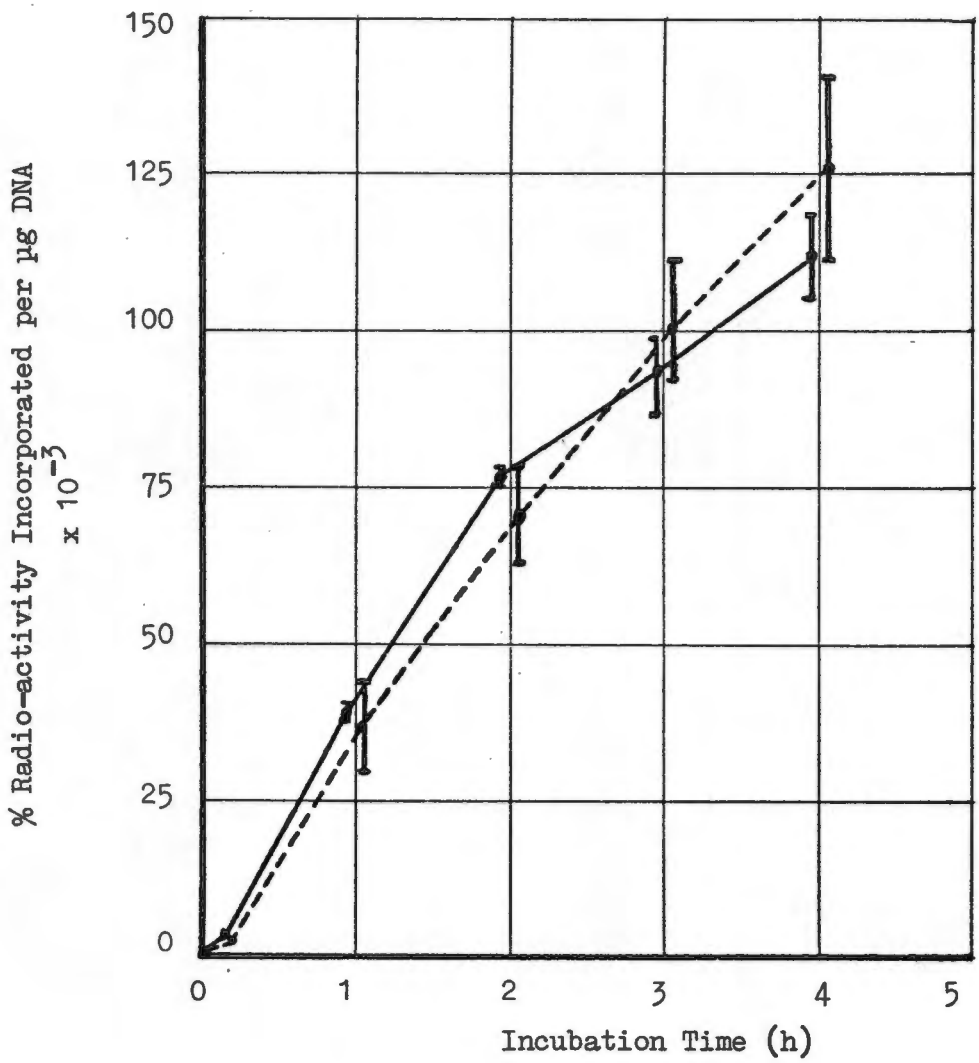


Fig. 4.3 Total RNA synthesis in hepatocytes after induction with TAA (———), control hepatocytes (– – – –).

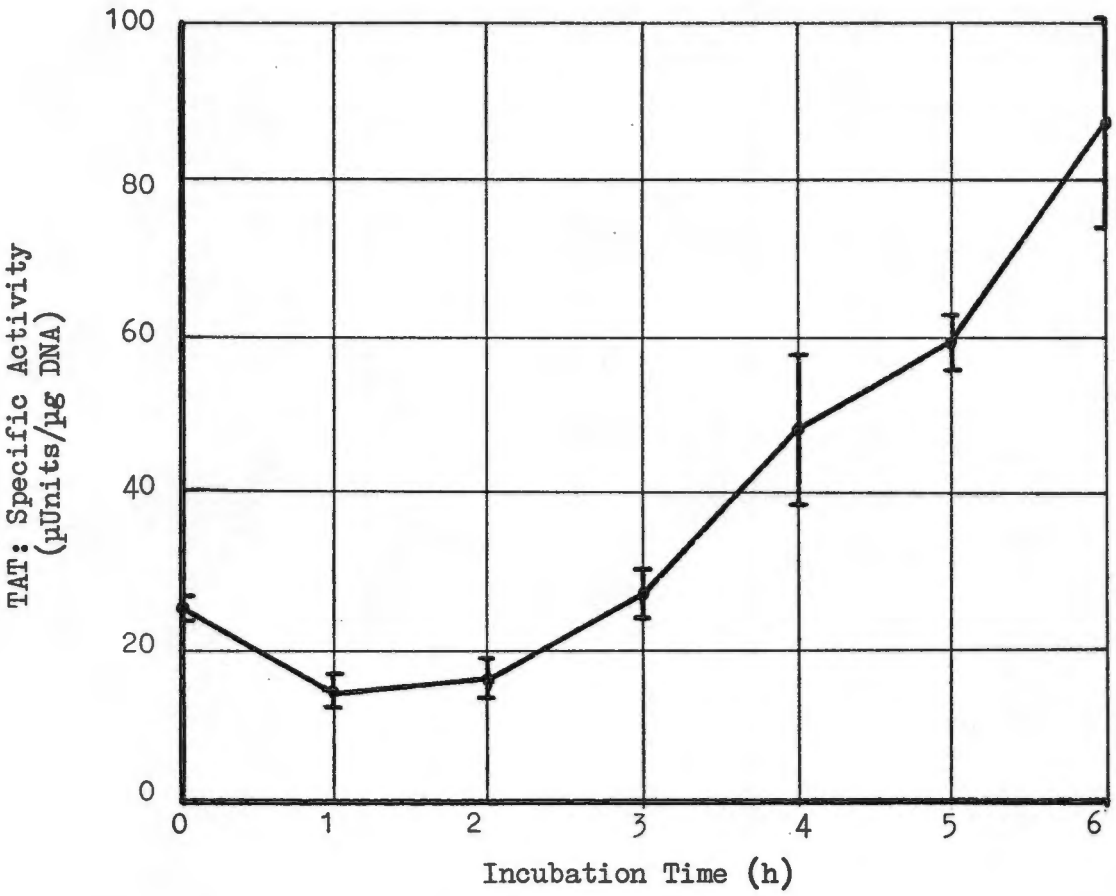


Fig. 4.4 Induction of TAT synthesis by TAA in hepatocytes.

increase in TAT activity. These values are in accordance with the literature (Gerchenson et al., 1974b). The initial drop in TAT activity to a general low level of protein synthesis is probably due to the conditions used in the hepatocyte preparation.

4.6 SEPARATION OF NUCLEAR PROTEINS ON A SDS GEL

Having established that TAT can be induced in hepatocytes prepared in the way described, the next task was to determine whether the methods available would be sufficiently sensitive and discriminating to measure changes in the enzymatic modification of nuclear proteins described previously to occur "in vivo".

A yield of approximately $0,2 \times 10^6$ nuclei could be expected after centrifuging 1×10^6 nuclei through a 2,3 M sucrose cushion. Separation of proteins presenting $0,12 - 0,26 \times 10^6$ nuclei was attempted on a 15% SDS gel (Fig.4.5). Some nuclear proteins could be visualised by Coomassie blue staining and good resolution of the histone bands was obtained.

It was not possible to simply run gels of the total cell content without separating the nuclei first. Cytoplasmic proteins obviously comigrated with certain nuclear proteins as is evident

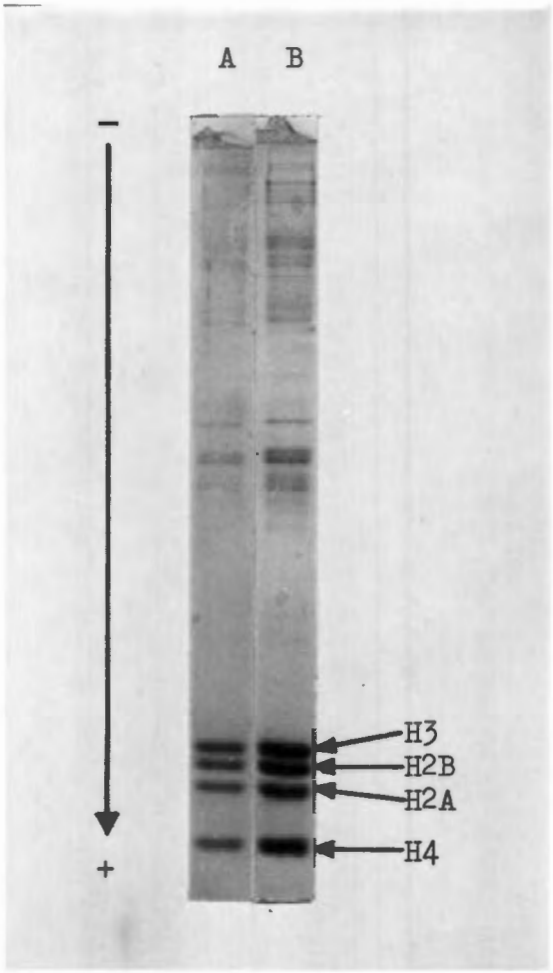


Fig. 4.5 SDS gel electrophoresis of nuclear proteins:

(A) $1{,}2 \times 10^5$ nuclei

(B) $2{,}6 \times 10^5$ nuclei

from the intensification of the histone H2A fraction in the electrophorectic pattern (Fig.4.6).

