Metabolic Effects of

Bordetella pertussis

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Abstract

The present work confirmed that B. pertussis infection or pertussis toxin produce hypoglycaemia in mice. The hypoglycaemia was associated with hyperinsulinaemia, and both were abolished by destruction of the pancreatic β cells with alloxan. Impaired glucose counterregulatory mechanisms may also contribute to pertussis-induced hypoglycaemia, as the hypoglycaemic action of insulin was prolonged in pertussis infected mice. On the other hand, impaired responsiveness to lower doses of insulin was found.

Pertussis-induced hyperinsulinaemia had two components. First, the increase in serum insulin in response to food intake was both greater and more prolonged in pertussis-infected mice. Second, infected or pertussis toxin-treated animals, unlike controls, showed a marked increase in serum insulin in response to certain stresses, such as ether, histamine, anoxia and 2-deoxyglucose. However, other stresses (LPS, cold and hypoxia) did not cause hyperinsulinaemia in pertussis infected mice.

Stress-induced hyperinsulinaemia was also seen in normal mice receiving the α_2-adrenoceptor blocking drug idazoxan. Stress-induced hyperinsulinaemia in α_2 adrenoceptor blocked mice, but not in pertussis-treated mice, was prevented by β adrenoceptor blockade using

propranolol.

Adrenal demedullation or ganglionic blockade (using hexamethonium) in normal mice also allowed stress-induced hyperinsulinaemia. Thus, adrenal medullary catecholamines may normally serve to prevent stress-induced hyperinsulinaemia, which becomes unmasked when they are absent or when their action is prevented.

Stress-induced hyperinsulinaemia in pertussis-treated mice was unlikely to involve autonomic, cholinergic or opioid mechanisms as it was not blocked by hexamethonium, atropine or naloxone.

Human infants with pertussis showed no hypoglycaemia compared with non-pertussis controls, although their plasma insulin concentrations were slightly but significantly raised. It remains possible that hyper-insulinaemia with resultant profound hypoglycaemia might occur in susceptible patients following exposure to pertussis-toxin (either during the disease or following vaccination).

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Dedication

To Mum and Dad - for putting up with me for so long!

DECLARATION

This thesis is the original work of the author except where otherwise stated.

FIONA M. SIDEY

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GLOSSARY

AC	Adenylate cyclase
FHA	Fimbrial haemagglutinin
HLT	Heat labile toxin
HSF	Histamine sensitising factor
IAP	Islet activating protein
LPF	Lymphocyte promoting factor
LPF-HA	Lymphocyte promoting factor haemagglutinin
LPS	Lipopolysaccharide
PT	Pertussis toxin
TCT	Tracheal cytotoxin

Section A

Whooping Cough

1. Clinical course of the disease

2. Complications of whooping cough and pertussis vaccination

3. Treatment of pertussis

4. Pathogenesis of pertussis

A. Whooping Cough

Whooping cough (pertussis) is a severe, highly contagious, respiratory disease affecting mainly infants and young children. The first account of pertussis came from Guillaume de Baillou in 1640, who reported an epidemic which had occurred in Paris in 1578, and gave details of a patient in a fit of paroxysmal coughing (Linnemann, 1979). The causative organism, *Bordetella pertussis*, was isolated by Bordet and Gengou in 1906. MacDonald and MacDonald (1933) demonstrated that *B. pertussis* organisms could produce typical pertussis in two of their children, but not in another two who had been previously immunised with killed bacteria. After this, vaccination against the disease became possible.

This thesis is concerned with some metabolic and endocrine effects of pertussis, especially those affecting insulin secretion and blood glucose regulation. This chapter will review, briefly, the disease and its pathogenesis followed by a more detailed consideration of how the disease and the toxins of *B. pertussis* may modify blood glucose regulation.

1. Clinical course of the disease

Pertussis, for which man is the only natural host, is an acute, localised disease of the respiratory tract (Pittman, 1970). The disease is spread by aerosol droplets from the respiratory tract of an infected case

(Linnemann, 1979) and is highly communicable amongst a susceptible population. The average duration of illness is about 50 to 60 days (Pittman, 1970), and during the course of the disease there are four distinct stages.

1.1 Incubation period

The incubation period of the disease lasts between 7 and 14 days (Court et al, 1953), the variability of this incubation period may depend on the size of the infecting dose or on the uncertainty as to when exposure first occurred (Olson, 1975).

1.2 Catarrhal stage

The onset of symptoms begins in this second stage, with anorexia, slight pyrexia, sneezing and an increasingly severe cough. During this time, which also lasts between 7 and 14 days, the disease is highly contagious producing attack rates of 80 to 90% in unvaccinated children exposed at home (MRC, 1951).

1.3 Paroxysmal stage

This is the most severe stage of the disease which can persist from 2 to 6 weeks, or longer. Fever is not usually present at this time (Dayal et al, 1969) but severe bouts of coughing or paroxysms can occur without warning. These bouts may be as frequent as 10 to 21 per day, and may be accompanied by vomiting, apnoea and cyanosis. The classical whoop, which gave the disease its common name, often accompanies the paroxysmal

coughing and is caused by the long inspiration of air following a coughing bout. Biochemical changes such as lymphocytosis (Lagergren, 1963) and relative hypoglycaemia have also been reported at this stage (Regan and Tolstoouhov, 1936).

Lung damage may result from the violent coughing episodes, and other complications (see later) associated with pertussis may also appear during this stage (Olson, 1975). The level of bacterial colonisation falls during this stage - B. pertussis is rarely isolated from the host after the second week of paroxysmal coughing (Linnemann, 1979). However, coughing and other symptoms can persist for several weeks after cultures for B. pertussis become negative (Pittman, 1970; Olson, 1975).

1.4 Convalescent stage

The symptoms of the paroxysmal stage subside gradually, but it may take up to six months for the paroxysms to stop (Olson, 1975).

2. Complications of whooping cough and pertussis vaccination

2.1 The disease

Many of the complications during or following pertussis infection can be related to the episodes of intense and prolonged expiratory spasms seen during the paroxysmal stage (Olson, 1975). These include

haemorrhagic events, hernias or prolapses resulting from increased intrathoracic and intraabdominal pressure, and pulmonary complications caused by either the pertussis infection itself or to suprainfections with other organisms.

More rarely however, the central nervous system may be affected leading to various degrees of neurological damage which may be permanent (Pittman, 1986). Convulsions, sometimes following a coughing seizure, can be present during the clinical stage of pertussis, and can lead to severe encephalopathy and death (Pittman, 1970). Up to 1% of patients can develop encephalopathy during the paroxysmal stage (Davis et al, 1984). Survivors of the disease may be left with neurologic deficits ranging in severity from character disorders (Pittman, 1956) to permanent brain damage (Prensky, 1985; Stetler et al, 1985).

Various factors such as cerebral anoxia after prolonged apnoea, hypoglycaemia, cerebral haemorrhages, viral infections or toxic factors from B. pertussis itself have been implicated in these neurologic sequalae (Lapin, 1943; Olson, 1975; Pittman, 1986). However, no certain causative factors have been identified.

2.2 Vaccination

On rare occasions similar complications are seen following vaccination against pertussis (Griffith, 1978;

Hennessen and Quast, 1979; Miller et al, 1981). Reactions attributed to pertussis vaccine have ranged from mild pain, swelling and redness at the site of injection to severe neurological reactions such as febrile or non-febrile convulsions, permanent brain damage or death (Robinson et al, 1985; Pittman, 1986). However, the incidence of spasms, convulsions and seizures of other aetiologies is relatively high in children of vaccination age, and some coincidental reactions may have been wrongly attributed to the vaccine (Griffith, 1978).

Despite this, reports of severe reactions following pertussis vaccination have led to fears over the safety of the vaccine, and consequently to a decrease in the vaccine acceptance rate after 1974 (Robinson et al 1985). This was probably responsible for the pertussis epidemics of 1978-79 and 1981-82 which were the most serious epidemics since the start of mass vaccination (Hirtz et al, 1983; Robinson et al, 1985).

3. Treatment of pertussis

Antibiotic therapy appears to be of little value in the treatment of pertussis, although it may modify the course of the disease if given early enough (Bass et al, 1969; Broomhall and Herxheimer, 1984). Erythromycin is the antibiotic of choice (Broomhall and Herxheimer, 1984), the main benefit of its use being the shortening of the

infectious period and the reduction in complications from secondary infections (Baraff et al, 1978; Pittman, 1984a). Antipertussis serum has been used both prophylactically and therapeutically, but its effectiveness is doubtful (Pittman, 1970).

4. Pathogenesis of pertussis

B. pertussis is a small, gram-negative cocobacillus which is non motile and non sporing. The organism is inspired by the host and lodges in the ciliated epithelium of the respiratory tract, where it multiplies during the catarrhal stage of the disease (Pittman, 1979). The organism does not invade tissues, including the blood, and any pathological changes seen are neither extensive nor general (Pittman, 1984a). However, the profound biological changes seen in experimental animals with a local respiratory tract infection of *B. pertussis* - such as lymphocytosis, hypoglycaemia, hyperinsulinaemia and sensitivity to histamine or cold stress - led Pittman to propose that the disease pertussis was toxin-mediated (Pittman 1979, 1984a).

B. pertussis toxins and virulence factors

B. pertussis produces several toxins and toxic substances. These, and their possible relevance to the disease pertussis, will be described in the following section. A more detailed account of the effect of *B. pertussis* and its products on blood glucose regulation

will be given in section E of the introduction.

4.1 Heat Labile Toxin (HLT)

HLT was the first toxin described from *B. pertussis* (Bordet and Gengou, 1909). It is a heat labile (56°C, 10 minutes) intracellular protein which has both dermonecrotising and lethal effects in mice, guinea pigs and rabbits, and causes atrophy of the spleen after intravenous injection (Wood, 1940). HLT has been implicated in the pathogenesis and symptomology of whooping cough (Munoz, 1971), but evidence does not support a role for HLT in immunity (Pittman, 1979, 1984a).

4.2 Adenylate Cyclase (AC)

Adenylate cyclase activity was discovered during investigation of the apparent sympathomimetic activity of pertussis vaccine (Wolff and Hope Cook, 1973). The AC of *B. pertussis* is unusual in that a high proportion of the cyclase activity is extracytoplasmic, and its regulation is markedly different to the classical membrane bound AC of eukaryotic cells (Hewlett et al, 1976).

In crude preparations the AC activity is sensitive to activation by mammalian calmodulin, a calcium dependent regulatory protein that is unique to eukaryotic cell systems (Goldhammer et al, 1981). Confer and Eaton (1982) have found that internalisation of *B. pertussis* products leads to a rapid intracellular cAMP production

in invaded human neutrophils, with subsequent inhibition of normal phagocytic function. These authors suggested that this effect was due to bacterial cyclase activity as opposed to activation of the intrinsic AC activity of the neutrophils by pertussis toxin. A definite role for AC in the pathogenesis of pertussis has still to be proven. However, transposon mutants deficient in AC and haemolysin activity were avirulent in an infant mouse respiratory infection model (Weiss et al, 1984).

4.3 Lipopolysaccharide (LPS)

The LPS of *B. pertussis* exhibits endotoxic activities similar to those of LPS from other gram negative bacteria. It is heat stable, pyrogenic, toxic and acts as an adjuvant (Wardlaw and Parton, 1983). Indeed, two different LPS have been isolated from *B. pertussis* (LeDur et al, 1978; Peppler, 1984) which are both chemically and serologically distinct. A definite role for LPS in the pathogenesis of pertussis has not been demonstrated, but its immunological and biological activities, as well as its contribution to local tissue damage, could play a role in the disease or in the rare neurological complications following disease or vaccination. Indeed, in experimental animals, the early hyperinsulinaemia following vaccination has been shown to be due to LPS (Hewlett et al, 1983).

4.4 Tracheal cytotoxin (TCT)

A small glycopeptide which is cytotoxic to hamster epithelial cells has been isolated and named tracheal cytotoxin (TCT). TCT produces ciliostasis and extrusion of the ciliated epithelial cells of hamster tracheal organ cultures (Goldman et al, 1982) and may therefore be involved in delaying clearance of the organisms from the respiratory tract or in the local tissue damage to the upper respiratory tract caused by *B. pertussis*.

4.5 Haemagglutinins

B. pertussis possesses two distinct haemagglutinins, known as fimbrial haemagglutinin (FHA) and lymphocyte promoting factor haemagglutinin (LPF-HA), which possess different chemical and biological properties. LPF-HA has been shown to be identical to pertussis toxin, whereas FHA may be derived from bacterial cell surface fimbriae. FHA may therefore have a role in the attachment of the organism to ciliated epithelial cells (Sato et al, 1979). Despite some doubts as to the fimbrial origin of FHA (Ashworth et al, 1982; Robinson et al, 1985), antibodies against FHA have been shown to inhibit attachment of *B. pertussis* to mammalian cells (Sato et al, 1979).

4.6 Agglutinogens

B. pertussis contains cell surface antigens which stimulate the production of antibodies, causing bacterial cell agglutination (Robinson et al, 1985). There are three major agglutinogens found (numbered agglutinogens

1, 2 and 3), and the importance of each agglutinogen in pertussis vaccine has been a matter of debate for many years (Wardlaw and Parton, 1983).

4.7 Haemolysin

Genetic studies have indicated that the haemolysin produced by B. pertussis may play a role in the pathogenesis of the disease, since mutants deficient in only haemolysin have a reduced virulence in the infant mouse respiratory infection model (Weiss et al, 1984).

4.8 Outer membrane proteins (OMP)

The OMP of a number of gram negative bacteria have been recognised as playing an important role in the pathogenesis of the organisms, and the hosts immune response is often directed against specific OMP (Levine et al, 1983; Robinson et al, 1985). In B. pertussis, Parton and Wardlaw showed that loss of virulence during phase variation (X mode to C mode) was associated with an altered envelope protein (Parton and Wardlaw, 1975).

4.9 Pertussis Toxin (PT)

Many of the biological activities caused by pertussis vaccine or pertussis infection have been found to be due to a protein toxin unique to B. pertussis. This protein has been isolated and purified by several groups (Yajima et al, 1978; Irons and MacLennan, 1979; Arai and Munoz, 1981; Sekura et al, 1983; Perera et al, 1985) and has been shown to be an "A-B" toxin, in that it

possesses an active (A) subunit and a binding (B) subunit (Tamura et al, 1982) The complete toxin, holotoxin, consists of five dissimilar subunits, numbered S_1 to S_5, which can be dissociated into two main parts (Ui et al, 1985). The A-protomer consists of a single S_1 subunit and contains the enzymic activity of the toxin. The B-oligomer is a pentameric arrangement of subunit S_2, S_3, S_5 and two S_4 subunits, and is the binding moiety of the toxin. The B-oligomer binds to cell membranes and allows transport of the A-protomer into target cells (Tamura et al, 1983). Indeed, some biological activities of PT require only the binding of the B subunit, e.g. mitogenic effects, leukocytosis promoting effect, histamine sensitisation and adjuvancy. However, other activities of PT require the ADP-ribosyltransferase activity of the A-protomer, e.g. islet activation, adenylate cyclase activation and haemagglutination (Nogimori et al, 1984; Ui et al, 1985).

PT is the most potent toxin produced by B. pertussis, being active in various cell systems in picogram doses (Munoz, 1985). It produces hypersensitivity to histamine (histamine sensitising factor, HSF) and to other shock inducing agents, acts as an adjuvant, induces leukocytosis with a predominance of small lymphocytes (lymphocytosis promoting factor, LPF) increases insulin secretion (islet activating protein, IAP) and alters sugar and fat metabolism. PT also enhances various

syndromes which have an immunological basis e.g. delayed type hypersensitivity (DTH), experimental allergic encephalomyelitis and some inflammatory responses. Indeed, PT appears to be the major protective antigen produced by B. pertussis, protecting against both intranasal and intracerebral infection in mice (Sato et al, 1981; Pittman, 1984a).

The biological effects and the mechanism of action of PT will be discussed in detail in later chapters.

Section B

Mechanism of action of Pertussis Toxin

1.1 ADP-ribosyltransferase activity of PT

1.2 GTP-binding regulatory proteins

1.3 Interaction of PT and N_i

2. Functions of the B-oligomer of PT

B. Mechanism of action of Pertussis Toxin

1.1 ADP-ribosyltransferase activity of PT

The S_1 subunit of pertussis toxin has ADP-ribosyltransferase activity, and catalyses the hydrolysis of NAD to ADP-ribose and nicotinamide. In the presence of N_i, transducin (or other ADP-ribose acceptor proteins), pertussis toxin catalyses the transfer of ADP-ribose from NAD to a specific residue on these proteins (Moss et al, 1985) as shown below:

NAD + protein ⟶ ADP-ribose-protein + nicotinamide + H^+

In the case of transducin, the residue which is the substrate for ADP-ribosylation by PT is an asparagine residue (Moss et al, 1985).

The ability of PT to catalyse ADP-ribosylation of N_i or transducin is enhanced by the presence of the $\beta\gamma$ subunit of each receptor (Moss et al, 1985). PT ADP-ribosylates the inactive form of GTP-binding proteins, since conditions which favour the inactive state of N_i or transducin (such as addition of GDP or dark rhodopsin), increase the levels of PT catalysed ADP ribosylation (Moss et al, 1985).

1.2 GTP-binding regulatory proteins

The pertussis toxin substrate, N_i or G_i, is a member of a family of GTP-binding regulatory proteins. These include N_s, which stimulates adenylate cyclase

(Hildebrandt et al, 1984) and is a substrate for ADP-ribosylation by cholera toxin, and transducin which transduces the signal from light-activated rhodopsin in retinal rods to a cGMP phosphodiesterase (Spiegel et al, 1985). Such proteins provide a means of signal transduction from the activated receptor to the effector system in many cell types (Spiegel, 1987). GTP-binding regulatory proteins are involved in receptor activation and inactivation of adenylate cyclase (N_s and N_i) transduction of signals from rhodopsin to cGMP phosphodiesterase ($transducin_1$ and $transducin_2$), stimulation of phospholipase C ($G_{'PI'}$) and possibly receptor-induced opening of ion channels (G_o) (Spiegel, 1987).

1.3 Interaction of PT and N_i

Both GTP regulatory proteins associated with adenylate cyclase are thought to consist of three subunits, α, β and γ (Hildebrandt et al, 1984). The α_i subunit is the substrate for ADP-ribosylation by PT, and also contains the GTP-binding site and GTP-ase activity of N_i (Ui, 1984).

Normally, when a hormone (H) interacts with a receptor (R) the RH complex activates the GTP-regulatory protein, causing the α subunit to bind GTP (Rodbell, 1980). The N protein then dissociates into the α subunit, and free βγ dimer. The α subunit interacts with the catalytic unit of adenylate cyclase (Rodbell, 1980)

and either stimulates (α_s) or inhibits (α_i) the production of cAMP.

Pertussis toxin stabilises N_i in its inactive state (Ui, 1984) by ADP ribosylation of the α_i subunit, thereby preventing the transduction of inhibitory signals to adenylate cyclase. This is in contrast to the effects on N_s of cholera toxin which stabilises α_s in the active state. Thus, cholera toxin produces an initial stimulation of cAMP production but prevents further stimulation by receptor agonists acting through N_s (Rodbell, 1980).

Because of the specificity of action of PT, it has been used as a probe for the presence of the α_i subunit of N_i in tissues (Ui, 1984). It does, however, ADP ribosylate other GTP-binding regulatory proteins (Spiegel, 1987), for example the α subunits of transducin, N_o and possibly $N_{'PI'}$.

1.4 Functions of the B-oligomer of PT

The binding activity of the B-oligomer subunit of PT is required to allow the transport of the enzymatically active A-protomer into whole cells. Antibodies directed against the B-oligomer prevented the action of PT in intact cells, but did not prevent activation of adenylate cyclase in membrane preparations (Katada and Ui, 1980). Katada and Ui (1980) showed that a lag time was required before the onset of action of PT in intact cells, which reflected the time required for internalisation of the

toxin molecule across the plasma membrane.

Controlled acetamidation of the lysine residues of PT prevented some activities of PT, but not others (Nogimori et al, 1984). Those activities prevented by acetamidation were presumed to be due to binding of the B-oligomer and did not require the ADP-ribosyltransferase activity of the A-protomer. These included mitogenic activity, lymphosytosis promoting activity, increases in vascular permeability, histamine sensitising ability, adjuvancy and the stimulation of glucose oxidation in adipocytes (Tamura et al, 1983).

Acetamidation of lysine residues prevented one point of attachment between PT and target cells, but did not affect the other (Nogimori et al, 1984). This suggested that the binding-dependent activities required divalent attachment of PT to cells, whereas monovalent attachment was sufficient to allow internalisation of the active A-protomer, and therefore to allow ADP-ribosylation-dependent activities to proceed.

Section C

B. pertussis infection of the mouse

1. Animal models of pertussis

2. Sublethal Intranasal Infection (SLINI) model of pertussis in the mouse

3. Comparison of SLINI in the mouse and human *B. pertussis* infection

C. B. pertussis infection of the mouse

1. Animal models of pertussis

Many attempts have been made to develop an animal model for pertussis which resembles the human disease. Pertussis infections in primates have been successful, producing a long lasting infection accompanied by a paroxysmal cough in some, but not all, species (Huang et al, 1962; Stanbridge and Preston, 1974).

However, the high cost of primates prohibits their routine use, and recourse to lower animals such as rodents is necessary. Attempts to infect rabbits (Preston et al, 1980), and rats (Hornibrook and Ashburn, 1939) with B. pertussis have been made. Only the rat was reported to develop a paroxysmal cough (Hornibrook and Ashburn, 1939).

2. Sublethal Intranasal Infection (SLINI) model of pertussis in the mouse

B. pertussis is not a natural pathogen for mice, but it may grow in the lungs or the brain of mice after incubation. Both these cell surfaces provide ciliated epithelial cells, amongst which the organism can multiply.

Respiratory tract infection can be initiated in mice by the instillation of live B. pertussis organisms intranasally (Burnet and Timmins, 1937), intratracheally (Bradford, 1938; Te Punga and Preston, 1958) or by

aerosol inhalation (Sato et al, 1980). The latter two techniques are considered to give more uniform and reproducible results (Sato et al, 1980).

In 1980, Pittman, Furman and Wardlaw classified the pathophysiological responses of B. pertussis infected mice into those changes seen in lethally or sublethally infected animals. Lethal infection of mice produced hypoglycaemia, hyperinsulinaemia, pronounced hypothermia, spleen atrophy, a loss of body weight and highly elevated levels of leukocytes. Sublethally infected mice showed less pronounced hypoglycaemia, a lesser degree of hyperinsulinaemia, near normal temperature, spleen enlargement, normal weight gain and a lesser degree of leukocytosis. These sublethally infected animals also became sensitive to the lethal effects of histamine (Pittman et al, 1980). The intensity of each reaction was related to the degree of lung involvement.

3. Comparison of SLINI and human B. pertussis infection

Both human and murine infections are typified by a localised, non invasive colonisation of the ciliated epithelium of the respiratory tract (Mallory and Hornor, 1912), a marked leukocytosis (Bradford, 1938; Cooper, 1952; Pittman et al, 1980; Sato et al, 1980) and hypoglycaemia (Regan and Tolstoouhov, 1936; Pittman et al, 1980). Moreover, the young infant of both species is the most susceptible host (Pittman, 1957; Pittman et al,

1980; Sato et al, 1980). Also, the onset and duration of the paroxysmal cough in the human infection, although having no direct equivalent in the mouse, parallels the development of histamine sensitisation in the infected mouse (Pittman, 1951; Pittman, 1957; Geller and Pittman, 1973; Pittman et al, 1980). The pathophysiological changes observed in fatal human B. pertussis infections are similar to those seen in lethal respiratory infections of mice (Burnet and Timmins, 1937).

Section D

Insulin Secretion

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D. Insulin Secretion

As much of the work presented in this thesis is concerned with the effects of pertussis on serum insulin concentration and blood glucose regulation, this section will review the relevant aspects of the physiological regulation of insulin secretion. The actions of insulin will also be considered.

The regulation of insulin secretion is a complex process involving several mediators, both hormonal and neuronal. Indeed, the synthetic and secretory mechanisms of the pancreatic β cells are separate processes, since some stimuli cause insulin release without affecting its synthesis.

In man, and many other species, the most important stimulus for insulin secretion is an increase in blood glucose concentration. However, some other hexoses and amino acids can also stimulate insulin release.

1. Glucose-Induced Insulin Secretion

The pancreatic β cell is freely permeable to glucose, in that it does not require the presence of insulin to take up glucose from the extracellular fluids (Taljedal, 1981). It was suggested that glucose is recognised by a specific receptor, the glucoreceptor, which when stimulated by glucose would cause insulin biosynthesis and release (Ashcroft, 1980). However, the 'glucoreceptor' appears to be the metabolism of

glucose within the pancreatic β cell, since inhibition of glucose metabolism with e.g. mannoheptulose prevents glucose-induced insulin secretion (Malaisse et al, 1968; Ashcroft, 1980). Also, non-metabolisable sugars do not cause insulin biosynthesis or secretion.

Exposure of pancreatic β cells in suspension, or perfused islets of Langerhans, to glucose produces a biphasic pattern of insulin release (Grodsky and Bennett, 1966; Hoshi and Shreeve, 1973; Idahl et al, 1976; Atwater et al, 1984). Glucose produces an initial prompt rise in insulin secretion, after a 1-2 minute latency period (Idahl et al, 1976), followed by a fall, which is in turn succeeded by a slower, progressive increase in insulin release to a plateau level (the second phase). These two phases of insulin release have been attributed to two separate pools of insulin within the β cell (Hedeskov, 1980) or to phasic changes in cation fluxes and accumulations (Wollheim and Sharp, 1981). Drugs which interfere with the microtubular microfilamentous system within the β cell prevented both phases of insulin secretion (Howell, 1984; Howell and Tyhurst, 1984), suggesting a role for these systems in the movement of insulin storage granules to the β cell membrane.

Exposure of islet β cells to glucose produces many effects which may be involved in the insulin secretory process. These glucose-induced alterations will now be considered.

1.1 Calcium

Extracellular calcium is required for glucose to stimulate insulin secretion (Grodsky and Bennett, 1966; Milner and Hales, 1967), and calcium may couple glucose recognition to insulin release (Malaisse, 1973). Stimulatory concentrations of glucose produce a biphasic pattern of radiolabelled calcium efflux from the β cell - an initial fall in efflux followed by a sustained rise (Herchuelz and Malaisse, 1980), this was interpreted by these authors as representing an increased entry of unlabelled calcium into the β cell, followed by intracellular calcium/calcium exchange (Herchuelz et al, 1980). However, glucose produces a decrease in intracellular free calcium under certain conditions which promote active sequestration of calcium within intracellular stores (Hellman, 1985). The balance between these two processes will determine the activity of calcium within the cytoplasm, and therefore the rate of insulin release (Hellman, 1985).

Both phases of glucose-induced calcium efflux depend on metabolism of glucose within the β cell, but only the first phase of calcium movement is present during exposure of islets to substimulatory concentrations of glucose (Herchuelz et al, 1980). Also, the second phase requires extracellular calcium, is associated with membrane depolarisation, and is inhibited under conditions which prevent calcium entry into the β cell (Herchuelz et

al, 1980). However, this second phase of calcium efflux does not correspond solely to the exocytosis of secretory granules as was first thought (Malaisse et al, 1973) since it can be dissociated from insulin release (Herchuelz and Malaisse, 1978).

Intracellular calcium may interact with the effector system for granule movement within the β cell (Howell, 1984; Howell and Tyhurst, 1984) through the calcium binding protein calmodulin, which has been identified in islet cells (Sugden et al, 1979; Howell and Tyhurst, 1984). Alternatively, calcium may regulate stimulus-secretion coupling in islet cells through cAMP independent protein phosphorylation (Kowluru and MacDonald, 1984).

1.2 Cyclic 3',5',-adenosine monophosphate (cAMP)

Agents known to increase the level of cAMP within the β cell (e.g. glucagon, enteroglucagon, dibutyryl cAMP, theophylline or caffeine) are unable to provoke sustained secretion of insulin themselves (Malaisse, 1973). Alterations in cAMP levels within the β cell are, however, accompanied by a parallel increase or decrease in the cells secretory response to glucose (Malaisse et al, 1967a). Agents which increase β cell cAMP levels exert their most marked insulinotropic action at high glucose levels (Malaisse et al, 1967a; Malaisse et al, 1970; Brisson et al, 1972)

Cyclic AMP may act itself to increase glucose-

induced insulin secretion, by activation of cAMP dependent protein kinases (Ashcroft, 1980; Christie and Ashcroft, 1985), through elevation of cytosolic Ca^{++} concentrations within the β cell (Brisson et al, 1972; Malaisse, 1973; Siegel et al, 1980; Henquin and Meissner, 1984), or by increasing the sensitivity of cellular targets to activation by calcium (Henquin, 1985).

High concentrations of glucose elevated cAMP levels in perifused pancreatic islets (Zawalich et al, 1975), but the time course in cAMP changes and insulin release differed. Therefore it is unlikely that glucose induces insulin release through elevation of cytosolic cAMP concentrations. However, both calcium and cAMP have been shown to phosphorylate separate proteins in islet tissue (Kowluru and MacDonald, 1984) and may use this mechanism to regulate the stimulus-secretion coupling in pancreatic cells.

1.3 Electrical Activity and Cation Fluxes

Glucose-induced insulin secretion is associated with the appearance of electrical activity in the pancreatic β cell (Ashcroft et al, 1984). Stimulatory glucose concentrations produce a slow membrane depolarisation followed by either oscillatory bursts of action potentials, or, at higher glucose concentrations, continuous spiking. Glucose metabolism is required for

the appearance of electrical activity (Dean et al, 1975). It appeared that this electrical activity could represent an early event in the stimulus-secretion coupling of glucose-induced insulin secretion, or could reflect the exocytotic fusion of granules with the β cell membrane (Atwater et al, 1984). However, cooling isolated islets of Langerhans from 37°C to 27°C prevents insulin release without affecting electrical activity, potassium permeability, calcium movements, or glucose metabolism (Atwater et al, 1984). Thus, there appears to be a highly temperature-dependent step in the process of secretion, located between the voltage dependent calcium entry and the exocytosis of secretory granules (Atwater et al, 1984).

Forskolin and cAMP analogues do not themselves alter membrane potential or electrical activity in the β cell, but markedly increase glucose-induced electrical changes (Henquin and Meissner, 1984).

Glucose-induced depolarisation of the β cell membrane is due to reduced K^+ permeability (Cook and Hales, 1984). Indeed, a potassium-channel sensitive to glucose metabolism within the cell has been identified in isolated rat pancreatic β cells (Ashcroft et al, 1984). Alternatively, protons, produced metabolically, could block a calcium-activated K^+ channel and depolarise the membrane (Cook et al, 1984). A third, ATP-sensitive, K^+ channel has also been identified in the β cell, and this

could link glucose metabolism to membrane K^+ permeability changes (Cook and Hales, 1984).

1.4 pH

Protons generated by the metabolism of glucose within the β cell have been proposed as a coupling factor between cationic changes and insulin secretory events (Smith and Pace, 1983). First phase changes in Ca^{++} efflux at substimulatory concentrations of glucose may be coupled to an increased generation of protons within the β cell (Malaisse et al, 1980). Indeed, alteration of intracelluar (but not extracellular) pH induced parallel changes in glucose-induced secretory and electrical events (Smith and Pace, 1983). These authors reported that increasing intracellular pH reduced glucose-induced insulin secretion, and decreasing intracellular pH increased glucose-induced insulin secretion.

However, glucose stimulation of pancreatic β cells appeared to cause either no change in intracellular pH, or internal alkalinisation. Also, it has been reported that intracellular alkalinisation of islet tissue produces an increase in Ca^{++} uptake and in insulin secretion (Lindstrom and Sehlin, 1986) at substimulatory concentration of glucose.

1.5 Phospholipid metabolism

An early event in the stimulus-secretion coupling of glucose-induced insulin release may be the rapid

hydrolysis of polyphosphoinositides (Montague et al, 1985; Rana et al, 1985) by phospholipase C (Best and Malaisse, 1984). Stimulatory concentrations of glucose cause increases in phosphatidic acid (PA), phosphatidyl-inositol (PI) and polyphospho-inositides (Farese et al, 1986). In particular, there is a breakdown of phosphatidyl inositol 4,5 bisphosphate (PIP_2) and phosphatidyl inositol 4 phosphate (PIP) and an accumulation of inositol triphosphate (ITP) and inositol bisphosphate (IP_2) (Montague et al, 1985).

These effects required metabolism of glucose, but were not dependent upon calcium (Montague et al, 1985). However, Morgan et al (1985) found that polyphosphoinositol metabolism, calcium mobilisation and insulin release in islet cells could be dissociated under certain conditions.

Hydrolysis of PIP_2 by phospholipase C to produce ITP increases cytosolic free calcium by mobilisation of Ca^{++} from the endoplasmic reticulum, and may therefore be involved in the process of insulin secretion (Farese et al, 1986). Diacylglycerol (DAG) production from PI, PIP, or PIP_2 may also be important in promoting insulin secretion (Malaisse et al, 1983; Farese et al, 1986).

Glucose metabolism within the pancreatic β cell produces <u>de novo</u> synthesis of phospholipids (Dunlop and Larkins, 1985; Farese et al, 1986) and this mechanism could contribute to the increased concentrations of ITP and DAG found after glucose stimulation (Farese et al,

1986).

1.6 Arachidonic Acid Metabolism

The presence in the endocrine pancreas of both cyclooxygenase and lipoxygenase metabolites of arachidonic acid was demonstrated in 1983 by Metz and co-workers. They showed that cyclooxygenase products inhibit induced insulin release, whereas lipoxygenase products increase stimulated insulin release. The overall effect of exogenously added arachidonic acid was to stimulate insulin release from pancreatic cells grown in a monolayer (Metz et al, 1983). Indeed, these authors later suggested that lipoxygenation of endogenously released arachidonic acid was a critical step in the stimulus-secretion coupling of the pancreatic β cell (Metz et al, 1984).

Prostaglandins (PG) of the E series (cyclooxygenase products) have been reported to be inhibitory to insulin secretion (Robertson, 1984) both *in vivo* in human volunteers (Giugliano et al, 1983) and *in vitro* in neonatal rat pancreatic islet cells (Fujimoto and Metz, 1984). However, studies with flurbiprofen, a potent inhibitor of prostaglandin synthesis, showed an augmentation of both basal and glucose-stimulated insulin release at low concentrations of the inhibitor, but an inhibition at high concentrations (MacAdams et al, 1984).

Glucose produces a biphasic pattern of insulin release, and this may be modulated by availability of arachidonic acid and its metabolites, particularly PGE

(Giugliano et al, 1983; Fujimoto and Metz, 1984; Metz, 1984). Exogenously applied phospholipase A_2 produced a concentration dependent insulin secretion (Yamamoto et al, 1983). Also, glucose-induced insulin secretion can be prevented by phospholipase A_2 inhibitors, and by lipoxygenase inhibitors (Yamamoto et al, 1982). However, interpretation of these studies is complicated because of the lack of specificity of the inhibitors used (Northover, 1977; Robertson, 1984).

Other products of phospholipase A_2 action, apart from arachidonic acid, are the lysophospholipids (Metz, 1986a). These compounds may be released when glucose metabolism stimulates phospholipase A_2, and may couple the glucose signal to mobilisation of membrane-associated Ca^{2+} stores and the initiation of exocytosis (Metz, 1986b). The simultaneous release of arachidonic acid by phospholipase A_2, and its lipoxygenase metabolites could then modulate - but not initiate - the insulin release process (Metz, 1986b).

1.7 Cytosolic Redox State

In 1973, Deery and Taylor reported that nutrient-induced insulin release from isolated islets was dependent on the maintenance of NAD concentrations. The cytosolic ratio of NADPH/$NADP^+$ appears to be important in determining Ca^{++} levels (Fain, 1984) and therefore may be important in determining insulin secretion. Glucose

metabolism may produce alterations in ion fluxes through the production of reduced pyridine nucleotides (Taljedal, 1981) which would in turn reduce a membrane redox protein and allow ion flux.

Glucose stimulation of rat pancreatic islets does produce an increased $NADPH/NADP^+$ ratio, as does L-Leucine (Sener et al, 1984). Also, the inhibition of insulin release from pancreatic islets caused by insulin itself is associated with a decrease in the ratio of NADPH to $NADP^+$ (Ammon and Versphol, 1976) without any changes in NADH and NAD^+ levels. However, Deery and Taylor (1973) suggested that maintenance of NAD concentrations was required for insulin release caused by sugars and amino acids, since depleting islets of NAD^+ and NADH prevented insulin secretion in response to glucose and other secretagogues.

1.8 Adenosine Triphosphate (ATP)

Metabolism of glucose and other nutrients within the β cell increases the rate of ATP generation. ATP is a substrate for many of the enzymatic reactions which are increased in stimulated β cells, and it may be that ATP availability is not only a permissive factor, but may regulate nutrient-induced insulin release (Malaisse et al, 1984). However, ATP content as measured by ATP/ADP ratio and adenylate charge is little affected by the glucose concentrations that cause stimulation of insulin

release (Malaisse et al, 1984).

2. Modulation of Glucose-Induced Insulin Secretion

Insulin secretion is modulated *in vivo* by both humoral and neurological mechanisms. Hormones from the other cell types of the islets of Langerhans, as well as other hormones predominantly of gastrointestinal origin, can either stimulate or inhibit insulin secretion (Hopcroft et al, 1985). The central nervous system provides a neuroregulatory system which adjusts the output of pancreatic endocrine hormones via the sympathetic and parasympathetic nervous systems (Rohner-Jeanrenaud et al, 1983).

2.1 Intraislet modulation of insulin release

Islet cell products are released into the interstitial space before their discharge into the islet vasculature. Thus, these products could interact with other islet cells to produce intraislet regulation of secretion (Pipeleers, 1984). Cell products such as prostaglandins, opioid peptides, biogenic amines and cyclic nucleotides have been detected in the effluent of isolated pancreatic preparations (Grill and Cerasi, 1974; Gylfe, 1980; Sachse et al, 1980; MacAdams et al, 1984). Administration of exogenous L-dopa and 5-HT *in vivo* produced a subsequent reduction in glucose-induced insulin secretion from pancreases removed and incubated *in vitro* (Wilson et al, 1974). This required

intracellular metabolism of L-dopa and 5-HT to biogenic amines, and could not be prevented by extracellular alpha-adrenoceptor blocking drugs which prevent the inhibitory action of extracellular monoamines. Morphine and β endorphin produce an increase in insulin secretion (Ipp et al, 1978), although this may be secondary to their inhibitory effect on somatostatin secretion. Prostaglandins also increase insulin release from isolated islets (Johnson et al, 1973).

Paracrine communication between islet cells may also occur, due to the localised feedback of secretory products. Within the islet, the three major cell types are arranged according to a precise topography (Orci, 1982), and are structurally connected by specialised membrane junctions. This microanatomy is important for normal intracellular communication and for intraislet regulation of insulin release (Halban et al, 1982; Hopcroft et al, 1985). Islet β cells were shown to possess surface receptors for glucagon (Van Schravendijk et al, 1985), a hormone which can augment glucose-induced insulin secretion both *in vitro* and *in vivo* (Hopcroft et al, 1985; Samols et al, 1965). Also, somatostatin may produce a local inhibitory action on adjacent β cells, since separation of β from D cells produced a rise in basal and glucose-stimulated insulin release (Hopcroft et al, 1985) which was prevented by somatostatin, but was not affected by glucagon.

Short,rhythmic cycles occur in the basal plasma concentrations of insulin, glucagon and glucose of monkeys (Goodner et al, 1977) and man (Lang et al, 1979; Lang et al, 1981). These were independent of central control, and of cholinergic and adrenergic innervation (Miller, 1981). Functional communication between islets, and a pancreatic pacemaker, may be responsible for this phenomena (Miller., 1981). Stagner and Samols (1985) suggested that an intrinsic, autonomously functioning, non-cholinergic, non-adrenergic pancreatic nervous system was responsible for coordination of islet secretion, and for the production of the periodic fluctuations in insulin secretion seen in the canine pancreas.

2.2 Central Control of Insulin Secretion

The central nervous system (CNS) integrates many signals arising from the periphery (e.g. hormones, visual, oropharyngeal and visceral sensory input, or blood substrates) to produce complex efferent modulation, the purpose of which is to maintain normoglycaemia. Normally, the CNS exerts a net inhibitory influence on insulin secretion, the sympathetic outflow being greater than the parasympathetic. However, it also appears to initiate a series of events aimed at efficient and rapid glucose disposal during ingestion of nutrients or during a plentiful glucose supply. The actual interrelationships between neuronal and hormonal responses in the CNS are

still poorly understood (Rohner-Jeanrenaud et al, 1983).

Efferent sympathetic and parasympathetic nerves reach the pancreas via the mixed pancreatic nerve, containing the vagus and postganglionic sympathetic fibres from the coeliac ganglion. The endocrine pancreas projects vagal and splanchnic afferents that are conveyed to the CNS (Miller, 1981).

Chemo-, mechano- and osmoreceptors present in the gastrointestinal tract - as well as glucoreceptors in the small intestine, portal vein and liver - influence the central control of insulin secretion via these afferent nerves. The CNS itself can sense central glucose levels via glucose sensing neurones in many areas of the brain. Also, some brain sites that are unprotected by the blood-brain barrier possess specific insulin receptors, suggesting that glucose and insulin levels together may affect the overall CNS integration process which ultimately modulates insulin secretion (Rohner-Jeanrenaud et al, 1983).

2.3 Neuronal control of insulin secretion

2.3.1 Cholinergic Innervation of the pancreas

The Islets of Langerhans receive efferent parasympathetic influences via the vagus, electrical stimulation of which produces a rise in plasma insulin concentrations *in vivo* (Kaneto et al, 1967; Sakaguchi and Yamaguchi, 1980). *In vitro*, acetylcholine stimulated

insulin output from isolated islets of Langerhans (Malaisse et al, 1967b) and from perfused pancreas preparations (Iversen, 1973a). Stimulation of the dorsal vagal trunk produces an increase in the plasma concentrations of glucagon and pancreatic polypeptide (Kaneto et al, 1974a and b), an effect which can also be reproduced *in vitro* by acetylcholine (Iversen, 1973a). However, vagotomy does not decrease basal insulin concentration in rats, cats and man (Miller 1981).

In most species pancreatic hormone release following vagal stimulation is under the control of cholinergic mechanisms and can be prevented by atropine. However, in some species (e.g. cats, pigs) peptidergic neurones may be involved (Miller, 1981).

2.3.2 Adrenergic innervation of the pancreas

Sympathetic innervation reaches the pancreas from the coeliac ganglion via the splanchnic nerves, stimulation of which has a direct effect on the endocrine pancreas to cause an inhibition of insulin release (Miller, 1975; 1981). The adrenoceptor responsible for this inhibition of insulin secretion, both *in vivo* (Nakadate et al, 1980) and *in vitro* (Nakaki et al, 1981) is of the α_2 subtype. Clonidine, an α_2 agonist, produced a marked inhibition of insulin secretion *in vitro* (Wollheim, 1981).

Stimulatory adrenoceptors of the β_2 subtype have been demonstrated on the β cell of the islets of Langerhans by

some workers (Loubatieres et al, 1971; Iversen, 1973b) but disputed by others (Malaisse et al, 1967b; Zielmann et al, 1985).

However, the predominant effect of physiological levels of catecholamines is that of α_2 adrenoceptor-mediated inhibition of insulin secretion (Miller, 1981).

3. The Action of Insulin

Insulin is the major hypoglycaemic hormone of man (Cahill, 1971), with wide ranging actions affecting carbohydrate, lipid and protein metabolism. Insulin produces a stimulation of glucose transport and metabolism, a stimulation of lipid synthesis, glycogen synthesis, amino acid influx and protein synthesis, and an inhibition of lipolysis (Kahn, 1985). These effects are observed at low concentrations of insulin, and occur rapidly after exposure of cells to insulin.

The growth-promoting effects of insulin are usually observed at higher concentrations, and are only observed after hours or even days of exposure to insulin (Kahn, 1985). Growth-promoting effects include, stimulation of RNA and DNA synthesis, inhibition of protein degradation, and a stimulation of cell growth and differentiation (Kahn, 1985).

Insulin circulating in the blood stream initiates its action by binding to a glycoprotein receptor on the surface of target cells (Kahn et al, 1981). This

initiates a chain of events leading to the characteristic biological effects of insulin.

3.1 The Insulin Receptor

The insulin receptor is not localised to target tissues, but is almost ubiquitous on mammalian cells (Kahn et al, 1981). The receptor itself is an insulin-sensitive tyrosine-specific protein kinase (Kasuga et al, 1982; Shia and Pilch, 1983) which autophosphorylates tyrosine residues on the β subunit of the receptor when stimulated by insulin. Other receptor-associated intracellular substrates for this kinase activity have not so far been found (Rees-Jones et al, 1984).

Other early events in insulin action include conformational changes in the receptor (Harman et al, 1981), aggregation of receptors (Kahn, 1985), changes in sodium and potassium fluxes (Czech, 1977) and internalisation of the receptor by receptor-mediated endocytosis (Carpentier et al, 1979).

3.2 Insulin stimulation of Glucose transport

Insulin stimulates the transport of glucose into cells within seconds of the addition of insulin (Kahn, 1985). This increase in glucose transport is caused by a translocation of glucose transporters from an intracellular pool to the plasma membrane (Cushman and Wardzala, 1980), or from other sites on the plasma membrane (Fain, 1984). Like the insulin receptor,

transporters are present in many cell types. However, in cells in which insulin has little or no effect there appears to be a smaller intracellular pool of receptors (Kahn, 1985).

The increase in glucose uptake caused by insulin may be linked to its rapid effects on ion flux and membrane potential, since altering membrane potential by other methods produces a similar change in glucose transport (Czech, 1977). *In vivo*, the major cell types in which insulin causes acceleration of glucose transport are those of skeletal muscle and adipose tissue (Fain, 1984).

