THE EFFECTS OF ALCOHOLS ON PINOCYTOSIS AND PROTEOLYSIS IN THE RAT YOLK SAC *IN VITRO*

by

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CONTENTS

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Dedication

This thesis is dedicated to the memory of John Taylor, who unfortunately did not see its completion.

Abbreviations

The abbreviations used throughout this thesis comply with the policy of the Biochemical Journal but in addition, the following are also used:

BzOH	Benzyl alcohol
c.p.m.	Counts per minute
E.I.	Endocytic Index
EtOH	Ethanol
FAS	Fetal Alcohol Syndrome
[^{125}I]fd BSA	Formaldehyde-treated ^{125}I-labelled bovine serum albumin
[^{125}I]PVP	^{125}I-labelled polyvinylpyrrolidone
r.p.m.	Revolutions per minute
TCA	Trichloroacetic acid
$\bar{x} \pm a$	Mean ± standard deviation

Abstract

This thesis investigates the effects of two alcohols, ethanol and benzyl alcohol, on the process of endocytosis in the rat visceral yolk sac *in vitro*. In Chapter 1, the process of endocytosis is introduced and the structure and function of the rat visceral yolk sac are described. The chapter is concluded by a review of the widespread effects of ethanol on cellular functions.

The data reported in the first experimental chapter indicate that ethanol is a rapid but readily reversible inhibitor of fluid-phase pinocytosis. The data are extended in Chapter 4 to include the effects of ethanol on adsorptive-phase pinocytosis and intralysosomal proteolysis. The latter chapter also introduces the Fetal Alcohol Syndrome and the role of the rat visceral yolk sac in the process of embryotrophic nutrition.

The effects of ethanol on pinocytosis at different gestational ages are reported in Chapter 5; the alcohol-induced inhibition of fluid-phase pinocytosis increases as the gestational age of the yolk sacs decreases from 19.5 to 13.5 days, while inhibition of adsorptive-phase pinocytosis is greater at 13.5 days than at 17.5 days of gestation.

The effects of benzyl alcohol (a preservative in a number of pharmaceutical formulations) on pinocytosis is reported in Chapter 7. The results indicate that the rat visceral yolk sac is more sensitive to the aromatic alcohol than the aliphatic alcohol. In Chapter 7, the effects of ethanol and benzyl alcohol on the endosomal compartment and the intracellular handling of endocytosed substrates are reported and discussed and a cellular mechanism of inhibition of pinocytosis by alcohols is suggested.

Finally, in Chapter 8, the possible biochemical mechanism(s) of action of ethanol are discussed together with ideas for extending the research.

CHAPTER ONE

GENERAL INTRODUCTION

1.1

The plasma membrane of the cell provides a semi-permeable limiting barrier which separates the cytoplasm from the surrounding environment. This helps the cell to maintain a constant micro-environment, to optimise cellular processes and to prevent entry of unwanted materials. Although certain low-molecular-weight, non-polar molecules (e.g. CO_2, O_2 and H_2O) are able to diffuse across the lipid bilayer which surrounds the cell, the passage of other compounds into the cell invariably requires active- or passive-transport processes involving membrane proteins. In recent years events surrounding the internalisation, processing and degradation of macromolecules and particles that enter the cell from the external environment have received considerable attention. This process is termed endocytosis.

1.2 Endocytosis

Endocytosis is the process whereby soluble, colloidal and particulate extracellular materials are internalised by the cell in membrane-bound vesicles derived from the plasmalemma. The process begins with an invagination of the plasma membrane to form a vesicle that is still in communication with the extracellular fluid. The process of vesiculation is completed by the plasma membrane "pinching off" to form a vesicle just below the cell surface. Any extracellular material in close proximity to the invaginating membrane can be captured within the vesicles formed.

Endocytosis has traditionally been divided into two categories: phagocytosis and pinocytosis. The first term is usually reserved for the ingestion of particulate material in a vesicle > 1 μm in diameter. Metchnikoff (1883) followed the ingestion of particles by leucocytes under the light microscope. This process enables macrophages to engulf invading micro-organisms as part of the host's defence system

and provides amoebae with food particles.

Pinocytosis differs from phagocytosis in two main ways. First, it involves the internalisation of extracellular fluid (not particles) and second the vesicles formed are 0.2 - 1.0 μm in diameter so are smaller than phagocytic vesicles.

Pinocytosis was first observed by Lewis (1931) who studied the formation of vesicles from the plasma membrane of rat macrophages under the light microscope (by phase-contrast techniques). With the introduction of the electron microscope, Palade (1953) showed that pinocytosis occurred at the sub-light microscope level. To differentiate between the two forms of pinocytosis, the gross movements of the cell membrane, that result in the larger fluid-filled vesicles seen under the light microscope, is termed macro-pinocytosis while the process that results in the formation of submicroscopic vesicles is termed micro-pinocytosis. Micro-pinocytosis has been demonstrated in virtually all mammalian cells except the mature erythrocyte. The diameter of vesicles formed via micro-pinocytosis is 70 - 110 nm. As well as being sub-divided on the basis of vesicle size, pinocytosis can also be sub-divided physicochemically into fluid-phase, adsorptive-phase and receptor-mediated endocytosis.

(A) Fluid-phase pinocytosis, as its name implies, is the internalisation of extracellular fluid within the pinocytic vesicle as it forms. Thus any solutes, present in the fluid and without any affinity for the plasma membrane, are taken up into the cell by this route. Their rate of uptake depends only upon their concentration in the extracellular fluid and also upon the rate of vesicle formation by the cell (Jacques, 1969; 1975; Williams et al., 1975a; Williams, 1981).

(B) Adsorptive-phase pinocytosis involves the interaction of the solute with the plasma-membrane surface by non-specific adsorption onto the surface of the cell (Williams et al., 1975b; Koolstra & Williams, 1981; Livesey & Williams, 1982). Thus the quantity of solute within the pinosome is increased and so is the rate of uptake by the cell. Jacques (1969; 1975) developed mathematical models, for both adsorptive-phase and mixed (fluid- and adsorptive-phase) pinocytosis, that permit the expression of the rate of uptake of extracellular material by these routes.

(C) Receptor-mediated endocytosis involves the substrate binding to a specific receptor molecule on the outer face of the plasma membrane (Willingham & Pastan, 1984). Such uptake is usually reserved for substrates with a specific fate other than degradation (Stahl & Schwartz, 1986).

In general, in receptor-mediated endocytosis once the macromolecular ligand binds to its specific cell-surface receptor (the involvement of calcium ions in ligand binding of certain substrates was reported by Eskild et al., 1986), the ligand-receptor complex moves to and clusters within specialised regions of the plasma membrane termed "coated pits" (Goldstein et al., 1979; 1985; Hollenberg, 1986). The coated pits contain the protein clathrin. They invaginate and pinch off to form coated vesicles which quickly lose their coat of clathrin and fuse with vesicular structures called endosomes (Dautry-Varsat, 1986). The peripheral/early endosome has vacuolar structures connected to tubular cisternae (Hopkins, 1986) with a low pH environment within its limiting membrane. Both clathrin-coated vesicles (Forgac et al., 1983; van Dyke et al., 1984) and endosomes (Wileman et al., 1985; Mellman et al., 1986) are thought to contain an electrogenic ATP-dependent proton pump. As well as coated-vesicles fusing with the endosome, non-coated vesicles

(containing substrates captured by fluid- and adsorptive-phase pinocytosis) are also thought to discharge their contents into the endosomal compartment (Helenius et al., 1983). In the perinuclear region of the cytoplasm the late/juxtanuclear endosome is located. This compartment has tubular cisternae which are frequently connected to multivesicular bodies.

The endosomal system is now believed to be the main site of receptor-ligand "sorting" and "routing" (Goldstein et al., 1985).

Route 1: receptor recycles but the ligand is degraded. This route is used in the uptake of LDL (low density lipoproteins), asialoglycoproteins, transcobalamin II, peptide hormones and α-2-macroglobulin. The ligands dissociate from their receptors within the endosome, apparently as a result of the decrease in pH. The ligand is then routed to the lysosomes where it is degraded. The receptor leaves the endosome, apparently by being incorporated into the membrane of vesicles that bud off from the endosome. These recycling vesicles may originate as tubular extensions of the endosome which accumulate receptors before pinching off.

Route 2: receptor and ligand both recycle. This pathway describes the route taken by transferrin (Dautry-Varsat, 1986) and Class I MHC (major histocompatibility) proteins in T-cells and Class II MHC proteins in macrophages. In the case of transferrin, iron dissociates from the protein, at acidic pH, in the endosome while the apo-transferrin remains attached to the receptor. The apo-transferrin:receptor complex appears to leave the endosome by a network of membrane tubules and vesicles that leads back to the cell surface. On the cell surface, at neutral pH, the apo-transferrin dissociates from the receptor.

Route 3: receptor and ligand both degraded. This pathway describes the uptake of EGF (epidermal growth factor) and immune complexes (Fc or C3b). Once the EGF:receptor-complex reaches the endosome the EGF dissociates from its receptor, at acidic pH, but both the ligand and receptor are subsequently degraded in the lysosomal system. In certain cells a small proportion of the EGF receptors may recycle back to the plasma membrane but the majority are degraded.

Route 4: receptor and ligand are transported across the cell. This pathway describes the route taken by polymeric IgA (immunoglobulin A) and IgM (immunoglobulin M) across epithelial surfaces, in liver cells (for secretion in bile) and mammary cells (for secretion in milk). In the hepatocyte, the receptor:IgA-complex enters the endosome from the sinusoidal surface. Then, during its transport (diacytosis) across the cell, the receptor is clipped proteolytically so that part of the receptor bound to the IgA is released into the bile canaliculi. In certain neonatal mammals, diacytosis of maternal IgG across the intestine is seen while in others late in gestation maternal IgG transfers across the yolk-sac epithelial cells to the fetus, mediated by a receptor that binds to the Fc domain of IgG.

Fusion of vesicles with lysosomes

How substrates are transferred to the lysosomal system is of current interest and may involve:

(1) Movement of the peripheral endosome to the perinuclear region of the cell (thus becoming a juxtanuclear endosome) before fusion with a primary/secondary lysosome.

Or, alternatively:

(2) By vesicles leaving the peripheral endosomes to fuse with the juxtanuclear endosomes, then either the juxtanuclear endosomes or vesicles from this compartment fuse with primary/secondary lysosomes.

Fate of materials within lysosomes

Once the substrates have entered the lysosomes, they encounter an acid pH environment which contains a wide variety of hydrolytic enzymes with acid pH optima. The contents of the resulting secondary lysosomes are degraded by the hydrolytic enzymes within the vacuole. As the lysosomal membrane is permeable to solutes with a molecular weight of approximately 200 - 240 daltons, free amino acids and possibly dipeptides produced by intralysosomal proteolysis can diffuse across the lysosomal membrane into the cytosol of the cell. However, this view may be an over-simplification, since a recent review of lysosomal-membrane permeability (Lloyd & Forster, 1986) reports evidence for specific membrane-transport systems for: cystine, lysine, arginine, histidine and ornithine and for sialic acid and cobalamin.

Primary lysosomes are believed to be formed in a specialised portion of the smooth endoplasmic reticulum (von Figura & Hasilik, 1986), which is adjacent to, but distinct from, the concave face of the Golgi complex. It is called GERL (Golgi-associated Endoplasmic Reticulum from which Lysosomes arise). Following numerous fusion events, a secondary lysosome will eventually lose the majority of its enzyme complement and such a vesicle is termed a "residual body" since it usually contains only indigestible material.

The lysosomal system plays a part in other cell functions not directly related to pinocytosis. The Golgi complex not only gives rise to primary lysosomes but also to vesicle-bound secretory products

which are directed to the surface of the cell where the vesicles fuse with the plasma membrane by a process called exocytosis which enables the secretory products to be released into the extracellular environment. However, if the cell produces an excess of secretory products, the secretory vesicles fuse with lysosomes before they reach the plasma membrane, resulting in the catabolism of the material. This process is termed "crinophagy". In secretory cells, pinocytosis may be the mechanism for the recovery of excess plasma membrane after secretory vesicles have discharged their contents. The process of "autophagy" also involves the vacuolar system. The function of autophagy is the turnover of intracellular organelles and proteins and requires regions of cytoplasm to become surrounded by membrane (probably of endoplasmic-reticulum origin) to form autophagic vacuoles which fuse with the lysosomal system. Grinde (1985) reviewed autophagy and lysosomal proteolysis in the liver and discussed mechanisms, requirements and regulation. The possibility that the lysosomal system may be involved in endogenous cell-protein turnover has been discussed by several authors (Dean, 1975a,b; Lloyd, 1976; Ballard, 1977; Dean, 1978; Grinde, 1985) but only Ballard discusses the possibility of a dual pathway of cell-protein catabolism.

1.3 Factors regulating the rate of pinocytosis

The polypeptide, EGF (epidermal growth factor) at a concentration of 5 ng/ml causes a threefold increase in the rate of [^{125}I]PVP uptake by human fibroblasts *in vitro* by a signal generated at the cell surface (Wiley & Cunningham, 1982). Similarly, the rate of fluid-phase pinocytosis (as measured by [^{14}C]sucrose uptake) in 3T3-L1 adipocytes *in vitro* was stimulated twofold, over a 2.0 h period, by the addition of insulin (50 nM) (Gibbs *et al.*, 1986). However, in rat-brain capillary endothelium, cryogenic injury not only stimulates ornithine decarboxylase activity and polyamine synthesis,

but also polyamine-mediated microvillus formation and endocytosis (Trout et al., 1986). In mouse J774.2 macrophages and phorbol ester-stimulated peritoneal macrophages, a structural modification of the "tubular lysosomal network" appears to accompany increased rates of pinocytic uptake of Lucifer Yellow, suggesting that elevated rates of solute accumulation accompany macrophage stimulation in vitro (Swanson et al., 1987).

In the 17.5-day rat visceral yolk sac in vitro, concentrations of trypan blue in the incubation medium that are below 100 μg/ml stimulate while concentrations above 200 μg/ml depress the rate of uptake of [^{125}I]PVP (Williams & Lloyd, 1973). However, this biphasic effect was later reported to arise from an interaction between trypan blue (which is an adsorptive substrate) and [^{125}I]PVP (normally a fluid-phase marker); this enables the latter substrate to enter the endodermal cells adsorptively by so-called "piggy-back" pinocytosis (Roberts et al., 1980). Once the temperature is decreased below 37^{o}C, the rate of pinocytic uptake of [^{125}I]PVP by 17.5-day rat yolk sacs decreases sharply; at 4^{o}C uptake is completely inhibited (Duncan & Lloyd, 1978). Pinocytosis also appears to be dependent on the presence of extracellular calcium and to be inhibited by the presence of microtubular or metabolic inhibitors (Duncan & Lloyd, 1978; Starling et al., 1983).

The process of fluid-phase pinocytosis was also strongly inhibited by glucagon (10 μM) in the culture medium (Brown & Segal, 1977). Cyclic-AMP levels in the yolk sacs were elevated and dibutyryl cyclic-AMP could replace glucagon as an effective inhibitor of fluid-phase pinocytosis. This is of interest since parathyroid hormone (0.12 μM) causes maximal stimulation of cyclic-AMP production in the rat visceral yolk sac (Gügi et al., 1986). However, insulin (1 μM) had no effect on the yolk-sac uptake either of invertase

(Brown & Segal, 1977) or of [^{125}I]PVP (Forster, 1982).

It was reported (Baenziger & Fiete, 1982) that hepatocytes cultured *in vitro* in a buffer devoid of Na^+ but containing 150 mM K^+ could not degrade [^{125}I]asialo-orosomucoid. A similar result was obtained by Clark (1986) when 17.5-day yolk sacs were incubated in a low [Na^+] : high [K^+] buffer in the presence of [^{125}I]fd BSA. Not only do low [Na^+]/[K^+] ratios inhibit the uptake and degradation of [^{125}I]fd BSA but ammonium, methylammonium, ethylammonium ions (20 mM) and chloroquine (500 μM) all inhibit both the uptake and the degradation of protein substrates in the rat visceral yolk sac. This inhibition does not necessarily relate to any increase in the lysosomal pH, since ammonium ion-induced inhibition is also accompanied by a decrease in the cellular [ATP]/[ADP] ratio (Livesey *et al.*, 1980).

The rate of fluid-phase pinocytosis is directly dependent upon the rate of vesicle formation. However, adsorptive-phase and receptor-mediated pinocytosis are not only dependent on this factor but also on both the type and number of binding sites/receptors on the cell surface and on the physicochemical characteristics of the ligand/substrate. Subtle changes in the conformation of a protein result in different rates of internalisation (Moore *et al.*, 1977; Agarwal & Moore, 1979). The composition of the incubation medium has also been shown to affect the rate of uptake of the substrate (Ibbotson & Williams, 1979; Forster & Williams, 1984).

1.4 The rat visceral yolk sac

The visceral yolk sac is not the only extraembryonic membrane present in the rat conceptus. The first membrane that surrounds the embryo is the amnion, then the visceral yolk sac; next is the parietal yolk sac which lines the inner surface of Reichert's membrane. However, after day 16 of gestation, the parietal yolk sac and

Reichert's membrane are no longer present. Over its capsular surface (the side furthest from the placental cap) the visceral yolk sac is smooth and lies adjacent to the uterine epithelial cells. However, the placental surface of the yolk sac is very folded with many macrovilli. The outer surface of the visceral yolk sac comprises a layer of columnar epithelial cells. These cells have a microvillous structure to their plasma membrane and are bathed in uterine fluid. The cells are tightly joined together at their junction thus preventing the passage of material between them. It is this layer of cells that is pinocytically active. The endodermal cells are attached to a fibrous basement-membrane below which is a layer of mesenchyme, containing the vitelline blood capillaries, collagen fibres and fibroblasts and macrophages.

The 17.5-day rat visceral yolk sac has already been used to investigate the rates of uptake of a diverse range of substrates and the various factors that control the rate of pinocytosis. Thus, the effects of removal of serum from the incubation medium (Ibbotson & Williams, 1979) and the presence of proteolytic inhibitors (Livesey *et al.*, 1980; Clark, 1986) have been investigated. Also, the uptake of a number of simple proteins (Livesey & Williams, 1981; Livesey & Williams, 1982) and immunoglobulins (Weisbecker, 1981; Seymour, 1985) have also been investigated. The effects of chemical denaturation and of galactose moieties (Moore *et al.*, 1977), of subtle changes in the chemical nature of a simple protein and of changes in the composition of the incubation medium (Forster, 1982) have all been studied.

Other groups of workers have looked at the uptake of vitamin B_{12} and vitamin B_{12}:intrinsic factor complex by the rat visceral yolk sac *in vitro* (Padykula *et al.*, 1966). McArdle & Priscott (1984) looked at the uptake and metabolism of transferrin in the rat conceptus at 9.5 days of gestation and in the rat visceral

yolk sac at 10.5 days of gestation *in vitro* and concluded that the uptake of transferrin was via receptor-mediated endocytosis. This work was further extended by Huxham & Beck (1985) who looked at the uptake of maternal transferrin and its transfer across the visceral yolk sac in 9.5 to 11.5-day rat conceptus *in vitro* and by Thiriot-Hebert (1987) who studied the uptake of transferrin by the rat visceral yolk sac and its materno-fetal transfer *in vivo* on day 15 of gestation. Both concluded that, like maternal IgG, transferrin uptake is followed by diacytosis of the protein across the yolk sac and its secretion into the fetal circulation. These results support the earlier observations of Priscott *et al.* (1983) that 9.5-day rat embryos cultured in rat serum selectively depleted the concentrations of four rat-serum proteins in the medium, these being: α_2-macroglobulin, transferrin, ceruloplasmin and another protein of molecular weight 214,000.

Rat conceptuses cultured from 9.5 to 11.5-days of gestation *in vitro* in 100% rat serum or 100% human serum were found to transport eighteen serum proteins from the extracellular medium across the visceral yolk sac into the exocoelomic fluid (Huxham & Beck, 1984). The same authors also reported that some of the proteins present in the exocoelomic fluid arose from protein synthesis within the conceptus. This was in accord with the findings of Rhinehardt *et al.* (1984) who reported that α-fetoprotein, synthesised in the visceral yolk sac of 9.5 to 11.5-day rat conceptuses, was also located in the visceral yolk-sac blood vessels, exocoelomic-cavity fluid and amniotic-sac fluid. Likewise, Liao *et al.* (1980) reported that the rat visceral yolk sac, not the fetal liver, was the main site of α-fetoprotein synthesis. Similarly, the synthesis and secretion of retinol-binding protein and transthyretin by the rat visceral yolk sac has also been reported (Soprano *et al.*, 1986; Sklan & Ross, 1987)

while Elshourbagy et al. (1985a & b) reported that the visceral yolk sac synthesises apolipoprotein E, A-IV and A-1 and Muglia & Locker (1984) reported that the rat visceral yolk sac was the site of synthesis of the insulin found in the fetal circulation.

Thus the rat visceral yolk sac seems to have a wide variety of anabolic functions as well as the catabolic role in uptake and intralysosomal proteolysis of exogenous proteins found in the extracellular fluid.

1.5 Ethanol effects on cellular functions

Reviews of the metabolic, behavioural and biomedical significance of ethanol are numerous (Kricka & Clark, 1979; Blum et al., 1980; Gadeholt et al., 1980; Reitz & Schilling, 1980; Tabakoff & Hoffman, 1980; Shaper et al., 1985).

Chiu et al. (1983) implicated the release of lysosomal enzymes as the cause of ethanol-induced gastric lesions in rats, while Skrzydlewski et al. (1985) reported the release of acid-proteinase activity from lysosomes and the subsequent degradation of protein in organs of rats intoxicated with ethanol. Furthermore, Sewell et al. (1986) found that in the perfused rat liver, ethanol (2 - 20 mM) markedly inhibited lysosomal enzyme release into the bile. It was also reported that ethanol ingestion alters the proton-pump activity in the rat liver, by causing a decrease in the pump activity in the lysosomes, but increases the activity in the plasma membranes (Schneider & Manara, 1985).

Maternal ethanol consumption was found to decrease maternal-, fetal- and neonatal-rat hepatic-protein synthesis (Rawat, 1976) while in rat liver in vitro Ohtake et al. (1986) reported that acute ethanol administration inhibited albumin synthesis, but that chronic ethanol administration had the opposite effect. The first finding is supported by the work of Girbés (1986) which indicates that acute

ethanol treatment of hepatocytes *in vitro* causes a pH-dependent inhibition of protein synthesis. Although acute ethanol treatment seems to inhibit protein synthesis in the liver, chronic ethanol feeding in the rat increases the synthesis of hepatic cholesterol and bile acids *in vivo* (Maruyama *et al.*, 1986).

The effects of ethanol intoxication on the endocrine system have been reviewed by Crabbe & Rigter (1980) since alcoholism has been associated with a hypermetabolic state and hypoglycaemia (Salaspuro *et al.*, 1977) and with: diabetes mellitus, ketoacidosis, lactic acidosis, hyperuricaemia, isolated ACTH deficiency, glucocorticoid excess, disturbed water and electrolyte metabolism, hypocalcaemia and magnesium depletion (Isselbacher, 1977) and androgen deficiency and feminisation (Anderson, 1978). Maternal alcoholism has also been associated with adverse effects on fetal development (the related literature is fully reviewed in Chapter 4).

Thus, the diverse effects of ethanol on metabolism, hormone balance and developmental abnormalities along with the known effects of alcohols on cell membranes (see Sections 3.1 & 6.1) make the identification of the primary effects of ethanol in different ethanol-induced disease states a formidable task.

The adverse effects of ethanol for which successful hypotheses of pathogenesis are most likely to be formulated are those for which:

1) A target tissue can be identified.
2) The essential function(s) of the tissue can be identified and characterised.
3) The function and its disturbance can be investigated meaningfully in an *in vitro* system.

4) Ethanol can be demonstrated to induce a specific and readily-evaluated disturbance of this function.

5) Clear-cut effects of ethanol are induced at concentrations of ethanol that fall within the range likely to obtain *in vivo*.

The hypothesis that ethanol may act to disrupt development in the rodent embryo, by disturbing yolk-sac function, coupled with the applicability of the first three of the above criteria, makes this hypothesis interesting to investigate experimentally. The investigation has been subdivided into five sub-areas for experimental convenience:

(1) The effects of ethanol on fluid-phase pinocytosis in the 17.5-day yolk sac.

(2) The effects of ethanol on adsorptive-phase pinocytosis in the 17.5-day yolk sac in relation to the possible disruption of embryotrophic nutrition.

(3) The effect of decreasing the gestational age of the yolk sac on ethanol-induced dysfunction.

(4) The effects of benzyl alcohol on fluid-phase and adsorptive-phase pinocytosis in the 17.5-day yolk sac.

(5) The induction, by ethanol and benzyl alcohol, of release of both fluid-phase and adsorptive-phase pinocytosis markers from the 17.5-day yolk sac.

It was hoped that these investigations would reveal whether alcohols, in particular ethanol, are likely to disrupt yolk-sac function, an action that may relate to the mechanism(s) of action of ethanol as a teratogen in humans and experimental animals.

CHAPTER TWO

MATERIALS AND METHODS

I MATERIALS

2.1.1 Equipment

Gamma Counters:	5124 Selektronic Gama Spectrometer & Modumatic 6 Gama Spectrometer, Packard Instrument Ltd., Caversham, Berks., U.K., LKB-Wallac 1282 Compugamma, Turku, Finland.
Centrifuge:	Mistral 4L, M.S.E., U.K.
Spectrophotometer:	CE 373 Linear Reading Grating Spectrophotometer, Cecil Instruments Ltd., Cambridge, U.K.
Fluorimeter:	Fluorescence Spectrometer Model 3000, Perkin-Elmer Ltd., Beconsfield, Bucks., U.K.
LP3 tubes:	L.I.P., Shipley, W. Yorkshire, U.K.
Waterbaths:	Type SS 30, Grant Instruments, Barrington, Cambs., U.K., Thermal Laboratory Equipment, A. Searle Co., Oldham, U.K.
Homogenizer:	Potter-Elvehjem type, Tri-R Instruments, Rockville Instruments, N.Y., U.S.A.

2.1.2 Incubation Requirements

Medium 199: with Earl's Salts, with L-glutamine and containing Penicillin-Streptomycin, Gibco Europe Ltd., Uxbridge, Middlesex, U.K

Calf Serum: Calf serum 1, Heat Inactivated (CS 07), Wellcome Reagents Ltd., Dartford, Kent, U.K., Heat-Inactivated Foetal Calf Serum (013-6290 H), Gibco Europe Ltd., Uxbridge, Middlesex, U.K.

Gas: O_2 95%/CO_2 5%, British Oxygen Co. Ltd., Medical Gases Division, London, U.K.

2.1.3 Chemicals

Na[^{125}I]: Supplied as Sodium Iodide in dilute NaOH solution, pH 7-11, free from reducing agents (preparation IMS.30), Amersham International Ltd., Amersham, Bucks., U.K.

[^{125}I]PVP: Supplied in succinate buffer solution (preparation IM.33P), Amersham International Ltd., Amersham, Bucks., U.K.

BSA: Bovine Serum Albumin, Crystallised and Lyophilized (preparation A-7638),

NAD: β-Nicotinamide adenine dinucleotide (N 7004), and

NADH: β-Nicotinamide adenine dinucleotide, reduced form (N 8129), all products of Sigma Chemical Co. Ltd., London, U.K.

Absolute Ethanol: Spectrograde (preparation E/0665), Fisons P.L.C., Loughborough, Leics., U.K.

Benzyl Alcohol: GPR(27354 4R), BDH Chemicals Ltd., Poole, U.K.

4-methyl-umbelliferyl 2-acetamido-2-deoxy β-D-glucopyranoside: Preparation (4048-00), and

4-methylumbelliferone: Preparation (4047-00), Koch-Light Ltd., Hollands Road, Haverhill, Suffolk, U.K.

II METHODS

2.2. Radiolabelling of Protein Substrates

2.2.1 Preparation of formaldehyde-treated [^{125}I]BSA

The methodology used for the radioiodination of bovine serum albumin was adapted from the method of Bocci (1969), which was in turn based on the original method of Hunter & Greenwood (1962). Protein (20 mg) was dissolved in 5 ml phosphate buffer (0.05 M Na_2HPO_4-KH_2PO_4, pH 8.0) and placed on ice whilst being continually stirred. A solution of Na[^{125}I] (10 µl; 1.0 mCi at reference date) was added via a Hamilton syringe and the mixture stirred for 2 min. [Before the reaction was started, the Chloramine-T and sodium metabisulphite solutions were checked for reactivity. To a test tube containing approximately 2 ml aqueous KI (2%, w/v) and 1 ml of chloroform were added a few drops of the chloramine-T solution. On shaking, the liberated iodine turned the dilute KI solution yellow and the chloroform pink. The subsequent additions of sodium metabisulphite solution led to both liquids being decolourised due to the reduction of iodine to KI.]

After 5.0 ml of chloramine-T solution (250 µg/ml) had been added, the reaction mixture was stirred for a further 8 min before the reaction was terminated by the addition of 5 ml of sodium metabisulphite solution (250 µg/ml). Stirring was continued for a further 2 min when approximately 100 mg of "cold" KI was introduced into the reaction mixture to displace and hence facilitate the removal of the remaining free Na[^{125}I]. The crude reaction mixture was added to an equal volume (15 ml) of 10% (w/v) solution of formaldehyde in 0.5 M $NaHCO_3$ buffer, pH 10, and kept at 4^{o}C for a minimum of 72 h. The solution was then transferred into Visking tubing (0.25" diameter) and dialysed at 4^{o}C against frequent changes of 5 l NaCl (1%, w/v) for

48 h. The ^{125}I-labelled protein solution was then removed from the tubing and stored at -20^{0}C. The labelling efficiency of formaldehyde-treated ^{125}I-labelled BSA was approximately 60 - 65%. [After the iodination procedure was complete, the reaction vessel was washed out with a little distilled water containing 10% (v/v) calf serum and the total- and acid-soluble radioactivity determined (see Section 2.4.2) in order to estimate the labelling efficiency of the protein and the quantity of unreacted Na[^{125}I] discarded.] The exact concentration of ^{125}I-labelled protein was measured spectrophotometrically via the A_{280} values using native BSA solutions (0 - 1 mg in 1%, w/v NaCl).

2.3 Incubation of Rat Visceral Yolk Sacs

The method used was that of Williams et al. (1975a; 1975b) with slight modifications. Female Wistar rats from an inbred colony were caged separately in grid cages and left overnight with a stud male. The detection of a sperm plug underneath the cage the next morning was taken as an indication of pregnancy. Mating was assumed to have taken place at midnight (day 0). After 13.5, 15.5, 17.5 and 19.5 days, the rats were killed by CO_2 asphyxiation and the uterus removed as quickly as possible and placed in a Petri dish containing medium 199 (that had been previously gassed with 95% O_2 : 5% CO_2 mixture) at 37^{0}C. The uterine wall was opened via a longitudinal cut and the conceptuses gently freed from the wall. The conceptuses were removed and placed in a Petri dish containing fresh, warm (37^{0}C) medium; the placental caps were removed and the cut edge of the visceral yolk sacs enlarged by a single cut to permit removal of the fetus. Finally, the yolk sacs were placed in a Petri dish containing fresh, warm medium, and any remaining amniotic membrane was dissected away. The whole of the dissection process (from removing the uterus to the removal of the amnion) was completed within 15 min to avoid tissue death through anoxia. When dissecting yolk sacs at 13.5 and 15.5 days of gestation, it was also necessary to remove the parietal yolk sac and Reichert's membrane.

Yolk sacs were individually placed into sterile 50 ml Erlenmeyer flasks which contained 9.0 ml of medium 199 (either alone or containing heat-inactivated calf/fetal-calf serum), that had been previously gassed with a mixture of 95% O_2 : 5% CO_2 for 10 sec and sealed with a sterile silicone rubber bung. The flasks were incubated in a water bath set at 37 ± 0.4^{0}C and shaken (stroke length 3.6 cm) at a frequency of 85 ± 5 strokes per min. Following a pre-incubation period of 15 - 45 min, the ^{125}I-labelled substrate, dissolved in

1.0 ml of medium 199, was added to each flask. The flasks were then regassed (95% O_2 : 5% CO_2) and stoppered.

However, when the effect of some compound on the pinocytosis of radiolabelled substrate was investigated, the flasks initially contained only 8.0 ml of medium. After gassing and a pre-incubation period of at least 15 min, the radiolabelled substrate was added to each flask as a solution in 1.0 ml of medium and the flasks regassed and stoppered. After a further period of time (usually 1.0 h) the agent was added (in 1.0 ml of medium) to each flask (the control simply had 1.0 ml of medium only added to each flask); the flasks were then regassed and stoppered. Alternatively, after the pre-incubation period of 15 min, the compound was added as a solution in 1.0 ml of medium quickly followed by the radiolabelled substrate (likewise added in 1.0 ml of medium) and the flasks regassed and stoppered.

Yolk sacs were removed at different time intervals during 5.0 h incubation periods and washed three times for 2 min in approximately 30 ml of 1% (w/v) NaCl at 4°C to remove any externally-bound substrate. The yolk sacs were stored in 5.0 ml volumetric flasks at -20°C until assayed. For the non-degradable radiolabelled substrate, [^{125}I]PVP, duplicate 1.0 ml samples of medium were pipetted into disposable 3 ml (LP3) tubes which were stoppered and stored at 4°C until assayed. However, for degradable radiolabelled substrates (^{125}I-labelled proteins) the duplicate medium samples were stored at -20°C until assayed.

2.4 Assay of Incubation Medium and Yolk-Sac Tissue

2.4.1 Assay of medium containing non-degradable substrates (e.g. [^{125}I]PVP)

Duplicate 1.0 ml samples of incubation medium containing the radiolabelled substrate were assayed for radioactivity in a gamma spectrometer using a constant geometrical relation of sample to detector.

2.4.2 Assay of medium containing degradable substrate (e.g. ^{125}I-labelled protein)

Duplicate 1.0 ml samples of incubation medium were assayed for their total radioactivity content as in Section 2.4.1 before 0.1 ml of calf serum was added to each sample tube. (If serum was present in the incubation medium then the above stage was missed out.) This acted as carrier protein in the precipitation procedure that follows. Next, 0.5 ml of 20% (w/v) trichloroacetic acid (TCA) was added to each tube and the contents of each tube were mixed thoroughly before being centrifuged for 30 min at 3,000 r.p.m. The supernatant from each tube was decanted into fresh tubes and then assayed for TCA-soluble radioactivity. However, as the total volume of the supernatant had become greater than 1.0 ml, the observed radioactivity had to be normalised. The reasons for this are: (i) loss of acid-soluble radioactivity occluded within the protein pellet after centrifugation, (ii) change in counting efficiency resulting from the increased sample volume and (iii) to normalise for the volume differences arising according to whether the original medium contained calf serum (10%, v/v) or serum was only added just prior to precipitation.

The radioactivity associated with the yolk-sac tissue was assayed by the procedure described in Section 2.4.1.

2.4.3 Assay of protein content of yolk-sac tissue

To each yolk sac in a 5 ml volumetric flask was added NaOH (1.0 M), and the flasks were made up to the mark. The yolk sacs were dissolved at 37°C over a 1.5 h period with the flasks being agitated at 0.5 and 1.0 h. Duplicate 1.0 ml samples of yolk-sac solutions were pipetted into 3.0 ml tubes (LP3) and assayed for radioactivity (as in Section 2.4.1). To obtain the protein content of each yolk-sac solution the method of Lowry et al. (1951) was used with bovine serum albumin as the reference protein. Duplicate 0.1 ml samples from each flask were made up to 1.0 ml by the addition of 0.4 ml 1.0 M-NaOH and 0.5 ml of distilled water. At the same time a calibration curve was constructed by assaying, in triplicate, a series of solutions containing

(1) 0.00, 0.05, 0.10, 0.15, 0.20, 0.25 (for 19.5-day yolk sacs)

(2) 0.00, 0.05, 0.10, 0.15, 0.20 (for 17.5- and 15.5-day yolk sacs)

(3) 0.000, 0.005, 0.010, 0.015, 0.020 (for 13.5-day yolk sacs) mg of BSA in 0.5 ml of distilled water and 0.5 ml of 1.0 M-NaOH.

To each solution was added 5 ml of Folin A solution (100 ml of 2% anhydrous Na_2CO_3, 1 ml of 1% $CuSO_4$, 1 ml of 2% potassium sodium tartrate). After at least 20 min, 0.5 ml of Folin B solution was added. (The Folin Ciocalteau's reagent was freshly diluted with an equal volume of distilled water.) After the addition of Folin B solution to each test tube, the contents were immediately mixed with a vortex-mixer. At least 45 min later (the developed colour is stable for up to 24 h) the absorbance of each solution was read against a water blank at 750 nm. The protein content (mg) of each sample was determined from the calibration curve and the value multiplied by 50 to give the total yolk-sac protein.

2.5 Determination of Acid-Soluble Radioactivity Correction Factors

As mentioned earlier (Section 2.4.2), precipitation of medium samples (1.0 ml), with 20% (w/v) TCA followed by centrifugation, results in the volume of the supernatant increasing to approximately 1.2 - 1.3 ml. To permit meaningful comparison of the observed radioactivity, a correction factor was determined that converted the observed count of the supernatant to the value that would have been obtained if the same amount of radioactivity had been assayed in 1.0 ml of solution.