4.7 ACETYLATION

In the literature acetylation studies are usually performed by routinely including the deacetylase inhibitor sodium butyrate in the incubation medium and isolation buffers. In a pilot experiment the efficiency of 10 mM sodium butyrate in preventing deacetylation was confirmed. Hepatocytes in the presence of sodium butyrate after a 4,5 hour incubation period had incorporated 1,5 times more ^{3}H-acetate than hepatocytes incubated in the absence of sodium butyrate. However, at the same time the induction of TAT is inhibited (Fig.4.7). This is in accord with findings by a number of authors, who also observed the suppression of TAT induction by glucocorticoids in the presence of butyrate (Tichonicky et al., 1981; Plesko et al., 1983).

In order to overcome this problem the short half-life of acetylation, i.e. 30 — 40 minutes (Covault and Chalkley, 1982) was taken into account and the acetate pulse shortened to 15 minutes. Sodium butyrate was omitted from the incubation medium,

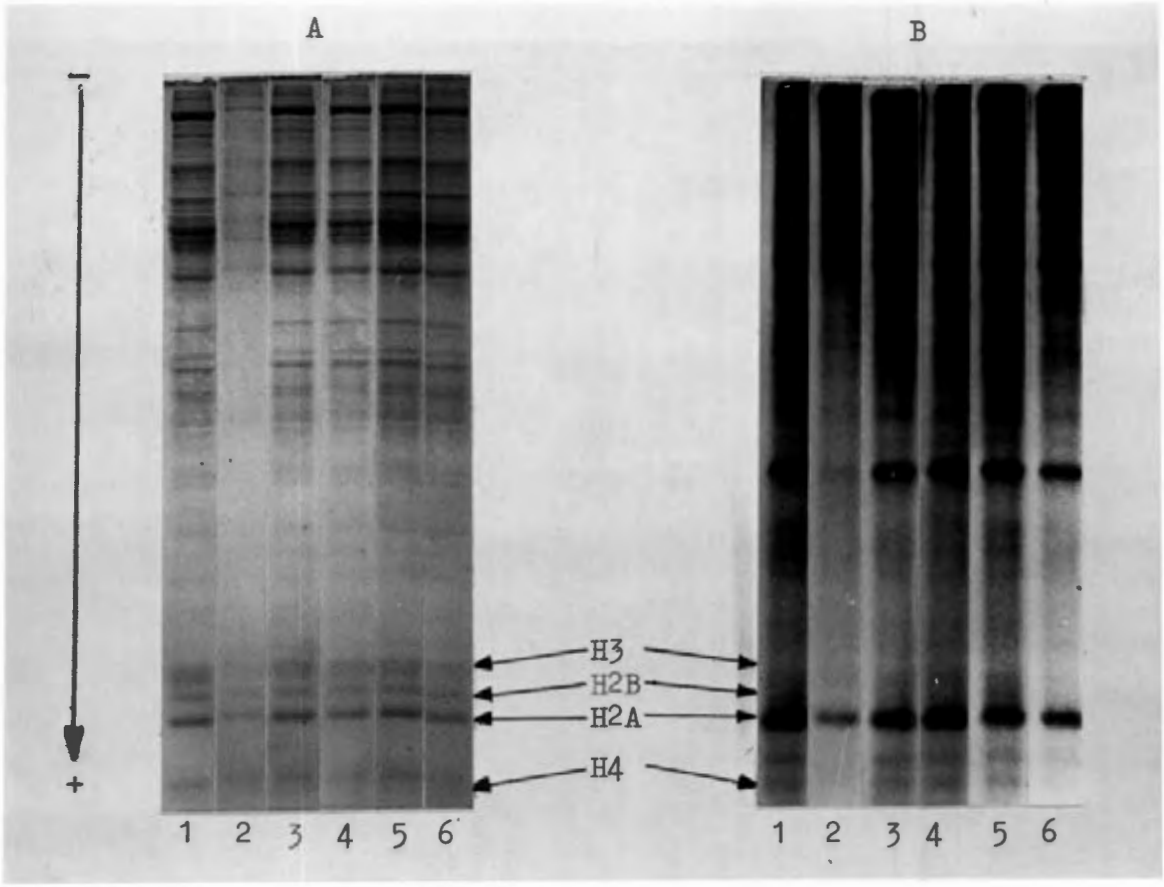


Fig. 4.6 SDS gel electrophoresis of (A) Total Rat Liver Proteins and (B) Autoradiograph of ^{32}P-labelled Proteins. The gel was exposed for 4 days at -70°C.

Incubation Times

Control

(1) 2,5 h
(2) 2,5 h - nuclear proteins
(3) 2,5 h - ^{32}P-labelled ATP
(4) 3,5 h

TAA Induced

(5) 2,5 h
(6) 3,5 h

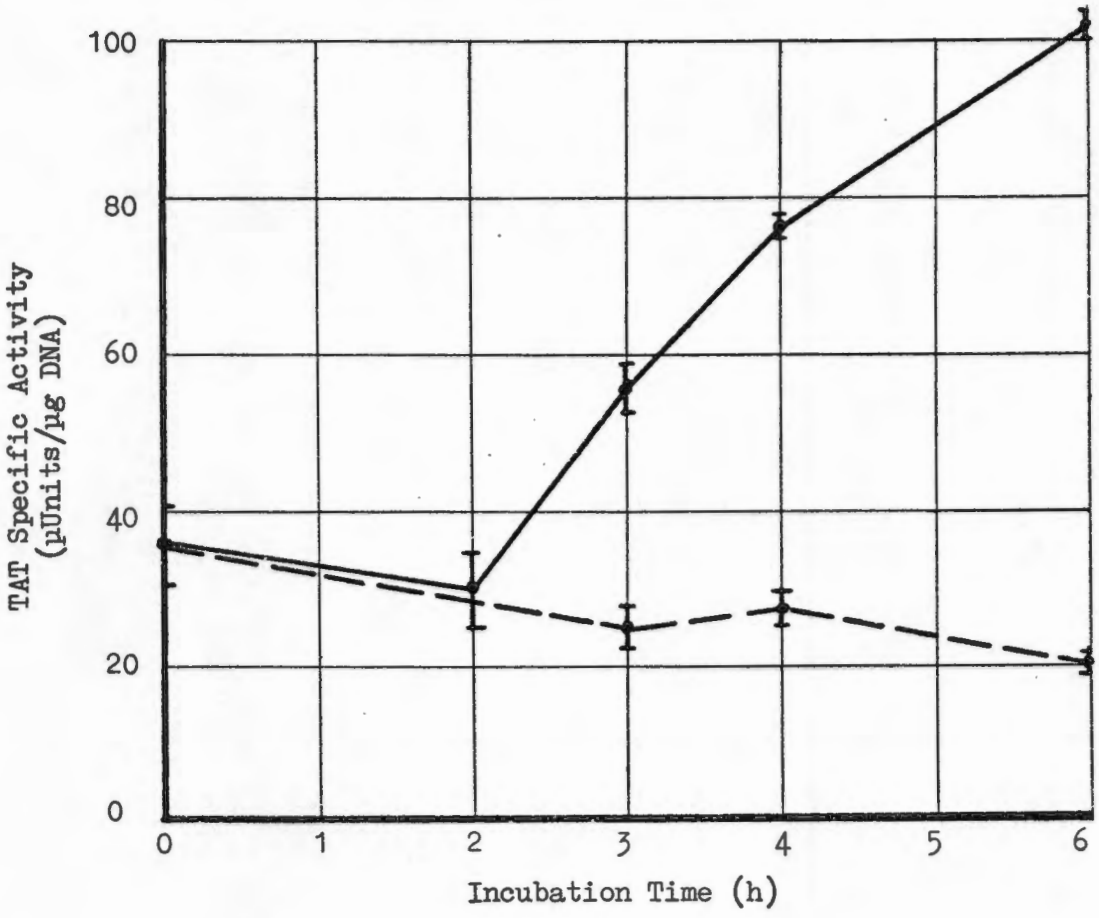


Fig. 4.7 Effects of sodium butyrate on the induction of TAT by TAA in hepatocytes.
0,2 nmole TAA (————).
0,2 nmole TAA and 10 mM sodium butyrate (– – – –).

but included in all the isolation buffers (Method 1)(6.5.5.1). To compensate for the loss of radio-activity due to deacetylation, the input radio-activity was raised 4 fold (Method 2) (6.5.5.2) This, however, resulted in only a 2 fold increase in the amount of radio-activity incorporated in the nuclear proteins. This relatively small improvement in radio-active incorporation, could thus not justify the extra cost involved, therefore, 600 µCi was routinely used.

This amount of input radio-activity required exposure times of the plates of approximately 1 month (Fig.4.8). The core histone bands were excised, their radio-activity determined in a liquid scintillation counter and the acetate incorporation expressed in terms of a percentage of the total incorporation into core histones. It was, therefore, decided to interpret the results by expressing the radio-activity present in the individual histone bands in terms of a percentage of the total radio-activity incorporated by the core histones.

Acetylation in the presence and absence of triamcinolone acetonide according to Method 3 (6.5.5.3), indicated that only histone H3 appeared to have incorporated more acetate as a result of the presence of the steroid (Fig.4.9).