3.3 Subreceptor mechanisms

Extracts of cells treated with insulin, but not insulin itself, may possess activity in cell free assay systems which resemble insulins effects in whole cells (Kahn, 1985). This suggested the presence of a subreceptor mediator of insulins action.

Many substances have been proposed as second messengers for insulins action, including cAMP, cGMP, Ca^{++}, H_2O_2, membrane phospholipids, degradation fragments of insulin and altered membrane potential (Kahn, 1985). Although a decrease in cAMP may explain some of the effects of insulin, a role for cGMP, Ca^{++} and H_2O_2 is thought to be unlikely (Cheng and Larner, 1985).

Insulin mediators have been isolated from many cell types (reviewed in Cheng and Larner, 1985), and all appear to be heat and acid stable, relatively low molecular

weight oligopeptides or glycopeptides (Cheng and Larner, 1985). There may indeed be multiple mediators of insulins action, allowing for amplification of the receptor signal and explaining the varied types and time-courses of insulins action (Cheng et al, 1980; Cheng and Larner, 1985).

Section E.

Metabolic alterations caused by *B. pertussis*

1. Glucose

2. Alterations in the hyperglycaemic effect of adrenaline

3. Insulin

4. In vitro alterations in insulin secretory responses

5. Free fatty acid concentrations

6. Other hormonal changes

6.1 Glucagon

6.2 Somatostatin

6.3 Catecholamines

6.4 Renin

E. Metabolic alterations caused by *B. pertussis*

1. Glucose

Pertussis vaccine causes hypoglycaemia in the mouse (Pafentjev and Schleyer, 1949; Stronk and Pittman, 1955; Furman et al 1981) and the rat (Gulbenkian et al, 1968; Ainapure et al, 1977). Isolated reports of very low blood or cerebrospinal fluid glucose concentrations have been reported in patients with encephalopathy following pertussis vaccination (Globus and Kohn, 1949).

Similarly, mice sublethally infected with *B. pertussis* show hypoglycaemia (Pittman et al, 1980, Furman et al, 1981). This hypoglycaemia was seen in fed animals, and in those subjected to a short fast (Szentivanyi et al, 1963), but was absent in animals fasted overnight (Hewlett et al, 1983). Hypoglycaemia in infants with pertussis has also been reported (Regan and Tolstoouhov, 1936).

Pertussis toxin produced long lasting hypoglycaemia in the normal mouse (Pittman et al, 1980) and in the NZO diabetic mouse (Re et al, 1984). A possible involvement of bacterial endotoxin in the hypoglycaemia seen in infection or after vaccination with pertussis organisms cannot be ruled out, since lipopolysaccharide from various organisms is known to produce hypoglycaemia (Filkins and Yelich, 1980).

2. Alterations in the hyperglycaemic effect of adrenaline

Both pertussis vaccine-treated (Szentivanyi et al, 1963; Keller and Fishel, 1967; Gulbenkian et al, 1968; Sumi and Ui, 1975) and *B. pertussis* infected mice (Furman et al, 1981) show a reduction in, or an abolition of, the hyperglycaemic effect of adrenaline. Also, the hyperglycaemic effect of agents thought to act through the release of adrenal medullary catecholamines is reduced by pertussis vaccine or infection (Szentivanyi et al, 1963; Yajima et al, 1981).

In humans, attenuation of adrenaline-induced hyperglycaemia has been reported in the infant with pertussis (Badr-El-Din et al, 1976) or following vaccination (Sen et al, 1974)

3. Insulin

Rats and mice treated with pertussis vaccine show marked increases in plasma insulin concentration (Gulbenkian et al, 1968; Sumi and Ui, 1975; Furman et al, 1981). Indeed, a biphasic pattern of increased insulin concentration was found in mice treated with pertussis vaccine (Hewlett et al, 1983). The second phase of increased insulin concentration could be prevented by heating the vaccine to destroy pertussis toxin, but the first phase was not sensitive to heat, and was attributed to endotoxin in the vaccine (Hewlett et al, 1983).

Hyperinsulinaemia was also seen in mice sublethally infected with B. pertussis organisms (Furman et al, 1981). However, this was shown to be dependent upon the use of anaesthesia at the time of blood sampling (Furman et al, 1981). A similar dependence upon anaesthesia was also found for vaccine-induced hyperinsulinaemia (Furman et al, 1981).

Glucose-induced hyperinsulinaemia *in vivo* has been shown to be enhanced by pretreatment with pertussis vaccine (Sumi and Ui, 1975). However, one report showed the increase in insulin induced by I.P. injection of glucose to be reduced by pertussis toxin pretreatment (Re et al, 1984).

The inhibitory action of adrenaline on insulin secretion is prevented, or even converted to a stimulatory effect, in pertussis vaccine-treated rats (Gulbenkian et al, 1968; Sumi and Ui, 1975) and in sublethally infected mice (Furman et al, 1981). Pertussis toxin treatment of rats (Yajima et al, 1978) or dogs (Nakamura et al, 1984) produced a similar reversal of the inhibitory effect of adrenaline on insulin secretion.

Hannik and Cohen (1979) showed a slight, but significant elevation of plasma insulin concentrations in children 8 hours after receiving pertussis vaccine.

4. In vitro alterations in insulin secretory responses

Both isolated pancreatic islets and perfused

pancreas preparations obtained from pertussis vaccine or toxin treated rats show a prevention of the inhibitory effect of adrenaline or noradrenaline on insulin secretion (Katada and Ui, 1977; Toyota et al, 1978; Furman and McMillan, 1985). Indeed, adrenaline was found to produce a marked stimulation of insulin secretion from the pancreas of pertussis-vaccinated rats (Katada and Ui, 1977).

Glucose-induced insulin secretion is increased from *in vitro* preparations isolated from pertussis vaccine-treated or toxin-treated animals (Sumi and Ui, 1975; Katada and Ui, 1977; Araki et al, 1981; Furman and McMillan, 1985). However, this may be secondary to the prevention of the inhibitory action of somatostatin on glucose-induced insulin secretion caused by pertussis toxin (Katada and Ui, 1979).

Insulin secretion induced by arginine, methylxanthines or sulphonylureas was increased in perfused pancreas isolated from pertussis vaccine treated rats (Sumi and Ui, 1975). This effect may be due to the prevention or reversal of the inhibitory effect of endogenous catecholamines on induced insulin secretion, since phentolamine increases glucose-induced insulin secretion *in vitro* (Furman and McMillan, 1985). However other α-adrenoceptor blocking drugs such as idazoxan and yohimbine did not increase glucose-induced insulin secretion *in vitro* (Furman and McMillan, 1985).

5. Free fatty acid concentrations

The body can utilize free fatty acids as metabolic fuels, particularly during periods of glucose deprivation. Tissues such as muscle can use these compounds directly, or after conversion to ketones, thereby preserving glucose for use by those tissues, such as the brain, which have an absolute requirement for glucose as a fuel (Cahill, 1971).

Pertussis vaccine elevated serum concentrations of both free fatty acids and ketone bodies in the hamster (Villalobos-Molina and Garcia-Sainz, 1981) but had no effect in the rat (Gulbenkian et al, 1968). *In vitro* however, adipose tissue from both species showed enhanced lipolysis (Gulbenkian et al, 1968; Garcia-Sainz, 1981) when removed from animals treated with pertussis vaccine.

Adipose tissue from pertussis treated animals had reduced sensitivity to the antilipolytic action of prostaglandins, α_2 adrenoceptor agonists and to adenosine (Gulbenkian et al, 1968; Garcia-Sainz, 1981) but the increase in lipolysis seen after vaccine treatment was still sensitive to supression by insulin (Gulbenkian et al, 1968).

Pertussis toxin treatment produced the same increase in basal lipolysis and inhibition of the antilipolytic action of adenosine as that produced by pertussis vaccine (Garcia-Sainz and Torner, 1985).

6. Other hormonal changes

Although insulin is the major hypoglycaemic hormone in man (Cahill, 1971), other hormones such as glucagon and adrenal medullary catecholamines can be important in regulating blood glucose concentrations. Pertussis treatment impaired the effect of exogenous catecholamines and somatostatin, as mentioned before, and reduced the hyperglycaemic response to glucagon (Szentivanyi et al, 1963), and it may also effect the release of these and other hormones.

6.1 Glucagon

Pretreatment of dogs with pertussis toxin produced an increase in basal plasma glucagon concentrations, and a marked augmentation of adrenaline-induced increases in plasma glucagon (Nakamura et al, 1984).

Treatment of cultured mouse islets with pertussis toxin produced an increase in glucagon secretion (Kikkawa et al, 1981). However this may also be due to impaired responsiveness of islet cells to somatostatin. The stimulatory effect of noradrenaline on glucagon secretion was impaired in perfused pancreas preparations from pertussis toxin-treated rats (Toyota et al, 1978).

6.2 Somatostatin

Seino et al (1983) reported that secretion of somatostatin in response to nutrient stimulation was increased in isolated pancreas preparations from

toxin-treated rats, but this has not been confirmed.

6.3 Catecholamines

Despite the quantity of information on the modification of responses to catecholamines caused by *B. pertussis*, there is little information about possible changes in the release of neural or medullary catecholamines. Szentivanyi et al (1963) found that the ability of histamine or 5-HT to deplete the adrenal gland of catecholamines appeared to be increased in the pertussis vaccinated mouse. Also, presynaptic α_2 adrenoceptor mechanisms were inactivated in pertussis toxin-treated perparations (Lai et al, 1983; Allgaier et al, 1985), suggesting that sympathetic nervous activity could be enhanced by pertussis toxin.

6.4 Renin

Plasma renin activity in response to adrenaline or anaesthesia was greatly increased in pertussis toxin-treated rats (Pedraza-Chaverri et al, 1984, 1985). Also, the α_2 adrenoceptor-mediated inhibition of renin secretion *in vitro* was prevented in pertussis toxin-treated cortical slices (Pedraza-Chaverri et al, 1986).

Whether these effects cause an increased activation of the renin-angiotensin system is unknown, but if so an augmented noradrenaline release from sympathetic nerves could result.

Object of the Research

Mice which have been infected sublethally with *Bordetella pertussis* exhibit hyperinsulinaemia and hypoglycaemia, the former being dependent on the use of anaesthesia at the time of blood sampling (Furman et al, 1981).

The objects of the present investigation were to determine:

1. the role, and mechanism, of stress as a factor in the hyperinsulinaemia in *B. pertussis*-infected mice
2. the role of insulin and of stress as factors responsible for the hypoglycaemia in these animals
3. whether hyperinsulinaemia and hypoglycaemia occur in infants with pertussis.

Materials and Methods

1. Microbiological Procedures

1.1 Media

1.2 Strain

1.3 Stock cultures

1.4 Growth of cultures

1.5 Challenge suspensions of *B. pertussis* strain 18-323

1.6 Identification of *B. pertussis*

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5. Experimental Design and Statistical analysis

5.1 Statistical analysis

6. List of drugs and reagents

1. Microbiological Procedures

1.1 Media

Bordet-Gengou agar (BG) plates were used for the cultivation of *B. pertussis* organisms. The agar base was obtained from Gibco Biocult Diagnostics Limited, Paisley, Scotland, and, after rehydration and autoclaving, was mixed with defibrinated horse blood at 45°C to give a final concentration of 15-20% blood. The horse blood was also obtained from Gibco.

1.2 Strain

B. pertussis strain 18-323 was used throughout.

1.3 Stock cultures

Aliquots of 10 opacity units (O.U.) *B. pertussis* strain 18-323 in 1% casamino acid (CAA) with 1% glycerol were stored frozen in heat-sealed ampoules under liquid nitrogen. For each infection experiment a new ampoule was removed, melted, and plated-out onto freshly prepared BG plates.

1.4 Growth of cultures

Stock culture suspensions were defrosted, innoculated onto BG plates and incubated at 37°C for 48 hours in a water-saturated atmosphere. The growth was then subcultured onto BG and incubated for a further 24 hours.

1.5 Challenge suspensions of *B. pertussis* strain 18-323

Twenty four hour growth of *B. pertussis* was scraped from BG plates and suspended in 1% CAA. This suspension was matched visually against the International Opacity Reference Preparation giving a 10 opacity unit (O.U.) suspension corresponding to approximately 2.5×10^9 colony forming units (cfu) per ml.

This suspension was diluted stepwise by a factor of 2500, and 0.05 ml was administered intranasally to each mouse. The bacterial concentration of this challenge suspension was therefore approximately 1×10^6 cfu per ml, and each mouse received approximately 1.25×10^5 cfu.

The viable count of each challenge suspension was determined by making a 1,000 fold and a 10,000 fold dilution in CAA from the challenge and spreading 0.1 ml on each of two BG plates. These were incubated at 37°C for five days after which the colonies were counted and a mean cfu per ml value calculated for the challenge suspension.

1.6 Identification of *B. pertussis*

The identity of organisms from stock and challenge cultures were checked using the following procedures.

1.6.1 Gram staining followed by light microscopy to show a small, non-motile, gram negative coccobacilli.

1.6.2 Slide agglutination tests with either anti-X-mode

rabbit antiserum, or purified anti-pertussis IgG. Two separate drops of B. pertussis organisms suspended in CAA were placed on a microscope slide. One drop of antiserum (or IgG) was added to the first drop, and one drop of CAA added to the second drop. The slide was gently tilted to allow the antiserum and organisms to mix, then inspected visually for agglutination. A positive agglutination test resulted in a visible precipitate of organisms and bound antibody in the test drop, whilst the control solution remained clear.

1.6.3 Growth characteristics and colony morphology.

B. pertussis is a slow growing organism, with visible single colony growth on solid media appearing in 3 to 5 days of incubation at 35°C. Colonies appear smooth, convex, pearly, glistening and nearly transparent (Pittman, 1984b). Zones of haemolysis may appear around colonies grown on BG agar.

1.7 Pertussis toxin

Pertussis toxin was isolated and purified by Dr. Y.V. Perera of Glasgow University by the method of Sekura et al (1983). Histamine sensitisation tests (HSD_{50}) were performed on each batch before use, and all toxin batches used had HSD_{50} values of between 25 and 60 ng per mouse.

2. Animals and Animal Procedures

2.1 Mice

Female mice from a randomly bred closed colony originally derived from the Ham 1/CR strain (Charles River UK Limited, Manston Road, Margate, Kent) were used routinely. The animals were housed in cages of 5 to 20, and kept in a room at 22°C with a twelve hour light/dark cycle. Standard pellet diet (Ogston Mills) and water were available at all times unless otherwise stated.

2.2 Intranasal infection procedures

The method of Burnet and Timmins (1937) was used for intranasal (IN) infection except that ether alone, rather than a mixture of ether and chloroform, was used as the anaesthetic. Female mice, aged three to four weeks, were anaesthetised in groups of five mice inside a glass jar with an ether soaked pad in the lid. Once the animals were unconscious they were removed one at a time and held so that the nose was vertical. The challenge suspension or control was administered in 0.05 ml volumes from an automatic pipette onto the nostrils. When this had been inhaled the animal was allowed to recover from the anaesthetic, then was marked to identify its treatment. The animals were then kept for a further fourteen days to allow the infection to become established (Pittman et al, 1980) before use in an experiment.

2.3 Autopsy

Mice were killed by exposure to ether vapour, dipped

in a beaker of disinfectant (chlorhexidin), and pinned out on a board. The thorax was opened with sterile scissors and forceps (sterilised by dipping in alcohol and flaming) and the lungs were inspected for signs of consolidation. A small portion of lung tissue was removed with sterile instruments and the cut surface was smeared onto a BG plate. After four to five days incubation at 37°C the plates were inspected for growth of B. pertussis (see plate 1).

2.4 Collection of serum

Mice were killed by cervical dislocation, either without anaesthetic or following exposure to ether vapour, then immediately decapitated. Blood from the trunk was collected into individually identified, cooled eppendorf tubes. The samples were allowed to clot at 4°C for up to five hours before the serum was separated by centrifugation using a Beckman 2 Microfuge (11500 g for 1½ mins.). Serum was decanted into a further set of eppendorf tubes and stored deep frozen (-20°C) for later analysis of glucose and immunoreactive insulin concentrations.

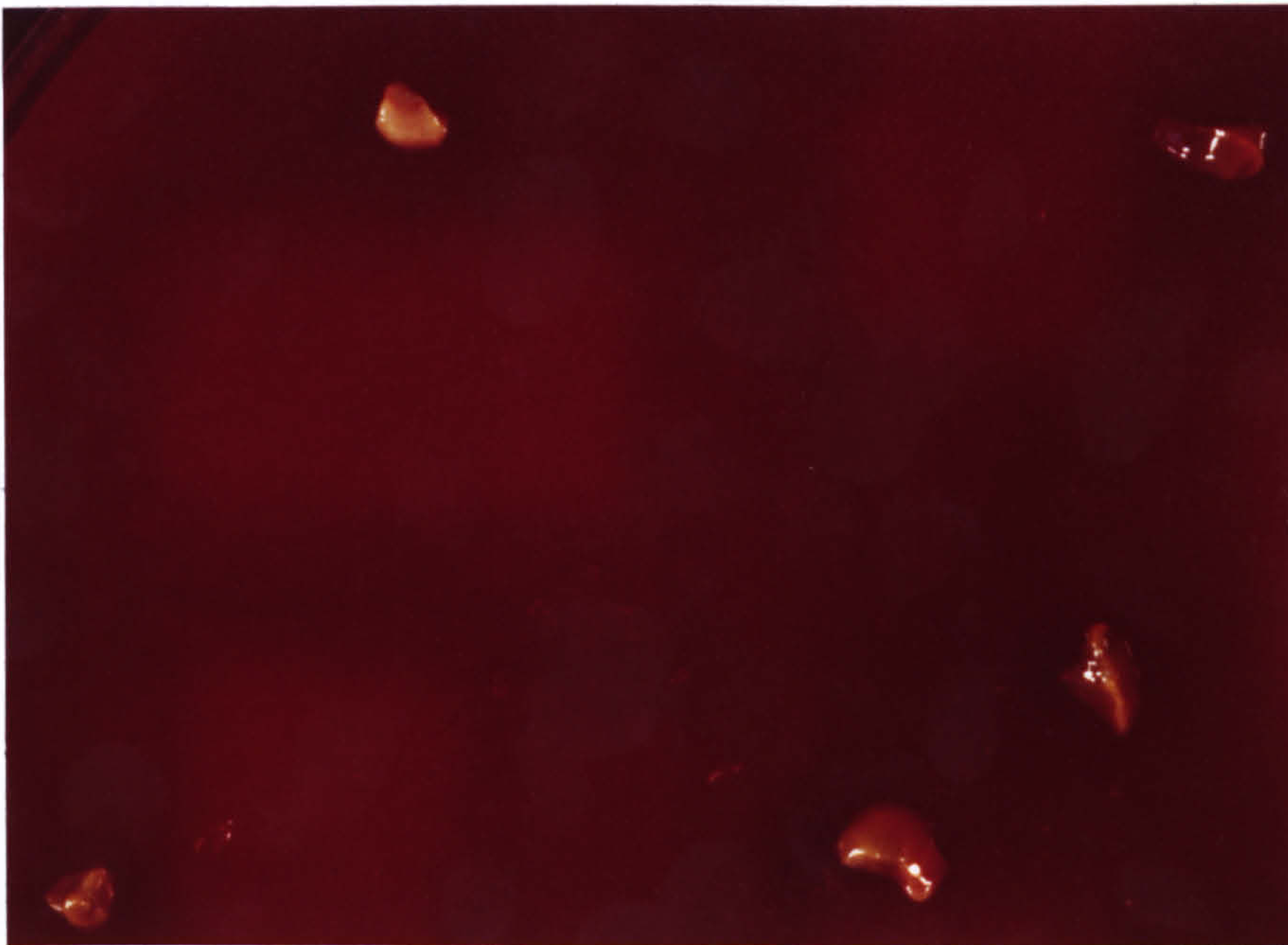
2.5 Routes of administration

2.5.1 Intravenous (I.V.)

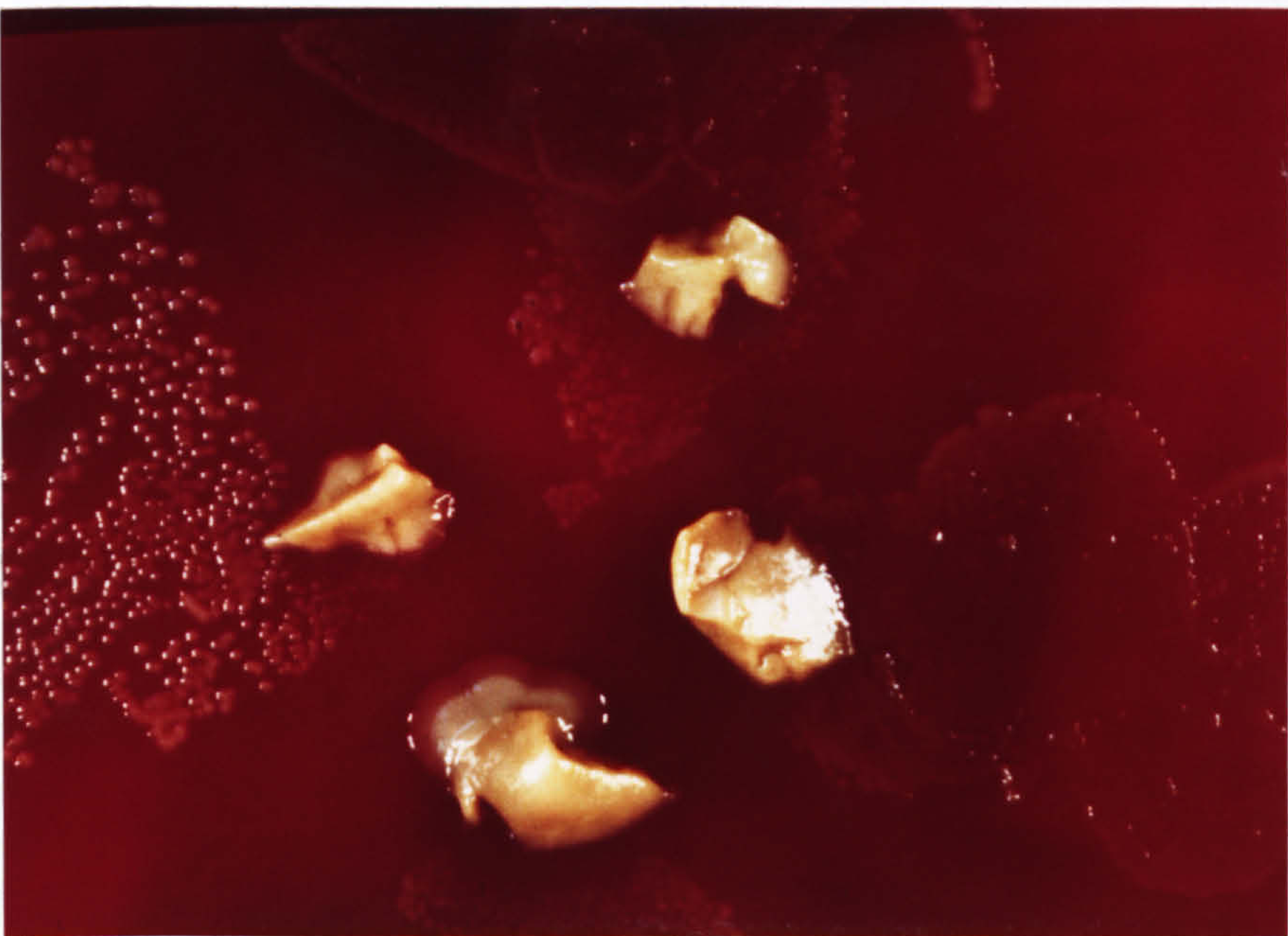
Intravenous injections were made into a tail vein whilst the animal was immobilised in a restraining tube.

Plate 1.

Lung-smear plates from control and B.pertussis infected mice, after 5 days incubation at 35 C.



a. Intranasal inoculation of saline 14 days previously.



b. Intranasal inoculation of live B.pertussis organisms 14 days previously.

The injection was facilitated by the following procedure:

A number 26 gauge needle was detached from its luer fitting and attached to a piece of fine polythene tubing. This was attached in turn to a syringe via a second needle. Using this device, the needle could be inserted into the approriate vein, whilst resting the loaded syringe on the bench. In very young mice, the tail was warmed to dilate the veins to facilitate injection.

2.5.2 Intraperitoneal (I.P.)

Mice were picked up by the scruff of the neck, and held with the ventral side uppermost. The animals were tilted back slightly before injection so that the liver would lie away from the site of injection.

2.5.3 Subcutaneous (S.C.)

Subcutaneous injections were made into the scruff of the neck.

2.6 Adrenal demedullation

Bilateral adrenal demedullation was performed on five week old mice under ether anaesthesia. The adrenal medullae were removed by the process of adrenal enucleation (Ingle and Higgins, 1938), which allows regeneration of the cortex. Sham-operated animals were subjected to the same operation, without removal of the glands. Both groups of mice were maintained on saline for one week and were allowed to recover from the

procedure for four weeks before being used for an experiment.

3. Collection of Human Plasma Samples

A single blood specimen was collected by venepuncture from each child during a routine blood sampling, normally at admission to Ruchill Hospital. The sample was divided into three portions, one into lithium-heparin (Sarstedt), one into heparin-fluoride (Sarstedt), and the third into EDTA for leukocyte count (Sigma).

The plasma was separated by centrifugation in a refrigerated centrifuge, and stored frozen (-20°C) for later analysis. Leukocyte determinations were performed by the Department of Laboratory Medicine at Ruchill Hospital.

4. Biochemical Determinations

4.1 Determination of glucose concentrations

Serum and plasma glucose levels were determined enzymatically using a Beckman Glucose Analyser 2 as follows:

1. The analyser was first calibrated using a Beckman aqueous glucose standard containing 8.3 mmol/l glucose.
2. A precise volume (10 µl) of sample was manually pipetted into an enzyme reagent cup containing glucose oxidase reagent and an electrode which

responds to oxygen concentration.

3. The rate of oxygen consumption during oxidation of the glucose in the sample was measured by the analyser. Since the maximum rate of change in oxygen consumption has been shown to be dependent upon the amount of glucose in the sample (Kadish et al, 1968) this is calculated by the analyser and displayed as glucose concentration in mmol/l.

This method is highly specific for β-D-glucose and is free from interference from agents used to prevent glycolysis or coagulation. Also, since the method does not depend on the optical properties of the solution it can be used even with uraemic, turbid or haemolysed samples.

However, 2-deoxyglucose (2-DG) was found to cross-react with glucose using the glucose oxidase method. To overcome this difficulty, sera from animals treated with 2-DG were assayed for glucose using a hexokinase enzymatic assay kit from Sigma Diagnostics (Fancy Road, Poole, Dorset BH17 7NH).

The hexokinase assay relies on the hexokinase-catalysed conversion of glucose to glucose-6-phosphate (Carroll et al, 1970), which is coupled to the subsequent reduction of NADP to NADPH by the action of glucose-6-phosphate dehydrogenase (G-6PDH). Since the 2 deoxy derivative of glucose-6-phosphate is not a substrate for G-6-PDH, 2-DG does not interfere with this assay.

4.2 Determination of Immunoreactive Insulin Concentration

Serum or plasma immunoreactive insulin (IRI) concentrations were determined by the double antibody radioimmunoassay method of Hales and Randle (1963), using antibody and I^{125} labelled insulin from a kit by Amersham UK, membrane filters (N25/45/UP Oxoid) and a crystalline rat insulin standard (Novo, Denmark) or freeze dried human insulin standard from the same kit by Amersham.

The radioimmunoassay method depends upon the competition between sample insulin and ^{125}I labelled insulin for a limited number of binding sites on an antibody raised against insulin. The insulin-specific antibody has been combined with a second antibody to give an insulin-binding reagent which is insoluble when bound to insulin, and can be separated from unbound insulin by filtration.

The procedure used was as follows:

i) Standards (human or rat insulin as appropriate) were prepared in duplicate, samples were assayed singly. 25 µl aliquots of both samples and standards were assayed.

ii) 25 µl of insulin binding reagent was added using a repeating pipette to each sample.

iii) Tubes were mixed twice on a vortex mixer, then incubated at 2-4°C for six hours.

iv) Each sample was mixed, 25 µl of ^{125}I labelled

insulin (diluted with buffer A as per manufacturers instruction) was added, and was then mixed twice.

v) Samples were incubated for a further 12 hours at 2-4°C.

vi) Each sample was mixed, then filtered through a 0.45 μm pore size membrane filter.

Each tube was rinsed twice with 0.5 ml buffer C (see appendix 1), which was also passed through the filter.

vii) Membrane filters were collected into plastic test tubes, which were then counted in a Wilj Gamma Counter 2001 for 100 seconds per tube. This reading is a measure of the bound ^{125}I labelled insulin, and is inversely proportional to the concentration of insulin in the original sample.

viii) Calibration curves from the duplicate standard readings were calculated using an Apple IIE computer which fits a line of formula

$$Y = K3/(X+K2)-K1$$

through the points. The concentration of IRI in each sample can then be calculated from this calibration curve.

This method was suitable for both serum and plasma samples, providing no haemolysis or fibrin strands were present. If the concentration of IRI in a sample was above that of the standards, it was diluted with buffer B (see appendix 1), then reassayed until within the range of the standards. It was shown that dilution produced a

linear reduction in IRI readings at dilutions of 1 in 2 to 1 in 6.

Standards

For mouse IRI determinations, rat insulin standards containing 0, 0.4, 1, 2, 3, 4, 6, 12 and 20 ng ml^{-1} were used. Rat and mouse insulins behave identically in this assay. Human standards were used at concentrations of 0, 10, 20, 40, 80 and 160 μU ml^{-1}.

4.3 Determination of Phosphorus

Inorganic phosphorus concentrations in the plasma samples from Ruchill Hospital were analysed by the method of Chen et al (1956). The samples were first diluted 1 in 20 with 10% trichloroacetic acid, mixed, then centrifuged at 1700 G for 30 minutes. Determinations were made on 1 ml volumes of the supernate thus prepared.

Potassium dihydrogen phosphate standards were prepared in duplicate and used for calibration at concentrations of 0, 0.1, 0.5, 1, 5 and 10 mg per 100 ml. The absorbance at 820 nm was read using a Shimadzu Recording Spectrophotometer UV240.

4.4 Determination of Calcium and Magnesium

The concentrations of calcium and magnesium in the plasma samples from Ruchill Hospital were determined by atomic absorption spectrophotometry, using a Perkin Elmer 3030 spectrophotometer. Samples were diluted with lanthanum chloride solution before determination.

4.5 Determination of glycogen deposition in the mouse hemidiaphragm

Mouse hemidiaphragms were dissected (Wardlaw and Moloney, 1961) 14 days after intranasal instillation of either CAA, or live *B. pertussis* organisms. Tissue was collected into a modified Gey and Geys buffer (Gey and Gey, 1936), prepared as described in Wardlaw and Moloney, 1961 and kept well gassed with 95% O_2, 5% CO_2 throughout the incubations.

Tissues were incubated at 37°C for 90 minutes in the same media, but containing 0, 0.1, 1, 10 or 100 mU per ml of insulin (Velosulin, Nordisk, Denmark). Samples were shaken at 120 strokes per minute throughout the incubation. The hemidiaphragms were removed, washed well, then assayed individually for glycogen using a modification (Wardlaw and Moloney, 1961) of the anthrone reagent method of Seifter et al (1950).

4.6 Estimation of adrenal catecholamines

The catecholamine content of the adrenal glands of mice were kindly determined by Dr. Harvey Dean of University of Leeds using high pressure liquid chromatography.

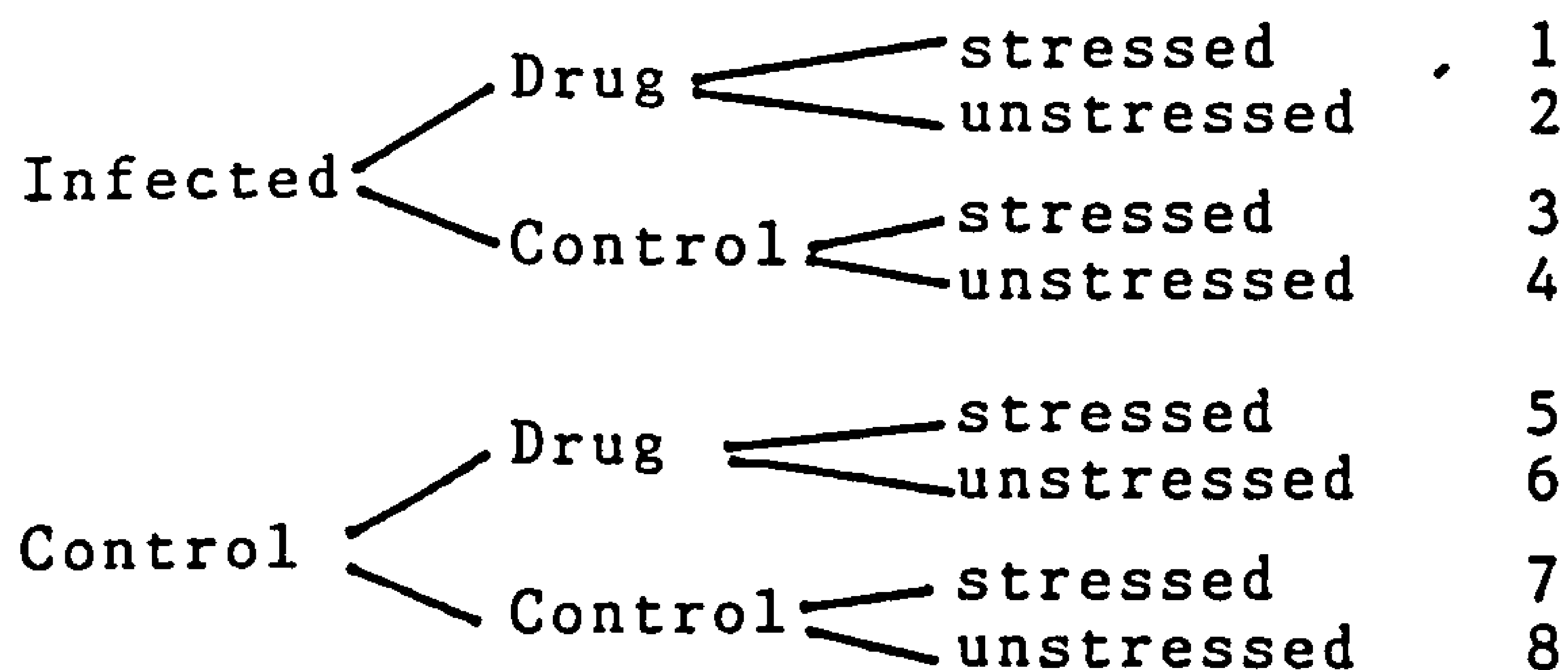
4.7 Serum Corticosterone concentrations

Serum corticosterone concentrations were measured by Drs. B.L. Furman and J. Pratt, using a kit from Radioassay Systems Laboratories, Inc, California. The

assay was specific for rat and mouse corticosterone. Serum samples were diluted 1 in 200 before assay, and results were expressed in ng corticosterone per ml of serum.

5. Experimental Design and Statistical analysis

To ensure unbiased collection of data, mice were allocated randomly to infection (or toxin) treatment or control groups. In most experiments a randomised block design was used, with each block containing at least one replicate of each variable. Thus, in an experiment with three independent variables (infection, drug and stress) each block would contain:-



i.e. eight different combinations of treatments.

The order in which each treatment was given within the block was also randomised to avoid bias.

Two way analysis of variance for such an experiment would tell us if any single variable had a significant effect on the result

e.g. Infection status

Drug treatment

or stress

Also, any significant interaction between the variables would be identified

e.g. in this case between

Infection state and drug treatment

or drug treatment and stress exposure

or Infection state and stress exposure

Finally, an interaction between all three variables would also be identified by the analysis of variance.

In many experiments reported here the third variable studied was time i.e. the effects of a drug (or control) was studied in infected and control animals at various times after administration.

5.1 Statistical analysis

Serum glucose concentrations are normally distributed and were therefore expressed as arithmetic mean ± standard error of the mean. Rankit analysis showed the distribution of serum immunoreactive insulin concentrations to be approximately log normally distributed. Therefore these values were expressed as geometric mean with 95% confidence limits (Furman, Stevenson and Wardlaw, 1981; Wardlaw, 1985).

Both glucose and insulin data were analysed using

two way analysis of variance (Wardlaw, 1985). This statistical method enabled significant interactions between variables to be identified, as well as significant effects of the variables themselves.

For this test to be valid certain conditions must be fulfilled:

i) There must be unbiased collection of data: an appropriate factorial experimental design was employed throughout to ensure unbiased results.

ii) The underlying populations must be normally, or approximately normally, distributed. Serum glucose values were analysed directly, but serum IRI concentration were converted to log IRI values before analysis to normalise the data (Furman, Stevenson and Wardlaw, 1981).

iii) More than one sample was required for each group i.e. replication was built into the experimental design.

iv) Equal numbers of observations were required in each group. This was also ensured by the design of the experiments.

6. List of Drugs and Reagents

Atropine sulphate	- Sigma
Carbachol	- B.D.H.
Diethyl ether (anaesthetic ether)	- May and Baker
Hexamethonium	- Sigma
Idazoxan	- Reckitt and Colman
Lanthanum chloride	- Sigma
Lipopolysaccharide *E. coli* 0111B4	- Sigma
Naloxone (Narcan$^{(R)}$)	- Dupont
Nicotine hydrogen (+) tartrate	- B.D.H.
Phentolamine HCl	- Ciba-Geigy
DL-propranolol	- Sigma
Trichloroacetic acid	- Sigma

All drugs were dissolved in and diluted with phosphate buffered saline, except lanthanum chloride and trichloroacetic acid which were diluted with distilled water.

Results Chapter 1

Role of insulin in the hypoglycaemia of *B. pertussis* infected mice

1.1 Introduction

1.2 Effect of Alloxan diabetes

1.3 Circadian variations in serum glucose and IRI concentrations

1.4 Effect of refeeding

1.5 *In vivo* insulin responsiveness

1.6 *In vitro* insulin sensitivity

1.7 Discussion

1.1 Treatment of rats with pertussis vaccine (PV) produced hypoglycaemia (Gulbenkian et al, 1968; Ainapure et al, 1977) and a prolonged increase in plasma immunoreactive insulin (IRI) concentrations (Gulbenkian et al, 1968). In mice treated with PV (Hewlett et al, 1983) or sublethally infected with B. pertussis (Pittman et al, 1980; Furman et al, 1981) marked hyperinsulinaemia accompanied a relatively modest hypoglycaemia. In humans, purified islet activating protein (pertussis toxin) (IAP) caused an increase in the insulin secretory response to glucose both *in vivo* (Toyota et al, 1980) and *in vitro* (Araki et al, 1981).

The lowering of blood sugar in rats caused by PV was not seen in diabetic animals (Ainapure et al, 1977). PV did not cause an increase in IRI in diabetic mice or rats (Gulbenkian et al, 1968). These observations suggest a role for insulin in the hypoglycaemia caused by pertussis.

However, Furman et al (1981) found that the hyperinsulinaemia seen in B. pertussis infected animals was a transient effect caused by the use of anaesthesia at the time of blood sampling. Animals bled *without* the use of anaesthetic showed hypoglycaemia, but without hyperinsulinaemia. In normal animals, anaesthetics did not increase serum immunoreactive insulin concentrations.

In this section, the role of insulin in the hypoglycaemic effect of sublethal B. pertussis infection in mice was investigated.

1.2 Effect of alloxan diabetes

Mice were rendered diabetic using alloxan monohydrate (Sigma, 80 mg kg^{-1} I.V.) 48 hours before the experiment. Alloxan produces selective destruction of the pancreatic β cell (Rerup, 1970).

Alloxan-diabetic animals, bled without ether anaesthesia, had very high serum glucose concentrations and low serum IRI concentrations relative to normal mice (Figure 1.1). Glucose or IRI concentrations in alloxan diabetic-infected mice were not significantly different from those found in alloxan diabetic-controls.

Urine from alloxan treated mice was tested for glucose using Clinistix ^{R} (Ames), and animals not showing glucosuria were rejected. Also, any animals whose serum glucose values were less than two standard deviations (2 x S.D.), greater than a previously determined mean value for freely-fed normal and infected mice were excluded. These criteria were necessary since alloxan is a highly unstable drug in solution. Moreover, some attempts at I.V. injection failed. Equal numbers of mice were rejected from control and infected groups.

The serum values taken as 'normal' were as follows:

control mice, mean - 9.04 mmol l^{-1}

+ 2 x S.D. (upper limit) - 13.60 mmol l^{-1}

infected mice, mean - 6.23 mmol l^{-1}

+ 2 x S.D. (upper limit) - 11.37 mmol l^{-1}

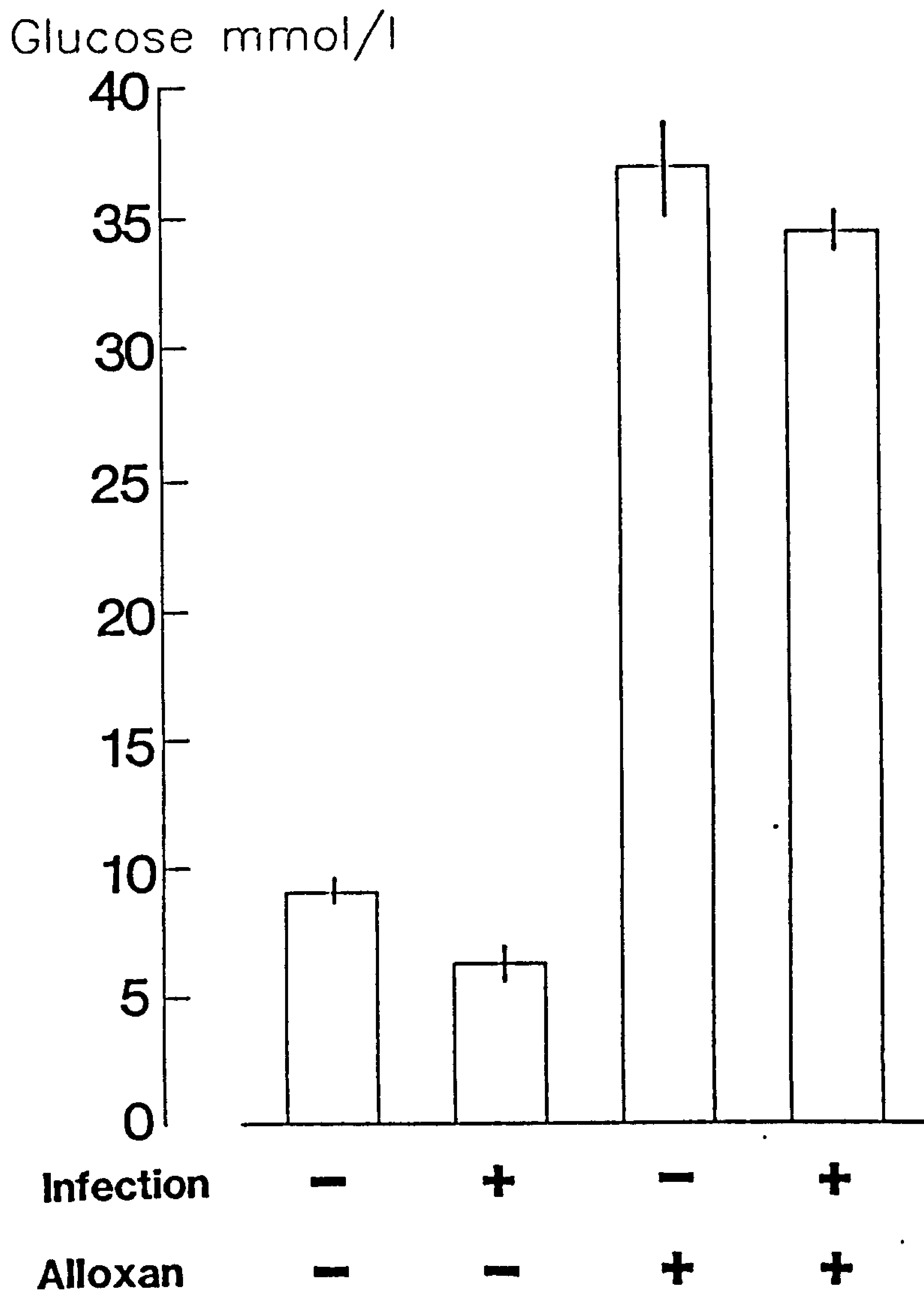


Figure 1.1.a.

Effect of alloxan-diabetes on the serum glucose concentrations in control and B.pertussis infected mice.

N= 19 to 21 for each treatment, glucose values expressed as arithmetic mean (S.E.),

Statistical analysis. Control vs infected.

Group	D.F.	t.	P.
Glucose			
Non-diabetic	37	7.58	0.01
Diabetic	38	1.147	N.S.

