

## Ethanol-induced inhibition of pinocytosis and proteolysis in rat yolk sac *in vitro*

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### Summary

Pinocytic capture of  $^{125}\text{I}$ -labelled polyvinylpyrrolidone and of formaldehyde-denatured  $^{125}\text{I}$ -labelled bovine serum albumin by 17.5-day rat visceral yolk sacs incubated *in vitro* was rapidly and strongly inhibited by low concentrations (0.01 and 0.05%, v/v) of ethanol. The induced inhibition of pinocytosis was readily reversible, but a marked lag was observed before ethanol-exposed tissue regained its full proteolytic capacity towards the exogenous protein.

These observations suggest that the acute administration of ethanol to a pregnant rat may give rise to concentrations of ethanol in the maternal blood

and/or uterine fluid that induce dysfunction of the yolk sac. In late gestation such inhibition of yolk-sac function may interfere with the transfer of passive immunity across the yolk sac. If similar dysfunction is induced earlier in gestation, in the period before the chorioallantoic placenta is functional, this could cause a transient period of inhibition of histiotrophic nutrition that may be important to the pathogenic mechanism of action of ethanol as a teratogen.

Key words: ethanol, yolk-sac function, teratogenesis, pinocytosis, proteolysis, rat embryo.

### Introduction

Excessive ingestion of ethanol, by either pregnant women or by pregnant experimental animals, is known to induce a characteristic pattern of developmental defects known as the fetal alcohol syndrome (Abel, 1982). In children the anatomical features of this syndrome include intrauterine growth retardation and characteristic facial features (flat with fissures, indistinct philtrum and small eyes); a high percentage of associated heart defects and limb and joint anomalies have also been reported (see Abel, 1982 (vol. 2)). Similarly, ethanol exposure of pregnant mice induces defects in their offspring which include facial clefts and ventricular septal defects. Moreover, it is now known that an isolated acute exposure to ethanol is sufficient to induce such defects in pregnant mice (Webster, Walsh, Lipson & McEwen, 1980; Webster, Walsh, McEwen & Lipson, 1983; Webster, Germain, Lipson & Walsh, 1984) raising the possibility that acute as well as chronic *in utero* exposure to ethanol may damage the human fetus.

The mechanism of induction of the syndrome of defects has yet to be established, but this task is made

difficult by the numerous effects of ethanol on tissues, some of which are probably adaptive changes during chronic exposure (Kricka & Clark, 1979; Abel, 1982). It would thus appear to be the simpler task to establish the mechanism of action of ethanol after acute rather than chronic exposure of experimental animals.

Ethanol or its metabolite, acetaldehyde, may act directly on the embryo proper. Alternatively, they may act indirectly by disturbing some facet of placental function. Studies of other known teratogens have suggested that histiotrophic nutrition, a placental process by which amino acids derived from maternal proteins are delivered to the growing embryo, may be disturbed when rats are exposed to certain teratogens (Williams, Roberts, Kidston, Beck & Lloyd, 1976; Freeman, Brent & Lloyd, 1982; Freeman & Lloyd, 1983). It was therefore of interest to determine whether ethanol, at concentrations similar to those found in the maternal bloodstream of a rodent following the administration of a single teratogenic dose of ethanol, had any profound effects on yolk-sac function *in vitro*, suggesting how ethanol may act *in vivo* during acute exposure.

A well-established *in vitro* technique that permits quantification of both the rate of pinocytic capture and of the rate of intralysosomal proteolysis (Williams, Kidston, Beck & Lloyd, 1975a,b) was used to establish the effects of ethanol on isolated rat yolk sacs.

## Materials and methods

Sodium [<sup>125</sup>I]iodide (preparation no. IMS 30) and <sup>125</sup>I-labelled polyvinylpyrrolidone (preparation no. IM 33P) were products of Amersham International, Amersham, Bucks, UK. Heat-inactivated calf serum (product CS 07) was from Wellcome Reagents, Dartford, Kent, UK, bovine serum albumin (preparation no. A-7638) was purchased from Sigma (London) Chemical Co. Ltd, London SW6, UK and absolute ethanol, spectrograde (preparation E/0665) was from Fisons PLC, Loughborough, Leicestershire, UK.

All other chemicals were of analytical grade.