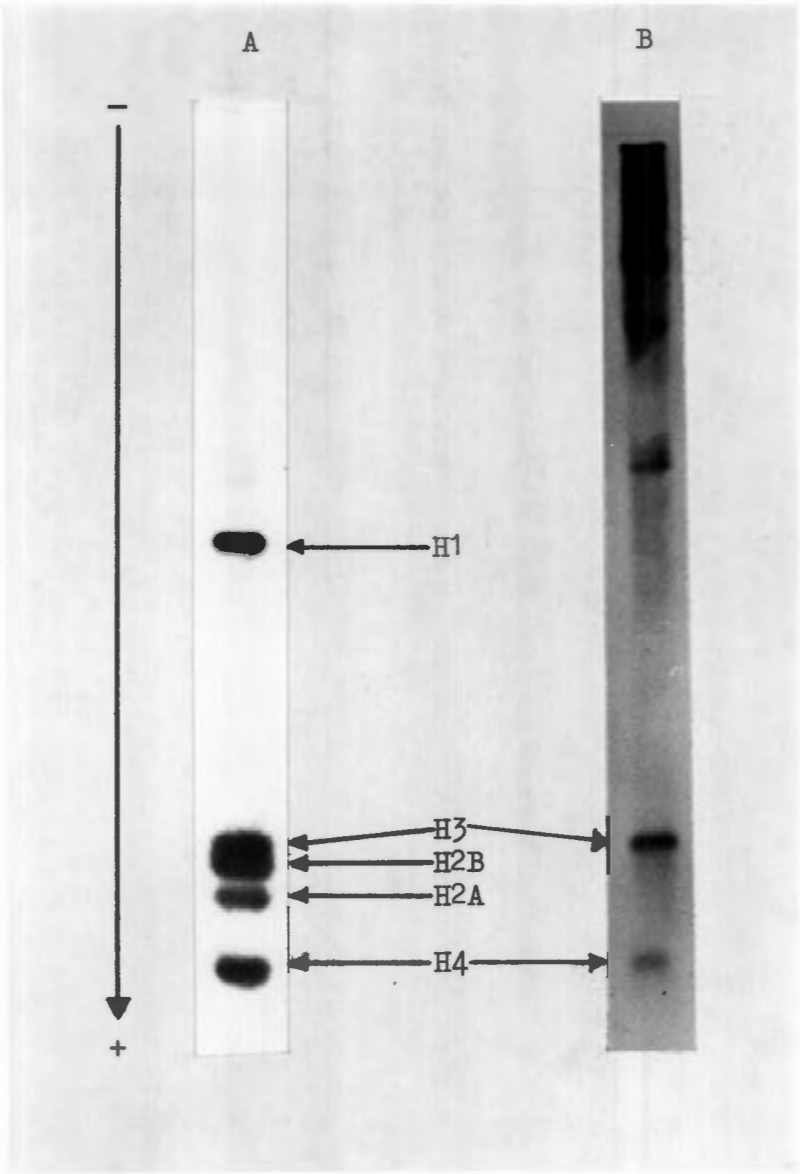


Fig. 4.8 SDS gel electrophoresis of (A) Chicken Erythrocyte Histones and (B) Fluorogram Of Acetylated Nuclear Rat Liver Proteins.

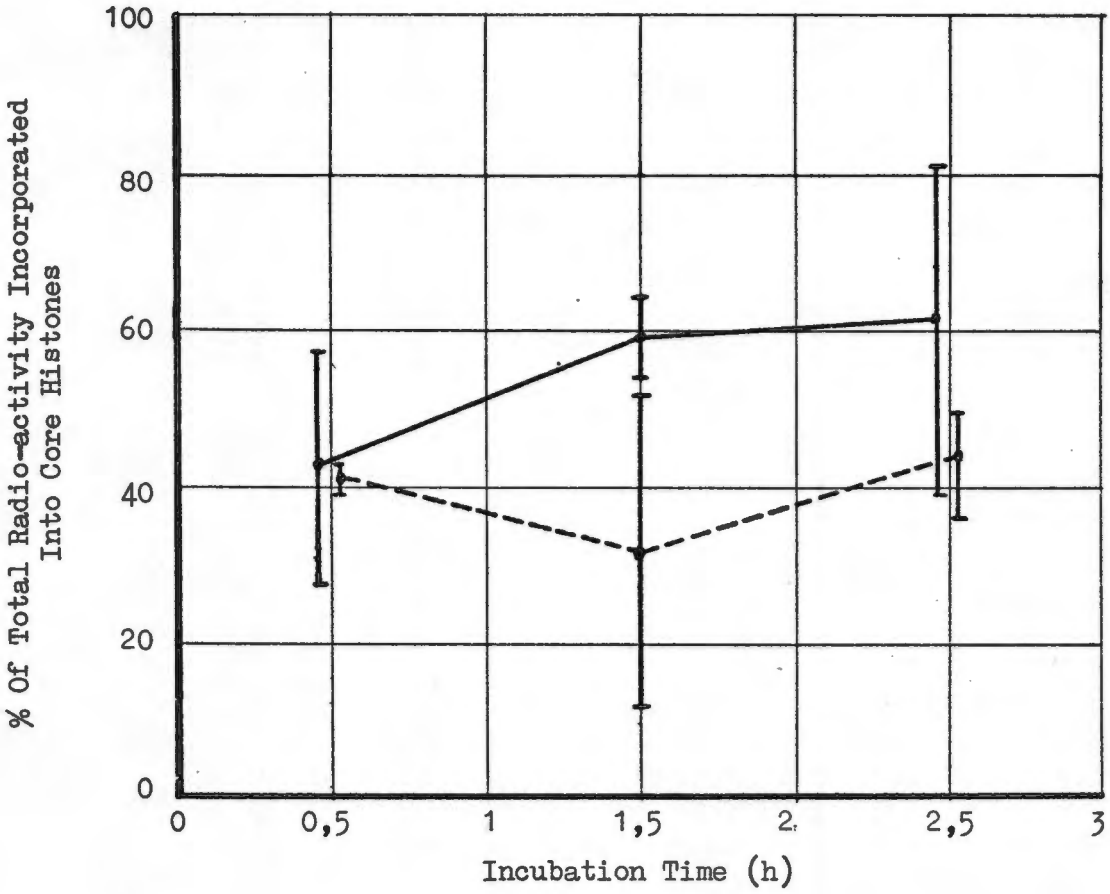


Fig. 4.9 Acetylation of histone H3 after induction with TAA (————), control hepatocytes (- - - -).

An attempt to increase the specific activity of the intracellular ^{3}H-sodium acetate by replacing the glucose present in the incubation medium with glutamine during the radio-active pulse, was also carried out (Method 4)(6.5.5.4). Glucose gives rise to acetyl co-A, diluting the radio-active sodium acetate, whereas glutamine following the α—ketoglutarate pathway should result in less dilution of the label. Although there was an 11 fold increase in the amount of radio-activity incorporated in the cells, there was not a significant increase in the amount of radio-activity incorporated in the nuclei (Table 4.4).

Table 4.4

Amount of Radio-activity Incorporated into Cells and Nuclei during Acetylation Studies

% Radioactivity Incorporated per µg DNA *	Methods	
	III	IV
Cells	$3,89 \times 10^{-4}$	$4,64 \times 10^{-3}$
Nuclei	$5,7 \times 10^{-6}$	$6,04 \times 10^{-6}$

* % of radio-active input incorporated per µg DNA representing viable hepatocytes.

4.8 PHOSPHORYLATION

To study the role phosphorylation plays in glucocorticoid induction, hepatocytes were incubated in the presence of ^{32}P or ^{32}P-labelled ATP for 2,5 hours (Method 1) (6.5.6.1)(Fig. 4.6). The same results were obtained with ^{32}P labelled ATP as with ^{32}P. This indicates that either the cell membrane structure has been modified during the isolation procedure allowing for the uptake of ATP or ATP has been dephosphorylated by phosphatases, liberated by broken cells or present in the FCS.

Cells were induced for different time periods with triamcinole acetonide, followed by a 30 minute radio-active pulse (Method 2) (6.5.6.2)(Fig.4.10). As can be seen from the Coomassie stained gel, differing amounts of protein were applied to the gel. This was because experiments yielded only small amounts of protein. In view of the limited half-life of the isotope, and the intensity of radio-activity required for autoradiography, it was decided to apply the differing amounts of protein, rather than to sacrifice some of the ^{32}P label present in the samples. All the core histones were phosphorylated. However, no conclusions can be drawn as to the presence or absence of increased phosphorylation due to the inducer. Even the most heavily phosphorylated histone H2A, did not show a difference in

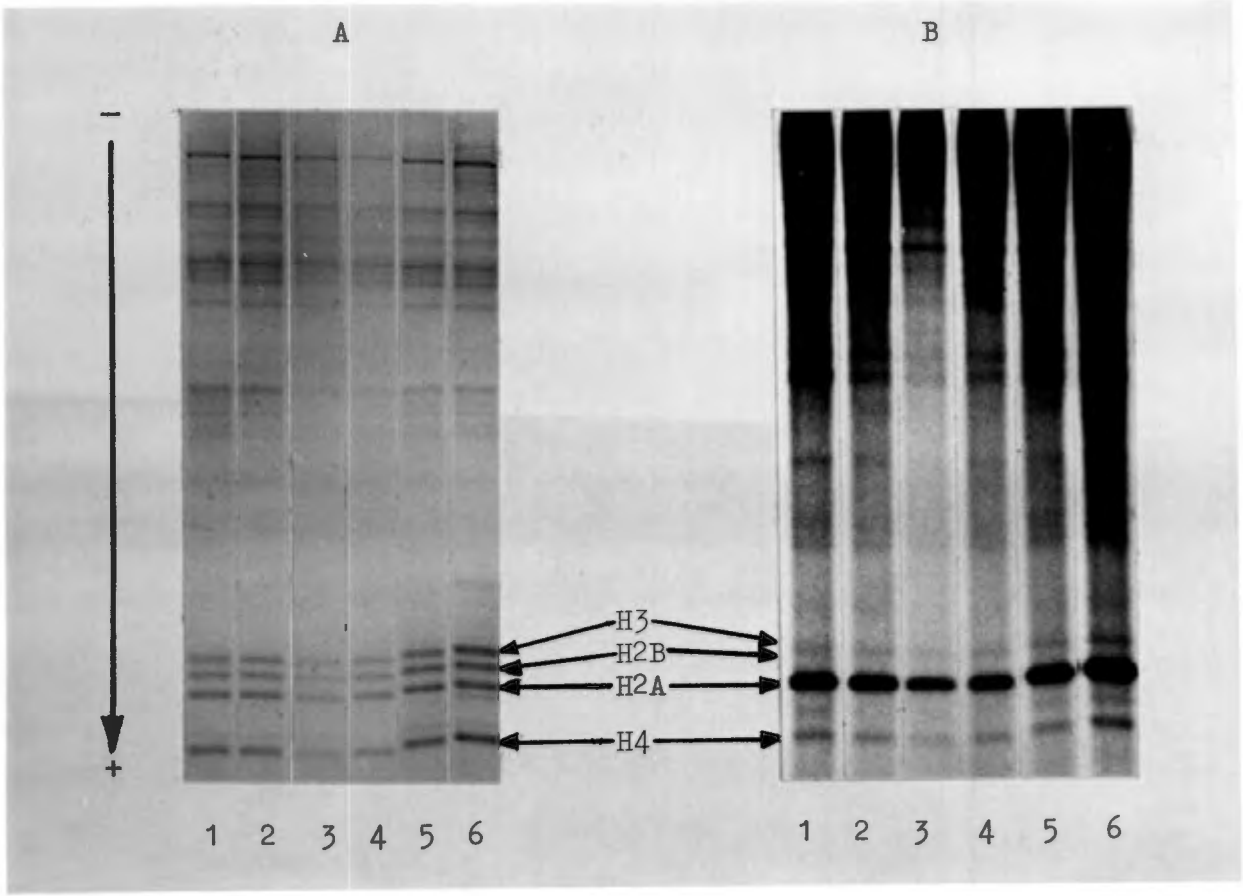


Fig. 4.10 SDS gel electrophoresis of (A) Nuclear Rat Proteins and (B) Autoradiograph of ^{32}P-labelled Proteins. The gel was exposed for 7 days at -70°C.