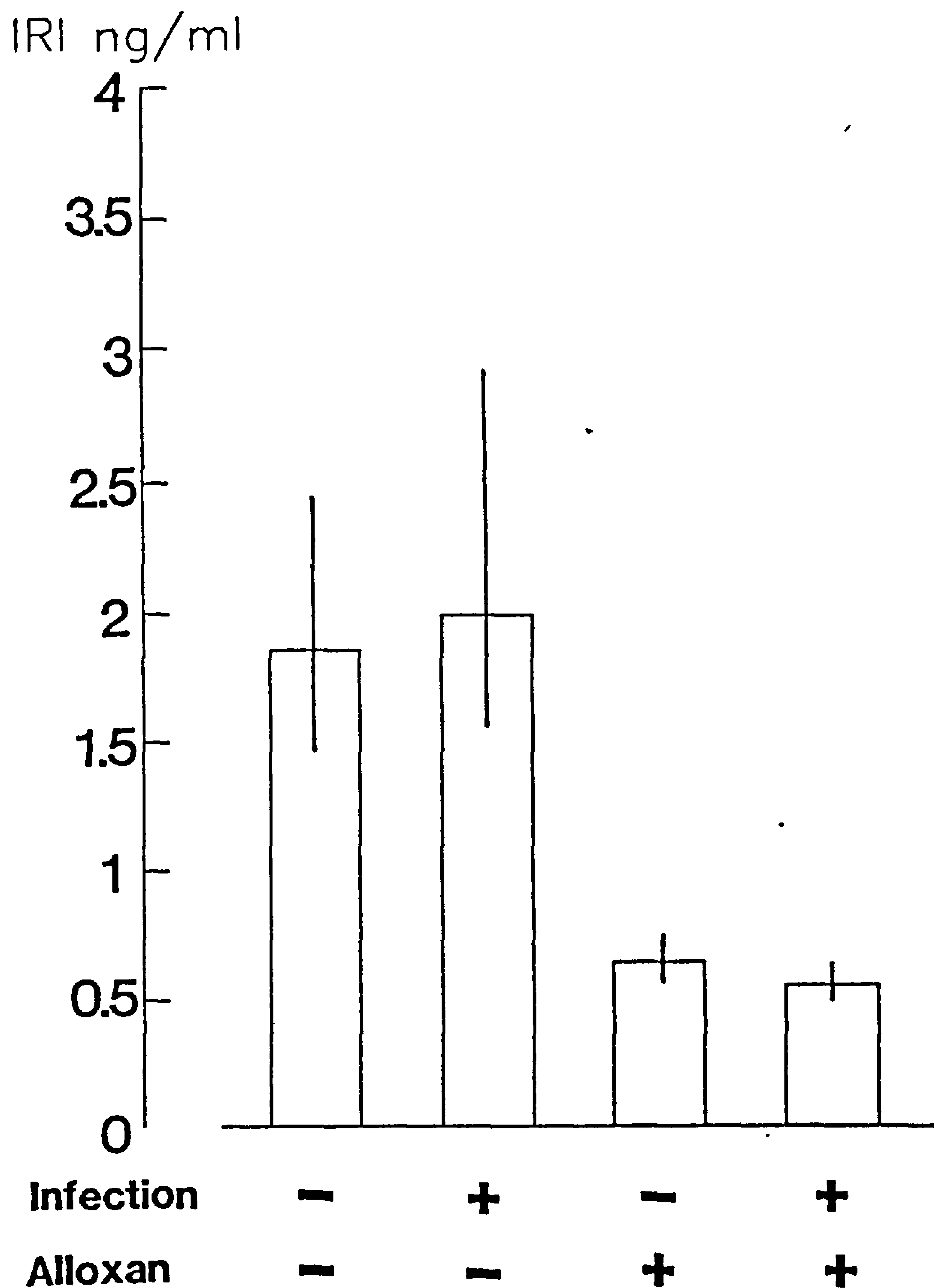


Figure 1.1.b.

Effect of alloxan-diabetes on the serum IRI concentrations in control and B.pertussis infected mice.

N= 19 to 21 for each treatment, IRI values expressed as geometric mean (95% C.L.).

Statistical analysis. Control vs infected.

Group	D.F.	t.	P.
IRI			
Non-diabetic	36	0.846	N.S.
Diabetic	38	1.266	N.S.

The serum glucose values of diabetic animals were much greater than these limits (Figures 1.1, 2.11 and 2.12).

1.3 Circadian variations in serum glucose and IRI concentrations

Groups of control and *B. pertussis* infected animals were bled *without* anaesthesia at three hour intervals across a twenty four hour period.

1.3.1 Glucose concentrations

Mice infected with *B. pertussis* had significantly lower serum glucose concentrations, compared to control animals, throughout the 24 hour period ($P < 0.01$). However, the pattern of glucose changes with time differed between the two groups (F value for interaction between infection and time = 2.54, $P < 0.05$). In both groups peak glucose concentrations occurred around midnight to 3 a.m. (Figure 1.2). These peak values were well maintained in control animals until 9 a.m., whereafter they declined gradually until 9 p.m. In infected animals, however, the peak values obtained during the night declined rapidly and remained low between 6 a.m. and 6 p.m. (Figure 1.2).

1.3.2 IRI concentrations

Infected mice had significantly elevated serum IRI concentrations compared to control animals (F value for effect of infection = 17.79, $P < 0.01$). However, this difference in IRI concentrations between the two groups

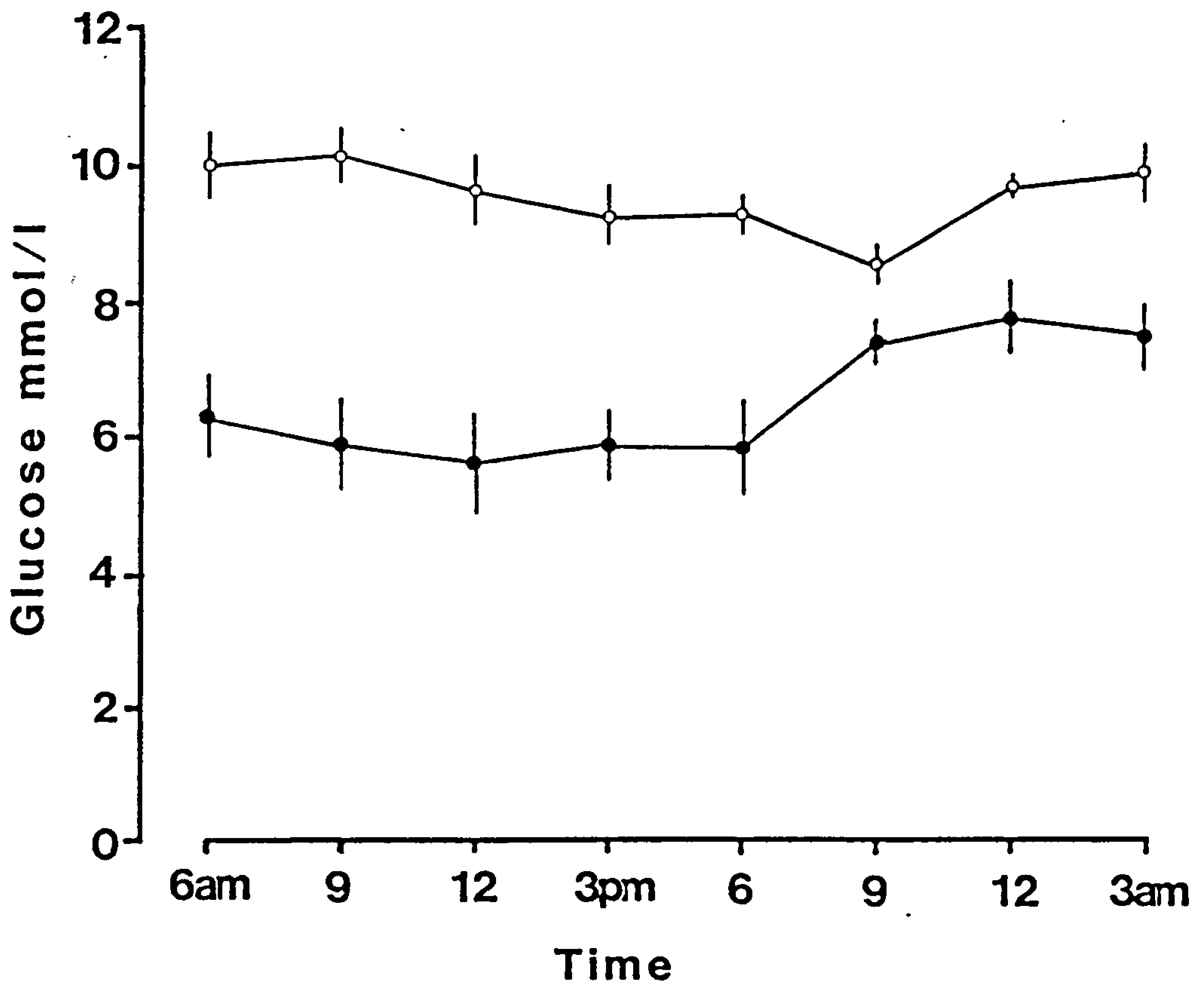


Figure 1.2

Circadian variations in serum glucose concentrations in control and B.pertussis infected mice.

N=9 for each treatment, values expressed as arithmetic mean (S.E.). o control, ● infected.

Analysis of variance.

Source	D.F.	F.	P.
Infection	1	155.54	0.01
Time	7	2.04	N.S.
Interaction	7	2.54	0.05

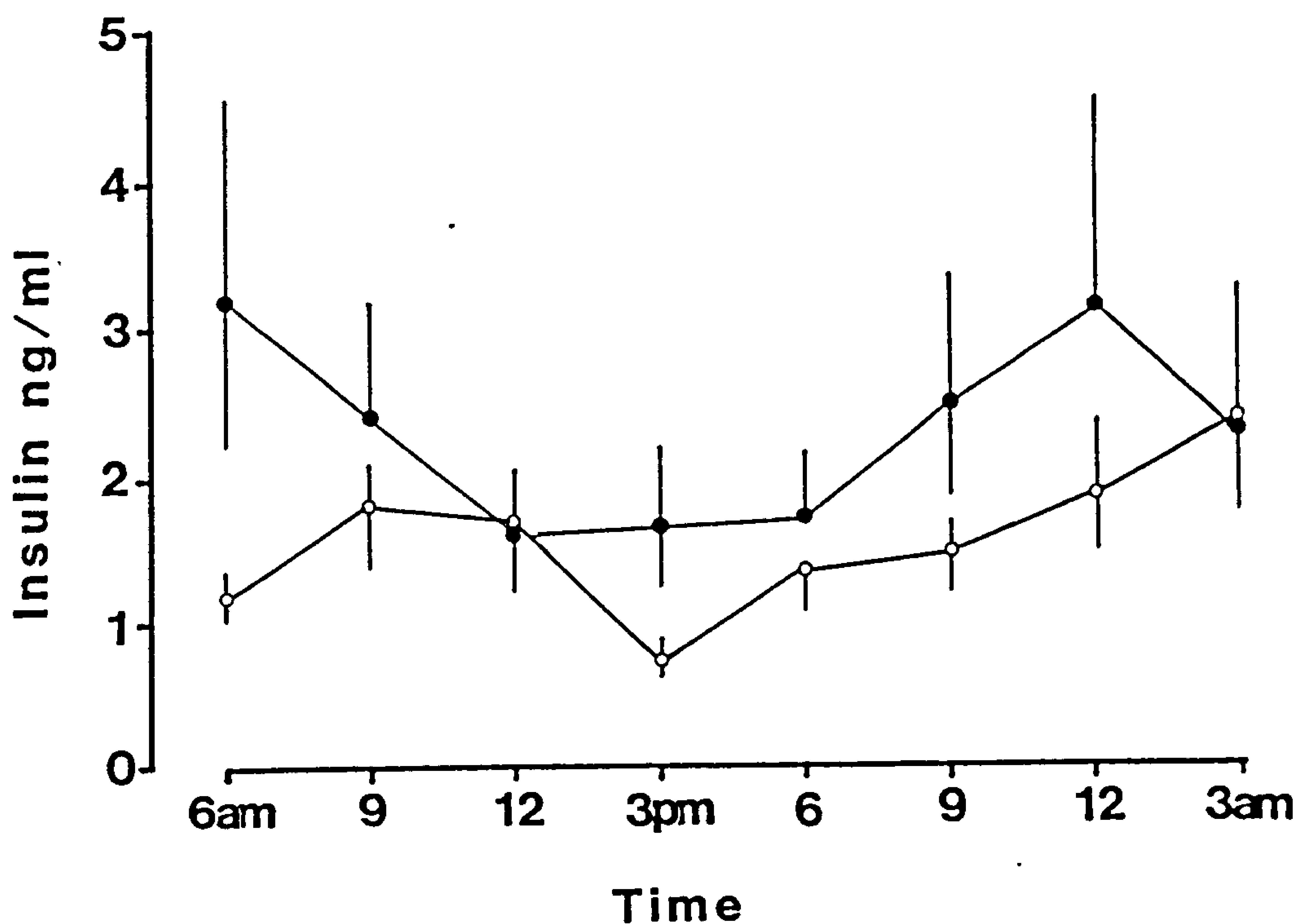


Figure 1.3

Circadian variations in serum IRI concentrations in control and B.pertussis infected mice.

N= 9 for each treatment, values expressed as geometric mean (95% C.L.) o control, ● infected.

Analysis of variance.

Source	D.F.	F.	P.
Infection	1	17.79	0.01
Time	7	3.09	N.S.
Interaction	7	1.33	N.S.

was not apparent at all the time points studied (F value for interaction between infection and time = 1.33, N.S.). Thus, the greatest divergence of IRI concentration between control and infected animals occurred during the 'night' phase of the light/dark cycle, whereas there was little difference between the groups at 9 a.m. and 12 noon (Figure 1.3).

1.3.3 Variation within treatment groups

The mice were randomly allocated to a bleeding time, and were removed for bleeding with the least possible disturbance to remaining animals. It was possible, however, that the order in which the mice were bled might have influenced the results. The serum glucose and IRI concentrations of animals were grouped depending on order of sampling within a cage (ie all animals removed first grouped together, all removed second grouped together etc.) irrespective of sample time during the day (Table 1.1). One way analysis of variance showed that sampling order had no significant effect on serum glucose or IRI concentrations.

1.4 Effect of refeeding

Groups of infected and control mice were starved for 18 hours, and then allowed access to their normal pellet diet. Blood samples were taken, without anaesthetic, before, and at various times after, the reintroduction of food.

Table 1.1

One way analysis of variance of experimental data from figures 1.2 and 1.3 investigating the effect of sampling order on the results obtained.

		M.S.	F	P
Control	Glucose	0.43	0.31	N.S.
	IRI	1.31	0.83	N.S.
Infected	Glucose	2.11	0.64	N.S.
	IRI	7.93	2.10	N.S.

Note: results were grouped depending on order of sacrifice within each cage, independent of time of sample.

Table 1.2

Analysis of glucose and IRI values at 90 minutes after the reintroduction of food in figure 1.4 compared to 1.5, and in figure 1.7 compared to 1.8.

	Experiment	Mean	t	P
Glucose Infected	1.4	7.11 (0.52)	0.48	N.S.
	1.5	6.8 (0.35)		
Glucose Control	1.4	10.96(0.44)	2.04	N.S.
	1.5	9.8 (0.35)		
IRI Infected	1.7	5.92 (3.8,9.24)	0.54	N.S.
	1.8	8.12 (5.06,13.02)		
IRI Control	1.7	6.23 (4.03,9.63)	1.71	N.S.
	1.8	3.86 (2.78,5.39)		

This experiment was performed in two separate parts, the first studying the time period from 0 to 90 minutes after the reintroduction of food, and the second studying the time period from 90 to 180 minutes. The results are shown separately, then as a continuous time span from 0 to 180 minutes after food.

The validity of combining these two separate series of experiments was checked by analysing the results at 90 minutes after food in both groups using Student's t-test for unpaired samples. No significant difference in glucose results for either control or infected animals was found between the two series of experiments. This was also true for the IRI data (Table 1.2).

Each series of results was analysed by two way analysis of variance. However, due to the unequal group numbers obtained when the experiments were combined, the composite data could not be analysed in its entirety by two way ANOVA. Student's t-test was therefore applied to each time point of the combined results, comparing the fasted glucose or IRI concentrations with the levels obtained at each time point after the reintroduction of food.

1.4.1 Glucose concentrations 0 to 90 minutes after feeding

There was no significant difference between the fasting serum glucose concentrations in control and

infected mice. Both groups showed the anticipated rise in serum glucose in response to food intake, but the increase seen in infected animals was significantly less than that of control mice (Figure 1.4). Thus, by 90 minutes after food the serum glucose concentrations in control mice were significantly higher than those of infected animals ($t = 5.68$, $P < 0.01$).

1.4.2 Serum glucose concentrations 90 to 180 minutes after feeding

The serum glucose concentrations in control animals remained significantly higher than those of infected animals throughout this time period (Figure 1.5).

1.4.3 Serum glucose concentrations 0 to 180 minutes after feeding

Taken together, these results showed that while there was no significant difference in fasting glucose concentrations in infected and control animals, the infected mice showed a reduced increase in serum glucose in response to food intake (Figure 1.6).

1.4.4 Serum IRI concentrations 0 to 90 minutes after feeding

In fasted mice, there was no significant difference in serum IRI concentrations between control and infected animals ($t = 1.33$, N/S). Both groups showed the expected increase in serum IRI in response to food intake (Figure

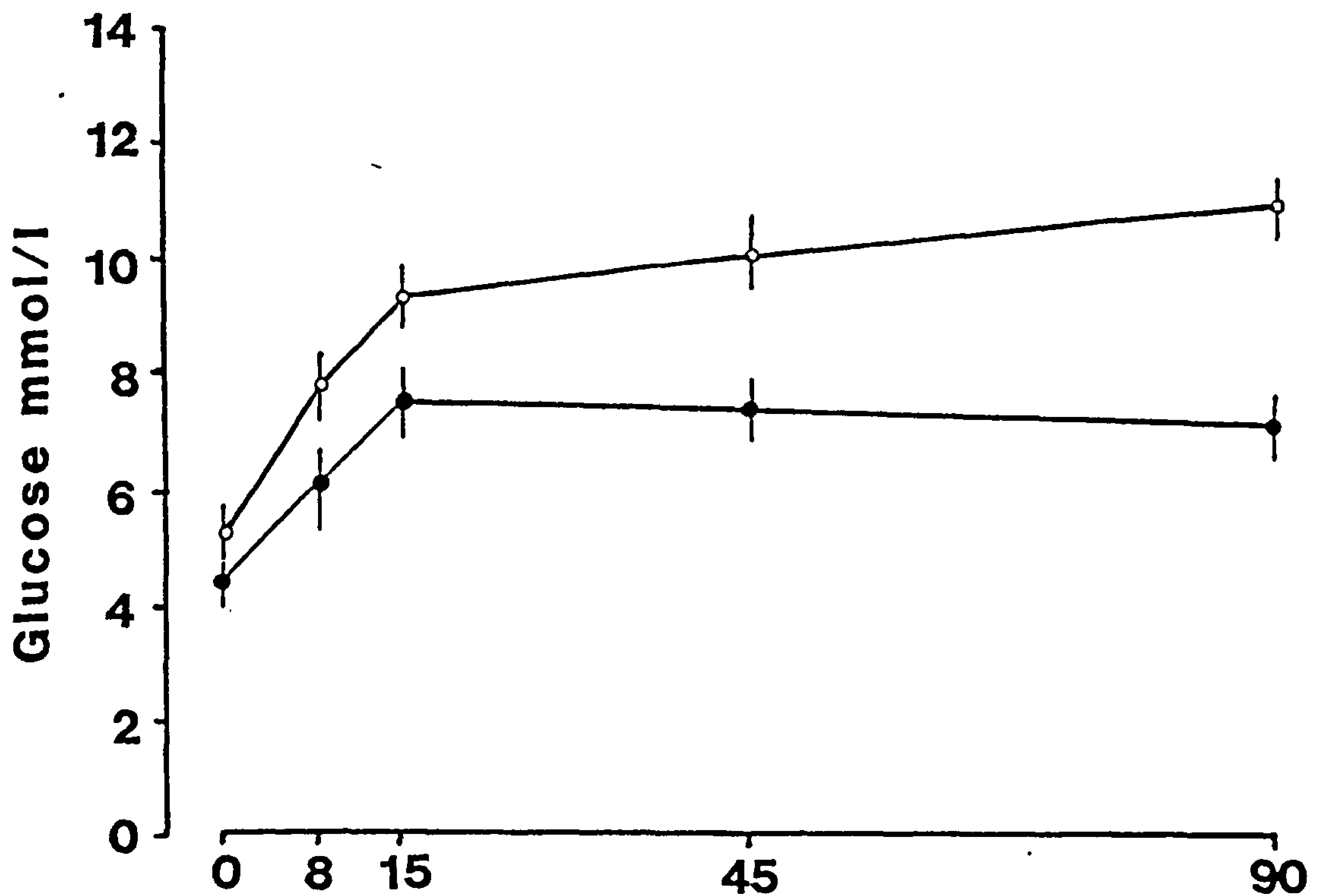


Figure 1.4

<u>Effect of refeeding on the serum glucose concentrations in control and B.pertussis infected mice 0 to 90 minutes after the reintroduction of food.</u>

N= 11 for each treatment, values expressed as arithmetic mean (S.E.) o control, ● infected.

<u>Analysis of variance.</u>

Source	D.F.	F.	P.
Infection	1	47.28	0.01
Time	4	23.74	0.01
Interaction	4	2.62	0.05

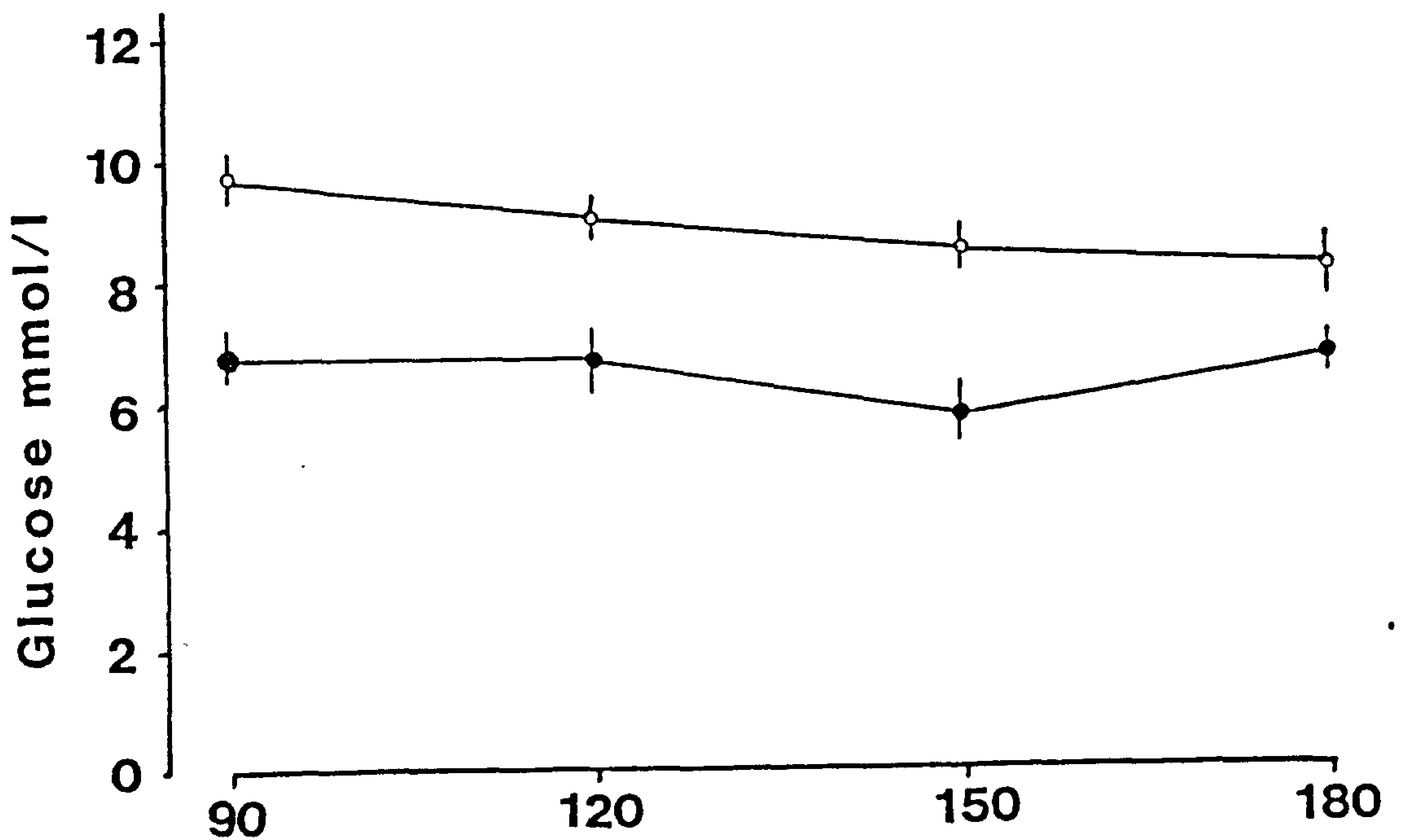


Figure 1.5

Effect of refeeding on the serum glucose concentrations in control and B.pertussis infected mice 90 to 180 minutes after the reintroduction of food.

N= 10 for each treatment, values expressed as arithmetic mean (S.E.). o control, ● infected.

Analysis of variance

Source.	D.F.	F.	P.
Infection	1	100.84	0.01
Time	3	4.47	0.05
Interaction	3	1.86	N.S.

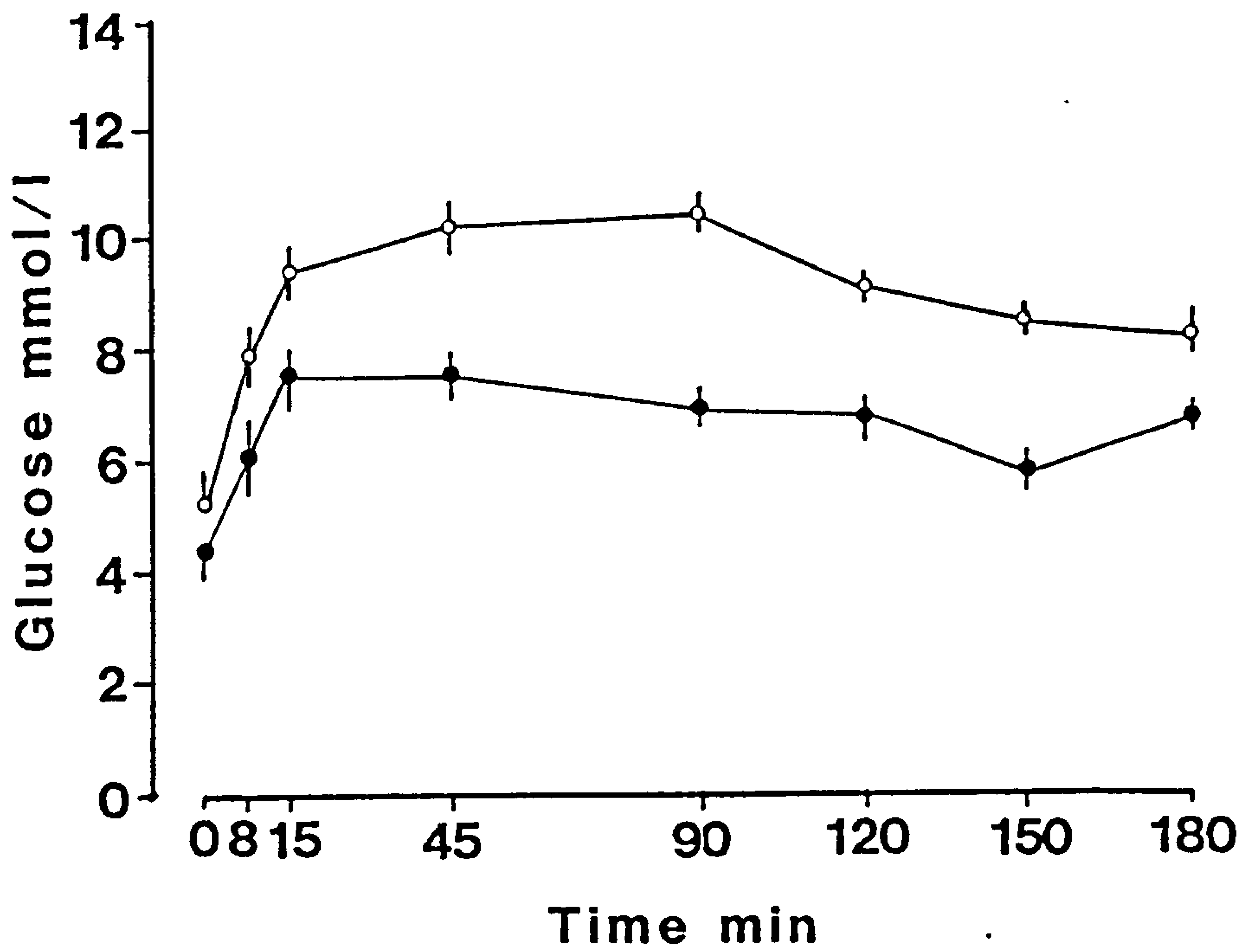


Figure 1.6

Effect of refeeding on the serum glucose concentrations in control and B.pertussis infected mice 0 to 180 minutes after the reintroduction of food.

The results shown in figures 1.4 and 1.5 were combined and are shown here as a single illustration. o control, ● infected.

1.7). However, whilst the rise seen in infected animals appeared to be greater than that seen in control mice, there was no significant effect of infection, nor interaction between infection and time in this time period.

1.4.5 Serum IRI concentrations 90 to 180 minutes after feeding

The serum IRI concentrations in infected animals were significantly greater than those in control animals at these time points after the reintroduction of food (Figure 1.8).

1.4.6 Serum IRI concentrations 0 to 180 minutes after feeding

Analysis of the combined results using Student's t-test showed that the rise in serum IRI above fasted levels seen after food intake was both greater, and more prolonged in infected animals than in controls (Figure 1.9).

Indeed, the serum IRI concentrations in infected animals were still significantly elevated 180 minutes after food intake when compared to mean freely-fed values (IRI in freely-fed, infected mice = 3.46 ng ml^{-1} (2.27, 5.29), IRI in infected mice at 180 minutes after reintroduction of food = 7.01 ng ml^{-1} (4.54, 10.82); $t = 4.501$, $P < 0.001$). However, in control animals the serum IRI had returned to freely-fed values at this time (IRI

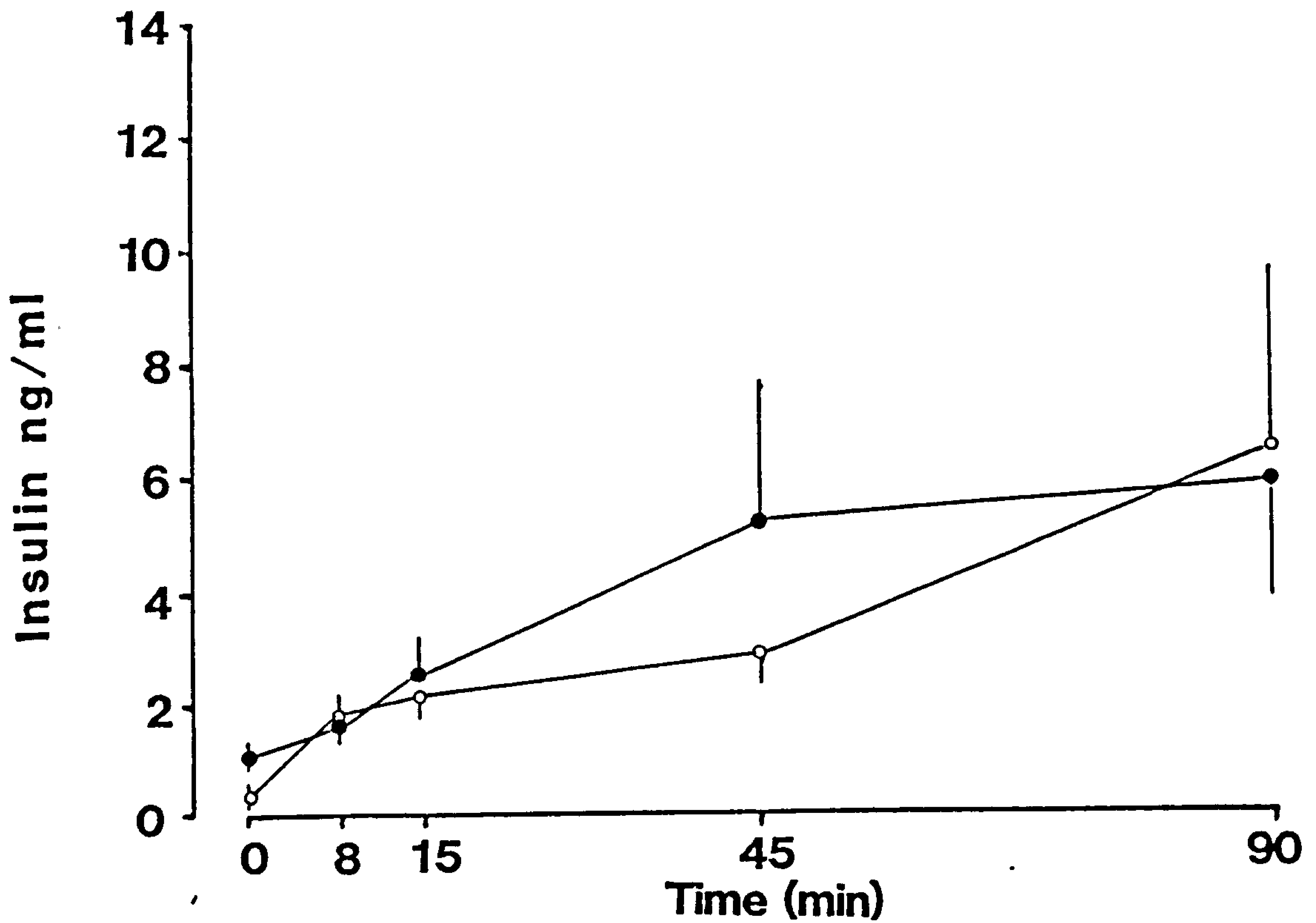


Figure 1.7

<u>Effect of refeeding on the serum IRI concentrations in control and B.pertussis infected mice 0 to 90 minutes after the reintroduction of food.</u>

N= 11 for each treatment, values expressed as geometric mean (95% C.L.). o control, ● infected.

<u>Analysis of variance</u>

Source	D.F.	F.	P.
Infection	1	0.63	N.S.
Time	4	3.80	0.01
Interaction	4	0.45	N.S.

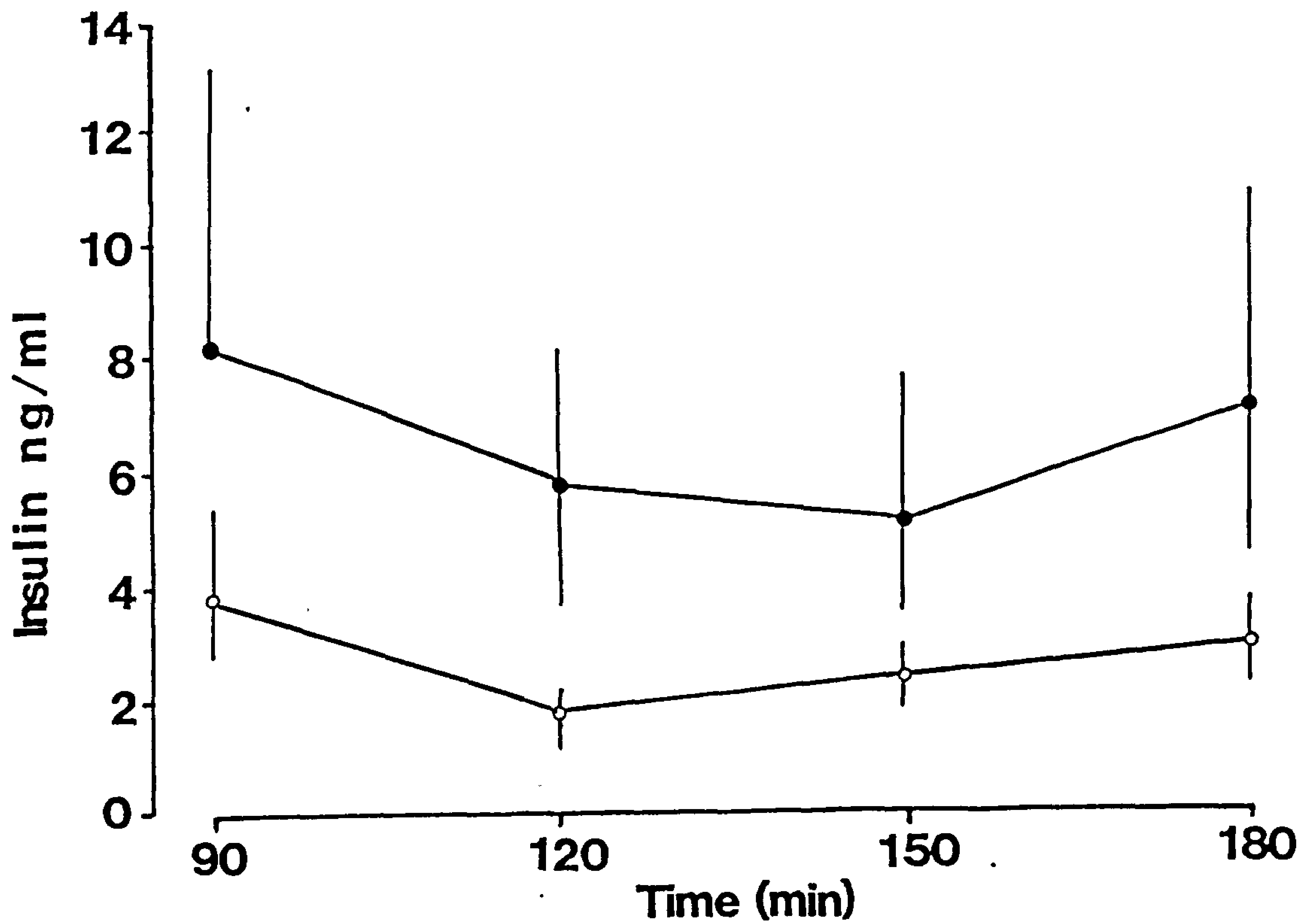


Figure 1.8

Effect of refeeding on the serum IRI concentrations in control and B.pertussis infected mice 90 to 180 minutes after the reintroduction of food.

N= 10 for each treatment, values expressed as geometric mean (95% C.L.). o control, ● infected.

Analysis of variance

Source	D.F.	F.	P.
Infection	1	17.12	0.01
Time	3	3.32	0.05
Interaction	3	1.06	N.S.

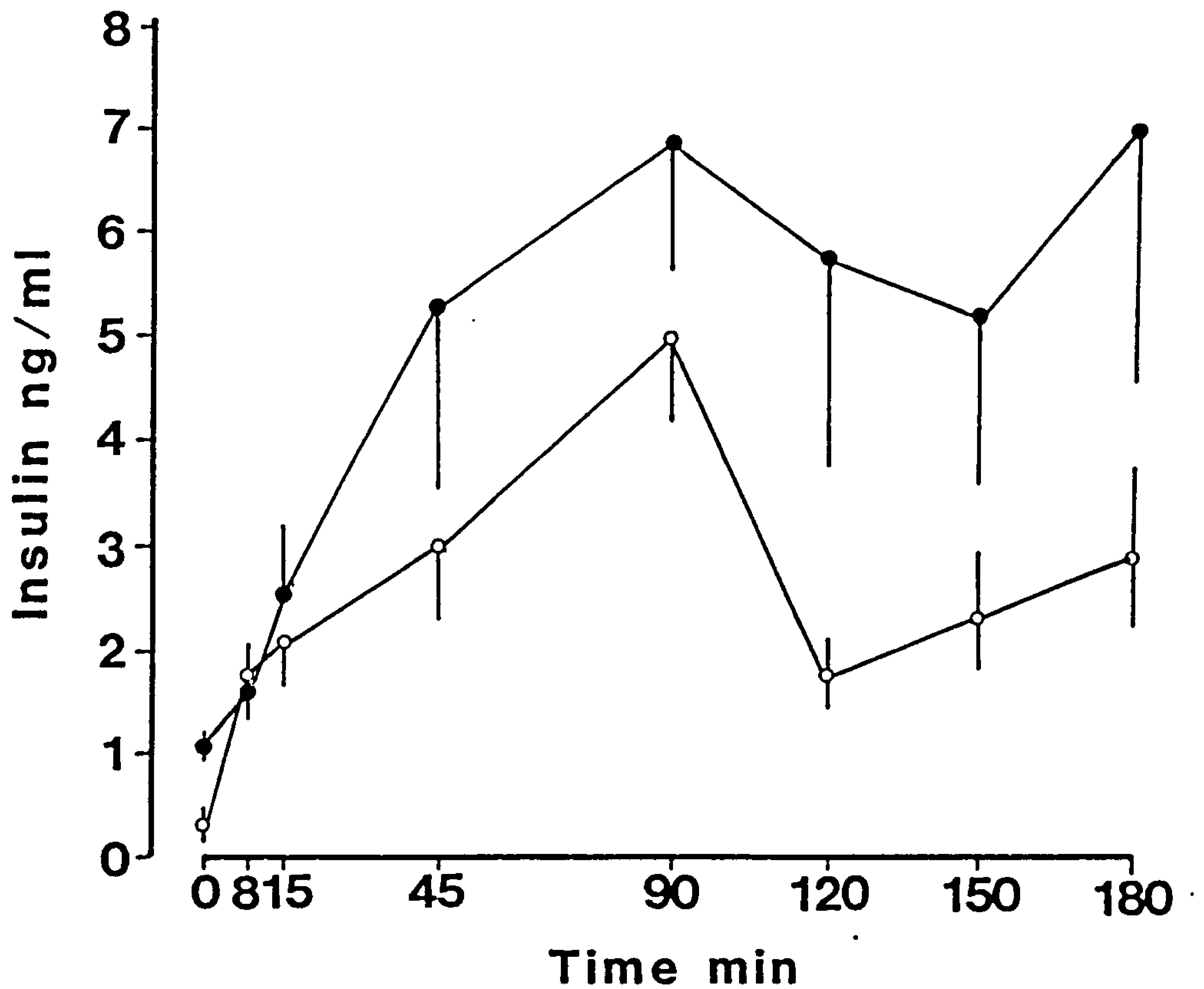


Figure 1.9

<u>Effect of refeeding on the serum IRI concentrations in control and B.pertussis infected mice at 0 to 180 minutes after the reintroduction of food.</u>

The results shown in figures 1.7 and 1.8 were combined and are shown here as a single illustration. o control, ● infected.

freely-fed controls = 2.65 ng ml^{-1} (1.89, 3.70), IRI in control mice at 180 minutes after reintroduction of food = 2.88 ng ml^{-1} (2.2, 3.71); $t = 0.991$, P = N/S).

1.4.7 Variation within treatment groups

Using the same procedure as used in section 1.3.3, one way analysis of variance showed there to be no significant effect of sequential removal of animals from the cages, on either glucose or IRI concentrations (Table 1.3).

1.5 In vivo insulin responsiveness

To determine the relative responsiveness of infected and control mice to exogenous insulin, animals were injected intravenously with saline or with various doses of insulin. Initially the dose-response relationship of serum glucose concentrations to increasing doses of insulin was investigated. Subsequently a more detailed study was made of the effect of a low dose and a high dose of insulin in both infected and control mice.

1.5.1 Serum glucose concentrations in response to various doses of insulin in normal mice

Velosulin, a highly purified, soluble pork insulin, was administered to normal mice (0.05, 0.5 and 5 U kg^{-1}, I.V.). Saline, given intravenously, served as treatment control. Groups of eight mice were bled by decapitation without anaesthetic at 15, 30, 60 and 120

Table 1.3

One way analysis of variance of experimental data from figures 1.6 and 1.9 investigating the effect of sampling order on the results obtained.

		M.S.	F	P
Control	Glucose	1.41	0.20	N.S.
	IRI	4.91	0.54	N.S.
Infected	Glucose	0.58	0.15	N.S.
	IRI	3.80	0.24	N.S.

Note: results were grouped depending on order of sacrifice within each cage, independent of sample time.

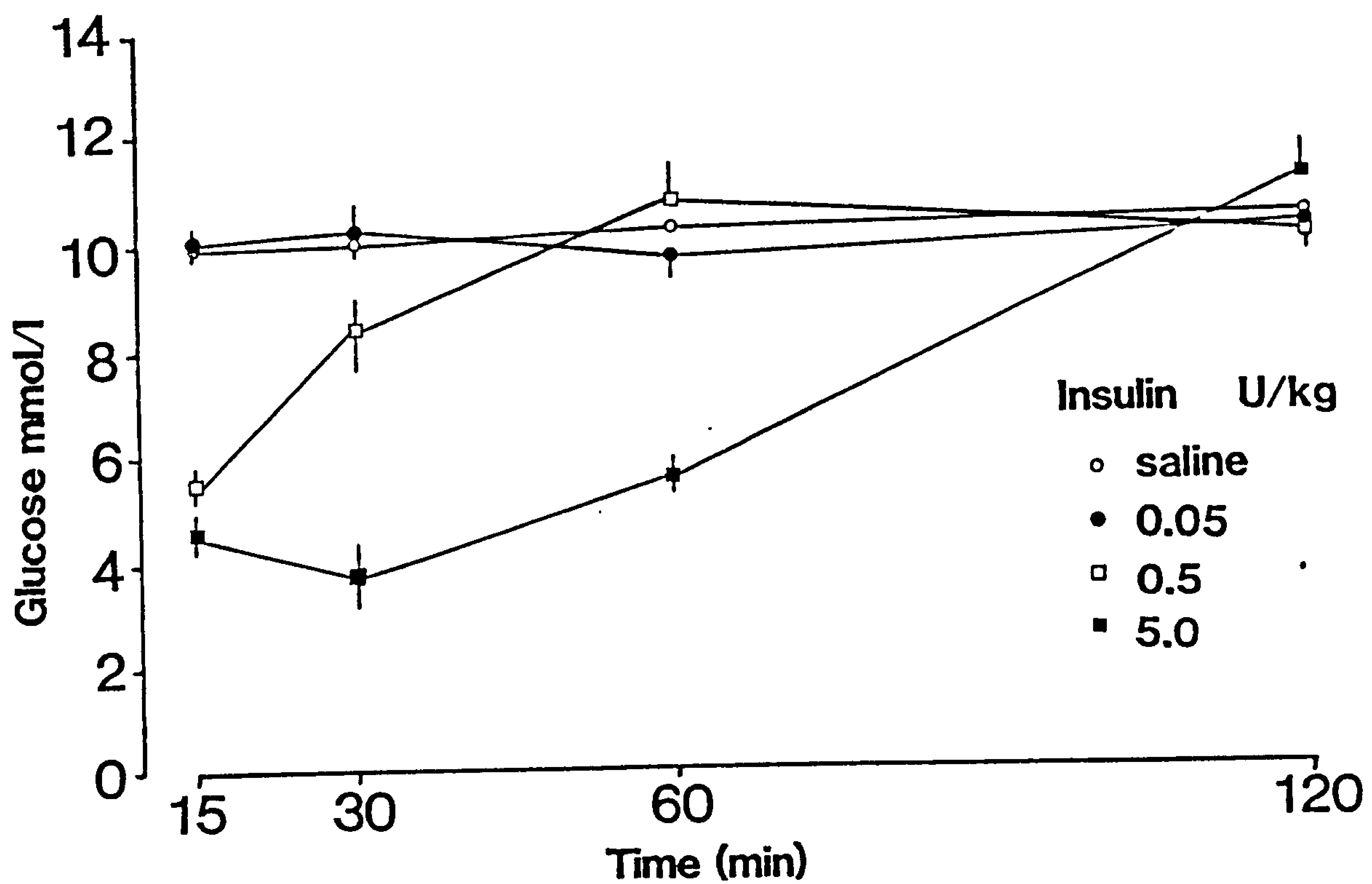


Figure 1.10

Hypoglycaemic effect of increasing doses of soluble insulin in normal mice.