Williams et al. (1975b) showed that the major degradation product following [^{125}I]fd BSA uptake into yolk-sac tissue was [^{125}I]-L-tyrosine. This compound was, therefore, used as the marker in establishing correction factors. Five samples (1.0 ml) of medium 199 containing [^{125}I]-L-tyrosine (with or without 10%, v/v calf serum) were assayed for radioactivity (see Section 2.4.1) in each of the gamma counters used. To those tubes containing no calf serum, 0.1 ml of calf serum was added. The protein in all the tubes was then precipitated by the addition of 0.5 ml (20%, w/v) TCA. All the tubes were centrifuged for 30 min at 3,000 r.p.m. (see Section 2.4.2) and the supernatants decanted and reassayed for radioactivity as in Section 2.4.1. It was assumed that no [^{125}I]-L-tyrosine was precipitated by the 20% (w/v) TCA and that the volume of fluid entrapped in the pellet was constant.

The correction factors were determined by the following expression:

$$\text{Correction Factor} = \frac{\text{Total counts (c.p.m., corrected for background)}}{\text{Observed acid-soluble counts (c.p.m., corrected for background)}}$$

The correction factors obtained are given in Table 2.5.1.

Table 2.5.1 Acid-soluble radioactivity correction factors

The correction factors were determined as described in Section 2.5. The values given are the means (± S.D.) of five determinations.

Gamma Counter	Medium containing 10% (v/v) calf serum	Calf-serum (0.1 ml) added prior to TCA precipitation
5142 Selektronic	1.35 ± 0.07	1.37 ± 0.05
Modumatic 6	1.25 ± 0.04	1.27 ± 0.06
LKB-Wallac 1282	1.33 ± 0.02	1.35 ± 0.03

2.6 Calculation of the Rate of Uptake of a Non-degradable Substrate

The non-degradable substrate used in this investigation was [^{125}I]PVP. To permit expression of the experimental results in a reproducible form, uptake (or more strictly net accumulation) of [^{125}I]PVP by the tissue was expressed in the manner described by Williams *et al.* (1975a). Uptake of a substrate was expressed as the volume of incubation medium whose contained substrate was captured by unit quantity of yolk-sac tissue (in microlitres per milligram of yolk-sac protein). In the case of a non-degradable substrate, the equation for the determination of its uptake is:

$$U = \frac{Y}{M.P} \quad \mu l/mg \text{ yolk-sac protein}$$

U = uptake (µl/mg yolk-sac protein)

Y = total radioactivity in yolk sac (c.p.m., corrected for background)

M = radioactivity per µl of incubation medium (c.p.m., corrected for background)

P = protein content of yolk sac in mg

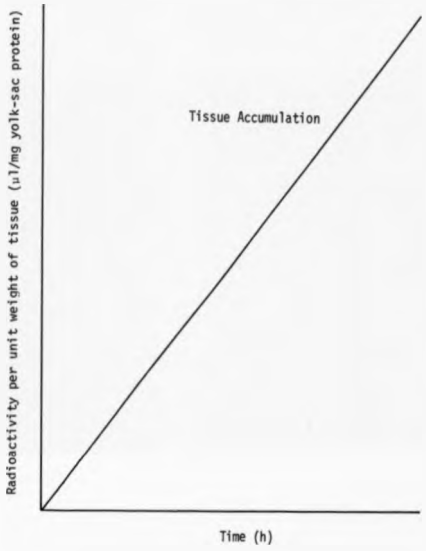
In the case of [^{125}I]PVP, the rate of decrease in the substrate concentration in the incubation medium (M) was approximately 0.1% per h (Williams *et al.*, 1975a), thus the concentration was effectively constant during the incubation period. Uptake values were calculated by a simple program (see Appendix II). Values of substrate uptake, so calculated, can be plotted against the period of incubation (h). If the resulting plot is linear, the rate of substrate uptake can be obtained from the gradient of the plot. This quantity is termed the Endocytic Index (E.I.). Each plot produced was inspected visually to

confirm linearity. However, the correlation coefficient, the intercept and the gradient (E.I.) for the best straight line fit were calculated using the appropriate statistical calculations.

See Figure 2.6.1 for a representative sketch obtained by the above calculations.

For substrates taken up entirely by fluid-phase pinocytosis, the concept of Endocytic Index is quite straightforward. However, for substrates that enter by adsorptive pinocytosis, the value of Endocytic Index will be greater than the actual rate of fluid uptake by the tissue. (Perhaps in these cases a less confusing unit of uptake would be nanogram (ng) substrate per mg of tissue protein per h; however this unit is dependent on the precise concentration of the substrate in the medium, so that each form of expression has its different merits and limitations.)

Fig. 2.6.1 Tissue accumulation of a non-degradable substrate



A radiolabelled substrate is taken up into yolk-sac tissue at a rate characteristic of the individual substrate. Upon reaching the lysosomal system, a non-degradable substrate is stored so that with time there is a linear increase in the quantity of radioactivity associated with the tissue.

2.7 Calculation of the Rate of Uptake of a Degradable Substrate

When ^{125}I-labelled proteins are used as substrates, the majority of the radiolabel taken up into the tissue is not retained within the lysosomal system of the yolk-sac tissue, but is released back into the incubation medium as acid-soluble degradation products (Williams et al., 1975b; Ibbotson & Williams, 1979; Livesey & Williams, 1979). Such substrates are also taken up at rates higher than that of [^{125}I]PVP. This results in a significant decrease in the amount of acid-insoluble radioactivity present in the medium during the incubation period. Both factors need to be taken into consideration when calculating the uptake of such substrates. The expression used in Section 2.6 thus has to be modified to:

$$U = \frac{Y + 10\,(S - F)}{M'.P} \quad \mu l/mg \text{ yolk-sac protein}$$

where U = uptake (µl/mg yolk-sac protein)

Y = total radioactivity retained in yolk-sac tissue (c.p.m., corrected for background)

S = acid-soluble radioactivity per ml of medium at end of incubation period (c.p.m., corrected for background)

F = acid-soluble radioactivity per ml of medium present at the beginning of incubation (c.p.m., corrected for background)

P = protein content of yolk sac (mg)

M' = mean acid-insoluble radioactivity per µl medium over the particular incubation period of an individual yolk sac (c.p.m., corrected for background)

The value of M' was calculated from M,

$$M' = M + \frac{(S - F)}{2 \times 10^3}$$

where M = acid-insoluble radioactivity per µl of medium at the end of the incubation period (c.p.m., corrected for background)

The values of S and F were calculated using the correction factors determined to correct for: i) the loss of acid-soluble radioactivity occluded within the protein pellet after precipitation, and ii) the change in counting efficiency from the increases in supernatant volume (see Section 2.5). The value of S was obtained from:

$$S = C.E$$

where S = acid-soluble radioactivity per ml of medium at the end of the incubation period (c.p.m., corrected for background)

C = measured acid-soluble radioactivity in whole medium sample at end of incubation period (c.p.m., corrected for background)

E = the appropriate correction factor

The value of F was calculated as:

$$F = D.E$$

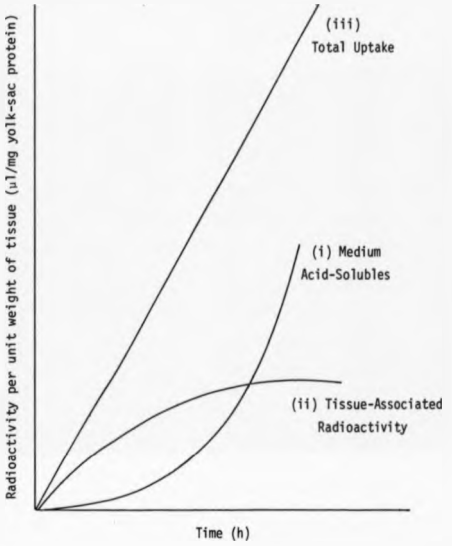
where F = acid-soluble radioactivity per ml of medium at the beginning of incubation, due to incomplete dialysis or deterioration during storage (c.p.m., corrected for background)

D = acid-soluble radioactivity in whole medium sample from control flask (c.p.m., corrected for background)

E = the appropriate correction factor

Results were processed by using a simple computer program shown in Appendix III. The net rate of uptake (E.I.) of the substrate was determined as in Section 2.6; its units are µl/mg yolk-sac protein per h. The quantity of tissue-associated radioactivity (T.A.R.) was calculated in the same units as uptake (see Section 2.6) and the values plotted against incubation period (h) to produce the characteristic form (see Figure 2.6.2).

Fig. 2.6.2 Time courses of tissue accumulation and of substrate degradation for a ^{125}I-labelled protein



Total Uptake = Medium Acid-Solubles + Tissue-Associated Radioactivity

(iii) (i) (ii)

When radiolabelled [^{125}I]fd BSA is taken up into yolk-sac tissue, it is degraded within the lysosomal system releasing [^{125}I]-Tyr.

2.8 The Release of a Non-Degradable Substrate from Yolk Sacs Induced by Various Agents

Yolk sacs were dissected as in Section 2.3, but were incubated 1 per 50 ml Erlenmeyer flask in a shaking water bath at 37°C. Each flask contained 10.0 ml of gassed medium 199 which contained [^{125}I]PVP (2 µg/ml). After an incubation period of 2.5 h, 2 x 1.0 ml samples of medium were removed and each group of three yolk sacs was washed for 3 x 2 min in warm (37°C) medium 199 (without [^{125}I]PVP), then re-incubated in a fresh Erlenmeyer flask containing 10.0 ml of gassed medium 199 in which was dissolved the agent. The yolk sacs were re-incubated for a further 2.75 h with 2 x 1.0 ml samples of medium being removed at intervals of 0.25 h for the first 0.75 h and then at 0.5 h intervals. After each sampling, 2 x 1.0 ml of medium 199 containing the agent (at 37°C) were added to the flasks and the flasks regassed. (This kept the volume in each flask constant.) At the end of the incubation period, the yolk sacs were washed 3 x 2 min in changes of ice-cold saline (1%, w/v). The yolk-sac samples and medium samples were assayed for radioactivity as in Section 2.4.1.

2.9 Calculation of Release of a Non-Degradable Substrate

The total radioactivity released into the incubation medium over the re-incubation period was obtained by the following equation:

$$T_n = 10\, C_{1(i=n)} + 2 \sum_{i=0}^{i=(n-1)} C_i$$

where T_n = total radioactivity released up to the time of the nth sampling (c.p.m., corrected for background)

C_i = radioactivity per ml of incubation medium of the ith sample (c.p.m., corrected for background)

The total quantity of radioactivity released into the incubation medium over the re-incubation period added to the quantity of radioactivity remaining associated with the tissue at the end of the re-incubation period is equal to the total amount of radioactivity associated with the yolk-sac tissue at the beginning of the re-incubation period. Using these values, it is possible to calculate the amount of radioactivity released during any part of the re-incubation period as a percentage of the quantity of radioactivity associated with the yolk sac at the beginning of the re-incubation period.

2.10 Recovery of Pinocytic Uptake Following Exposure of Yolk Sacs to Various Agents

Yolk sacs were dissected as in Section 2.3 and exposed to an agent by incubation in 50 ml Erlenmeyer flasks containing 10 ml medium 199 and agent. The yolk sacs were incubated in this manner for 0.5 h before being removed and washed 3 x 2 min in fresh, warm (37°C) medium 199 (which did not contain agent). They were then placed into fresh flasks containing 10.0 ml of agent-free medium containing [^{125}I]PVP (2 µg/ml). The flasks were gassed and re-incubated. The yolk sacs were usually removed at 0.5 h intervals for 2.0 h, then at 1.0 h intervals up to 4.0 h. The yolk-sac tissue and samples of incubation medium were assayed as described in Section 2.4.

2.11 Recovery of the Release of TCA-Soluble Radioactivity into the Incubation Medium Following Exposure of Yolk Sacs to Various Agents

Essentially the same procedure as outlined in Section 2.10 was followed except that, instead of [^{125}I]PVP dissolved in medium (10 ml), the re-incubation flasks held 14.0 ml medium 199 containing 5 µg/ml [^{125}I]fd BSA. The re-incubation medium was sampled (4 x 0.5 ml) immediately after the addition of a yolk sac (to give an initial % acid-soluble radioactivity value). Then 2 x 0.5 ml samples were removed every 0.25 h for the first 1.5 h and then every 0.5 h for the final 2.5 h of re-incubation. (Clark & Williams, unpublished data, have shown that yolk-sac function is unimpaired by incubation in 7.0 ml of medium 199.) The incubation medium samples were assayed for radioactivity as described in Section 2.4.2. The yolk-sac solution was assayed as described in Section 2.4.3.

2.12 Calculation of the Quantity of TCA-Soluble Radioactivity Released into the Incubation Medium

The TCA-soluble radioactivity released into the incubation medium by a given time (T_i, c.p.m. per mg yolk-sac protein) was calculated by the following equation:

$$T_i = \frac{2V.\Delta S_t^{i=n} + \sum_{i=0}^{i=(n-1)} \Delta S_t}{P}$$

where T_i = TCA-soluble radioactivity released into medium (c.p.m./mg yolk-sac protein)

V = total volume of medium at time of sampling (ml)

S_t = observed acid-soluble radioactivity in 0.5 ml of medium at time T_i (uncorrected c.p.m./0.5 ml)

$\Delta S_t^{i=n}$ = increase in acid-soluble radioactivity over initial acid-soluble radioactivity in medium sample (corrected c.p.m./0.5 ml)

$\sum_{i=0}^{i=(n-1)} \Delta S_t$ = total acid-soluble radioactivity removed in previous samples (c.p.m.)

$2V.\Delta S_t^{i=n}$ = total increase in acid-soluble radioactivity remaining in the medium in the flask (c.p.m.)

$2V.\Delta S_t^{i=n} + \sum_{i=0}^{i=(n-1)} \Delta S_t$ = total acid-soluble radioactivity produced by a given flask containing a yolk sac (c.p.m.)

P = protein content of yolk sac (mg)

2.13 Release of a Previously-Accumulated, Non-Degradable Substrate on Exposure of Yolk Sacs to Various Agents

Yolk sacs were dissected as in Section 2.3, but were incubated 3 per 50 ml Erlenmeyer flask in a shaking water bath at 37°C. Each flask contained 7.0 ml of gassed medium 199 which contained [^{125}I]PVP at a higher than usual concentration (10 µg/ml). After a brief incubation period (pulse period) of 0.25 or 0.5 h, the yolk sacs were removed. Each group of three was washed for 3 x 2 min in warm (37°C) medium 199 (without [^{125}I]PVP), then re-incubated in fresh Erlenmeyer flasks containing 10.0 ml of gassed medium 199. The yolk sacs were then incubated for 0.0 - 2.0 h (chase period). Immediately the chase period expired, 1.0 ml of medium was removed from each flask (t_0 sample) and 1.0 ml of medium 199 containing the agent (at 37°C) was added to the flasks (this kept the volume in each flask constant) and the flasks were regassed. The yolk sacs were re-incubated for a further 2.75 h with samples being removed as in Section 2.8 (except that only single 1.0 ml samples were removed and replaced). Yolk sacs and medium samples were assayed for radioactivity as in Section 2.4.1.

2.14 Modified Method for the Release of a Previously-Accumulated, Non-Degradable Substrate on Exposure of Yolk Sacs to Various Agents

The methodology was essentially as described in Section 2.13, except that the 3 x 2 min wash period was reduced to 3 x 15 sec.

2.15 Release of a Previously-Accumulated, Degradable Substrate on Exposure of Yolk Sacs to Various Agents

The methodology was essentially as described in Section 2.13, except that, during the pulse period, the tissue was exposed to [^{125}I]fd BSA (25 µg/ml) and the wash period was reduced from 3 x 2 min to 3 x 15 sec. The incubation medium samples were assayed for radioactivity as described in Section 2.4.2, except that only a single 1.0 ml medium sample was removed at each time point. The yolk-sac solution was assayed for radioactivity as described in Section 2.4.3.

2.16 Modified Method for the Release of a Previously-Accumulated, Degradable Substrate on Exposure of Yolk Sacs to Various Agents

Yolk sacs were dissected as in Section 2.3, but were incubated 3 per 50 ml Erlenmeyer flask in a shaking water bath at 37°C. Each flask contained 7.0 ml of gassed medium 199 in which was dissolved non-radioactive [^{127}I]fd BSA (25 µg/ml). After an incubation period of 0.25 h (pre-pulse period) the yolk sacs were removed. Each group of three was washed for 3 x 15 sec in warm (37°C) medium (without [^{127}I]fd BSA), then re-incubated for 0.25 h (pulse period) in fresh flasks containing 7.0 ml of gassed medium 199 in which was dissolved radioactive [^{125}I]fd BSA (25 µg/ml). Each group of yolk sacs was removed and washed for 3 x 15 sec in warm (37°C) medium 199 (without [^{125}I]fd BSA). The methodology was then as described in Section 2.13. The medium samples were assayed for radioactivity as described in Section 2.4.2 and the yolk sacs assayed for radioactivity as described in Section 2.4.3.

2.17 Release of a Previously-Accumulated, Non-Degradable Substrate along with the Enzymes Lactate Dehydrogenase and N-Acetyl-β-Glucosaminidase, on Exposure of Yolk Sacs to Various Agents

The methodology was essentially as described in Section 2.13, except that the 3 x 2 min wash period was reduced to 3 x 15 sec and after the incubation period was complete the yolk sacs were given 3 x 2 min wash in ice-cold saline (1%, w/v) and homogenized at 4°C for 60 sec in 5 ml of 0.01 M K_2HPO_4 buffer, pH 7.4, by using a Tri-R homogenizer set at speed 2. The homogenates were assayed immediately for lactate dehydrogenase, N-acetyl-β-glucosaminidase activity and radioactivity as described in Sections 2.18, 2.19 and 2.4.1.

2.18 Assay of Lactate Dehydrogenase Activity

Lactate dehydrogenase activity was measured by the method of Lowry et al. (1957). A substrate mixture was prepared which contained 10 mM NADH and 10 mM sodium pyruvate in 50 mM Tris buffer at pH 7.6. To 10 µl of this mixture was added 10 µl of sample (incubation medium or yolk-sac homogenate), and the mixture was incubated for 5 min at 37°C. The reaction was terminated by the addition of 10 µl of 0.6 M HCl, and, after 1 min incubation at 37°C, 300 µl of 7.0 M NaOH was added and the incubation continued for 30 min at 37°C. The reaction mixture was added to 2.0 ml of distilled water and the fluorescence was determined in a Perkin-Elmer 3000 Fluorescence Spectrometer using an excitation wavelength of 365 nm and an emission wavelength of 465 nm. Medium samples were centrifuged at 5,000 r.p.m. for 20 min to remove cell debris and the supernatant assayed for enzymic activity.

Standards containing up to 100 nmole NAD were assayed as described above.

2.19 Assay of N-Acetyl-β-Glucosaminidase Activity

N-acetyl-β-glucosaminidase was assayed by using 2.5 mM 4-methyl-umbelliferyl 2-acetamido-2-deoxy β-D-glucopyranoside as substrate (Livesey, 1978) in 0.1 ml of citrate/phosphate buffer, pH 4.3 (0.1 M citric acid adjusted to pH 4.3 with 0.2 M Na_2HPO_4). To this mixture was added 10 µl of incubation medium (centrifuged as in Section 2.15) or yolk-sac homogenate, and the mixture was incubated at 37^{0}C for 15 min. The reaction was terminated by the addition of 2.5 ml of 0.17 M glycine/carbonate buffer, pH 10.5. The fluorescence was determined in a Perkin-Elmer 3000 fluorescence spectrometer, using an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

Standards containing up to 1 nmole of 4-methylumbelliferone in glycine/carbonate buffer, pH 10.5, were also assayed.

2.20 Clearance of [^{125}I]fd BSA from the Rat Bloodstream After An Injection of Ethanol

Male Wistar rats weighing 300 - 500 g were anaesthetised with ether and 1% (w/v) NaCl (0.022 ml/g body weight) or 25% (v/v) EtOH in physiological saline (0.022 ml/g body weight) at 37^{o}C was administered by intraperitoneal injection. The animal was placed in a recovery cage and 15 or 45 min later the animal was again anaesthetised with ether and the femoral vein exposed for injection of [^{125}I]fd BSA solution (1.0 ml containing 150 µg). The X-ray scintillation probe of a hand monitor was held above the heart; this served to indicate a successful injection (t_0 for sampling of blood). The wound was then closed and an incision made into the footpad of the hind leg opposite to that used for injection. A sample of blood (10 µl) was taken from the incision as soon as possible after the injection, using a 10 µl heparinised capillary tube. Further samples were taken frequently (every 3 - 4 min after the initial sample) during the first 15 min, then at 10 min intervals up to 1.0 h. Bleeding between samples was prevented by restricting the blood supply to the foot with an elastic band and an artery clamp. Blood samples were washed into 3.0 ml (LP3) tubes with 1.0 ml of a 10% (v/v) aqueous solution of heat-inactivated calf serum and stored at -20^{o}C until assayed. Radioactivity was assayed as in Section 2.4.2.

2.21 Presentation of Blood-Clearance Data

Each blood sample was assayed for both total- and TCA-soluble radioactivity (see Section 2.4.2 for details), the observed values were corrected for background radiation then normalized for geometric factors (see Table 2.5.1). These values represent the "total" radioactivity content (T, c.p.m.) and the "TCA-soluble" radioactivity content (S, c.p.m.) of each blood sample. The corresponding value of "TCA-insoluble" radioactivity is given as the difference of these two values (I = T - S, c.p.m.).

However, after storage, the [^{125}I]fd BSA preparations were always found to contain a small but measurable percentage of TCA-soluble radioactivity, usually equivalent to 1 - 2% of the total radioactivity. This was termed the "percentage initial TCA-soluble" radioactivity (S_o, %) and was determined at the start of each experiment by analysing a small sample of the [^{125}I]fd BSA injection solution in the same manner as described above for blood samples. This value was used to correct each value of the "TCA-soluble" radioactivity (S), calculated as described in the first paragraph, for TCA-soluble radioactivity initially present at the time of injection:

$$\text{Corrected TCA-soluble radioactivity, } S' \text{ (c.p.m.)} = S - \frac{(T \times S_o)}{100}$$

The corresponding values of "TCA-insoluble" radioactivity at any time point do not require correction and are calculated as:

$$\text{Corrected TCA-insoluble radioactivity, } I \text{ (c.p.m.)} = (T - S)$$

Values of I, calculated in this manner, were plotted against time and, by extrapolating back to time zero through the initial 2 - 3 points in the time-course, the initial value of I, I_0 (c.p.m.), could be estimated.

In each of the different experiments the above values were calculated and the quantities of "TCA-soluble" and "TCA-insoluble" radioactivity present in the blood samples at a given time were expressed as a percentage of the "TCA-insoluble" radioactivity present at time zero:

i.e. "TCA-insolubles" in blood at a given time (%) $= \frac{I}{I_0} \times 100$

and "TCA-solubles" in blood at a given time (%) $= \frac{S'}{I_0} \times 100$

The merit of reporting the data in this format is that the data do not need further correction for the effects of radioactive decay (due to the experiments being performed over a period of time) as would be required if the data had been expressed in c.p.m. Furthermore, no assumptions need be made about the blood volume of individual animals in order to perform these calculations.

CHAPTER THREE

THE EFFECT OF ETHANOL ON FLUID PHASE PINOCYTOSIS IN THE RAT VISCERAL YOLK SAC

3.1 INTRODUCTION

The effects of ethanol on cells have been well documented by many workers. In Chapter 1 the pharmacological effects on the phospholipid bilayer (Grenell, 1975) and the fluidizing effect on the plasma membrane (Ross, 1980) were reported. Indeed, the precise mechanisms of action of drugs that act as general depressants of the central nervous system are still unknown. However, the physical characteristics which they share, particularly lipid-solubility, suggest (Littleton, 1983) that they act, at least partly, by entering the hydrophobic regions of the synapse causing disruption of both postsynaptic receptor-mediated events and Ca^{2+}-dependent presynaptic release of neurotransmitters. The "fluidizing action" can be measured at pharmacologically-relevant concentrations (Chin et al., 1979). It has also been shown that animals made tolerant to ethanol have plasma membranes which are resistant to ethanol-induced fluidization. This resistance is believed to be achieved via changes in membrane-lipid composition which include a decrease in the proportion of unsaturated fatty acyl chains in membrane phospholipids (Taraschi & Rubin, 1985) and an increase in the content of cholesterol (Chin et al., 1978). It should be mentioned that, in the various experiments, differences in certain variables: such as diet, the means by which ethanol was administered and the duration of ethanol treatment may partly explain the conflicting conclusions drawn by researchers in this still controversial field.

In their study on chronic effects of ethanol on liver plasma membranes, Polokoff et al. (1985) found that the content of sphingomyelin and phosphatidylserine were decreased significantly (by 23% and 34%) after ethanol treatment, whereas the ratio of sphingomyelin to phosphatidylcholine was increased strikingly (by 42%). From model membrane studies, this increase in

phosphatidylcholine/sphingomyelin ratio would be expected to enhance bilayer fluidity (Shinitzky & Barenholz, 1974). However, the decrease in plasma-membrane phosphatidylserine-content may also contribute to the change in other physical properties of membranes. For example, it is now known that the lipid composition of the bilayer also affects membrane-transport proteins (Carruthers & Melchior, 1984). Other effects that have been described include the fluidization effects of ethanol on prolactin binding sites (Dave et al., 1985) and vertical displacement of proteins within the membrane mediated by changes in microviscosity (Borochov & Schinitzky, 1976). Increasing membrane microviscosity resulted in an appreciable increase in the amount of membrane protein that becomes exposed to the aqueous environment surrounding the plasma membrane.

An indirect effect of ethanol on plasma-membrane protein composition of liver cells was reported by Mailliard et al. (1984) who studied the effect of acute ethanol exposure on the assembly and incorporation of glycoproteins into hepatocyte plasma-membrane in the rat. The results obtained demonstrated that ethanol administration impaired the insertion of glycoproteins into the plasma membrane. Possibly the later stages of insertion of glycoproteins, following terminal glycosylation in the Golgi complex, are important in the action of ethanol. In a later study, Tuma et al. (1986) found in the same system that this inhibition of insertion of glycoproteins into hepatocyte plasma-membranes was caused by ethanol interfering with the flow of membrane components from the Golgi apparatus to the surface membrane.

Isolated hepatocytes in culture were used by Nunes et al. (1984) in a study of the effect of ethanol on iron uptake. Hepatic siderosis is frequently associated with alcohol-induced liver disease, but the exact influence of ethanol on iron uptake in hepatocytes is

unclear. These authors demonstrated that cells took up significantly less $^{59}Fe^{3+}$ in the presence of 10 mM ethanol over a 90 min period than cells incubated in the absence of ethanol. Total transferrin binding was equivalent in both groups, but the molecular ratio of iron : transferrin on cell membranes after 90 min was markedly decreased in the presence of ethanol. This suggests an increase in the retention of apotransferrin at the plasma-membrane surface.

An interesting observation was that, in the presence of ethanol, there was a decrease in the pH of the incubation medium. This observation is compatible with the notion that iron uptake from transferrin is mediated by a pH-dependent transferrin receptor (see Beloqui et al., 1986). In this study, 10 mM ethanol in weakly-buffered medium caused a marked decrease in iron uptake by hepatocytes with a greater pH depression than in ethanol-free controls. Further investigation of iron uptake over the pH range 6 - 8.5 revealed a marked dependency of iron uptake on the extracellular pH. By increasing the buffering capacity of the system or by the addition of 4-methylpyrazole (an inhibitor of alcohol dehydrogenase), iron uptake returned to control values.

In addition to the effects of ethanol on plasma-membrane binding events, ethanol has also been shown to interfere with amino-acid transport in cultured hepatocytes (Dorio et al., 1984). Exposure of hepatocytes for 24 h to 100 mM ethanol selectively decreased (by 40 - 70%) neutral amino-acid uptake by the Na^+-dependent A- and N-systems, but had no significant effect on the Na^+-dependent ASC or Na^+-independent L-systems. The ethanol treatment failed to prevent cells from increasing the A-system activity in response to the hormones insulin and glucagon, but the magnitude of hormone-stimulated uptake was reduced relative to controls. The ethanol-induced depression of the A-system was due to a decrease in

the V_{max}, with no significant change in the apparent K_m. It has been shown that 100 mM ethanol inhibits protein synthesis by approximately 35% by interfering with Leu-t RNA synthetase (David et al., 1983). Thus the partial inhibition of protein synthesis may lead to a decrease in the number of transport units in the plasma membrane, without affecting the affinity of the translocator.

Thus, if ethanol has a direct effect on the plasma membrane of cells, it may be possible to alter certain activities of cells. Indeed, Saxena et al. (1986) reported that in vitro addition of ethanol (in concentrations equivalent to those found in the blood of chronic alcoholics) inhibited the natural killer-activity of human peripheral blood mononuclear cells. The observed inhibition was not caused by a direct toxic effect of ethanol nor by a change in the target binding capacity of the cells. Moreover, ethanol has been reported to inhibit pinocytosis and phagocytosis in the white blood cells of alcoholics (Chanarin, 1982).

The main aim of the experimental work reported in this chapter was to establish the effect on the pinocytic uptake of [^{125}I]PVP by rat visceral yolk sacs of acute exposure to different concentrations of ethanol.

3.2 MATERIALS AND METHODS

All the materials and equipment used in this chapter were as described in Section 2.1. The medium was either 199 alone or, if calf serum was present, it was added at concentrations of 10%, 20% or 50% (v/v). All uptake experiments were performed as described in Section 2.3 and assays as described in Section 2.4. [^{125}I]PVP was used at a concentration of 2 μg/ml; ethanol was added 1.0 h after the addition of the radiolabelled substrate. (The pattern of agent addition and ^{125}I-labelled substrate concentrations employed are given in the legends to the figures and tables.)

The only new method employed was that for assaying the release of [^{125}I]PVP from yolk sacs in the presence of ethanol at various concentrations (Section 2.8). At ethanol concentrations of 20 mM and 100 mM the medium was sampled as stated in Section 2.8. The assayed radioactivity was termed "total radioactivity". The LP3 tubes were then centrifuged at 3,000 r.p.m. for 30 min, to remove any tissue-derived cell debris, and the supernatant was then decanted into fresh LP3 tubes and assayed for radioactivity (Section 2.4.1). The results were corrected by the appropriate correction factor and termed the "non-sedimentable radioactivity". Both non-sedimentable- and total-radioactivity were then expressed as the percentage of the total tissue-associated radioactivity that was released during the re-incubation period and the values were plotted against time.

3.3 RESULTS

3.3.1 The effect of ethanol on fluid-phase pinocytosis

The effects of ethanol on the pinocytic uptake of the fluid-phase marker, [^{125}I]PVP, by yolk sacs incubated in serum-free medium 199 were studied. The initial range investigated was 0.02 - 100.0 mM (Table 3.3.1.1). It can be seen that ethanol concentrations of 0.02 and 0.20 mM in the incubation medium had no effect on the pinocytic capture and accumulation of [^{125}I]PVP. However, the 100 mM concentration proved to be cytotoxic, with the tissue being extensively damaged beyond the 3.0 h point in a 5.0 h incubation experiment. (The incubation medium became more and more "milky" in appearance with cell debris becoming detached from the yolk sacs during the last 2 h of incubation.) Of more interest were the effects of 2.0 and 10.0 mM ethanol; so the effects of these two concentrations were investigated in detail (see Fig. 3.3.1.1). The rates of uptake were expressed in the form of Endocytic Indices (E.I.) as defined by Williams et al. (1975a;b). The Endocytic Index of the control was 2.67 ± 0.14 µl/mg yolk-sac protein/h which was in agreement with the findings of Ibbotson & Williams (1979). At both of these ethanol concentrations, pinocytosis was seen to be inhibited almost immediately after exposure of the tissue to ethanol (Fig. 3.3.1.1). By 5.0 h, the residual rates of uptake as a percentage of the control values were 34.9 ± 3.9% for 2.0 mM and 6.41 ± 0.01% for 10.0 mM ethanol.

In the case of 10.0 mM ethanol, there appears to be either a "leakage" or exocytosis of [^{125}I]PVP from the tissue so that there is no net uptake (see Fig. 3.3.1.1 and Table 3.3.1.2). A rate of leakage of 1.43 ± 0.86%/h was reported by Roberts et al. (1976) in equivalent control experiments, a value approximately twice that reported for controls in the current study (Table 3.3.1.2). Relative to

ethanol-free controls, the rate of progressive loss of [^{125}I]PVP approximately doubled on exposure to 2.0 mM ethanol and was 2.5 x the control rate with 10.0 mM ethanol. When the ethanol concentrations were increased further, there was an 18.5-fold and 19.5-fold increase in the loss of [^{125}I]PVP with 20.0 mM and 100.0 mM ethanol, respectively. Time-courses of release are given in Figs. 3.3.1.2 and 3.3.1.3. Both figures indicate that, during the first 15 min of ethanol exposure, there is a greater initial release of [^{125}I]PVP. As the ethanol concentration increases from 2.0 mM to 100.0 mM, the increases above that of the control values are: x 2.5, x 3.5, x 3.1 and x 5.2, respectively. In Fig. 3.3.1.3 the use of ethanol at concentrations that may be cytotoxic was investigated. Again the time-courses of release were plotted as in Fig. 3.3.1.2, but additionally the release patterns of [^{125}I]PVP into the incubation medium following centrifugation of the medium samples (to remove radioactivity associated with cell debris) were also plotted (see Section 3.2). With 20.0 mM ethanol, little difference between the release patterns was seen. In the case of 100.0 mM ethanol, after 45 min, visual inspection indicated an approximate difference of 5% between the "total radioactivity" and the "non-sedimentable radioactivity" for the final 120 min of the incubation period (see Fig. 3.3.1.3). This indicates that at ethanol concentrations of 2.0 mM and 10.0 mM there was no sharp increase in release. However, at the higher ethanol concentrations of 20.0 mM and 100.0 mM up to 40 - 45% of the tissue-associated radioactivity was released within 2.75 h.

To answer the question of whether ethanol (2.0 or 10.0 mM) was causing cell death, recovery of the pinocytic capacity of the tissue was examined (Fig. 3.3.1.4). From the results, it can be seen that the recovery of pinocytic capacity after the treatment of the tissue with 2.0 mM ethanol for 0.5 h was effectively immediate and complete.

The E.I. of the control was 2.66 ± 0.03 µl/mg yolk-sac protein/h compared with an E.I. of 2.52 µl/mg yolk-sac protein/h for the 2.0 mM ethanol study. However, visual inspection indicated that a period of approximately 1.5 h passed before the tissue fully recovered from the 10.0 mM ethanol treatment but, by 3.0 h, the tissue had regained its full pinocytic capacity; so no permanent tissue damage was caused, even by the higher of these two ethanol concentrations. (No recovery studies were carried out at the 20.0 mM and 100.0 mM ethanol concentrations.)

3.3.2 The effects of serum on fluid-phase pinocytosis

This work was carried out mainly to establish whether increasing concentrations of serum in the incubation medium modified the effects of ethanol on pinocytosis. In this investigation, the concentration range of serum employed in the incubation medium was 10, 20 and 50% (v/v), and the effects on the E.I. are reported in Table 3.3.1.3. The relative rates of uptake of the controls were in agreement with those reported by Forster & Williams (1984); as the serum concentration increases the E.I. decreases. [It should be noted that when the concentration of serum was 100% (v/v) of heat-inactivated whole rat serum (Dunton et al., 1986) the rate of uptake of [^{125}I]PVP by 17.5-day rat visceral yolk sacs was 1.03 ± 0.02 µl/mg yolk-sac protein/h (mean ± S.E.M.). Thus, the uptake of [^{125}I]PVP in 100% (v/v) rat serum was greatly decreased compared with serum-free controls, but not abolished.] At the 10% (v/v) serum concentration there is little relative difference in the effects of 2.0 or 10.0 mM ethanol on the patterns of uptake in comparison with matched serum-free controls (see Figs. 3.3.1.1 and 3.3.1.5). However, with 20% (v/v) serum, even though the relative effect on the 10.0 mM ethanol results were the same as in the serum-free studies, the results for 2.0 mM ethanol show a decrease in the relative percentage inhibition of pinocytosis from 60% in the

serum-free study to only 38% (Table 3.3.1.3 and Fig. 3.3.1.6). Finally, when the concentration of serum in the incubation medium reached 50% (v/v), the relative effect of 2.0 mM ethanol on fluid-phase pinocytosis was completely removed, but the effect of 10.0 mM ethanol was not. The relative percentage inhibition was decreased from approximately 94% in serum-free medium to approximately 34% in the 50% (v/v) serum medium, showing that, even at the highest serum concentration, ethanol at the higher concentration still caused a partial inhibition of fluid-phase pinocytosis (Table 3.3.1.3 and Fig. 3.3.1.7).

Fig. 3.3.1.1 Effects of different concentrations of ethanol on the accumulation of [^{125}I]PVP by yolk sacs

Yolk sacs were incubated in serum-free medium 199 in the presence of [^{125}I]PVP (2 μg/ml). At 1.0 h the appropriate concentration of ethanol was added in TC199 to give a final concentration of 2.0 mM or 10.0 mM ethanol in each flask. Each point is the mean ± S.D. of > 4 yolk sacs.

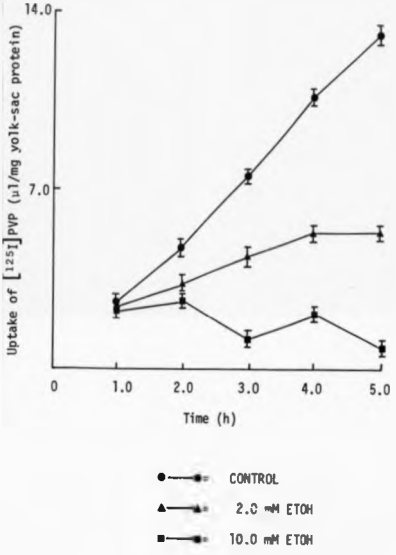


Fig. 3.3.1.2 Release of [^{125}I]PVP into the incubation medium

Yolk sacs were incubated in serum-free TC199 containing [^{125}I]PVP (2 µg/ml) for 2.5 h then washed and transferred to substrate-free medium containing either 2.0 mM or 10.0 mM ethanol and incubated for a further 2.75 h. Each point is the mean ± S.D. of 9 yolk sacs.