Incubation Times

Control		TAA Induced	
(1)	0,5 h	(4)	0.5 h
(2)	1,5 h	(5)	1,5 h
(3)	2,5 h	(6)	2,5 h

incorporation of the label (Fig.4.11). Histone H1 could not be identified on the gel. The results were analysed by solubulizing the individual histone bands and counting them.

To increase the radio-active labelling of the core histones, the input radio-activity would have had to be increased. This step would have been undesirable for economic reasons and even more important, the increased risks involved in working with such a high concentration of ^{32}P.

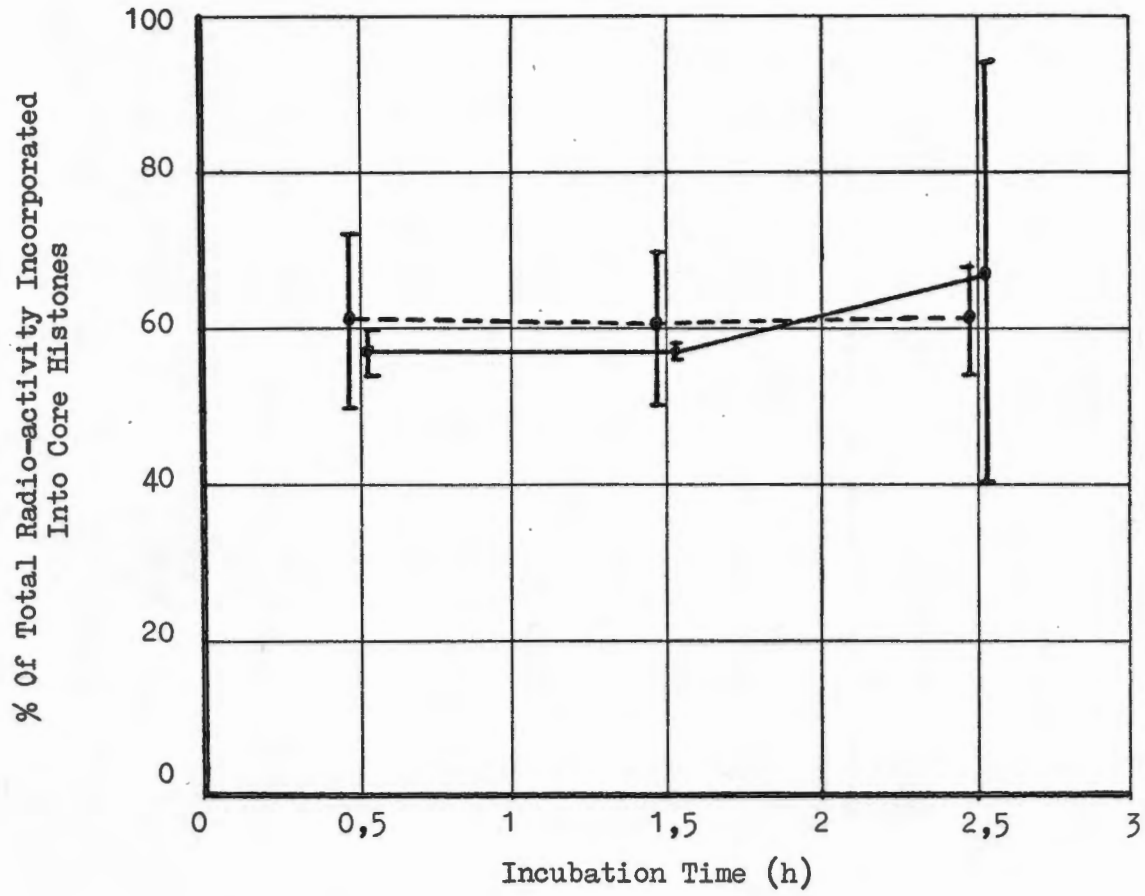


Fig. 4.11 Phosphorylation of histone H2A after induction with TAA (———), control hepatocytes (- - - -).

PART 5

CONCLUSIONS

The methodology for the isolation of hepatocytes, using the collagenase perfusion technique was successfully established. A compact perfusion system with a small dead volume allowing uninterrupted flow of the perfusate during buffer changes, was designed.

Performing the perfusion at a high flow rate in the physiological direction, resulted in the efficient removal of Ca^{++} and penetration of the whole liver by collagenase, absolute prerequisites for a cell preparation with a high viability. Leaving the connective tissue support of the portal vein intact during excision of the liver prevented the premature rupture of the portal vein resulting in termination of the collagenase perfusion step. Hence the duration of collagenase perfusion could be controlled, allowing the liberation of hepatocytes from the connective tissue framework without application of any mechanical force (Table 2.1). However, one factor, the variability in the quality of different collagenase batches, cannot be controlled and unfortunately this plays a very important role in the success achieved (Table 2.2). Although

the varying protease content of the different collagenase preparations has been considered to be a possible cause, no correlation could be demonstrated between the quality of a collagenase preparation and the latter.

The tying off of the intestines and the extracorporeally collagenase perfusion together with the presence of gentamicin in the perfusion buffers, resulted in routinely sterile preparations.

On average, in the excess of 80% of the isolated cells were structurally intact as determined by the trypan blue exclusion test. The cells were also functioning metabolically as proven by their ability to support the following metabolic functions, i.e. protein and RNA synthesis, hormonal induction and post-translational modifications to histone proteins.

The isolation of hepatocytes remains a complicated and tedious procedure and although there was a vast improvement in the success rate with practice, "it still remains an art" (Seglen, 1976).

Establishing the cells in monolayer culture was complicated. The cells only survived 1 — 2 days in culture and that prompted the decision to rather establish a short term suspension culture,

which was less cumbersome. The cells in the latter culture mode were metabolically active for at least 6 hours.

Maximal uptake of triamcinolone acetonide was attained within 10 minutes (Fig. 4.2), which is in good accord with the literature. No increase in total RNA synthesis was observed during induction. However, this phenomenon is not surprising, since amplification takes place during the translation process and TAT represents only 0,005% — 0,01% of the total protein synthesized in the liver (Ernest and Feigelson, 1979).

The modification effected to the spectrophotometrical TAT assay (Diamondstone, 1966 and Spencer and Gelehrter, 1974) using ^{3}H labelled tyrosine, increased the sensitivity of the assay, making the detection of pmole quantities of tyrosine transaminated by the TAT activity present in 1 x 10^{6} cells, possible.

TAT was induced with the characteristic 2 hour lag period. During the induction process, only histone H3 appeared to have incorporated more acetate as a result of the presence of the steroid. However, not even the most heavily phosphorylated histone H2A, showed a difference in incorporation of the label. Changes at the chromatin level, e.g. an increase in post-translational acetylation (Libby, 1972, 1973; De Villiers Graaf

and von Holt, 1973) and phosphorylation (Murthy et al., 1970; Liew et al., 1973; Prentice et al., 1978) of histones, during hormone induction, have been reported in literature. The majority of these studies have been conducted "in vivo". In the present study a small number of cells was used, resulting in a very low level of incorporation of radio-activity. The methodology used was not sensitive enough to study the above-mentioned changes, hence no clear cut conclusion could be drawn from the observations made.

In an attempt to increase the specific activity of the label intracellularly, the radio-active input was raised ten fold, resulting in no major improvement. It can be argued that by increasing the number of cells per experiment ten times, better results would have been achieved. However, culture conditions would have had to be adapted to accommodate the bigger number of cells involved and overloading of the gels would have resulted in bad resolution of the histone bands. On average approximately 100×10^6 viable hepatocytes were liberated, implying that cells from more than one animal would have had to be pooled to perform one experiment. This would defeat one of the advantages of cell culture studies, namely eliminating individual differences.

The cumbersome process of isolating the cells, problems

experienced with varying quality of collagenase and the short lifetime of the cells in culture, all contribute to making this system not very suitable for studies at the chromatin level.

Although cell lines could solve the problem of the availability of a limited number of cells, the crux of the matter is the low level of chromatin protein change required to switch on one gene in eucaryotes. A possible solution might be to employ gene families in these studies, e.g. histone, actin, tubulin and collagen genes.

PART 6

MATERIALS AND METHODS

6.1 MATERIALS

Item	Source
Radio-active Chemicals	Radiochemical Centre, Amersham
Nutrient Agar	Difco
Dulbecco's Modification of Eagle's Medium (DMEM)	Flow Chemical Company
Penicillin	Flow Chemical Company
Streptomycin	Flow Chemical Company
Sagatal Solution	May and Baker Ltd., Dagenham, England
Pularin	Glaxo Company
Ampicillin	Sigma Chemical Company
Gentamicin	Sigma Chemical Company
Tetracycline	Sigma Chemical Company
Zwitterionic Buffers	Sigma Chemical Company
Unlabelled Triamcinolone Acetonide	Sigma Chemical Company
Collagenase	Sigma and Worthington Chemical Companies
Fetal Calf Serum	State Vaccine Institute
Other Chemicals and Reagents	Merck, Germany

6.2. MAINTENANCE OF RATS

Rats used were female Wistar, with a 200 to 210g body-mass, aged 3 – 4 months, bred in the Department of Biochemistry at the University of Cape Town, kept on Epol Rat Cubes and tap water ad libitum under controlled light conditions (12 h light,12 h darkness).