### Preparation of formaldehyde-denatured <sup>125</sup>I-labelled albumin

Formaldehyde-treated <sup>125</sup>I-labelled bovine serum albumin was prepared by first labelling bovine serum albumin with [<sup>125</sup>I]iodide, by using a chloramine-T method, then treating the labelled protein with formaldehyde before exhaustive dialysis. [Formaldehyde treatment greatly enhances the rate of clearance of albumin by rat yolk-sac tissue (see Moore, Williams & Lloyd, 1977, for further discussion).]

### Incubation of rat yolk sacs

Pairs of Wistar rats from an inbred colony were mated overnight in grid cages and if a copulation plug was observed next morning, the female was termed 0.5 days pregnant. At 17.5 days, yolk sacs (8–12) were removed and each was incubated separately at 37°C under 95% O<sub>2</sub>+5% CO<sub>2</sub> in 10 ml of medium (see Williams *et al.* 1975a,b for full details). After 15 min of preincubation in 9.0 ml of medium, 1.0 ml of a stock solution (×10) of radiotracer in medium was added to give a final tracer concentration of 2 μg ml<sup>-1</sup>. At regular intervals yolk sacs were removed from the medium, rinsed (3×2 min) in changes of ice-cold saline, then assayed for both protein content (Folin–Lowry method) and for contained radioactivity. The radioactivity content of the medium was also assayed.

### Uptake of <sup>125</sup>I-labelled polyvinylpyrrolidone

Uptake of <sup>125</sup>I-labelled polyvinylpyrrolidone (<sup>125</sup>I-PVP) from serum-free medium 199, by 17.5-day yolk sacs was determined by using the method described by Ibbotson & Williams (1979). Similarly, uptake of <sup>125</sup>I-PVP from medium 199 containing from 10% to 50% (v/v) of calf serum was determined by using the method described by Williams *et al.* (1975a). Reported values were normalized for differences in tissue size using the protein content of each yolk sac and the time course of uptake plotted. From the gradient of this plot the clearance rate could be determined. It represents the volume (μl) of medium whose contained substrate is captured per mg of yolk-sac protein during 1 h of incubation.

### Uptake of denatured <sup>125</sup>I-labelled bovine serum albumin

Uptake and digestion of the labelled protein were determined by the method of Williams *et al.* (1975b) as modified by Livesey & Williams (1979). The quantities of albumin that would have accumulated in the tissue had there been no digestion of the captured albumin and release back into the medium of digestion products, were calculated and plotted against time. This permitted rates of uptake to be calculated as for <sup>125</sup>I-PVP.

### Release of <sup>125</sup>I-PVP from incubated yolk sacs

The release of <sup>125</sup>I-PVP was monitored by reincubating washed, <sup>125</sup>I-PVP-laden tissue in fresh medium that initially contained no radioactivity and removing samples of the medium at regular intervals for assay of radioactivity (see Williams *et al.* 1975a for details).

### Recovery of pinocytic and proteolytic capacities of ethanol-treated tissue

The recovery of the pinocytic capacity of ethanol-exposed tissue was determined with <sup>125</sup>I-PVP, essentially as described above for fresh tissue. Recovery of proteolytic capacity simply required incubation of the ethanol-exposed tissue in medium containing <sup>125</sup>I-labelled albumin and the regular removal of samples of medium, addition of trichloroacetic acid (to precipitate any undigested protein) and assay of the acid-soluble radioactivity present in the supernatant after centrifugation (30 000 g·min). This permitted calculation of the quantity of low-molecular-weight digestion products of <sup>125</sup>I-labelled albumin released from the lysosomal compartment of the cells (Livesey & Williams, 1979).