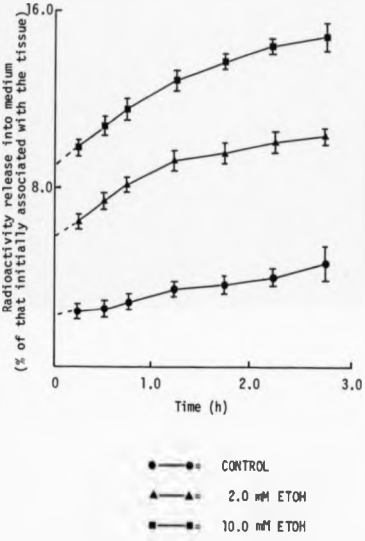
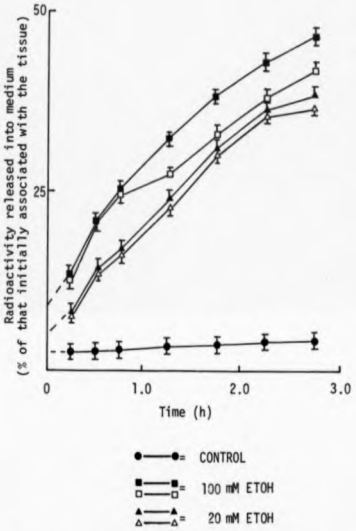


Fig. 3.3.1.3 Release of [^{125}I]PVP into the incubation medium

Yolk sacs were incubated as described in Fig. 3.3.1.2 but were then transferred into substrate-free, serum-free medium containing 20.0 mM and 100.0 mM ethanol for a further 2.75 h. Each point is the mean ± S.D. of 9 yolk sacs.



Closed symbols are the "total radioactivity" but open symbols are "non-sedimentable" radioactivity" (see text for experimental details).

Fig. 3.3.1.4 Uptake of [^{125}I]PVP following ethanol pre-treatment of the tissue

Yolk sacs were pre-incubated in serum-free TC199 containing only 2.0 mM or 10.0 mM ethanol for 0.5 h then washed and transferred to inhibitor-free medium containing [^{125}I]PVP (2 µg/ml) for a further 4.0 h of incubation. Each point is the mean ± S.D. of 5 yolk sacs.

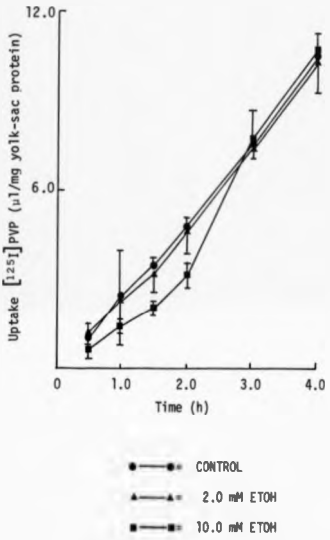


Fig. 3.3.1.5 Effect of different concentrations of ethanol on the accumulation of [^{125}I]PVP in the presence of 10% (v/v) calf serum

Yolk sacs were incubated in TC199 containing both 10% (v/v) calf serum and [^{125}I]PVP (2 µg/ml). At 1.0 h the appropriate concentration of ethanol was added in TC199 to give a final concentration of either 2.0 mM or 10.0 mM ethanol in each flask. Each point is the mean ± S.D. of 6 yolk sacs.

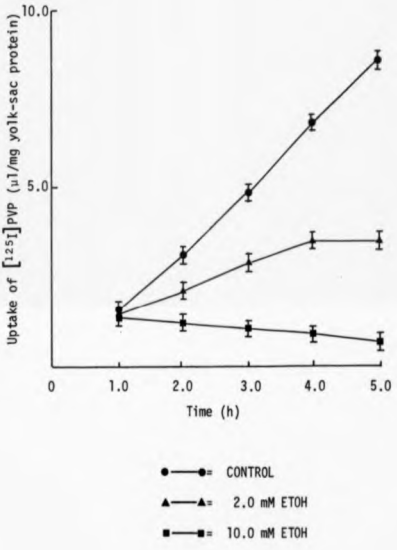


Fig. 3.3.1.6 Effect of different concentrations of ethanol on the accumulation of [^{125}I]PVP in the presence of 20% (v/v) calf serum

Yolk sacs were incubated in TC199 containing both 20% (v/v) calf serum and [^{125}I]PVP (2 ug/ml). At 1.0 h the appropriate concentration of ethanol was added in TC199 to give a final concentration of either 2.0 mM or 10.0 mM ethanol in each flask. Each point is the mean ± S.D. of 6 yolk sacs.

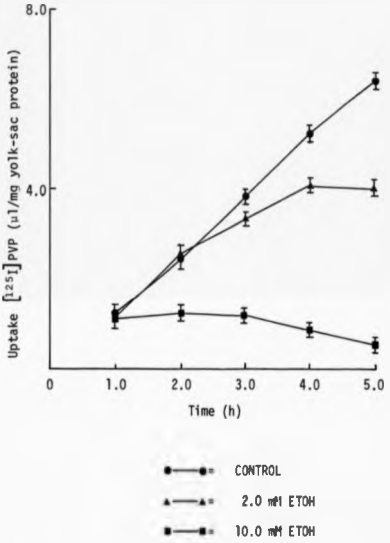


Fig. 3.3.1.7 Effect of different concentrations of ethanol on the accumulation of [^{125}I]PVP in the presence of 50% (v/v) calf serum

Yolk sacs were incubated in TC199 containing both 50% (v/v) calf serum and [^{125}I]PVP (2 µg/ml). At 1.0 h the appropriate concentration of ethanol was added in TC199 to give a final concentration of either 2.0 mM or 10.0 mM ethanol in each flask. Each point is the mean ± S.D. of 6 yolk sacs.

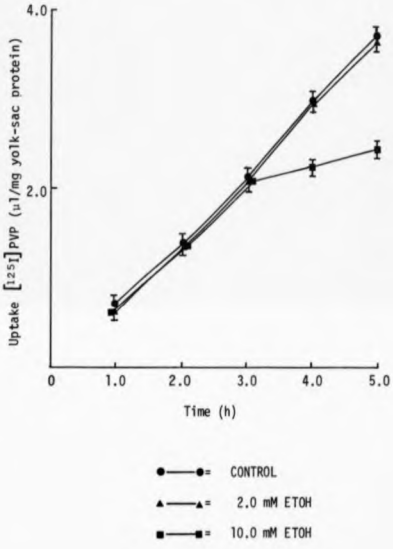


Table 3.3.1.1 Effects of different concentrations of ethanol on the rate of accumulation of [^{125}I]PVP by yolk sacs incubated in vitro

Yolk sacs from a 17.5-day pregnant rat were incubated in 9.0 ml of serum-free medium 199 containing [^{125}I]PVP (2 µg/ml of medium) (see Section 2.3 for details). At 1.0 h, medium 199 (1.0 ml) containing ethanol at x 10 the final concentration was added to give final concentrations of either 0.02, 0.2, 2.0, 10.0 or 100.0 mM ethanol in each flask.

Each value reported is the mean (± S.D.) from at least 4 experiments, except for those marked (*) for which n = 1.

Ethanol Concentration (mM)	Endocytic Index (µl/mg yolk-sac protein/h)	Correlation Coefficient (r)
0 (Control)	2.67 ± 0.14	0.99
*0.02	2.55	0.99
*0.20	2.41	0.99
2.00	0.76 ± 0.27	0.96
10.00	- 0.32 ± 0.64	- 0.67
*100.00	This concentration was cytotoxic, causing visible tissue disintegration towards the end of the 5.0 h incubation period.	

Table 3.3.1.2 Rates of release of [^{125}I]PVP from yolk sacs incubated in the presence of different concentrations of ethanol

Yolk sacs were incubated in serum-free medium containing [^{125}I]PVP (2 µg/ml) for 2.5 h, then washed and transferred to substrate-free medium containing 2.0 mM, 10.0 mM, 20.0 mM or 100.0 mM ethanol and incubated for a further 2.75 h. Both the initial loss (0 - 0.25 h) and the rate of progressive loss (0.25 - 2.75 h) are expressed as a percentage of the radioactivity initially associated with the tissue.

Each value reported is the mean (± S.D.) of 9 observations.

Ethanol Concentration (mM)	Initial loss over period 0 - 0.25 h (% of radioactivity released)	Rate of loss of [^{125}I]PVP over period 0.25 - 2.75 h (%/h)
0 (Control)	2.56 ± 0.03	0.78 ± 0.30
2.0	6.38 ± 0.20	1.56 ± 0.10
10.0	9.67 ± 0.23	2.08 ± 0.45
20.0	7.96 ± 0.35	14.54 ± 0.51
100.0	13.41 ± 0.70	15.44 ± 0.47

Table 3.3.1.3 Effects of increasing the concentration of calf serum in the incubation medium on the degree of inhibition of fluid-phase pinocytosis induced by ethanol

The time-courses of uptake of [^{125}I]PVP by 17.5-day yolk sacs incubated in medium 199 containing [^{125}I]PVP (2 μg/ml), ethanol (2.0 or 10.0 mM) and different concentrations of heat-inactivated calf serum were determined. The values reported are means (± S.D.) from at least three similar experiments.

Concentration of serum in the medium (%, v/v)	Uptake after 5 h of incubation (μl/mg yolk-sac protein)			Percentage uptake relative to ethanol-free matched controls (%)	
	Control	Ethanol (2.0 mM)	Ethanol (10.0 mM)	Ethanol (2.0 mM)	Ethanol (10.0 mM)
0	13.22 ± 0.35	4.59 ± 1.05	0.85 ± 0.17	34.7 ± 7.93	6.42 ± 1.38
10	8.59 ± 0.11	3.51 ± 0.04	0.63 ± 0.01	40.9 ± 0.56	7.33 ± 0.11
20	6.46 ± 0.09	4.05 ± 0.01	0.61 ± 0.02	62.6 ± 0.82	9.47 ± 0.30
50	3.70 ± 0.02	3.68 ± 0.01	2.45 ± 0.01	99.3 ± 0.50	65.9 ± 0.43

3.4 DISCUSSION

The results reported in this chapter indicate that pinocytic activity in the visceral yolk sac was insensitive to ethanol at concentrations of 0.2 mM or lower. However, the potency of ethanol as an inhibitor of pinocytosis became evident at a concentration of 2.0 mM in the serum-free medium. At this concentration fluid-phase pinocytosis was reduced to approximately 30% of the control value after 5 h of incubation. When the ethanol concentration was increased to 10.0 mM, fluid-phase pinocytosis was decreased to approximately 6% of the control value at the 5 h incubation point (Fig. 3.3.1.1). Neither 2.0 mM nor 10.0 mM ethanol concentrations were overtly cytotoxic, since after the first 15 min of exposure to ethanol the rate of release of the non-degradable fluid-phase pinocytosis marker, [^{125}I]PVP, was not greatly increased above that of the control (Table 3.3.1.2). However, if the ethanol concentration was raised to 20.0 mM or 100.0 mM then these concentrations were overtly cytotoxic (Fig. 3.3.1.3 and Table 3.3.1.2) with approximately 45 - 50% of the tissue-associated radioactivity released during a 2.75 h incubation period.

At both 10.0 mM and 2.0 mM ethanol concentrations, the agent was a powerful inhibitor of fluid-phase pinocytosis, but subsequent studies showed that such inhibition was reversible. Recovery from the lower concentration of ethanol was immediate and complete. In contrast, recovery from the higher ethanol concentration required a "lag-period" of approximately 1.5 h before pinocytosis began to recover, but after 2.0 - 3.0 h of re-incubation the rate of fluid-phase pinocytosis was elevated to 4.55 µl/mg yolk-sac protein/h, as compared with the control rate over the same period of 2.65 µl/mg yolk-sac protein/h (see Fig. 3.3.1.4), a 1.7-fold increase in the basal rate of fluid-phase pinocytosis above that of the control.

One of the few other reports of stimulation of pinocytosis is that of Goldstone et al. (1983) who showed that testosterone and the calcium ionophore A23187 caused a 2.0- and 1.6-fold increase in the fluid-phase pinocytosis of horseradish peroxidase by mouse kidney-cortex slices. This increase in the rate of fluid-phase pinocytosis was accompanied by an increased influx of ^{45}Ca into the cortex slices. If this influx of ^{45}Ca was prevented, by chelation of extracellular calcium with E.G.T.A. (ethyleneglycol-bis(β-aminoethylether)-N,N′-tetracetic acid) or by inhibiting the transport of calcium into the cells with verapamil, then the increase in the rate of fluid-phase pinocytosis was abolished. However, the results of Goldstone et al. (1983) must be regarded with some reservations. First, the culture system used slices of mouse-kidney cortex which may not have been of uniform thickness, thus preventing some cells from being exposed to the substrate. Second, horseradish peroxidase is a protein and so is susceptible to lysosomal degradation within 20 - 30 min of the uptake experiment starting, making total uptake difficult to quantify. Third, the uptake data were calculated using only one (a 60 min) time point, thus giving "one-point-kinetics" and making it impossible to distinguish linear from non-linear uptake.

Intracellular calcium concentrations and the influx of extracellular calcium into cells have been well documented as possible secondary messengers in cells (Rubin, 1982). It was suggested (Hoek et al., 1987) that in isolated hepatocytes ethanol caused a rapid transient activation of phosphorylase accompanied by a transient increase in cytosolic free calcium. Since the effects of ethanol were comparable to those produced by a physiological dose of vasopressin (1 nM), the results indicate that ethanol activates hormone-sensitive phosphoinositide-specific phospholipase C. The resulting changes in

inositol 1, 4, 5-trisphosphate can then account for the mobilisation of intracellular calcium and the activation of phosphorylase by ethanol.

On the other hand, Fenn & Litteton (1983), in describing the interactions of ethanol with membrane lipids in human blood platelets, reported that ethanol inhibited platelet aggregation. When platelets were exposed to the calcium ionophore A23187 it caused aggregation, but when ethanol was also present aggregation was prevented. This suggested that, in the case of ionophore A23187, ethanol may act to inhibit aggregation at some point between the rise in cytosolic calcium and the cleavage of membrane phospholipids associated with the platelet release reaction. In the case of A23187, it was thought that the ionophore acted indirectly by making Ca^{2+} available to activate membrane phospholipase A_2 which causes the release of arachidonic acid. This is metabolised with the formation of the aggregatory substance, thromboxane A_2, since aggregation caused by arachidonic acid was unaffected by ethanol.

In the heavy sarcoplasmic reticulum of skeletal muscle, ethanol appeared to render the sarcoplasmic reticulum more permeable to Ca^{2+} (Ohnishi et al., 1984), thus decreasing the amount of intracellular calcium by enhancing its release from the sarcoplasmic reticulum. However, in rat- or mouse-brain synaptosomes (Harris & Hood, 1980) ethanol inhibited the depolarization-dependent influx of calcium in a concentration-dependent manner over the range 45 - 720 mM. Similar results were obtained with the drug pentobarbital. Calcium has been reported to be important in pinocytosis by amoeba proteus (Prusch, 1986). Similarly, Duncan & Lloyd (1978) reported that removal of extracellular calcium, by the addition of 5 mM E.G.T.A., inhibited the uptake of [^{125}I]PVP by rat visceral yolk sac incubated in vitro. (If 5 mM $CaCl_2$ and 5 mM E.G.T.A. were added simultaneously, the effect was

not seen.)

In equivalent experiments investigating the inhibition of pinocytosis by trypan blue, Williams et al. (1976) observed that the percentage inhibition of pinocytosis, relative to matched controls, by a fixed concentration of trypan blue fell sharply as the serum concentration of the medium was increased. (Presumably, free trypan blue is the species that inhibits pinocytosis and more trypan blue binds to serum proteins as the serum content of the medium is raised.) For 2 mM ethanol, the relative inhibition of pinocytosis disappeared completely as the serum concentration of the medium was raised; however, in the case of 10.0 mM ethanol the effect appears to be less marked than the corresponding decrease with trypan blue. [This work was carried out since serum albumins are known to bind small molecules, including propanol, used to remove fatty acids from albumin (Spector, 1975; Foster, 1960); hence binding of ethanol would be expected.]

The results gathered in this chapter are compatible with ethanol being capable of inducing a substantial inhibition of pinocytosis in yolk sacs in vivo when its concentration in the uterine fluid exceeds approximately 10.0 mM. However, in vivo the effect would be expected to be transient as the liver metabolises the ethanol so that its level in the blood, and hence in the uterine fluid, falls. In rats given an intraperitoneal injection of ethanol, the blood-ethanol concentration reached a maximum by the 1.0 h period post-injection and then declined rapidly over the next 5.0 h period (Abrams & Cooper, 1976).

CHAPTER FOUR

THE EFFECT OF ETHANOL ON ADSORPTIVE PHASE PINOCYTOSIS *IN VITRO* AND *IN VIVO*

4.1 INTRODUCTION

Ethanol is known to induce cirrhosis of the liver (Lieber, 1980) and hepatomegaly in the rat (Baraona et al., 1977), but there is still disagreement as to whether ethanol is a primary hepatotoxin or whether its effects on the liver are secondary to an associated nutritional disturbance. However, Wickramasinghe et al. (1986) found that human monocyte-derived macrophages, grown in the presence of 2.17 - 43.4 mM ethanol for 72 h, released into the culture medium a non-dialysable cytotoxic protein which caused the detachment of mouse A9 tumour cells and a decrease in the incorporation of [^{3}H]thymidine into the DNA of K562 cells. These observations suggested the protein could play some role in the pathogenesis of ethanol-induced tissue damage in vivo.

Ethanol not only affects amino-acid uptake in hepatocytes, as described in Chapter 3, but also has been shown, at concentrations of 2 - 20 mM, to inhibit the uptake of [^{14}C]α-aminoisobutyric acid by human placental-villus tissue in vitro (Fisher et al., 1981). A further study (Lin, 1981) again used [^{14}C]α-aminoisobutyric acid, but pregnant rats were fed a liquid diet, containing 30% ethanol-derived calories, from day 6 to day 21 of gestation. [Control rats were pair-fed identical diets except sucrose was substituted for ethanol isocalorically.] Maternal plasma and liver uptake of [^{14}C]α-aminoisobutyric acid, 90 min after administration on day 21 of gestation, were the same in both groups. However, placental and fetal tissues showed a significant (20 - 40%) decrease in the uptake of substrate in the ethanol group compared with the control group, suggesting that ethanol intake during pregnancy causes placental dysfunction. Acute and chronic ethanol exposure have produced similar effects on [^{14}C]-L-valine uptake by placental-villus fragments both in vivo (Patwardhan et al., 1981) and in vitro (Henderson et al., 1981).

The adverse effects of maternal ethanol consumption on fetal growth and development have been the subject of active investigation for the past seventeen years. At the whole-animal level, both chronic and acute ethanol ingestion are known to induce malformation in man and experimental animals, producing an effect known as the FETAL ALCOHOL SYNDROME (FAS). The FAS has been associated with craniofacial abnormalities along with growth and developmental deficiencies, joint abnormalities, cardiac defects, fine motor dysfunction, microcephaly, neural-tube defects and renal problems. However, this pattern of abnormalities was probably first recognised after the Gin Epidemic (1720 - 1750) in England and was only re-discovered by Jones et al. (1973) who coined the term FAS to describe the pattern of reported deformities.

In mice (strain C57BL/6J), acute ethanol exposure was found to cause exencephaly on day 7, maxillary hypoplasia on day 8 and limb defects on days 9, 10 or 11 (Webster et al., 1980). A fetus with maxillary hypoplasia also had defects of the eyes, brain and hypophysis. The ethanol was administered as a single intraperitoneal injection (25% v/v, in normal saline; 0.015, 0.022 or 0.030 ml/g body weight) at one of the above days of gestation. Control mice were given the same volume of isotonic saline solution, but otherwise were treated exactly like the ethanol group. The highest ethanol doses were embryotoxic as well as teratogenic, causing a high resorption rate. Maternal blood-ethanol concentrations fell from 800 mg/100 ml (30 min after injection) to 50 mg/100 ml (12 h after injection). At the intermediate concentration of ethanol used, the incidence of malformations was decreased and the blood-ethanol concentration (maternal) was 600 mg/100 ml, 30 min after injection. At the lowest concentration, both resorption and malformation rates were the lowest with a maternal blood-ethanol concentration of 350 mg/100 ml being

recorded 30 min after injection. However, all ethanol concentrations used were teratogenic and produced the above-mentioned abnormalities. In a further study (Webster et al., 1983), doses of ethanol (given orally either as two doses of the lowest ethanol concentration mentioned above, but 4 h apart, or as a single dose of the highest ethanol concentration mentioned above) were found to be teratogenic and indicated that ethanol treatment on day 7 - 8 caused facial abnormalities, comparable with those seen in FAS children. Ethanol exposure on day 9/10 produced limb defects.

This suggests that one or more episodes of heavy maternal drinking at critical periods of organogenesis may be sufficient to induce FAS. These two initial studies were followed by a third, to determine the critical periods for ethanol-induced heart defects (Webster et al., 1984). In this study, pregnant mice were exposed to a single dose of ethanol (0.030 ml/g body weight of a 25%, v/v, ethanol solution) or to two doses (0.015 ml/g body weight of a 25%, v/v, ethanol solution) 4 h apart by intraperitoneal injection or gavage on days 7, 8, 9 and 10 of gestation. It was found that ethanol exposure on day 8, 9 or 10 of gestation caused a high incidence of ventricular-septal defects of both the membranous and muscular parts of the septum, as well as more complex ventricular-septal defects involving the great vessels.

In general, two patterns of maternal ethanol consumption have been associated with the FAS: (1) Chronic ethanol intake (which induces moderately high blood-ethanol concentrations over long time periods) and (2) "Binge" or "Spree" ethanol intake, resulting in very high blood-ethanol concentrations lasting for short time periods (as reported by Chernoff, 1977; Madden & Jones, 1972; Clarren & Smith, 1978). A fuller review of FAS in relation to alcohol embryopathy in humans is given by Majewski & Goecke (1982) and Pratt (1984). The

former authors produced a classification of the FAS (or Alcohol Embryopathy as it was termed). In their classification (see Table 4.1.1) 25 symptoms were scored and the sum of the points used to estimate the degree of severity (see bottom of Table 4.1.1).

Alcohol Embryopathy I, the mild form of FAS (10 - 29 points), consists of: oligosymptomatic cases, intrauterine growth retardation, low weight and microcephaly with few or no associated craniofacial anomalies or internal malformations. Mental development and nervous co-ordination are normal in approximately 50% of the cases, with others being only slightly retarded. If there is no detailed history of maternal ethanol abuse, these cases may be overlooked.

Alcohol Embryopathy II, the moderate degree of FAS (30 - 39 points): characterised by intrauterine growth retardation, low birth-weight, microcephaly and mild neurological anomalies, especially hypotonicity and hyperactivity. Mental development is retarded in almost all cases, but not as pronounced as in Alcohol Embryopathy III (see below). There are some facial anomalies, but they are often not diagnostic and internal malformations are rare.

Alcohol Embryopathy III. Patients with the severe form of FAS (>40 points) demonstrate almost all the symptoms listed in Table 4.1.1. The facial abnormalities are so typical that diagnosis of FAS is possible without prior knowledge of maternal ethanol abuse. All patients are severely mentally retarded and demonstrate gross neurological disturbances.

Majewski and Goecke concluded from their study that the severity of embryonic damage was not dependent on the daily amounts of

Table 4.1.1 Symptoms seen in Alcohol Embryopathy (FAS)

Below are the common features associated with the FAS. The frequency (%) of occurrence as well as the points given to each defect allow classification of the degree of ethanol-induced damage into three classes (n = 108).

a = observed symptom

b = number of patients in which the symptom was sought.

Points awarded	Symptoms	a/b	Frequency (%)
4	Intrauterine growth retardation	93/105	89
4	microcephaly	87/103	84
2/4/8	mental retardation	88/99	89
4	hyperactivity	69/101	68
2	muscular hypotonia	58/100	58
2	epicanthic folds	71/108	66
2	ptosis	41/107	38
2	blepharophimosis	8/71	11
-	antimongoloid palpebral fissures	39/105	37
3	short, upturned nose	52/106	49
1	nasolabial furrows	74/104	71
1	small lips	64/104	61
2	hypoplasia of mandible	80/107	74
2	high-arched palate	41/106	39

4	cleft palate	8/108	7
3	anomalous palmar creases	70/102	69
2	clinodactyly V	54/106	51
2	camptodactyly	16/104	16
1	hypoplasia of terminal phalanges	13/105	12
2	limited supination	15/105	14
2	hip dislocation	9/98	9
-	pectus excavatum	30/107	28
4	heart defects	29/102	28
2/4	genital abnormalities	49/106	46
1	sacral dimple	45/102	44
-	hemangiomas	12/108	11
2	hernias	12/105	12
4	genitourinary-tract malformations	7/71	10

Classification based on total scores:

Alcohol Embryopathy I (n = 41) : 10 - 29 points

Alcohol Embryopathy II (n = 32) : 30 - 39 points

Alcohol Embryopathy III (n = 35) : >40 points

[Taken from Abel, E. L. (1982): "Fetal Alcohol Syndrome", Vol. II: Human studies, p67, CRC Press, Inc.]

ethanol consumed, but on the stage of ethanol illness in the mothers, since mothers showing only prodromal alcohol disease gave birth to children showing AEI in only 2.0% of the cases studied. On the other hand, women in the critical phase of alcohol disease gave birth to children showing AEI (21%), AEII (11%) and AEIII (4%) in all the cases studied. Finally, women in the chronic phase of alcohol disease gave birth to children showing AEI (10%), AEII (23%) and AEIII (25%) in the cases studied. In fact, later on Majewski (1984) stated that he believed that ethanol alone was not teratogenic but that it is a secondary teratogen, becoming teratogenic only when other factors are present.

Neither the use of animal models of the FAS nor the study of human patients has elucidated the mechanism(s) of action of ethanol as a teratogen. Mechanisms of action so far suggested include:

(1) Ethanol-induced inhibition of aminoacyl-tRNA synthetases (Fleming et al., 1975; David et al., 1983).

(2) Alterations of the nuclear membrane resulting in reduced transport of newly-synthesized RNA to the cytoplasm (Tewari et al., 1975).

(3) Reduced RNA polymerase activity (Tewari et al., 1975).

(4) Defective association of messenger RNA with ribosomes (Tewari et al., 1977).

(5) Fetal carbohydrate metabolism is disturbed by ethanol in utero since fetal-growth retardation is associated with hypoglycemia, hypoinsulinemia and liver glycogen depletion. Decreases in both glycogen synthase (active and total) and phosphorylase (active and total) activities have been reported (Singh et al., 1986; Witek-Janusek, 1986).

(6) Alcoholism-induced auto-immune response in the mother may produce antibodies against proteins released by the

breakdown of brain tissue in severe alcoholics. If the mother became pregnant, her immune response may cross-react with components of the fetal brain (Foster, 1986). An alternative mechanism (Henderson et al., 1982) proposed that ethanol affects fetal growth by disturbing the systems which enable the developing embryo to accrue protein (by the protein synthetic machinery) and that this in turn retards cell replication and growth. Thus in utero exposure to ethanol may cause fetal defects by decreasing the net rate of protein synthesis. However, the observed decrease in protein synthesis in fetal rats and mice has been suggested to be an effect secondary to ethanol-induced maternal hypothermia (since, if the mothers were placed in an incubator at 37°C, the decrease in the rate of fetal-protein synthesis disappeared) rather than by direct effects on protein synthesis. Alterations in placental function and alterations in hormone balances (Colbern et al., 1985; Handa et al., 1985; Pohl et al., 1987) governing development at a cellular level may also be induced by ethanol.

One general mechanism that has been suggested for the induction of malformations in rodents is the disturbance of embryotrophic nutrition (Beck & Lloyd, 1968; Lloyd & Beck, 1968; Lloyd et al., 1974; Williams et al., 1976; Freeman et al., 1981; Freeman et al., 1982; Freeman & Lloyd, 1983a & b; Freeman & Lloyd, 1985; Lloyd et al., 1985; Freeman & Lloyd, 1986). After implantation in the uterine wall, the blastocyst differentiates into an inner-cell mass and a trophoblastic layer. (The inner-cell mass develops into the embryo proper and, in the rodent, the trophoblastic layer eventually gives rise to the visceral yolk-sac endoderm.) In the period before the chorioallantoic

placenta forms, embryotrophic nutrition is thought to supply the embryo with amino acids required for protein synthesis (Freeman et al., 1981) by pinocytosis and intralysosomal proteolysis within the yolk sac. (The nutrients are obtained from the plasma transudate surrounding the implanted "egg cylinder".) The amino acids so generated pass to the developing embryo (Freeman & Lloyd, 1983a) for protein synthesis. If this process is disturbed at either the level of pinocytosis (Williams et al., 1976; Freeman et al., 1983 or proteolysis (Freeman & Lloyd, 1983b; Freeman & Lloyd, 1986), the resulting partial or transient inhibition of intralysosomal proteolysis will result in starvation of the developing embryo thus causing manformations. (The transport of nutrients to the embryo before the formation of a vitelline circulation in the yolk sac would be by diffusion through a system of intercellular spaces which pervades the underlying mesenchyme and is continuous with the extra-embryonic coelom.)

As shown earlier (Webster et al., 1980; 1983 & 1984), the administration of ethanol, on days of gestation before the formation of the chorioallantoic placenta, caused malformations. Thus ethanol exposure at critical periods of organogenesis may cause a disruption of embryotrophic nutrition and this may be the mechanism of ethanol action in inducing the FAS. The main aim of the work reported in this chapter was to seek experimental support for the suggestion that ethanol may disturb normal development in the rat by interfering in the process of embryotrophic nutrition.

4.2 MATERIALS AND METHODS

All the materials and equipment used in this chapter were as described in Section 2.1. The medium usually used was 199 alone; if calf serum was present, it was at a concentration of 10% (v/v) as was the fetal-calf serum. All uptake experiments were performed as described in Section 2.3 and assays as described in Section 2.4. [^{125}I]fd BSA (preparation I, II or III) was used at a concentration of 5 µg/ml. The agent was added at time zero (t_0) directly following the addition of the radiolabelled substrate. The pattern of agent addition and ^{125}I-labelled substrate concentrations employed were as described in the text and the legends to figures.

The only new method used was that for studying the uptake of [^{125}I]fd BSA following ethanol pretreatment. In this case the only difference between the methods (see Section 2.10) was that the time points of sampling were 1.5, 2.0, 2.5, 3.0 and 4.0 h, respectively.

4.3 RESULTS

4.3.1 The effects of ethanol on uptake and intralysosomal proteolysis of [^{125}I]fd BSA

In contrast to [^{125}I]PVP, individual [^{125}I]fd BSA preparations have previously been shown to exhibit different rates of uptake in the rat visceral yolk sac (Ibbotson & Williams, 1979), probably as a result of differing degrees of denaturation (see Moore et al., 1977). In the current study, the absolute uptake rates in both control and ethanol-treated tissues varied markedly between preparations; there were corresponding inter-batch differences in the quantities of tissue-associated radioactivity. Intra-batch variability was much less marked. Provided ethanol effects (on the rates of uptake and on the quantities of tissue-associated radioactivity) were expressed relative to corresponding data from matched (ethanol-free) controls, the patterns of data for the effects of ethanol were reproducible.

The effects of 2.0 mM and 10.0 mM ethanol on the uptake and intralysosomal degradation of [^{125}I]fd BSA (batches I & II) were investigated in serum-free medium (Fig. 4.3.1.1; Fig. 4.3.1.2) and in serum-containing medium (Fig. 4.3.1.3; Fig. 4.3.1.4; Fig. 4.3.1.5 & Fig. 4.3.1.6). In Fig. 4.3.1.1 the control shows that, in the absence of serum, the substrate was captured rapidly (by adsorptive-phase pinocytosis) and had an E.I. of 85.0 ± 2.1 µl/mg yolk-sac protein/h while the rates of capture by the tissue incubated in the presence of ethanol (2.0 mM and 10.0 mM) were only 18.4 ± 0.66 and 18.5 ± 0.53 µl/mg yolk-sac protein/h, respectively. The quantity of radioactivity associated with the control tissue (Fig. 4.3.1.2) shows the typical steady-state kinetics as reported by other workers (Williams et al., 1975b; Ibbotson & Williams, 1979; Livesey & Williams, 1981). However, the quantity of radioactivity associated with tissue exposed to either of the two ethanol concentrations rose linearly up to 3.0 h (Fig.

4.3.1.2) before eventually levelling off during the interval 3 - 4 h.

When equivalent experiments were performed, but with 10% (v/v) calf serum present in the incubation medium, a different pattern of data was obtained. The uptake of [^{125}I]fd BSA I in 10% (v/v) calf serum (Fig. 4.3.1.3) for the ethanol-free control gave an E.I. of 20.63 µl/mg yolk-sac protein/h, while that for 2.0 mM ethanol was 1.33 ± 0.03 and that for the 10.0 mM ethanol treatment, 0.05 ± 0.05 µl/mg yolk-sac protein/h. The decrease in the E.I. of the controls caused by the presence of 10% (v/v) calf serum was in agreement with the report of Forster & Williams (1984). The quantity of tissue-associated radioactivity in these experiments (Fig. 4.3.1.4) showed the typical steady-state pattern as seen in the serum-free experiments. However, in the 10.0 mM ethanol-treated tissue the quantity of tissue-associated radioactivity was very low (approximately 3% of the control value at the 5.0 h incubation point). In the case of the 2.0 mM ethanol-treated tissue, the tissue-associated radioactivity rose linearly during the 1.0 - 3.0 h incubation period, but then plateaued at approximately 18% of the control value at the 5.0 h incubation point.

Unfortunately, at this point Wellcome PLC stopped producing the heat-treated, newborn-calf serum that was routinely used, so an alternative serum preparation had to be substituted. It was decided to use fetal-calf serum. The patterns of uptake of [^{125}I]fd BSA (preparation II) in the presence of 10% (v/v) fetal-calf serum are shown in Figs. 4.3.1.5 & 4.3.1.6. The controls show that in 10% (v/v) fetal-calf serum [^{125}I]fd BSA II was captured rapidly and progressively with an E.I. of 66.5 ± 1.0 µl/mg yolk-sac protein/h. In the presence of the 2.0 mM and 10.0 mM ethanol, uptake was 2.4% and 1.2% of the control value at the 5.0 h incubation point, showing virtually no uptake of the substrate in the presence of either of

these ethanol concentrations (Fig. 4.3.1.5). The E.I. of 66.5 ± 1.0 µl/mg yolk-sac protein/h for [^{125}I]fd BSA II in 10% (v/v) fetal-calf serum compares well with the value reported by Moore et al. (1977) of 65.0 ± 11.0 µl/mg yolk-sac protein/h for heated calf serum (10%, v/v), showing that substitution of fetal-calf serum for newborn-calf serum has no effect on the uptake of [^{125}I]fd BSA. The low quantities of tissue-associated radioactivity (Fig. 4.3.1.6) indicate that very little substrate was associated with the tissue when both serum and ethanol were present. At the 5.0 h incubation point the 10.0 mM ethanol-treated tissue possessed only 0.60% of the radioactivity (expressed as µl/mg yolk-sac protein) of the control, but the 2.0 mM ethanol-treated tissue showed a figure of 3.95% of the control radioactivity at the same time point.

Since the effect of ethanol on fluid-phase pinocytosis had been shown to be reversible (see Chapter 3), it was decided to establish whether adsorptive-phase pinocytosis showed similar recovery following ethanol pre-treatment, by monitoring the production of TCA-soluble radioactivity by the yolk sac. Figs. 4.3.1.7 & 4.3.1.8 show the control recovery for each batch of albumin to be linear over a 4.0 h period, but the ethanol-exposed tissue showed a lag in recovery of approximately 0.75 h after exposure to 2.0 mM ethanol and 1.5 h for tissue exposed to 10.0 mM ethanol. Essentially the same results were obtained when these experiments were carried out with either [^{125}I]fd BSA preparation (see Figs. 4.3.1.7 & 4.3.1.8). An interesting point to note from these results was that ethanol-treated tissue showed an increased rate of production of TCA-soluble radioactivity above that of the matched control before the rate of production stabilised, by 3.5 h, to that of the control. A similar effect was seen in the equivalent recovery experiment with 10.0 mM ethanol (Fig. 3.3.1.6) which shows the rate of uptake of [^{125}I]PVP to

increase above that of the matched control before stabilising, within 3 h, at the same rate as the control. [A similar over-shoot effect was reported by Clark (1986) when the recovery of adsorptive-phase pinocytosis was studied following the exposure of yolk sacs to decreased temperature.]

As it has been reported that benzyl alcohol inhibits the fusion between lysosomes and endocytic vesicles in isolated hepatocytes (Tolleshaug & Berg, 1982), ethanol may induce a similar effect in yolk sacs. A series of experiments was therefore conducted in which the uptake by adsorptive-phase pinocytosis following ethanol pretreatment was studied. In Fig. 4.3.1.9, the uptake of [^{125}I]fd BSA III following 0.5 h pre-treatment in the presence or absence of ethanol can be seen. The control tissue showed that the substrate was captured rapidly and progressively at a rate of 362 ± 11 µl/mg yolk-sac protein/h which is similar to the value 382 ± 25 µl/mg yolk-sac protein/h reported for serum-free medium by Ibbotson & Williams (1979). The 2.0 mM ethanol-treated tissue also showed linear uptake with an E.I. of 386 ± 14 µl/mg yolk-sac protein/h, i.e. a relative rate of 106% with respect to the control. The 10.0 mM ethanol-treated tissue showed a linear response in its uptake of [^{125}I]fd BSA III.