N= 8 for each treatment, values expressed as arithmetic mean (S.E.)

o Saline

● 0.05 U/kg

□ 0.5 U/kg

■ 5.0 U/kg

minutes post-injection.

Insulin caused a dose-dependent decrease in serum glucose concentration within the dose range of 0.5 to 5 U kg^{-1} (Figure 1.10). This dose dependency was related to both the degree of hypoglycaemia obtained, and the duration of the hypoglycaemia.

1.5.2 Insulin responsiveness in *B. pertussis* infected mice

Two doses of insulin (0.25 and 5 U kg^{-1}) were used to determine the hypoglycaemic effect of insulin in infected and control animals. Intravenous saline served as treatment control. Animals were bled at 15, 30, 60 and 90 minutes after injection, and groups of eight were used at each point.

1.5.2a Hypoglycaemic effect of 0.25 U kg^{-1} insulin

This dose of insulin produced hypoglycaemia in control mice at 15 minutes, which returned to saline-control levels by 30 minutes. There was little or no response in the infected mice (Figure 1.11). Due to the complexity of this experiment, a further experiment was performed to examine the effect of 0.25 U kg^{-1} of insulin in both control and infected animals at 15 minutes post injection (Table 1.4).

Both infected and control mice were hypoglycaemic at this time point. However, analysis of variance showed that the hypoglycaemic effect of this dose of insulin was significantly attenuated in infected mice (F for inter-

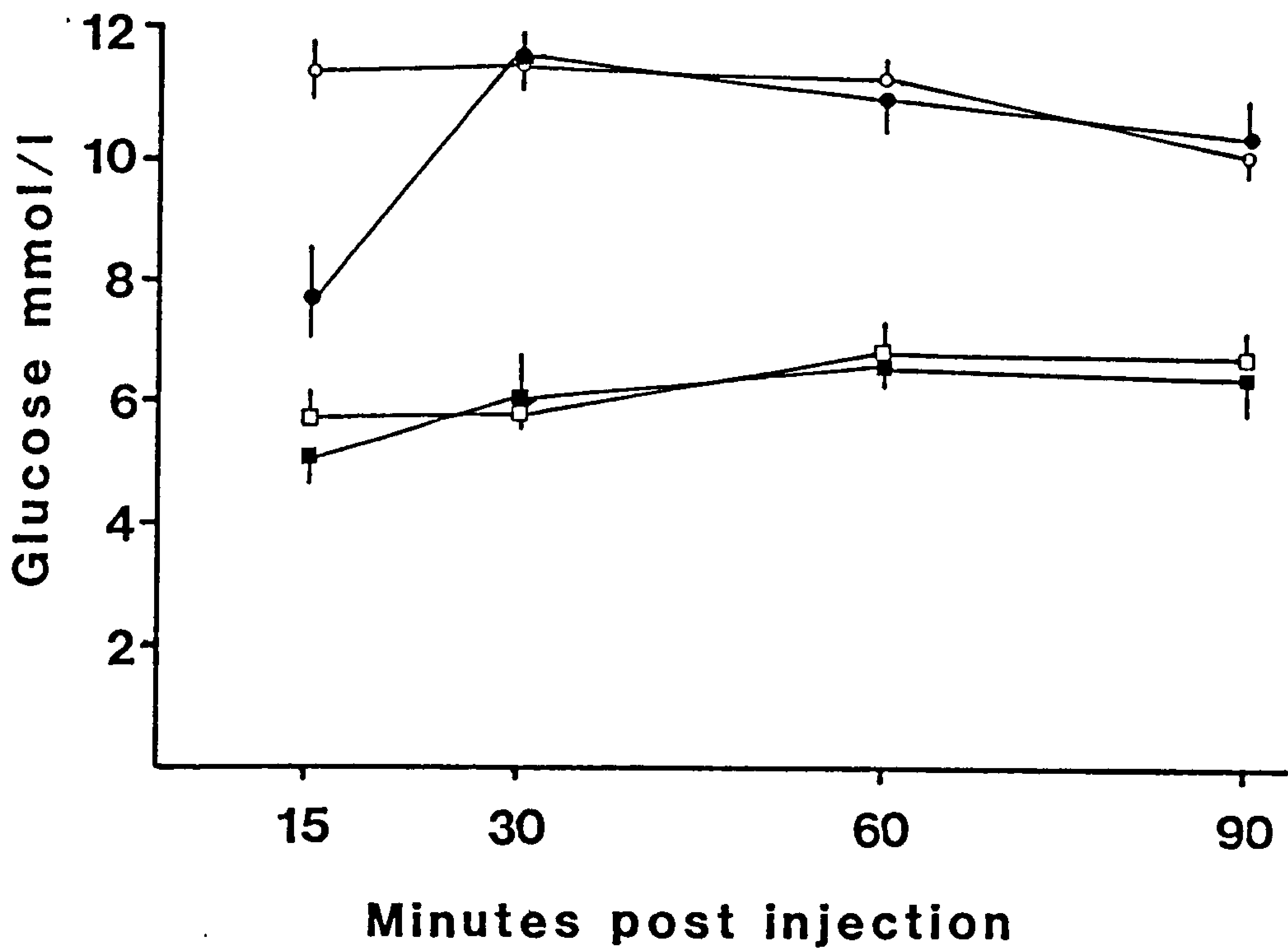


Figure 1.11

Hypoglycaemic effect of 0.25 U/kg of soluble insulin.

N=8 for each treatment, values expressed as arithmetic mean (S.E.).

o saline, non-infected

● insulin, non-infected

□ saline, infected

■ insulin, infected.

Table 1.4.

Effect of soluble insulin on the serum glucose concentrations of control and B.pertussis infected mice (II).

Dose of insulin. (U/kg)	Bleeding time. (min.)	Serum glucose	
		Control	Infected.
Saline	15	11.74 (0.56)	6.83 (0.37)
0.25	15	7.40 (0.31)	4.33 (0.47)
Saline	90	10.08 (0.18)	6.39 (0.43)
5.0	90	9.76 (0.40)	4.19 (0.51)

Note: values given in mmol/l and expressed as arithmetic mean (S.E.). N= 12 for each treatment.

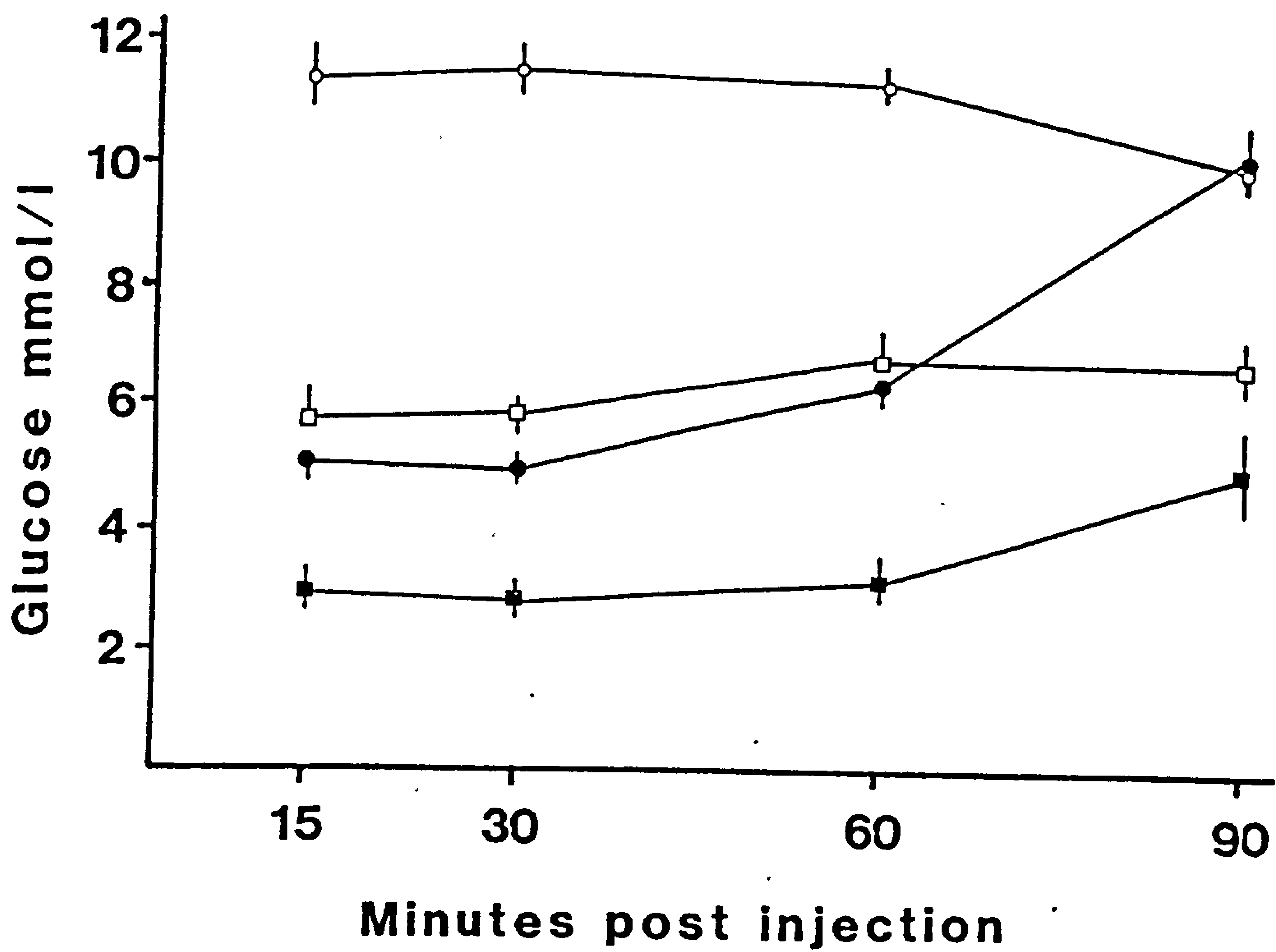


Figure 1.12

Hypoglycaemic effect of 5.0 U/kg of soluble insulin.

N=8 for each treatment, values expressed as arithmetic mean (S.E.).

○ saline, non-infected

● insulin, non-infected

□ saline, infected

■ insulin, infected.

action = 4.3, $P < 0.05$).

1.5.2b Hypoglycaemic effect of 5.0 U kg^{-1} insulin

This dose of insulin produced hypoglycaemia in both infected and control mice (Figure 1.12). However, whereas the glucose concentrations in control mice had returned to saline-control levels by 90 minutes, those of infected mice still remained depressed. This was confirmed in a separate experiment where the effect of 5.0 U kg^{-1} insulin I.V. was studied at 90 minutes post injection (Table 1.4). This confirmed that the hypoglycaemia caused by a high dose of insulin was prolonged in infected animals.

1.6 In vitro insulin sensitivity

The ability of insulin to stimulate glycogen synthesis in mouse hemidiaphragm preparations has been used as a measure of free insulin (Wardlaw and Moloney, 1961) and to determine both the qualitative and quantitative presence of insulin (Wardlaw and Van Belle, 1964).

The effect of a range of insulin concentrations on the glycogen content of hemidiaphragms removed from *B. pertussis* infected and control mice was investigated. Hemidiaphragms were incubated in a media containing a standard glucose concentration (16.7 mmol l^{-1}) with 0, 0.1, 1, 10, or 100 mU ml^{-1} of soluble insulin. Tissue was incubated for 90 minutes in the presence of insulin,

Table 1.5

Insulin-induced glycogen deposition in mouse hemidiaphragms isolated from control or B.pertussis infected mice.

Insulin Concentration (mU/ml)	Change in optical density (OD650)			
	Infected	Control	t	P
100	0.39 (0.14)	0.66 (0.10)	1.53	N.S.
10	0.38 (0.07)	0.45 (0.04)	1.32	N.S.
1	0.33 (0.06)	0.49 (0.14)	1.10	N.S.
0.1	0.23 (0.08)	0.36 (0.13)	0.89	N.S.

Note: Change in optical density calculated as OD650 of test minus OD650 without insulin.

then removed and the glycogen content assayed. The experiment was repeated three times and the results are summarised in Table 1.5

There was no significant decrease in insulin-induced glycogen deposition between hemidiaphragms from control and B. pertussis infected mice.

1.7 Discussion

1.7.1 Mechanism of pertussis induced hypoglycaemia

These results have confirmed previous findings that sublethal, intranasal infection with B. pertussis produces hypoglycaemia in mice (Pittman et al, 1980; Furman et al, 1981). This hypoglycaemia persisted throughout the day (Figure 1.2). The circadian pattern of serum glucose concentration found was similar to that reported by Nowell (1970), who also showed a nadir in serum glucose between 8 and 9 p.m.

It is possible that reduced food intake in the week prior to the experiment could contribute to the hypoglycaemia seen in infected mice. Indeed, infected mice consumed significantly less food (g per mouse) during the 14 days following infection ($P < 0.05$), and gained significantly less weight during the same time ($P < 0.01$) than did control mice (Figure 1.13). However, this is unlikely to be the sole cause of the hypoglycaemia seen since, during the feeding experiments, both control and B. pertussis infected animals consumed

Figure 1.13

Food intake and weight gain of control and B.pertussis infected mice.

o control

● infected

a) Food intake, mean of four experiments.

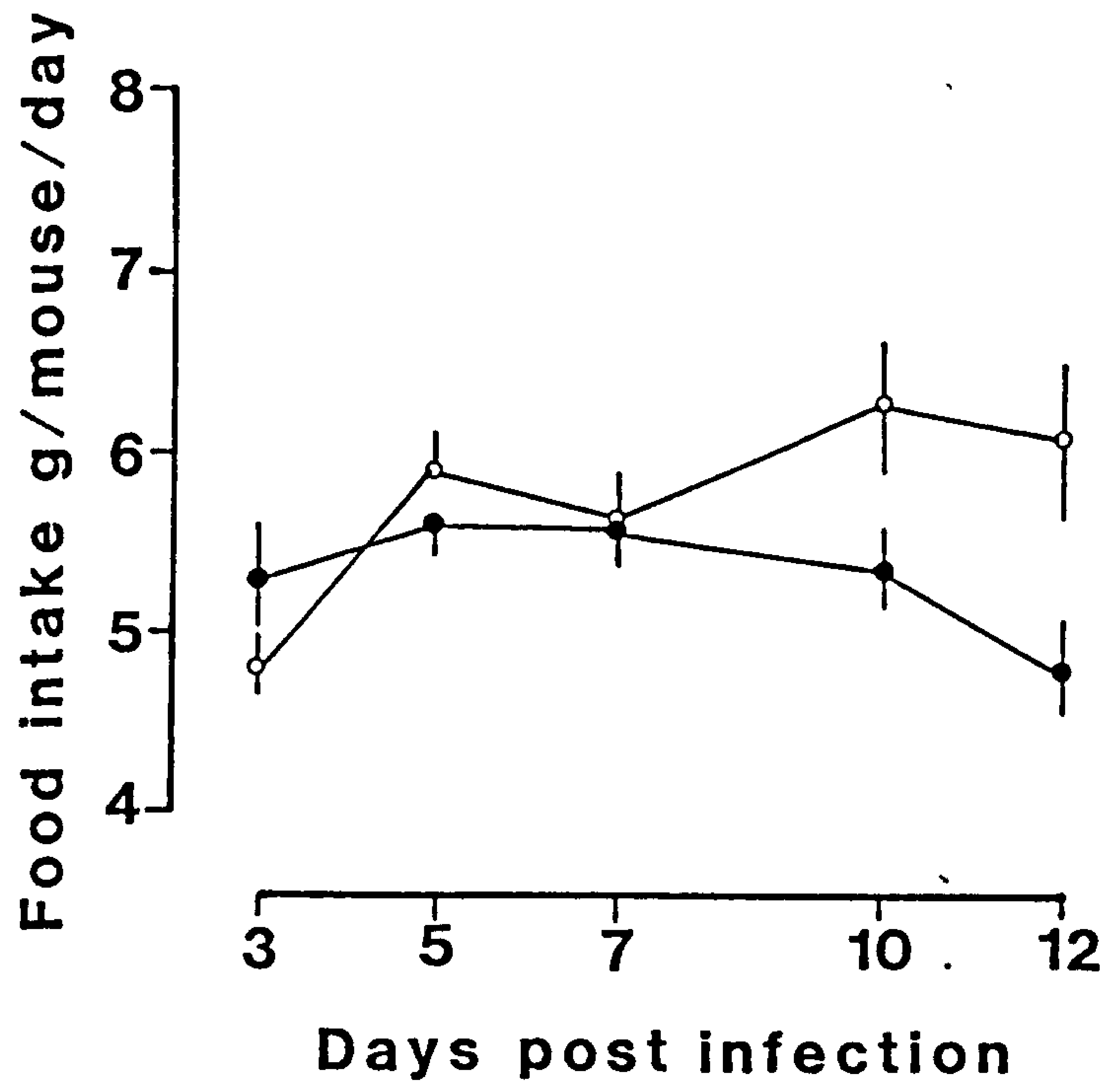
Analysis of variance.

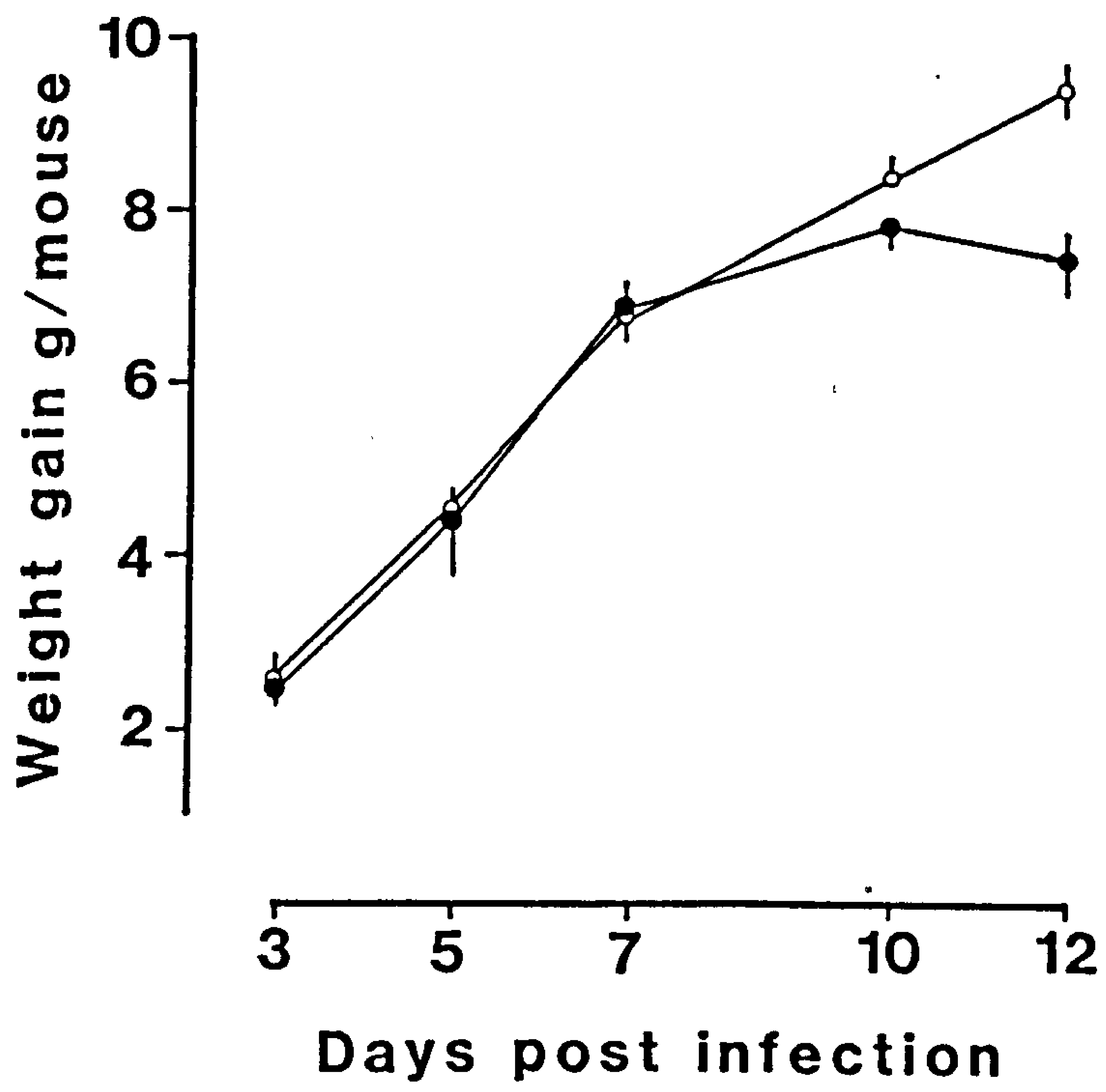
Source	D.F.	F.	P.
Infection	1	6.31	0.05
Time	4	2.93	0.05
Interaction	4	3.72	0.05

b) Weight gain, mean of four experiments.

Analysis of variance.

Source	D.F.	F.	P.
Infection	1	10.35	0.01
Time	4	140.74	0.01
Interaction	4	3.853	0.05


Food intake g/mouse/day
8
7
6
5
4
3
5
7
10
12
Days post infection


Weight gain g/mouse
10
8
6
4
2
3
5
7
10
12
Days post infection

similar amounts of food when allowed access to diet after an 18 hour fast (Table 1.6). Despite this, the infected animals were hypoglycaemic relative to controls after refeeding.

The moderately, but persistently, elevated serum IRI concentrations observed in infected mice appeared at first to be at variance with earlier reports that hyperinsulinaemia was only present in B. pertussis infected mice which had been exposed to ether, or some other anaesthetic (Furman et al, 1981). These authors argued that anaesthesia might cause the release of adrenal catecholamines which, in B. pertussis infected animals, would stimulate insulin secretion (Gulbenkian et al, 1968; Furman et al, 1981). Thus, pertussis-infected mice did not appear to be chronically hyperinsulinaemic. This would cast doubt on the role of insulin in the pertussis-induced hypoglycaemia. However, it was noteworthy that, in the results presented here, the difference between serum IRI concentrations in control and infected mice was minimal between the hours of 9 a.m. and 12 noon (Figure 1.3), this being the time at which the previous experiments had been performed (Furman et al, 1981)

The role of insulin in the hypoglycaemia seen in B. pertussis infected mice was further supported by the prevention by alloxan diabetes of pertussis-induced hypoglycaemia (Figure 1.1). This, taken with the fact that pertussis-infected mice had significantly higher

Table 1.6

Data from control and B.pertussis infected mice subjected to an eighteen hour fast.

a. Food consumption after reintroduction of food (g/mouse).

Time after food (min)	8	15	45	90	120	150	180
Control	0.5	0.69	1.81	2.24	1.31	1.76	1.86
Infected	0.38	0.81	1.81	1.92	2.29	2.12	1.66

b. Weight of mice before starvation and weight loss during fast.

	Control	Infected	t.	P.
Weight before fast (g)	23.26 (0.12) n=376	20.74 (0.19) n=374	7.99	0.01
Weight loss during fast (g)	2.36 (0.14) n=55	2.13 (0.18) n=54	0.18	N.S.

Note: weights expressed as mean (S.E.)

serum IRI concentrations than did controls, suggests that pertussis-induced hypoglycaemia is secondary to hyper-insulinaemia.

1.7.2 Mechanism of pertussis-induced hyperinsulinaemia

The difference between serum IRI concentrations in control and infected mice was greatest during the dark phase of the light-dark cycle (see Figure 1.3). As mice are predominantly nocturnal feeders (Nowell, 1970), it was possible that an exaggerated serum insulin response to feeding contributed to the hyperinsulinaemia seen in pertussis-infected mice. This was confirmed by the results shown in Figures 1.7 to 1.9, which show an exaggerated and prolonged serum IRI response to food intake in pertussis-infected animals, despite similar fasted IRI concentrations (Figure 1.7). Also, fasted serum glucose concentrations in infected and control animals were not significantly different, but relative hypoglycaemia in the infected groups was seen after food intake (Figures 1.4 to 1.6).

Peak glucose values obtained in Figure 1.6 correspond to peak glucose values in Figure 1.2 (the circadian study). Also, the relative hypoglycaemia in infected mice (compared to control mice) at 90 minutes after the reintroduction of food was similar to that seen in the circadian study.

The exaggerated feeding-induced hyperinsulinaemia seen in pertussis-infected mice may be due to a pertussis-

induced augmentation of glucose and/or amino acid-induced insulin secretion. This suggestion is supported by previous work in which pretreatment of rats with pertussis vaccine augmented glucose and arginine-induced insulin secretion from the subsequently isolated and perfused pancreas (Katada and Ui, 1977). Also, pertussis toxin augmented glucose-induced insulin secretion from perfused rat pancreas (Toyota et al, 1978), and from rat islets (Katada and Ui, 1981) and human islets (Araki et al, 1981) *in vitro*.

The inhibitory effect of catecholamines on glucose-induced insulin secretion is prevented, or indeed converted to a stimulatory effect in pertussis vaccinated rats (Gulbenkian et al, 1968; Sumi and Ui, 1975), *B. pertussis* infected mice (Furman et al, 1981) and pertussis toxin-treated rats (Yajima et al, 1978). Thus, adrenaline released in response to stress could contribute to the elevated serum IRI concentrations in infected mice. Although much effort was made to avoid stressing the animals, the handling required during the blood sampling procedure could produce stress, and therefore catecholamine release (Halter et al, 1984; Young et al, 1984).

The effect upon insulin release of adrenaline released as a glucose counterregulatory hormone could also be altered in *B. pertussis* infected mice, and could contribute to the postprandial hyperinsulinaemia seen in

infected mice. Although glucagon is the primary counterregulatory hormone, adrenaline is also important in preventing the late hypoglycaemia seen after glucose ingestion (Tse et al, 1983), particularly in the absence of glucagon. Adrenaline may contribute to restoring glucose concentrations after insulin-induced hypoglycaemia in man (Garber et al, 1976; Gerich et al, 1979).

Altered sensitivity to circulating insulin could affect the degree of hypoglycaemia which would develop in response to the modest hyperinsulinaemia seen in *B. pertussis*-infected mice. Impaired insulin-sensitivity is suggested by the decreased hypoglycaemic response to a low dose of insulin seen in infected mice (Figure 1.11). Pertussis-toxin treated rat adipocytes show a reduced sensitivity to insulin (Goren et al, 1985). Moreover treatment of hepatocytes with pertussis toxin prevented insulin-induced inhibition of adenylate cyclase activity in intact hepatocytes and broken plasma membranes (Heyworth et al, 1986).

The mechanism of the reduced responsiveness of pertussis-infected mice to a low dose of insulin is unknown. Chronically raised insulin concentrations can lead to down-regulation of insulin receptors (Olefsky, 1981) and therefore to a reduced hypoglycaemic response to exogenous insulin. However, this is not supported by the observation that there was no difference in the *in vitro* sensitivity of a skeletal muscle preparation to

insulin between control and B. pertussis infected mice (Table 1.5). It is, of course, possible that there may have been a decrease in the responsiveness to insulin of the other insulin-sensitive tissues (adipose tissue, liver). On the other hand, Trimble et al (1984) reported that chronic hyperinsulinaemia in vivo increased the sensitivity of rats to injected insulin.

An alternative explanation of the decreased sensitivity of B. pertussis infected mice to insulin could be an increased production of glucose counterregulatory hormones. This might blunt any hypoglycaemia produced by exogenous insulin (Garber et al, 1976; Gerich et al, 1979). However, the hypoglycaemic effect of a high dose of insulin was prolonged in B. pertussis-infected mice, suggesting a defective, rather than overactive, glucose counterregulatory mechanism. This defect in glucose counterregulation might involve the absence of adrenaline-induced hyperglycaemia seen in B. pertussis infected mice (Furman et al, 1981), as discussed previously. Adrenaline is important in restoring the glucose concentrations in man following insulin-induced hypoglycaemia (Garber et al, 1976). Increased sensitivity to injected insulin has also been reported following pertussis vaccine (Lane, 1969) and pertussis toxin (Re et al, 1984).

Results Chapter 2

Stress-induced hyperinsulinaemia in *B. pertussis* infected and pertussis toxin-treated mice

2.1 Introduction

2.2 Effect of ether exposure

2.3 I.P. injection of 36 mg kg^{-1} histamine

2.4 Exposure to cold

2.5 I.V. injection of *E. Coli* endotoxin 0111B4

2.6 2-Deoxyglucose

2.7 Exposure to 8% oxygen, 92% nitrogen (Hypoxia)

2.8 30 minute exposure to 8% oxygen

2.9 Exposure to 100% N_2 or 100% CO_2 (anoxic stimuli)

2.10 Effect of alloxan on the metabolic changes induced by ether exposure

2.11 Effect of ether stress on pertussis toxin-treated mice

2.12 Effect of repeated exposures to ether vapour

2.13 Discussion

2.1 Furman et al (1981) showed that hyperinsulinaemia could be induced in B. pertussis infected animals using the anaesthetics ether, pentobarbitone and trichloroethylene. They proposed that the hyperinsulinaemic response was due to anaesthetic-induced catecholamine release from the adrenal medulla (Greene, 1963), which, in pertussis-infected animals, would cause an increase in serum IRI.

In support of this theory, adrenaline produced hyperinsulinaemia in vivo (Furman et al, 1981) in pertussis-infected mice. The inhibitory effect of adrenaline on insulin secretion was absent, or converted to a stimulation in vivo in pertussis vaccinated rats (Sumi and Ui, 1975). This effect of pertussis sensitisation is thought to be due to a modification of the adrenoceptor mechanisms on the insulin-secretory β-cell. In pertussis-treated islets, the normally dominant α-adrenoceptor-mediated inhibition of insulin release is prevented, and the β adrenoceptor-mediated stimulation of insulin release is revealed (Katada and Ui, 1977).

Since adrenaline is released from the adrenal medulla in response to a variety of stressful stimuli (Young, Rosa and Landsberg, 1984; Halter, Beard and Porte, 1984), stress could lead to a marked hyperinsulinaemia, with resultant hypoglycaemia in pertussis-sensitised animals.

A variety of stressful stimuli has been studied in

relation to their effects on the serum glucose and insulin concentrations of pertussis treated mice, and the results are presented in this section.

2.2 Effect of ether exposure

Groups of control or infected mice were killed by decapitation after either exposure to ether vapour or air for 60 sec. Mice infected with B. pertussis were significantly hypoglycaemic compared to non-infected controls ($P < 0.01$), but exposure to ether vapour produced no significant change in the serum glucose concentrations in either group (P N.S.) (Figure 2.1a).

There was no significant difference in the serum IRI concentrations between infected and control mice when blood was collected without the use of anaesthesia. However, whereas in non-infected mice ether exposure did not alter IRI concentrations, it produced a marked increase in IRI in infected mice (P for interaction < 0.01) (Figure 2.1b).

The effect of varying both the duration of ether exposure, and the time allowed for recovery after removal from the ether atmosphere was also investigated in infected animals. Groups of seven mice were bled by decapitation either immediately, or after 30, 60 or 90 seconds exposure to ether vapour. This was repeated allowing 30 seconds recovery after removal from ether vapour before bleeding. The results are shown in Figure

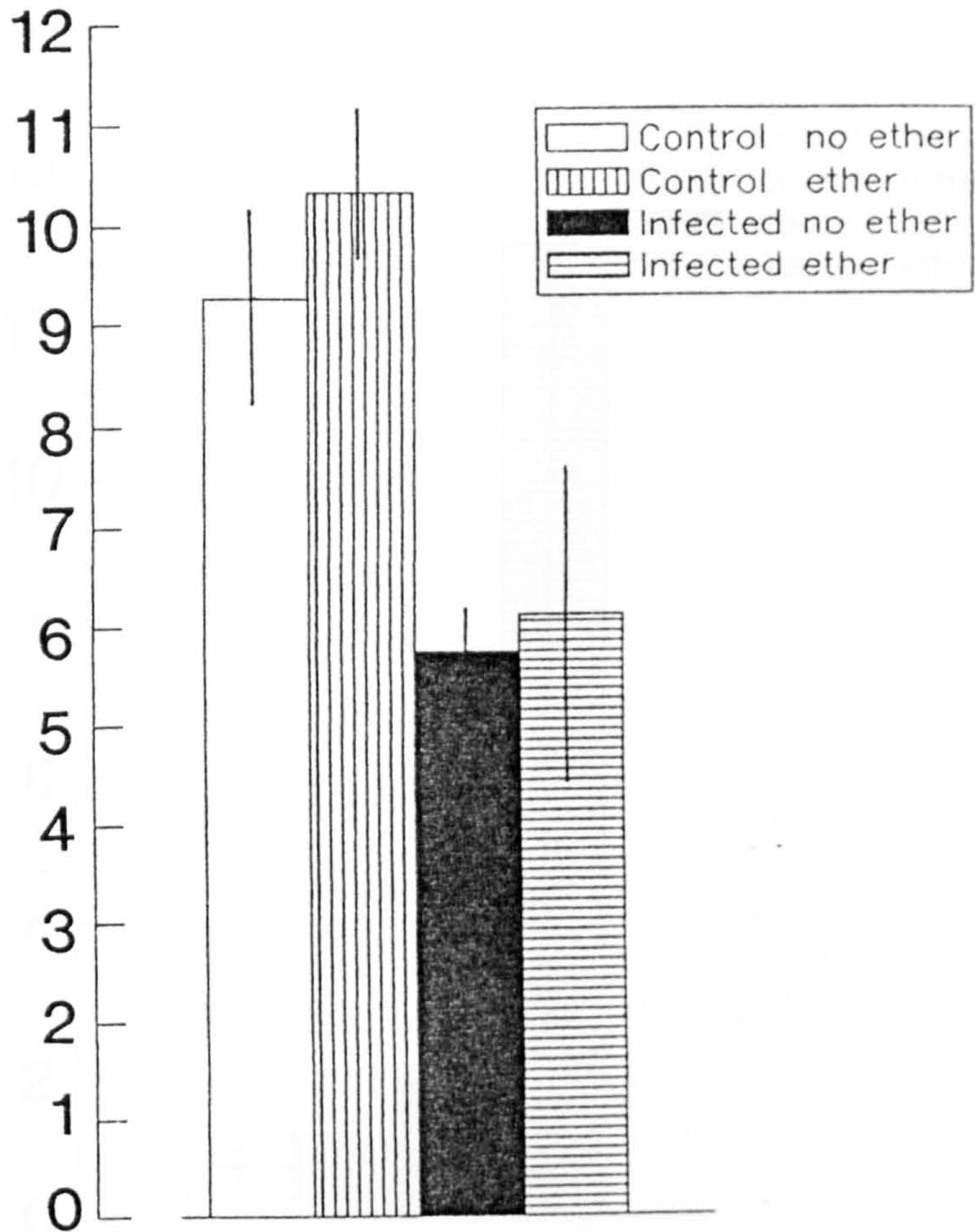


Figure 2.1a

Effect of exposure to ether vapour on the serum glucose concentrations of control and B.pertussis infected mice.

N= 5 for each treatment, values expressed as arithmetic mean (S.E.)

Analysis of variance.

Source	D.F.	F.	P.
Ether	1	2.83	N.S.
Infection	1	79.41	0.01
Interaction	1	0.65	N.S.

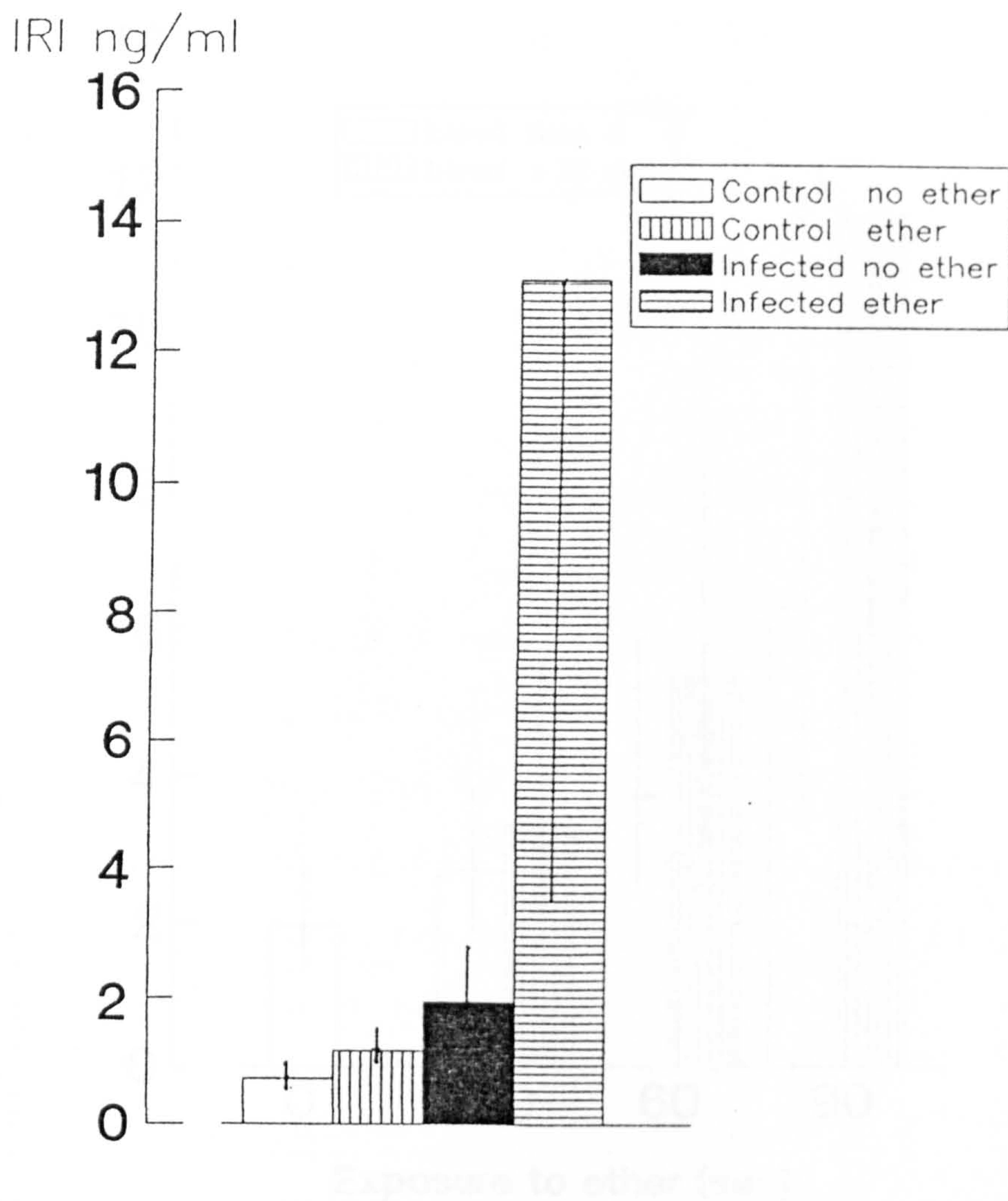


Figure 2.1b

Effect of exposure to ether vapour on the serum IRI concentrations in control and B.pertussis infected mice.

N= 5 for each treatment, values expressed as geometric mean (95% C.L.)

Analysis of variance.

Source	D.F.	F.	P.
Ether	1	62.45	0.01
Infection	1	31.53	0.01
Interaction	1	11.65	0.01

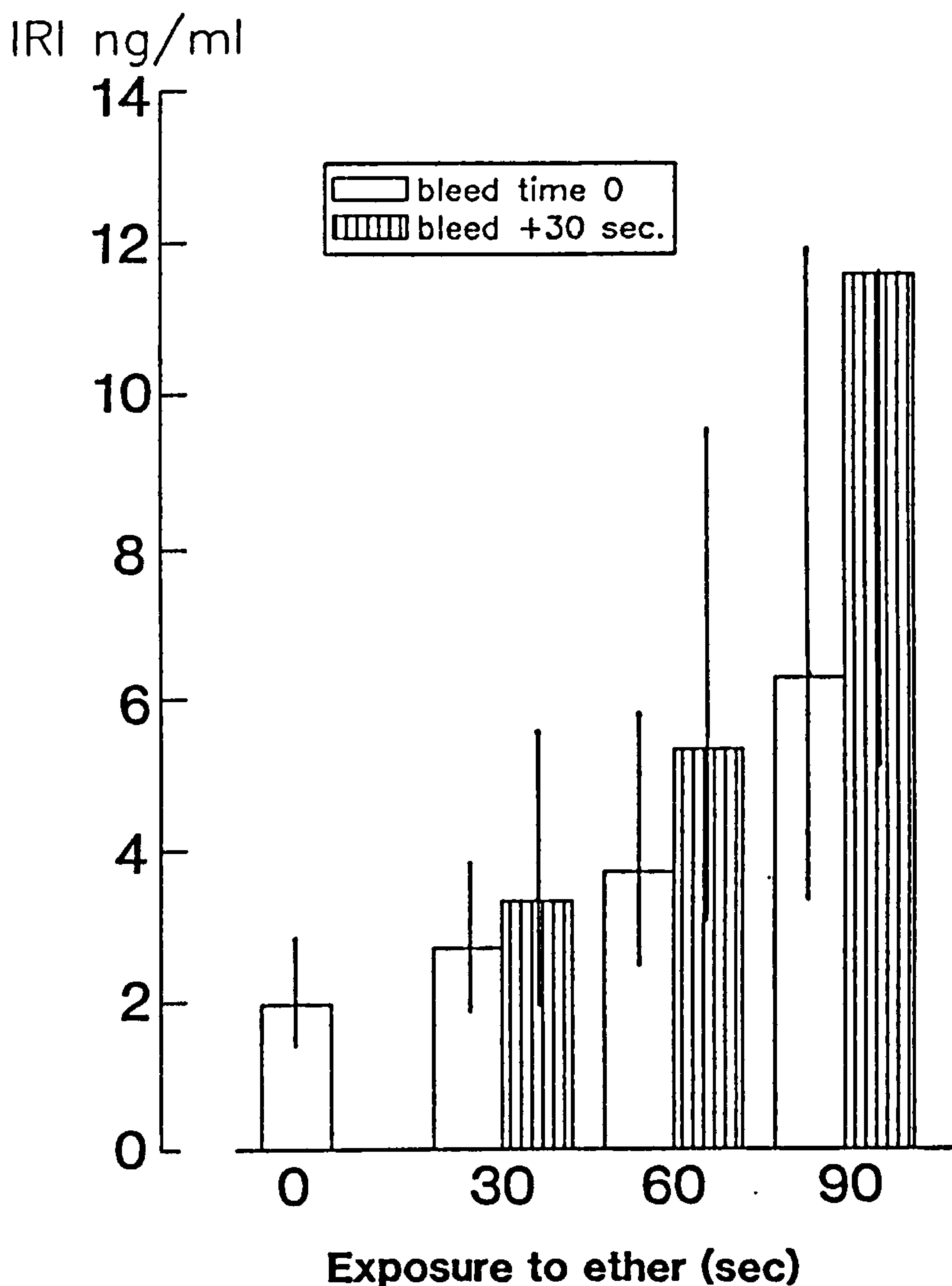


Figure 2.2

Effect of varying duration of exposure to ether vapour on the hyperinsulinamia seen in B.pertussis infected mice.

N= 7 for each treatment, values expressed as geometric mean (95% C.L.).

Analysis of variance.

Source	D.F.	F.	P.
Exposure time	2	6.23	0.01
Recovery time	1	1.55	N.S.
Interaction	2	1.69	N.S.

2.2.

Analysis of variance showed that varying the time of exposure to ether has a significant effect to increase serum glucose concentrations ($P < 0.01$). However, the recovery period did not alter the serum glucose concentrations, and there was no interaction between recovery time and ether exposure (results not shown).

Serum IRI concentrations were significantly increased by lengthening the time of exposure to ether ($F = 6.23$, $P < 0.01$), although there was no significant effect of recovery time or interaction between recovery time and ether exposure. The highest IRI values were obtained in the animals exposed for 90 seconds to ether then allowed 30 seconds recovery, so this was used as the standard stimulus in later experiments.

2.3 I.P. injection of 36 mg kg^{-1} histamine

A preliminary experiment was performed to find a dose of histamine which caused hyperglycaemia in normal mice and which was not toxic to infected animals. A dose of 36 mg kg^{-1} (calculated as histamine base) was chosen as giving significant hyperglycaemia at 15 minutes after injection without causing death.

Treatment of mice with pertussis vaccine produces sensitisation to the lethal effects of histamine (Parfentjev and Schleyer, 1949) however, the standard challenge used in the histamine-sensitisation test is 3

mg per mouse (approximately 120 mg kg^{-1}). Therefore, the chosen dose of histamine was not expected to be toxic in pertussis-infected mice.

2.3.1 Experimental protocol

Histamine, 36 mg kg^{-1}, or phosphate-buffered saline (PBS) as a control, was injected I.P. into infected or non-infected mice. Groups of 10 animals were bled, without exposure to ether, either before, or at 5, 15 or 30 minutes after the injection. All animals given histamine were difficult to bleed at 30 minutes after injection, probably due to the haemodynamic changes caused by histamine (Ganong, 1979).