## Results

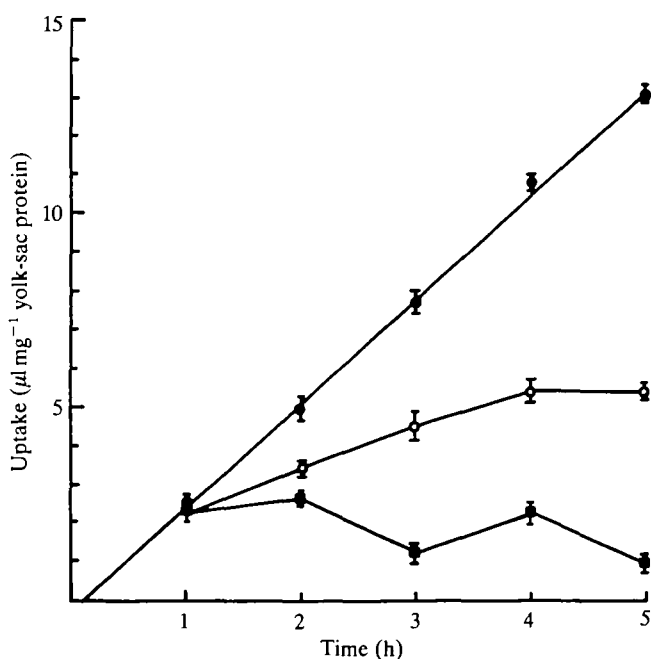
### The effect of ethanol on pinocytosis

The effects of different concentrations of ethanol on the rate of pinocytic capture of a fluid-phase marker, <sup>125</sup>I-PVP, by yolk sacs incubated in serum-free medium 199 were studied. The initial concentration range of ethanol investigated was 0.0001–0.05% (v/v). At 0.0001% and 0.001% (v/v) there was no detectable effect on the rate of pinocytic capture of <sup>125</sup>I-PVP (results not reported in detail). However, the effects of 0.01% and 0.05% (v/v) ethanol were marked (see Fig. 1). The mean clearance rate (±S.D.) for controls was 2.67 ± 0.14 μl h<sup>-1</sup> mg<sup>-1</sup> yolk-sac protein, in agreement with the findings of Ibbotson & Williams (1979). After addition of ethanol at either of these concentrations, pinocytosis was rapidly inhibited (Fig. 1). Over the period 1.0 to 5.0 h the residual uptake as a percentage of the control value was approx. 30% for 0.01% (v/v) ethanol. At a concentration of 0.05% (v/v), ethanol appears either to prevent the formation of pinosomes or to modify membrane properties so that nascent pinosomes

recycle their contents to the plasma membrane ('futile cycling') or causes preformed pinosomes to discharge their contents from the cell (induces exocytosis) so that there is no net uptake (Fig. 1).

#### Effect of ethanol on cell integrity

To establish whether ethanol caused any gross damage to yolk-sac cells, the rates of loss of radioactivity from tissues preloaded with  $^{125}\text{I}$ -PVP were monitored (Table 1). For tissues exposed to either 0.01 or 0.05 % (v/v) ethanol, the progressive rate of loss of  $^{125}\text{I}$ -PVP was approximately double that for control tissues not exposed to ethanol. [None of these values is strikingly different from the value of  $1.43 \pm 0.9\% \text{ h}^{-1}$  reported from similar experiments with ethanol-free medium by Roberts, Nicholls, Griffiths, Williams & Lloyd (1976), but the rates of progressive loss rise sharply on exposing the tissues to higher concentrations of ethanol.] Thus incubation of tissues in serum-free medium containing concentrations of ethanol below 0.05 % (v/v) is unlikely to cause major cell damage. This suggestion was substantiated by an examination of the recovery of the



**Fig. 1.** Effect of ethanol on the time course of uptake of  $^{125}\text{I}$ -labelled polyvinylpyrrolidone by rat yolk sacs. Yolk sacs from 17.5-day rats were incubated separately in serum-free medium 199 (9.0 ml) containing  $^{125}\text{I}$ -PVP ( $2 \mu\text{g ml}^{-1}$ ). After 1.0 h of incubation, either a further 1.0 ml of medium alone or of ethanol in medium 199 was added to each flask; the final ethanol concentrations in the uptake medium were 0.01 and 0.05 % (v/v). Uptake of  $^{125}\text{I}$ -PVP was then monitored for up to 5.0 h (see text for details). Each point represents the mean uptake ( $\pm$ s.d.) by at least four yolk sacs, each from a different pregnant female animal; ●, control (no ethanol present); ○, ethanol (0.01 %, v/v) and ■, ethanol (0.05 %, v/v).

pinocytic capacity of ethanol-exposed tissue (Fig. 2). Recovery of pinocytic capacity after exposure of the tissue to 0.01 % (v/v) ethanol was immediate and complete. Exposure to 0.05 % (v/v) ethanol caused some delay in the regain of full pinocytic capacity, but beyond 3.0 h of recovery there appeared to be no difference between ethanol-exposed and control tissue in the quantity of  $^{125}\text{I}$ -PVP that had been captured.