The quantities of tissue-associated radioactiviy in the same recovery experiments are shown in Fig. 4.3.1.10. The mean quantity of tissue-associated radioactivity for the control tissue was 210 ± 25 µl/mg yolk-sac protein. But comparison of the quantities of tissue-associated radioactivity for the 2.0 mM and 10.0 mM ethanol-treated tissue with those of matched controls shows a marked

increase in tissue-associated radioactivity in ethanol-treated tissue. This increase was seen to reach a maximum by 2.5 h, before rapidly declining to a point just above that of the control (see Fig. 4.3.1.10). By 2.5 h the 10.0 mM ethanol-treated tissue had a mean relative value of tissue-associated radioactivity that was 226% with respect to matched controls, whereas the 2.0 mM ethanol-treated tissue showed an equivalent relative value of 168%. This observation indicates a transient and reversible increase in the level of tissue-associated radioactivity above that of the matched control. When the release of TCA-soluble radioactivity following 0.5 h ethanol pre-treatment was studied (see Fig. 4.3.1.11), the characteristic pattern of "lag phase" in the release of TCA-soluble radioactivity into the medium following 2.0 mM and 10.0 mM ethanol pre-treatment was seen, which compares favourably with those seen earlier (Figs. 4.3.1.7 & 4.3.1.8). The control tissue showed a constant rate of release of TCA-soluble radioactivity into the incubation medium during the period 1.5 h - 4.0 h. However, both the 2.0 mM- and 10.0 mM-treated tissue showed a lag in the release of TCA-soluble radioactivity before an increase in the rate of TCA-soluble radioactivity release was seen (between 2.0 h and 4.0 h) to a rate above that of the control tissue (Fig. 4.3.1.11).

In the final series of experiments, evidence for inhibition of adsorptive phase pinocytosis by ethanol *in vivo* was sought. In these experiments adult male Wistar rats were given an intraperitoneal injection of ethanol or of 1% aq. NaCl (controls); then, 15 or 45 min later, an intravenous injection of [^{125}I]fd BSA II. Samples of blood were removed (via foot pad incision) at various time intervals up to 1 h and analysed for TCA-insoluble and TCA-soluble radioactivity. The clearance of [^{125}I]fd BSA II from the bloodstream of control animals (see Fig. 4.3.1.12) was in agreement with Moore *et al.* (1977). Most

of the TCA-insoluble radioactivity was cleared from the bloodstream of control animals by 20 min. A similar pattern of clearance was seen when animals were given an intraperitoneal injection of ethanol 25% (v/v) either 15 min or 45 min previously (Figs. 4.3.1.13 & 4.3.1.14). When the time-course of appearance of TCA-soluble radioactivity in the bloodstream was examined (Fig. 4.3.1.15), control animals showed a "lag phase" of approximately 15 min before there was a rapid increase in TCA-soluble radioactivity in the blood which reached 90 - 100% by 1.0 h. Again, when the TCA-soluble radioactivity patterns for the rats given ethanol 15 min and 45 min prior to the administration of the substrate were studied (see Figs. 4.3.1.16 & 4.3.1.17), a maximum release of TCA-soluble radioactivity (following an initial "lag phase" of 15 min) by 1.0 h was seen. However, by 1.0 h the release from ethanol-exposed tissue was approximately 60 - 80% compared with 90 - 100% for the controls.

Fig. 4.3.1.1 The effect of ethanol on the uptake of [^{125}I]fd BSA I by yolk sacs incubated in serum-free medium

Yolk sacs were incubated separately in serum-free medium 199 (8.0 ml). At 0.0 h of incubation 1.0 ml of medium containing [^{125}I]fd BSA I was added which resulted in a substrate concentration of 5 µg/ml and 1.0 ml of medium containing ethanol (final concentration of 2.0 or 10.0 mM) was also added. Uptake of [^{125}I]fd BSA I was then monitored up to 5.0 h. Each point represents the mean uptake (± S.D.) by at least six yolk sacs each derived from a different pregnant animal.

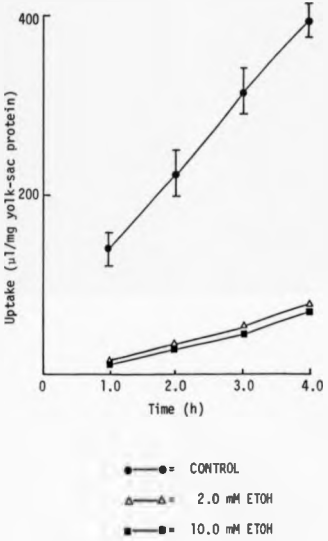


Fig. 4.3.1.2 The effect of ethanol on the quantity of tissue-associated radioactivity derived from [^{125}I]fd BSA I

The data reported are derived from the same experiments described in Fig. 4.3.1.1. Each point represents the mean quantity of tissue-associated radioactivity values (± S.D.) of at least six yolk sacs.

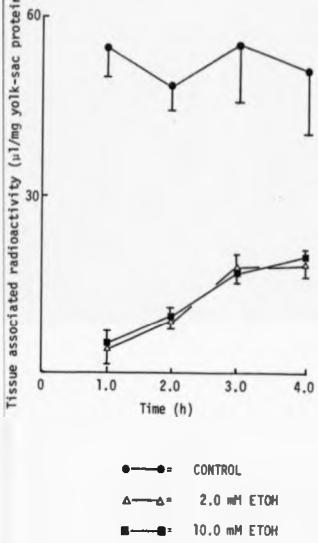


Fig. 4.3.1.3 The effect of ethanol on the uptake of [^{125}I]fd BSA I by yolk sacs when incubated in serum-containing medium

Yolk sacs were incubated separately in medium 199 (8.0 ml) containing 10% (v/v) calf serum. At 0.0 h of incubation 1.0 ml of medium containing [^{125}I]fd BSA I was added which resulted in a substrate concentration of 5 µg/ml and 1.0 ml of medium containing ethanol (final concentration of 2.0 or 10.0 mM) was also added. Uptake of [^{125}I]fd BSA I was then monitored up to 5.0 h. Each point represents the mean (± S.D.) by at least six yolk sacs each derived from a different pregnant animal.

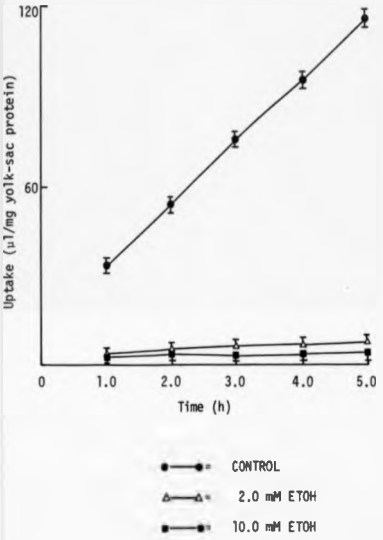


Fig. 4.3.1.4 The effect of ethanol on the quantity of tissue-associated radioactivity derived from [^{125}I]fd BSA I

The data reported are derived from the same experiments described in Fig. 4.3.1.3. Each point represents the mean tissue-associated radioactivity values (± S.D.) of at least six yolk sacs.

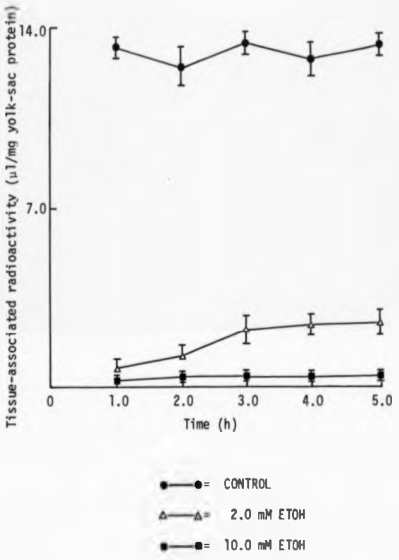


Fig. 4.3.1.5 The effect of ethanol on the uptake of [^{125}I]fd BSA II by yolk sacs incubated in medium containing fetal-calf serum

Yolk sacs were incubated separately in medium 199 (8.0 ml) containing 10% (v/v) fetal-calf serum. At 0.0 h of incubation 1.0 ml of medium containing [^{125}I]fd BSA II was added which resulted in a substrate concentration of 5 µg/ml and 1.0 ml of medium containing ethanol (final concentration of 2.0 or 10.0 mM) was also added. Uptake of [^{125}I]fd BSA II was then monitored up to 5.0 h. Each point represents the mean (± S.D.) by at least six yolk sacs each derived from a different pregnant animal.

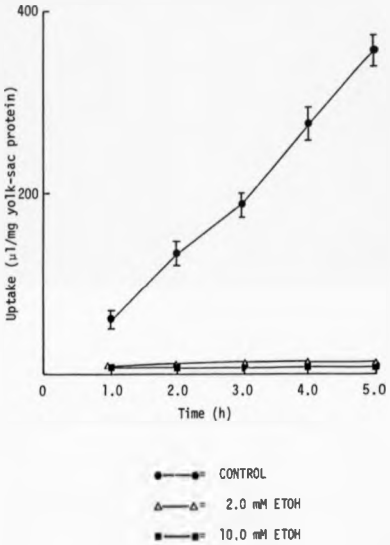


Fig. 4.3.1.6 The effect of ethanol on the quantity of tissue-associated radioactivity derived from [^{125}I]fd BSA II

The data reported are derived from the same experiments described in Fig. 4.3.1.5. Each point represents the mean tissue-associated radioactivity values (± S.D.) of at least six yolk sacs.

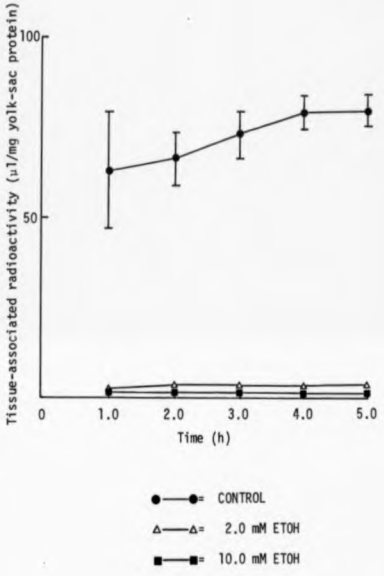


Fig. 4.3.1.7 The effect of ethanol pre-treatment of yolk sacs on the release of TCA-soluble radioactivity into the incubation medium

Yolk sacs were incubated separately (but in triplicate) in serum-free medium containing 2.0 or 10.0 mM ethanol for 2.5 h. The yolk sacs were then removed, rinsed (3 x 2.0 min) in fresh TC199 at 37°C and re-incubated in 14.0 ml of ethanol- and serum-free medium containing [^{125}I]fd BSA I (5 µg/ml) for 2.75 h. Each point represents the mean value (± S.D.) of at least nine yolk sacs, derived from at least three different animals. (The results reported here have been normalized to account for radioactivity decay.)

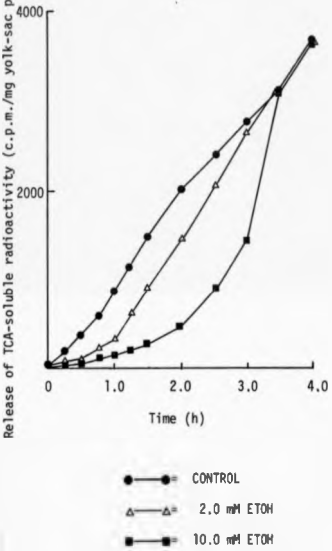


Fig. 4.3.1.8 The effect of ethanol pre-treatment of yolk sacs on the release of TCA-soluble radioactivity into the incubation medium

Yolk sacs were incubated separately (but in triplicate) in serum-free medium containing 2.0 or 10.0 mM ethanol for 2.5 h. The yolk sacs were then removed, rinsed (3 x 2.0 min) in fresh TC199 at 37°C and re-incubated in 14.0 ml of ethanol- and serum-free medium containing [^{125}I]fd BSA II (5 µg/ml) for 2.75 h. Each point represents the mean (± S.D.) of at least nine yolk sacs, derived from at least three different animals. (The results reported here have been normalized to account for radioactivity decay.)

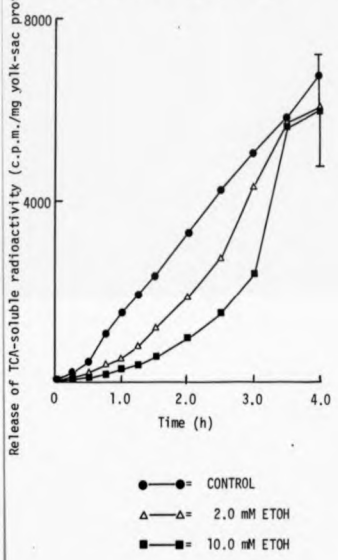


Fig. 4.3.1.9 The effect of ethanol pre-treatment of yolk sacs on the uptake of [^{125}I]fd BSA III

Yolk sacs were incubated separately in serum-free medium 199 containing ethanol (2.0 or 10.0 mM) for 0.5 h. The yolk sacs were removed, rinsed (3 x 2.0 min) in fresh TC199 at 37°C and re-incubated in serum- and ethanol-free medium containing [^{125}I]fd BSA III (5 µg/ml) for 4.0 h. Each point represents the mean (± S.D.) of at least six yolk sacs, derived from at least 2 pregnant animals.

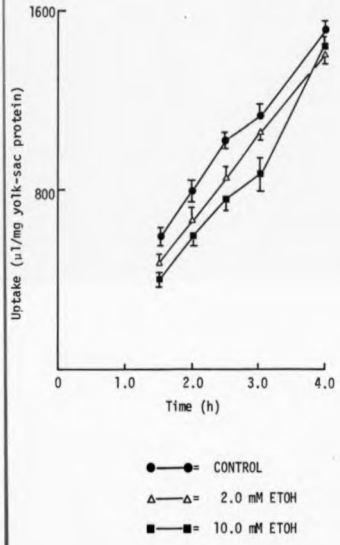


Fig. 4.3.1.10 The effect of ethanol pre-treatment of yolk sacs on the quantity of tissue-associated radioactivity derived from [^{125}I]fd BSA III

Yolk sacs were incubated as in Fig. 4.3.1.9. Each point represents the mean (± S.D.) quantity of tissue-associated radioactivity from at least six yolk sacs.

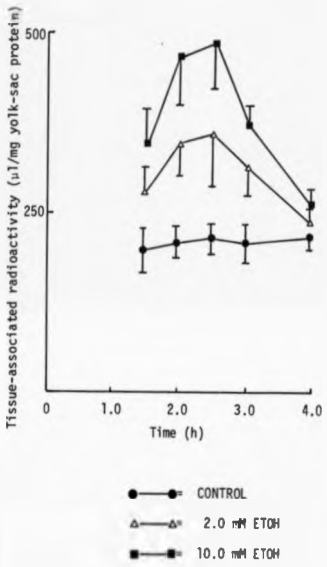


Fig. 4.3.1.11 The effect of ethanol pre-treatment of yolk sacs on the release of TCA-soluble radioactivity into the incubation medium

Yolk sacs were incubated as in Fig. 4.3.1.9. Each point represents the mean (± S.D.) quantity of TCA-soluble radioactivity released into the medium from at least six yolk sacs.

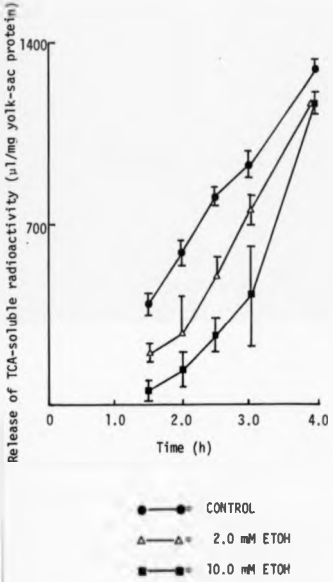


Fig. 4.3.1.12 Clearance of [^{125}I]fd BSA II from the bloodstream of a non-pregnant adult rat

Control rats were given an intraperitoneal injection of 1% (w/v) NaCl (0.022 ml/g body weight) and 15 min later an intravenous injection of [^{125}I]fd BSA II (150 µg). Blood samples were then taken over a 60 min period. Each graph represents data derived from one animal.

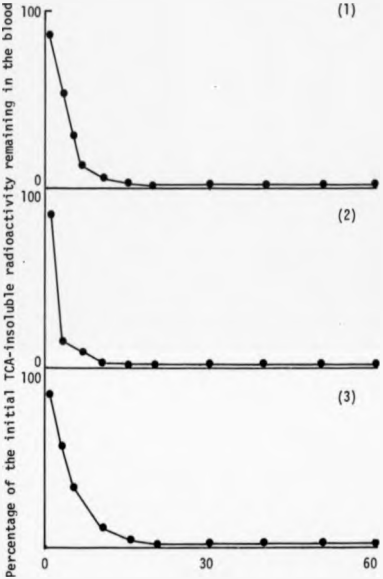


Fig. 4.3.1.13 Clearance of [^{125}I]fd BSA II from the bloodstream of an ethanol-treated adult rat

Rats were given an intraperitoneal injection of 25% (v/v) ethanol in physiological saline (0.022 ml/g body weight) and 15 min later an intravenous injection of [^{125}I]fd BSA II (150 µg). Blood samples were taken over a 60 min period. Each graph represents data derived from one animal.

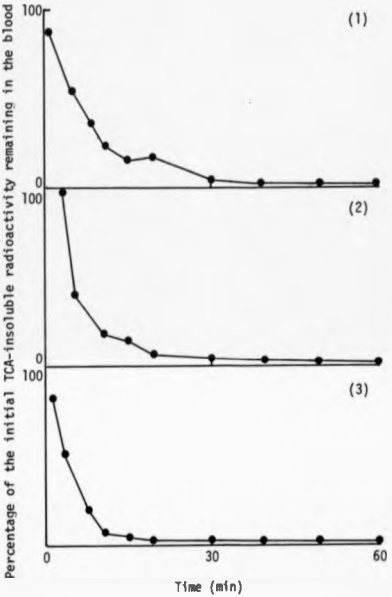


Fig. 4.3.1.14 Clearance of [^{125}I]fd BSA II from the bloodstream of an ethanol-treated adult rat

Rats were given an intraperitoneal injection of 25% (v/v) ethanol in physiological saline (0.022 ml/g body weight) and 45 min later an intravenous injection of [^{125}I]fd BSA II (150 µg). Blood samples were taken over a 60 min period. Each graph represents data derived from one animal.

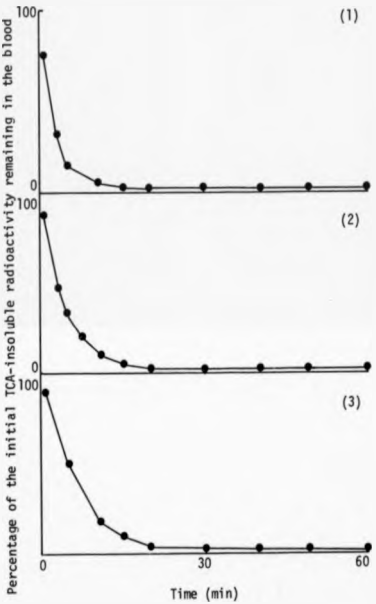


Fig. 4.3.1.15 Release of TCA-soluble radioactivity into the bloodstream of an untreated adult rat

The blood samples obtained in the experiments reported in Fig. 4.3.1.12 were analysed for their content of TCA-soluble radioactivity, the data normalized for counting geometry and reported in the same format as the data for TCA-insoluble radioactivity.

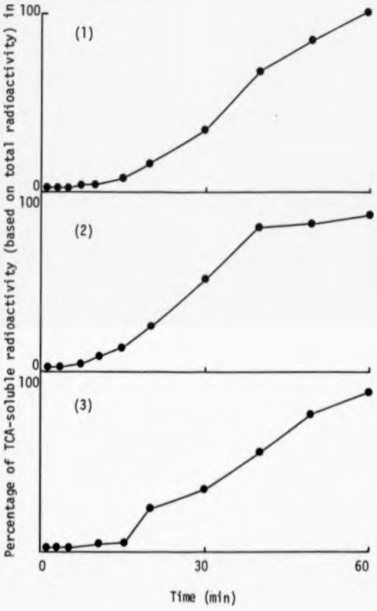


Fig. 4.3.1.16 Release of TCA-soluble radioactivity into the bloodstream of an ethanol-treated adult rat

The blood samples obtained in the experiments reported in Fig. 4.3.1.13 were analysed for their contained TCA-soluble radioactivity, the data normalized for counting geometry and reported in the same format as the data for TCA-insoluble radioactivity.

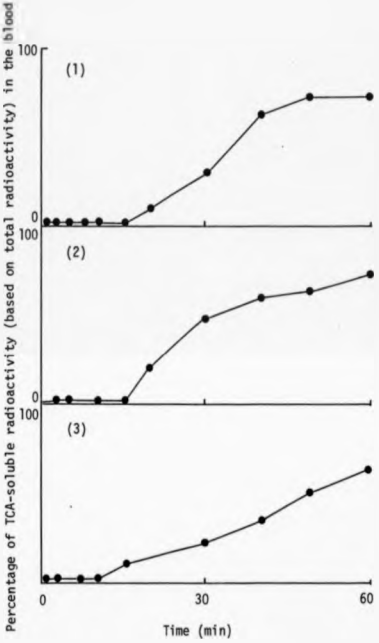
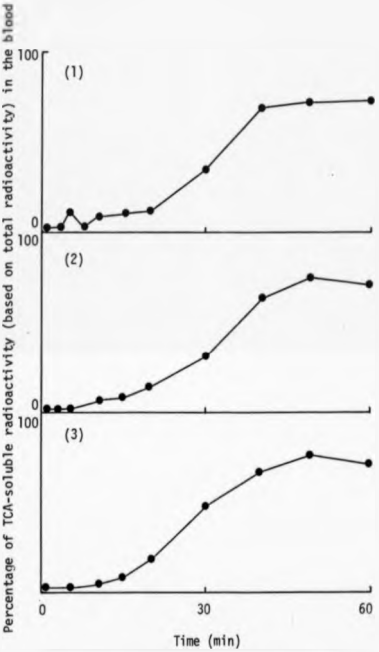


Fig. 4.3.1.17 Release of TCA-soluble radioactivity into the bloodstream of an ethanol-treated adult rat

The blood samples obtained in the experiments reported in Fig. 4.3.1.14 were analysed for their contained TCA-soluble radioactivity, the data normalized for counting geometry and reported in the same format as the data for TCA-insoluble radioactivity.



4.4 DISCUSSION

In this chapter the effects of ethanol on adsorptive-phase pinocytosis *in vitro* and *in vivo* were studied. The first series of experiments examined the uptake and intralysosomal degradation of different batches of [^{125}I]fd BSA. Both 2.0 mM and 10.0 mM ethanol caused rapid inhibition of adsorptive-phase pinocytosis (Fig. 4.3.1.1), although the inhibition produced was not as complete as seen in the case of fluid-phase pinocytosis (Fig. 3.3.1.2). The results presented in Figs. 4.3.1.1 & 4.3.1.2 pose a problem. How can fluid-phase pinocytosis of [^{125}I]PVP be completely inhibited at the higher of the two ethanol concentrations used (Fig. 3.3.1.2) when, under these conditions, adsorptive-phase pinocytosis still occurs, albeit at 21 - 22% of the rate of controls (Fig. 4.3.1.1)? One answer could be that ethanol causes the labelled albumin to bind to the yolk-sac surface, as seen in the case of hepatocytes with other protein substrates (Nunes *et al.*, 1984; Dave *et al.*, 1985), and what is reported as uptake is, in fact, external binding without internalisation. [The rat yolk sac has been shown to bind formaldehyde-treated bovine serum albumin to hydrophobic binding sites on the outer surface of the plasma membrane (Livesey & Williams, 1982).]

It was reported by Casey *et al.* (1987) that ethanol caused significantly less ligand to be bound, internalised and degraded by hepatocytes isolated from chronically ethanol-fed rats. The decreased binding was a result of a decrease in the number of cell-surface receptors for [^{125}I]asialo-orosomucoid rather than the result of a lowered affinity of the receptor. However, the above results are not in accord with those of Sharma & Grant (1986) who found that isolated hepatocytes from chronically ethanol-fed rats showed a decrease in the endocytic rate for

[^{125}I]-D-galactosyl-β-bovine serum albumin and not a decrease in the number of cell-surface receptors or a change in receptor affinity.

To try to substantiate the suggestion that ethanol causes [^{125}I]fd BSA preparations to bind externally without being internalised, a series of experiments was performed with [^{125}I]fd BSA I in the presence of 10% (v/v) calf serum and [^{125}I]fd BSA II in the presence of 10% (v/v) fetal-calf serum. The uptake rates in serum-containing control experiments were decreased relative to those from equivalent serum-free controls (see Figs. 4.3.1.3 & 4.3.1.4) in agreement with the findings of Forster & Williams (1984) and, in the case of the controls in 10% (v/v) fetal-calf serum, with the results of Moore et al. (1977) (see Figs. 4.3.1.5 & 4.3.1.6). However, the relative rates of uptake of [^{125}I]fd BSA in the presence of 10% (v/v) calf serum and ethanol as well as those of [^{125}I]fd BSA in the presence of 10% (v/v) fetal-calf serum and ethanol at both concentrations were very small indeed relative to matched controls (see Figs. 4.3.1.3 & 4.3.1.5). Also, the small accumulation of radioactivity within the tissue relative to matched controls (Figs. 4.3.1.4 & 4.3.1.6) showed that pinocytosis was strongly inhibited, indicating that adsorptive-phase pinocytosis like fluid-phase pinocytosis was fully inhibited by these concentrations of ethanol.

As well as being a rapid and effective inhibitor of fluid-phase pinocytosis, ethanol inhibition of fluid-phase pinocytosis was found to be reversible (see Chapter 3). Similarly, the production of TCA-soluble radioactivity after exposure of tissue to ethanol (see Figs. 4.3.1.7 & 4.3.1.8) showed lag periods of approximately 1.5 h for tissue exposed to 10.0 mM ethanol, and of approximately 0.75 h for tissue exposed to 2.0 mM ethanol, before regaining by 3.0 h the same rate of production of TCA-soluble radioactivity as controls. In the

above studies, pinocytic function (i.e. both fluid-phase pinocytosis and TCA-soluble radioactivity production) after ethanol pre-treatment of the tissue, showed a lag period before recovering and also showed a period of either increased pinocytic rate (Fig. 3.3.1.6) or of increased production of TCA-soluble radioactivity (Figs. 4.3.1.7 & 4.3.1.8) above that of the controls. Similarly, Clark (1986) observed an overshoot in the rate of release of TCA-soluble radioactivity following recovery of yolk sacs exposed to low temperature ($6^{o}C$). Again a "lag phase" in TCA-soluble radioactivity release was observed when compared with the matched controls. This was followed by a period of enhanced rate of release of TCA-soluble radioactivity.

The recovery of fluid-phase pinocytosis of [^{125}I]PVP following 2.0 mM ethanol pre-treatment (Fig. 3.3.1.4) was immediate and complete. In contrast, the equivalent experiments with labelled albumin show recovery of TCA-soluble production following 2.0 mM ethanol to be subject to a lag period of approximately 0.75 h (Figs. 4.3.1.7 & 4.3.1.8). This difference suggests ethanol is possibly having effects on both pinocytosis (fluid-phase and adsorptive-phase) and on the processes that lead to the production of TCA-soluble radioactivity. To investigate the second possibility further, the recovery of adsorptive-phase pinocytosis was investigated in greater depth. The recovery of [^{125}I]fd BSA III uptake following ethanol pre-treatment (Fig. 4.3.1.9) showed a similarity to the recovery of [^{125}I]PVP uptake following ethanol pre-treatment (Fig. 3.3.1.4). In each case the 2.0 mM ethanol-treated tissue showed immediate and complete recovery of rates to values comparable with those of the matched controls. The 10.0 mM ethanol-treated tissue (in both the fluid-phase and adsorptive-phase pinocytosis experiments) showed a "lag period" in recovery before fully regaining its pinocytic activity. However, when the quantities of tissue-associated

radioactivity were compared (Fig. 4.3.1.10), it was obvious that both concentrations of ethanol (10.0 mM and 2.0 mM) caused an increase in the amount of tissue-associated radioactivity above that of the controls before these tissue levels rapidly decreased to the control values. Similarly, the time-courses of TCA-soluble radioactivity production (Fig. 4.3.1.11) show a "lag period" for the ethanol-treated tissue (which corresponds to the time periods when the ethanol-treated tissue shows an increased level of tissue-associated radioactivity above that of the matched controls) before a period of TCA-soluble radioactivity release at a rate above that of the matched controls (Figs. 4.3.1.7 & 4.3.1.8). This increased tissue accumulation above that of the controls suggests that one of the effects of ethanol was either on pinosome-lysosome fusion (Tolleshaug & Berg, 1981) or on intra-lysosomal proteolysis. However, the transient increase in the rate of pinocytosis of [^{125}I]PVP (Fig. 3.3.1.6) following ethanol pre-treatment must be due to an increase in the rate of pinosome formation. (The basis of this effect has yet to be established.) It should also be noted that ethanol (Baraona et al., 1979) caused a decrease in the volume density of microtubules per hepatocyte, and this inhibitory effect of ethanol on tubulin polymerisation may explain the increased quantities of tissue-associated radioactivity in yolk sacs (Fig. 4.3.1.10) through inhibition of endosomal-vesicle movements from the peripheral region to the perinuclear region of the cell. But, once the effects of the ethanol pre-treatment are removed, an increase in the rate of pinosome-lysosome fusion could explain the increased rates of TCA-soluble radioactivity production. The relative increase in the rate of release of TCA-soluble radioactivity was greater than could be explained by an equivalent increase in uptake alone. This suggests an increase in the rate of proteolysis within the lysosomes (Figs. 4.3.1.7, 4.3.1.8 & 4.3.1.11).

Since ethanol, in an acute dose, rapidly inhibited pinocytosis *in vitro*, an acute dose of ethanol *in vivo* might also inhibit adsorptive-phase pinocytosis by the liver. As can be seen (Figs. 4.3.1.12, 4.3.1.13 & 4.3.1.14) ethanol administered 15 min or 45 min before 150 µg of [^{125}I]fd BSA II was injected intravenously, had no effect on the clearance of [^{125}I]fd BSA II from the bloodstream by the liver. [The rate of clearance of [^{125}I]fd BSA was in agreement with Buys *et al*. (1973) & Moore *et al*. (1977).] But a small difference was observed (Figs. 4.3.1.15, 4.3.1.16 & 4.3.1.17) in the time-course of appearance of TCA-soluble radioactivity into the bloodstream. Abrams & Cooper (1976) showed that circulating serum ethanol concentrations reached a peak 45 - 60 min after an intraperitoneal injection of a 20% (v/v) ethanol solution. Thus, [^{125}I]fd BSA II administration (15 - 45 min after the ethanol) would coincide with the serum-ethanol concentration being close to its peak value. However, neither regime resulted in a real difference in the uptake and degradation of [^{125}I]fd BSA II. (The marginally lower concentrations of TCA-soluble radioactivity in the bloodstream of ethanol-treated animals could possibly reflect a lowered rate of catabolism of albumin in the liver, but could equally well result from the diuretic effect of ethanol causing an enhanced rate of clearance of TCA-soluble fragments by the kidney.) A similar result was also observed in the isolated perfused rat liver (Poso *et al*., 1987), in which the uptake and intralysosomal degradation of [^{125}I]-asialofetuin was only marginally affected by the addition of 50 mM ethanol. In another study, Okanoue *et al*. (1984) found that ethanol administration (5g per kg body wt.), as a 20% (w/v) solution by gastric tube 2 h prior to the injection of horseradish peroxidase, had no effect on the uptake of the substrate by the liver. It appears from these experiments that the liver, *in vivo*, is not as sensitive as the visceral yolk sac, *in vitro*, to the effects of acute

ethanol administration on adsorptive-phase pinocytosis and intralysosomal degradation.

The effects of ethanol on rat embryos *in vitro* have been studied (Brown *et al.*, 1979; Beck *et al.*, 1984) and both studies found that ethanol was teratogenic and growth retarding. In the first study ethanol was used at concentrations of 300 mg/100 ml and 150 mg/100 ml of culture medium, while, in the second, ethanol was used at 120 mg/100 ml and 115 mg/100 ml of culture medium. Blood concentrations of ethanol in mice after acute exposure to ethanol (Webster *et al.*, 1980 & 1983) were reported to be 500 - 600 mg/100 ml, 4.5 h after the initial injection of ethanol but only 50 mg/100 ml after 12 h. It was reported (Abel & Greizerstein, 1980) that when pregnant rats (gestation age, 16 - 21 days) were intubated with 6g/kg of ethanol (30%, w/v), the maternal-blood ethanol concentrations 1.0 h and 4.5 h later were 236 mg/100 ml and 194 mg/100 ml, respectively. However, the ethanol concentrations in the amniotic fluid at the two time points were 243 mg/100 ml and 198 mg/100 ml and fetal-blood ethanol concentrations at 1.0 h and 4.5 h were 293 mg/100 ml and 208 mg/100 ml. It was also reported that blood-ethanol concentrations were significantly higher in 16 - 17 day fetuses at 1.0 h and 4.5 h compared with 20 - 21 day fetuses.

The concentrations of ethanol used in this study were 50 mg/100 ml (10.0 mM) and 10 mg/100 ml (2.0 mM) respectively. Thus the *in vitro* and *in vivo* concentrations of ethanol employed by other workers are far greater than the concentrations of ethanol in the medium in these *in vitro* experiments. However, blood-ethanol concentrations of between 500 - 700 mg/100 ml have been recorded in patients in a casualty ward at the Sahigren's Hospital (Webster *et al.*, 1983). The possibility that either a maternal or fetal metabolite, produced via the detoxification of ethanol, may be the

active compound has been investigated. The effects of acetaldehyde and butan-2,3diol (Veech et al., 1981) were studied by Priscott (1985) on rat embryos in vitro. The results showed that 25 mM butan-2,3diol and 100 μM and 200 μM acetaldehyde had no effect on embryonic development, but that 800 μM acetaldehyde was overtly embryotoxic rapidly causing death. In vivo, one or two doses of 4% (w/v) acetaldehyde (administered intraperitoneally) induced teratogenesis in mice (Webster et al., 1983).

Evidence that the yolk sac produces amino acids for embryonic development via intralysosomal proteolysis of pinocytosed proteins was obtained by Freeman et al. (1981) and Freeman & Lloyd (1983). Also, Freeman et al. (1982) provided evidence that teratogenic anti-rat visceral yolk-sac antiserum decreased pinocytosis of protein by the visceral yolk sac and thus decreased the availability of amino acids for protein synthesis in both the yolk sac and embryo in early organogenesis-stage rat embryos.

All the work reported in this chapter deals with rat visceral yolk sacs at 17.5 days of gestation. It would be of interest to decrease the gestational age of the visceral yolk sacs to see if ethanol-induced inhibition of pinocytosis varies with gestational age. This would better permit an evaluation of the relevance of the above observations to the mechanism of action of ethanol as a teratogen.

In conclusion, the results described in this chapter indicate that:

i) Ethanol strongly and rapidly inhibits adsorptive-phase pinocytosis in the visceral yolk sac.

ii) The inhibition is readily reversible since both adsorptive-phase pinocytosis and the rate of release of TCA-soluble radioactivity recovered following ethanol pre-treatment.

iii) The inhibition of adsorptive-phase pinocytosis was accompanied by either: (a) inhibition of lysosomal enzymes, or by: (b) inhibition of pinosome-lysosome fusion.

iv) The rat visceral yolk sac *in vitro* is more sensitive than the adult rat liver *in vivo* to the acute effects of ethanol on adsorptive phase pinocytosis.

v) It would be interesting to extend these experiments to earlier stages of gestation.

CHAPTER FIVE

THE EFFECTS OF GESTATIONAL AGE OF YOLK SACS ON ETHANOL-INDUCED DYSFUNCTION

5.1 INTRODUCTION

Although the ultrastructure of the yolk sac is, in many respects, qualitatively similar at 8.5 days and 17.5 days of gestation (Beck, unpublished work), extrapolation of findings with 17.5-day tissue to events at 8.5 days is somewhat speculative. Thus the work reported in this chapter extends the direct investigation of yolk-sac function to earlier ages of gestation.

In the rat, the chorioallantoic placenta probably begins to contribute to embryonic nutrition from the time of formation of the placental blood circulation (17-somite stage/11 days of gestation, see New, 1978), but organogenesis has already started by day 8 (see Fig. 5.1.1). The early evidence for regarding the visceral layer of the yolk sac as contributing to pre-placental nutrition in the rat (see Beck *et al.*, 1967; Beck & Lloyd, 1968) was obtained using horseradish peroxidase (injected intravenously into rats on 8.5, 9.5, 10.5, 11.5, 12.5 and 20.5 days of gestation). The enzyme was found to localise predominantly in the visceral yolk-sac endoderm. The same vesicles in this tissue also gave a positive acid-phosphatase reaction, suggesting that the exogenous protein enters secondary lysosomes. The requirement of serum for the normal development of rat embryos *in vitro* was reported by New (1978). [Embryos cultured in medium containing no serum show poor growth and development.] The role of the yolk sac in embryotrophic nutrition has since been more convincingly demonstrated by Freeman *et al.* (1981) and by Freeman & Lloyd (1983a & b) for the period of 9.5 to 11.5 days of gestation in the rat. The visceral yolk sac appears to play a focal role by the pinocytic capture and intralysosomal degradation of serum proteins to provide amino-acid monomers that are incorporated into the developing embryo.