2.3.2 Serum glucose concentrations

Histamine produced a highly significant hyperglycaemic effect in normal animals ($P < 0.005$) which was attenuated in pertussis-infected animals (F for interaction pertussis and histamine = 34.49, $P < 0.01$). Infection state itself caused significant hypoglycaemia, as noted before.

The effect of histamine varied with time of sampling (Figure 2.3). (Interaction between histamine and time $P < 0.01$).

2.3.3 Serum IRI concentrations

Infection and histamine each had a significant effect on serum insulin concentrations (Figure 2.4). However, in non-infected animals histamine caused a

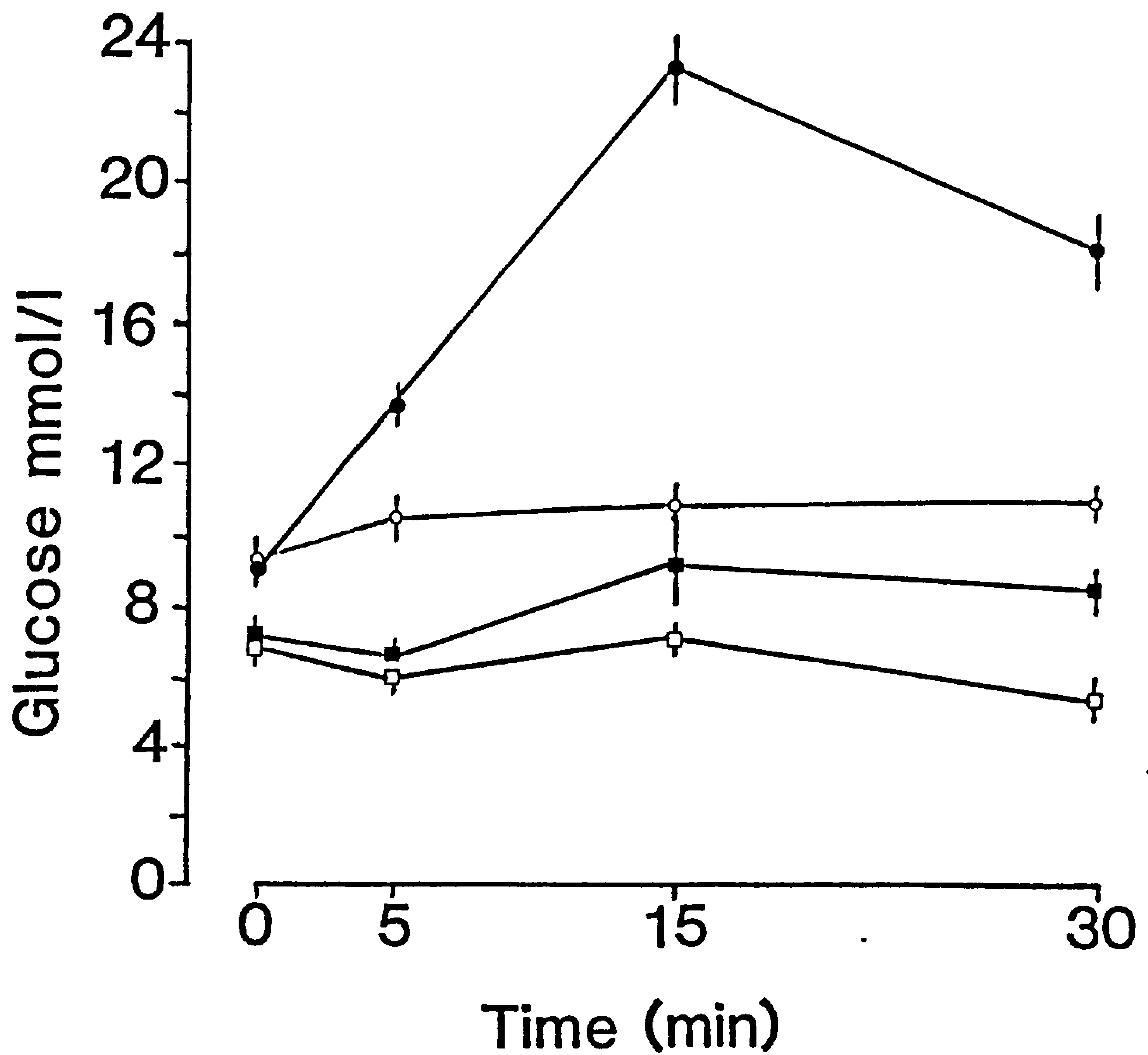


Figure 2.3

Effect of histamine on the serum glucose concentrations in control and B.pertussis infected mice.

Results given in mmol/l and expressed as arithmetic mean (S.E.). o control-saline, ● control-histamine, □ infected-saline, ■ infected-histamine.

Analysis of variance.

Source	D.F.	F.	P.
Infection	1	278.2	0.01
Histamine	1	99.81	0.005
Time	3	28.42	0.01
Infection/time	3	15.2	0.01
Histamine/time	3	20.4	0.01
Infection/histamine	1	34.49	0.01
Infect/hist/time	3	9.61	0.01

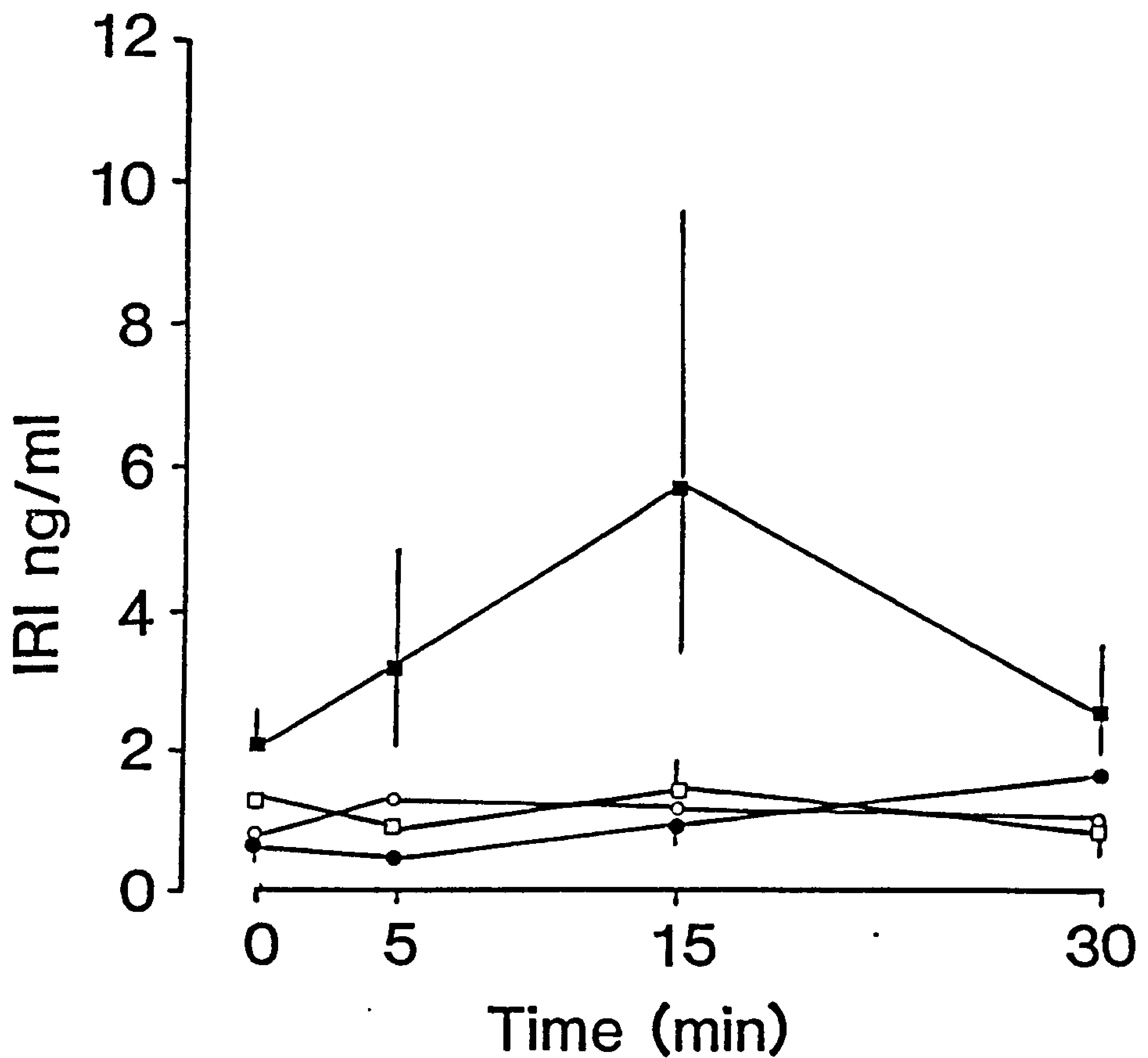


Figure 2.4

Effect of histamine on the serum IRI concentrations in control and B.pertussis infected mice.

Results given in ng/ml and expressed as geometric mean (95% C.L.). o control-saline, ● control-histamine, □ infected-saline, ■ infected-histamine.

Analysis of variance.

Source	D.F.	F.	P.
Infection	1	22.41	0.01
Histamine	1	15.55	0.01
Time	3	3.45	0.05
Infection/time	3	3.03	0.05
Histamine/time	3	2.09	0.05
Infection/histamine	1	23.26	0.01
Infect/hist/time	3	2.36	N.S.

slight reduction in IRI at 5 minutes, whereas in infected animals histamine caused a large increase in serum IRI which remained elevated even 30 minutes after injection. This is shown statistically as a significant interaction between infection state and histamine ($F = 23.26$, $P < 0.01$).

2.4 Exposure to cold

Pertussis vaccinated mice were shown to be more susceptible to the lethal effects of cold than the non-sensitised controls (Munoz and Schuchardt, 1957). Here serum glucose and insulin concentrations were measured in control and *B. pertussis* infected mice following a sublethal exposure to cold.

2.4.1 Experimental protocol

Groups of 9 infected or non-infected mice were exposed to a temperature of 2-4°C (mean 3.4 ± 0.2°C) in a container placed in a bath of melting ice with a chilled air supply. Animals used as a temperature control were housed in a similar container at room temperature.

Mice were bled by decapitation without anaesthetic after 30, 60, 120 or 180 minutes exposure to the appropriate temperature.

2.4.2 Serum glucose concentrations

As before, *B. pertussis* infected mice were significantly hypoglycaemic relative to non-infected mice (Figure 2.5). Exposure to cold produced a further

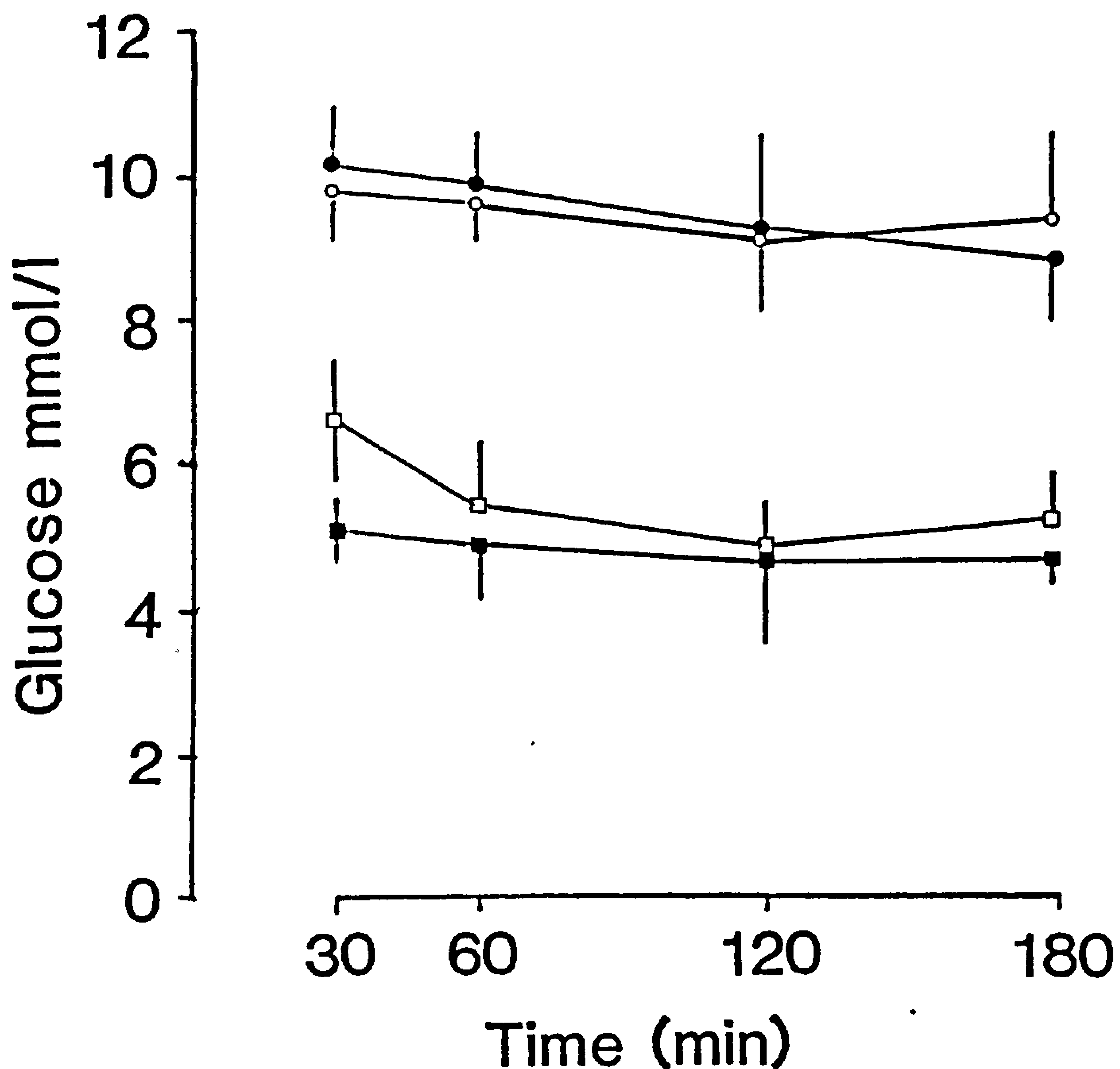


Figure 2.5

Effect of cold exposure on the serum glucose concentrations in control and B.pertussis infected mice.

Results given in mmol/l and expressed as arithmetic mean (S.E.). o control-room temperature, ● control-2 to 4°C, □ infected-room temperature, ■ infected-2 to 4°C.

Analysis of variance.

Source	D.F.	F.	P.
Infection	1	471.9	0.01
Cold	1	2.12	N.S.
Time	3	8.96	0.01
Infection/time	3	0.41	N.S.
Cold/time	3	1.09	N.S.
Infection/cold	1	5.21	0.05
Infect/cold/time	3	79.08	0.01

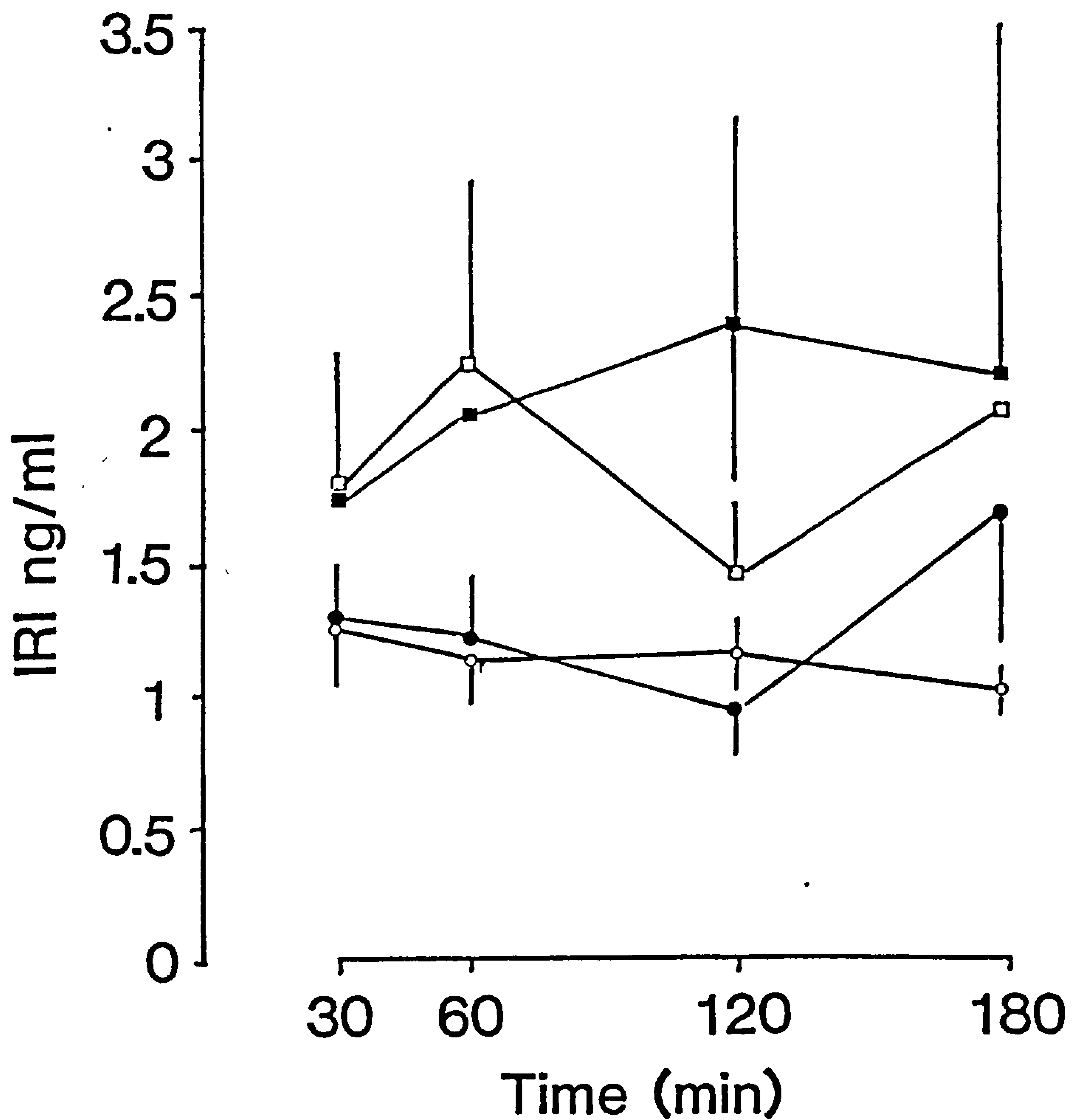


Figure 2.6

Effect of cold exposure on the serum IRI concentrations in control and B.pertussis infected mice.

Results given in ng/ml and expressed as geometric mean (95% C.L.). o control-room temperature, ● control-2 to 4°C, □ infected-room temperature, ■ infected-2 to 4°C.

Analysis of variance.

Source	D.F.	F.	P.
Infection	1	24.59	0.01
Cold	1	1.00	N.S.
Time	3	0.74	N.S.
Infection/time	3	0.62	N.S.
Cold/time	3	3.02	N.S.
Infection/cold	1	0.92	N.S.
Infect/cold/time	3	0.00	N.S.

lowering of serum glucose levels in B. pertussis infected mice which was absent in non-infected animals (F for interaction between cold and infection = 5.21, $P < 0.05$). Cold exposure did not produce a clear hyperglycaemic response in non-infected animals such as was seen after histamine or ether exposure (Figure 2.5).

2.4.3 Serum IRI concentrations

Exposure to cold produced no consistent effect on serum IRI concentrations in either infected or non-infected animals (Figure 2.6). Infected animals did however show hyperinsulinaemia relative to non-infected controls ($P < 0.01$).

2.5 I.V. injection of E. coli endotoxin 0111B4

Endotoxaemia is a stress factor which would be present in both B. pertussis infected and pertussis vaccinated subjects. B. pertussis liberates two lipopolysaccharides (LPS) similar to those of other gram-negative bacteria (Peppler, 1984), and these LPS are not destroyed during the production of conventional whole cell vaccine (Larter et al, 1984). Experimentally, the early phase of hyperinsulinaemia in mice following pertussis vaccination has been shown to be due to pertussis LPS (Hewlett et al, 1983) and could be mimicked by E. coli LPS.

2.5.1 Experimental protocol

E. coli endotoxin 0111B_4 (LPS) was dissolved in pyrogen free saline (PFS), sonicated for five minutes, then boiled for ten minutes to ensure even dispersion. Initially a pilot experiment was performed to find a dose of LPS which produced hyperglycaemia in normal mice. A dose of 0.5 mg kg^{-1} was selected, and was given I.V. at time 0 to non-infected or infected mice. Groups of eight mice were bled without anaesthetic before, and at 10, 30, 60, 120 and 180 minutes after LPS injection. PFS was used as treatment control, and LPS and PFS were given in 0.1 ml per 10 g body weight volumes.

2.5.2 Serum glucose concentrations

B. pertussis infection produced highly significant hypoglycaemia ($F = 456.1$, $P < 0.01$), as did administration of LPS ($F = 7.33$, $P < 0.01$). However, there was no significant difference in the responses of non-infected and infected animals to LPS injection (F for interaction = 0.045, N.S.) (Table 2.1).

2.5.3 Serum IRI concentrations

Infected animals showed significantly elevated serum IRI concentrations compared to non-infected animals. LPS itself did not have a statistically significant effect on serum IRI, but there was a significant interaction between LPS and infection state ($F = 3.603$, $P < 0.01$). This showed that infected and

Table 2.1

Effect of E.coli endotoxin (LPS) on the serum glucose concentrations of control and B.pertussis infected mice.

Time after LPS	Control		Infected	
	Saline	LPS	Saline	LPS
0	9.5 (0.89)	9.4 (1.33)	6.26 (0.79)	6.77 (1.10)
10	10.64 (1.25)	11.93 (1.08)	6.92 (1.70)	7.24 (2.79)
30	10.29 (0.92)	11.19 (1.69)	6.28 (1.31)	6.69 (1.62)
60	10.06 (1.00)	10.22 (1.32)	6.10 (1.11)	4.83 (0.94)
120	9.71 (1.11)	7.96 (0.63)	6.02 (1.11)	4.83 (0.81)
180	10.34 (0.79)	6.70 (1.01)	5.74 (0.76)	4.26 (0.82)

Analysis of variance.

Source	D.F.	F.	P.
Infection	1	456.1	0.01
LPS	1	7.33	0.01
Time	5	16.82	0.01
Infection/time	5	2.19	0.01
LPS/time	5	9.04	0.01
Infection/LPS	1	0.045	N.S.
Infection/LPS/time	5	2.22	0.01

Note: values given in mmol/l and expressed as arithmetic mean (S.E.)
N=8 for each treatment

Table 2.2

Effect of E.coli endotoxin (LPS) on the serum IRI concentrations of control and B.pertussis infected mice.

Time after LPS	Control Saline	Control LPS	Infected Saline	Infected LPS
0	1.08 (0.83,1.40)	1.27 (1.01,1.59)	2.00 (1.48,2.69)	1.41 (1.16,1.70)
10	0.82 (0.62,1.08)	0.99 (0.79,1.24)	1.07 (0.83,1.39)	1.00 (0.71,1.42)
30	0.78 (0.68,0.90)	0.80 (0.66,0.98)	1.36 (1.09,1.69)	1.10 (0.97,1.24)
60	1.28 (1.11,1.47)	0.95 (0.80,1.12)	1.65 (1.32,2.06)	1.17 (0.99,1.37)
120	0.91 (0.79,1.05)	1.90 (1.43,2.52)	2.54 (1.76,3.66)	2.01 (1.56,2.60)
180	1.37 (1.04,1.80)	0.89 (0.71,1.11)	2.23 (1.67,2.98)	1.99 (1.48,2.67)

Analysis of variance.

Source	D.F.	F.	P.
Infection	1	21.68	0.01
LPS	1	0.471	N.S.
Time	5	4.878	0.01
Infection/time	5	0.787	N.S
LPS/time	5	0.570	N.S.
Infection/LPS	1	3.603	0.01
Infection/LPS/time	5	0.301	N.S.

Note: values given in ng/ml and expressed as geometric mean (95% C.L.)
N=8 for each treatment.

normal animals differed in their response to LPS. From Table 2.2, it can be seèn that LPS caused a reduction in serum IRI in infected mice, but no change or an increase in serum IRI in non-infected animals.

2.6 2-Deoxyglucose (2-DG)

2-DG is a non metabolisable analogue of glucose which blocks glycolysis by competitive inhibition of phosphohexoseisomerase, and produces neuroglucopenia associated with hyperglycaemia (Frohman et al, 1973). Systemic administration of 2-DG leads to sympathetic activation and adrenaline release from the adrenal medulla (Brown and Bachrach, 1959; Hokfelt and Bydgeman, 1961).

Both 2-DG administration and insulin-induced hypoglycaemia result in central nervous system glucopenia, and produce similar counterregulatory hormonal responses (Asplin et al, 1985). These include activation of both parasympathetic and sympathetic nervous systems, stimulation of glucagon and pancreatic polypeptide secretion, inhibition of insulin secretion and release of growth hormone, prolactin and ACTH from the anterior pituitary (Asplin et al, 1985). Inhibition of insulin secretion by 2-DG has been demonstrated both *in vivo* (Kilo et al, 1962), and *in vitro* (Malaisse et al, 1967a) where it is due to inhibition of islet β-cell glucose metabolism.

2.6.1 Experimental protocol

2-DG at a dose of 400 mg kg^{-1} was injected I.V. to non-infected and infected mice 10 or 30 minutes before blood sampling. PBS was given as treatment control.

2.6.2 Serum glucose concentrations

Pertussis-infected animals were hypoglycaemic relative to non-infected animals as before (Figure 2.7). 2-DG produced marked hyperglycaemia in both normal and infected mice, but the increase caused by 2-DG in infected animals was not as great as that seen in normal mice (F for interaction between infection state and 2-DG = 27.34, $P < 0.01$).

2-DG cross-reacts with glucose when glucose oxidase methods are used to measure glucose (Marks and Rose, 1981). This was found to be the case for the method using the Beckman Glucose Analyser in the present work. Glucose values were also determined by the hexokinase assay method. Both the absolute values and the pattern of results obtained were the same, whether the hexokinase or the glucose oxidase method of determination was used.

2.6.3 Serum IRI concentrations

Injection of 2-DG produced a significant rise in serum IRI in non-infected mice (Figure 2.8), and this was augmented in pertussis-infected animals (F for interaction between infection state and 2-DG = 14.57, $P < 0.01$).

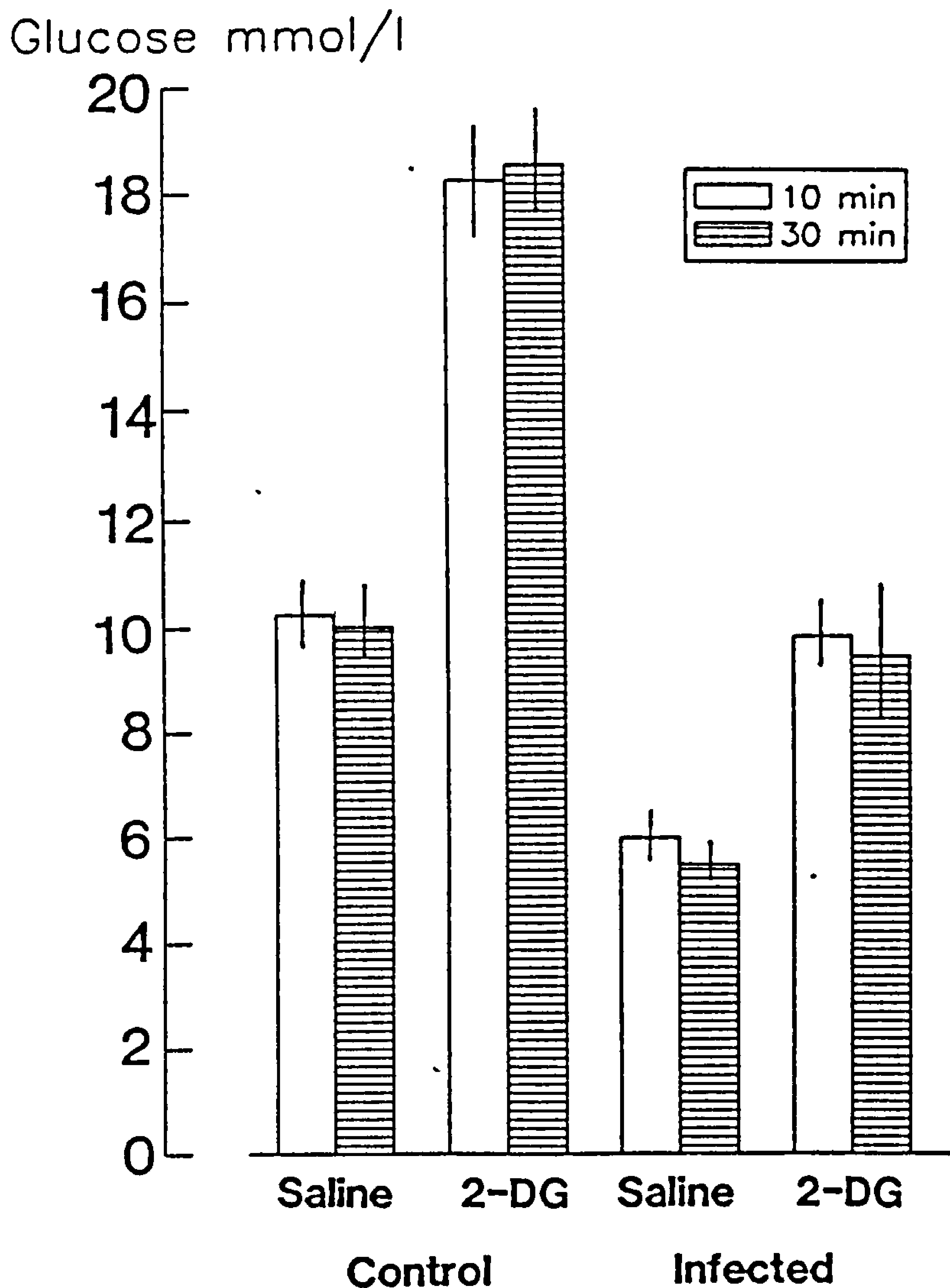


Figure 2.7

Effect of 2-deoxy glucose on the serum glucose concentrations of control and B.pertussis infected mice.

N= 11 for each treatment, values expressed as arithmetic mean (S.E.).

Analysis of variance.

Note: only significant factors shown.

Source	D.F.	F.	P.
Infection	1	253.3	0.01
2-DG	1	222.3	0.01
Infection/2-DG	1	27.34	0.01

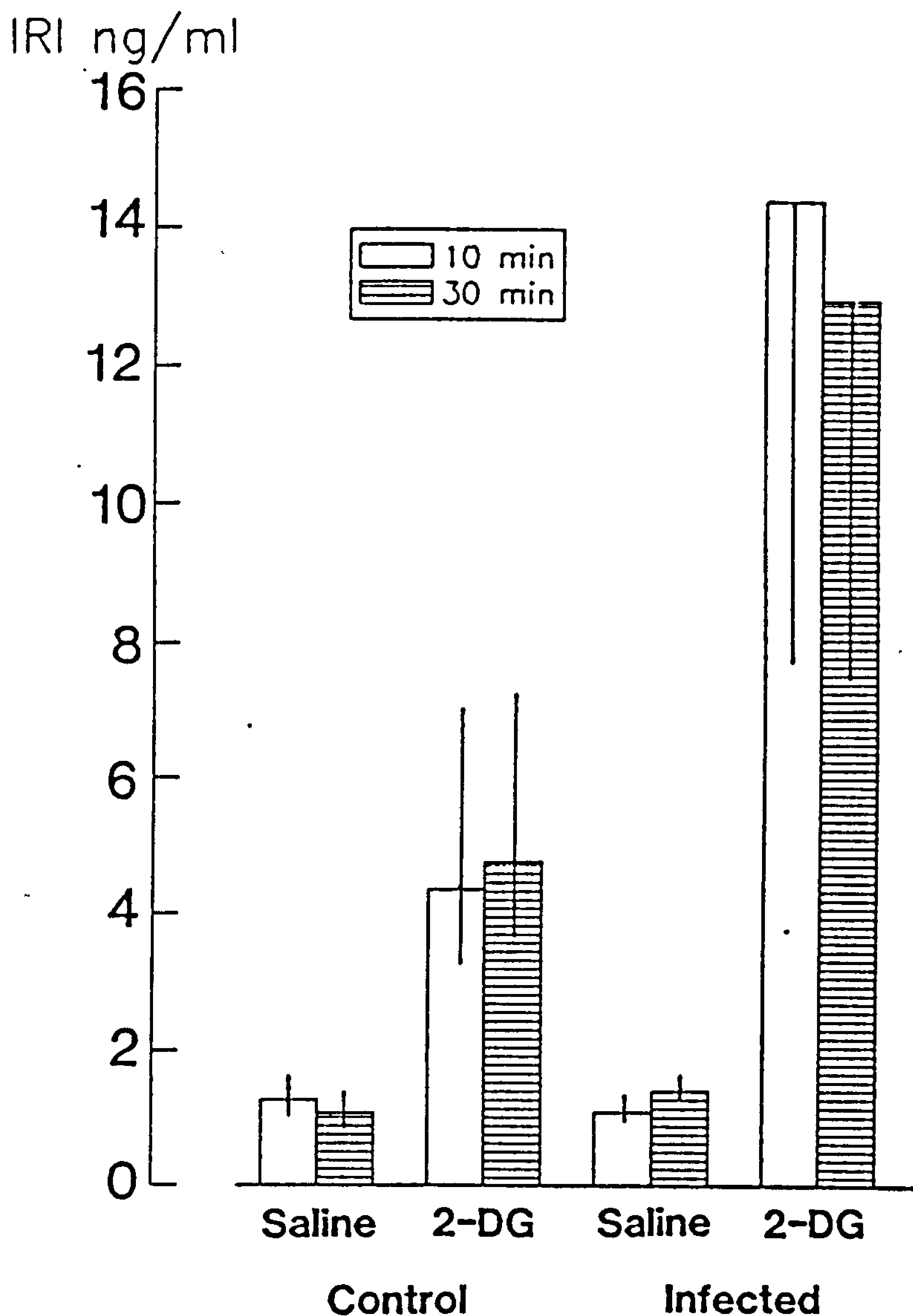


Figure 2.8

Effect of 2-deoxy glucose on the serum IRI concentrations in control and B.pertussis infected mice.

N= 11 for each treatment, values expressed as geometric mean (95% C.L.).

Analysis of variance.

Note: only significant factors shown.

Source	D.F.	F.	P.
Infection	1	18.15	0.01
2-DG	1	213.37	0.01
Infection/2-DG	1	14.57	0.01
Infection/2-DG/time	1	7.91	0.01

2.7 Exposure to 8% oxygen, 92% nitrogen (Hypoxia)

During the paroxysmal stage of the disease whooping cough, children are susceptible to coughing bouts during which cyanosis and apnoea can occur. Thus, cerebral anoxia has been implicated in the neurological effects of the disease (Olson, 1975; Cavanagh et al, 1981).

Hypoxia may also activate the adrenal medulla (Young et al, 1984) and inhibit insulin secretion in normal animals (Baum et al, 1979).

2.7.1 Experimental protocol

Non-infected and infected mice were exposed to an atmosphere of 8% O_2 and 92% N_2 (hypoxia), or air as a control. Atmos bags ^{R} (Sigma) were adapted to contain one mouse cage, and to allow addition and removal of animals without loss of atmosphere. Mice were exposed to the appropriate atmosphere for 5, 15, 30 or 60 minutes, then removed and bled without anaesthetic immediately.

2.7.2 Serum glucose concentrations

B. pertussis infected mice showed the expected hypoglycaemia relative to non-infected controls (Table 2.3), and this was further lowered by exposure to the hypoxic atmosphere. In non-infected animals, however, hypoxia also produced hypoglycaemia. There was no significant interaction between infection state and

Table 2.3

Effect of exposure to 8% oxygen, 92% nitrogen (hypoxia) on the serum glucose concentrations of control and B.pertussis infected mice.

Time in atmosphere	Control		Infected	
	Air	Hypoxia	Air	Hypoxia
5	10.39 (0.25)	8.74 (0.91)	7.08 (0.81)	6.35 (0.61)
15	10.33 (0.65)	8.60 (0.75)	8.19 (1.15)	6.51 (0.43)
30	10.53 (0.36)	9.08 (1.01)	7.42 (0.77)	5.27 (0.37)
60	10.05 (0.53)	8.80 (0.72)	8.19 (0.84)	5.89 (0.70)

Analysis of variance.

Source	D.F.	F.	P.
Infection	1	56.30	0.01
Hypoxia	1	20.22	0.01
Time	3	0.16	N.S.
Infection/time	3	0.67	N.S.
Hypoxia/time	3	0.16	N.S.
Infection/hypoxia	1	0.07	N.S.
Infection/hypoxia/time	3	0.37	N.S.

Note: values given in mmol/l and expressed as arithmetic mean (S.E.)
N=8 for each treatment

Table 2.4

Effect of exposure to 8% oxygen, 92% nitrogen (hypoxia) on the serum IRI concentrations of control and B.pertussis infected mice.

Time in atmosphere.	Control		Infected	
	Air	Hypoxia	Air	Hypoxia
5	1.99 (1.55,2.62)	1.70 (1.33,2.17)	1.23 (1.10,1.38)	1.91 (1.44,2.53)
15	1.99 (1.45,2.74)	1.42 (1.04,1.94)	1.62 (1.26,2.08)	1.45 (1.21,1.73)
30	1.34 (1.16,1.54)	1.33 (1.13,1.55)	1.43 (1.08,1.96)	3.28 (2.02,5.32)
60	1.41 (1.09,1.83)	1.79 (1.39,2.30)	1.87 (1.32,2.64)	1.07 (0.93,1.24)

Analysis of variance.

Source	D.F.	F.	P.
Infection	1	0.082	N.S.
Hypoxia	1	0.02	N.S.
Time	3	0.686	N.S.
Infection/time	3	0.53	N.S.
Hypoxia/time	3	0.64	N.S.
Infection/hypoxia	1	1.87	N.S.
Infect/hypox/time	3	3.64	0.05

Note: values given in ng/ml and expressed as geometric mean (95% C.L.)
N=8 for each treatment.

hypoxia.

2.7.3 Serum IRI concentrations

Neither infection nor hypoxia produced a significant change in serum IRI concentrations (Table 2.4). However, there was an increase in serum IRI in infected animals exposed to hypoxia for 30 minutes, which was not statistically significant.

A further experiment was performed to study this 30 minute time point in more detail.

2.8 30 Minute exposure to 8% oxygen

2.8.1 Experimental protocol

Infected and non-infected mice were exposed to 8% O_2, 92% N_2 for 30 minutes before bleeding without anaesthetic.

2.8.2 Serum glucose concentrations

The hypoglycaemia seen in B. *pertussis* infected mice was further lowered by a 30 minute period of hypoxia. In normal mice however, exposure to the hypoxic atmosphere produced a rise in serum glucose concentrations (Figure 2.9). This is reflected by the significant interaction between infection state and hypoxia ($F = 4.81$, $P < 0.05$).

2.8.3 Serum IRI concentrations

Neither infection state nor exposure to the hypoxic atmosphere produced a significant effect on serum IRI

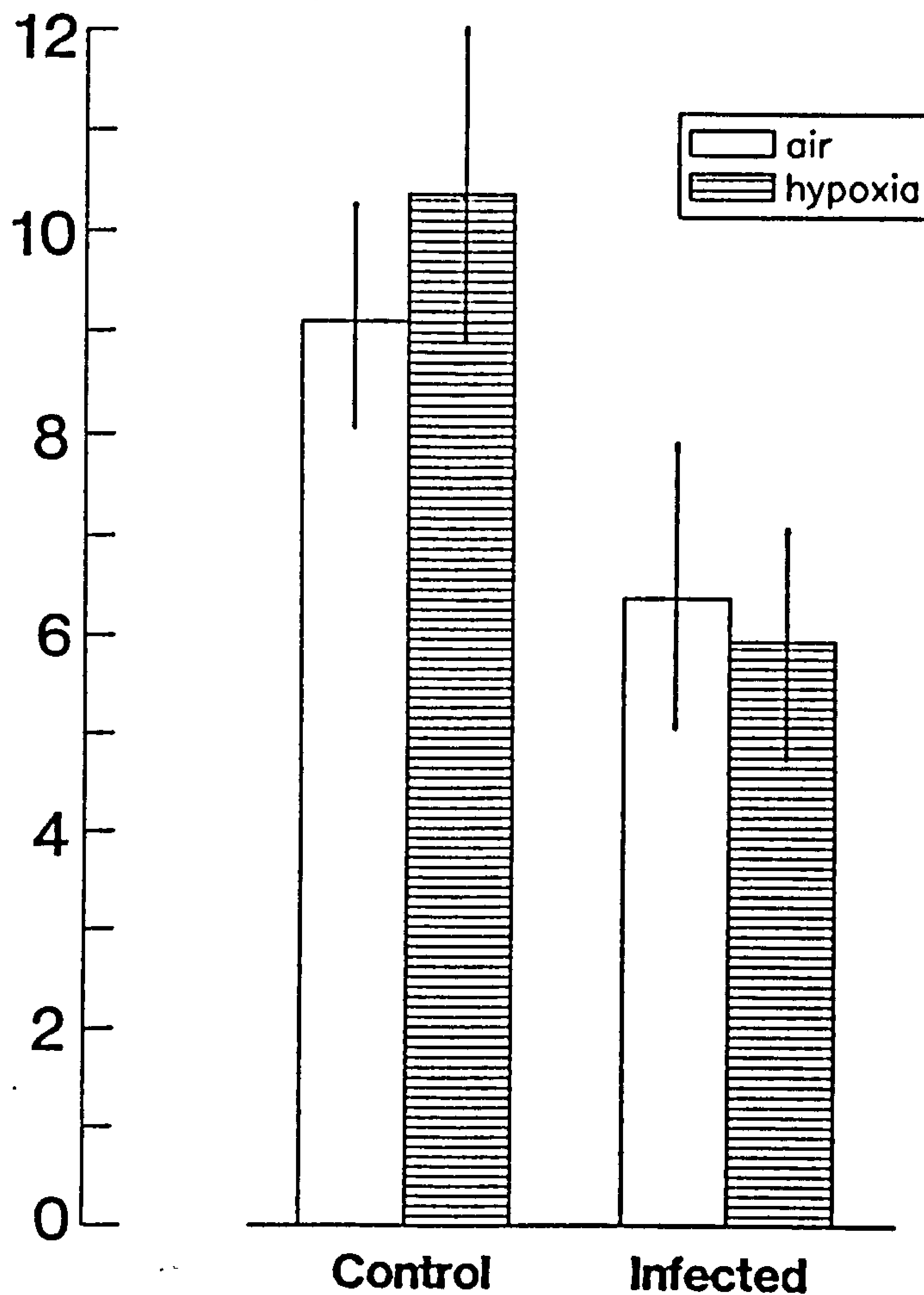


Figure 2.9

Effect of exposure to 8% oxygen for thirty minutes on the serum glucose concentrations of control and B.pertussis infected mice.

N= 12 for each treatment, values expressed as arithmetic mean (S.E.)

Analysis of variance.

Source	D.F	F.	P.
Infection	1	89.02	0.01
Hypoxia	1	1.09	N.S.
Interaction	1	4.81	0.05

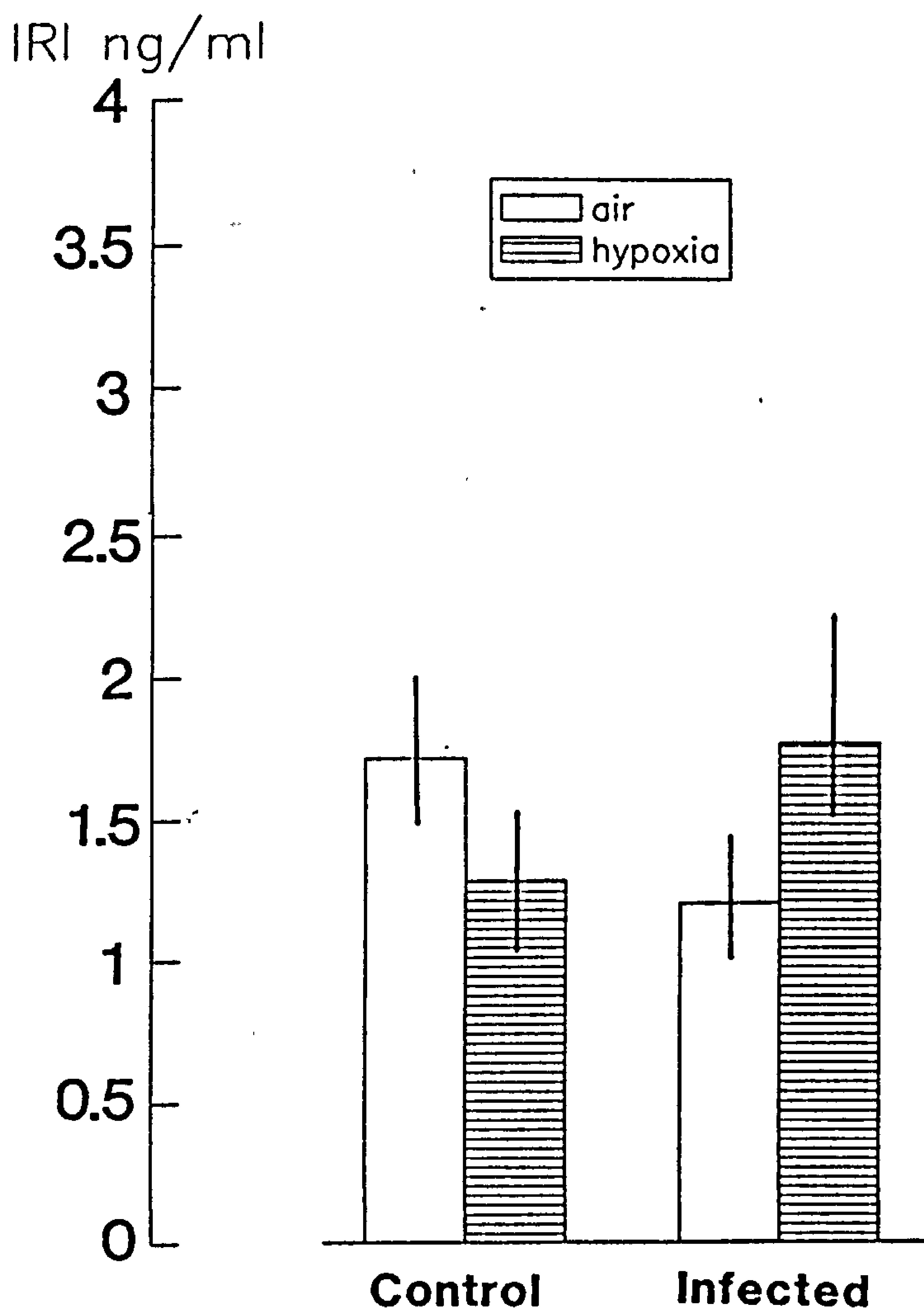


Figure 2.10

Effect of exposure to 8% oxygen for thirty minutes on the serum IRI concentrations of control and B.pertussis infected mice.