6.3 ISOLATION PROCEDURES

6.3.1 Isolation of Hepatocytes

Hepatocytes were isolated according to the methods described in Part 2.

6.3.1.1 Isolation Buffers

As used by (Seglen, 1976)

Perfusion Buffers			
Chemical	Ca^{++}– Free	Colla-genase	Washing Buffer
NaCl	142	67	142
KCl	6,7	6,7	6,7
$CaCl_2$ H_2O	-	4,76	1,22
Hepes	10	100	10
Collagenase	-	0,035	-
pH	7,4	7,4	7,4

Concentrations are given in mM and the collagenase concentration in percentage % (w/v). The pH of the buffers was adjusted to 7,4 at 37°C in the cases of both the perfusion buffers and at 4°C for the washing buffer.

6.3.2 ISOLATION OF NUCLEI

6.3.2.1. Method 1

Nuclei were isolated from hepatocytes according to a modified method of Blobel and Potter (1966).

The cell pellet was resuspended in 0,5 mℓ ice-cold 0,25 M sucrose, 10,48 mM Hepes (pH 7,5); 0,025 M KCl; 0,0015 M $MgCl_2$ and transferred with the aid of a siliconized Pasteur pipette to a 1 mℓ Pierce Micro-Tissue tapered mortar (Product No.20049), suspended from a rubber support in ice-water. The centrifuge tube and Pasteur pipette were rinsed out with an additional 0,5 mℓ 0,25 M sucrose buffer.

The cells were broken up by 25 to 60 strokes (1 stroke comprises one upwards and downwards movement) of a pre-cooled teflon pestle rotating at 1500 rpm. During the homogenization procedure the temperature of the homogenate was maintained at 4°C.

The homogenate and 1 mℓ 0,25 M sucrose buffer used to rinse out the homogenization vessel were pooled and transferred to a pre-cooled Sm24 centrifuge tube. The tube was centrifuged at 770 g for 10 minutes in a Sorvall Superspeed RC2-B Centrifuge.
The supernatant was discarded and the pellet resuspended in 0,5mℓ 0,25 M sucrose buffer on a whirlly mix at low speed. 9 mℓ

of ice-cold 2,3 M sucrose, 10,48 mM Hepes (pH 7,5); 0,025 M KCl; 0,0015 M $MgCl_2$ were added and the tube covered with parafilm. The contents of the tube were mixed by mechanical mixing and inversion of the tube. The nuclei were spun down at 49 500 g for 40 minutes. When the supernatant was decanted, approximately 1 mℓ was left behind in the tube so as not to disturb the nuclear pellet.

Any cell debris adhering to the inner walls of the tube was wiped off with a paper tissue before resuspending the nuclear pellet in 9 mℓ of 0,25 M sucrose buffer. The nuclei were spun down at 2986 g in a benchtop centrifuge and resuspended in 0,2 mℓ 0,25 M sucrose buffer before further processing.
The sucrose buffers were kept frozen and thawed just before use. During acetylation studies, the buffers were supplemented with 10 mM sodium butyrate and in the case of phosphorylation studies with 50 mM sodium bisulfite.

6.3.2.2 Method 2

Nuclei were isolated according to the method of Woll (1981). Cells were washed once in ice-cold Ca^{++}- and Mg^{++}-free phosphate buffered saline (137 mM NaCl; 2,68 mM KCl; 0,9 mM $CaCl_2$; 1,05 mM $MgCl_2$; 8,1 mM Na_2HPO_4; 1,47 mM KH_2PO_4 and twice

with modified homogenization buffer (0,25 M sucrose; 2 mM Na_2EDTA; 0,5 mM Na_2EGTA; 60 mM KCl; 15 mM NaCl; 0,15 mM spermine; 0,5 mM spermidine; 15 mM Tris-HCl pH 7,4) before being resuspended in swelling buffer (0,15 mM spermine; 0,5 mM spermidine; 2 mM Na_2EDTA; 0,5 mM Na_2EGTA; 15 mM Tris-HCl, pH 7,4). After different time periods, ranging from 10 minutes to 3 hours, the cell suspension was transferred to various homogenizing vessels and homogenized in a volume of swelling buffer determined by the size of the vessel.

6.3.2.3. Method 3

Nuclei were isolated essentially according to the method of Mory and Gefter (1977).

Cells were washed twice in ice-cold swelling buffer (30 mM Tris-HCl pH 7,5); 120 mM KAc; 5 mM MgAc; 7 mM β—mercaptoethanol), before being resuspended in 0,3 M sucrose; 2 mM MgAC; 3 mM $CaCl_2$; 10 mM Tris-HCl (pH 7,5); 0,5 mM β—mercaptoethanol; 0,1% (v/v) Triton—X—100 and left on ice. After 10 minutes the cells were transferred to the homogenizing vessel and homogenized in a volume of swelling buffer, determined by the size of the vessel.

6.4. STERILE PRECAUTIONS

6.4.1. Cell Culture Laboratory

Bench tops were always wiped with 70% (v/v) ethanol and the floors and other surfaces in the room were cleaned once a week with 1% (w/v) Biocide D. Ultraviolet irradiation was used overnight to sterilize the tissue culture room. The sterile room also had microbial filters at all inlets and outlets of the airconditioning system and was kept under positive pressure.

Experiments were performed in a hood, fitted with an ultraviolet light.

Occasional sterility checks were performed with nutrient agar plates.

6.4.2. Perfusion Apparatus

6.4.2.1 Waterbath

Biocide D was added to the water to prevent bacterial growth and the water was also replaced at regular intervals. The perspex lid and sieves were irradiated overnight with ultraviolet light.

6.4.2.2 Pump

The pump was rinsed with 500 mℓ of 1% Biocide D followed by 2,5 ℓ of autoclaved distilled water. The tubing leading to the Ca^{++}- free and collagenase buffer containers were removed for autoclaving and the open ends of the pump wrapped in autoclaved tinfoil.

6.4.2.3 Glassware

All glassware was sterilized by autoclaving with steam from deionised water (121°C, 20 min., 1,2 Kg/cm^2) in a Hirayama Automatic Autoclave.

All glassware was siliconized according to the method of Parker (1979).

The glassware was washed thoroughly, rinsed with distilled water, dried and immersed in a solution of 2% dichlorodimethylsilane in carbon tetrachloride. The glassware was baked at 120°C for 3 hours, cooled and again immersed in the dichlorodimethylsilane solution and baked. This procedure was repeated, and after the final baking, the tubes were rinsed with and then boiled in distilled water. After drying, the glassware was

autoclaved.

A less cumbersome method was adopted later on. The clean glassware was immersed in a solution of 2% dichlorodimethylsilane in carbon tetrachloride, rinsed with technical methanol, dried in the oven and autoclaved.

6.4.2.4 Surgical Instruments and Plasticware

The surgical instruments (forceps, needles and thread) were autoclaved wrapped in tinfoil. Disposable, sterile plasticware (syringes, filters, tissue culture flasks and tubes and catheters) was employed.

The wrapping from sterile catheters was removed and a short piece of silicon tubing slipped over the catheter in position to secure the 3rd ligature. Afterwards the catheter was returned to its plastic sheath, wrapped in tinfoil and autoclaved. Although the catheter (needle and the surrounding plastic tubing) could be reautoclaved several times, it was preferably only used once. With several autoclavings the plastic tube started to fray at the edges and with time the needle became blunt making the insertion of the catheter in the portal vein more difficult.

6.4.2.5 Buffers and Medium

6.4.2.5.1 Buffers

All perfusion buffers were autoclaved and stored at room temperature. The sucrose containing buffers were stored at -20°C.

6.4.2.5.2 Culture Media

6.4.2.5.2.1 Commercial Medium

DMEM, buffered with 25 mM Hepes, 25 mM Tes and 25 mM Tricine, was dissolved in distilled water and the pH adjusted with NaOH to 7,4 at 37°C. The medium was filtered under negative pressure through a 0,45 µm prefilter and a 0,2 µm filter using a Satorius membrane filter, aliquoted and stored at -20°C.

6.4.2.5.2.2 Medium A

The medium was made up according to the recipe given in the Flow catalogue, except for the following modifications:

Volume 11:

56,78 mg L-Cystine disodium salt was replaced by 24,03 mg L—

Cysteine-HCl.

4 mg Pyridoxal HCl was replaced by 4,05 mg Pyridoxine HCl.

L-Cystine disodium salt and pyridoxal HCl were not available

6,4 g NaCl was replaced by 4,21 g NaCl to lower the osmolality of the medium.

The medium was buffered with 25 mM Hepes, 25 mM Tes, 25 mM Tricine and the pH adjusted to 7,4 at 37°C.

6.4.2.5.2.3 Diluted Commercial Medium (Medium B)

Dilution 1

405 mℓ DMEM, buffered with 30 mM Hepes was diluted to 500 mℓ with distilled H_2O. Final concentration of Hepes was 24,4 mM.

The medium was supplemented with 25 mM Tes, 25 mM Tricine, and the pH adjusted to 7,4 at 37°C.

Dilution 2

370 mℓ DMEM was diluted to 500 mℓ with distilled H_2O. The medium was supplemented with 25 mM Hepes, 25 mM Tes, 25 mM Tricine and the pH adjusted to 7,4 at 37°C.

6.4.2.5.2.4 Hormone and Radio-active Media

Triamcinolone acetonide (TAA) was dissolved at a concentration of 1 nmole/µℓ in absolute ethanol and stored at -20°C. Just before use, it was mixed with a small volume of medium (DMEM; 10% (v/v) FCS; 100 µg/mℓ gentamicin) and passed through a 0,2 µm filter.