Other studies have shown that, when the rat visceral yolk sac

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was incubated *in vitro* with [^{125}I]IgG (Williams & Ibbotson, 1979), the fate of the protein within the yolk sacs was atypical, since a significant release of tissue-associated [^{125}I]IgG in a macromolecular form was observed. Moreover, this capacity appeared to be more developed in 17.5-day tissue compared with 15.5-day tissue. This suggested the maturation of a transport system towards term for the pre-natal transfer of passive immunity from mother to fetus (Wild, 1975). Possibly there is an associated change in the pinocytic and proteolytic capacities of the tissue.

A number of studies have investigated endocytosis in the rat yolk sac at earlier stages of gestation. Thus the isolated visceral yolk sac has been investigated *in vitro* at 11.5 days (Marlow & Freeman, 1987) and pinocytic activity has been studied in the whole post-implantation stage rat conceptus *in vitro* at 10.5 days (Freeman *et al.*, 1981). Moreover, *in vivo*, Beck *et al.* (1967) reported the endocytosis of horseradish peroxidase at 8.5 days of gestation in the rat at the "egg cylinder" stage of development. Fleming (1986) has discussed the maturation of the endocytic organelles in the mouse embryo at the late cleavage stage (8 - 16 cell stage) and at the early blastocyst stage (>32-cell stage). At the 8-cell stage (2.5 days post-fertilization) the principal events, in compaction and polarization of cytocortical and cytoplasmic constituents, included the restriction of microvilli to the apical surface and a redistribution of endocytic organelles from a symmetrical location in the cortical cytoplasm to an apical polar cluster above the nucleus in the perinuclear region of the cytoplasm. But, endocytic activity occurred only after compaction and the organelles involved lacked cytochemical reactivity for acid phosphatase and for aryl sulphatase although electron-dense markers were rapidly ingested. When the 8-cell stage progressed to the 16-cell stage (3 days

post-fertilization), a definitive lysosomal compartment developed which was acid-phosphatase positive and participated both in the degradation of endocytosed substances and in the autolysis of storage granules. Both horseradish peroxidase and fluorescein isothiocyanate-labelled concanavalin-A were used as substrates to follow the endocytic processes from the 8-cell to the 16-cell stage of development.

It has been shown (Beck et al., 1967) that horseradish peroxidase and trypan blue, injected intravenously before 16 days of gestation in the rat, can accumulate in the visceral yolk sac without any significant accumulation in the other extra-embryonic membranes. These observations were supported by Jollie (1986) who reported that ferritin traversed the trophoblast cells by transcytosis and passed directly through Reichert's membrane. A full list of the various receptors found on the extraembryonic membranes and their functions is found in Table 5.1.2.

The effects of ethanol during the gastrulation stage of embryogenesis in the mouse have been investigated (Sulik et al., 1981). A 25% (v/v) ethanol solution (given intraperitoneally at 7 days 0 hours and again 4 h later) produced craniofacial abnormalities in mouse embryos closely resembling those seen in FAS. It was concluded that decreased development of the neural plate and its derivatives accounted for the craniofacial malformations seen. A further study (Sulik & Johnston, 1983) showed that ethanol administration (Sulik et al., 1981) caused obvious size reduction in the neural plate which was particularly noticeable in the forebrain region as detected by scanning electron-microscopic analysis of embryos at 8.0 days of gestation. Also, cleared skeletal preparations showed a marked premaxillary bone deficiency. Thus, an acute insult of ethanol during the time that the anterior neural plate was forming

Table 5.1.2 The receptors/functions of various extraembryonic membranes

The receptors, enzymes and functions of the extraembryonic membranes in various mammals are listed below, together with the method of detection and the workers responsible for their discovery.

Species	Extraembryonic Membrane	Receptor/Function	Method of Detection	Workers
Rat	Parietal Yolk Sac (Day 14.0 of gestation)	Biosynthesis of Basement Membrane Collagen and Glycoprotein	Incorporation of radiolabelled precursors	Clark et al., 1975
Rat	Parietal Yolk Sac (Day 14.5 of gestation)	Biosynthesis of Proteoglycans	Incorporation of radiolabelled precursors	Iozzo & Clark, 1986
Rat	Parietal Yolk Sac (Day 13.5 & 16.5 of gestation)	Glucocorticoid Receptor Progesterone Receptor	Ligand Binding to receptors	Carbone et al., 1986
Rat	Parietal Yolk Sac (Days 10.5 to 14.5 of gestation)	Δ^5, 3β-Hydroxysteroid dehydrogenase activity	Enzyme Studies	Sheth et al., 1978

Table 5.1.2 (continued)

Species	Extraembryonic Membrane	Receptor/Function	Method of Detection	Workers
Rat	Visceral Yolk Sac (Days 9.5 to 20.5 of gestation)	Progesterone Metabolism	Enzyme Studies	Sheth *et al.*, 1982
Rat	Visceral Yolk Sac (Day 13.5 & 16.5 of gestation)	Glucocorticoid Receptor	Ligand Binding to receptor	Carbone *et al.*, 1986
Rat	Visceral Yolk Sac (Day 15.0 & 18.0 of gestation)	Cytosolic 1,25-Dihydroxyvitamin D_3 receptor protein	Ligand Binding to receptor	Danan *et al.*, 1981
Rat	Visceral Yolk Sac (Day 18.0, 19.0 & 20.0 of gestation)	25-hydroxyvitamin D_3 & 1,25-dihydroxyvitamin D_3 24-hydroxylase activity	Enzyme Studies	Danan *et al.*, 1982
Rat	Visceral Yolk Sac (Days 13.5 to 21.5 of gestation)	Parathyroid Hormone Receptor	Ligand Binding to receptor	Gügi *et al.*, 1986

Table 5.1.2 (continued)

Species	Extraembryonic Membrane	Receptor/Function	Method of Detection	Workers
Rat	Visceral Yolk Sac (Day 10.4 & 11.6 of gestation)	Insulin Receptors	Ligand Binding to receptor	Unterman et al., 1986
Rat	Visceral Yolk Sac (Day 17.0 of gestation)	Cytosolic Ceruloplasmin	cDNA probe to mRNA	Aldred et al., 1987
Rat	Visceral Yolk Sac (Days 14.0, 16.0, 18.0 & 20.0 of gestation)	Cytosolic Retinol-Binding Protein and Transthyretin	cDNA probe to mRNA	Soprano et al., 1986
Rat	Visceral Yolk Sac (Days 12.0, 14.0, 16.0 & 18.0 of gestation)	Cytosolic Insulin mRNA Cytosolic pro-Insulin and Insulin	cDNA probe to mRNA Radioimmunoassay	Muglia & Locker, 1984
Mouse	Visceral Yolk Sac (Days 9.5, 11.5, 13.5, 15.5 & 17.5 of gestation)	Synthesis and Secretion of α-Fetoprotein and Transferrin	Radiolabelled precursor incorporation into proteins & cDNA probe to mRNA	Janzen et al., 1982

Table 5.1.2 (continued)

Species	Extraembryonic Membrane	Receptor/Function	Method of Detection	Workers
Mouse	Visceral Yolk Sac (Day 10.5 of gestation)	Synthesis and Secretion of Apolipoprotein AI and Low Density Lipoprotein	Metabolic Labelling and Immunoprecipitation and Immunolocalisation	Shi & Heath, 1984
Human	Secondary Fetal Yolk Sac (Weeks 5 - 11 of gestation)	Synthesis and Secretion of Apolipoprotein AI & B, α-Fetoprotein, Albumin & Transferrin	Metabolic Labelling and Immunoprecipitation and Immunolocalisation	Shi *et al.*, 1985
Human	Secondary Fetal Yolk Sac Endodermal Cells (Weeks 6 - 12 of gestation)	Synthesis and Secretion of Apolipoprotein B 100	Metabolic Labelling and Immunoprecipitation	Hopkins *et al.*, 1987

(during gastrulation) in the mouse results in abnormal development of the brain. Finally, it was concluded (Sulik, 1984) that ethanol interference with the rapid proliferation required to establish the mesoderm-cell layer is responsible for the craniofacial abnormalities; a similar observation was reported by Nakatsuji & Johnson (1984) in mouse embryos.

The work reported in this chapter describes an *in vitro* investigation of ethanol-induced inhibition of fluid-phase pinocytosis in yolk sacs at 13.5, 15.5 and 19.5 days of gestation and of ethanol-induced inhibition of adsorptive-phase pinocytosis in 13.5-day yolk sacs to see whether tissues at these earlier gestational ages respond differently relative to 17.5-day tissue.

5.2 MATERIALS AND METHODS

The materials and methods used were as described in Chapter 2. The only difference being the precise method of dissection of the yolk sacs at 13.5 and 15.5 days of gestation. The method given in Section 2.3 was followed, except that both Reichert's membrane and the parietal yolk sac must first be dissected away before the placental cap can be removed from the visceral yolk sac.

5.3 RESULTS

5.3.1 The effects of ethanol on fluid-phase pinocytosis in 13.5 - 19.5-day yolk sacs

The effect of decreasing gestational age of the rat visceral yolk sac on fluid-phase pinocytosis can be seen in Table 5.3.1.1a. At 19.5 days of gestation the rate of uptake of [^{125}I]PVP in control tissue was 2.99 ± 0.01 µl/mg yolk-sac protein/h, an increase in [^{125}I]PVP uptake of 112% relative to that for 17.5-day tissue. But the rate of [^{125}I]PVP uptake at 15.5 days was 2.29 ± 0.01 µl/mg yolk-sac protein/h, only 86% of that at 17.5 days. Finally, when the rate of uptake of [^{125}I]PVP at 13.5 days of gestation was determined, it showed a value of 1.61 ± 0.02 µl/mg yolk-sac protein/h, only 60% of the rate of uptake by 17.5 day yolk sacs.

The time courses of uptake for yolk sacs at each stage of gestation (i.e. days 13.5, 15.5, 19.5) are shown in Figs. 5.3.1.1 - 5.3.1.3. For 13.5-day yolk sacs (Fig. 5.3.1.1) uptake by the controls was linear over the time period 1.0 to 5.0 h and showed a rate of 1.61 ± 0.02 µl/mg yolk-sac protein/h. When ethanol was added at 1.0 h, a dramatic effect was seen. Ethanol (2.0 mM) caused an immediate and complete inhibition of fluid-phase pinocytosis for the final 4.0 h of the uptake period. The percentage uptake after 5.0 h of incubation, relative to ethanol-free matched controls, was 22% (see Table 5.3.1.2). In contrast, in the period 1.0 - 2.0 h of incubation 10.0 mM ethanol caused an immediate release of 74% of the [^{125}I]PVP-derived radioactivity accumulated during the first 1.0 h of incubation. A further, smaller release of radioactivity was seen during the 2.0 to 5.0 h period so that, by 5.0 h, the percentage uptake relative to ethanol-free matched controls was only 3% (see Table 5.3.1.2).

Fig. 5.3.1.2 shows the effects of ethanol on fluid-phase

pinocytosis by 15.5-day yolk sacs. The uptake of [^{125}I]PVP by the controls was linear over the 1.0 to 5.0 h uptake period with an E.I. of 2.29 ± 0.01 µl/mg yolk-sac protein/h. The addition of ethanol at 1.0 h caused, in the case of 2.0 mM ethanol, a rapid but incomplete inhibition of fluid-phase pinocytosis. After 5.0 h, the percentage uptake, relative to ethanol-free matched controls, was 38% (Table 5.3.1.2). In the case of the 10.0 mM ethanol a rapid release of [^{125}I]PVP was again caused by the addition of ethanol after 1.0 h of incubation. By 2.0 h a decrease of 40% in the radioactivity, accumulated by the tissue within 1.0 h, was seen while the percentage uptake by 5.0 h, relative to ethanol-free matched controls, was 10% (Table 5.3.1.2).

Finally, the effects of ethanol on fluid-phase pinocytosis in 19.5-day visceral yolk sacs are reported in Fig. 5.3.1.3. The control tissue showed linear uptake of [^{125}I]PVP at a rate of 2.99 ± 0.01 µl/mg yolk-sac protein/h over the period 1.0 to 5.0 h. The effects of ethanol administered at the 1.0 h incubation period were examined; 2.0 mM ethanol took approximately 3.0 h after its addition to produce any effects. Between 1.0 h (when the ethanol was added) and 4.0 h there was no discernible effect of ethanol on fluid-phase pinocytosis. However, between 4.0 h and 5.0 h of incubation, ethanol completely arrested fluid-phase pinocytosis. The percentage uptake (at the 5.0 h point), relative to ethanol-free matched controls, was 80% (Table 5.3.1.2). A similar effect was seen with 10.0 mM ethanol; between 1.0 and 3.0 h there was no effect of ethanol on fluid-phase pinocytosis, but between 3.0 and 5.0 h this concentration of ethanol completely inhibited the uptake of [^{125}I]PVP. The percentage uptake, relative to ethanol-free matched controls, at the 5.0 h incubation period was 60% (Table 5.3.1.2).

A summary table (Table 5.3.1.2) shows the effects of ethanol on

fluid-phase pinocytosis as gestational age increases from 13.5 to 19.5 days.

The effects of ethanol on the rate of release of [^{125}I]PVP from 13.5-day yolk sacs are shown in Fig. 5.3.1.4 and Table 5.3.1.1b. The effects of 2.0 and 10.0 mM ethanol on the rate of progressive loss of [^{125}I]PVP are shown in Table 5.3.1.1b. The control tissue shows an initial loss, after 15 min in medium containing neither ethanol nor serum, of 3.96% and a rate of progressive loss of 0.78 ± 0.03%/h. The 2.0 mM ethanol-treated tissue showed an initial loss (after 15 min in the presence of ethanol) of 11.8%, a 3.0-fold increase over that of the controls, but showed a rate of progressive loss of 1.81 ± 0.02%/h, a 2.3-fold increase relative to that of controls. The situation with regard to the 10.0 mM ethanol-treated tissue was somewhat similar. After 15 min in the presence of ethanol the tissue showed an initial loss of radioactivity of 15.8% and a rate of progressive loss of 1.74 ± 0.08%/h, increases of 3.98- and 2.23-fold relative to matched controls. It can be seen (Fig. 5.3.1.4) that the time-courses of release of radioactivity (in the matched controls, as well as in tissues exposed to 2.0 mM and 10.0 mM ethanol) were linear over the 2.75 h incubation period. However, it was observed that by 2.75 h approximately 20% of the tissue-associated radioactivity present at the start of the ethanol exposure had been released into the medium (in the 10.0 mM ethanol-treated tissue) and approximately 16% in the case of the 2.0 mM ethanol-treated tissue. These values must be compared with only 6% release in the ethanol-free matched controls.

Since 16 - 20% of the tissue-associated radioactivity was released by the two concentrations of ethanol used in this study, the possibility that 2.0 - 10.0 mM ethanol may be cytotoxic to 13.5-day yolk sacs was investigated. In Fig. 5.3.1.5 the results of ethanol pre-treatment on the subsequent uptake of [^{125}I]PVP by 13.5-day yolk

sacs can be seen. The control tissue shows linear uptake over the 4.0 h re-incubation period with an E.I. of 1.61 ± 0.01 µl/mg yolk-sac protein/h. However, both the 2.0 mM and 10.0 mM ethanol-treated tissue showed a "lag period" in the uptake of the substrate of 1.5 and 2.0 h respectively before the pinocytic activity of the tissue was regained. Furthermore, the 2.0 mM ethanol-treated tissue showed a period of uptake of [^{125}I]PVP above that of the control (from 1.5 - 4.0 h incubation). A similar result was seen with the 10.0 mM ethanol-treated tissue except that the increased rate of pinocytic uptake fell in the period 2.0 - 4.0 h. These results should be compared with those seen in Fig. 3.3.1.6 in which only the 10.0 mM ethanol-treated tissue showed a "lag phase" before recovering with an increased rate of uptake above that of the matched controls.

5.3.2 The effects of ethanol on adsorptive-phase pinocytosis and intralysosomal proteolysis of [^{125}I]fd BSA in 13.5-day visceral yolk sacs

Since the effects of ethanol on fluid-phase pinocytosis were more markedly shown at 13.5 days of gestation, the investigation into the effects of ethanol on adsorptive-phase pinocytosis was confined to 13.5-day yolk sacs. Fig. 5.3.2.1 shows the time-course of uptake of [^{125}I]fd BSA II in the presence of 10% (v/v) fetal-calf serum by 13.5-day yolk sacs. Uptake by the control yolk sacs was linear over the incubation period and had an E.I. of 40.3 ± 0.95 µl/mg yolk-sac protein/h. Adsorptive-phase pinocytosis was rapidly and completely inhibited by both 2.0 and 10.0 mM ethanol. The uptake of [^{125}I]fd BSA II at the 5.0 h incubation period for the 2.0 mM and 10.0 mM ethanol-treated yolk sacs was 5.0 and 2.3 µl/mg yolk-sac protein compared with 202 µl/mg yolk-sac protein for the matched controls, values which compare favourably with equivalent data for the uptake of [^{125}I]fd BSA II in 10% (v/v) fetal-calf serum by 17.5-day

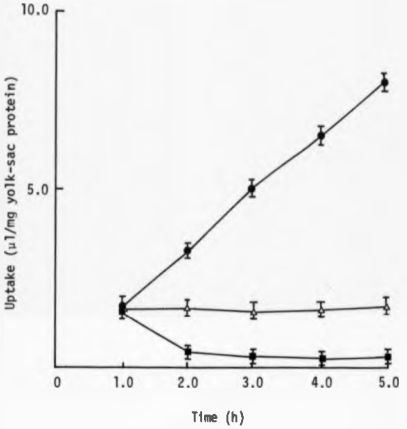
yolk sacs (Fig. 4.3.1.5). A corresponding inhibition was observed (Fig. 5.3.2.2) in the quantities of tissue-associated radioactivity. The control yolk sacs had a mean content of tissue-associated radioactivity of 36.0 ± 2.8 µl/mg yolk-sac protein and showed the typical steady-state kinetics pattern (c.f. Williams et al., 1975b; Moore et al., 1977; Livesey & Williams, 1981). Ethanol, at 2.0 mM and 10.0 mM, strongly inhibited the tissue association of the radioactive substrate. [At 5.0 h of incubation, the 2.0 mM ethanol-treated tissue showed a value of 3.9 µl/mg yolk-sac protein and the 10.0 mM ethanol-treated tissue a value of 1.8 µl/mg yolk-sac protein compared with the matched control value of 37.8 µl/mg yolk-sac protein.] These results indicate that the principal effect of ethanol is inhibition of adsorptive uptake of [^{125}I]fd BSA II rather than inhibition of lysosomal degradation.

Finally, in Fig. 5.3.2.3, the recovery of adsorptive-phase pinocytosis (as measured by the release of TCA-soluble radioactivity into the incubation medium) was investigated. The control tissue showed a linear pattern of release of TCA-soluble radioactivity. The ethanol-treated 13.5-day tissue showed a "lag phase" in the release of TCA-soluble radioactivity of approximately 1.5 h (2.0 mM ethanol) and of 3.0 h (10.0 mM ethanol). (These results are equivalent to an increase of 0.75 h and 1.5 h on the corresponding "lag phases" seen at 17.5 days of gestation with 2.0 mM and 10.0 mM ethanol-treated tissue, c.f. Figs. 4.3.1.7 & 4.3.1.8.) However, both the 2.0 mM- and 10.0 mM ethanol-treated tissues showed a period of release of TCA-soluble radioactivity at rates above those of the control tissue once they had

recovered from the ethanol pre-treatement. These observations are similar to those reported at 17.5 days of gestation (Figs. 4.3.1.7 & 4.3.1.8) showing that in 13.5-day yolk sacs, ethanol pre-treatment causes a period of inhibition of adsorptive-phase pinocytosis and/or intralysosomal proteolysis before full recovery of the tissue was observed.

Fig. 5.3.1.1 The effect of ethanol on the uptake of [^{125}I]PVP by 13.5-day yolk sacs incubated in serum-free medium

Yolk sacs were incubated separately in 9.0 ml of serum-free medium 199 containing [^{125}I]PVP (2 ug/ml). After 1.0 h incubation, 1.0 ml of medium only or medium containing ethanol (final concentration 2.0 or 10.0 mM) was added. Uptake was monitored up to 5.0 h. Each point represents the mean uptake (± S.D.) by at least six yolk sacs, each from a different pregnant animal.



●——● = CONTROL

△——△ = 2.0 mM ETOH

■——■ = 10.0 mM ETOH

Fig. 5.3.1.2 The effect of ethanol on the uptake of [^{125}I]PVP by 15.5-day yolk sacs incubated in serum-free medium

Yolk sacs were incubated as in Fig. 5.3.1.1. Each point represents the mean (± S.D.) uptake by at least six yolk sacs, each from a different pregnant animal.

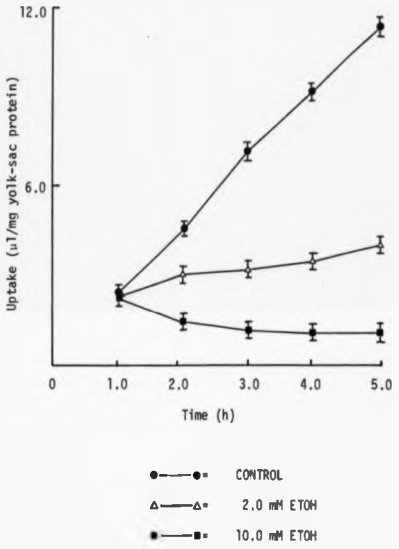


Fig. 5.3.1.3 The effect of ethanol on the uptake of [^{125}I]PVP by 19.5-day yolk sacs incubated in serum-free medium

Yolk sacs were incubated as in Fig. 5.3.1.1. Each point represents the mean uptake (± S.D.) by at least six yolk sacs, each from a different pregnant animal.

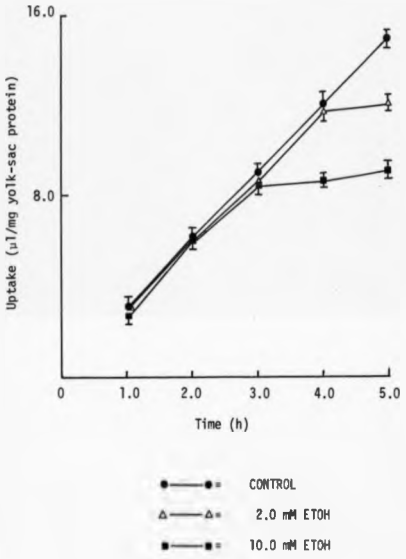


Fig. 5.3.1.4 The release of [^{125}I]PVP from 13.5-day yolk sacs into serum-free medium containing ethanol

Yolk sacs were incubated separately (but in triplicate) in 10.0 ml of serum-free medium containing [^{125}I]PVP (2 µg/ml) for 2.5 h, then rinsed in fresh medium (3 x 2 min), at 37°C, before being transferred to either serum-free medium or serum-free medium containing ethanol (2.0 or 10.0 mM) and incubated for a further 2.75 h. Each point represents the mean release (± S.D.) by at least nine yolk sacs.

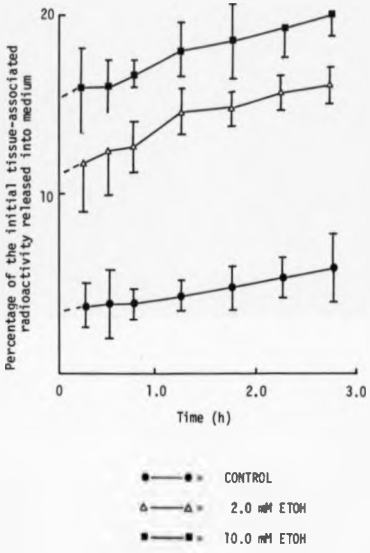


Fig. 5.3.1.5 The effect of ethanol pre-treatment on the uptake of [^{125}I]PVP by 13.5-day yolk sacs incubated in serum-free medium

Yolk sacs were incubated separately in 10.0 ml of serum-free medium containing ethanol (2.0 or 10.0 mM) for 0.5 h, then removed and rinsed in fresh medium (3 x 2 min) at 37°C, before being transferred into 10.0 ml of serum-free medium containing [^{125}I]PVP (2 µg/ml) and incubated for a further 4.0 h. Each point represents the mean (± S.D.) uptake by at least six yolk sacs.

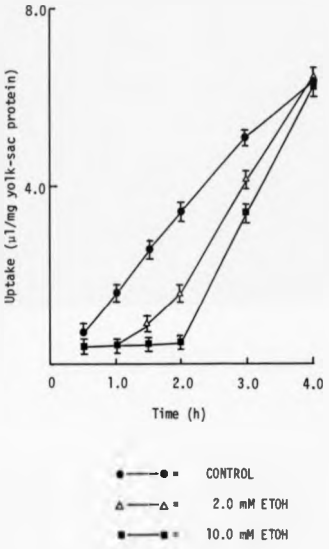


Fig. 5.3.2.1 The uptake of [^{125}I]fd BSA II from medium 199 containing 10% (v/v) fetal-calf serum by 13.5-day yolk sacs

Yolk sacs were incubated separately in 8.0 ml of medium containing 10% (v/v) fetal-calf serum. After 0.0 h incubation, 1.0 ml of medium containing [^{125}I]fd BSA II (final concentration 5 µg/ml) followed by 1.0 ml of medium containing ethanol (final concentration 2.0 or 10.0 mM) was added. Uptake was monitored for 5.0 h. Each point represents the mean uptake (± S.D.) by at least six yolk sacs.

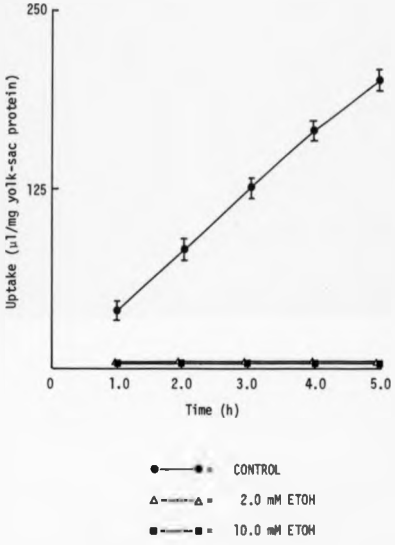


Fig. 5.3.2.2 Tissue-associated radioactivity derived from [^{125}I]fd BSA II for 13.5-day yolk sacs incubated in medium containing 10% (v/v) fetal-calf serum

The tissue-associated radioactivity is indicated for the yolk sacs whose net uptake is reported in Fig. 5.3.2.1.

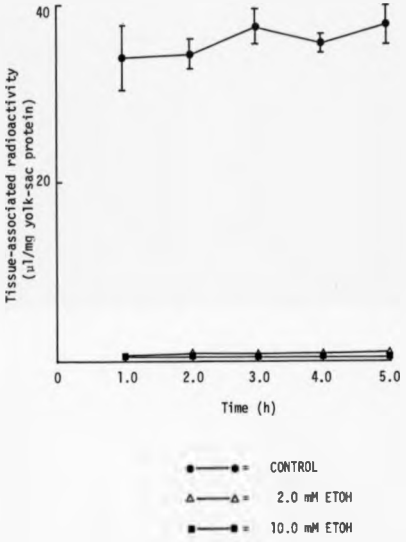


Fig. 5.3.2.3 The effect of ethanol pre-treatment on the release of TCA-soluble radioactivity by 13.5-day yolk sacs incubated in serum-free medium

Yolk sacs were incubated separately (but in triplicate) in 10.0 ml of serum-free medium containing ethanol (2.0 or 10.0 mM) for 0.5 h, then removed and rinsed in fresh medium (3 x 2.0 min) at 37°C, before being transferred into 14.0 ml of serum-free medium containing [^{125}I]fd BSA II (5 µg/ml) and incubated for a further 4.0 h. Each point represents the mean (± S.D.) quantity of TCA-solubles produced by at least nine yolk sacs. As the data are reported as c.p.m. they have been normalized for the effects of isotope decay.

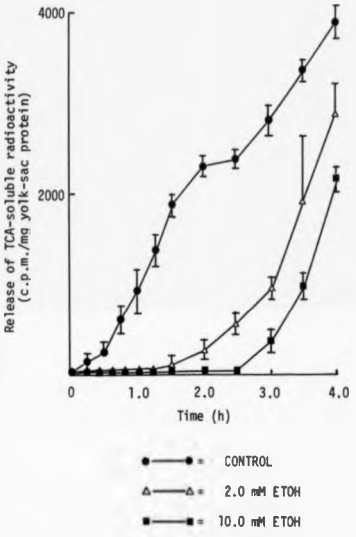


Table 5.3.1.1a Rate of uptake of [^{125}I]PVP by 13.5 - 19.5 day yolk sacs

The yolk sacs were incubated in serum-free medium containing [^{125}I]PVP (2 μg/ml) for 5.0 h. Each value reported is the mean ± S.D. from at least three similar experiments each using yolk sacs from a different animal.

Gestational Age (days)	Endocytic Index (μl/mg yolk-sac protein/h)
13.5	1.61 ± 0.02
15.5	2.29 ± 0.01
17.5	2.67 ± 0.14
19.5	2.99 ± 0.01

Table 5.3.1.1b Rate of release of [^{125}I]PVP from 13.5-day yolk sacs incubated in the presence of different concentrations of ethanol

Yolk sacs were first incubated in serum-free medium containing [^{125}I]PVP (2 μg/ml) for 2.5 h then washed and transferred to fresh medium containing no substrate but 2.0 mM and 10.0 mM ethanol for a further 2.75 h. Each value reported is the mean ± S.D. of at least nine similar experiments.

Concentration of EtOH in re-incubation medium (mM)	Initial Loss over period 0 - 0.25 h (%)	Rate of Loss over period 0.25 - 2.75 h (%/h)
0.00 (Control)	3.96 ± 0.14	0.78 ± 0.03
2.00	11.75 ± 0.17	1.81 ± 0.02
10.00	15.79 ± 0.13	1.74 ± 0.08

Table 5.3.1.2 The effect of increasing gestational age of the yolk sac on the degree of inhibition of fluid-phase pinocytosis induced by ethanol

Yolk sacs of different gestational ages were incubated separately in serum-free medium containing [^{125}I]PVP (2 μg/ml). After 1.0 h of incubation, either a further 1.0 ml of medium alone or of ethanol in medium was added to each flask; the final ethanol concentrations in the uptake medium were 2.0 mM or 10.0 mM. Each value represents the mean ± S.D. of at least 6 determinations, each with yolk sacs from a different animal.

Gestational Age (days)	Uptake after 5 h of incubation (μl/mg yolk-sac protein)			Percentage uptake relative to ethanol-free matched controls (%)	
	Control	2.0 mM Ethanol	10.0 mM Ethanol	2.0 mM Ethanol	10.0 mM Ethanol
13.5	8.05 ± 0.21	1.82 ± 0.12	0.22 ± 0.02	22.62 ± 1.48	2.79 ± 0.41
15.5	11.02 ± 1.00	4.11 ± 0.14	1.09 ± 0.07	37.68 ± 4.70	9.89 ± 1.32
17.5	13.22 ± 0.35	4.59 ± 1.05	0.85 ± 0.17	34.70 ± 7.93	6.42 ± 1.38
19.5	15.12 ± 0.18	12.12 ± 0.08	9.17 ± 0.13	80.16 ± 0.59	60.65 ± 1.50

5.4 DISCUSSION

This chapter reports the effects of ethanol on fluid-phase pinocytosis in visceral yolk sacs from 13.5 to 19.5 days of gestation and on adsorptive-phase pinocytosis and intralysosomal proteolysis in 13.5-day yolk sacs.

In the absence of ethanol, as gestational age decreases from 19.5 to 13.5 days, the rate of fluid-phase pinocytosis in serum-free controls (Table 5.3.1.1a) decreases from 3.0 to 1.6 µl/mg yolk-sac protein/h. This observation was in agreement with those of Williams et al. (1976) and Jollie (1986) but was in contrast with those of Record et al. (1982) and Marlow & Freeman (1987) who reported that rates of fluid-phase pinocytosis in 9.5-, 10.5- and 11.5-day visceral yolk sacs were similar to that of the 17.5-day visceral yolk sac. However, the effects of ethanol inhibition on fluid-phase pinocytosis are greatly increased as gestational age decreases. [At 19.5 days of gestation, 2.0 mM and 10.0 mM ethanol produced only a 20% and a 40% inhibition of pinocytosis by 5.0 h of incubation. At 17.5 days this inhibition was increased to 65% and 93%, respectively, for 2.0 mM and 10.0 mM ethanol. At 15.5 days of gestation, the percentage inhibition was little changed, at 62% and 90%, but by 13.5 days of gestation inhibition increased to 77% and 97% for these same ethanol concentrations.] Thus ethanol-induced inhibition of fluid-phase pinocytosis was more marked at earlier stages of gestation. At 19.5 days, ethanol-induced inhibition of pinocytosis took 2.0 - 3.0 h to manifest itself (Fig. 5.3.1.3). At 17.5 days, the effect of ethanol on the uptake of [^{125}I]PVP was immediate (Fig. 3.3.1.1) with the highest concentration of ethanol (10.0 mM) causing release of the accumulated radioactivity during the first hour of incubation. By 15.5 days (Fig. 5.3.1.2), the effect is similar to that seen at 17.5 days of gestation except that the release of [^{125}I]PVP by 15.5-day

yolk sacs, following the addition of 10.0 mM ethanol, was greater over the 1.0 - 2.0 h incubation period.

Finally, at 13.5 days of gestation, 2.0 mM ethanol completely and immediately inhibited fluid-phase pinocytosis (Fig. 5.3.1.1) while 10.0 mM ethanol caused an immediate release of [^{125}I]PVP during its first hour in the incubation medium, resulting in 74% of the accumulated radioactivity, present after 1.0 h of incubation, being released. Thus, as the gestational age of the tissue decreases from 19.5 to 13.5 days, the yolk sac becomes more sensitive to ethanol-induced inhibition of fluid-phase pinocytosis (Table 5.3.1.2) with progressive increases in the release of [^{125}I]PVP caused by 10.0 mM ethanol during its first hour in the incubation medium (Figs. 3.3.1.1; 5.3.1.2 & 5.3.1.1).

Because ethanol (10.0 mM) caused substantial release of [^{125}I]PVP in 13.5-day yolk sacs within an hour of its addition, the effect of ethanol on the release of previously-accumulated [^{125}I]PVP in 13.5-day yolk sacs was studied (Table 5.3.1.1b & Fig. 5.3.1.4). The control 13.5-day tissue showed a loss, over the initial 0.25 h of re-incubation (in medium containing neither substrate nor ethanol) of 4.0% of the total radioactivity present at the start of the re-incubation period. This compares with an initial loss of 2.6% for tissue at 17.5 days of gestation (Table 3.3.1.2), a 1.5-fold increase. But the rate of progressive loss of [^{125}I]PVP was 0.78%/h at 13.5 days compared with 0.79%/h at 17.5 days of gestation (Table 3.3.1.2). At 13.5 days of gestation, 2.0 mM ethanol caused an initial loss of radioactivity (over the first 0.25 h of re-incubation) of 11.8% compared with 6.4% over the same time period at 17.5 days of gestation, a 1.8-fold increase. The rates of progressive loss of radioactivity were 1.56%/h and 1.81%/h for yolk sacs at 17.5 days and 13.5 days of gestation.

When data for the release of radioactivity following exposure to 10.0 mM ethanol at 13.5 days (Table 5.3.1.1b) and 17.5 days (Table 3.3.1.2) of gestation are studied, it can be seen that the initial loss of radioactivity at 13.5 days was 15.8% while at 17.5 days of gestation it was 9.7%, a 1.6-fold increase for a 4-day decrease in gestational age. However, if the rate of progressive loss of [^{125}I]PVP from the tissue was observed, it was found that in 13.5-day and 17.5-day yolk sacs the values were 1.74%/h and 2.08%/h, respectively. Thus, the main difference in the action of ethanol (at 2.0 mM and 10.0 mM concentrations) on yolk sacs at 13.5 days of gestation, compared with those at 17.5 days, was to increase the release of [^{125}I]PVP during the first 0.25 h of re-incubation in the presence of the inhibitor; the rates of progressive loss of [^{125}I]PVP caused by both concentrations of ethanol at 17.5- and 13.5-days of gestation were very similar.

In Chapter 3, it was shown that ethanol-induced inhibition of pinocytosis was readily reversible (Fig. 3.3.1.6) showing a biphasic response. Firstly, there was a period of no pinocytic activity; this was followed by a second period of enhanced pinocytic activity, at a rate above that of the control tissue. To see if the same was true for yolk sacs at earlier days of gestation, the equivalent recovery experiments were performed with tissue at 13.5 days (Fig. 5.3.1.5). The control tissue showed the characteristic linear uptake of [^{125}I]PVP over the 4.0 h re-incubation period, but both the 2.0 mM- and the 10 mM-ethanol-treated tissue showed a "lag-phase" before the resumption of pinocytic activity. The "lag period" for the 10.0 mM ethanol-treated tissue was approximately 2.0 h, 1.25 h longer than that for 17.5-day yolk sacs (Fig. 3.3.1.6). The 2.0 mM ethanol-treated tissue at 17.5 days of gestation recovered immediately and completely, but 13.5-day yolk sacs showed a "lag period" of

approximately 1.5 h. Once both sets of tissue had regained their pinocytic activity, a period of uptake of [^{125}I]PVP at rates above that of the control tissue was observed which resulted in control, 2.0 mM and 10.0 mM ethanol-treated tissues showing similar uptake values at the 4.0 h incubation point (Fig. 5.3.1.5). These results confirm those reported earlier in this chapter that, as the gestational age of the yolk sacs decreases, the tissue becomes more sensitive to the action of ethanol. The inhibition of fluid-phase pinocytosis was greatest at 13.5 days of gestation and the tissue took more time to recover its pinocytic activity following ethanol pre-treatment. Even for 13.5-day yolk sacs, the ethanol-induced inhibition of fluid-phase pinocytosis was slowly but fully reversed after the inhibitor was removed.