#### The effect of ethanol on proteolysis

The effects of 0.01 % and 0.05 % (v/v) ethanol on the uptake and intralysosomal degradation of formaldehyde-denatured  $^{125}\text{I}$ -labelled bovine serum albumin were investigated (Fig. 3A,B). The control shows that the substrate was captured rapidly (by adsorptive-phase pinocytosis) and showed a mean clearance rate of  $85.0 \pm 2.1 \mu\text{l h}^{-1} \text{ mg}^{-1}$  yolk-sac protein while the mean clearance rates of tissues incubated in the presence of ethanol (0.05 % and 0.01 %) were  $18.4 \pm 0.66$  and  $18.5 \pm 0.53 \mu\text{l h}^{-1} \text{ mg}^{-1}$  yolk-sac protein, respectively (Fig. 3A). The quantities of radioactivity associated with the tissue in control experiments (Fig. 3B) are indicative of steady-state kinetics, as previously reported (Williams *et al.* 1975b). In contrast, the quantities of radioactivity associated with tissues exposed to either of the two ethanol concentrations rose linearly over the interval 1 h to 3 h before eventually approaching a maximal value (Fig. 3B). The difference between corresponding time points in Fig. 3A,B (note scale differences of ordinate in these figures) represents the net amount of proteolysis that has occurred up to a given time. Both concentrations of ethanol inhibit proteolysis by

**Table 1.** Rate of release of  $^{125}\text{I}$ -PVP from yolk sacs incubated in the presence of different concentrations of ethanol

Concentration of ethanol in the reincubation medium (% v/v)	Initial loss (%)	Progressive rate of loss ( $\% \text{ h}^{-1}$ )
0.00 (Control)	$2.58 \pm 0.09$	$0.79 \pm 0.36$
0.01	$6.38 \pm 0.20$	$1.56 \pm 0.10$
0.05	$9.67 \pm 0.23$	$2.08 \pm 0.45$
0.10	$7.96 \pm 0.35$	$14.5 \pm 0.51$
0.50	$13.4 \pm 0.70$	$15.4 \pm 0.47$

Yolk sacs were first incubated in serum-free medium 199 containing  $^{125}\text{I}$ -PVP ( $2 \mu\text{g ml}^{-1}$  of medium) for 2.5 h then washed and transferred to fresh medium that initially contained no radioactivity, but different concentrations of ethanol. The tissues were incubated for a further 2.75 h and both the initial loss of radioactivity (0–0.25 h) and the progressive rate of loss (0.25–2.75 h) were monitored. Data are expressed as the percentage of the radioactivity associated with the tissue at the start of the reincubation released per h. The values reported are means ( $\pm$ s.d.) from nine similar experiments.

approx. 90 % and, since there is a marked decrease in the association of labelled albumin with the ethanol-exposed tissue relative to controls (Fig. 3B), the major cause of the decrease in proteolysis must be inhibition of pinocytic uptake of the labelled protein rather than inhibition of intralysosomal proteolysis. (If the latter effect was dominant, the quantity of radioactivity found within ethanol-treated tissue would exceed that in corresponding controls as labelled protein accumulated in the lysosomal system.)

Since the effect of ethanol on the capture of  $^{125}\text{I}$ -PVP (a nondegradable marker macromolecule that is captured entirely in the fluid phase by yolk sacs; Williams *et al.* 1975a) was reversible, it was of interest to establish whether the proteolytic capacity of yolk sacs showed an equivalent recovery after exposure to ethanol (Fig. 4). Tissues not exposed to ethanol