N= 12 for each treatment, values expressed as geometric mean (95% C.L.)

Analysis of variance.

Source	D.F.	F.	P.
Infection	1	0.061	N.S.
Hypoxia	1	0.007	N.S.
Interaction	1	3.019	N.S.

concentrations. There was no interaction between infection and hypoxia (Figure 2.10).

2.9 Exposure to 100% N_2 or 100% CO_2 (anoxic stimuli)

In the previous experiment, a chronic exposure to an hypoxic atmosphere was used as the stress. During exposure to the hypoxic atmosphere the animals appeared to acclimatise to the conditions, and returned to their normal behaviour. It was thought that the 8% O_2 might not have constituted a sufficiently marked stress. A brief exposure to anoxia (100% N_2 or 100% CO_2) was therefore chosen as a severe, short lasting stress.

2.9.1 Experimental protocol

Infected and non-infected mice were individually exposed to an atmosphere of air, 100% CO_2 or 100% N_2 for 15, 10 and 15 seconds respectively. The atmospheres were produced in lidded jars identical to those used for ether exposure, and were maintained with a constant flow of air from an aquarium pump, or N_2 or CO_2 from their respective cylinders.

Animals were removed from the jars and bled by decapitation either immediately, or after 5, 10 or 15 minutes recovery from gas exposure.

The experiment was later repeated with times of sampling being 60 or 150 seconds after removal from gas.

2.9.2 Serum glucose concentrations

B. pertussis infected mice were hypoglycaemic relative to non-infected mice (Table 2.5). Exposure to 100% CO_2 produced no overall significant change in glucose concentration ($F = 0.445$, N.S.). However, the pattern of glucose response varied with time after exposure (F for interaction between stress and time = 14.5, $P < 0.01$), with initial hyperglycaemia in both normal and infected animals, followed by a return to basal concentrations in normal mice. In infected mice, additional hypoglycaemia was seen 15 minutes after exposure to CO_2 (Table 2.5).

Exposure to 100% N_2 produced a gradual increase in serum glucose concentrations in normal animals, but a gradual decrease in infected animals (Table 2.5). However, neither of these effects were significantly different from the pattern of responses seen in mice exposed to air.

When the recovery period after removal from gas was shortened to 60 and 150 seconds, there was no significant alteration in serum glucose concentrations of nitrogen exposed animals, but hyperglycaemia was seen in both infected and non-infected mice after exposure to 100% CO_2 (Table 2.6).

2.9.3 Serum IRI concentrations

Exposure to 100% N_2 or 100% CO_2 did not significantly

Table 2.5

Effect of exposure to 100% nitrogen or 100% carbon dioxide on the serum glucose concentrations in control and B.pertussis infected mice.

Time after atmosphere	Air		Nitrogen		Carbon dioxide	
	control	infected	control	infected	control	infected
0	9.2 (0.27)	6.27 (0.55)	8.56 (0.32)	6.02 (0.51)	8.99 (0.21)	5.80 (0.44)
5	9.32 (0.27)	6.38 (0.45)	9.94 (0.48)	6.36 (0.33)	12.11 (0.48)	8.88 (0.67)
10	10.17 (0.50)	5.95 (0.77)	10.65 (0.51)	5.49 (0.39)	9.57 (0.27)	5.42 (0.53)
15	10.44 (0.35)	5.96 (0.15)	10.92 (0.62)	4.92 (0.77)	10.05 (0.37)	4.08 (0.37)

Note: Values given in mmol/l and expressed as arithmetic mean (S.E.)
N=6 for each treatment.

Table 2.6

Effect of exposure to 100% nitrogen or 100% carbon dioxide on the serum glucose concentrations of control and B.pertussis infected mice (II).

Treatment	Time (sec)	Air	Nitrogen	Carbon dioxide
Control	60	10.04 (0.12)	10.95 (0.28)	11.25 (0.45)
Control	150	10.08 (0.44)	10.59 (0.40)	11.68 (0.33)
Infected	60	6.21 (0.23)	5.68 (0.24)	6.76 (0.59)
Infected	150	6.25 (0.56)	7.10 (0.66)	8.18 (0.66)

Note: values given in mmol/l and expressed as arithmetic mean (95% C.L.)
N=8 for each treatment.

alter the serum IRI concentrations after 5, 10 or 15 minutes of recovery (Table 2.7). However, it appeared that B. pertussis infected mice showed hyperinsulinaemia at 5 minutes after exposure to both CO_2 or N_2. It was possible that the large confidence limits found at this time point were due to an earlier, marked hyperinsulinaemia which the sampling protocol had missed. Therefore the earlier sampling points of 60 and 150 seconds were chosen for further study.

The serum IRI concentrations in normal animals were not altered by the brief exposure to N_2 or CO_2 at 60 or 150 seconds after exposure (Table 2.8). However, in infected mice a very marked hyperinsulinaemia was detected at both time points after exposure to 100% CO_2 (F for interaction between CO_2 and infection = 20.19, $P < 0.01$), and to 100% N_2 (F for interaction between N_2 and infection = 32.10, $P < 0.01$) (Table 2.8). In both cases, the hyperinsulinaemia was more marked at 60 seconds compared with 150 seconds, showing that the hyperinsulinaemic response to these anoxic stimuli is transient.

2.10 Effect of alloxan on the metabolic changes induced by ether exposure

In Figure 1.1 it was shown that alloxan diabetes prevented the hypoglycaemia seen in pertussis-infected mice. In order to determine whether the stress-induced hyperinsulinaemia seen in pertussis-infected animals was

Table 2.7

Effect of exposure to 100% nitrogen or 100% carbon dioxide on the serum IRI concentrations in control and B.pertussis infected mice.

Time after atmosphere	Air control	Air infected	Nitrogen control	Nitrogen infected	Carbon dioxide control	Carbon dioxide infected
0	1.32 (1.04) (1.67)	2.51 (1.45) (4.35)	0.97 (0.84) (1.12)	2.52 (1.42) (4.50)	1.87 (1.21) (2.89)	2.17 (1.30) (3.62)
5	1.15 (0.79) (1.67)	3.06 (1.67) (5.63)	0.94 (0.63) (1.40)	3.68 (1.85) (7.32)	3.17 (1.63) (6.17)	9.24 (2.97) (28.7)
10	1.43 (1.07) (1.93)	1.61 (1.10) (2.36)	1.30 (0.66) (2.55)	1.58 (1.09) (2.29)	0.73 (0.57) (0.92)	1.93 (1.19) (3.15)
15	1.52 (1.14) (2.02)	2.21 (1.30) (3.71)	1.05 (0.78) (1.41)	1.25 (0.98) (1.60)	0.75 (0.54) (1.04)	1.60 (1.21) (2.11)

Note: values given in ng/ml and expressed as geometric mean (95% C.L.)
N=6 for each treatment.

Table 2.8

Effect of exposure to 100% nitrogen or 100% carbon dioxide on the serum IRI concentrations of control and B.pertussis infected mice (II).

Treatment	Time (sec)	Air	Nitrogen	Carbon dioxide
Control	60	0.82 (0.74,0.91)	1.80 (1.39,2.33)	3.51 (2.27,5.44)
Control	150	1.09 (0.98,1.22)	1.46 (1.20,1.77)	7.53 (4.10,13.84)
Infected	60	1.31 (1.13,1.52)	21.74 (8.82,53.6)	47.21 (15.9,140.1)
Infected	150	1.32 (1.10,1.58)	8.92 (4.72,16.9)	16.79 (1.53,37.4)

Note: values given in ng/ml and expressed as geometric mean (95% C.L.)
N=8 for each treatment.

of pancreatic origin, alloxan-diabetic animals were used in an ether-stress experiment.

2.10.1 Experimental protocol

Infected mice received alloxan (80 mg kg^{-1} I.V.) 48 hours before the experiment. Mice were bled by decapitation after 60 seconds exposure to ether vapour, or to air (control).

Mice were accepted as being diabetic if their serum glucose values were higher than two standard deviations above the mean value for non-alloxanised, infected mice (see results section 1.2).

2.10.2 Serum glucose concentrations

Alloxan-treated mice were markedly hyperglycaemic relative to infected-non diabetic animals ($F = 620.1$, $P < 0.01$) (Figure 2.11). Ether had no significant effect on the serum glucose values of either group.

2.10.3 Serum IRI concentrations

Alloxan-diabetic mice had very low serum IRI concentrations, and serum IRI was not elevated by ether exposure in these mice. However, the expected increase in the non diabetic-infected mice was seen after ether exposure (F for interaction between ether and alloxan = 21.75, $P < 0.01$) (Figure 2.12).

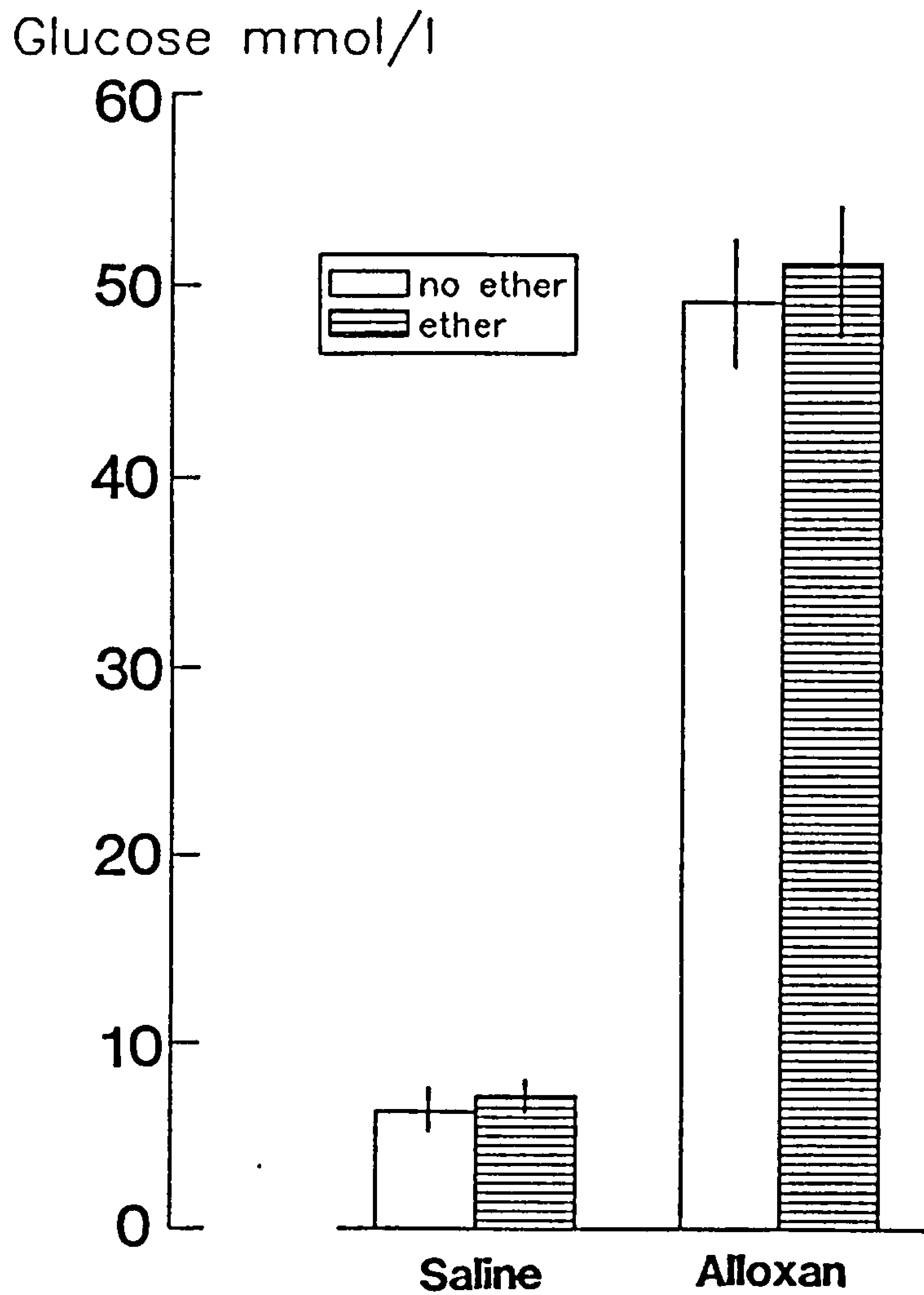


Figure 2.11.

Effect of alloxan diabetes on the serum glucose response to ether exposure in B.pertussis infected mice.

N= 12 for each treatment, values expressed as arithmetic mean (S.E.).

Analysis of variance.

Source	D.F.	F.	P.
Alloxan	1	620.1	0.01
Ether	1	1.20	N.S.
Interaction	1	0.38	N.S.

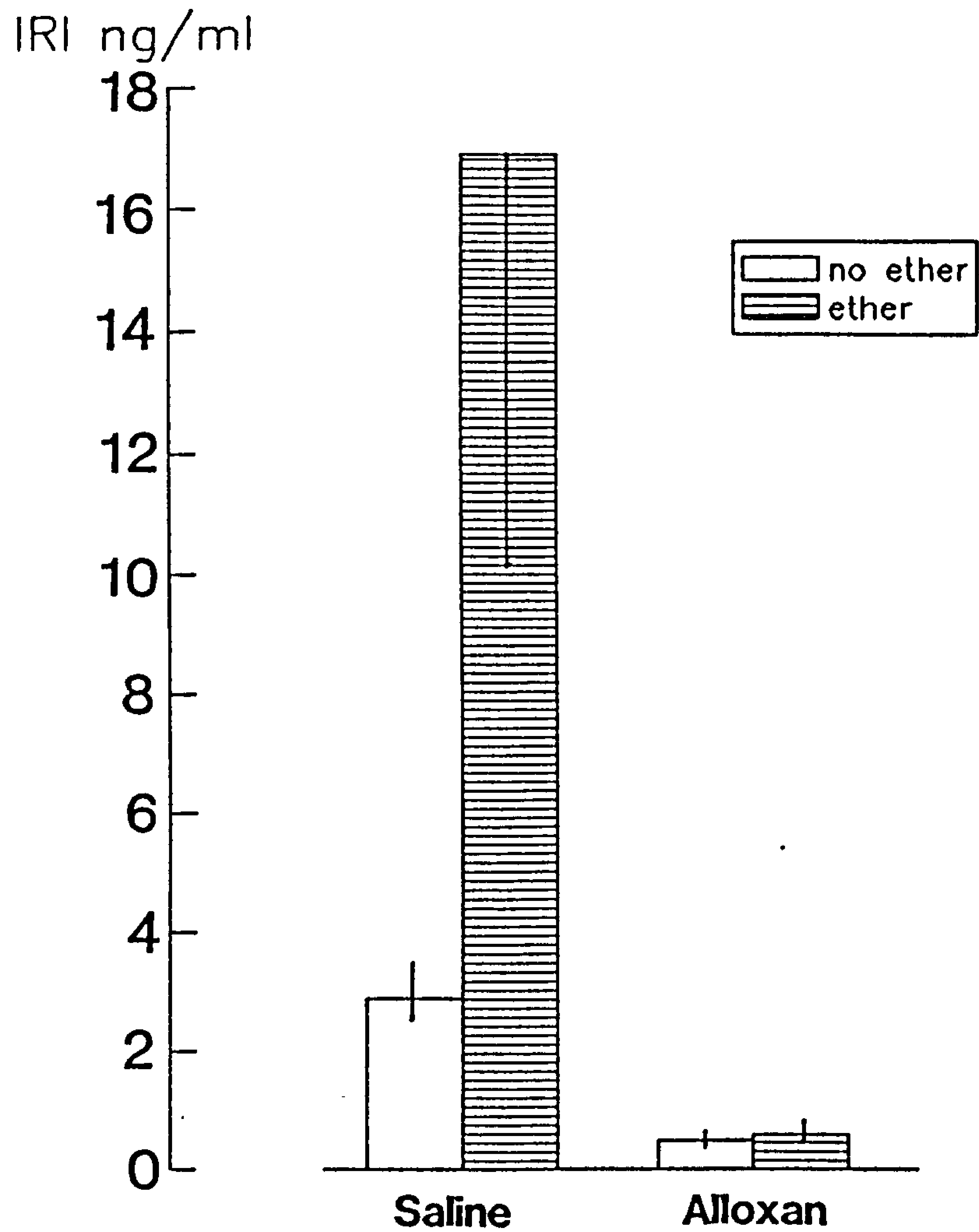


Figure 2.12.

Effect of alloxan diabetes on the serum IRI response to ether exposure in B.pertussis infected mice.

N= 12 for each treatment, values expressed as geometric mean (95% C.L.).

Analysis of variance.

Source	D.F.	F.	P.
Alloxan	1	36.15	0.01
Ether	1	22.41	0.01
Interaction	1	21.75	0.01

2.11 Effect of ether stress on pertussis toxin-treated mice

Pertussis toxin, otherwise known as islet activating protein, is the product of B. pertussis thought to be responsible for the long lasting metabolic changes brought about by both pertussis vaccine, and pertussis infection (Pittman, 1979; Katada and Ui, 1981; Hewlett et al, 1983). The toxin was purified by Dr. Y. Perera at Glasgow University Microbiology Department (Perera et al, 1985), and this preparation was used to investigate the effect of ether stress on pertussis toxin-treated mice.

2.11.1 Experimental protocol

Mice were injected with P T (150 ng per mouse) or saline intravenously, five days before the experiment. Animals were bled by decapitation after 60 seconds exposure to air or ether vapour. The experiment was performed in three, five, six and seven week old mice.

2.11.2 Serum glucose concentrations

PT produced significant hypoglycaemia in mice of all ages, and the degree of hypoglycaemia was not affected by exposure to ether vapour (Figure 2.13) (P value for interaction between ether and PT not significant for all ages).

2.11.3 Serum IRI concentrations

PT increased serum IRI concentrations in all ages of

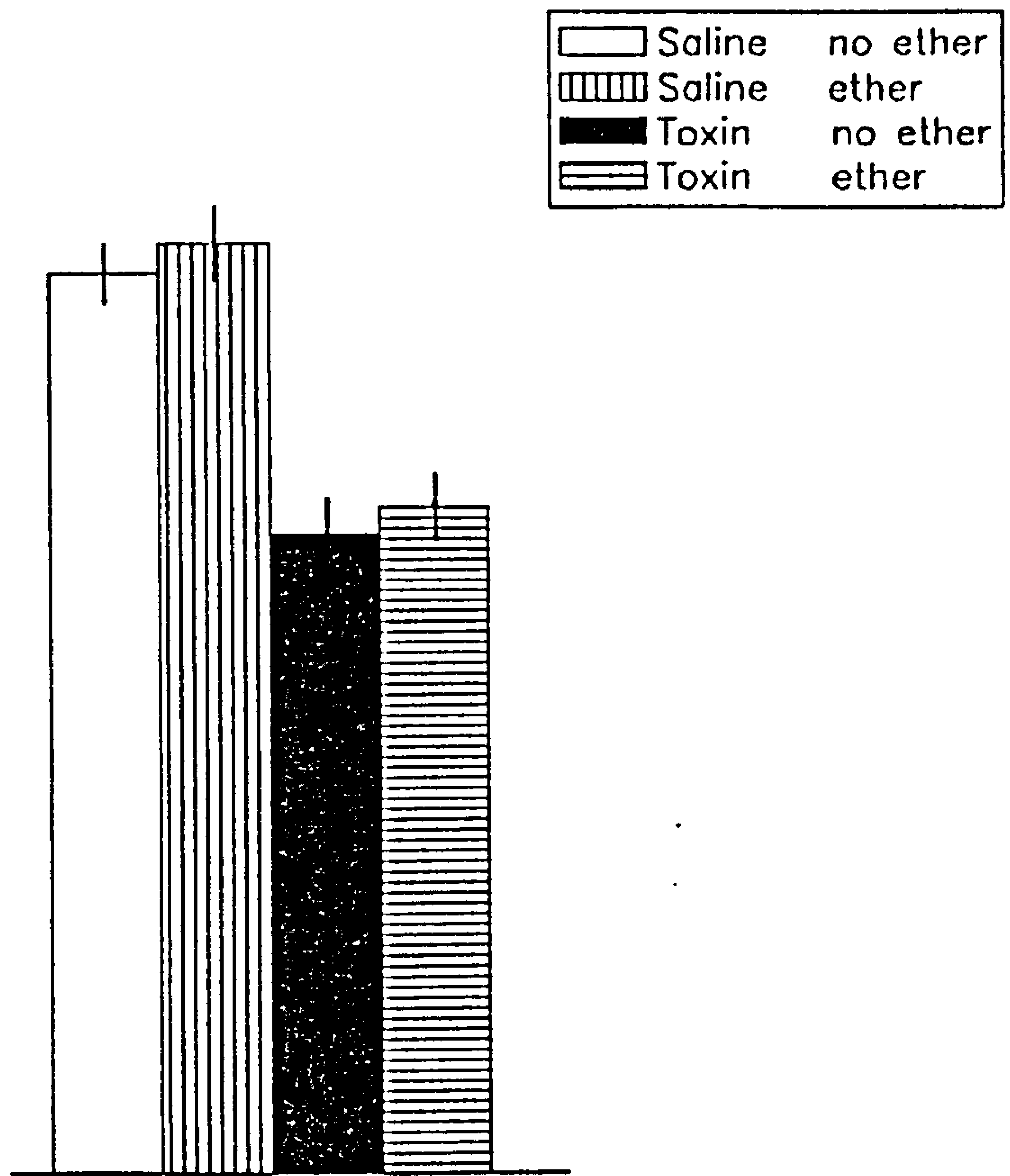


Figure 2.13.a

Hypoglycaemic effect of pertussis toxin in three week old mice.

N= 12 for each treatment, values expressed as arithmetic mean (S.E.).

Analysis of variance.

Source.	D.F.	F.	P.
Pertussis toxin	1	102.15	0.01
Ether	1	1.07	N.S.
Interaction	1	0.000	N.S.

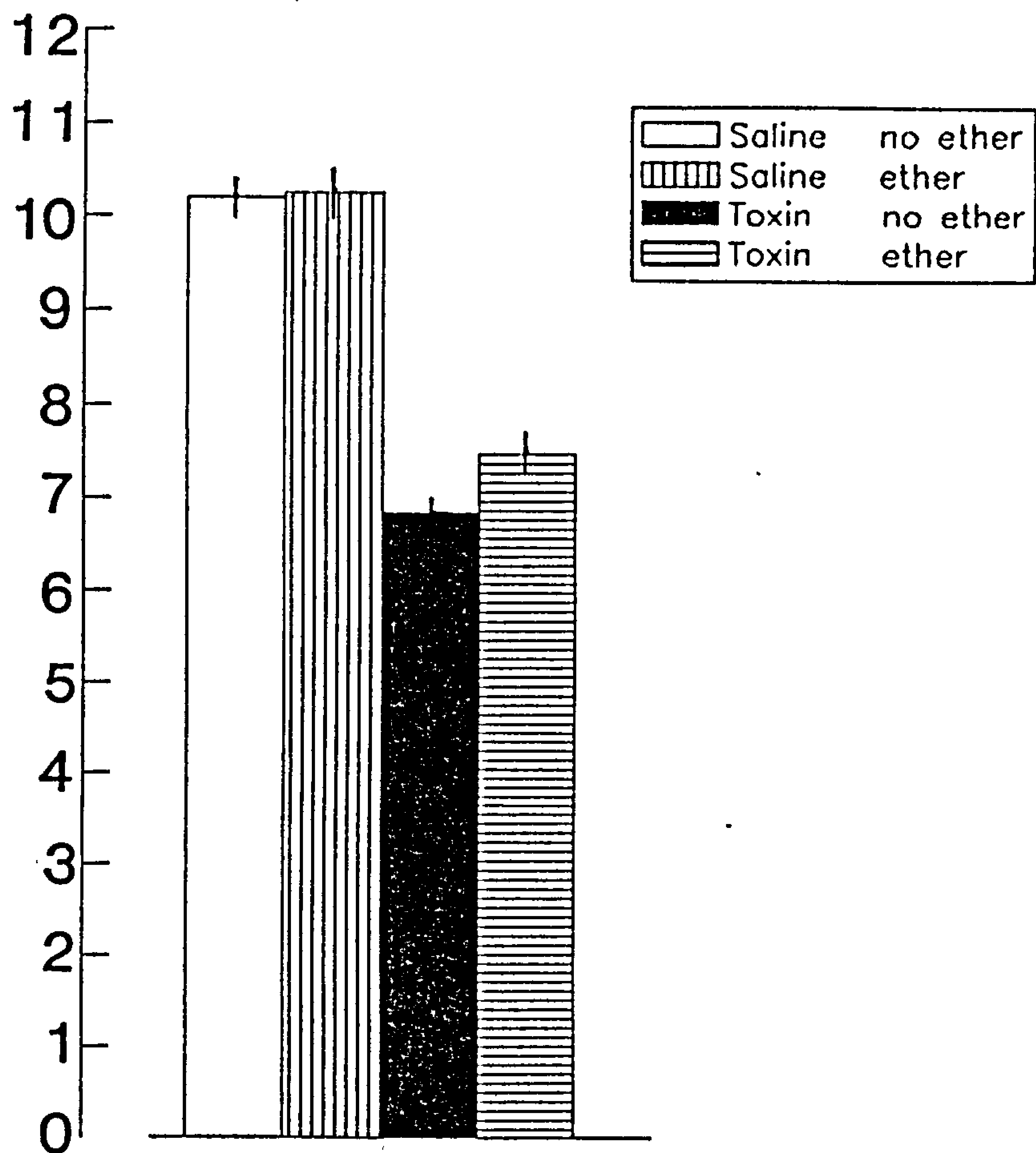


Figure 2.13.b

Hypoglycaemic effect of pertussis toxin in five week old mice.

N= 12 for each treatment, values expressed as arithmetic mean (S.E.).

Analysis of variance.

Source.	D.F.	F.	P.
Pertussis toxin	1	260.92	0.01
Ether	1	3.35	N.S.
Interaction	1	2.18	N.S.

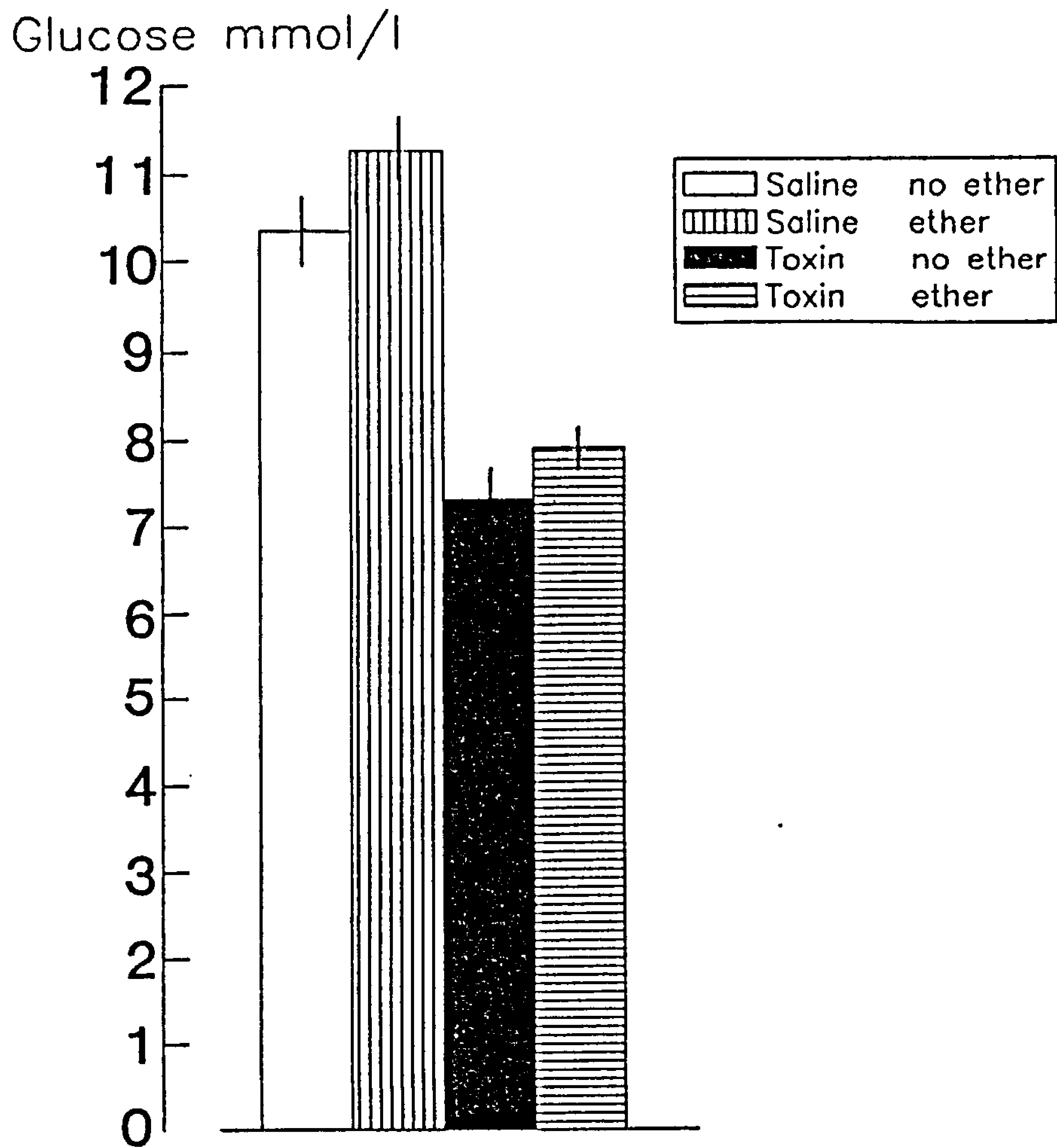


Figure 2.13.c

Hypoglycaemic effect of pertussis toxin in six week old mice.

N= 12 for each treatment, values expressed as arithmetic mean (S.E.).

Analysis of variance.

Source.	D.F.	F.	P.
Pertussis toxin	1	320.86	0.01
Ether	1	17.86	0.01
Interaction	1	0.88	N.S.

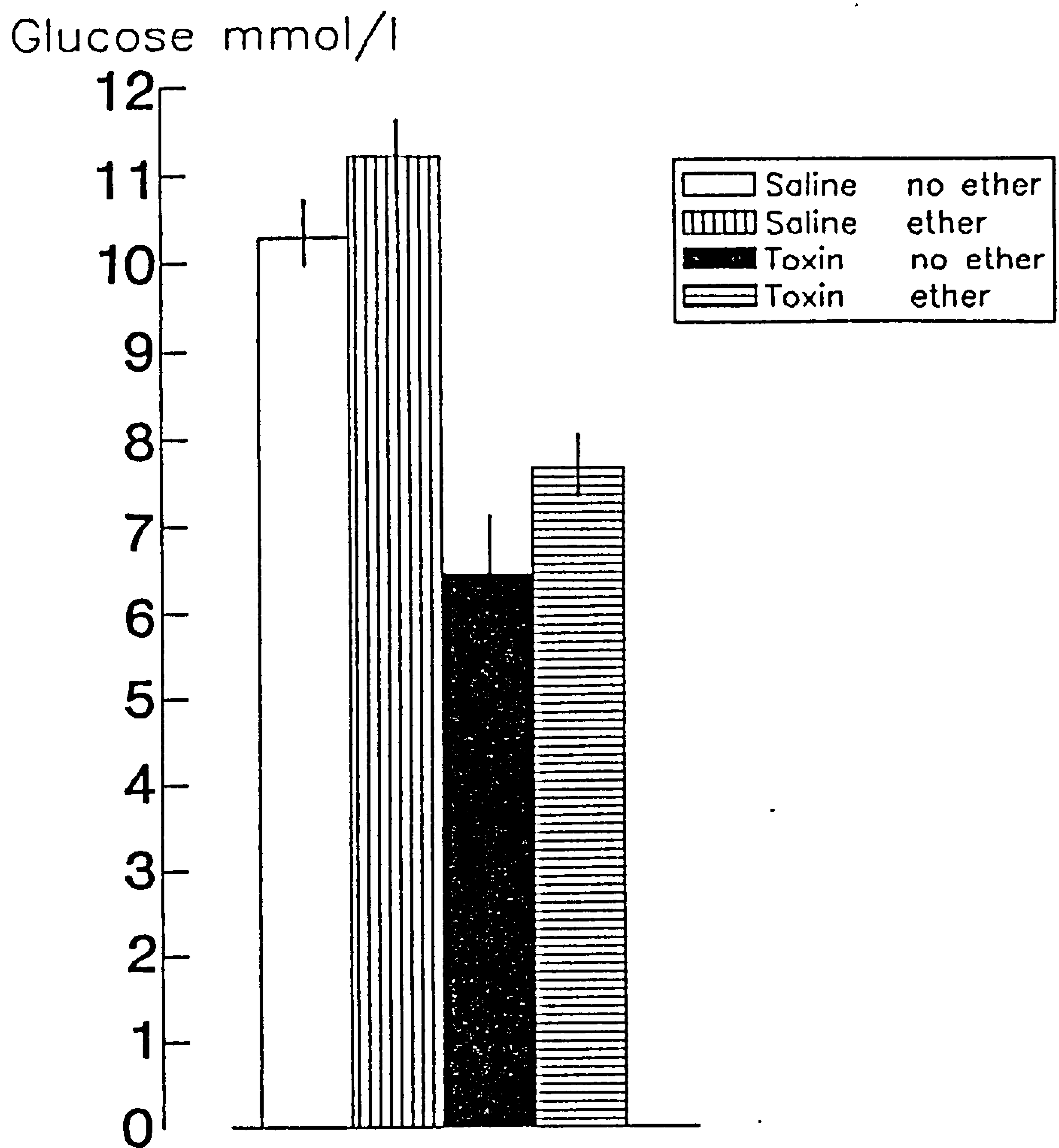


Figure 2.13.d

Hypoglycaemic effect of pertussis toxin in seven week old mice.

N= 12 for each treatment, values expressed as arithmetic mean (S.E.).

Analysis of variance.

Source.	D.F.	F.	P.
Pertussis toxin	1	194.74	0.01
Ether	1	10.86	0.01
Interaction	1	0.07	N.S.

mice ($P < 0.01$) (Figure 2.14).

Ether exposure did not affect the serum IRI concentrations in normal mice, but produced significant hyperinsulinaemia in PT treated animals of all ages (F for interaction between toxin and ether = 10.70 (3 week), 9.85 (5 week), 12.91 (6 week), 17.24 (7 week); $P < 0.01$).

The maximum values for IRI concentration in ether exposed, PT treated mice increased as the age of the mouse increased, to a maximum at 6-7 weeks of age (Figure 2.14). This is interesting since the development of histamine sensitisation following PT (or pertussis whole cell vaccine) is also dependent upon the age of the animal. In this case, histamine sensitisation only develops in animals of at least 8 weeks of age.

2.11.4 Effect of heat treatment of pertussis toxin

The activity of pertussis toxin can be destroyed by heating at 80°C for 30 minutes (Wardlaw and Parton, 1983b). The hypoglycaemic effect of pertussis toxin and heated pertussis toxin was investigated, and the results are shown in Figure 2.15. Pertussis toxin produced significant hypoglycaemia compared to saline-treated controls, and this was prevented by heat treatment of the toxin (Figure 2.15). The hyperinsulinaemic effect of ether was markedly reduced in mice treated with heated pertussis toxin (Figure 2.16).

2.11.5 Weight changes during PT sensitisation

The body weights of the mice used were recorded

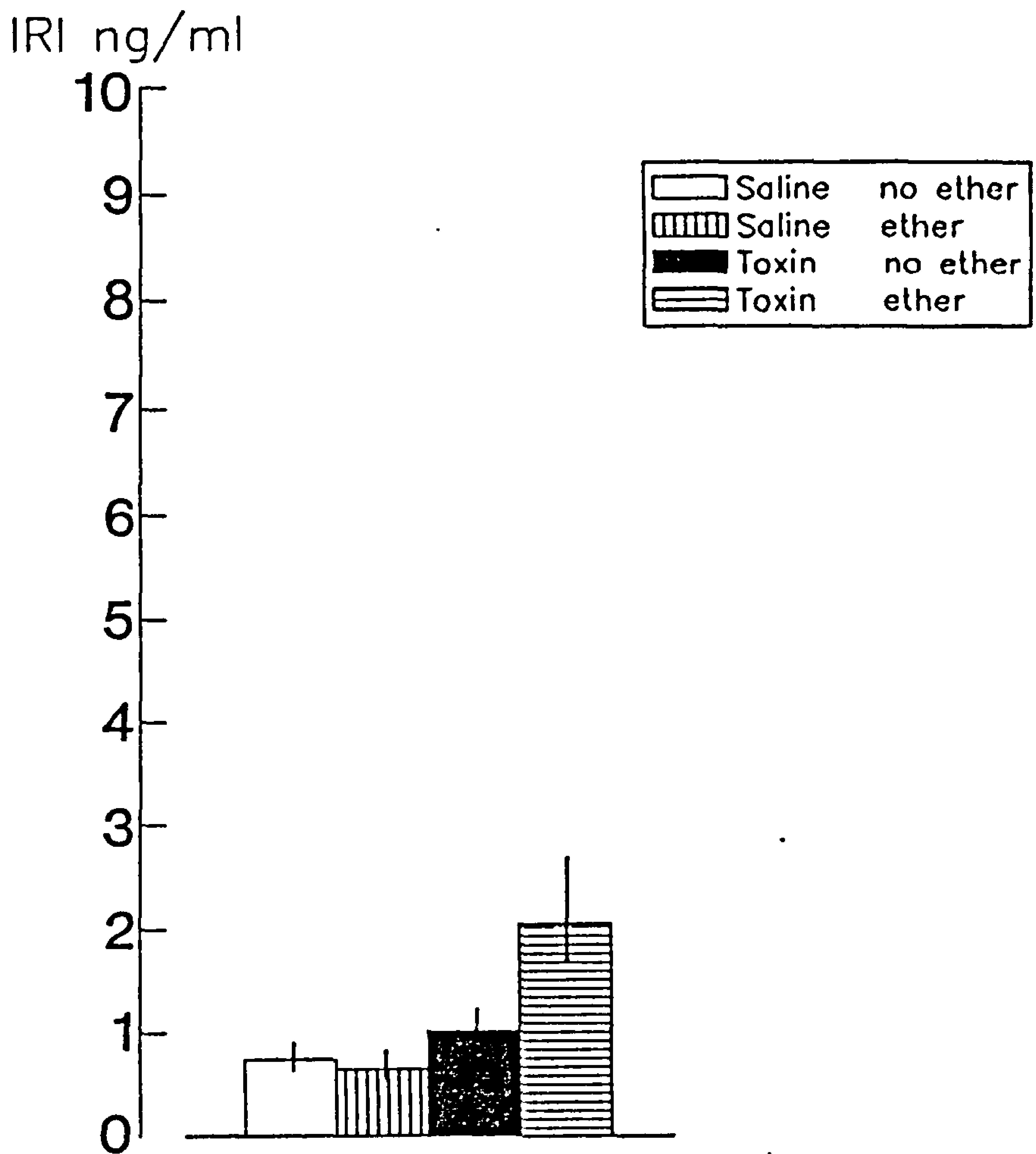


Figure 2.14.a

Hyperinsulinaemic effect of ether in three week old mice treated with pertussis toxin.

N= 12 for each treatment, values expressed as geometric mean (95% C.L.).

Analysis of variance.

Source.	D.F.	F.	P.
Pertussis toxin	1	0.66	N.S.
Ether	1	0.09	N.S.
Interaction	1	10.70	0.01

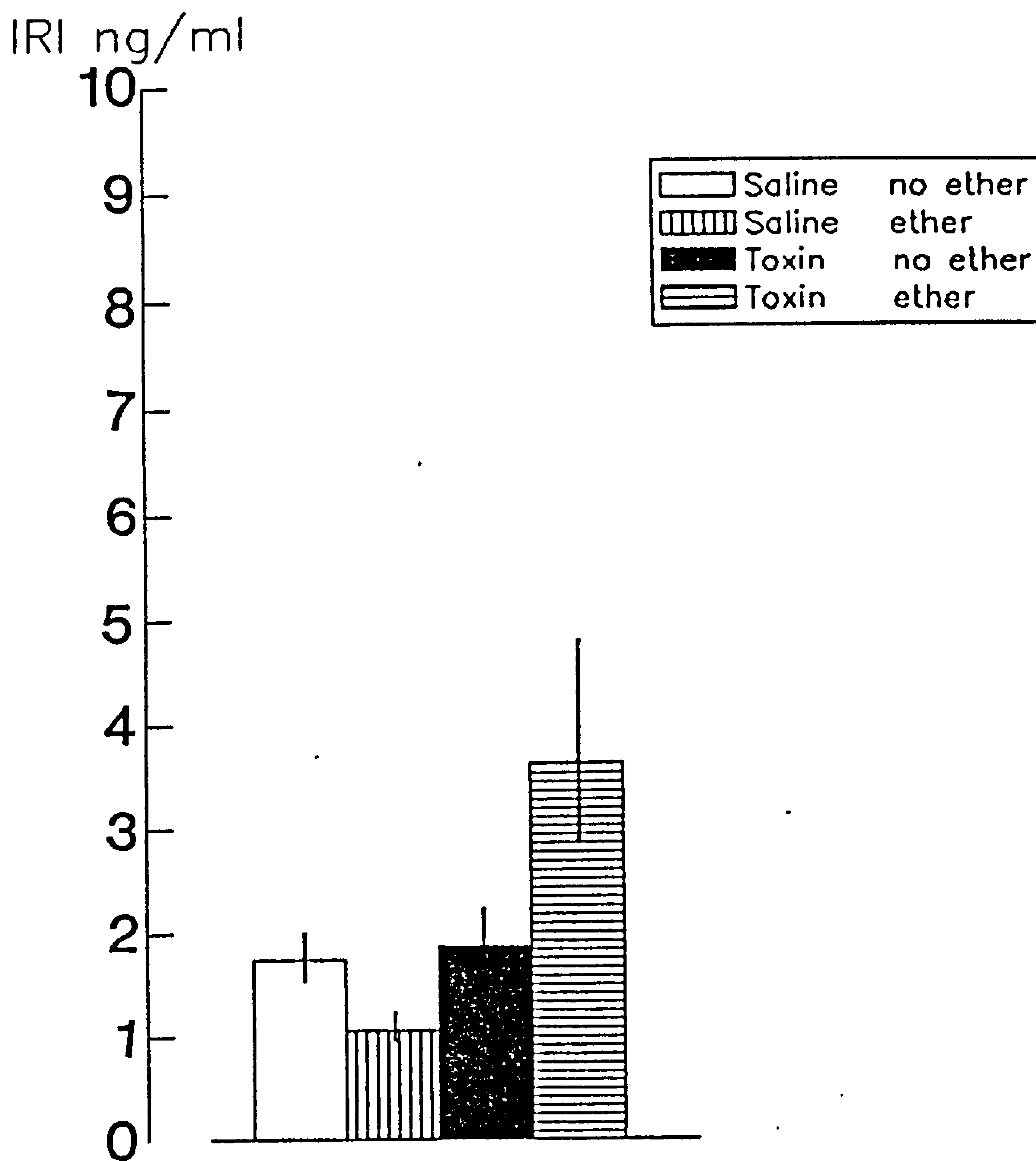


Figure 2.14.b

<u>Hyperinsulinaemic effect of ether in five week old mice treated with pertussis toxin.</u>

N= 12 for each treatment, values expressed as geometric mean (95% C.L.).