When the effect of ethanol on adsorptive-phase pinocytosis was investigated in 13.5-day yolk sacs (Fig. 5.3.2.1), the control yolk sacs showed a linear uptake of [^{125}I]fd BSA II in 10% (v/v) fetal-calf serum with an E.I. of 40.3 µl/mg yolk-sac protein/h. The E.I. of [^{125}I]fd BSA II in 10% (v/v) fetal-calf serum for control 17.5-day yolk sacs was 66.5 µl/mg yolk-sac protein/h. This decrease of approximately 40% in the rate of adsorptive-phase pinocytosis was in agreement with the observations of Jollie (1986). At both 17.5 days and 13.5 days of gestation (Figs. 4.3.1.5 & 5.3.2.1), ethanol (2.0 mM and 10.0 mM) caused a rapid and complete inhibition of net uptake of [^{125}I]fd BSA II with little association of substrate with the tissue (Figs. 4.3.1.6 & 5.3.2.2). At the 5.0 h incubation point, the 2.0 mM and 10.0 mM ethanol-treated yolk sacs showed an uptake of approximately 2.5% and 1.1% of the control value. These results are supported by those of the tissue-associated radioactivity data (Fig. 5.3.2.2). The control tissue showed a mean value of tissue-associated radioactivity of 36 µl/mg yolk-sac protein compared

with a mean value of 73 µl/mg yolk-sac protein for 17.5-day yolk sacs, a decrease of 50% in the tissue-associated radioactivity with a decrease in gestational age. However, at both 13.5 and 17.5 days of gestation, ethanol (at both concentrations used) completely inhibited the tissue-association of the substrate.

Since adsorptive-phase pinocytosis was rapidly and completely inhibited by 2.0 mM and 10.0 mM ethanol in 13.5-day visceral yolk sacs, the recovery of adsorptive-phase pinocytosis following ethanol pre-treatment was investigated (Fig. 5.3.2.3). At 17.5 days of gestation the "lag phases", for the release of TCA-soluble radioactivity into the incubation medium, were 0.75 h and 1.5 h for 2.0 mM and 10.0 mM ethanol-treated tissue (Figs. 4.3.1.7 & 4.3.1.8). These periods of apparent pinocytic inactivity were greatly increased in 2.0 mM and 10.0 mM ethanol-treated yolk sacs at 13.5 days of gestation (to approximately 1.5 h and 2.5 h, respectively). These results also show that, following the "lag phases" in uptake and lysosomal degradation of substrate in ethanol-treated tissue, there was a period of pinocytic uptake and/or lysosomal proteolysis at a rate above that of the control tissue. This increase was also seen in 17.5-day yolk sacs (Figs. 4.3.1.7 & 4.3.1.8). These results show that as the gestational age decreases (down to 13.5 days) the yolk sac becomes more sensitive to ethanol-induced inhibition of fluid- and adsorptive-phase pinocytosis but that, once the inhibitor was removed, this inhibition was reversible.

These results are of great interest since the dose-response threshold of ethanol during critical periods of organogenesis _in utero_ are being actively investigated (Abel & Sokol, 1986; Ernhart _et al._, 1987). The results reported in this chapter suggest that, as the gestational age decreases, the visceral yolk sac becomes more sensitive to ethanol-induced dysfunction. If this same trend holds

good for rat yolk sacs at 9.5 - 11.5 days of gestation, then the supply of nutrients during organogenesis will be interrupted. In fact, it has been suggested (Lieber, 1982) that ethanol may alter the metabolism, transport and storage of many essential nutrients and that nutritional deprivation due to maternal alcoholism may result in the pattern of malformations known as the FAS.

Interestingly, Zawoiski (1975) reported that a diet rich in L-glutamic acid and L-valine on days 2 to 17 of gestation in albino mice prevented trypan blue-induced exencephaly and otocephaly (trypan blue was administered subcutaneously on day 8) when compared with trypan blue-treated animals on the normal diet. However, in a study of the relation of ethanol and maternal nutritional status to fetal development, Weinberg (1985) found that neither an optimal diet (25% of calories as protein) nor a "supra-optimal/protein-enriched diet" (32% of calories as protein) prevented the characteristic deformities of the FAS and concluded that maternal nutritional status had no major effect on developmental outcome in chronic ethanol-fed rats. But Oreosti (1984) reported that supplementary dietary zinc provided some protection against the teratogenic effects of ethanol.

The last observation is an interesting one since Record *et al.* (1982) reported that, in rat visceral yolk sacs *in vitro*, cadmium inhibited the uptake of [^{125}I]PVP in 9.5- and 10.5-day yolk sacs by approximately 55%. When zinc was included in the incubation medium as well as cadmium, the rate of pinocytosis was restored to normal. This work was further extended by Marlow & Freeman (1987) who found that zinc not only decreased cadmium-induced inhibition of pinocytosis (as measured by the uptake of [^{125}I]PVP), but also decreased the inhibition of pinocytosis produced by anti-yolk-sac antiserum in 11.5-day yolk sacs *in vitro*. When the same experiments were tried with trypan blue, zinc had no effect on the trypan blue-induced

inhibition of [^{125}I]PVP uptake. These results suggest that cadmium and anti-yolk-sac antiserum act via a similar mechanism which is different from that of trypan blue.

In summary, the results described in this chapter indicate that:

(i) As gestational age decreases from 19.5 to 13.5 days the rates of fluid-phase and of adsorptive-phase pinocytosis in control tissues both decrease.

(ii) As gestational age decreases from 19.5 to 13.5 days the degree of inhibition of fluid-phase pinocytosis, induced in yolk sacs by a given concentration of ethanol, increases.

(iii) Although such inhibition was readily reversible in 13.5-day yolk sacs the recovery period is longer than for 17.5-day yolk sacs.

(iv) Ethanol (2.0 mM and 10.0 mM) causes an immediate and complete inhibition of adsorptive-phase pinocytosis in 13.5-day yolk sacs.

(v) Again, inhibition was readily reversible but recovery took a longer time period in 13.5-day yolk sacs, compared with 17.5-day yolk sacs.

The results described in this chapter add further support to the suggestion that ethanol may owe part of its teratogenic effect to inducing yolk-sac dysfunction. If events at 13.5 days can be extrapolated back to 8.5 - 9.5 days of gestation the findings are compatible with ethanol inhibiting the process of embryotrophic nutrition during the period of organogenesis.

CHAPTER SIX

THE EFFECTS OF BENZYL ALCOHOL ON FLUID-PHASE AND ADSORPTIVE-PHASE PINOCYTOSIS IN THE 17.5-DAY RAT VISCERAL YOLK SAC

6.1 INTRODUCTION

The effects of ethanol on fluid-phase and adsorptive-phase pinocytosis in the visceral yolk sac are well documented in Chapters 3 and 4. It was reported (Almeida *et al.*, 1986) that, as the chain lengths of primary aliphatic alcohols (ethanol, butan-1-ol and hexan-1-ol) increase, individual alcohols interact with and perturb different domains of the sarcoplasmic reticulum membrane. This is of interest since another alcohol, benzyl alcohol, has been reported to possess local anaesthetic properties, possibly as a result of its ability to perturb membrane fluidity. Ashcroft *et al.* (1977) reported that benzyl alcohol (7.5 mM) increased the "thickness" of the hydrocarbon region of an artificial lecithin bilayer membrane by approximately 25% (1.2 nm) probably by causing a straightening out of the hydrocarbon tails. In contrast, Turner & Oldfield (1979), found no such increases in the thickness of black lipid membranes at concentrations of benzyl alcohol that induce local anaesthesia in rat phrenic nerve (4 mM) and frog sciatic nerve (12 mM). But, as the concentration of benzyl alcohol was increased, the membrane thickness was slightly decreased. However, these authors suggest that such results are strongly dependent on the amount of residual solvent in the lipid membrane preparations used, so the relevance of such findings to events in intact cells is difficult to assess.

A further study investigated the effects of benzyl alcohol on erythrocyte shape, membrane-hemileaflet fluidity and membrane viscoelasticity in human red cells and red-cell ghosts (Chabanel *et al.*, 1985). These authors found that concentrations (non-haemolytic) of benzyl alcohol up to 50 mM caused progressive stomatocytic shape change but induced no change in membrane viscoelasticity. When the alcohol concentration was increased above 50 - 60 mM, intact red blood cells became echinocytic, but no change was observed in membrane

viscoelasticity. Thus concentrations of benzyl alcohol up to 50 mM primarily affect the inner leaflet of the red blood cell membrane but higher concentrations affect both the inner and outer leaflets. These increases in membrane fluidity were not associated with changes in membrane viscoelasticity.

The increases in membrane fluidity associated with benzyl alcohol treatment also affect the activity of intrinsic membrane enzymes (Gordon et al., 1980). At concentrations in the range 10 - 40 mM, benzyl alcohol caused an increase in membrane fluidity of rat-liver plasma membranes and caused activation of glucagon-stimulated adenylate cyclase, basal adenylate cyclase, 5'-nucleotidase, cyclic AMP phosphodiesterase and Na^+,K^+-ATPase; higher concentrations induced reversible inhibition of these enzymes. Similarly, another study (Needham & Houslay, 1982) indicates that benzyl alcohol (50 mM) causes a marked activation of the adenylate cyclase activity in rat brain striata membrane preparations but at higher concentrations caused an inhibition of enzyme activity. Such effects are possibly induced by the benzyl alcohol competing for sites on the protein that are normally occupied by annular phospholipids. However, Carrière & Le Grimellec (1986) showed that in kidney brush-border membranes 50 mM benzyl alcohol had little effect on the activities of alkaline phosphatase and γ-glutamyltranspeptidase, but markedly inhibited the uptake of D-glucose. At a concentration of 30 mM, benzyl alcohol decreased the value of J_{max} (the maximal value of the saturable contribution to the total rate of glucose uptake) without a significant effect on the K_m of the glucose transporter, or on the $[^{22}Na^+]$ uptake or on the vesicular volume of kidney brush-border membranes. The effect on glucose transport was accordingly attributed to a direct effect of the alcohol on either the glucose carrier or its immediate environment. The addition of 5 mM

octan-1-ol to the membrane preparations induced similar effects.

Not only does benzyl alcohol (10 mM) affect membrane fluidity and the activities of various membrane enzymes and transporters, it also interferes with intracellular processes (Tolleshaug & Berg, 1982). In isolated hepatocytes, benzyl alcohol at a concentration of 10 - 20 mM affects the degradation of [^{125}I]asialo-orosomucoid by reducing the rate of transfer of endocytosed protein from the endosomal compartment to the lysosomes. Since the alcohol neither decreased the binding of the substrate to its receptor at the plasma-membrane surface nor affected intralysosomal pH (as do other local anaesthetics), the primary mode of action of benzyl alcohol on pinosome/endosome-lysosome fusion was unclear. Other events are known to interfere with vesicle-lysosome fusion. The presence of certain microorganisms, namely Mycobacterium tuberculosis, Toxoplasma gondii and some Trypanosoma species, in the vacuolar system (Hart & Young, 1978) has been shown to interfere with phagosome-lysosome fusion in macrophages in culture. This inhibition of fusion could possibly be due to the secretion of a chemical by the microorganism (Mauel, 1984) since poly-D-glutamic acid both inhibited phagosome-lysosome fusion and promoted intracellular growth of L. mexicana mexicana in macrophages in vitro. Alternatively, the microbial cell-wall components may inhibit fusion (Goren et al., 1976). Thus, when sulphatides of M. tuberculosis (anionic trehalose glycolipids) were administered to mouse peritoneal macrophages in vitro they inhibited phagosome-lysosome fusion (possibly by ionic interactions between the polyanionic bacterial sulphatides and organelle membranes) so inducing dysfunction.

A second intracellular action of benzyl alcohol was reported by Grinde (1984) who reported that benzyl alcohol (10 mM) caused a complete and selective inhibition of the methylamine-sensitive pathway

of protein degradation in isolated rat hepatocytes. This inhibition was readily reversible within 30 min of removing the agent, and morphometric examination of electron micrographs revealed that benzyl alcohol-induced inhibition of protein degradation within lysosomes coincided with a block in the formation of autophagic vacuoles while the number of acidic vacuoles (those induced to swell by the addition of methylamine) was not drastically reduced.

As benzyl alcohol seems to inhibit endosome-lysosome fusion and/or formation of autophagic vacuoles (as well as its known effects on membrane fluidity), it was decided to investigate the effects of benzyl alcohol on fluid-phase and adsorptive-phase pinocytosis and on intralysosomal proteolysis in the rat visceral yolk sac *in vitro*.

6.2 MATERIALS AND METHODS

All the methods reported in this chapter are similar to those reported in Chapter 2 except for the time intervals used in the recovery of pinocytosis experiments (Section 2.10). In this chapter the experimental procedure involved pre-treating yolk sacs for 0.5 h with benzyl alcohol, then incubating them with [^{125}I]fd BSA (5 μg/ml) for 4.0 h in the absence of benzyl alcohol. Samples of medium were removed at 1.5, 2.0, 2.5, 3.0 and 4.0 h for analysis.

The benzyl alcohol used in this chapter was BDH General Purpose Reagent rather than spectroscopically-pure grade (due to the high cost of the spectrograde benzyl alcohol). This resulted not only in the presence of benzyl alcohol in the final incubation medium (0.4 mM and 1.0 mM), but also approx. 0.00002% (w/v) benzaldehyde (2 μM) and 0.000012% (w/v) chlorinated compounds. Finally, after the section of work on the effects of benzyl alcohol on fluid-phase pinocytosis was completed, it was noticed that [^{125}I]PVP from Amersham PLC contained 1% (w/v) benzyl alcohol as a preservative. This contributed to the total benzyl alcohol concentration in the incubation medium. Thus, the addition of [^{125}I]PVP alone to the incubation medium at tracer concentrations resulted in exposure of the tissue to a benzyl alcohol concentration of approx. 90 μM. This problem was not encountered when [^{125}I]fd BSA was used as a substrate.

6.3 RESULTS

6.3.1 The effects of benzyl alcohol on fluid-phase pinocytosis

Fig. 6.3.1.1 shows the results of a preliminary experiment in which the dose-response curve of benzyl alcohol on fluid-phase uptake (after 5.0 h of incubation) was established in outline. The notional range of benzyl alcohol concentrations used was 0.02 - 20.0 mM (actual concentration range: 0.02 mM + 0.093 mM to 20 mM + 0.093 mM due to the presence of benzyl alcohol in [^{125}I]PVP preparations). The uptake of [^{125}I]PVP is expressed as the percentage of the matched controls after a single period of 5.0 h of incubation (i.e. "single-point kinetics"). At concentrations of 1.093 mM and 0.493 mM, benzyl alcohol caused uptake to be decreased to only approximately 15% and 40%, respectively relative to controls. These decreases were of similar magnitude to those produced by 10.0 mM and 2.0 mM ethanol in 17.5-day yolk sacs (see Chapter 3) so these concentrations of benzyl alcohol were used throughout the remainder of the experiments.

The detailed effects of 0.493 mM and 1.093 mM benzyl alcohol on the uptake of [^{125}I]PVP in 17.5-day yolk sacs are reported in Fig. 6.3.1.2. The matched control yolk sacs show linear uptake of [^{125}I]PVP over the incubation period with an E.I. of 2.95 ± 0.13 µl/mg yolk-sac protein/h. The 0.493 mM benzyl alcohol-treated tissue produced an uptake value between the 1.0 - 5.0 h incubation period that was 50% of that of the matched controls whereas the 1.093 mM benzyl alcohol-treated tissue had an uptake value that was only 23% of that of the matched controls. Thus benzyl alcohol induces a rapid but incomplete inhibition of fluid-phase pinocytosis at these concentrations. The effects of higher concentrations of benzyl alcohol were also investigated in detail (Fig. 6.3.1.3). The matched control tissue showed a linear uptake of [^{125}I]PVP over the incubation period with an E.I. of 3.04 ± 0.19 µl/mg yolk-sac protein/h. However,

when either 2.093 mM or 10.093 mM benzyl alcohol was added at the 1.0 h incubation point, both concentrations of inhibitor caused an immediate and complete inhibition of fluid-phase pinocytosis. Over the 1.0 - 5.0 h incubation period the 2.093 mM and 10.093 mM benzyl alcohol-treated tissues had uptake values that were 1.9% and 2.9% of the matched-control values. An interesting difference between these time-courses (Figs. 6.3.1.2 & 6.3.1.3) and that for 10 mM ethanol (see Chapter 3, Fig. 3.3.1.1) was that there was no release of radioactivity induced by even the highest concentration of benzyl alcohol, whereas 10.0 mM ethanol caused a dramatic release of [^{125}I]PVP from the tissue upon its addition to the incubation medium.

Fig. 6.3.1.4 shows the pattern of results when 17.5-day yolk sacs were allowed to accumulate [^{125}I]PVP before being washed and transferred to substrate-free medium containing either 0.40 mM or 1.0 mM benzyl alcohol. By 15 min the control tissue released 2.70 ± 0.38% of the accumulated radioactivity present at the start of the re-incubation period and showed a rate of progressive loss of 0.98 ± 0.09%/h. The 0.40 mM benzyl alcohol-treated tissue released 8.04 ± 0.35% of its accumulated radioactivity within the first 15 min of re-incubation and gave a rate of progressive loss of 0.81 ± 0.64%/h while the values for the 1.0 mM benzyl alcohol-treated tissue were 9.91 ± 1.0% and 0.96 ± 0.79%/h, respectively. Thus, from these results, both 0.4 mM and 1.0 mM benzyl alcohol had no effect on the rate of progressive loss of [^{125}I]PVP from yolk sacs, but, relative to controls, caused a 3.0- to 3.5-fold increase in the loss of [^{125}I]PVP during the first 15 min of exposure of the tissue to 0.4 mM and 1.0 mM benzyl alcohol, respectively.

The final series of experiments on the effects of benzyl alcohol on fluid-phase pinocytosis aimed to establish whether, like ethanol-induced inhibition of pinocytosis, such inhibition was

reversible. In Fig. 6.3.1.5, the effects of 0.5 h pre-treatments with both 0.4 mM and 1.0 mM benzyl alcohol can be seen. The control tissue showed a linear uptake of [^{125}I]PVP during the re-incubation period with an E.I. of 2.80 ± 0.19 µl/mg yolk-sac protein/h. The 0.4 mM benzyl alcohol-treated tissue showed an immediate and complete recovery of [^{125}I]PVP uptake which was linear over the re-incubation period with an E.I. of 2.84 ± 0.19 µl/mg yolk-sac protein/h. The 1.0 mM benzyl alcohol-treated tissue, however, showed a "lag period" in the recovery of [^{125}I]PVP uptake of approximately 1.0 h before the rate of uptake of the substrate accelerated to above that of the matched controls and remained elevated until the final hour of incubation when the rate of pinocytosis decreased to that of the controls. These results are somewhat similar to those reported in Fig. 3.3.1.4 for the effect of 10.0 mM ethanol pre-treatment on the uptake of [^{125}I]PVP by 17.5-day yolk sacs.

6.3.2 The effects of benzyl alcohol on adsorptive-phase pinocytosis and intralysosomal proteolysis

Exploratory experiments (results not reported in detail in graphical form, but uptake values given below) on the effects of benzyl alcohol at 0.4 mM and 1.0 mM concentrations on the uptake of [^{125}I]fd BSA IV, suggested that, like ethanol, benzyl alcohol caused surface binding of the labelled protein without internalisation. The uptake of [^{125}I]fd BSA IV by control yolk sacs in serum-free medium was linear over the incubation period with an E.I. of 408 µl/mg yolk-sac protein/h. Both series of benzyl alcohol-treated tissues showed linear time-courses of uptake over 5.0 h. The 0.4 mM benzyl alcohol-treated yolk sacs had an E.I. of 82 µl/mg yolk-sac protein/h while the 1.0 mM benzyl alcohol-treated yolk sacs had an E.I. of 76 µl/mg yolk-sac protein/h, rates that are approximately 20% and 18% relative to the matched controls. The patterns of tissue-associated

radioactivity were similar to those of the ethanol-treated yolk sacs in serum-free medium (see Chapter 4, Fig. 4.3.1.2).

In order to remove this non-specific tissue-associated binding of the substrate, it was decided to conduct detailed studies of the uptake of [^{125}I]fd BSA in the presence of 10% (v/v) fetal-calf serum (as in Chapter 4). The control yolk sacs (Fig. 6.3.2.1) showed a linear uptake of [^{125}I]fd BSA IV in the presence of 10% (v/v) fetal-calf serum with an E.I. of 98 ± 24 µl/mg yolk-sac protein/h. At the 5.0 h incubation point the control uptake value was 492 ± 101 µl/mg yolk-sac protein while those for the 0.4 mM and 1.0 mM benzyl alcohol-treated yolk sacs were 41.5 ± 11 and 21.0 ± 7.6 µl/mg yolk-sac protein, rates that are only 8.4% and 4.3% relative to matched controls. So benzyl alcohol at 0.4 mM and 1.0 mM concentrations caused an immediate and almost complete inhibition of adsorptive-phase pinocytosis (Fig. 6.3.2.1). However, when the quantities of tissue-associated radioactivity are inspected (Fig. 6.3.2.2) it can be seen that the control yolk sacs show the typical steady-state kinetics pattern with a mean value of 83.9 ± 13.8 µl/mg yolk-sac protein. At the 5.0 h incubation point the quantity of tissue-associated radioactivity in control yolk sacs was 79.6 ± 16.2 µl/mg yolk-sac protein but for 0.4 mM and 1.0 mM benzyl alcohol-treated yolk sacs the equivalent values were 25.9 ± 7.4 and 12.8 ± 4.7 µl/mg yolk-sac protein, values that are 32% and 16% of the matched-control values, respectively. Thus, although benzyl alcohol at 0.4 mM and 1.0 mM concentrations caused rapid and almost complete inhibition of adsorptive-phase pinocytosis (Fig. 6.3.2.1), the quantity of radioactivity associated with the tissue did not decrease as markedly.

Since 17.5-day yolk sacs were able to recover their ability to pinocytose [^{125}I]PVP once benzyl alcohol (0.4 mM and 1.0 mM) was

removed from the incubation medium (Fig. 6.3.1.5), it was decided to investigate the effects of benzyl alcohol pre-treatment on the recovery of adsorptive uptake and intralysosomal proteolysis of [^{125}I]fd BSA (Figs. 6.3.2.3, 6.3.2.4 & 6.3.2.5).

The effects of 0.5 h pre-treatment with benzyl alcohol (0.4 mM and 1.0 mM) on the net uptake of [^{125}I]fd BSA IV in serum-free medium are shown in Fig. 6.3.2.3. The control yolk sacs show linear uptake over the 4.0 h re-incubation period with a mean E.I. of 415 ± 25 µl/mg yolk-sac protein/h. The 0.4 mM and 1.0 mM benzyl alcohol-treated yolk sacs also showed linear uptake of the substrate during the re-incubation period with mean E.I. values of 392 ± 22 and 472 ± 52 µl/mg yolk-sac protein/h, rates of uptake that were 94% and 114% relative to matched controls. Thus, at the concentrations of benzyl alcohol used, adsorptive-phase pinocytosis appears to recover completely following 0.5 h pre-treatment with the agent.

However, when the corresponding tissue-associated radioactivity plots were inspected (Fig. 6.3.2.4) an interesting feature emerged. The control yolk sacs showed the typical steady-state kinetics pattern seen in earlier chapters (4, 5 & 6) with a mean quantity of tissue-associated radioactivity of 198 ± 23 µl/mg yolk-sac protein. But, the 0.4 mM and 1.0 mM benzyl alcohol-treated yolk sacs showed peaks in their tissue-associated radioactivity values at 2.5 h that were 166% and 202% of the corresponding control value. These values quickly decreased to near normal by 4.0 h of incubation in a manner that was similar to the effects of ethanol pre-treatment of yolk sacs ingesting [^{125}I]fd BSA (Fig. 4.3.1.10). These results suggest that benzyl alcohol reversibly inhibits the transfer of substrate from the endosomal to the lysosomal compartments as well as the uptake of the substrate from the extracellular medium into the endosomal compartment. This suggestion was given more support by examining the

pattern of release of TCA-soluble radioactivity into the incubation medium following 0.5 h benzyl alcohol pre-treatment (Fig. 6.3.2.5). The control yolk sacs showed a linear release of TCA-soluble radioactivity over the entire re-incubation period studied (1.5 - 4.0 h). The 1.0 mM benzyl alcohol-treated yolk sacs showed a "lag phase" in the release of TCA-soluble radioactivity of 1.5 h before recovering their ability to release TCA-soluble radioactivity into the incubation medium at a rate above that of the controls. These results are similar to those seen in Fig. 4.3.1.11 for release of TCA-soluble radioactivity following ethanol pre-treatment. It is interesting to note that the period of enhanced release of TCA-soluble radioactivity by benzyl alcohol-treated yolk sacs coincided with the time of decrease in the elevated quantity of tissue-associated radioactivity in the same yolk sacs. Considered together, the results obtained in Figs. 6.3.2.3, 6.3.2.4 & 6.3.2.5 would seem to be compatible with the suggestion of Tolleshaug & Berg (1982) that benzyl alcohol inhibits the transfer of substrate from endosomes to lysosomes.

Fig. 6.3.1.1 Dose response curve for the effects of benzyl alcohol on the uptake of [^{125}I]PVP

Yolk sacs were incubated separately in 9.0 ml of TC199 containing [^{125}I]PVP (2 µg/ml). At the 1.0 h incubation point 1.0 ml of medium containing benzyl alcohol was added (giving a final concentration of 0.02 - 20.0 mM). The incubation was allowed to proceed for a further 4.0 h before uptake was terminated. Each point is the mean of two yolk sacs.

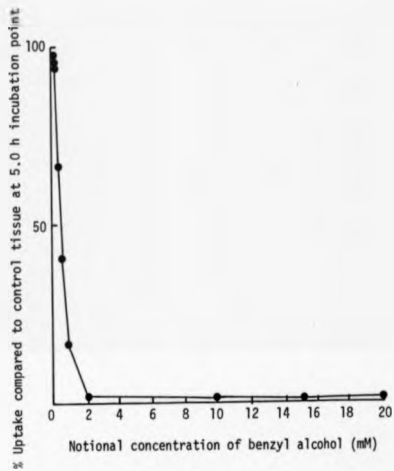


Fig. 6.3.1.2 The effect of benzyl alcohol on the uptake of [^{125}I]PVP

Yolk sacs were incubated separately in serum-free medium 199 (9.0 ml) containing [^{125}I]PVP (2 μg/ml). After 1.0 h incubation, either a further 1.0 ml of medium or of benzyl alcohol (final concentration of 0.493 or 1.093 mM) in medium was added to each flask. Uptake of [^{125}I]PVP was then monitored up to 5.0 h. Each point represents the mean uptake (± S.D.) by at least six yolk sacs.

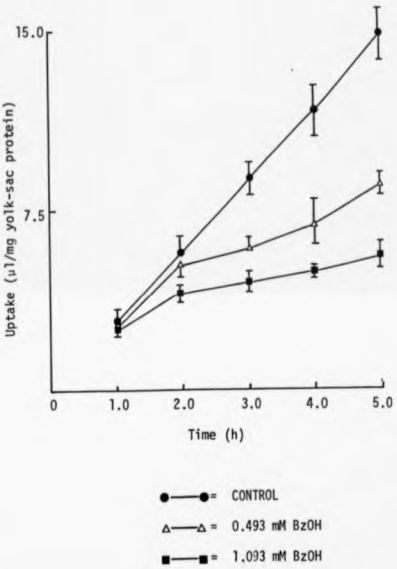


Fig. 6.3.1.3 The effect of benzyl alcohol on the uptake of [^{125}I]PVP

Yolk sacs were incubated separately in serum-free medium 199 (9.0 ml) containing [^{125}I]PVP (2 ug/ml). After 1.0 h incubation, either a further 1.0 ml of medium or of medium containing benzyl alcohol (final concentration 2.093 or 10.093 mM) was added to each flask. Uptake of [^{125}I]PVP was monitored up to 5.0 h. Each point represents the mean uptake (± S.D.) by at least six yolk sacs.

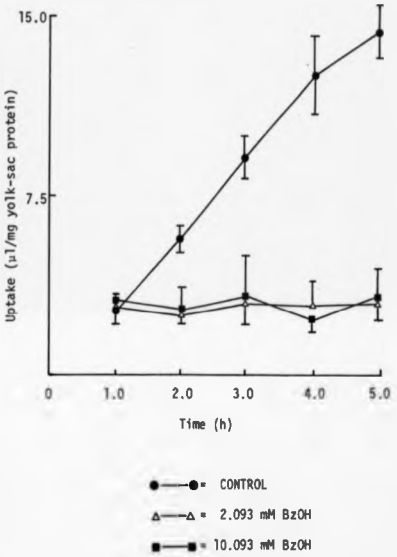


Fig. 6.3.1.4 The effects of benzyl alcohol on the release of [^{125}I]PVP

Yolk sacs were incubated separately (but in triplicate) in serum-free medium 199 (10.0 ml) containing [^{125}I]PVP (2 μg/ml) for 2.5 h, then removed and rinsed (3 x 2 min) in fresh serum-free, substrate-free medium at 37°C. The yolk sacs were then re-incubated for a further 2.75 h in 10.0 ml of TC199 containing either 0.4 or 1.0 mM benzyl alcohol. Each point represents the mean release values (± S.D.) of at least nine yolk sacs.

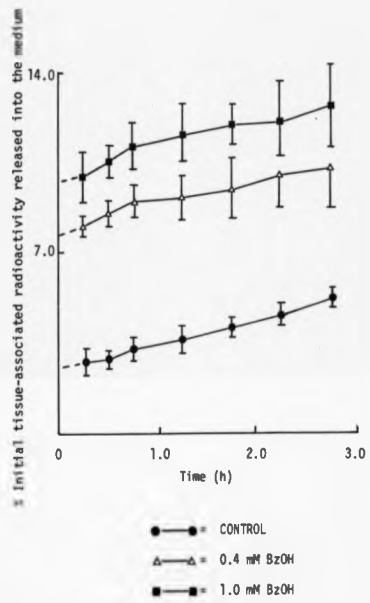


Fig. 6.3.1.5 The effect of benzyl alcohol pre-treatment on the uptake of [^{125}I]PVP

Yolk sacs were incubated separately in serum-free medium 199 containing benzyl alcohol (0.4 or 1.0 mM) for 0.5 h. The yolk sacs were then removed and rinsed (3 x 2.0 min) in fresh TC199 at 37°C before being re-incubated in serum- and benzyl alcohol-free medium containing [^{125}I]PVP (2 µg/ml) for 4.0 h. Each point reporesents the mean uptake (± S.D.) by at least six yolk sacs.

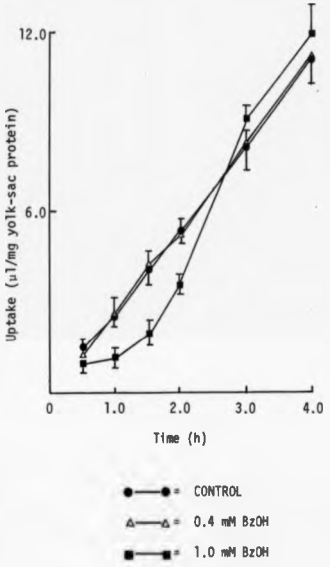


Fig. 6.3.2.1 The effect of benzyl alcohol on the uptake of [^{125}I]fd BSA IV

Yolk sacs were incubated separately in medium 199 (8.0 ml) containing 10% (v/v) fetal-calf serum. At 0.0 h of incubation 1.0 ml of medium containing [^{125}I]fd BSA IV was added, resulting in a substrate concentration of 5 ug/ml, and 1.0 ml of medium containing benzyl alcohol (final concentration 0.4 or 1.0 mM) was also added. Uptake of [^{125}I]fd BSA IV was then monitored up to 5.0 h. Each point represents the mean uptake (± S.D.) by at least six yolk sacs.

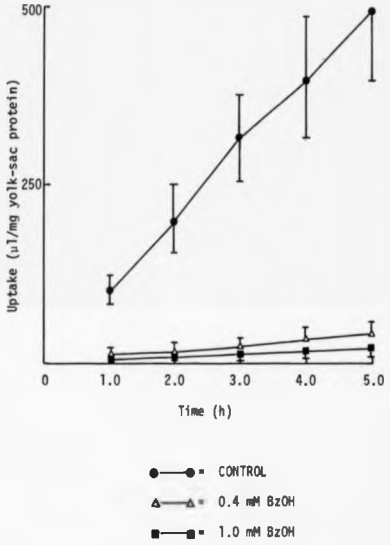


Fig. 6.3.2.2 The effect of benzyl alcohol on the tissue-associated radioactivity of [^{125}I]fd BSA IV

Yolk sacs were incubated as in Fig. 6.3.2.1. Each point represents the mean tissue-associated radioactivity values (± S.D.) of at least six yolk sacs.

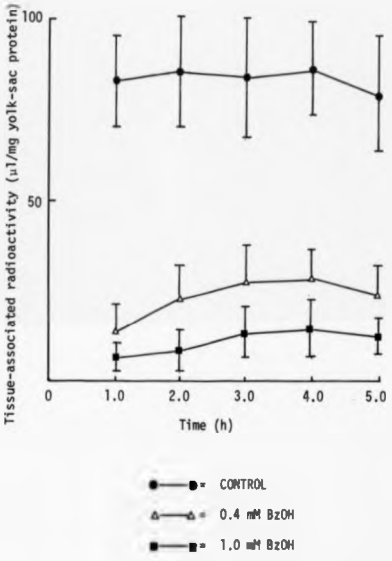


Fig. 6.3.2.3 The effect of benzyl alcohol pre-treatment on the uptake of [^{125}I]fd BSA IV

Yolk sacs were incubated separately in serum-free medium 199 containing benzyl alcohol (0.4 or 1.0 mM) for 0.5 h. The yolk sacs were removed and rinsed (3 x 2.0 min) in fresh TC199 at 37°C before being re-incubated in serum- and benzyl alcohol-free medium containing [^{125}I]fd BSA IV (5 µg/ml) for 4.0 h. Each point represents the mean net uptake (± S.D.) of at least six yolk sacs.

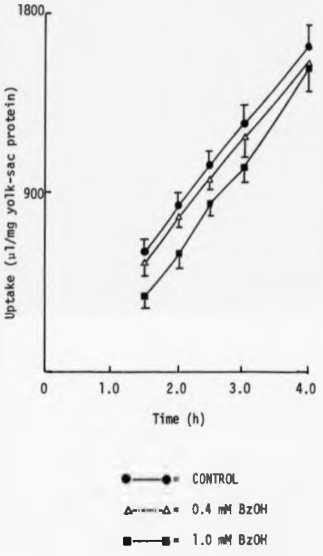


Fig. 6.3.2.4 The effect of benzyl alcohol pre-treatment on the quantity of tissue-associated radioactivity derived from [^{125}I]fd BSA IV

Yolk sacs were incubated as in Fig. 6.3.2.3. Each point represents the mean quantity (± S.D.) of tissue-associated radioactivity from at least six yolk sacs.

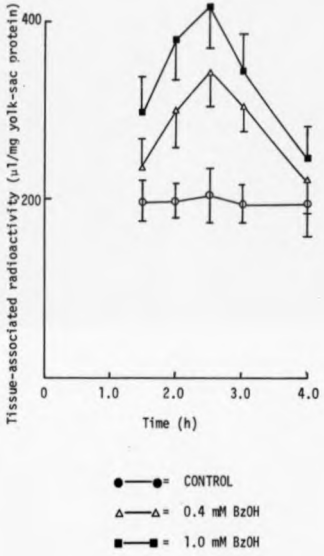
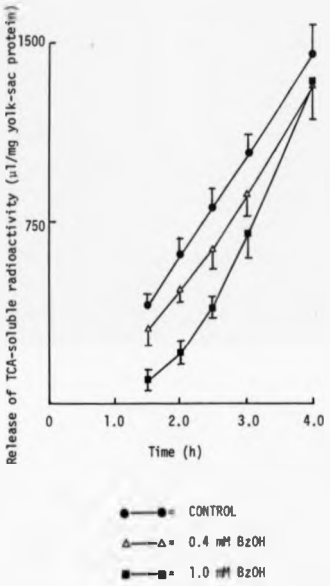


Fig. 6.3.2.5 The effect of benzyl alcohol pre-treatment of yolk sacs on the release of TCA-soluble radioactivity into the incubation medium

Yolk sacs were incubated as in Fig. 6.3.2.3. Each point represents the mean (± S.D.) TCA-soluble radioactivity values of at least six yolk sacs.



6.4 DISCUSSION

Benzyl alcohol is used as a preservative in pharmaceutical formulations because of its stability, antibacterial activity and supposed low toxicity (Novak et al., 1972). But, Gershanik et al. (1982) forward evidence to indicate that benzyl alcohol, at a bacteriostatic concentration of 0.9% (w/v) in aqueous sodium chloride (for intravenous administration into premature babies) caused death via the "Gasping Syndrome". The clinical symptoms of this condition include gradual neurological deterioration, severe metabolic acidosis, rapid onset of gasping respiration, hematological abnormalities, skin breakdown, hepatic and renal failure, hypotension and cardiovascular collapse. Infants with the Gasping Syndrome received an average daily quantity of benzyl alcohol (via the bacteriostatic sodium chloride solution) of 99 - 234 mg/kg body weight. Analysis of blood samples showed benzyl alcohol concentrations ranging from 0.61 - 1.38 mM (66 - 149 mg/100 ml), and urine samples contained benzoic acid and hippuric acid (metabolites of benzyl alcohol) ranging from 88 - 685 μM (1.0 - 8.0 mg/100 ml) and 0.85 - 2.1 mM (15 - 38 mg/100 ml), respectively. It was also reported that, since discontinuing the use of bacteriostatic sodium chloride and water in their nurseries in June 1981, no further cases of the Gasping Syndrome were seen.