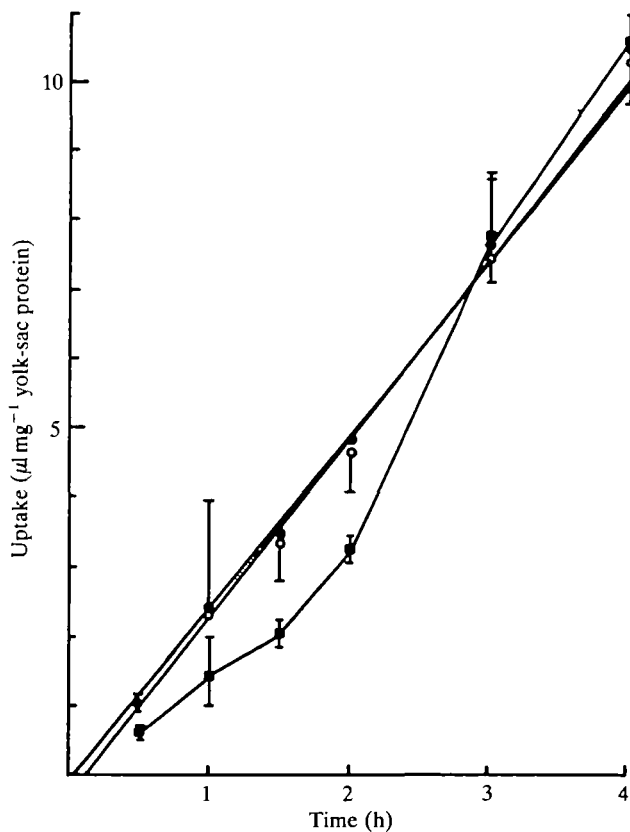


Fig. 2. Recovery of pinocytic capacity of yolk sacs previously exposed to ethanol. Yolk sacs were incubated for 0.5 h in serum-free medium 199 containing ethanol, but no  $^{125}\text{I}$ -PVP, essentially as described in the legend to Fig. 1. The tissues were then removed and washed in medium 199 at 37°C before being reincubated in ethanol-free medium containing  $^{125}\text{I}$ -PVP ( $2 \mu\text{g ml}^{-1}$ ). Uptake was monitored over the 4 h period of reincubation using tissues exposed to: ●, no ethanol (control); ○, ethanol (0.01 %, v/v) and ■, ethanol (0.05 %, v/v).

showed a linear release into the medium of acid-soluble radioactivity over a 4 h period (Fig. 4). However, ethanol-exposed tissues showed a lag in recovery of approximately 1 h after exposure to 0.01 % (v/v) ethanol; this lag increased to 2 h for tissues exposed to 0.05 % (v/v) ethanol. Moreover, examination of the gradients of these plots indicates that ethanol-treated tissues show an increase in proteolytic rate above that of the control tissues before the rate stabilized after approximately 3.5 h. The effect was qualitatively similar to that observed for the rate

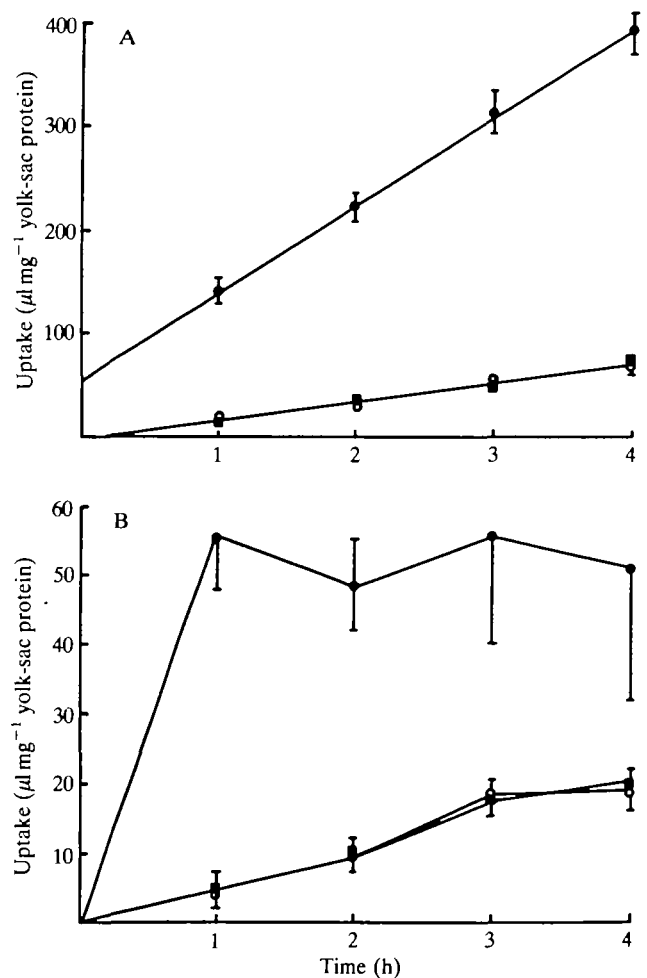
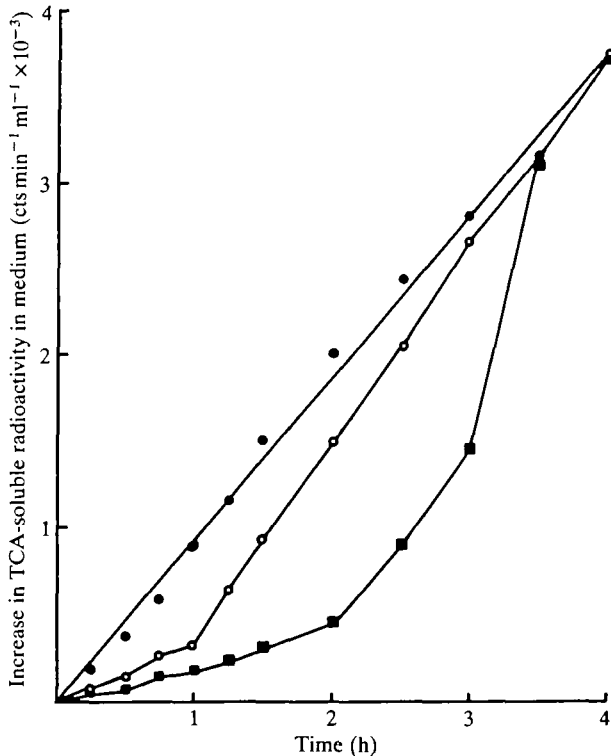