Media containing ^{3}H-methionine and ^{3}H-orotic acid respectively were filtered, but media containing ^{3}H-sodium acetate and ^{32}P respectively were used unfiltered because of the shorter incubation times involved.

6.4.2.5.2.5. Fetal Calf Serum (FCS)

Sterile, virus screened fetal calf serum was heat-inactivated at 56°C for 30 minutes on arrival, aliquoted and stored at -20°C.

6.4.2.5.2.6 Antibiotics

Sterile penicillin (5 000 IU/mℓ) and streptomycin (5 mg/mℓ) were obtained in solution and stored at -20°C. Gentamicin, ampicillin and tetracycline were obtained in a powdered form and made up to 100 mg/mℓ and 3 mg/mℓ in 0,1 M phosphate buffer, pH 8 in the case of the first two antibiotics respectively and to

0,625 mg/mℓ in water in the case of tetracycline. The antibiotic solutions were passed through a 0,2 µm filter, aliquoted and stored at -20°C.

6.4.3 Sterility Control

6.4.3.1 Preparation of Agar Plates

Plates were poured under a sterile hood.

1 ℓ of distilled water was added to 23 g of nutrient Agar, autoclaved and kept in an oven at 50°C. 20 mℓ aliquotes were poured into smaller containers, already containing the antibiotics, mixed and poured into petri dishes. The agar plates were left at room temperature to solidify and dried at 37°C for 2 hours in an inverted position.

Aliquots of filtered medium and new batches of FCS were plated out on agar plates in the presence and absence of antibiotics, incubated for 3 days at 37°C and monitored daily for bacterial growth.

6.4.3.2 Disk Method

This method was employed to determine the sensitivity of the bacteria present in the incubation media obtained from 1 day old cell cultures to different antibiotics and a range of antibiotic concentrations.

The contaminated medium was plated on an agar plate, incubated for 2 days and a bacterial colony scraped up with a flamed wire-loop and resuspended in 1 mℓ of sterile saline. 0,1 mℓ of the suspension were transferred to an agar plate and spread out with a sterile glass spreader. The paper disks, containing the antibiotics were applied aseptically and the plates incubated overnight at 37°C.

6.5 METABOLIC STUDIES

6.5.1 Protein Synthesis

The incorporation of a radio-active amino acid into protein was measured according to the disk method of Mans and Novelli (1961).

0,5 x 10^6 viable hepatocytes were preincubated at 37°C in 1 mℓ incubation medium (DMEM; 10% (v/v) FCS; 100 µg/mℓ gentamicin) for

30 minutes. 3 µCi (0,366 nmoles) of ^{3}H — methionine with a specific activity of 8,2 Ci/nmole was added to the cell suspension to bring the final volume to 2 mℓ and the incubation continued for different time periods.

Protein synthesis was stopped by placing the cell culture tube on ice, immediately followed by the addition of ice-cold saline. The cells were centrifuged in a benchtop centrifuge and the cell pellet resuspended in 0,25 mℓ cold saline, the maximum volume that can be absorbed by the filter. A siliconized Pasteur pipette was used to transfer the cell suspension to a 12 cm^2 GFC (glass fibre cellulose) filter. The filter was dried before transferring an additional 0,25 mℓ of saline, used to rinse out the tissue culture tube and pipette, to it. The protein was precipitated on the filter during a 10 minute period in 10 mℓ ice-cold 10% (w/v) TCA, (trichloro-acetic acid) supplemented with 10 mM methionine, followed by three 2 minute washes in 10 mℓ cold 5%(w/v) TCA, 10 mM methionine. The filter was soaked twice for 2 minutes at a time in 10 mℓ ethanol (technical grade), dried overnight at 37°C, transferred into scintillation fluid and counted in a scintillation counter.

6.5.2 RNA Synthesis

$0,5 \times 10^6$ viable hepatocytes were pre-incubated at 37°C in 1 mℓ incubation medium (DMEM; 10% (v/v) FCS; 100 µg/mℓ gentamicin) for 30 minutes. 6,27 µCi (0,242 pmole) of ^{3}H-orotic acid with a specific activity of 26 Ci/mmole was added to the cell suspension to bring the final volume to 2 mℓ and the incubation continued for different time periods.

The same method as for protein was followed to measure the incorporation of the radio-active nucleotide into RNA.

6.5.3 Time Study of ^{3}H-Triamcinolone Acetonide Uptake

The incorporation of the radio-active glucocorticoid analogue was essentially measured according to the disk method used by Rao et al.(1976).

$0,6 \times 10^6$ viable hepatocytes were pre-incubated at 37°C in 0,5 mℓ incubation medium (DMEM; 10% (v/v) FCS; 100 µg/mℓ gentamicin) in an Eppendorf vial for 30 minutes, 25 µCi (0,68 pmole of [6,7 ^{3}H (N)] triamcinolone acetonide with a specific activity of 37 Ci/mmole was added to the cell suspension to bring the final volume to 0,75 mℓ and the incubation continued for different time periods. The cells were centrifuged for 30 seconds in an Eppendorf

Centrifuge, the supernatant aspirated off, and the cells resuspended in 0,25 mℓ of ice-cold saline. The cells were washed a second time with 0,5 mℓ saline, before transferring it in 0,25 mℓ saline to a 12 cm^2 GFC filter. The filter was dried, before transferring an additional 0,25 mℓ of saline, used to rinse out the Eppendorf vial and pipette, to it. The filter was dried overnight at 37°C, transferred into scintillation fluid and counted in a scintillation counter. The same method was followed for the study of ^3H-triamcinilone acetonide uptake conducted at 4°C.

6.5.4 Tyrosine Aminotransferase Assay

The assay was done according to a modification of the methods of Diamondstone (1966), Spencer and Gelehrter (1974).

1×10^6 viable hepatocytes, suspended in incubation medium (DMEM; 10%(v/v) FCS; 100µg/mℓ gentamicin) were pre-incubated for 30 min. 0,2 nmoles of cold TAA were added in 1 mℓ medium, 10 minutes were allowed for uptake of the hormone by the cells and the incubation continued for different time periods.

6.5.4.1 Method I

The cells were spun down and the tubes placed on ice before adding 0,5 mℓ of isolation buffer. The cell suspension was transferred with the aid of a siliconized Pasteur pipette to a 1 mℓ Pierce Micro-Tissue tapered mortar, suspended from a rubber

support in ice-water. The centrifuge tube and Pasteur pipette were rinsed out with an additional 0,5 mℓ isolation buffer. The cells were broken up by 25 to 60 strokes of a pre-cooled teflon pestle rotating at 1500 r/min.

The homogenate and 0,25 mℓ isolation buffer used to rinse out the homogenizing vessel were pooled and transferred to a pre-cooled SM 24 centrifuge tube. The tube was centrifuged at 49 500 g for one hour. The supernatant was heated at 65°C for 6 minutes in a waterbath. The tubes were cooled on ice and centrifuged at 49 500 g for 20 minutes. The supernatant was kept on ice.

6.5.4.1.1 Assay

0,8 mℓ of TAT assay incubation medium reaction mixture was added to 0,2 mℓ supernatant in an Eppendorf vial and incubated for 22 minutes at 37°C. A 0,3 mℓ aliquot was transferred to a clean Eppendorf vial and 50 µℓ of p-hydroxyphenylpyruvate carrier in MeOH added. The tube was inverted once before addition of 0,1 mℓ of DNPH reagent. The incubation at 37°C was continued for another 12 minutes. The tubes were left at room temperature for 1,5 hours, followed by centrifugation for 5 minutes in an Eppendorff centrifuge. The precipitate was washed twice in 0,8 mℓ of 0,6 N H_2SO_4, before being dissolved in 0,5 mℓ of MeOH at 50°C for 15 minutes.

A 0,2 mℓ aliquot was counted in 10 mℓ of commercial scintillation fluid (Packard-Emulsifier Scintillator 299 TM).

6.5.4.1.2 TAT Assay Incubation Medium

Solutions	Volumes (mℓ)
A*	5
B*	3
C	0,2
D	0,1
E	0,2
^{3}H Tyrosine*	0,01
Total Volume	8,51

*pH of AB mixture must be 7,6

*^{3}H Tyrosine (45 mCi/ µmol)

6.5.4.1.3. Stock Solutions

Isolation Buffer (kept for 1 week at 4°C in the dark)

0,2 mM pyridoxal phosphate

1 mM α-ketoglutarate

1 mM EDTA

Dissolved in 50 mM potassium phosphate buffer, pH 6,5.

A

125 mM $K_2HPO_4 \cdot 3H_2O$

2,125 mM tyrosine

0,05 N KOH

B

125 mM KH_2PO_4

Solutions A an B were kept for 1 week at 4°C.

C (Kept frozen at -20°C)

0,5 M α-Ketoglutarate

D (Kept frozen at -20°C in the dark).

20 mM pyridoxal phosphate

E (Made fresh)

0,5% BSA (crystalline bovine serum albumin) in water.

2,4-Dinitrophenylhyrazone (DNPH) Reagent (Mann and Saunders, 1974)

1,67 mℓ concentrated H_2SO_4 was added with stirring to 80 mg 2,4 - dinitrophenylhydrazine in 20 mℓ absolute methanol. This solution was stored for 1 month in a dark bottle.

p-Hydroxiphenylpyruvate carrier (made fresh)

0,1% p-hydroxyphenylpyruvate in methanol

6.5.4.2 Method 2

The cells were spun down and the tubes placed on ice before adding 0,5 mℓ of lysing buffer. The lysis process was allowed to continue for 20 minutes before the tubes were placed in the freezer at -20°C.

6.5.4.2.1 Day of Assay:

The samples were thawed at room temperature and spun at 20 000 r/min(49 500 g) for 20 minutes at 4°C in a SM 24 rotor.