The single dose of 0.9% (w/v) benzyl alcohol that is considered safe for "healthy human adults" is 30 ml or approximately 0.5 ml/kg body weight or 480 mg/kg body weight (Gershanik et al., 1982). McCloskey et al. (1986) reported that in the CD-1 strain of mice (both adult and neonatal) benzyl alcohol and not its metabolite, benzaldehyde, was the cause of sedation, dyspnea and loss of motor function following an acute intraperitoneal injection of benzyl alcohol into adult (23 - 28 g) and neonatal (2 - 7 g) CD-1 mice. It should be noted that, in humans, benzoic acid and hippuric acid and

not benzaldehyde are the major metabolites of benzyl alcohol. Further reports on the use of benzyl alcohol as a preservative in pharmaceutical preparations were made by Jordan et al. (1986) and van der Hal et al. (1987).

The effect of 10.0 mM and 2.0 mM benzyl alcohol on fluid-phase pinocytosis was to inhibit immediately the uptake of [^{125}I]PVP. The concentrations of benzyl alcohol needed to induce degrees of inhibition of fluid-phase pinocytosis equivalent to that caused by 2.0 mM and 10.0 mM ethanol were 0.49 mM and 1.09 mM. Thus, relative to ethanol, 17.5-day rat visceral yolk sacs appear to be more sensitive to benzyl alcohol as an inhibitor of fluid-phase pinocytosis. Also, benzyl alcohol (Figs. 6.3.1.2 & 6.3.1.3), unlike ethanol (Fig. 3.3.1.1), appears to cause no extensive release of the tissue-associated [^{125}I]PVP into the incubation medium.

When the effects of 0.4 mM and 1.0 mM benzyl alcohol on the release of [^{125}I]PVP by 17.5-day yolk sacs were investigated (Fig. 6.3.1.4), it was found that the two concentrations used both had no effect on the rate of progressive loss of [^{125}I]PVP from yolk sacs, but increased the initial (over the first 15 min) release of substrate to values that were 300% and 370% above that of the matched controls. These results are higher than those reported for 2.0 mM and 10.0 mM ethanol (Fig. 3.3.1.2 & Table 3.3.1.2) again indicating that the tissue was more sensitive to benzyl alcohol than to ethanol.

Like ethanol-induced inhibition of fluid-phase pinocytosis, benzyl alcohol inhibition was readily reversible (Fig. 6.3.1.5). The 0.4 mM benzyl alcohol-treated yolk sacs recovered their capacity to take up [^{125}I]PVP completely and immediately while the 1.0 mM benzyl alcohol-treated tissue first showed a period of pinocytic inactivity (for approximately 1.0 h) then pinocytosed [^{125}I]PVP at a rate above that of the matched controls before finally adopting the control rate

of uptake during the last hour of re-incubation. These results are similar to those with 10.0 mM ethanol pre-treated tissues (see Fig. 3.3.1.4).

Unlike 2.0 mM and 10.0 mM ethanol, which completely inhibited the adsorptive-phase uptake of [^{125}I]fd BSA in 10% (v/v) fetal-calf serum, benzyl alcohol (0.4 mM and 1.0 mM) caused an almost complete inhibition of uptake, with benzyl alcohol-treated tissues showing a rate of uptake that was between 4 and 8% of that of the matched controls (Fig. 6.3.2.1). This resulted in the tissue-associated radioactivity values that were 16 - 32% of that of the controls (Fig. 6.3.2.2) unlike those of the ethanol-treated yolk sacs (Fig. 4.3.1.6) which showed almost no tissue-associated radioactivity. This could possibly be due to benzyl alcohol, which is known to displace bilirubin from albumin-binding sites (Gershanik et al., 1982), having a greater affinity for binding to serum proteins than ethanol, thus resulting in a greater decrease in alcohol concentration in the incubation medium for benzyl alcohol than ethanol (this problem would not arise in serum-free incubations). When the effects of 0.5 h pre-treatment with benzyl alcohol (0.4 mM and 1.0 mM) on the uptake and intralysosomal proteolysis of [^{125}I]fd BSA IV were investigated (Figs. 6.3.2.3, 6.3.2.4 & 6.3.2.5), no effect on the net uptake of the substrate could be seen (Fig. 6.3.2.3) other than the induction of a lag period. But, when the tissue-associated radioactivity values and TCA-soluble radioactivity release values were separated and inspected, a pattern of results similar to that caused by 2.0 mM and 10.0 mM ethanol pre-treatment of yolk sacs emerged (Figs. 4.3.1.10 & 4.3.1.11). Both 0.4 mM and 1.0 mM benzyl alcohol pre-treatment caused elevated quantities of radioactivity to become associated with the tissue relative to the matched controls (Fig. 6.3.2.4). These values increased over the period 1.5 - 2.5 h, then reached a peak before

decreasing to control values over the period 2.5 - 4.0 h. These periods of elevated tissue-associated radioactivity coincided with the "lag periods" in the release of TCA-soluble radioactivity by the benzyl alcohol-treated yolk sacs (Fig. 6.3.2.5).

Moreover, following the "lag phases", periods of release of TCA-soluble radioactivity at rates above those of the matched controls followed. These periods of enhanced TCA-soluble radioactivity production coincided with the decrease in the quantities of tissue-associated radioactivity from the elevated values of the benzyl alcohol-treated yolk sacs (Fig. 6.3.2.4). Thus these results support the suggestion of Tolleshaug & Berg (1982) that benzyl alcohol decreases/inhibits the rate of transfer of endocytosed protein from the endosomes to the lysosomes. The results are also compatible with the observation (Grinde, 1984) that benzyl alcohol reversibly inhibits the methylamine sensitive pathway of protein degradation.

In summary, the data gathered in this chapter indicate that the 17.5-day rat visceral yolk sac was more sensitive to inhibition by benzyl alcohol relative to ethanol. The main findings were:

(i) Benzyl alcohol inhibits fluid-phase pinocytosis (of [^{125}I]PVP) at concentrations below those required by ethanol to produce equivalent results.

(ii) Benzyl alcohol causes a greater initial release of [^{125}I]PVP over the first 15 min of incubation of yolk sacs in 0.4 mM and 1.0 mM benzyl alcohol relative to that produced by higher ethanol concentrations.

(iii) The benzyl alcohol-induced inhibition of fluid-phase pinocytosis was readily reversible once the agent was removed. (In the case of the 0.4 mM benzyl alcohol-treated tissue, the recovery was immediate and complete but the 1.0 mM benzyl alcohol-treated yolk sacs required a recovery

period of 1.0 h before pinocytic activity resumed.)

(iv) Benzyl alcohol (0.4 - 1.0 mM) produced a 92 - 96% inhibition of adsorptive-phase pinocytosis.

(v) Benzyl alcohol pre-treatment induced an inhibition of degradation of albumin within the lysosomal system which was reversible once the agent was removed and a recovery period of 0.75 - 1.5 h had passed.

In conclusion, the findings in this chapter suggest that alcohols in general can be expected to interfere with the functions of the visceral yolk sac and some may thus be more effective teratogens than ethanol. The use of benzyl alcohol and other alcohols (e.g. chlorobutanol) as preservatives in pharmaceutical preparations needs to be given a more rigorous investigation in order to establish their possible mechanism(s) of action as teratogens or cytotoxic agents in both adult and neonatal animals and humans.

CHAPTER SEVEN

THE EFFECTS OF ALCOHOLS ON THE RELEASE OF [^{125}I]PVP AND [^{125}I]fd BSA FROM THE ENDOSOMAL COMPARTMENT OF THE VISCERAL YOLK SAC

7.1 INTRODUCTION

The fate of internalised plasma membrane following endocytosis is one of great interest since the cell must either: (1) dissociate the internalised plasma membrane into its monomer units prior to catabolism or re-synthesis or (2) recycle the intact plasma membrane back to the cell surface via "budding off" of excess membrane either from fusing pinosomes (Duncan & Pratten, 1977) or from lysosomal membranes. If the cell did not carry out one or more of these functions it would rapidly internalise its excess plasma membrane. The total degradation of the constituent monomers would be extremely wasteful from an energetic standpoint, while the recycling of the membrane via pinosomes (Duncan & Pratten, 1977) or via lysosomes (Dean, 1977) will be energetically more favourable. However, both these hypotheses lack experimental evidence to support them.

Work by Schneider et al. (1979) started to rectify this situation, by using rat-embryo fibroblasts labelled with fluorescein-tagged-anti-plasmamembrane IgG, and showed that plasma membrane internalised by endocytosis was recycled/shuttled back to the cell curface. Also, Dean & Jessup (1982) reported that [^{3}H]sucrose previously endocytosed by normal human-skin fibroblasts showed a slow phase of linear exocytosis after an initial burst of release. Such release was not due to cell lysis since it was virtually abolished at 4^{o}C. It was also reported (Besterman et al., 1981) that [^{14}C]sucrose, internalised via fluid-phase pinocytosis, was released by pulmonary-alveolar macrophages and by fetal-lung fibroblasts. This release of the substrate from pre-loaded cells was temperature-sensitive, but not dependent on extracellular calcium or magnesium. Furthermore, results indicated that the process of fluid-phase pinocytosis and subsequent exocytosis required at least two intracellular compartments in series, one being of a small

capacity with a very rapid turnover ($t_{0.5}$ = 5 min in macrophages, 6 - 8 min in fibroblasts) and the other compartment being larger in capacity and showing a very slow turnover ($t_{0.5}$ = 180 min in macrophages, 430 - 620 min in fibroblasts). When Chinese-hamster ovary-cells were cultured in the presence of horseradish peroxidase, the substrate was shown to be internalised via fluid-phase pinocytosis (Adams et al., 1982). After an horseradish peroxidase pulse of 2.5 - 10 min, a rapid decrease of approximately 30 - 50% in cell-associated substrate was observed within 10 - 20 min at 37°C. During this period of loss of cell-associated horseradish peroxidase, an equivalent increase in extracellular horseradish peroxidase activity was seen. Following this period of rapid loss, the remaining cell-associated substrate activity decreased with a $t_{0.5}$ of 6 - 8 h (known lysosomal half-life for horseradish peroxidase). When pulse-chase experiments with the substrate were conducted, the horseradish peroxidase was chased into a non-exocytic compartment. Based on cell-fractionation and electron-microscopy work, the non-exocytic compartment was identified as lysosomal while the exocytic compartment was pinosomal/endosomal.

The uptake and intracellular processing of [^{3}H]inulin (a marker of fluid uptake) by rat hepatocytes in culture or by perfused rat liver (Scharschmidt et al., 1986) showed that the rates of uptake and of efflux of the substrate were kinetically compatible with the entry of [^{3}H]inulin into a rapidly ($t_{0.5}$ = 1 - 2 min) turning-over endosomal compartment, that exchanged contents with the extracellular space and which comprised approximately 3% of the hepatocyte volume, before entering and concentrating within a slowly ($t_{0.5}$ > 1.0 h) turning-over storage compartment. Thus it can be estimated that hepatocytes endocytosed the equivalent of 20% or more of their volume and 5 or more times their plasma-membrane surface-area each hour. Chloroquine

(1 mM) and 200 μM taurocholate (an inhibitor of biliary secretion) had no effect on [^{3}H]inulin handling by cultured hepatocytes, but colchicine (10 μM) inhibited the transfer of the substrate to storage compartments by more than 50%. These results suggest that [^{3}H]inulin, endocytosed across the basolateral membrane, was largely (80%) exocytosed back into the plasma while smaller amounts were transported to intracellular storage compartments (18%) or to the bile (2%). The results described in the last few pages of this paper (Scharschmidt et al., 1986) seem to support the hypothesis that plasma membranes internalised via endocytosis are returned to the cell surface via exocytosis from an endosomal/pinosomal compartment.

In the rat visceral yolk sac, the kidney proximal-tubules of arctic lampreys and golden-hamster ductuli efferentes unique and highly-ordered structures were discovered (Hatae et al., 1986a). These absorbing epithelia share a number of common features including a brush border of numerous microvilli and a well-developed system of endocytic vesicles and tubules in the apical cytoplasm. These apical tubules show a cylindrical structure limited by a unit membrane, their diameter is approximately 80 nm while their length varies from 500 to 1500 nm. The larger tubules bend in different directions and occasionally branch or anastomose with one another. They are not seen to be continuous with the apical plasma membrane, but are frequently connected with the large endocytic vacuoles lying at some distance from the cell surface. In longitudinal sections, the apical tubules are shown to contain parallel bands of electron-dense material in close proximity to the limiting membrane. These wind in the form of a left-hand helix within the tubule, with the bands being approximately 14 nm apart and at an angle of 70^0 to the axis of the tubule. These tubule-vacuole complexes described above are reminiscent of CURL (compartment of uncoupling of receptor and ligand) as described by

Geuze et al. (1984) in receptor-mediated endocytosis.

Moreover, when rat-kidney proximal-tubule cells were exposed in vivo to horseradish peroxidase (Hatae et al., 1986b) it was found that, 0.5 min after injection, invaginations of the plasma membrane and small, apical endocytic vesicles, including coated vesicles, were labelled with reaction product. Almost all the large endocytic vacuoles and the apical tubules were negatively stained. Immediately (1 min) after injection, the endocytic vacuoles and about 18% of the apical tubules were labelled. The reaction product in the large endocytic vacuoles was usually seen on the luminal surface of the vacuoles. The apical tubules appeared as a branched network frequently connected to the labelled endocytic vacuoles. After 3 min, the reaction product was detected in about 38% of the apical tubules; the percentage increased to 74% after 7 min. These results indicate that the apical tubules are probably formed by "budding off" from the large endocytic vacuoles rather than being formed directly in the endocytic process.

This is of interest since Kugler & Miki (1985) looked at the internalisation and intracellular movements of apical cell-membrane material in the endodermal cells of cultured visceral yolk sacs of rats. The yolk sacs were explanted at 10.5 days of gestation and cultured for 24 h in the presence of horseradish peroxidase- and ferritin-labelled concanavalin-A (Con-A HRP, Con-A Fer). When visceral yolk-sac endoderm was exposed to Con-A HRP or Con-A Fer for 5 min at 4^{o}C, the apical cell membranes were heavily labelled but apical vacuoles, lysosomes and apical canaliculi were not. Incubation of Con-A-labelled endoderm for 5 - 60 min at 20^{o}C and 37^{o}C in Con-A-free serum resulted in a temperature-dependent internalisation of membrane-bound lectin into coated vesicles, apical vacuoles and lysosomes. After 30 and 60 min at 37^{o}C, most of the apical canaliculi

contained high concentrations of the markers. With increasing incubation time, the number of labelled vacuolar structures and the intensity of their labelling decreased, but the number of labelled apical canaliculi increased. These findings suggest that the apical canaliculi do not fuse with, but detach from, the apical vacuoles and lysosomes and that apical plasma membrane internalised via endocytosis is then brought back to the plasma membrane by the apical canaliculi which concentrate and store the membrane material. This form of membrane recycling complies with the model proposed by Steinman et al. (1983).

A further study was carried out by Miki & Kugler (1986) on the effect of exposure to leupeptin (25.0 µg/ml) on the endocytic activity and membrane-flow of apical-cell membranes in endodermal cells of 10.5-day rat visceral yolk sacs cultured for 24 h. Again Con-A HRP and Con-A Fer were used to label yolk-sac plasma-membranes. Significantly-fewer labelled apical vacuoles, lysosomes and apical canaliculi were present after leupeptin treatment than in controls at the corresponding times. At all times examined, the giant lysosomes found in leupeptin-treated cells exhibited no labelling. These findings indicate that, after leupeptin treatment, both endocytic activity and membrane recycling decreased and that fusions of the apical vacuolar system with giant lysosomes were retarded or inhibited.

This idea, that apical tubules or canaliculi in the rat visceral yolk sac are involved in membrane recycling, is of great interest. In Chapter 3 it was reported that yolk sacs pre-loaded with [^{125}I]PVP released 9.7% of their tissue-associated radioactivity into the extracellular medium during the first 15 min of exposure to 10.0 mM ethanol. To explain this phenomenon, an hypothesis: "The Nascent-Pinosome Hypothesis", was evolved. The hypothesis states that

ethanol induces only nascent pinosomes to release their contained [^{125}I]PVP since, after a 2.50 h "loading period", only approximately 10% of the total tissue-associated radioactivity was discharged and this comes only from "nascent pinosomes". (Such pinosomes are envisaged as being formed in the final 10% of 2.50 h, i.e. the final 0.25 h.) If the hypothesis is valid it permits three predictions to be made.

Following a pulse period of [^{125}I]PVP uptake (either 0.25 h or 0.50 h exposure to x 5 the normal [^{125}I]PVP concentration):-

I Complete protection of [^{125}I]PVP against ethanol-induced release, following a pulse uptake period of 0.25 h, will be afforded by a chase period equal to or greater than 0.25 h, since only pinosomes formed within 0.25 h are susceptible to ethanol-induced release.

II If the pulse period is 0.25 h and the tissue is washed and transferred almost immediately to 10.0 mM ethanol-containing medium, the fraction of the total tissue-associated [^{125}I]PVP discharged should be close to 100%, since all the tissue-associated substrate should be in "nascent pinosomes" and therefore susceptible to release.

III If the pulse period is increased to 0.50 h and the chase period cut to a minimum (i.e. a few minutes) then approximately 50% of the total tissue-associated [^{125}I]PVP should be discharged on re-incubation in the presence of 10.0 mM ethanol (i.e. approximately 50% should be present in "nascent pinosomes" and hence susceptible to release).

The work reported in this chapter attempts to provide experimental evidence to test the "Nascent-Pinosome Hypothesis" by use of the pulse-chase experiments outlined above. Most of the results reported in this chapter deal with the effects of 10.0 mM ethanol on

[^{125}I]PVP release, but some results on the effects of 2.0 mM ethanol and 0.4 mM and 1.0 mM benzyl alcohol on [^{125}I]PVP release and 10.0 mM ethanol effects on the release of [^{125}I]fd BSA are also reported.

7.2 MATERIALS AND METHODS

The materials and methods used in this chapter are described in full in Chapter 2: see Sections 2.13, 2.14, 2.15 & 2.16 for the release of [^{125}I]PVP and [^{125}I]fd BSA; see Section 2.17 for the release of lactate dehydrogenase and of N-acetyl-β-glucosaminidase.

The format of the pulse-chase experiments was modified slightly following the initial scan of "one-off" experiments. The normal 3 x 2 min wash period, to remove any extracellular [^{125}I]PVP loosely associated with the yolk sacs, contributed a significant fraction (at least 6 - 8 min) to the chase period. [If the chase period was 2.00, 1.00 or 0.50 h then it added an extra 5%, 10% and 20% to the chase periods, respectively. When the chase period was only 0.25 h, it added an extra 45%, and when notionally no chase period was used, the error was even greater.] Thus a "quick-wash" method, as used by Thoene et al. (1985), for rinsing rat yolk sacs was employed. Three 0.25 min washes ensured that the total time elapsed before the tissue was transferred to fresh medium was only approximatley 1.5 - 2.0 min. In this way, in the modified method the wash-period accounted for a far smaller percentage of the chase-period.

Since the "loading" or pulse-period was reduced from the ususal 2.50 h (see Chapter 3) to 0.50 h (a 5.0-fold decrease), to maintain good counting statistics the [^{125}I]PVP concentration in the medium during the pulse-period was increased from 2.0 µg/ml to 10.0 µg/ml (a 5.0-fold increase). Also, the volume of incubation medium in the pulse flasks and the number of yolk sacs were altered from 1 yolk sac in 10.0 ml of TC199 to 3 yolk sacs in 7.0 ml of medium to obtain adequate quantities of radioactivity in the yolk-sac tissue and medium samples without excessive use of the labelled substrate.

7.3 RESULTS

7.3.1 The effects of ethanol on the release of [^{125}I]PVP from yolk sacs

In Table 7.3.1.1 the effects of various pulse-chase regimes on the release of [^{125}I]PVP are reported.

The effect of a 0.50 h pulse with [^{125}I]PVP (10.0 µg/ml) and 2.00, 1.00 or 0.50 h chase-periods (Table 7.3.1.1) was to give complete protection, from 10.0 mM ethanol-induced release of [^{125}I]PVP, to the yolk sacs. The percentages of the total tissue-associated radioactivity released at 0.00 and 0.25 h were similar, regardless of the chase period, while the rate of progressive loss of [^{125}I]PVP from control and ethanol-treated tissue remained in the range 0.56 - 0.65%/h. The losses of [^{125}I]PVP from control tissue at 0.00 and 0.25 h are in agreement with those of Williams et al. (1975a) and Roberts et al. (1976) while the rate of progressive loss was in agreement with Williams et al. (1975a) but below that reported by Roberts et al. (1976) for control tissues. These initial results give some support to prediction I of the hypothesis.

Support for prediction III can also be found in Table 7.3.1.1. When a pulse-period of 0.50 h was followed by immediate exposure to 10.0 mM ethanol (after effectively zero washing- or chase-period), the control and ethanol-treated tissue showed similar values for the initial (relative to ethanol addition) loss of radioactivity (3.88 ± 0.19% compared with 3.99 ± 0.28%). However, by 0.25 h the values for the loss of [^{125}I]PVP from the tissue were 4.09 ± 0.26 and 48.8 ± 2.7% for the control and ethanol-treated yolk sacs, respectively. Also, there was an increase in the rate of progressive loss of substrate (over the period 0.25 - 2.75 h) following ethanol treatment.

When the pulse-period was decreased to 0.25 h and no

chase-period was allowed, the initial losses by both control and ethanol-treated tissues were very similar (see Table 7.3.1.1). However, by 0.25 h the percentage of the total tissue-associated [^{125}I]PVP released was 4.0 ± 0.2% and 91.5 ± 1.9% for the control and 10.0 mM ethanol-treated tissues, respectively. Furthermore, the rate of progressive loss of radioactivity from the ethanol-treated yolk sacs was 2.2%/h compared with 0.68%/h for the matched controls. These results give some support to prediction II of the "Nascent-Pinosome Hypothesis". Finally, the effects of 0.25 h pulse and 0.25 h chase can be seen in Table 7.3.1.1. Both control and 10.0 mM ethanol-treated tissues show similar values for the initial loss of [^{125}I]PVP, both at zero time and at 0.25 h; the rates of progressive loss of [^{125}I]PVP are also similar. These preliminary results indicate that, after a 0.25 h pulse-period, a 0.25 h chase-period in ethanol-free medium effectively prevents ethanol-induced release of [^{125}I]PVP from yolk sacs.

A further series of experiments was conducted with both short pulse-periods (0.25 - 0.50 h) and short chase-periods (0.00 - 0.25 h), using the modified method with only a 3 x 0.25 min wash period, to further test the hypothesis. In Table 7.3.1.2 & Fig. 7.3.1.1, the effects of a 0.50 h pulse-period and a 0.00 h chase-period can be seen. At time zero (relative to when ethanol was added to the incubation medium) the values for the percentage of the total tissue-associated radioactivity released are all very similar, but by 0.25 h the values are 4.1 ± 0.2% and 49.7 ± 5.0% for the control and 10.0 mM ethanol-treated yolk sacs (i.e. a 12.0-fold increase over the control value at the same time point). Also, the rate of progressive loss of [^{125}I]PVP during the re-incubation period showed a 2.0-fold increase.

If the pulse-period was decreased to 0.25 h and the

chase-period kept at 0.00 h (Table 7.3.1.2 & Fig. 7.3.1.2) the percentage of the total tissue-associated radioactivity released at zero time was nearly equal for the control and 10.0 mM ethanol-treated yolk sacs, but by 0.25 h the values were 4.1 ± 0.2% for the control tissue and 90.1 ± 4.6% for the 10.0 mM ethanol-treated tissue (a 22-fold increase relative to the control). A 4.0-fold increase in the rate of progressive loss of [^{125}I]PVP from ethanol-treated yolk sacs compared with the matched controls could also be seen (2.80 ± 0.72%/h compared with 0.70 ± 0.09%/h). Furthermore, if a 0.25 h pulse-period and a 0.00 h chase-period were used (Table 7.3.1.2 & Fig. 7.3.1.3) before exposure to 2.0 mM ethanol, a different pattern of results was found. The percentages released at zero time did not differ between control and 2.0 mM ethanol-treated yolk sacs, but the 0.25 h values were 4.22 ± 0.21% and 40.7 ± 2.3/%, respectively (a 9.5-fold increase for the ethanol-treated tissue). The values for the rate of progressive loss of [^{125}I]PVP were 0.69 ± 0.07%/h and 4.92 ± 0.48%/h for the matched controls and 2.0 mM ethanol-treated tissues (an increase of 7.0-fold on that of the matched controls). These results show that the effect of ethanol on the release of [^{125}I]PVP from the yolk sacs is concentration dependent.

Finally, when the tissue was exposed to [^{125}I]PVP for a 0.25 h pulse-period then a 0.25 h chase-period incorporated before 10.0 mM ethanol treatment, it provided complete protection against ethanol-induced release of [^{125}I]PVP (see Table 7.3.1.2 & Fig. 7.3.1.4). The control-tissue and ethanol-treated tissue values for the rate of progressive loss and for loss of [^{125}I]PVP at 0.00 and 0.25 h do not differ. These results give good experimental support to predictions I - III of the "Nascent-Pinosome Hypothesis".

7.3.2 The effects of ethanol on the release of lactate dehydrogenase and N-acetyl-β-glucosaminidase from yolk sacs

In this section of work, the pulse-chase regime and the ethanol concentration that caused maximum release of [^{125}I]PVP were used and the simultaneous release of [^{125}I]PVP, of a marker cytosolic enzyme (lactate dehydrogenase) and of a marker lysosomal enzyme (N-acetyl-β-glucosaminidase) were monitored to determine whether the release of [^{125}I]PVP was accompanied by leakage of cytosol or exocytosis of the lysosomal contents. The conditions employed were: a 0.25 h pulse-period, a 0.00 h chase-period, then exposure to 10.0 mM ethanol.

In Table 7.3.2.1, the effects of this regime on the release of [^{125}I]PVP and of lactate dehydrogenase are reported. At zero time, the control and ethanol-treated tissues show similar values for the percentage loss of the total tissue-associated radioactivity, but by 0.25 h the values are 4.2 ± 0.2% and 87.3 ± 4.4%, respectively, while the control yolk sacs show a rate of progressive loss of [^{125}I]PVP of 0.64 ± 0.06%/h and the ethanol-treated tissue a value of 4.11 ± 1.24%/h. (These results are in accord with those in Table 7.3.1.2.) However, when the loss of the total tissue-associated lactate dehydrogenase activity in the same experiment was investigated, the matched controls and 10.0 mM ethanol-treated yolk sacs did not differ at any time (see Table 7.3.2.1) indicating that the pulse-chase and 10.0 mM ethanol exposure regime did not induce release of this cytosolic marker.

Equivalent experiments were performed, using the same pulse-chase regime and the same ethanol concentration, but monitoring both the release of [^{125}I]PVP and of the lysosomal enzyme (N-acetyl-β-glucosaminidase). The results are reported in

Table 7.3.2.2. Again, by 0.25 h the 10.0 mM ethanol-treated yolk sacs had released 84.1 ± 2.2% of their total tissue-associated radioactivity whereas the control yolk sacs had released only 4.35 ± 0.36%. During this period, the release of N-acetyl-β-glucosaminidase by control and by the 10.0 mM ethanol-treated yolk sacs did not differ at any time (see Table 7.3.2.2). The control rate of progressive loss of the enzyme was below the 3%/h value reported by Roberts et al. (1976) for 17.5-day yolk sacs in 10% (v/v) calf serum over a 6.0 h incubation period. These results also support the idea that the release of [^{125}I]PVP was not from the lysosomal system, but only from nascent pinosomes or the endosomal system.

7.3.3 The effects of ethanol on the release of [^{125}I]fd BSA from yolk sacs

In preliminary experiments (data not reported in Figs. & Tables), when yolk sacs were given a 0.25 h pulse of [^{125}I]fd BSA (25.0 μg/ml) followed by a 3 x 0.25 min wash-period then a 0.00 h chase-period, the control yolk sacs released into the medium 8.0% of their total tissue-associated radioactivity by 0.25 h and 14.0% by 0.50 h. The value subsequently rose to approximately 16.0% before "plateauing off" for the rest of the re-incubation period. However, the 10.0 mM ethanol-treated yolk sacs showed a different pattern of results. By 0.25 h the percentage of the total tissue-associated radioactivity released into the medium was approximately 40% and rose to 44% by 0.50 h before remaining at this value for the rest of the incubation period. [No entirely satisfactory explanation could be found for this 2.75-fold increase in the release of [^{125}I]fd BSA from yolk sacs induced by 10.0 mM ethanol. Possibly, during a short (0.25 h) pulse-exposure of the tissue to formaldehyde-treated albumin, quite a high percentage of the radioactivity associated with the

tissue is bound to non-specific sites on the tissue surface that are not involved in the pinocytic uptake of the protein. If ethanol induces a decrease in the affinity of albumin for such sites, a rapid dissociation of radioactivity from the tissue could occur on exposure to ethanol. If this explanation is valid, pre-exposure of the tissue to a non-radiolabelled form of formaldehyde-treated albumin would be expected to fill such sites preventing them from releasing radioactive albumin after subsequent exposure first to the tracer and then to ethanol.]

Indeed, when yolk sacs were pre-pulsed for 0.25 h with non-radioactive [^{127}I]fd BSA V (25.0 µg/ml) and washed for 3 x 0.25 min before being pulsed for 0.25 h with [^{125}I]fd BSA IV and then treated in the usual manner, the results reported in Figs. 7.3.3.1, 7.3.3.2 & 7.3.3.3 were obtained. [As soon as the tissues had been washed after the radioactive-pulse period an initial sample of the re-incubation medium was removed and either fresh medium or medium containing ethanol was added to each flask.] From Fig. 7.3.3.1 it can be seen that both the control and 10.0 mM ethanol-treated yolk sacs released similar quantities (approximately 14%) of radioactivity in a rapid and linear manner up to 0.75 h before each "levelled off" at a maximum value of approximately 18.0% by 2.75 h. Thus ethanol does not appear to induce as extensive a degree of release of the adsorptive-phase substrate as it does for the fluid-phase substrate.

However, if the release of TCA-soluble radioactivity during the same experiments (Fig. 7.3.3.2) was investigated then a clear trend emerges. The release of total tissue-associated radioactivity as TCA-soluble radioactivity by the control yolk sacs closely followed the time-course for total radioactivity as in Fig. 7.3.3.1, indicating that the substrate had reached the lysosomal system and had become degraded. In contrast, the 10.0 mM ethanol-treated yolk sacs released

only 0.4 - 0.5% of the total tissue-associated radioactivity in the form of TCA-soluble radioactivity over the 2.75 h incubation period suggesting that the protein had been prevented from reaching the lysosomal system. (Fig. 7.3.3.3 shows the release of tissue-associated radioactivity in the form of TCA-insoluble radioactivity, i.e. undegraded substrate, in these experiments.) Control yolk sacs released only 0.5 - 1.0% of the total tissue-associated radioactivity in the form of TCA-insoluble material during the 2.75 h re-incubation period, whereas the 10.0 mM ethanol-treated yolk sacs effectively released only TCA-insoluble radioactivity. These results taken together (Figs. 7.3.3.1, 7.3.3.2 & 7.3.3.3) indicate that 10.0 mM ethanol-treatment following a 0.25 h pulse-period with [^{125}I]fd BSA caused approximately 18% of the total tissue-associated radioactivity to be released in a TCA-insoluble form whereas control tissues released 18% of the total tissue-associated radioactivity but in a TCA-soluble form.

7.3.4 The effects of benzyl alcohol on the release of [^{125}I]PVP by yolk sacs

The results of a set of preliminary (single) experiments on the effects of benzyl alcohol on the release of [^{125}I]PVP, in which the experimental format incorporated pulse-periods of 0.25 or 0.50 h and chase-periods of 0.00 or 0.25 h, are shown in Table 7.3.4.1. (The experiments follow the same format as the equivalent ethanol experiments reported in Table 7.3.1.2.)

The control yolk sacs and 0.4 mM benzyl alcohol-treated yolk sacs both released similar quantities of [^{125}I]PVP immediately after the benzyl alcohol was added. By 0.25 h the control yolk sacs had released 4.28 ± 0.14% of the total tissue-associated radioactivity into the medium, but the 0.4 mM benzyl alcohol-treated tissue had released 82.8 ± 2.7% (a 19.0-fold increase over the control values).

The benzyl alcohol-treated tissue also showed a rate of progressive release of [^{125}I]PVP that was more than 10 times that of the controls. When 1.0 mM benzyl alcohol was used instead of 0.4 mM (Table 7.3.4.1) the percentage of the total tissue-associated radioactivity released at 0.25 h by the 1.0 mM benzyl alcohol-treated yolk sacs increased to 93.6 ± 2.2% (a 22.4-fold increase in release of [^{125}I]PVP relative to matched controls). A smaller corresponding increase (3.9-fold) in the rate of progressive loss of the substrate from 1.0 mM benzyl alcohol-treated yolk sacs was observed. However, when the pulse-period was increased to 0.50 h (Table 7.3.4.1) but the chase-period kept at 0.00 h (i.e. immediate addition of benzyl alcohol) the 1.0 mM benzyl alcohol-treated tissue released only 64.8 ± 6.5% of the total tissue-associated [^{125}I]PVP (an increase of 15.5-fold). The 1.0 mM benzyl alcohol-treated tissue also showed a 10.2-fold increase in the rate of progressive loss of radioactivity relative to matched controls. Finally, when the 0.25 h pulse-period was followed by a 0.25 h chase-period before the addition of 1.0 mM benzyl alcohol (Table 7.3.4.1), the control and 1.0 mM benzyl alcohol-treated yolk sacs showed no marked differences in their subsequent loss of radioactivity. These preliminary results indicate that, for similar pulse-chase regimes, the 17.5-day rat visceral yolk sac was more susceptible to release of [^{125}I]PVP by benzyl alcohol than by ethanol. However, more work is required to substantiate this claim.

Fig. 7.3.1.1 The release of radioactivity by yolk sacs exposed to ethanol (10.0 mM) after a 0.5 h pulse-uptake of [^{125}I]PVP

Three flasks, each containing yolk sacs (x 3) and [^{125}I]PVP (10 µg/ml) dissolved in 7.0 ml of TC199, were incubated for 0.5 h, then the yolk sacs removed and rinsed (3 x 0.25 min) in fresh medium at 37°C. The yolk sacs were then re-incubated (3 per flask) in 10.0 ml of serum-free medium containing ethanol (10.0 mM) for a further 2.75 h. Each plot shows the mean quantity of radioactivity released (± S.D.) by at least 27 yolk sacs (from 3 experiments) expressed as a percentage of the total initially associated with the tissue.

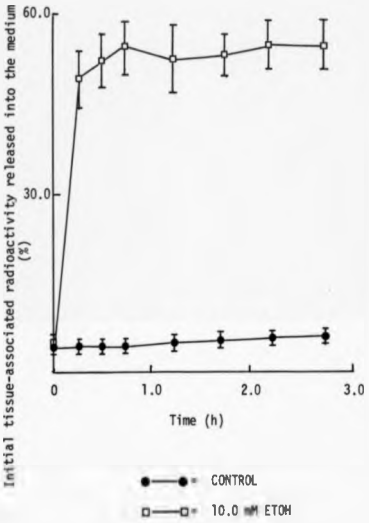


Fig. 7.3.1.2 The release of radioactivity by yolk sacs exposed to ethanol (10.0 mM) after a 0.25 h pulse-uptake of [^{125}I]PVP

Yolk sacs were incubated as described in the legend to Fig. 7.3.1.1 except that the incubation period in the presence of [^{125}I]PVP was only 0.25 h. Each plot shows the mean quantity of radioactivity released (± S.D.) by at least 27 yolk sacs (in 3 experiments).

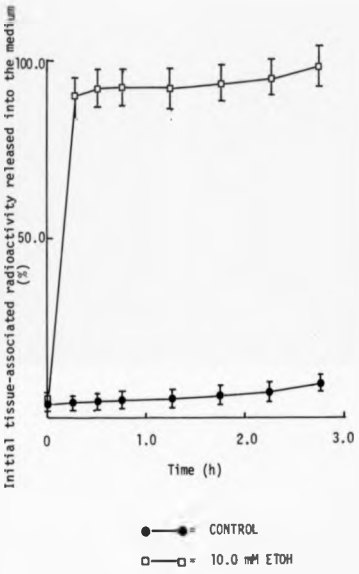


Fig. 7.3.1.3 The release of radioactivity by yolk sacs exposed to ethanol (2.0 mM) after a 0.25 h pulse-uptake of [^{125}I]PVP

Yolk sacs were incubated as described in the legend to Fig. 7.3.1.1 except that the incubation period in the presence of [^{125}I]PVP was only 0.25 h and the concentration of ethanol used was 2.0 mM. Each plot shows the mean quantity of radioactivity released (± S.D.) by at least 27 yolk sacs (in 3 experiments).