Fig. 3. Effects of ethanol on the rate of uptake of formaldehyde-denatured  $^{125}\text{I}$ -labelled bovine serum albumin and on the quantity of radioactivity associated with yolk sacs. Yolk sacs were incubated as described in the legend to Fig. 1 except that formaldehyde-denatured  $^{125}\text{I}$ -labelled albumin ( $2 \mu\text{g ml}^{-1}$ ) was used in place of  $^{125}\text{I}$ -PVP and, if ethanol was present in the serum-free medium, it was added at the start of the incubation. (A) Net uptake (including TCA-soluble digestion products released into the medium); (B) the quantity of radioactivity associated with the tissue. Each point represents mean values ( $\pm$ s.d.) from at least four yolk sacs from different animals; ●, no ethanol (control); ○, ethanol (0.01 %, v/v) and ■, ethanol (0.05 %, v/v).

of capture of  $^{125}\text{I}$ -PVP by tissues exposed to 0.05 % (v/v) ethanol (Fig. 2), but was more marked.

#### The effects of serum on fluid-phase pinocytosis

Increasing the concentration of serum in the medium decreased the effectiveness of ethanol as an inhibitor



**Fig. 4.** Recovery of proteolytic capacity of yolk sacs previously exposed to ethanol. Yolk sacs were exposed to ethanol for 0.5 h (see legend to Fig. 2 for details), washed and transferred to serum-free medium 199 containing formaldehyde-treated  $^{125}\text{I}$ -labelled albumin ( $2\ \mu\text{g ml}^{-1}$ ). The time course of release of TCA-soluble radioactivity into the medium over the 4 h reincubation period was established by sampling the medium at each time point. Values are means from nine similar incubations each with a different yolk sac. Key: the same as in Fig. 2.

of pinocytosis (Table 2). The concentrations of serum in the incubation medium were 10, 20 and 50 % (v/v). The relative rates of uptake of the controls were in agreement with those reported by Forster & Williams (1984); as the serum concentration increases the rate of capture of  $^{125}\text{I}$ -PVP decreases. However, with increasing serum concentration the relative effect of 0.01 % (v/v) ethanol decreases so that at a concentration of 50 % (v/v) calf serum this concentration of ethanol no longer inhibits pinocytosis. The effect of 0.05 % (v/v) ethanol on fluid-phase pinocytosis was not completely abolished, even by 50 % (v/v) calf serum; the residual uptake of  $^{125}\text{I}$ -PVP at 5 h was approximately 66 % of that from corresponding incubations in ethanol-free medium containing the same concentration of serum (Table 2).