0,6 mℓ of TAT assay incubation medium was added to 0,4 mℓ of supernatant in an Eppendorf vial and incubated for 22 minutes at 37°C. 0,05 mℓ of p-hydroxyphenolpyruvate carrier in MeOH was added and the tube inverted once before addition of 0,1 mℓ of DNPH reagent. The incubation at 37°C was continued for another 12 minutes. The tubes were left at room temperature for 1 1/2 hours, followed by centrifugation for 5 minutes in an Eppendorf centrifuge. The precipitate was washed twice in 0,8 mℓ of 0,6 N H_2SO_4, before being dissolved in 0,5 mℓ of MeOH at 50°C for 15 minutes.

A 0,4 mℓ aliquot was counted in 10 mℓ of Commercial Scintillation

fluid (Packard-Emulsifier Scintillator 299 TM).

6.5.4.2.2. TAT Incubation Medium

Solutions	Volumes (mℓ)
A	5,880
B	3,530
C	0,157
D	0,157
E	0,275
^{3}H Tyrosine	0,016
Total Volume	10,015

6.5.4.2.3 Stock Solutions

Isolation Buffer

0,122 mM pyridoxal phosphate

5 mM α—ketoglutarate

5 mM MgAC . $4H_2O$

150 mM KCl

Dissolved in 50 mM potassium phosphate buffer, pH 7,6.

A

167 mM $K_2HPO_4 \cdot 3H_2O$

2,83 mM Tyrosine

0,05 N KOH

B

167 mM KH_2PO_4

C

1 M α-Ketoglutarate

D

20 mM pyridoxal phosphate

E

distilled water

BSA carrier

0,3% BSA (crystalline bovine serum albumin) in isolation buffer

Lysing Buffer

0,1% (v/v) N.I.P. - 40 (Nonidet P-40) and 0,018% (w/v) BSA in isolation buffer

DNPH reagent and p-hydroxyphenylpyruvate carrier are the same as for Method 1.

6.5.5 ^{3}H Acetate Labelling of Rat Liver Nuclear Proteins

1 x 10^{6} viable hepatocytes were pre-incubated for 30 minutes in 1 mℓ DMEM and 10% (v/v) FCS.

6.5.5.1 Method 1

Cells were pulsed for 15 minutes with 600 µCi ^{3}H sodium acetate (specific activity 3,17 Ci/nmole) added in 1 mℓ DMEM.

6.5.5.2 Method 2

Cells were pulsed for 35 minutes with 2,3 mCi ^{3}H sodium acetate.

6.5.5.3 Method 3

0,8 mℓ DMEM containing 0,2 nmoles of TAA was added to the cells and incubated for different time periods. An additional 10 minute period was allowed in all cases for the uptake of the hormone. This was followed by a 30 minute pulse with ^{3}H-sodium acetate (added in 0,2 mℓ DMEM).

6.5.5.4 Method 4

1 mℓ of DMEM containing 0,2 nmoles of TAA was added to the cells, 10 minutes allowed for uptake of the hormone in the cells and the incubation continued for different time periods.

The cells were spun down and the medium replaced with 2 mℓ acetylation medium in the presence of 600 µCi of ^{3}H sodium acetate and the incubation continued for an additional 30 minutes.

In all 4 methods, acetylation was stopped by adding 4 mℓ of ice-cold 0,25 M sucrose buffer in the presence of 10 mM sodium butyrate (deacetylase inhibitor) (Seally and Chalkley, 1978; Perry et al., 1979; Jiakuntorn and Mathias,1982) to the cultures. The cells were spun down, resuspended in 0,5 mℓ of cold 0,25 M sucrose buffer and frozen.

6.5.5.5 Acetylation Medium

Suspension buffer used by Seglen (1976) during the isolation of hepatocytes, supplemented with glutamine (Zielke et al., 1984).

Chemicals	Final Concentration (mM)
NaCl	68
KCl	5,36
$CaCl_2.2H_2O$	1,22
$MgCl_2.6H_2O$	0,639
KH_2PO_4	1,102
Na_2SO_4	0,704
Hepes	30
Tes	30
Tricine	36
Glutamine	4
pH	7,4

The pH of the medium was adjusted to 7,4 at 37°C.

6.5.6. ^{32}P Labelling of Rat Liver Nuclear Proteins

1 x 10^6 viable hepatocytes were pre-incubated for 30 minutes in 1mℓ DMEM and 10% (v/v) FCS.

6.5.6.1 Method 1

Cells were pulsed for different time periods with 165 µCi ^{32}P or $\gamma-^{32}P$-ATP and 0,2 nmoles of cold TAA added in 1 mℓ medium. An additional 10 minute period was allowed in all cases for the uptake of the hormone.

6.5.6.1.1 Preparation of $\gamma-^{32}P$-ATP

$\gamma-^{32}P$-ATP was made using a Gamma Prep-A synthesis system (Promega Biotec)

2 mCi (50 µℓ) of ^{32}P in dilute HCl, pH 2-3, carrier free was added to a reaction mixture containing a series of enzymes and vortexed gently. After an hour incubation at room temperature, the reaction was stopped by heating for 5 minutes at 90°C and cooling in ice. The $\gamma-^{32}P$-ATP, divided into two 50 µℓ 1 mCi aliquots, each having a specific activity of 3 000 — 6 000 Ci/mmole, was quick frozen in liquid nitrogen and stored at —20°C.

6.5.6.2 Method 2

The same as Method 3 for acetylation. 400 µCi ^{32}P was added. In both methods, phosphorylation was stopped by adding 4 mℓ of ice-cold 0,25 M sucrose buffer in the presence of 50 mM sodium bisulfite (protease inhibitor), (Gurley et al., 1975) to the cultures.

6.6. ASSAYS

6.6.1 Cell Viability Test

The trypan blue exclusion test for cell viability was done according to the method of Phillips (1973).

0,4 mℓ of the cell suspension (3 x $10^6$6 cells/mℓ) in washing buffer was mixed with 0,2 mℓ of dye solution (0,4% (w/v) trypan blue; 4,4mM KH_2PO_4; 140mM NaCl; pH 7,3) to give a final trypan blue concentration of 0,133% (w/v). A drop of the suspension was applied to a hemocytometer and a viability count made 4 minutes later.

6.6.2. Harvesting of Cells

6.6.2.1. Method 1

0,9% (w/v) Saline

The incubation medium was poured off. The tissue culture flask was rinsed out twice with saline, 0,48 (w/v) trypan blue solution and saline in a ratio of 1:1 were added. After 2 minutes the dye was poured off and the bottle rinsed twice with saline. Cells were dislodged by squirting saline on them with a Pasteur pipette. The cells were spun down for 2 minutes at 747 g in a bench top centrifuge, resuspended in 1 mℓ saline and counted.

6.6.2.2. Method 2

EDTA - Solution	
Chemicals	Final Concentration (mM)
NaCl	122
KCl	3
$Na_2HPO_4.2H_2O$	1
Glucose	4
Na_2EDTA	0,54
Tris (hydroxymethyl-aminomethane)	0,165
pH	7,6

Cells were treated as described in method 1.

6.6.2.3 Method 3

The tissue culture flask was rinsed out twice with EDTA solution. Viability test was performed as already described. 3 mℓ of trypsin solution was added to the tissue culture flask. The flask was incubated at 37°C with occasional agitation. After 15 minutes the action of the trypsin was stopped by the addition of culture medium containing fetal calf serum (10% v/v final concentration). The cells were spun down for 2 minutes at 747 g in a bench top centrifuge, resuspended in 1 mℓ saline and counted.

6.6.2.3.1 Trypsin Solution

0,01% (w/v) Trypsin (Sigma Type XI) was dissolved in the EDTA — solution used in Method 2.

6.6.3. Determination of Osmolality of Culture Media and Buffers

The osmolalities of culture media and buffers were determined with a Knaver Halb Micro Freezing Point Osmometer.

6.7 ANALYTICAL METHODS

6.7.1 DNA Determination

6.7.1.1 Method 1

DNA was determined according to the method of Burton (1956) using salmon testes Type III DNA (Sigma) as standard.
Range of standard curve: 0 - 100 µg DNA/mℓ

6.7.1.2. Method 2

DNA was determined according to the method of Ogur and Rosen (1950). Range of standard curve: 0 - 100 µg DNA/mℓ. This method is 3 times more sensitive than Method 1.

2 mℓ of ice-cold 0,5 N $HClO_4$ was added to the cell pellet and 30 minutes allowed for the nucleic acids and proteins to precipitate. The tube was centrifuged at 2986 g for 5 minutes, the supernatant removed and 2 mℓ of 0,3 N KOH added. The RNA was alkaline hydrolysed for 16 hours at 30°C in acid-cleaned glass centrifuge tubes. 2 mℓ of ice-cold 1 N $HClO_4$ (final concentration 0,5 N $HClO_4$) was added and 30 minutes allowed for the DNA to precipitate . The DNA pellet wash washed with 2 mℓ

ice-cold 0,5 N HCl_4 before being solubulized at 80°C for 15 minutes in 1 mℓ 0,5 N $HClO_4$ and the supernatant was read at 260 nm in a Unicam SP 1800 Ultraviolet Spectrophotometer.

6.7.2. Protein Determination

6.7.2.1. Method 1

Protein was determined according to the method of Lowry et al. (1951) using crystalline bovine serum albumin (BSA) (Sigma) as standard. Range of standard curve: 0 - 100 µg BSA/mℓ.

6.7.2.2. Method 2

The protein determination was done according to the methods of Butcher et al. (1976); Viets et al. (1979), modified by Peterson (1983).

6.7.2.2.1 Solutions

6.7.2.2.1.1 Stock OPA (o-Phthalaldehyde) Solution

1,2 mg OPA was dissolved in 0,2 mℓ methanol (analar-HPLC-grade). 10 µℓ 30% (v/v) Brij 35 was added and the solution made up to 10

mℓ with 1,0 M borate buffer, pH 10,4 (1,0 M boric acid titrated to pH 10,4 with 10 N KOH). This solution was stable for three weeks at room temperature.