<u>Analysis of variance.</u>

Source.	D.F.	F.	P.
Pertussis toxin	1	12.77	0.01
Ether	1	1.83	N.S.
Interaction	1	9.85	0.01

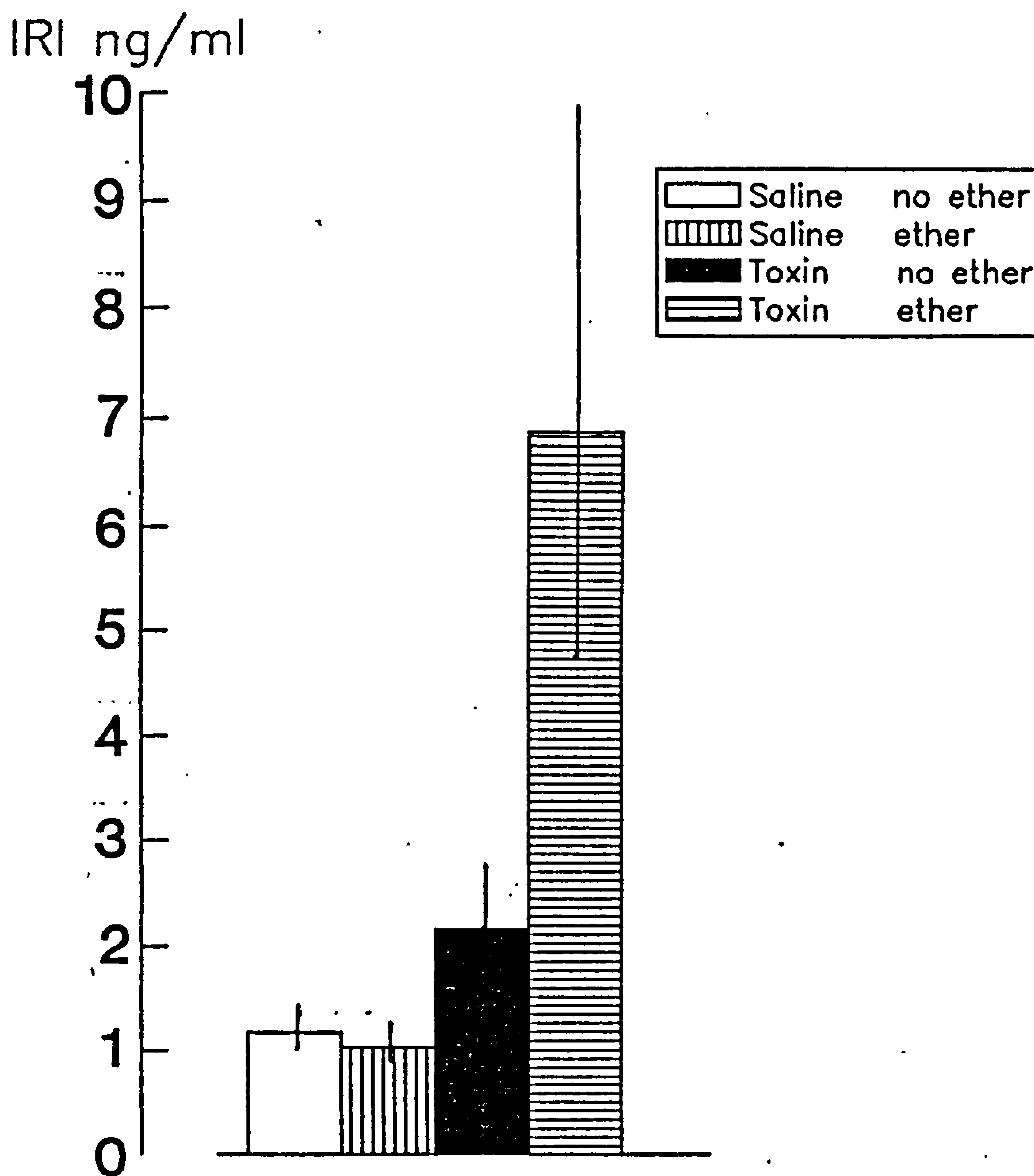


Figure 2.14.c

Hyperinsulinaemic effect of ether in six week old mice treated with pertussis toxin.

N= 12 for each treatment, values expressed as geometric mean (95% C.L.).

Analysis of variance.

Source.	D.F.	F.	P.
Pertussis toxin	1	47.19	0.01
Ether	1	8.64	0.05
Interaction	1	12.91	0.01

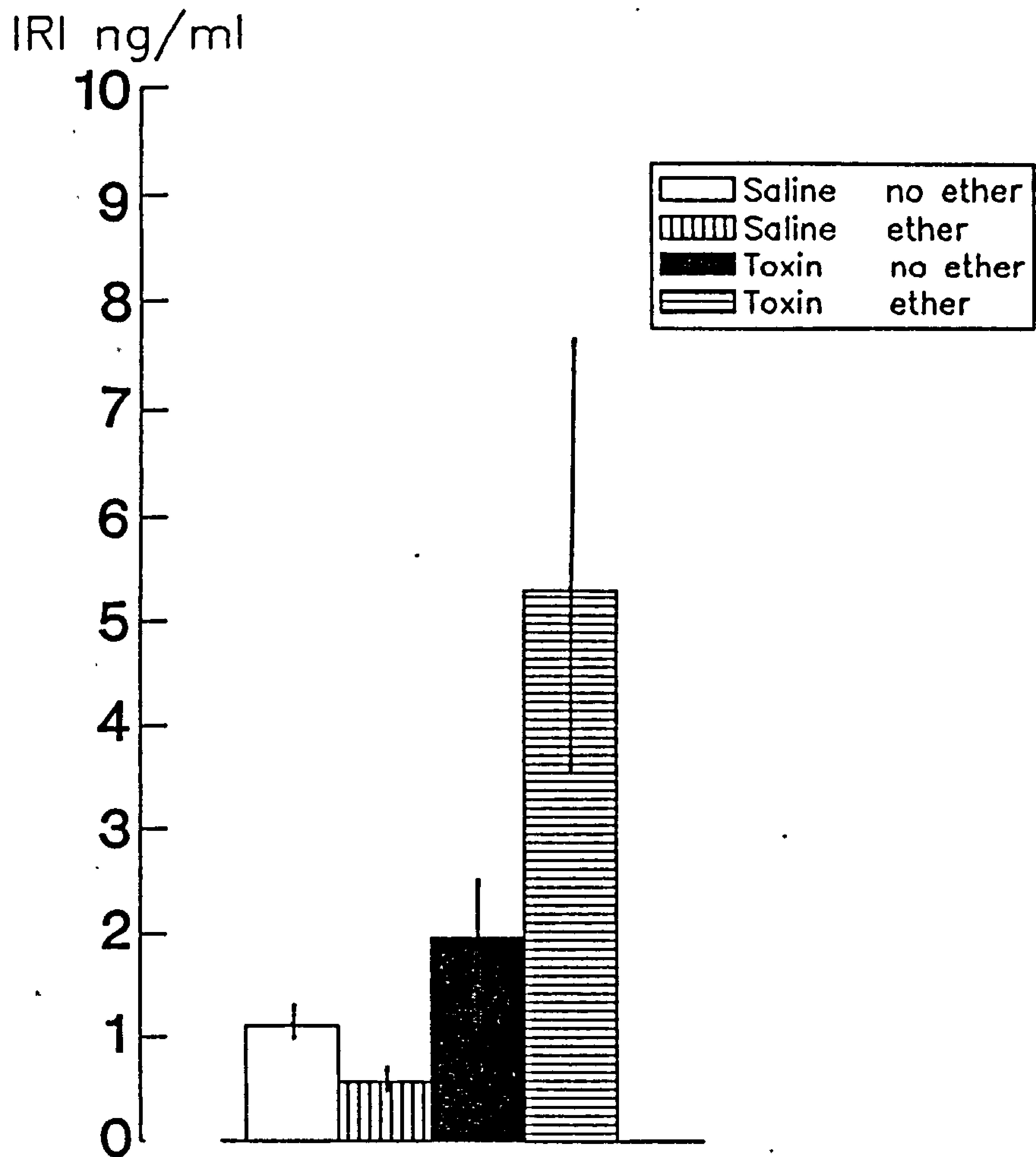


Figure 2.14.d

Hyperinsulinaemic effect of ether in seven week old mice treated with pertussis toxin.

N= 12 for each treatment, values expressed as geometric mean (95% C.L.).

Analysis of variance.

Source.	D.F.	F.	P.
Pertussis toxin	1	47.95	0.01
Ether	1	4.98	0.05
Interaction	1	17.24	0.01

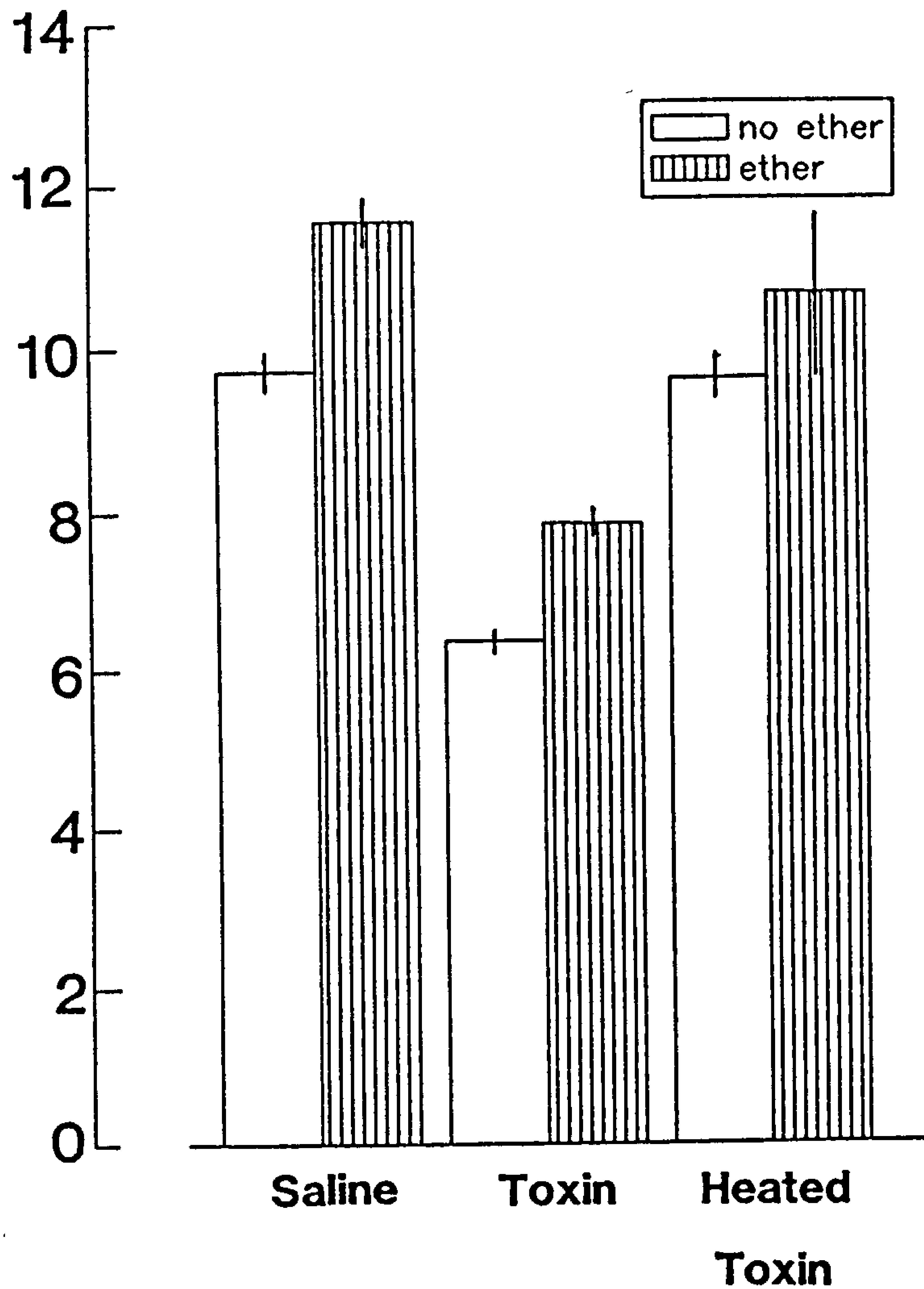


Figure 2.15.

Effect of heat treatment (80°C, 30 mins) on the hypoglycaemic effect of pertussis toxin.

N= 8 for each treatment, values expressed as arithmetic mean (S.E.).

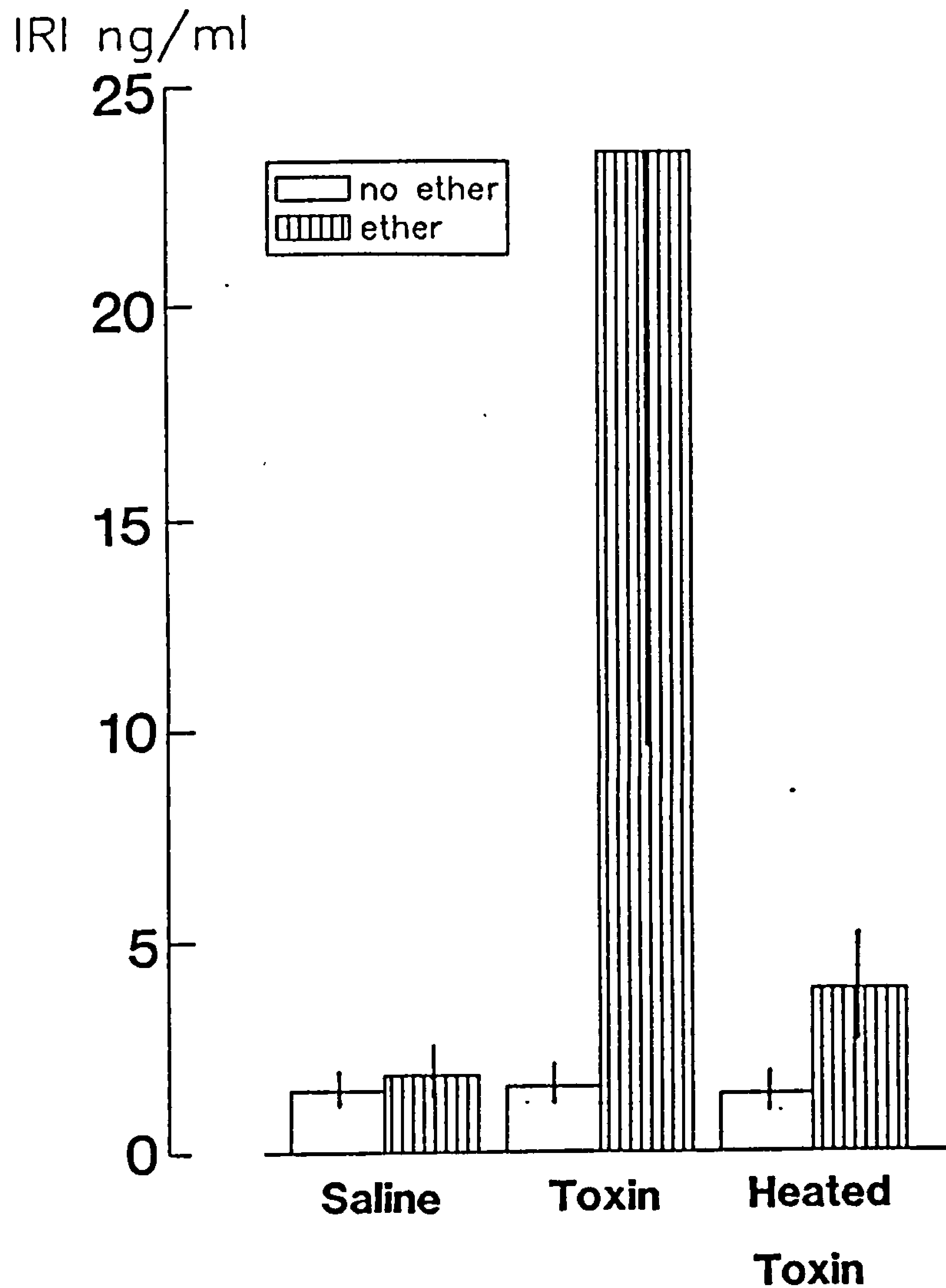


Figure 2.16.

Effect of heat treatment (80°C, 30 mins) on the ether induced hyperinsulinaemia in pertussis toxin-treated mice.

N= 8 for each treatment, values expressed as geometric mean (95% C.L.).

before (day 0) injection of PT, and on the day of the experiment (day 5). The data obtained is shown in Table 2.9.

2.12 Effect of repeated exposures to ether vapour

Exposure to ether produced hyperinsulinaemia in pertussis infected mice (Figure 2.1b) but did not cause any hypoglycaemia additional to that seen in all *B. pertussis* infected animals. This may be due to the transient nature of the hyperinsulinaemic response, therefore the effect of repeated exposures to ether was investigated.

2.12.1 Experimental protocol

B. pertussis infected mice were exposed to ether vapour on three or six occasions at 25 minute intervals. Blood was obtained 30 seconds after the third or sixth exposure, and serum glucose and IRI concentrations were compared with those from mice subjected to a single exposure to air for 90 seconds, followed by a 30 second recovery period before bleeding.

2.12.2 Serum glucose concentrations

After the third exposure to ether serum glucose concentrations were slightly, but not significantly increased (Table 2.10), and six exposures to ether did not alter the serum glucose compared to animals exposed to air.

Table 2.9

Weight gain in saline and pertussis toxin-treated mice in the 5 day period between injection and experiment.

Age of mice. (weeks)	Saline treated (g/mouse)	Pertussis toxin treated (g/mouse)	t.	P.
3	4.66 (0.35)	3.71 (0.35)	1.92	N.S.
5	1.51 (0.28)	3.08 (0.40)	3.20	0.05
6	1.85 (0.24)	3.62 (0.21)	5.60	0.01
7	0.93 (0.16)	3.28 (0.12)	11.5	0.01

Note: N=12 for each treatment, mean weight gain per mouse over 5 days calculated.

Table 2.10

Effect of repeated exposure to ether vapour on the serum glucose and IRI concentrations of B.pertussis infected mice.

Exposure	Glucose (mmol/l)	t.	P.	IRI (ng/ml)	t.	P.
Air	6.18 (0.22)	-	-	1.52 (1.28,1.79)	-	-
3x	7.25 (0.65)	1.57	N.S.	9.93 (4.95,19.94)	2.44	0.05
6x	6.53 (0.68)	0.49	N.S.	8.34 (4.51,5.42)	5.08	0.01

Note: mice were exposed to ether three or six times at 25 minute intervals. Ether exposed animals were compared statistically with animals exposed once to air.

2.12.3 Serum IRI concentrations

The serum IRI concentrations were not different between animals exposed to ether three or six times (Table 2.10), but were significantly higher than those of animals bled without exposure to ether.

2.13 Discussion

Furman et al (1981) showed that *B. pertussis* infected mice, but not control mice, showed marked hyperinsulinaemia after exposure to ether vapour and other anaesthetics. They suggested that any stress factor which caused release of adrenal catecholamines would produce a similar effect. Here, the study was extended to investigate the effect of various stresses, all of which have been shown, or inferred, to release catecholamines from the adrenal medulla (Baum et al, 1979; Yajima et al, 1981; Vallerand et al, 1983; Young et al, 1984).

2.13.1 Changes in serum insulin concentrations

The hyperinsulinaemia in *B. pertussis* infected mice following ether exposure (Furman et al, 1981) was confirmed here, and was also present after a brief exposure to anoxia, or after injection of histamine or 2-DG. It was also noted (Figures 2.1, 3.1, 3.3, 3.9, 3.16) that ether frequently caused slight hyperinsulinaemia in non-infected mice. This was also seen after 2-DG injection, or after exposure to 100% CO_2 (Figure 2.8

and Table 2.8). Such slight hyperinsulinaemia was probably secondary to the hyperglycaemia produced by these stimuli.

The treatments causing hyperinsulinaemia in *B. pertussis* infected mice (anoxia, histamine and 2-DG) are thought to cause release of adrenal catecholamines (Yajima et al, 1981; Young et al, 1984). Therefore, the marked hyperinsulinaemia in response to these agents in *B. pertussis* infected mice could be explained by the reversal of the inhibitory effect of adrenaline on insulin secretion seen after pertussis vaccine (Gulbenkian et al, 1968) or infection with *B. pertussis* (Furman et al, 1981).

However, cold exposure, LPS injection and hypoxia, despite causing activation of the adrenal medulla (Young et al, 1984; McKechnie et al, 1985) and inhibiting insulin secretion in normal animals (Baum et al, 1979; Vallerand et al, 1983), did not result in hyperinsulinaemia in infected mice. Possibly, the pattern of catecholamine release following hypoxia, cold or LPS may be delayed - or may be less marked than that caused by the stresses which produce hyperinsulinaemia in infected animals.

The lack of effect of cold exposure or hypoxia on the serum IRI concentrations in *B. pertussis* infected mice could be due to a rapidly developing tolerance to any hyperinsulinaemic response to the stresses during the

longer periods of exposure used here. Indeed, the behaviour of mice exposed to a hypoxic atmosphere quickly returned to normal. However, the hyperinsulinaemic response of _B. pertussis_ infected mice to ether exposure was fully evident even after six successive exposures (Table 2.10). Admittedly, these exposures to ether were carried out at 25 minute intervals, which may obviate the development of tolerance.

The type and duration of stimuli used could also alter the serum IRI responses seen. Not all stimuli which produce a sympathetic discharge alter insulin secretion. For example, bilateral carotid occlusion in puppies produced a sympathetic discharge, with an increase in heart rate and blood pressure but _without_ any inhibition of insulin secretion (Lee and Miller, 1985). Varying the intensity or type of stimuli can lead to dissociation between sympathetic and adrenal activation (Johnson et al, 1983; Young et al, 1984). Indeed, Young et al (1984) showed that stress-induced inhibition of insulin secretion occurs principally when sympathetic activity is reduced, and adrenal secretion is greatest. They concluded that certain physiological processes (such as insulin secretion) may respond differently to neurally released noradrenaline as opposed to adrenaline from the adrenal medulla. However, these authors found 2-DG and hypoxia to produce a similar pattern of sympathetic suppression and adrenal

activation, whereas in the experiments reported here 2-DG caused marked hyperinsulinaemia in pertussis-infected mice, whereas hypoxia failed to do so.

Hyperventilation caused by hypoxia could also affect the serum IRI response to such stress. Hyperventilation would produce respiratory alkalosis (Bowman and Rand, 1980), and this has been shown to alter the metabolic response of animals to adrenergic stimuli (Yajima and Ui, 1975; 1977). During alkalosis, the hyperglycaemic effect of adrenaline is prevented (Yajima and Ui, 1975) and the hypoglycaemia which is present during alkalosis is not due to insulin (Yajima and Ui, 1977). These authors showed that the metabolic changes during alkalosis were due to potentiated α adrenoceptor function, and reduced β adrenoceptor function. Thus, in pertussis-infected mice the proposed β-adrenoceptor mediated stimulation of insulin secretion may be reduced or prevented by hypoxia-induced alkalosis. This may explain the lack of hyperinsulinaemia in pertussis-infected mice exposed to an hypoxic atmosphere (Table 2.4).

Endotoxin (LPS) produced a more complex picture. LPS caused an increase in IRI concentrations in normal animals (as has been reported previously by Hewlett et al, 1983), but caused a decrease in serum IRI concentrations in infected mice (Table 2.2). The mechanism of this latter response is unknown, but is

unlikely to be related to the release of adrenal catecholamines caused by LPS (McKechnie et al, 1985). This would be expected to produce an increase in serum IRI concentrations in infected mice. The interaction between LPS and pertussis is complicated, and some actions of LPS (eg stimulation of interleukin production by macrophage) may be prevented by pertussis toxin (Jakway and DeFranco, 1986).

2.13.2 Changes in serum glucose concentrations

The prevention or attenuation of the hyperglycaemic effects of histamine and 2-DG in *B. pertussis* infected mice was probably due to the abolition of the hyperglycaemic effect of adrenaline released from the adrenal medulla. Attenuation of adrenaline-induced hyperglycaemia is a well documented effect of pertussis vaccine, infection or toxin (Szentivanyi et al, 1963; Gulbenkian et al, 1968; Furman et al, 1981; Yajima et al, 1978). On the other hand, the hyperglycaemia caused by exposure to 100% CO_2 was not altered in *B. pertussis* infected mice, suggesting that such hyperglycaemia is not mediated by catecholamines.

Injection of LPS, or exposure to cold, or to a hypoxic atmosphere, produced a further lowering of the serum glucose concentrations in infected mice. This could not, however, be related to any stress-induced alterations in serum IRI since LPS caused a decrease, and cold and hypoxia caused no change, in serum IRI

in infected mice. In normal mice cold produced no change in serum glucose concentrations, and hypoxia and LPS produced similar decreases to those seen in infected mice. There was, however, a significant interaction between cold exposure and pertussis infection (Figure 2.5) in that cold produced a greater lowering of serum glucose concentrations in infected mice than in control mice. This may be related to the findings of Vallerand et al (1983) who showed that exposure to cold lowered basal glucose concentrations in rats, and improved the rate of glucose disappearance despite a reduced plasma insulin response to glucose.

Despite the marked hyperinsulinaemia caused by histamine, ether and 2-DG in B. pertussis infected animals, no additional hypoglycaemia (other than that seen in pertussis-sensitised animals) was found. This is in contrast to the profound hypoglycaemia reported by Yajima et al (1981) in pertussis toxin-treated rats given histamine. Several explanations for this lack of hypoglycaemia are possible. First, the time course over which the blood samples were taken may not have been sufficiently prolonged for the effect of high insulin concentrations to become manifest as hypoglycaemia. However, blood samples up to 30 minutes were taken, and hypoglycaemia would have been expected within this time.

Secondly, the transient nature of the hyperinsulinaemia (eg after anoxic stimuli) may have been such that

no effect on serum glucose would occur. However, multiple exposures to ether, each of which is presumed to produce hyperinsulinaemia, did not cause any further lowering of serum glucose concentrations in B. pertussis infected mice.

Thirdly, the hypoglycaemic effect of insulin may be impaired in pertussis-infected animals. This is supported by the data presented in Figure 1.11 and Table 1.4(a), which showed diminished responsiveness to insulin. The serum concentration of insulin produced by the dose of insulin used can be estimated as approximately 1250 $\mu U\ ml^{-1}$, assuming the dose of insulin to be distributed in the extracellular fluid. Exposure to stress produced serum insulin concentrations equivalent to between 300 and 1000 $\mu U\ ml^{-1}$, thus the serum insulin concentrations achieved following stress are probably similar to those achieved in the experiment showing insulin resistance.

Alternatively, although the stress-induced elevation in IRI was prevented by alloxan indicating the pancreatic origin of the immunoreactivity, the measured IRI concentration may not represent fully biologically active insulin. The assay detects proinsulin, and possibly a greater proportion of proinsulin is released from the β cell in infected mice under these conditions. This has previously been suggested to contribute to the glucose intolerance of hypokalaemia (Gorden et al,

1972).

2.13.3 Role of pertussis toxin

Pertussis toxin was found to mimic the effects of infection in that pretreatment of 3-7 week old mice with PT resulted in hypoglycaemia, and a marked hyperinsulinaemia in animals exposed to ether. Heating the PT preparation to 80°C for 30 minutes markedly reduced these activities, showing that they are not due to contamination of the toxin with trace amounts of LPS which would be stable at this temperature.

Results Chapter 3

Role of the adrenal medulla in stress-induced hyperinsulinaemia

3.1 Introduction

3.2 Alpha-adrenoceptor blockade

3.3 Beta-adrenoceptor blockade

3.4 Adrenal demedullation

3.5 Ganglion blockade

3.6 Muscarinic cholinoceptor blockade

3.7 Opiate receptor blockade using naloxone

3.1 The work presented in the previous section clearly showed that a variety of stressful stimuli (ether, histamine, 2-DG and anoxia) produced hyperinsulinaemia in B. pertussis infected or pertussis toxin-treated mice. Common to all these stressors is presumed to be the release of adrenal medullary catecholamines. As discussed previously, B. pertussis infection, or treatment with pertussis vaccine or toxin, prevents the inhibitory action of catecholamines on insulin secretion and, indeed, converts it to a stimulatory one.

In this section the role of the adrenal medulla in the ether-induced hyperinsulinaemia seen in B. pertussis infected or toxin-treated mice was investigated. The effects of drugs altering parasympathetic, sympathetic and opiate influences on the pancreatic islets was also investigated, to determine the mechanism of the ether-induced hyperinsulinaemia in pertussis-treated mice.

The reversal of the effect of adrenaline on insulin secretion seen in pertussis-treated animals has been explained by the prevention of α-adrenoceptor mediated inhibition of insulin secretion, and the unmasking of a β-adrenoceptor mediated stimulation of insulin secretion (Sumi and Ui, 1975; Katada and Ui, 1977). If adrenal medullary catecholamines act in this way to mediate the hyperinsulinaemia seen after stress in pertussis-treated mice, then α-adrenoceptor blockade in normal mice should mimic the effect of pertussis treatment.

Similarly, the hyperinsulinaemia caused by ether in pertussis-treated mice should be prevented by β-adrenoceptor blockade, by adrenal demedullation or by blockade of the sympathetic ganglia.

Stimulation of the vagus *in vivo* (Kaneto et al, 1967) and addition of acetylcholine *in vitro* (Malaisse et al, 1967b; Iversen, 1973a) increase insulin release. Also, opioid peptides are released during stress (Madden et al, 1977; Rossier et al, 1977) and both morphine and β endorphin produced an increase in insulin secretion *in vivo* (Ipp et al, 1978). Because of this, the effects of parasympathetic blockade (both muscarinic and nicotinic) and opiate receptor blockade with naloxone on the ether-induced hyperinsulinaemia in pertussis-infected mice was investigated.

3.2 Alpha adrenoceptor blockade

3.2.1 Effect of α-adrenoceptor antagonists in normal mice

(a) Phentolamine 40 mg kg^{-1}

Phentolamine hydrochloride (40 mg kg^{-1} I.P.) or saline was given to normal mice 10.5 minutes before the animals were exposed to ether vapour, or air. Phentolamine produced significant hypoglycaemia ($F = 77.3$, $P < 0.01$) (appendix 2a), and ether produced a significant increase in serum glucose ($F = 5.5$, $P < 0.05$). There was, however, no significant interaction

between ether and phentolamine, showing that ether had the same effect in saline and phentolamine treated mice (appendix 2a).

Serum IRI concentrations in the same animals are shown in Figure 3.1. In these normal mice phentolamine had no significant effect on serum IRI concentrations, and there was no interaction between ether and phentolamine. Ether itself produced hyperinsulinaemia in both saline and phentolamine treated mice ($F = 34.28$, $P < 0.01$).

(b) Phentolamine 5 mg kg^{-1}

The above experiment was repeated using a lower dose of phentolamine i.e. 5 mg kg^{-1}. The same pattern of serum glucose results were obtained (appendix 2b).

Phentolamine caused significant hypoglycaemia ($F = 73.43$, $P < 0.01$) and ether significant hyperglycaemia ($F = 5.66$, $P < 0.05$). As before, ether caused the same degree of hyperglycaemia in both saline treated and phentolamine treated mice (F for interaction = 0.43, N.S.).

The serum IRI concentrations of the same animals are shown in Figure 3.2. Here, neither phentolamine nor ether had a significant effect on serum IRI, and there was no interaction between phentolamine and ether.

(c) Idazoxan 5 mg kg^{-1}

The α_2 adrenoceptor-specific antagonist idazoxan

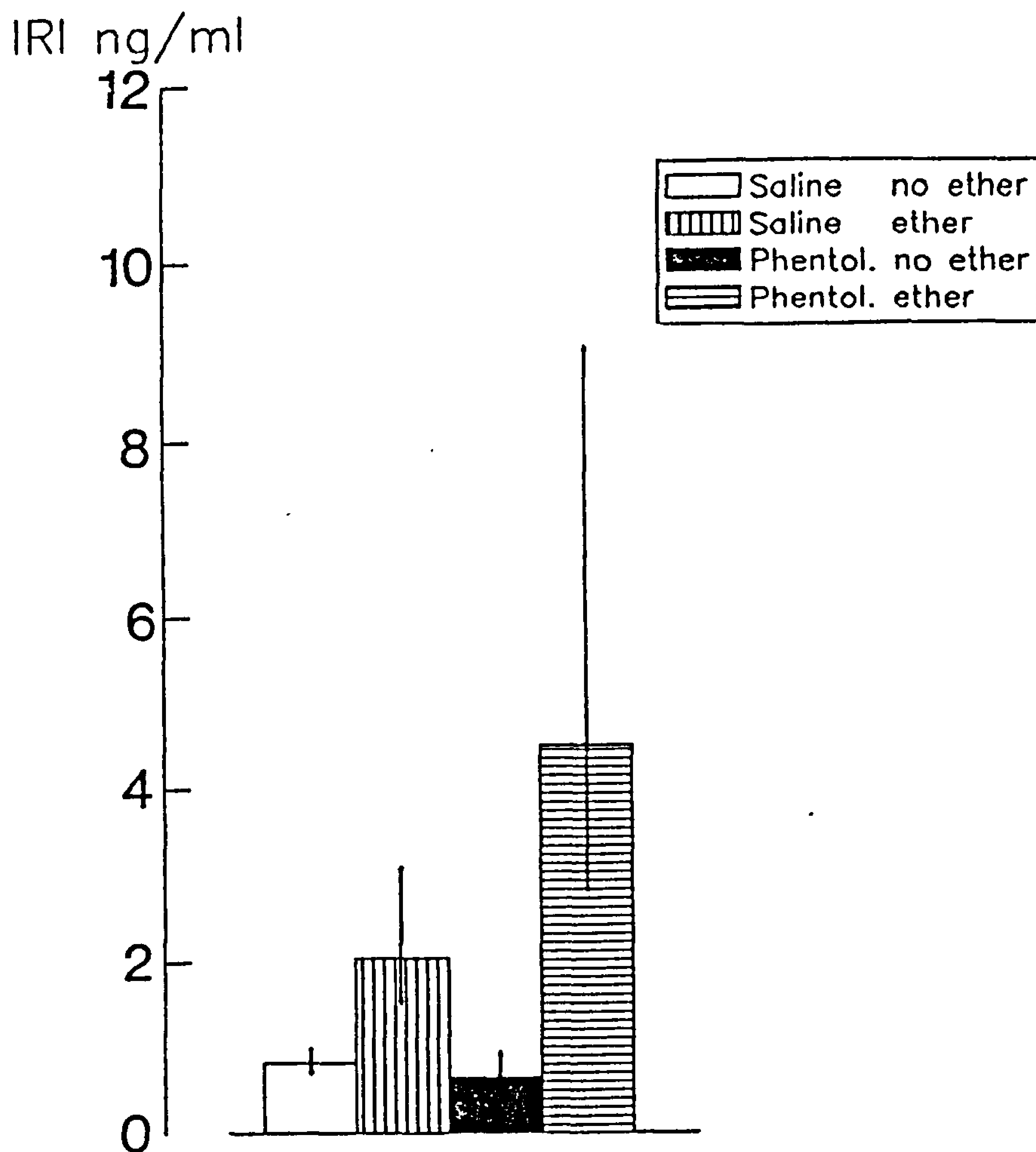


Figure 3.1

Effect of Phentolamine 40mg/kg on the serum immunoreactive insulin concentrations of normal mice.

N=6 for each treatment, values expressed as geometric mean with 95% C.L.

Analysis of variance.

Source	D.F.	F.	P.
Ether	1	34.28	0.01
Phentolamine	1	1.12	N.S.
Interaction	1	3.76	N.S.

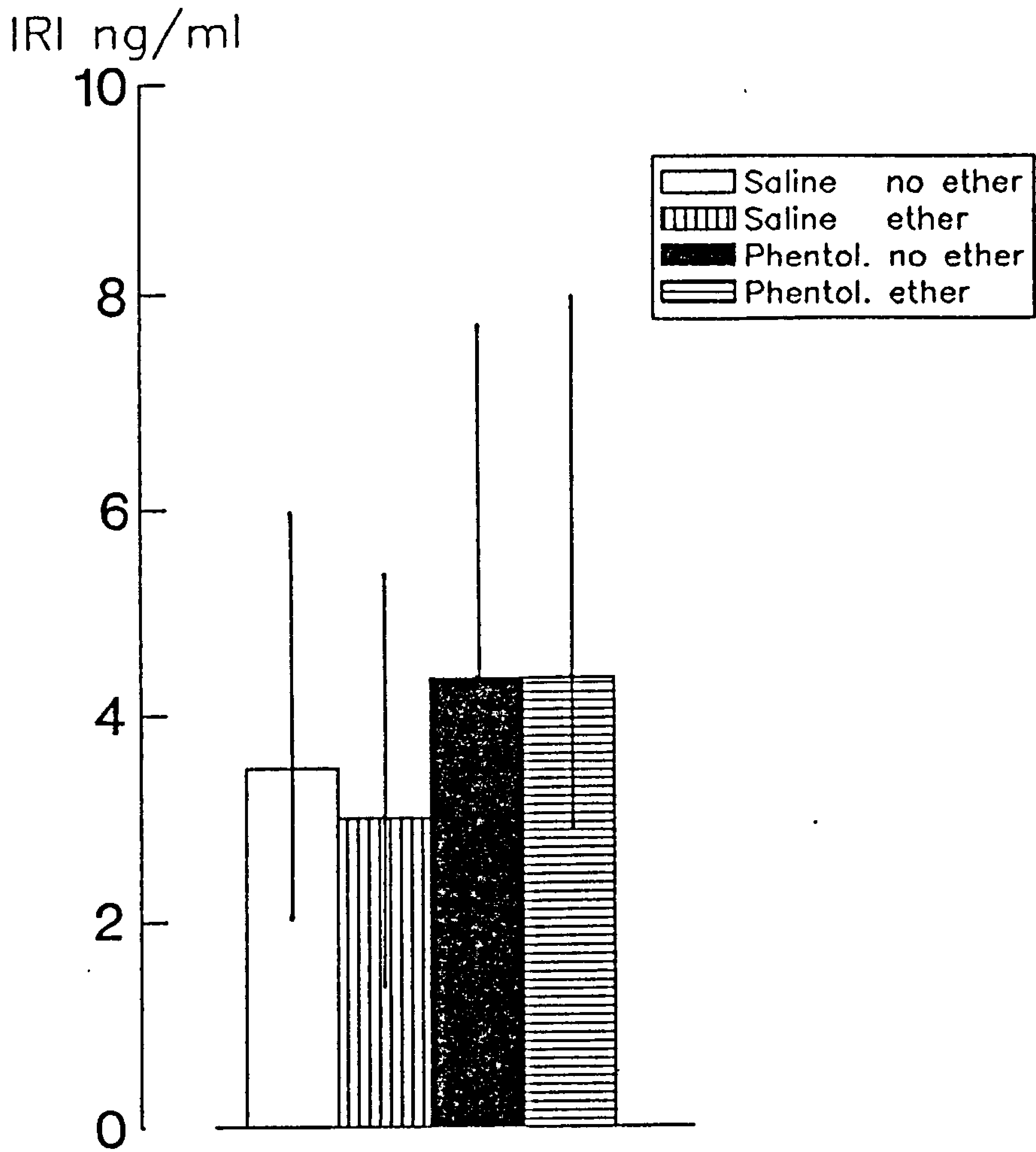


Figure 3.2

Effect of Phentolamine 5mg/kg on the serum immunoreactive insulin concentrations of normal mice.

N=6 for each treatment, values expressed as geometric mean with 95% C.L.

Analysis of variance.

Source	D.F.	F.	P.
Ether	1	0.1	N.S
Phentolamine	1	2.18	N.S.
Interaction	1	0.09	N.S.

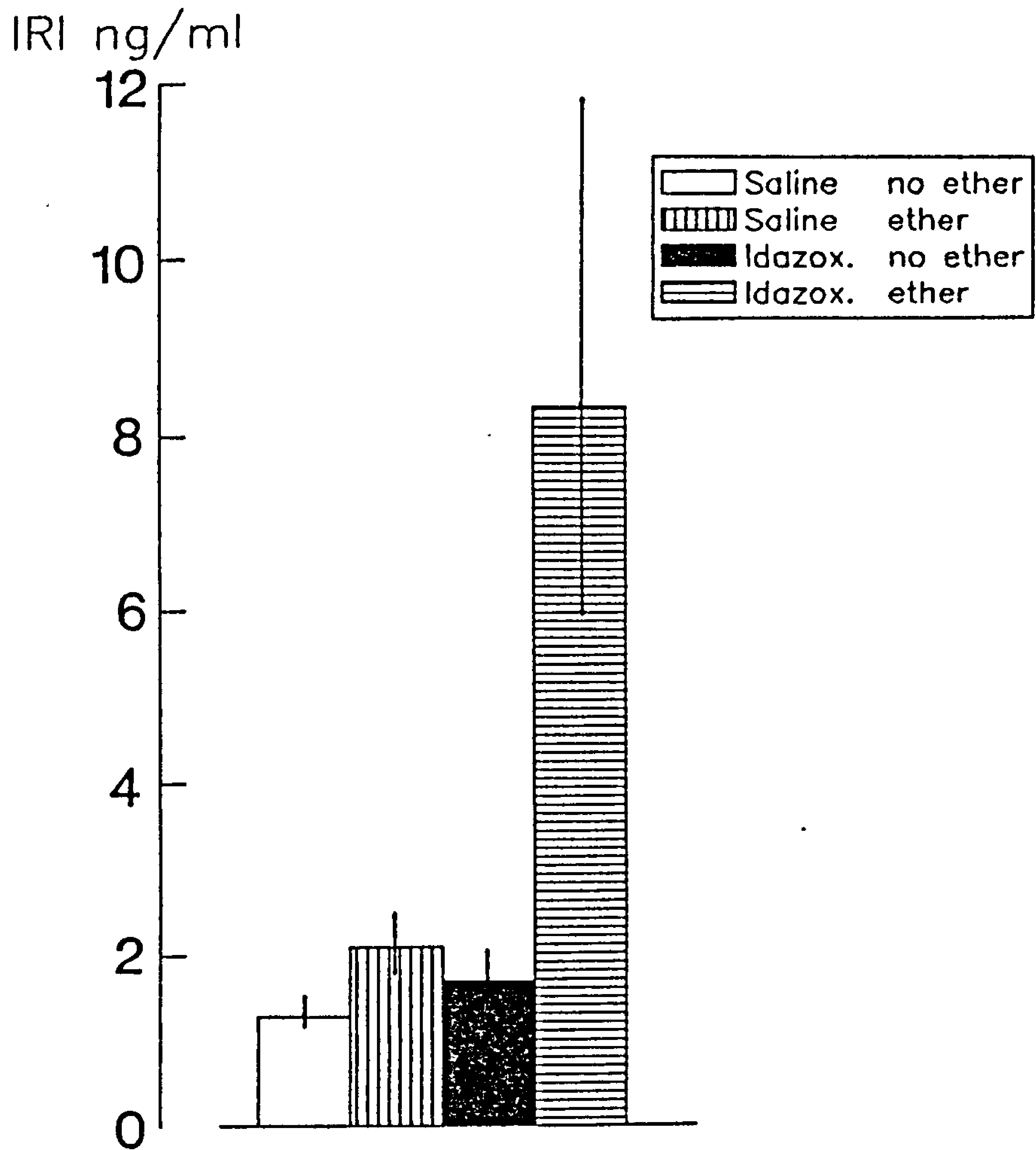


Figure 3.3

Effect of Idazoxan on the serum immunoreactive insulin concentrations of normal mice.

N=14 for each treatment, values expressed as geometric mean with 95% C.L.

Analysis of variance.

Source	D.F.	F.	P.
Ether	1	26.66	0.01
Idazoxan	1	16.69	0.01
Interaction	1	7.35	0.01

or saline was given I.P. to normal mice 20 minutes before exposure to ether or air. Idazoxan caused significant hypoglycaemia ($F = 76.79$, $P < 0.01$), and ether produced significant hyperglycaemia ($F = 17.86$, $P < 0.01$) (appendix 2c). The effect of ether was similar in both saline treated and idazoxan treated mice (F for interaction = 2.61, N.S.).

The serum IRI concentrations for the same animals are shown in Figure 3.3. Ether exposure or idazoxan treatment produced significant hyperinsulinaemia, but the hyperinsulinaemic effect of ether was augmented in idazoxan-treated mice (F for interaction = 7.35, $P < 0.01$). Thus, α-adrenoceptor blockade in normal mice mimicked the effect of pertussis treatment.

3.2.2 Effect of α adrenoceptor antagonists in pertussis-treated mice

(a) Phentolamine 40 mg kg^{-1}

Phentolamine hydrochloride (40 mg kg^{-1} I.P.) or saline was given to B. pertussis infected mice 10.5 minutes before exposure to ether or air. Neither phentolamine nor ether had a significant effect on serum glucose concentrations in those infected animals (appendix 2d). There was no interaction between ether and phentolamine (F for interaction = 0.825, N.S.).

The corresponding serum IRI concentrations are shown in Figure 3.4. Phentolamine produced hyperinsulin-

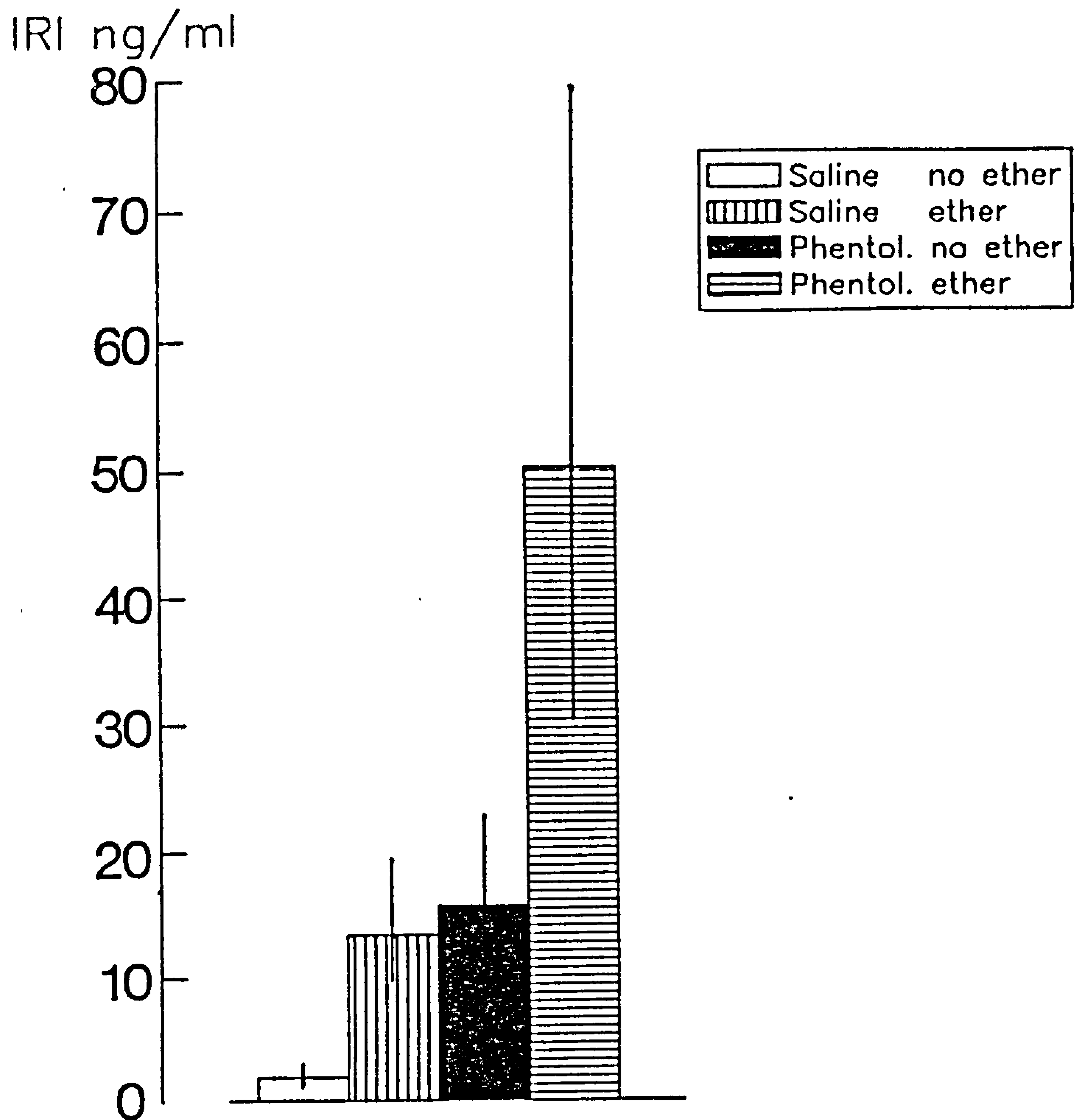


Figure 3.4

Effect of Phentolamine on the serum immunoreactive insulin concentrations of B. pertussis infected mice.