#### Discussion

The results reported in this study indicate that two constitutive cellular activities, pinocytosis and proteolysis of an exogenous protein, both of which are well quantitated in the 17.5-day rat yolk sac *in vitro* (Williams *et al.* 1975a,b), are rapidly and severely disrupted, in a dose-dependent manner, by the presence of ethanol in the incubation medium (Figs 1, 3A). Inhibitions of approx. 90 % are induced by an ethanol concentration of 0.05 % (v/v) in serum-free medium. However, concentrations of ethanol of up to 0.05 % (v/v) in serum-free medium had little effect on the rate of progressive loss of  $^{125}\text{I}$ -PVP from tissues that had previously captured this marker macromolecule by pinocytosis (Table 1). This suggests that such concentrations of ethanol neither lyse nor permanently damage the endocytic cells of yolk-sac tissue incubated in serum-free medium 199. This contention is further supported by the observed regain of both the pinocytic and the proteolytic capacities of the tissue after its exposure to 0.05 %

**Table 2.** Effect of increasing the concentration of calf serum in the incubation medium on the degree of inhibition of pinocytosis induced by ethanol

Concentration of serum in the medium (% v/v)	Uptake after 5 h of incubation ( $\mu\text{g mg}^{-1}$ of yolk-sac protein)			Percentage uptake relative to ethanol-free matched controls (%)	
	Control	Ethanol 0.01 % (v/v)	Ethanol 0.05 % (v/v)	Ethanol 0.01 % (v/v)	Ethanol 0.05 % (v/v)
0	13.22 $\pm$ 0.35	4.59 $\pm$ 1.05	0.85 $\pm$ 0.17	34.7 $\pm$ 7.93	6.42 $\pm$ 1.38
10	8.59 $\pm$ 0.11	3.51 $\pm$ 0.04	0.63 $\pm$ 0.01	40.9 $\pm$ 0.56	7.33 $\pm$ 0.11
20	6.46 $\pm$ 0.09	4.05 $\pm$ 0.01	0.61 $\pm$ 0.02	62.6 $\pm$ 0.82	9.47 $\pm$ 0.30
50	3.70 $\pm$ 0.02	3.68 $\pm$ 0.01	2.45 $\pm$ 0.01	99.3 $\pm$ 0.50	65.9 $\pm$ 0.43

The time courses of uptake of  $^{125}\text{I}$ -labelled PVP by 17.5-day yolk sacs incubated in medium 199 containing  $^{125}\text{I}$ -PVP ( $2\ \mu\text{g ml}^{-1}$ ), ethanol (0.01 or 0.05 %, v/v) and different concentrations of heat-inactivated calf serum were determined (see Fig. 1 and text for details).

The values reported are means ( $\pm$ s.d.) from at least three similar experiments.

(v/v) ethanol (Figs 2, 4). Thus ethanol, at low concentrations, acts as an effective, rapid, but reversible inhibitor of pinocytosis and proteolysis of exogenous proteins in this tissue.

Since it is well established that formaldehyde-denatured  $^{125}\text{I}$ -labelled albumin is captured by adsorptive pinocytosis before it is degraded within the lysosomal system (Williams *et al.* 1975*b*; Livesey & Williams, 1979) it appears difficult to explain why a concentration of ethanol (0.05%, v/v), that completely inhibits the uptake of  $^{125}\text{I}$ -PVP, does not fully inhibit the association of the  $^{125}\text{I}$ -labelled protein with yolk-sac tissue (Fig. 3B). The most likely explanation of this observation is that this concentration of ethanol modifies the properties of the plasma membranes of yolk-sac cells so that nonspecific surface binding of labelled albumin occurs rather than uptake into the vacuolar system. This explanation finds support in the observation (not reported in detail) that in equivalent experiments to those reported in Fig. 3, but with medium containing 10% (v/v) of heat-inactivated calf serum, the progressive increase in tissue-associated radioactivity by tissues exposed to ethanol (as seen in Fig. 3B) was abolished. Presumably nonradioactive serum proteins occupy the surface sites so preventing the nonspecific binding of labelled albumin to the ethanol-exposed tissue.