6.7.2.2.1.2 Working Stock OPA Solution

At 30 minutes prior to use, 3 µℓ of 2-mercaptoethanol was added per 1 mℓ aliquot of the stock OPA solution.

6.7.2.2.1.3 Standard Solution

Crystalline bovine serum albumin was used as standard.
Range of standard curve: 0 - 5000 ng BSA/mℓ.

The tubes, as well as the 10 mℓ volumetric flasks used in this assay, were acid-cleaned and baked at 110°C for two hours before use. All the tubes were pre-read on the fluorometer and those registering more or less the same background reading were used.

50 µℓ of a 0,2% (w/v) SDS solution was added to 50 µℓ of nuclei suspension in 0,25 M sucrose buffer 0,1 mℓ of working stock OPA solution was added to the lysate. The contents of the tube were carefully mixed on a whirlly mix so as to avoid air bubble formation. 15 minutes later, 3 mℓ of a 0,5 N NaOH solution was

added, the tubes covered with parafilm and mixing was accomplished by inverting the tube twice. During this step all the air bubbles, which might have been present were removed. Fluorescence relative to a reagent blank was read on a Turner fluorometer. An excitation wave-length near 340 nm and an emission wave-length between 440 nm and 455 nm were used.

6.7.3 Gel Electrophoresis

Slab gels were made by clamping two glass plates (25,5 cm x 25 cm), held apart by teflon spacers (1 mm), together with wide fold back clips. The glass plates and spacers were sealed with 1% (w/v) agarose solution. A comb with eighteen wells (1,2 cm x 0,9 cm x 0,10 cm) was used.

The power supply used was a Shandon Vokam SAE 2761 power supply with a variable voltage (0 - 400 V) and current (0 - 80 mA) output.

6.7.3.1 SDS Polyacrylamide Gel Electrophoresis

SDS polyacrylamide gel electrophoresis (one dimensional) was performed according to the method of Panyim and Chalkley (1971). This electrophoresis was employed to identify acetylated and

phosphorylated histones and some non-histone proteins of rat liver nuclei, which were radio-actively labelled during the induction of hepatocytes with triamcinolone acetonide.

6.7.3.2 Stock Solutions

6.7.3.2.1 Stacking Gel

A 0,5 M Tris-HCl; pH 6,8
0,4% (w/v) SDS

B 29,2% acrylamide
0,8% (w/v) N,N^{1}—methylene bisacrylamide

C TEMED

D 10% (w/v) ammonium persulphate in H_2O (made up fresh)

6.7.3.2.2 Running Gel

A 1,5 M Tris — HCl; pH 8,8
0,4% (w/v) SDS

B 30% acrylamide
0,8% (w/v) N,N^{1}—methylene bisacrylamide

C TEMED

D 10% (w/v) ammonium persulphate in H_2O (made up fresh)

6.7.3.2.3 Running Buffer

0,025 M Tris – HCl; pH 8

0,192 M glycine

0,1% (w/v) SDS

6.7.3.2.4 Sample Application Buffer

0,0625 M Tris –HCl; pH 6,8

3% (w/v) SDS

10% (w/v) glycerol

0,01% (w/v) bromophenol blue

5% (v/v) β–mercaptoethanol (added just prior to use)

6.7.3.2.5 Staining Solution

0,1% (w/v) Coomassie Brilliant Blue R250

50% (v/v) methanol (technical)

10% (v/v) acetic acid (technical)

6.7.3.2.6 Destaining Solution

7% (v/v) acetic acid (technical)

25% (v/v) ethanol (technical)

6.7.3.3 Gel Preparation

The running gel consisted of 15% (w/v) acrylamide; 0,4% (w/v) N,N^{1}—methylene bisacrylamide; 0,375 M Tris-HCl, pH 8,8; 0,14% (v/v) TEMED and 0,05% (w/v) ammonium persulphate. The gel was poured leaving enough space (2 1/2 times the depth of the well) for a stacking gel. A straight interface was obtained by layering distilled water onto the gel solution. The gel was allowed about 20 minutes to set, before pouring the water layer off. The stacking gel solution 4,38% (w/v) acrylamide; 0,12% (w/v) N,N^{1} methylene bisacrylamide; 0,125 M Tris-HCl pH 8,8; 0,1% (v/v) TEMED and 0,05% (w/v) ammonium persulphate was poured and the comb inserted. The gel was left for 4 — 6 hours at room temperature before application of the samples.

All the empty wells were filled with an equivalent volume of sample application buffer in order to ensure uniform current distribution. Electrophoresis was carried out at 130 volts (constant) for 16 hours and the gel was cooled continuously by a fan. During that period the bromophenol blue marker was run right off the gel in order to obtain good separation of the histone bands.

6.7.3.4 Preparation of the Sample

The sample was solubulized in 30 µℓ application buffer, followed by a 3 minute incubation period in a boiling waterbath. The undissolved particles were removed by centrifugation and the supernatant applied to the gel with a 50 µℓ Hamilton syringe. Boiled, frozen samples were warmed up, clarified by centrifugation and applied to the gel.

6.7.3.5 Staining and Destaining of the gel

The gels were first stained in recycled staining solution for 1 hour at room temperature in order to remove SDS leeching out of the gel, followed by a 2 hour staining period in fresh staining solution.

Destaining of the gel was carried out at 37°C for a few hours with regular changes of destaining solution followed by destaining overnight at room temperature. By the next morning the gel background was sufficiently clear for the gel to be photographed.

6.7.3.6 Fluorography

Fluorography was done according to the method of Chamberlain (1979). The SDS polyacrylamide gels containing the ^{3}H-acetylated proteins were soaked for 30 minutes in several changes of deionized water in order to remove the methanol and acetic acid. The gel was transferred to 10 volumes of 1 M sodium salicylate, pH 5-7 at room temperature and was allowed to soak for 30 minutes before being laid on Photra C41 clear cronar film (0,1mm thick) with water wetted Whatman 3 M paper covering it.

Drying of the gel was achieved at about 70 — 80°C on a gel dryer under a vacuum (4 - 6 hours). The dried gel was exposed to 4 times preflashed Chronex 4 X-ray film in an X-ray cassette at -70°C. Exposure time was 1 month.

6.7.3.7 Autoradiography

The SDS polyacrylamide gels containing the ^{32}P-labelled proteins were dried the same way as the gels treated with sodium salicylate, but a shorter drying period was required (2 — 3 hours).

The dried gel was exposed to 4 times preflashed Chronex 4 X-ray

film in the presence of an intensifying screen in an X-ray cassette at -70°C. Exposure time varied from 4 — 7 days.

6.7.3.8 Densitometry

X-ray plates were scanned, at a speed of 3 cm/minute on a Vitatron densitometer in the negative logarithmic mode using a 0,25 mm diaphragm and a transparent U3 filter. The recorder speed was varied between 2 — 4 cm/minute.

6.7.3.9 Solubulization of SDS Polyacrylamide Gels

SDS polyacrylamide gels were solubulized according to a modification of the method of Jackson et al.(1975).

6.7.3.9.1 Wet Gel (Acetylation)

A surgical blade was used to cut the protein bands out. The histone bands, cut into small pieces, were solubulized in 0,5 mℓ 30% (v/v) H_2O_2. The bigger gel pieces, comprising all the non-histone bands, were solubulized in 4 mℓ of the above acid. Solubulization was performed in glass scintillation vials at 80°C for 2 hours in a waterbath. Scintillation counting was performed in 10 mℓ Insta Gel (Packard) scintillation fluid for 20 minutes.

6.7.3.9.2 Dry Gel

A pair of scissors was used to cut the protein bands out. The bands, cut into smaller pieces and pieces of the clear cronar film covering the gel were incubated under the same conditions as the wet gel. During this period, rehydration of the gel pieces occurred. Complete solubulization of the gel pieces were achieved by adding 0,5 mℓ and 3 mℓ of fresh 30% (v/v) H_2O_2 to the respective vials and continuing the incubation for a further 2 hours.

The vials were left with their caps off at 80°C in an oven for a few hours, allowing the excess H_2O_2 to evaporate.

Scintillation counting was performed in 10 mℓ scintillation fluid for 20 minutes.

6.7.3.10 Scintillation Counting

Scintillation counting was performed in a Packard Tricarb Liquid Scintillation Spectrometer model 3385.

6.7.3.10.1 Scintillation Fluids

I.

A. 25 g of SDS was dissolved in 220 mℓ H_2O

B. 25 g of PPO (2,5 — diphenyl oxazole) was dissolved in a small volume technical toluene

C. 1,5 kg of Triton X-100

The three solutions were mixed together and made up to 5ℓ with technical toluene. Counts were corrected to dpm by experimentally established quench factors.

This scintillation cocktail can accommodate 10% water and sucrose buffer.

II.

5 g of PPO were dissolved in 1ℓ technical toluene.

6.7.4 Photography

6.7.4.1 Operation on Rat

The operation on the rat was photographed with a Pentax camera fitted with a 50mm macro-lens. Ilford FP 4 (Asa 125) moderate film was used. Film was developed with Kodak 110 diluted (1 + 7) for 4 1/2 minutes at 20°C.

Negatives were printed on high contrast 4,1 M Ilfospeed paper.

6.7.4.2 Gels and X-ray Plates

Gels and the X-ray plates were placed on an opaque light box and photographed with a Pentax camera fitted with a 50 mm macrolens and a Vivitar red filter (25 A) for gels and Vivitar blue filter (25 A) for X-ray plates. Technical Pan film (black and white) was used.

Film was developed with Kodak HC 110 diluted (1 + 9) for 6 minutes at 20°C.

Negatives were printed on high contrast 3,1 M and 4,1 M Ilfospeed paper.

6.7.4.3 Microphotography

Microphotography was done on a Zeiss standard microscope fitted with a C35 camera body. Both bright field and phase contrast modes were used and a yellow-green filter was inserted in the base of the camera.

Technical Pan film (Asa 100, black and white) was used. The film was developed with Kodak HC 110 diluted (1 + 9) for 6 minutes at 20°C. Negatives were printed on high contrast 4,1 M Ilfospeed paper.

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