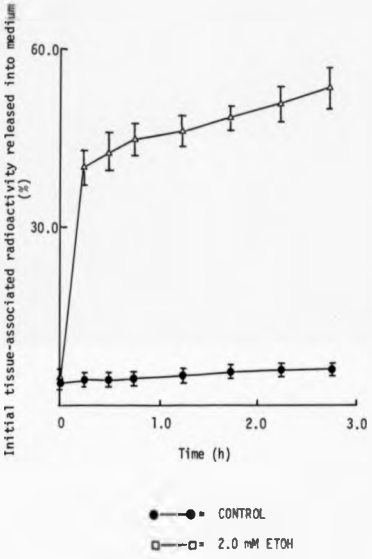


Fig. 7.3.1.4 The release of radioactivity by yolk sacs exposed to ethanol (10.0 mM) after a 0.25 h pulse-uptake of [^{125}I]PVP followed by a 0.25 h chase period

Yolk sacs were incubated 3 per flask in 7.0 ml of TC199 containing [^{125}I]PVP (10 µg/ml) for 0.25 h, then removed and rinsed (3 x 0.25 min) in fresh medium at 37°C. The yolk sacs were then re-incubated in 10.0 ml of serum-free medium for 0.25 h. At this point ethanol (final concentration 10.0 mM) was added and the yolk sacs incubated for a further 2.75 h. Each plot shows the mean quantity of radioactivity released (± S.D.) by at least 27 yolk sacs (in 3 experiments).

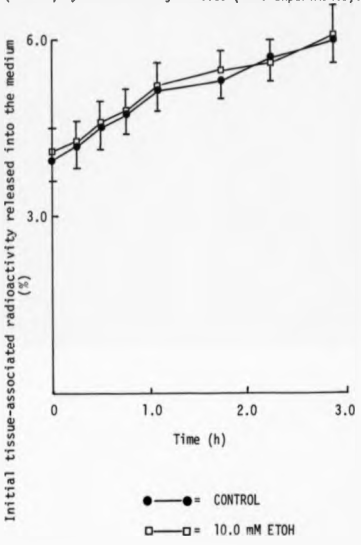


Fig. 7.3.3.1 The release of total radioactivity by yolk sacs exposed to ethanol (10.0 mM) after a 0.25 h pulse-uptake of [^{125}I]fd BSA

Yolk sacs were incubated 3 per flask in 7.0 ml of TC199 containing [^{127}I]fd BSA V (25 µg/ml) for 0.25 h, then removed and rinsed (3 x 0.25 min) in fresh medium at 37°C before being re-incubated in 7.0 ml of TC199 containing [^{125}I]fd BSA IV (25 µg/ml) for 0.25 h. The yolk sacs were then removed and rinsed (3 x 0.25 min) in fresh medium at 37°C before being re-incubated in serum-free medium containing ethanol (10.0 mM) for 2.75 h. Each plot shows the mean quantity of radioactivity released (± S.D.) by at least 27 yolk sacs or 3 experiments.

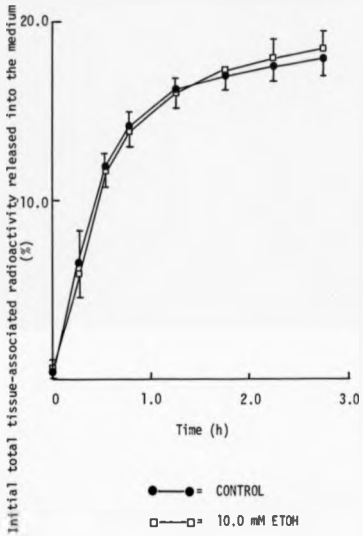


Fig. 7.3.3.2 The release of TCA-soluble radioactivity by yolk sacs exposed to ethanol (10.0 mM) after a 0.25 h pulse-uptake of [^{125}I]fd BSA

The same experiments described in the legend to Fig. 7.3.3.1 were analysed, but only the release of TCA-soluble radioactivity is reported.

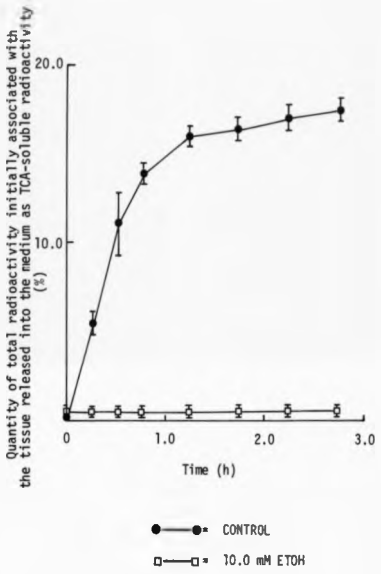


Fig. 7.3.3.3 The release of TCA-insoluble radioactivity by yolk sacs exposed to ethanol (10.0 mM) after a 0.25 h pulse-uptake of [^{125}I]fd BSA

The same experiments described in the legend to Fig. 7.3.3.1 were analysed, but only the release of TCA-insoluble radioactivity is reported.

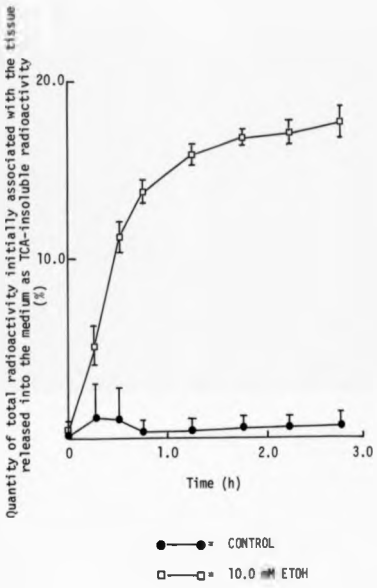


Table 7.3.1.1 The effects of various pulse and chase periods on ethanol-induced release of [^{125}I]PVP from yolk sacs

Yolk sacs (3 per flask) were incubated in 7.0 ml of serum-free medium 199 containing [^{125}I]PVP (10 µg/ml) for 0.25 - 0.5 h then removed, washed (3 x 2 min) in fresh changes of medium 199 (37°C) and then incubated for a chase period of 0.0 - 2.0 h in medium 199 at 37°C before ethanol was added to give the appropriate concentration for the final 2.75 h incubation. Each value given is the mean of 9 yolk sacs or 1 experiment.

Incubation Conditions (pulse & chase periods)	Concentration of Ethanol in medium (mM)	Initial Loss of Radioactivity (%) at: 0.0 h	0.25 h	Rate of Progressive Loss 0.25 - 2.75 h (%/h)
0.5 h pulse and 2.0 h chase	0 (Control)	3.94 ± 0.17	4.00 ± 0.24	0.56
	10.0	3.90 ± 0.25	4.14 ± 0.25	0.59
0.5 h pulse and 1.0 h chase	0 (Control)	3.96 ± 0.27	4.13 ± 0.22	0.57
	10.0	3.87 ± 0.17	4.11 ± 0.43	0.59
0.5 h pulse and 0.5 h chase	0 (Control)	3.95 ± 0.19	4.07 ± 0.25	0.62
	10.0	3.94 ± 0.14	3.98 ± 0.42	0.65
0.5 h pulse and 0.0 h chase	0 (Control)	3.88 ± 0.19	4.09 ± 0.26	0.66
	10.0	3.99 ± 0.28	48.8 ± 2.8	1.71
0.25 h pulse and 0.0 h chase	0 (Control)	3.92 ± 0.21	4.02 ± 0.21	0.68
	10.0	4.04 ± 0.23	91.5 ± 1.9	2.22
0.25 h pulse and 0.25 h chase	0 (Control)	3.92 ± 0.35	3.95 ± 0.25	0.61
	10.0	3.86 ± 0.25	3.92 ± 0.31	0.76

Table 7.3.1.2 The effects of ethanol on the release of [^{125}I]PVP from yolk sacs

Yolk sacs (3 per flask) were incubated in 7.0 ml of medium 199 with [^{125}I]PVP (10 μg/ml) for 0.25 - 0.50 h then removed, washed (3 x 0.25 min) in fresh changes of medium 199 (37°C) and then incubated for a chase period of 0.0 - 0.25 h in ethanol-free medium 199 (37°C) before the addition of ethanol for the final 2.75 h of incubation. Each value given is the mean ± S.D. of 27 yolk sacs or 3 experiments.

Incubation Conditions (pulse & chase periods)	Concentration of Ethanol in medium (mM)	Initial Loss of Radioactivity (%) at: 0.0 h	0.25 h	Rate of Progressive Loss 0.25 - 2.75 h (%/h)
0.25 h pulse and 0.0 h chase	0 (Control)	3.95 ± 0.29	4.22 ± 0.21	0.69 ± 0.07
	2.0	3.93 ± 0.26	40.7 ± 2.3	4.92 ± 0.48
0.5 h pulse and 0.0 h chase	0 (Control)	3.88 ± 0.33	4.14 ± 0.24	0.69 ± 0.08
	10.0	4.03 ± 0.21	49.7 ± 5.0	1.41 ± 0.78
0.25 h pulse and 0.0 h chase	0 (Control)	3.97 ± 0.22	4.12 ± 0.22	0.70 ± 0.09
	10.0	3.92 ± 0.24	90.1 ± 4.6	2.80 ± 0.72
0.25 h pulse and 0.25 h chase	0 (Control)	4.01 ± 0.35	4.19 ± 0.34	0.70 ± 0.09
	10.0	4.11 ± 0.34	4.23 ± 0.30	0.71 ± 0.02

Table 7.3.2.1 The effects of ethanol on the release of [^{125}I]PVP and lactate dehydrogenase from yolk sacs

Yolk sacs (3 per flask) were incubated in 7.0 ml of medium 199 with [^{125}I]PVP (10 µg/ml) for 0.25 h then removed, washed (3 x 0.25 min) in changes of fresh medium 199 (37°C) and then immediately 10.0 mM ethanol was added and the tissues re-incubated for 2.75 h. Each value given is the mean ± S.D. of 27 yolk sacs or 3 experiments.

Incubation Conditions (pulse & chase periods)	Concentration of Ethanol in medium (mM)	Initial Loss of Radioactivity (%) at: 0.0 h	0.25 h	Rate of Progressive Loss 0.25 - 2.75 h (%/h)
0.25 h pulse and 0.0 h chase	0 (Control)	3.98 ± 0.25	4.19 ± 0.23	0.64 ± 0.06
	10.0	3.90 ± 0.23	87.3 ± 4.4	4.11 ± 1.24

Incubation Conditions (pulse & chase periods)	Concentration of Ethanol in medium (mM)	Initial Loss of Lactate Dehydrogenase (%) at: 0.0 h	0.25 h	Rate of Progressive Loss 0.25 - 2.75 h (%/h)
0.25 h pulse and 0.0 h chase	0 (Control)	0.59 ± 0.15	0.69 ± 0.13	0.60 ± 0.09
	10.0	0.61 ± 0.21	0.78 ± 0.19	0.59 ± 0.10

Table 7.3.2.2 The effects of ethanol on the release of [^{125}I]PVP and N-acetyl-β-glucosaminidase from yolk sacs

Yolk sacs (3 per flask) were incubated in 7.0 ml of medium 199 with [^{125}I]PVP (10 μg/ml) for 0.25 h then removed, washed (3 x 0.25 min) in fresh medium 199 (37°C) and then immediately 10.0 mM ethanol was added and the tissue re-incubated for 2.75 h. Each value given is the mean ± S.D. of 27 yolk sacs or 3 experiments.

Incubation Conditions (pulse & chase periods)	Concentration of Ethanol in medium (mM)	Initial Loss of Radioactivity (%) at: 0.0 h	0.25 h	Rate of Progressive Loss 0.25 - 2.75 h (%/h)
0.25 h pulse and 0.0 h chase	0 (Control)	4.13 ± 0.39	4.35 ± 0.36	0.54 ± 0.04
	10.0	4.00 ± 0.28	84.1 ± 2.2	5.24 ± 0.42

Incubation Conditions (pulse & chase periods)	Concentration of Ethanol in medium (mM)	Initial Loss of N-Acetyl-β-Glucosaminidase at: 0.0 h	0.25 h	Rate of Progressive Loss 0.25 - 2.75 h (%/h)
0.25 h pulse and 0.0 h chase	0 (Control)	2.83 ± 0.77	3.30 ± 0.80	1.30 ± 0.08
	10.0	2.82 ± 0.91	3.36 ± 0.91	1.17 ± 0.13

Table 7.3.4.1 The effects of benzyl alcohol on the release of [^{125}I]PVP from yolk sacs

Yolk sacs (3 per flask) were incubated in 7.0 ml of medium 199 with [^{125}I]PVP (10 μg/ml) for 0.25 - 0.50 h then removed, washed (3 x 0.25 min) in changes of fresh medium 199 (37°C) and then incubated for a chase period of 0.0 - 0.25 h before the addition of benzyl alcohol for the final 2.75 h of incubation. Each value given is the mean of 9 yolk sacs or 1 experiment.

Incubation Conditions (pulse & chase periods)	Concentration of Benzyl Alcohol in medium (mM)	Initial Loss of Radioactivity (%) at: 0.0 h	0.25 h	Rate of Progressive Loss 0.25 - 2.75 h (%/h)
0.25 h pulse and 0.0 h chase	0 (Control)	4.12 ± 0.25	4.28 ± 0.14	0.56
	0.4	3.98 ± 0.37	82.8 ± 2.7	5.83
0.25 h pulse and 0.0 h chase	0 (Control)	3.95 ± 0.33	4.16 ± 0.29	0.58
	1.0	4.17 ± 0.29	93.6 ± 2.2	2.31
0.5 h pulse and 0.0 h chase	0 (Control)	3.98 ± 0.33	4.16 ± 0.26	0.58
	1.0	3.88 ± 0.44	64.8 ± 6.5	5.94
0.25 h pulse and 0.25 h chase	0 (Control)	3.91 ± 0.26	4.04 ± 0.30	0.57
	1.0	6.02 ± 0.37	6.24 ± 0.37	0.50

7.4 DISCUSSION

The fate of endocytosed ligands immediately after their internalisation by cells has been under active investigation for some time. For example, the uptake and intracellular sorting of [^{125}I]asialo-orosomucoid/horseradish peroxidase conjugate and [^{131}I]transferrin in HepG2 cells has recently been studied (Stoorvogel et al., 1987). After receptor-mediated uptake the asialo-glycoprotein was routed to the lysosomes while the transferrin was returned to the medium as apotransferrin. At 37^{o}C, sorting of the two ligands occurred with a $t_{0.5}$ of approximately 2 min and was completed within 10 min. Similarly, Merion & Sly (1983) reported that, 5 min after internalisation by human fibroblasts, the ligands: epidermal growth factor, β-hexosaminidase and low-density lipoprotein could be identified in membrane-bound structures that were essentially free from plasma-membrane marker-enzymes and were more buoyant than secondary lysosomes (on Percoll density-gradient fractionation).

These intermediate structures were in all probability endosomes. A study of the role of endosomes in both fluid- and adsorptive-phase pinocytosis in rat pheochromocytoma cells concluded that the endosomes were the recipients of both fluid- and adsorptive-phase pinocytosis markers (Gonatas et al., 1984). The role of the endosome in endocytosis was reviewed by Helenius et al. (1983). These authors preferred the neutral term "endosome" to either: pinosome, intermediate vacuoles, receptosome or CURL (compartment of uncoupling of receptor and ligand) because it does not imply any specific fate or function of such a compartment.

Ultrastructurally, endosomes can be differentiated into "early" (or "peripheral") and "late" endosomes. The former are usually located close to the plasma membrane, often have an electron-lucent interior and consist partly of tubular elements which can form

anastamosing networks. The second population of endosomes, the "late" endosomes, are concentrated in the perinuclear region and tend to be spherical and larger than the peripheral endosomes. There is growing evidence that endosomes are involved in membrane recycling, since fluid-phase pinocytosis markers can be channelled into many of the same endosomes as those ligands ingested by receptor-mediated endocytosis. Likewise, ligands ingested by different receptors enter the same coated pits and consequently the same endosomes. Delivery of the substrate into the lysosomes only occurs after a typical "lag period" which varies from one cell type to another. [In cultured fibroblasts it is 20 - 50 min but in macrophages it is only 5 - 10 min.]

Several considerations suggest that membrane recycling occurs from the endosomes (Helenius et al., 1983):

(1) The relatively long half-lives of plasma-membrane proteins and receptors imply that they are not routinely exposed to and degraded by lysosomal enzymes.

(2) The very rapid recycling times (< 5 min) for several receptors contrasts with the slower delivery of substrates to the lysosomes.

(3) Transferrin is internalised into endosomes and returned to the extracellular medium without transition into and out of secondary lysosomes.

(4) Receptor-mediated uptake of extracellular ligands and receptor recycling continues at 16 - 20°C, but transfer of substrates into the lysosomes was inhibited.

When Livesey (1978) investigated the adsorptive-phase uptake of [^{125}I]-labelled glucagon, insulin B-chain, calcitonin, insulin, ribonuclease, formaldehyde-denatured bovine serum albumin, lysozyme and rat IgG it took approximately 2.5, 9.5, 10.0, 13.5, 15.0, 17.5, 25.0, 28.0 min before the TCA-soluble radioactivity (digestion

products) appeared in the incubation medium. As the rates of uptake and probably those of intralysosomal degradation vary from one protein to another, such data do not permit firm conclusions to be drawn on the speed of entry of exogenous proteins into lysosomes. However, the results in Tables 7.3.1.1, 7.3.1.2, 7.3.2.1, 7.3.2.2 and Figs. 7.3.1.1, 7.3.1.2, 7.3.1.3, 7.3.1.4 indicate that the time taken for [^{125}I]PVP to reach a compartment (probably the "late" endosome or lysosomal compartment) that will not release its contents following exposure to 10.0 mM ethanol was approximately 15 min, hence ethanol appears to induce dysfunction of the uptake and intracellular "handling" of the substrate in the peripheral region of the endodermal cells of the 17.5-day rat visceral yolk sac. However, the preliminary results obtained with [^{125}I]fd BSA IV in place of [^{125}I]PVP as marker (Figs. 7.3.3.1 - 7.3.3.3) indicate that none of the labelled albumin, captured by adsorptive-phase pinocytosis over a 0.25 h period, passes into the lysosomal compartment when the tissue is subsequently exposed to 10.0 mM ethanol. Unfortunately, even when the 17.5-day rat visceral yolk sacs were given a pre-pulse-period of 0.25 h with non-radioactive [^{127}I]fd BSA V (25.0 µg/ml) and washed for 3 x 0.25 min before being given the 0.25 h pulse with [^{125}I]fd BSA IV (25.0 µg/ml) and exposed to 10.0 mM ethanol (Figs. 7.3.3.1, 7.3.3.2 & 7.3.3.3), only 18.0% of the total tissue-associated radioactivity was released over the 2.75 h incubation period (either as TCA-soluble or TCA-insoluble radioactivity). The fate of the remaining 82.0% of the total tissue-associated radioactivity was unknown. This somewhat clouds the fine interpretation of such experiments. However, Livesey (1978) found that a pulse-period of 2.0 h with [^{125}I]fd BSA (5.0 µg/ml), [^{125}I]insulin (5.0 µg/ml), [^{125}I]lysozyme (5.0 µg/ml) or [^{125}I]ribonuclease A (5.0 µg/ml), before a 3 x 2.0 min wash-period and re-incubation in medium containing no substrate, resulted in the

release by 2.0 h of only 76.8%, 75.0%, 99.5% and 67.0% of the total tissue-associated radioactivity for each substrate, suggesting that non-specific adsorption may be the cause of the low release. Possibly, more conclusive results would have been obtained if [^{125}I]lysozyme had been used in place of [^{125}I]fd BSA, since Livesey showed almost 100% release of this protein in his experiments. A further interesting experiment would have been to use a 0.25 h pre-pulse-period with non-radioactive [^{127}I]fd BSA (25.0 µg/ml followed by a 0.25 h pulse-period with [^{125}I]fd BSA (25.0 µg/ml) and to include a 0.25 h chase-period to see whether the ingested protein passed into lysosomes and was released entirely as TCA-soluble radioactivity whether or not ethanol (10.0 mM) was present in the re-incubation medium.

In summary it can be reported that:

(1) The release of [^{125}I]PVP, immediately the tissue was exposed to 10.0 mM ethanol, was not the result of general cell leakage since no significant release of lactate dehydrogenase accompanied that of the [^{125}I]PVP.

(2) The release of [^{125}I]PVP is from a non-lysosomal compartment. (Since release of [^{125}I]PVP was not accompanied by a significant release of a marker lysosomal enzyme.)

(3) The release of [^{125}I]PVP caused by 10.0 mM ethanol was dependent on the pulse-chase periods used. (Only the [^{125}I]PVP pinocytosed during the last 0.25 h of the pulse-period was susceptible to ethanol-induced release, implying a selective effect of ethanol on peripheral endosomes.)

(4) The magnitude of the effect on the endosomal compartment was dependent on the concentration of ethanol.

(5) Preliminary investigations with benzyl alcohol indicate that yolk sacs are more sensitive to benzyl alcohol than to ethanol.

Thus, the effects of ethanol, and possibly benzyl alcohol, seem to be specific to only the peripheral endosomes/"nascent pinosomes" since there is neither detectable alcohol-induced permeability of the plasma membrane to lactate dehydrogenase nor any detectable release of lysosomal enzymes. Thus the "recycling"/"perturbation of trafficking" of vesicles in the apical region of the cell seems likely to be the cellular mechanism of inhibition of uptake in the continuous presence of either of the alcohols (see Sections 3.3 & 6.3). Furthermore, the discharge of the contents of "nascent pinosomes"/endosomes also appears to be the explanation of the elevated initial release (0.00 - 0.25 h) when yolk sacs pre-loaded with [^{125}I]PVP are re-incubated in the presence of either ethanol or benzyl alcohol (see Sections 3.3, 5.3 & 6.3).

CHAPTER EIGHT

GENERAL DISCUSSION

8.1 General Discussion

In Chapters 1, 4 and 5 the detailed literature on the involvement of the yolk sac in : endocytosis, intralysosomal proteolysis, diacytosis and the synthesis and secretion of a number of proteins and compounds was reported. The dysfunction of the yolk sac and its role in teratogenesis in the rat are still of great interest. For example, Freeman & Brown (1986) claim to have identified a yolk-sac antigen (a single 30 kd glycopeptide) whose antibody causes yolk-sac dysfunction. This work was followed up by Jensen et al. (1987) who raised monoclonal antibodies against different antigens from the rat visceral yolk sac and injected them into pregnant rats on the 9th day of gestation. Some monoclonal antibodies were not teratogenic but clone B-3 (IgG 2b type) induced CNS malformations, clone D-4 (IgG 2a type) induced growth retardation and clone B-10 induced skeletal anomalies and delayed ossification. Also, teratogenic monoclonal antibodies decreased pinocytosis of proteins by the visceral yolk sac while the non-teratogenic monoclonal antibodies did not affect pinocytosis. This is of interest, since Lerman et al. (1986) found that teratogenic anti-yolk-sac antisera inhibited pinocytosis in 17.5-day yolk sacs by up to 40% (when compared with matched controls) but non-teratogenic antisera did not affect pinocytosis. Furthermore, the teratogenic antisera did not interfere with the uptake of [^{14}C]α-aminoisobutyric acid or [^{3}H]2-deoxyglucose into the 17.5-day rat visceral yolk sac. These results suggest that teratogenic antisera/monoclonal antibodies decrease the availability of proteins to the developing embryo in the rat.

Yolk-sac dysfunction due to hyperglycemia was reported in 9.5-day rat conceptuses when they were cultured, in vitro, for 48 h (Pinter et al., 1986a). When the conceptuses were exposed to D-glucose (750 mg/100 ml) the visceral yolk-sac capillaries and

vitelline vessels were sparse, patchy and non-uniformly located. The endodermal cells also had reduced areas of rough endoplasmic reticulum and reduced numbers of ribosomes and mitochondria, suggesting that hyperglycemia during organogenesis in the rat has a deleterious effect on yolk-sac function. In a related study, Pinter et al. (1986b) found that rat conceptuses cultured in vitro from 9.5 days of gestation for 48 h in hyperglycemic serum (D-glucose concentration of 950 mg/100 ml) developed the following malformations: open neural tube and advanced neuropil formation in the neuroepithelium. These effects were accompanied by a significant reduction of the rough endoplasmic reticulum, decreased size and number of lipid droplets and an increased number of lysosome-like structures in the visceral yolk-sac endodermal cells. However, addition of arachidonic acid (20 µg/ml) to teratogenic hyperglycemic serum prevented both the induction of malformations and of yolk-sac dysfunction. The mechanism of action of arachidonic acid in protecting the conceptus against the adverse effects of hyperglycemia is unknown, but the observation suggests that the effect of these high glucose concentrations (42 - 53 mM) is not simply to induce damage by some non-specific (e.g. osmotic) effect.

The development of the embryo during organogenesis is still poorly understood in biochemical terms and is not, of course, solely dependent on yolk-sac uptake and digestion of exogenous proteins. For example, in the mouse Fozard et al. (1980) showed that administration (via the drinking water) of substantial doses of DL-α-difluoromethylornithine (an irreversible inhibitor of L-ornithine decarboxylase) on days 5 to 9 of gestation greatly decreased ornithine decarboxylase activity (which normally peaks on day 8 of gestation) and decreased the concentrations of putrescine and spermidine (both of which normally peak close to day 8 of gestation). Implantation appeared to have taken place normally, but there was an arrest of

embryonic development (at the day 6/day 7 stage) and later such embryos were resorbed. Similar observations were also made in pregnant rats and rabbits. There thus appears to be an essential role for L-ornithine decarboxylase in early mammalian embryogenesis, but at present this has not been defined.

The results relating to ethanol-induced yolk-sac dysfunction reported in this thesis indicate that a continued interest in the endocytosis of various substrates by this tissue is justified (from a physiological/developmental standpoint). In particular, the ability of the established teratogens ethanol and anti-yolk-sac antisera to inhibit yolk-sac pinocytosis suggests that continued studies of the mechanism(s) of action of these inhibitors are of interest.

The main findings of the thesis (at the cell-biology level) are that ethanol:

(1) Has strong effects on pinocytosis (both fluid-phase and adsorptive-phase).

(2) Shows associated effects on proteolysis, possibly by interfering with endosome-lysosome fusion.

(3) Shows no signs of non-specific cell damage, since the dysfunction was readily reversible on removal of the alcohol and leakage of a cytosolic enzyme marker was not detectable.

(4) Shows stronger effects on yolk-sac function at the earlier stages of gestation.

The next important task is to formulate a mechanistic explanation of these observations in biochemical terms.

Although lack of time prevented an experimental investigation of ethanol effects on calcium metabolism in the rat yolk sac, a number of observations in the literature provide circumstantial evidence to suggest that a study of the possible role of calcium in mediating ethanol-induced abnormalities of yolk-sac function, and thus of

embryonic development, would be worthwhile. Thus, Moore & Stainisstreet (1986) investigated the requirement for extracellular calcium in neural-fold elevation in rat embryos at 10.4 days of gestation *in vitro*. Extracellular calcium concentrations below 3 mM caused the elevated cephalic neural-folds to re-open and then to flatten before collapsing at calcium concentrations of 0.1 mM. The involvement of extracellular calcium in neural-tube closure has been shown in explanted early chick embryos (Lee & Nagele, 1979). It was reported that papaverine (50 µg/ml) inhibited the uplifting of neural folds in explanted stage-8 (gestational age 24 h) chick embryos. Papaverine is known to lower the intracellular concentration of free calcium ions. The inhibition produced by papaverine was due, at least in part, to the impaired ability of apical microfilaments to contract. The inhibition caused by papaverine was reversed by subsequent treatment of the chick embryos with the calcium ionophore A23187 (which is known to promote an increased influx of extracellular calcium and thus to increase intracellular calcium concentrations). A further study by Lee & Nagele (1986), in early chick embryos explanted at stage 8 of development, showed that the calcium channel blocker, verapamil, at a concentration of 15 µg/ml caused a significant incidence of neural-tube closure defects in the treated chick embryos. The neuroepithelial cells of verapamil-treated embryos had smoother apical surfaces and less conspicuous microfilament bundles. The deleterious effects of verapamil (15 µg/ml) could be reversed by subculturing affected embryos on a nutrient medium containing chlorotetracycline (25 µg/ml), a calcium agonist. These studies suggest that intracellular and extracellular calcium ions play an important role in neural-tube elevation and folding in the rat and chicken *in vitro*.

Ethanol, at concentrations ranging from 45 - 720 mM, inhibited

calcium uptake by mouse synaptososomes *in vitro* in a concentration-dependent manner (Harris & Hood, 1980), and it was also reported (Hassler & Moran, 1986a) that ethanol in the concentration range 10 - 40 mM caused disruption of the organisation of the neural-crest cell actin cytoskeleton in embryos of the salamander *Ambrystoma maculatum* at stages 21 - 26 *in vitro*. A further *in vitro* study (Hassler & Moran, 1986b), in posterior cranial neural-tube and crest cells at stages 21 - 26 (day 6 of gestation) from *Ambrystoma maculatum*, showed that ethanol (10 - 40 mM) caused rapid cell retraction and alterations in cell-to-cell contacts. Monoclonal antibodies against tubulin and actin revealed that these ethanol-induced morphological changes are related to disruption of microtubules and microfilaments.

The above observations may well relate to the suggestion (Salisbury *et al.*, 1980) that calmodulin and microfilaments have functional roles in receptor-mediated endocytosis by B lymphoblastoid cells *in vitro* since Shirazi & Dean (1983) found that, in human fibroblasts and mouse peritoneal macrophages *in vitro*, trifluoperazine (a calmodulin antagonist) inhibited fluid-phase, adsorptive-phase and receptor-mediated endocytosis. A further study (Dijkstra *et al.*, 1985) showed that trifluoperazine (14 μM) inhibited the internalization of liposomes by Kupffer cells *in vitro*. Nielsen *et al.* (1987) reported the association of calmodulin and calmodulin-acceptor proteins with lysosomes of primary thyroid-follicular cells, thyroid C-cell tumour cells and kidney cells. Similarly, Nemere *et al.* (1986) reported the identification of lysosomes containing calcium and calcium-binding protein in chick duodenal cells *in vivo*. However, when Iacopetta *et al.* (1986) investigated the role of intracellular calcium and protein kinase C in the endocytosis of transferrin and insulin by HL60 cells *in vitro*,

they concluded that intracellular calcium played only a minor role in regulating receptor-mediated endocytosis, but that protein kinase C can selectively modulate receptor-internalization depending on the receptor type and occupancy.

Calcium has also been shown to be required for vesicle fusion in the rat liver (Judah & Quinn, 1978) and in small unilamellar vesicles made of phosphatidyl serine (Deleers et al., 1986). Furthermore, ethanol has been reported to inhibit the secretion of hepatic albumin and transferrin by inhibiting microtubule functions in the rat liver in vivo and in vitro (Baraona et al., 1975; Baraona et al., 1980 & Volentine et al., 1986) and of lipoprotein (both HDL and LDL) in the rat liver in vivo (Koga & Hirayama, 1986).

Future work with ethanol and its effects on rat yolk-sac dysfunction should concentrate on:

(1) The relation of calcium metabolism to membrane-fusion events in yolk-sac tissue and the possible role of calmodulin in regulating endocytic rate.

(2) The possible effects of ethanol on microfilament and microtubule activity and the relation of such effects to uptake, intracellular- and transcellular movement of endocytosed substrates within the endodermal cells.

(3) Establishing whether the rat visceral yolk sac possesses the requisite enzymes to metabolise ethanol (via either alcohol dehydrogenase and aldehyde dehydrogenase, or the microsomal ethanol oxidation system (MEOS) or the catalase/hydrogen peroxide pathways). Beck (1984) reported that rat embryos cultured between 9.5 - 11.5 days of gestation in vitro were not susceptible to acetaldehyde-induced congenital malformations, but no one has as yet investigated the ability of the rat visceral yolk sac

to detoxify ethanol via oxidative metabolism. The use of a specific inhibitor of alcohol dehydrogenase (e.g. 4-methylpyrazole) may settle the question of whether ethanol itself or its proximal metabolite, acetaldehyde, is the species that causes the observed inhibition of pinocytosis.

Finally, the majority of the results reported in this thesis are compatible with the suggestion that the mechanism of induction of malformations by ethanol in the rat embryo is by inhibition of the process of embryotrophic nutrition. However, if ethanol more generally disrupts yolk-sac function it could also inhibit: the synthesis and secretion of yolk-sac proteins, the diacytosis of maternal IgG and transferrin from the mother to the fetus and affect other feto-maternal exchanges of essential metabolites.

It is therefore important that future studies aim to define more specifically and in biochemical terms the precise effects of ethanol on yolk-sac function. If the detailed biochemical disturbances induced by ethanol are also induced by teratogenic antisera, but not by non-teratogenic ones, then, for the first time we may have constructed a detailed biochemical picture of the mechanism of action of a teratogen. In view of the widespread abuse of ethanol and the now well-established importance of the Fetal Alcohol Syndrome in humans, such goals are of clinical as well as of academic interest.

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APPENDIX I

PUBLISHED PAPERS NOT SCANNED AT THE REQUEST OF THE UNIVERSITY.

APPENDIX II

PVP UPTAKE PROGRAM

```
0010  DIM C£30
0020  REM KEW 125-IPVP PROGRAM,MODIFIED NOV.,1974
0027 REM FURTHER MODIFIED DECEMBER 1980 BY LEN
0030 DIM#(30),I(30),K(30),L(30),R(30)
0060  PRINT "NON-DIGESTIBLE PROG. ENTERED"
0070 % "EXPERIMENT CODE =";
0080 INPUT Z£
0090  PRINT "BACKGROUND IN CPM=";
0100  INPUT A
0110  PRINT "COUNTING TIME FOR EACH ML OF MEDIUM, SECS=";
0120  INPUT C
0130  PRINT "COUNTING TIME FOR EACH ML OF YS SOLUTION, SECS=";
0140  INPUT D
0150  PRINT "NO. OF POINTS IN PLOT=";
0160  INPUT G
0165  PRINT "NAME OF DATA FILE:";
0167  INPUT F1£
0170  PRINT "NAME OF REGRESSION FILE:";
0174  INPUT F2£
0176  FILES 125I-BSA_rd,F1£,University of Keele,,F2£
0180  PRINT "NAME OF RESULTS FILE:";
0184  INPUT F3£
0186  FILES Keele,,F3£
0190  FOR X=1 TO G
0200  INPUT 125I-BSA_rd, H(X),I(X),K(X),L(X),
0210  NEXT X
0220  FOR X=1 TO G
0230  LET M=(I(X)*60/C)-A
0240  LET Q=((K(X)*60/D)-A)*5
0250  LET N=M+Q/20
0260  LET R(X)=(Q*1000)/(M*L(X))
0265
0266
0270 % University of Keele,,H(X),",",R(X)
0280  NEXT X
0310  PRINT Keele,, " "
0315 % Keele,,"THIS IS EXPT CODED ";Z£
0320  PRINT Keele,, " "
0330 % Keele,,"INCUBATION TIME";"   PROTEIN IN Y.S. ";"  UPTAKE "
0332 % Keele,,"(HOURS)"
0340  PRINT Keele,,
0350  FOR X=1 TO G
0360 % Keele,,H(X),L(X),R(X)
0370  NEXT X
0380  STOP
```

APPENDIX III

BSA UPTAKE PROGRAM

```
0020  REM PROTEIN PROGRAM ,REVISED OUTPUT ,NOV. 1974
0030  DIM E(12),H(12),I(12),J(12),K(12),L(12),S(12)
0040  DIM C£30
0045 DIM V£30
0060  PRINT "PROTEIN PROGRAM ENTERED"
0070 PRINT "EXPERIMENT CODE =";
0080 INPUT V£
0090  PRINT  "BACKGROUND IN CPM=";
0100  INPUT  A
0110  PRINT  "PERCENT SOLUBLES IN PREP=";
0120  INPUT  B
0130  PRINT  "COUNTING TIME MEDIUM TOTALS,SECS=";
0140  INPUT  C
0150  PRINT  "COUNTING TIME MEDIUM SOLUBLES,SECS=";
0160  INPUT  Z
0170  PRINT  "YS COUNTING TIME,SECS=";
0180  INPUT  D
0190  PRINT  "CORRECTION FACTOR FOR MEDIUM TOTALS=";
0200  INPUT  Y
0210  PRINT  "CORRECTION FACTOR FOR MEDIUM SOLUBLES=";
0220  INPUT  R
0230  PRINT  "NO. OF POINTS IN PLOT=";
0240  INPUT  G
0264  PRINT "NAME OF DATA FILE:";
0265  INPUT F1£
0266  PRINT "NAME OF REGRESSION FILE:";
0267  INPUT F2£
0269  FILES  "I-BSA",F1£,University of Keele,,F2£
0270  PRINT "NAME OF RESULTS FILE:";
0272  INPUT F3£
0275  FILES Keele,,F3£
0277  FOR X=1 TO G
0280   INPUT "I-BSA",H(X),I(X),J(X),K(X),L(X),
0290  NEXT X
0300  FOR X=1 TO G
0310  LET M=(I(X)*60/C-A)*Y
0320  LET N=(J(X)*60/Z-A)*R
0330  LET O=N-(M*B/100)
0340  LET Q=((K(X)*60/D)-A)*5
0350  LET P=(M-N)+O/2
0360  LET F=((10*O)+Q)*1000
0370  LET E(X)=(Q*1000)/(L(X)*P)
0380  LET S(X)=F/(L(X)*P)
0385
0387 % Keele,,
0390 % University of Keele,,H(X),",",S(X)
0400  NEXT X
0405 % Keele,,
0410 % Keele,,"GOOD MORNING! THIS IS EXPT CODED ";V£
0430  PRINT Keele,, " "
0435
0440  PRINT Keele,, " "
0445 % Keele,,
0450  PRINT Keele,, " INCUBATION ","PROTEIN","MICROLITRES","
UPTAKE"
0460  PRINT Keele,, "TIME (HOURS)"," IN YS "," PER MG YS "
0465 % Keele,,
0470  FOR X=1 TO G
0480 % Keele,,H(X),L(X),E(X),S(X)
0490  NEXT X
0500  STOP
```