In experiments (Table 2) that were performed with  $^{125}\text{I}$ -PVP as substrate and that were designed to establish whether increasing the serum content of the medium modulated the effect of ethanol on pinocytosis, a sharp decrease was observed in the effects of both 0.01 and 0.05% (v/v) ethanol, relative to the uptake in matched ethanol-free control experiments in which the medium contained the same concentration of serum. This suggests that serum components associate with ethanol and thereby decrease the concentration of free ethanol in the medium. The ability of serum albumin to bind fatty acids and other small molecules is well established (Foster, 1960; Spector, 1975), hence such binding of ethanol would not be surprising. A marked example of the ability of serum to relieve the inhibition of pinocytosis in rat yolk sacs (induced by a fixed concentration of the dye trypan blue) has been reported previously (Williams *et al.* 1976). From these results, it is predicted that *in vivo* the precise ethanol-to-protein ratio in the fluid that surrounds the yolk sac is likely to be more important than simply the ethanol concentration in determining the magnitude of the disturbance of yolk-sac function.

The observations reported in this paper are of interest from the standpoint of yolk-sac function during both late and early gestation. In some of the mammalian species (e.g. rat and rabbit) that show prenatal transmission of passive immunity, it is the

vacuolar system of the yolk sac late in gestation that is almost certainly used for the transport of intact molecules of IgG (immunoglobulin G) from the mother to the fetus (Wild, 1975). A study of the fate of IgG in 17.5-day rat yolk sacs *in vitro* (Weisbecker, Ibbotson, Livesey & Williams, 1983) showed that agents that inhibited pinocytosis also prevented the uptake and release of macromolecular IgG by yolk sacs in a manner that mimics the process of trans-yolk-sac transport. Thus, if the ratio of ethanol to protein in uterine fluid was maintained at a sufficiently high level for a prolonged period, as the result of chronic exposure to ethanol late in pregnancy, transfer of passive immunity could be impaired as a result of either prevention of pinosome formation or as a result of disrupting related processes that cause vesicles carrying IgG to undergo transcellular movement. Uterine fluid is probably an ultrafiltrate of serum, but its small volume in the rat prevents accurate estimation of its precise protein composition and comparison with that of serum. It is thus difficult to predict the magnitude of the ethanol effect *in vivo* from these *in vitro* data. It would appear easier to follow up this suggestion by direct *in vivo* determination of the effect of ethanol on the transfer of IgG from mother to fetus.

Second, if rat yolk sacs at earlier stages of gestation are equally sensitive to the effects of ethanol, a suggestion that is supported by preliminary experiments (Steventon & Williams, unpublished data), an ethanol concentration of approximately 0.05% (v/v) in the maternal blood at the organogenesis stage of development would be sufficient to partially impair histiotrophic nutrition, a process suggested to be the target of several teratogens including: trypan blue (Williams *et al.* 1976) anti-yolk-sac antiserum (Freeman *et al.* 1982) and leupeptin (Freeman & Lloyd, 1983). As pinocytosis is known to be the rate-limiting step in the overall process of pinocytic capture of proteins by rat yolk sacs (Williams *et al.* 1975*b*), embryotrophic nutrition can be inhibited in either of two ways. It can be inhibited by arresting pinocytosis (the suggested mode of action of trypan blue and of anti-yolk-sac antisera) or by preventing intralysosomal proteolysis (the proposed mode of action of leupeptin). The evidence obtained in this study of 17.5-day yolk sacs indicates that ethanol is more likely to act in the first manner. As the ethanol is oxidized by the maternal liver its concentration in the blood (see Abrams & Cooper, 1976) and thus in the yolk sac will rapidly decrease. Pinocytosis would be expected to resume so that only a temporary interruption of the flow of nutrients to the embryo would be induced after acute ingestion of ethanol.

Although these results suggest that ethanol may act as a teratogen by inhibiting embryotrophic nutrition

at the pinocytic stage, this effect could be compounded by any attendant inhibition, within fetal tissues, of processes such as amino acid transport (Fisher, Atkinson, Thiel, Rosenblum, David & Holzman, 1981; Dorio, Hoek & Rubin, 1984) or of protein synthesis (Henderson, Patwardhan, McLeroy & Schenker, 1982). The overall effect of such multiple actions could be to retard protein synthesis and embryonic growth at a critical stage of development and thus induce malformations.

These initial findings with 17.5-day yolk sacs are sufficiently interesting to suggest that a detailed study should be made of the effects of acute exposure of yolk sacs to ethanol at earlier stages of gestation, before the formation of a chorioallantoic placenta has occurred and when the embryo is more directly dependent on the yolk sac for its nutritional requirements. Also, the possible effects of chronic exposure to ethanol late in gestation on the transfer of passive immunity should be investigated in the rat and the rabbit.

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