N=18 for each treatment, values expresses as geometric mean with 95% C.L.

Analysis of variance.

Source	D.F.	F.	P.
Ether	1	67.81	0.01
Phentolamine	1	69.80	0.01
Interaction	1	2.90	N.S.

aemia compared to saline treated controls ($F = 69.80$, $P < 0.01$), and ether produced hyperinsulinaemia in both saline and phentolamine treated animals ($F = 67.81$, $P < 0.01$). The hyperinsulinaemic effects of phentolamine and ether were no more than additive (F for interaction = 2.90, N.S.).

(b) <u>Phentolamine 5 mg kg^{-1}</u>

The previous experiment was repeated using a lower dose of phentolamine (5 mg kg^{-1}) in pertussis toxin-treated mice. Whilst ether had a significant hyperglycaemic effect ($F = 16.82$, $P < 0.01$), phentolamine had no effect (at this dose) on serum glucose ($F = 1.38$, N.S.). There was no interaction between ether and phentolamine ($F = 0.82$, N.S.) (appendix 2c).

Ether produced a highly significant hyperinsulinaemia in both saline treated and phentolamine treated mice ($F = 87.67$, $P < 0.01$) (Figure 3.5). Phentolamine did not affect serum IRI, and had no effect on the ether-induced hyperinsulinaemia seen in these toxin-treated mice (F for interaction = 0.51, N.S.).

(c) <u>Idazoxan 5 mg kg^{-1}</u>

Idazoxan (5 mg kg^{-1} I.P.) or saline was given to <u>B. pertussis</u> infected mice 20 minutes before exposure to ether or air.

Ether produced significant hyperglycaemia ($F = 8.87$, $P < 0.01$), and idazoxan significant hypoglycaemia

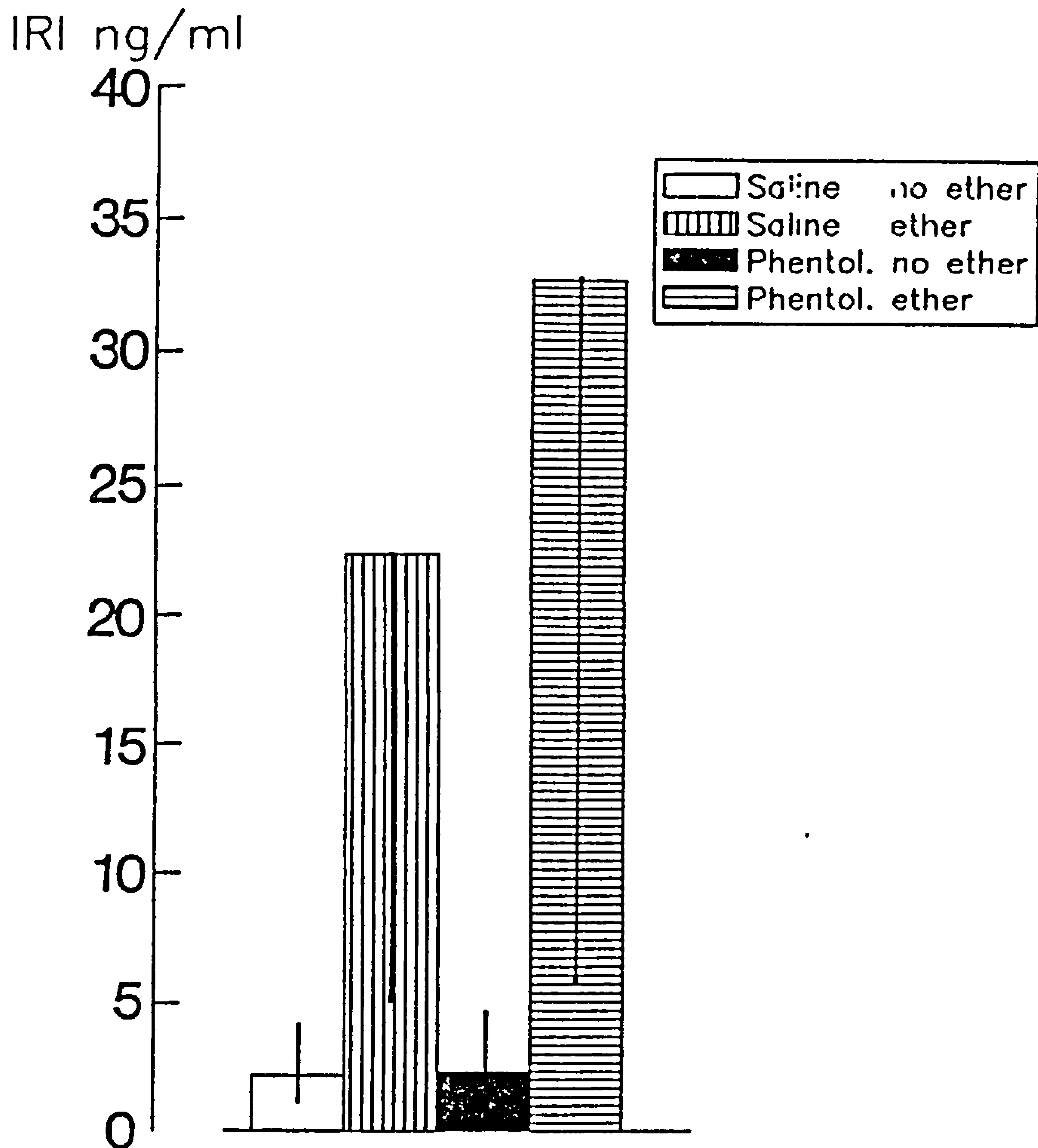


Figure 3.5

<u>Effect of Phentolamine 5mg/kg on the serum immunoreactive insulin concentrations of Pertussis toxin-treated mice.</u>

N=5 for each treatment, values expressed as geometric mean with 95% C.L.

<u>Analysis of variance.</u>

Source	D.F.	F.	P.
Ether	1	87.67	0.01
Phentolamine	1	1.099	N.S.
Interaction	1	0.51	N.S.

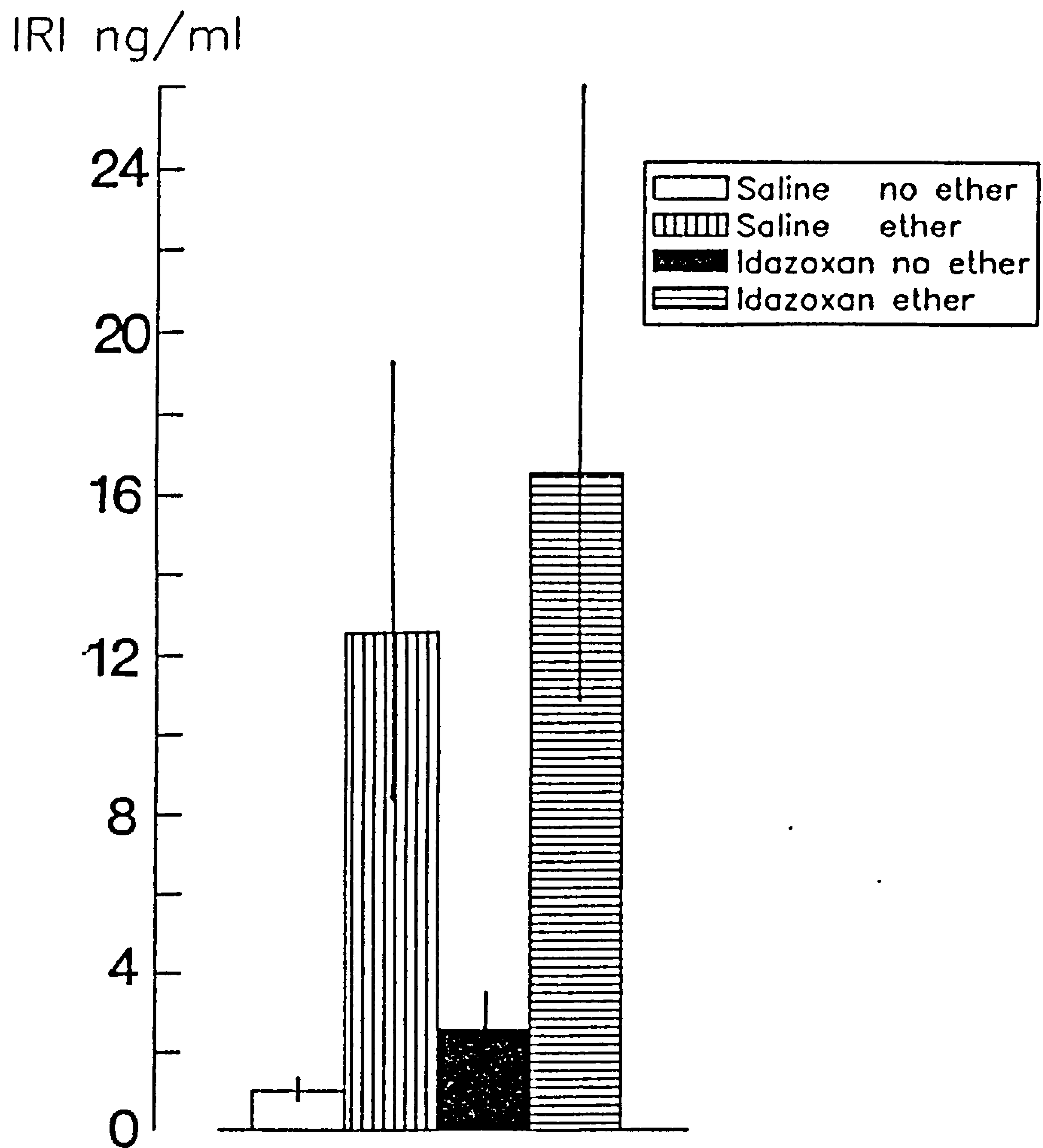


Figure 3.6

Effect of Idazoxan on the serum immunoreactive insulin concentrations of B. pertussis infected mice.

N=14 for each treatment, values expressed as geometric mean with 95% C.L.

Analysis of variance.

Source	D.F.	F.	P.
Ether	1	131.37	0.01
Idazoxan	1	9.88	0.01
Interaction	1	2.90	N.S.

($F = 10.85$, $P < 0.01$) in these infected mice (appendix 2f). There was no interaction between ether and idazoxan ($F = 0.41$, N.S.).

The corresponding serum IRI values are shown in Figure 3.6. Idazoxan produced slight hyperinsulinaemia ($F = 9.88$, $P < 0.01$), and ether caused marked hyper-insulinaemia in both saline and idazoxan treated mice ($F = 131.37$, $P < 0.01$). However, the effects of idazoxan and ether were no more than additive (F for interaction = 2.90, N.S.).

3.3 <u>Beta-adrenoceptor blockade</u>

(a) <u>Effect of propranolol in pertussis-infected mice</u>

Propranolol (20 mg kg^{-1}) or saline was given I.P. to *B. pertussis* infected mice 20 minutes before exposure to ether vapour or air. Ether caused significant hyperglycaemia ($F = 5.68$, $P < 0.05$) but propranolol did not affect serum glucose concentrations ($F = 3.63$, N.S.). There was no interaction between ether exposure and propranolol (F for interaction = 1.79, N.S.) (appendix 2g).

Propranolol produced significant hypoinsulinaemia, and reduced the degree of hyperinsulinaemia seen in ether exposed animals (Figure 3.7). However, the effects of propranolol and ether were no more than additive (F for interaction = 0.02, N.S.), showing that β blockade has not affected the increase in insulin

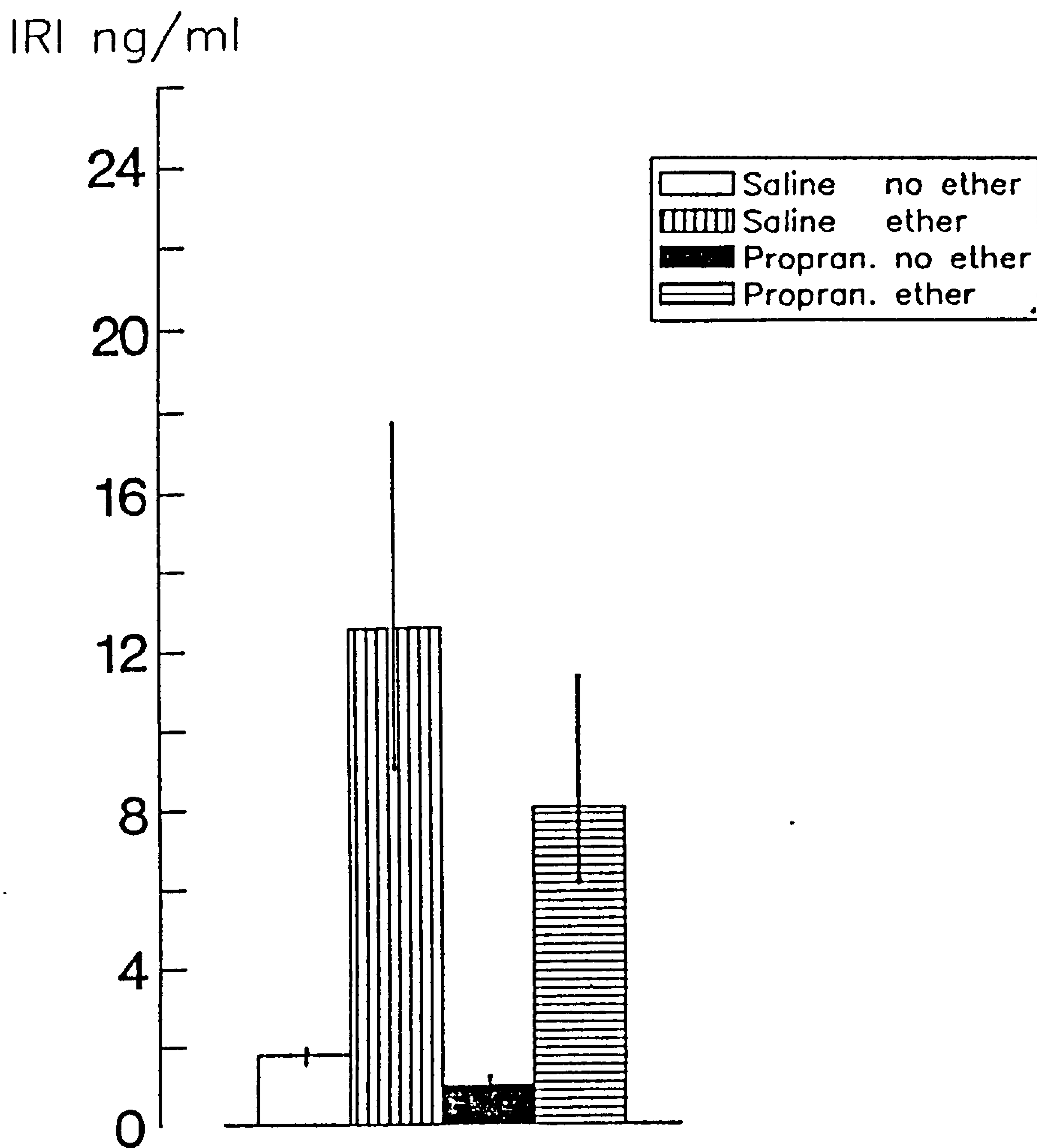


Figure 3.7

Effect of Propranolol on serum immunoreactive insulin concentrations in B. pertussis infected mice.

N=18 for each treatment, values expressed as geometric mean with 95% C.L.

Analysis of variance.

Source	D.F	F.	P.
Ether	1	97.81	0.01
Propranolol	1	5.68	0.05
Interaction	1	0.02	N.S.

concentrations caused by exposure of B. pertussis infected mice to ether.

(b) Effect of propranolol in pertussis toxin-treated mice

The previous experiment was repeated using mice injected 5 days previously with pertussis toxin (150 ng per mouse I.V.). The serum glucose concentrations showed a similar pattern of responses to that seen in the infected mice (appendix 2h). Thus, ether exposure is the only significant factor causing a change in glucose concentrations ($F = 29.19$, $P < 0.01$).

Beta-adrenoceptor blockade with propranolol did not prevent the hyperinsulinaemic effect of ether in pertussis toxin-treated mice (Figure 3.8) (F for interaction = 0.01, N.S.).

(c) Effect of propranolol in idazoxan-treated mice

Figures 3.1, 3.2 and 3.3 show that α-adrenoceptor blockade produced hyperinsulinaemia when mice were subsequently exposed to ether vapour. The effect of β adrenoceptor blockade on the hyperinsulinaemia caused by ether in idazoxan treated mice was investigated.

Idazoxan (5 mg kg^{-1}) and propranolol (20 mg kg^{-1}) were given together I.P. to mice 20 minutes before exposure to ether vapour or air. Saline was given by the same route as control. Ether exposure caused significant hyperglycaemia ($F = 8.43$, $P < 0.01$) and the

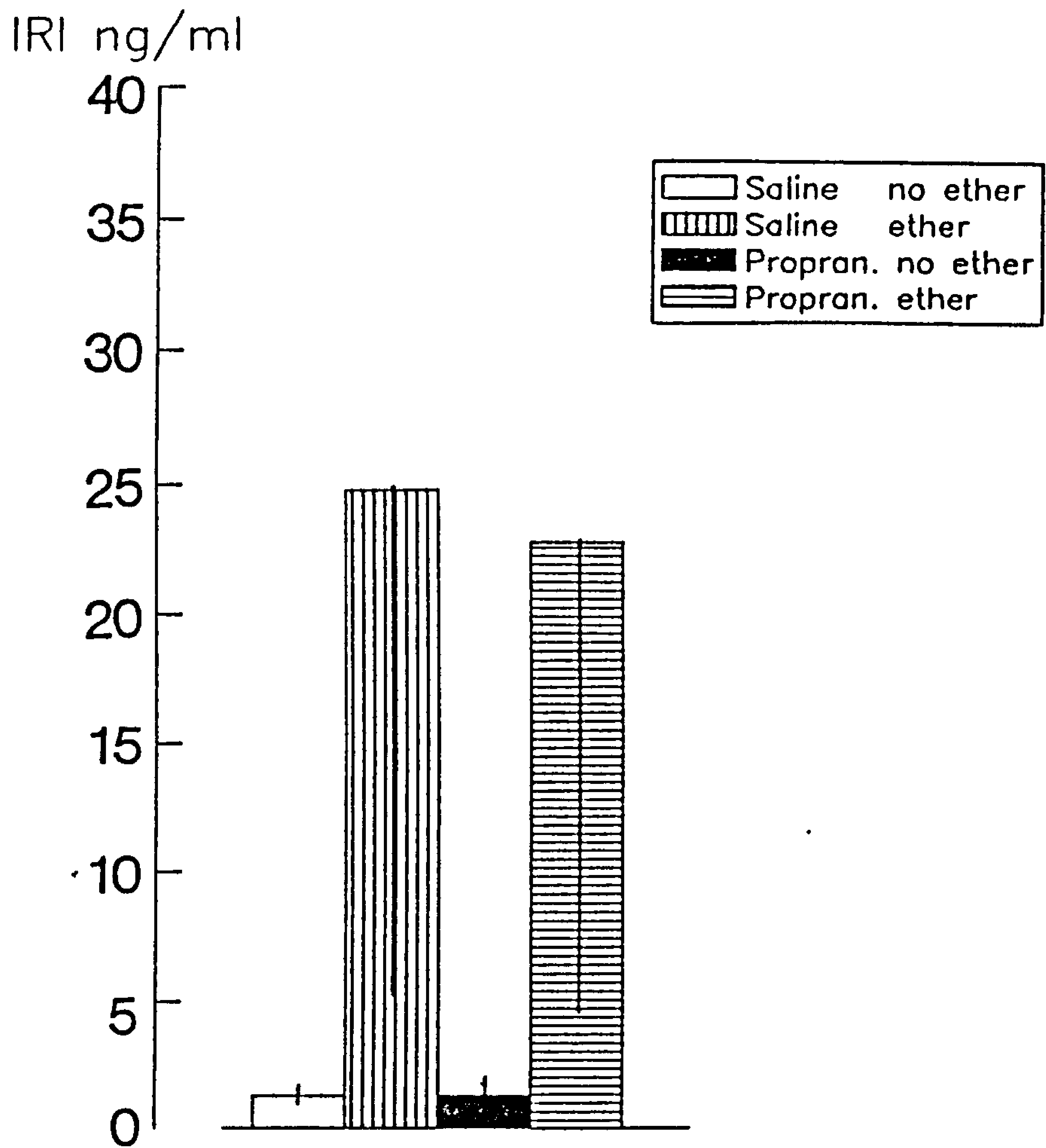


Figure 3.8

Effect of Propranolol on the serum immunoreactive insulin concentrations of Pertussis Toxin-treated mice.

N=5 for each treatment, values expressed as geometric mean with 95% C.L.

Analysis of variance.

Source	D.F.	F.	P.
Ether	1	118.6	0.01
Propranolol	1	0.00	N.S.
Interaction	1	0.01	N.S.

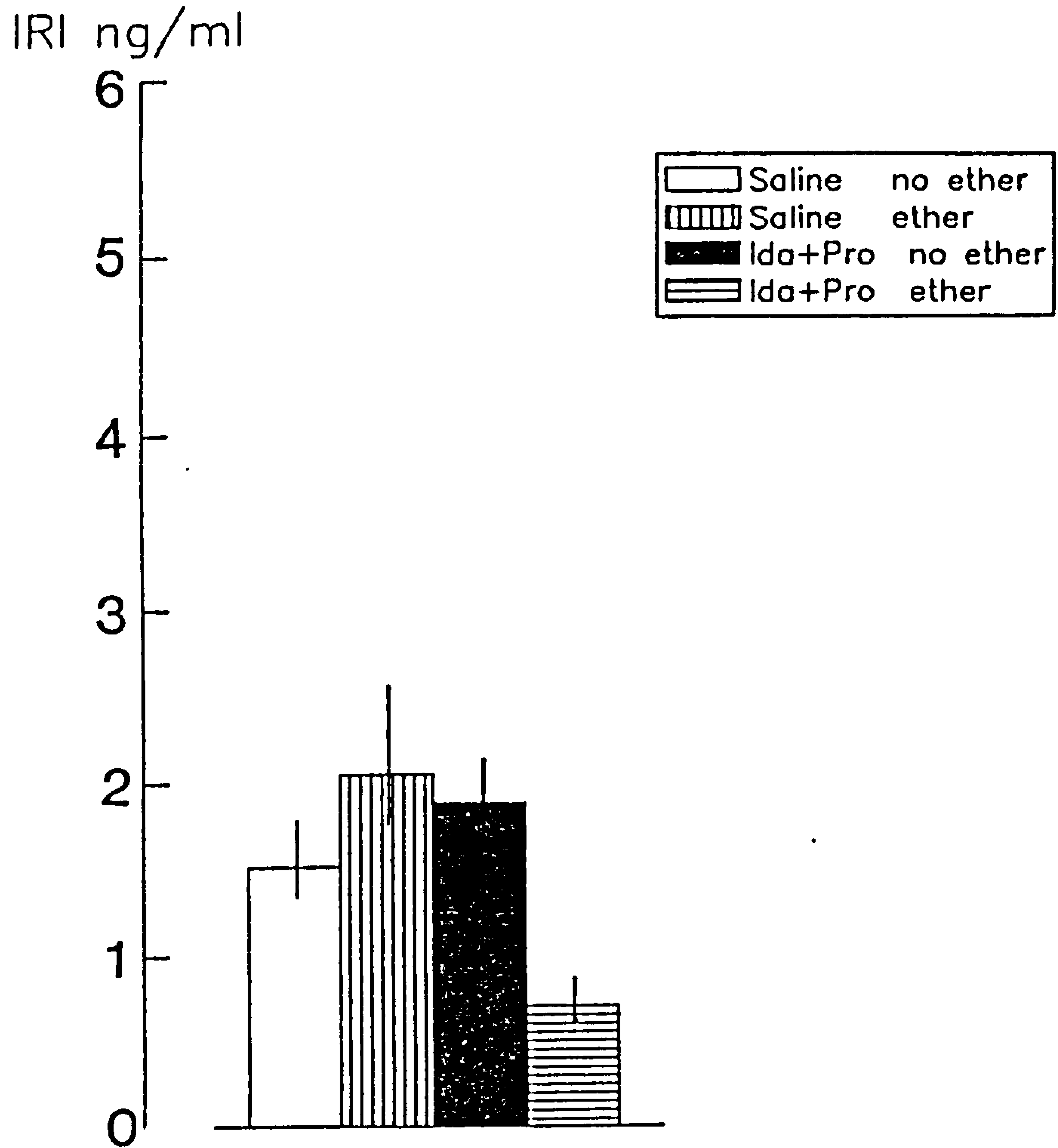


Figure 3.9

Effect of Idazoxan + Propranolol on the serum immunoreactive insulin concentrations of normal mice.

N=14 for each treatment, values expressed as geometric mean with 95% C.L.

Analysis of variance.

Source	D.F.	F.	P.
Ether	1	2.54	N.S.
Idazoxan+Propranolol	1	3.36	N.S.
Interaction	1	6.39	0.05

idazoxan and propranolol mixture caused significant hypoglycaemia ($F = 36.97$, $P < 0.01$) (appendix 2i). There was no interaction between ether exposure and drug treatment (F for interaction = 3.10, N.S.).

The corresponding serum IRI concentrations are shown in Figure 3.9. Propranolol completely prevented the hyperinsulinaemia seen in idazoxan-treated mice (F for interaction = 6.39, $P < 0.05$) (c.f. Figure 3.3).

(d) <u>Effect of propranolol on carbachol-induced hyperinsulinaemia</u>

The increase in serum IRI concentrations caused by carbachol (1 mg kg^{-1} I.P. 5 minutes before bleeding) was completely prevented by atropine (6 mg kg^{-1} I.P. 20 minutes before carbachol) (Figure 3.10). The dose of propranolol used in the previous experiments did not affect the carbachol-induced increase in IRI concentrations ($t = 1.71$, N.S.).

3.4 Adrenal demedullation

(a) <u>Effect of adrenal demedullation in normal and pertussis toxin-treated mice</u>

Both control (sham operated) and adrenal demedullated mice were injected with PBS or pertussis toxin (150 ng per mouse) I.V. 5 days before the experiment. Mice were exposed to ether or air for 90 seconds, then removed and bled by decapitation after 30 seconds recovery. Serum glucose concentrations are

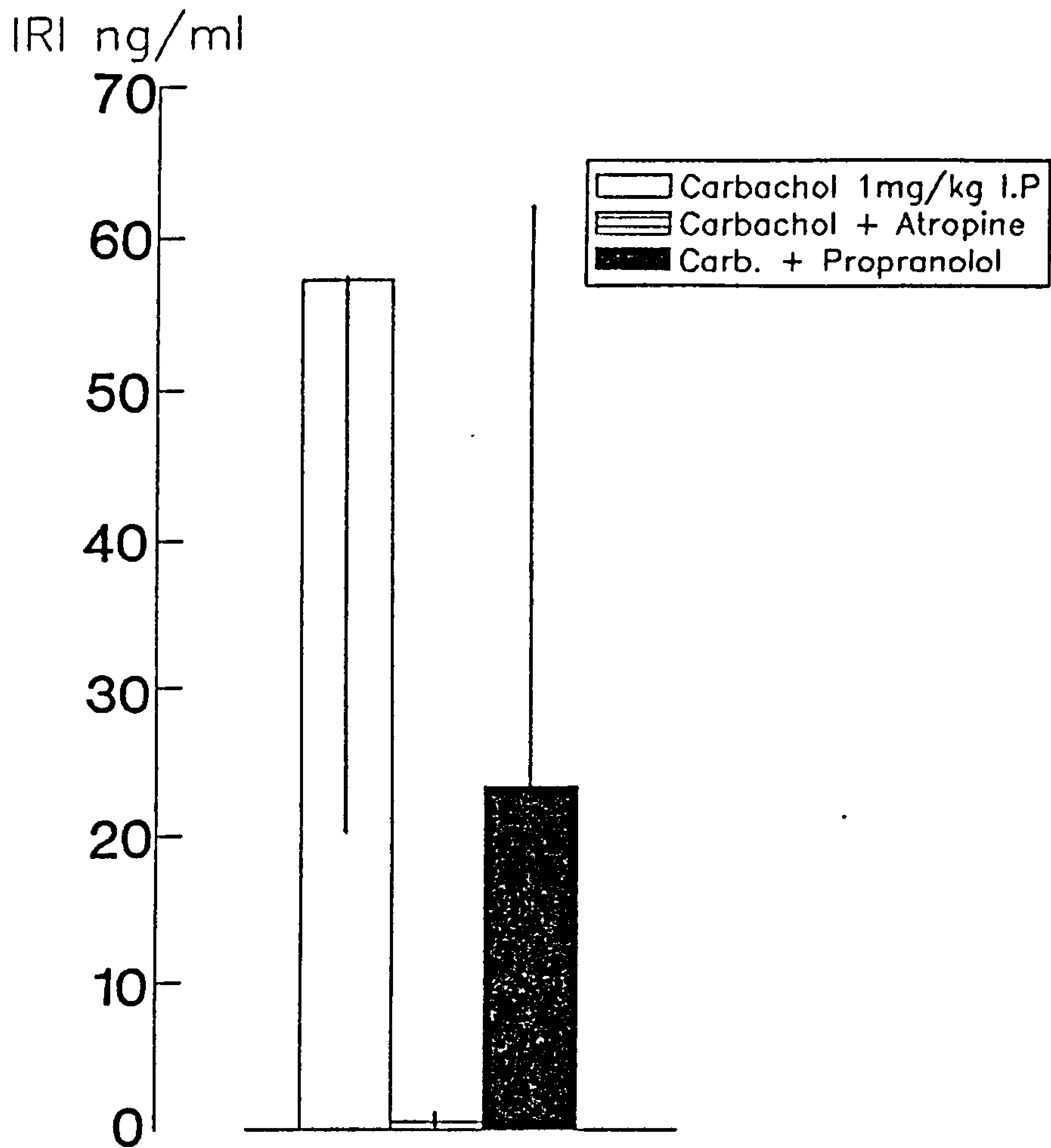


Figure 3.10

Effect of Atropine and Propranolol on Carbachol-induced immunoreactive insulin concentrations in normal mice.

N=9 for each treatment, values expressed as geometric mean with 95% C.L.

Statistical analysis.

Groups	D.F.	T.	P.
Atropine vs. carbachol	16	5.48	0.01
Propranolol vs. carbachol	15	1.71	N.S.

given in appendix 2j.

In toxin-treated animals ether produced significant hyperinsulinaemia (Figure 3.11), and this was not affected by adrenal demedullation (F for interaction = 0.207, N.S.). However, in normal mice, adrenal demedullation caused hyperinsulinaemia after exposure to ether, which was not present in sham operated animals (F for interaction = 4.454, $P < 0.05$).

This unexpected hyperinsulinaemia in adrenal demedullated, normal mice was investigated in more detail in a further experiment.

(b) Effect of adrenal demedullation in normal mice

In normal mice, ether exposure caused hyperglycaemia (appendix 2k), and this was not affected by adrenal demedullation.

The serum IRI concentrations are shown in Figure 3.12. Here, ether did not alter the serum IRI of sham operated animals, but caused hyperinsulinaemia in adrenal demedullated mice (F for interaction = 10.26, $P < 0.01$).

(c) Adrenal catecholamine content in sham operated and adrenal demedullated mice

The adrenal glands from both sham operated and adrenal demedullated mice were removed under light ether anaesthesia and homogenised separately in 0.4M perchloric acid. The adrenaline and noradrenaline content of each

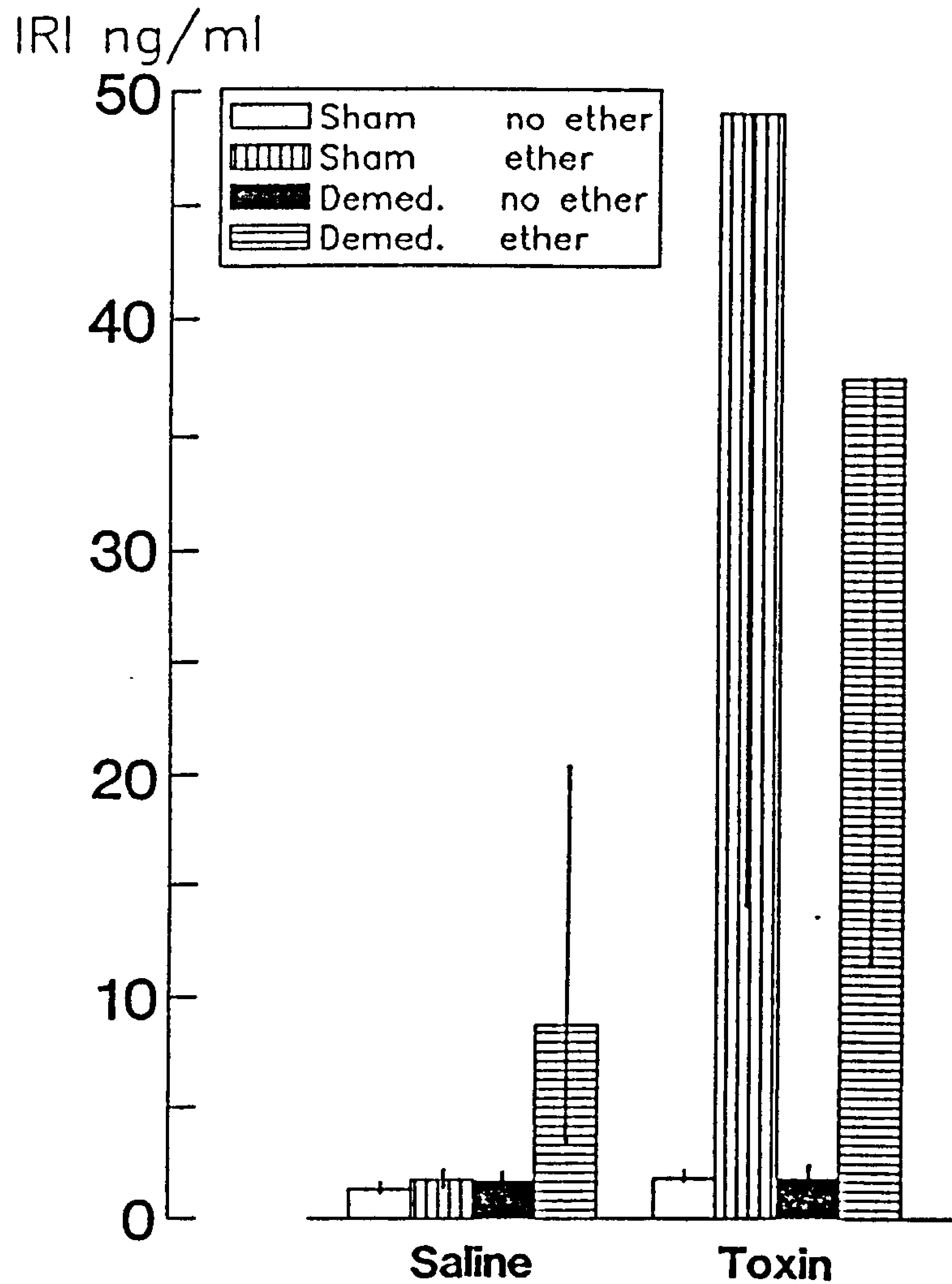


Figure 3.11.

Effect of adrenal demedullation on the serum IRI concentrations of control and pertussis toxin-treated mice.

N= 7 for each treatment, values expressed as geometric mean (95% C.L.).

Analysis of variance.

Source.	D.F.	Saline-treated F.	Saline-treated P.	Toxin-treated F.	Toxin-treated P.
Ether	1	8.30	0.01	125.39	0.01
Demedullation	1	7.90	0.01	0.26	N.S.
Interaction	1	4.454	0.05	0.207	N.S.

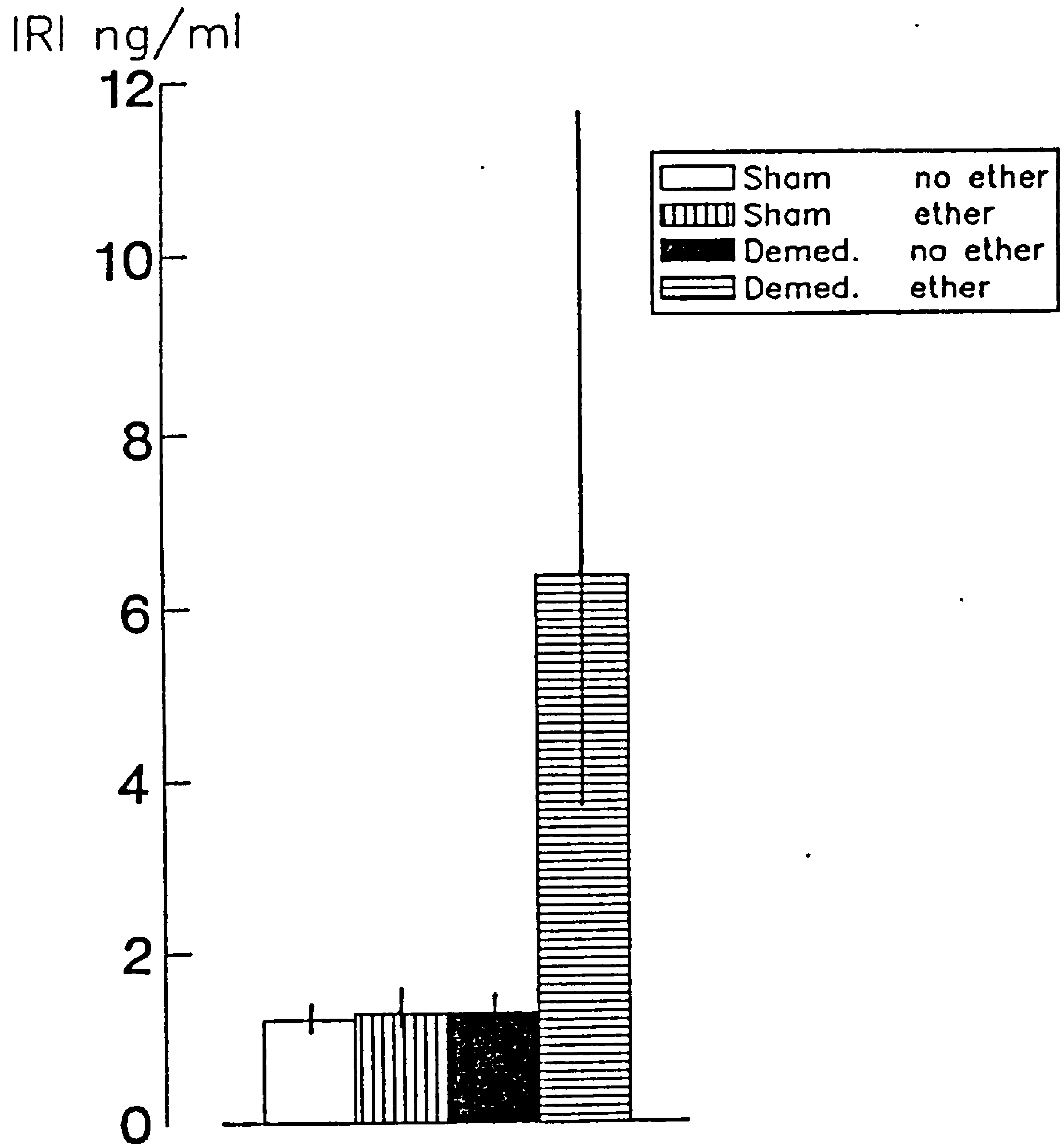


Figure 3.12

Effect of ether exposure on the serum immunoreactive insulin concentrations of adrenal demedullated mice.

N=8 for each treatment, values expressed as geometric mean with 95% C.L.

Analysis of variance.

Source	D.F.	F.	P.
Ether	1	11.95	0.01
Adrenal demedullation	1	12.97	0.01
Interaction	1	10.26	0.01

gland was assayed by HPLC (Dr. H.G. Dean, University of Leeds) and the results are given in Table 3.1(a).

Adrenal demedullation produced a very marked reduction of both adrenaline and noradrenaline content. Adrenaline content per gland was reduced by a factor of 2800, and noradrenaline content by at least 2000 times (Table 3.1(a)).

(d) Serum corticosterone responses of sham operated and adrenal demedullated mice to ether

In a preliminary experiment sham operated and adrenal demedullated animals were exposed to ether or air for 90 seconds, then bled by decapitation after 30 seconds recovery. Ether did not alter the serum corticosterone concentrations of sham or adrenal demedullated animals, and there was no difference between the corticosterone concentrations of sham operated or adrenal demedullated mice. (Serum corticosterone concentrations in ng ml^{-1}: sham/air = 175.4 (40.6); sham/ether = 174.2 (36.91); demed/air = 198.1 (34.33); demed/ether = 141.5, (39.77)).

The corticosterone concentrations of sham operated animals exposed to air (basal levels) were high because of the time of day at which the experiment was performed. Corticosterone concentrations in mice are lowest during the early hours of the morning, and rise steadily throughout the day (Engeland et al, 1977). Also, 30 seconds after ether exposure might not have

Table 3.1

Effect of adrenal demedullation on the adrenal catecholamine content, and the serum corticosterone response to ether in mice.

a) Catecholamine content of single adrenal glands (per gland) (S.E.)

	Adrenaline	Noradrenaline
Sham operated	2.24 μg (0.26)	0.91 μg (0.11)
Adrenal demedullated	0.80 ng (0.08)	< 0.5 ng

b) Corticosterone response to 90 seconds exposure to ether .

ng/ml	No Ether	Ether
Sham operated	17.2 (10.8)	59.6 (17.8)
Adrenal demedullated	37.7 (14.8)	58.3 (15.4)

Analysis of variance.

Source.	D.F.	F.	P.
Adrenal demedullation	1	0.08	N.S.
Ether	1	6.13	0.05
Interaction	1	1.17	N.S.

been the appropriate time to detect stress-induced increases in corticosterone. A further experiment was performed investigating the corticosterone concentrations at several time points after ether exposure. Mice were bled immediately or at 2.5, 5 and 10 minutes after exposure to ether. The greatest corticosterone concentrations were attained in mice bled 2.5 minutes after ether exposure (serum corticosterone concentrations ng ml^{-1} (S.E.) at bleeding time (min), 0 = 180.8 (61.3); 2.5 = 272.0 (46.51); 5 = 138 (25.9); 10 = 270 (37.1)).

A final experiment was performed in sham operated and adrenal demedullated mice to determine whether ether stress caused an increase in serum corticosterone concentrations. The experiment was performed early in the morning (all animals bled by 6 a.m.), and a recovery time of 2.5 minutes was allowed between exposure to air or ether and bleeding. The results are shown in Table 3b.

Ether caused a significant increase in serum corticosterone concentrations in both groups of mice ($F = 6.13$, $P < 0.05$), and adrenal demedullation did not affect this increase (F for interaction = 1.17, N.S.).

3.5 Ganglion blockade

(a) Effect of nicotinic cholinoceptor blockade in normal mice

Hexamethonium (6 mg kg^{-1}) or saline was given I.P.

to normal mice 20 minutes before exposure to ether vapour, or air. Hexamethonium caused a significant decrease in serum glucose (appendix 21) ($F = 7.07$, $P < 0.05$), and ether caused hyperglycaemia ($F = 6.75$, $P < 0.05$). There was no interaction between ether exposure and hexamethonium in these animals (F for interaction = 0.48, N.S.).

The corresponding serum insulin concentrations are shown in Figure 3.13. Ether caused no significant change in serum IRI in saline treated mice, but caused significant hyperinsulinaemia in hexamethonium treated mice (F for interaction = 7.42, $P < 0.05$).

(b) Effect of nicotinic cholinoceptor blockade on pertussis toxin-treated mice

The previous experiment was repeated in mice treated 5 days previously with pertussis toxin. Ether and hexamethonium had no significant effect on serum glucose (appendix 2m), nor was there an interaction between them ($F = 3.74$, 2.30, 0.08 respectively).

The serum IRI concentrations are shown in Figure 3.14. Hexamethonium did not prevent the ether-induced hyperinsulinaemia seen in toxin-treated animals (F for interaction = 0.717, N.S.).

(c) Prevention of nicotine-induced increases in insulin concentration with hexamethonium

Ganglionic stimulation with nicotine causes the

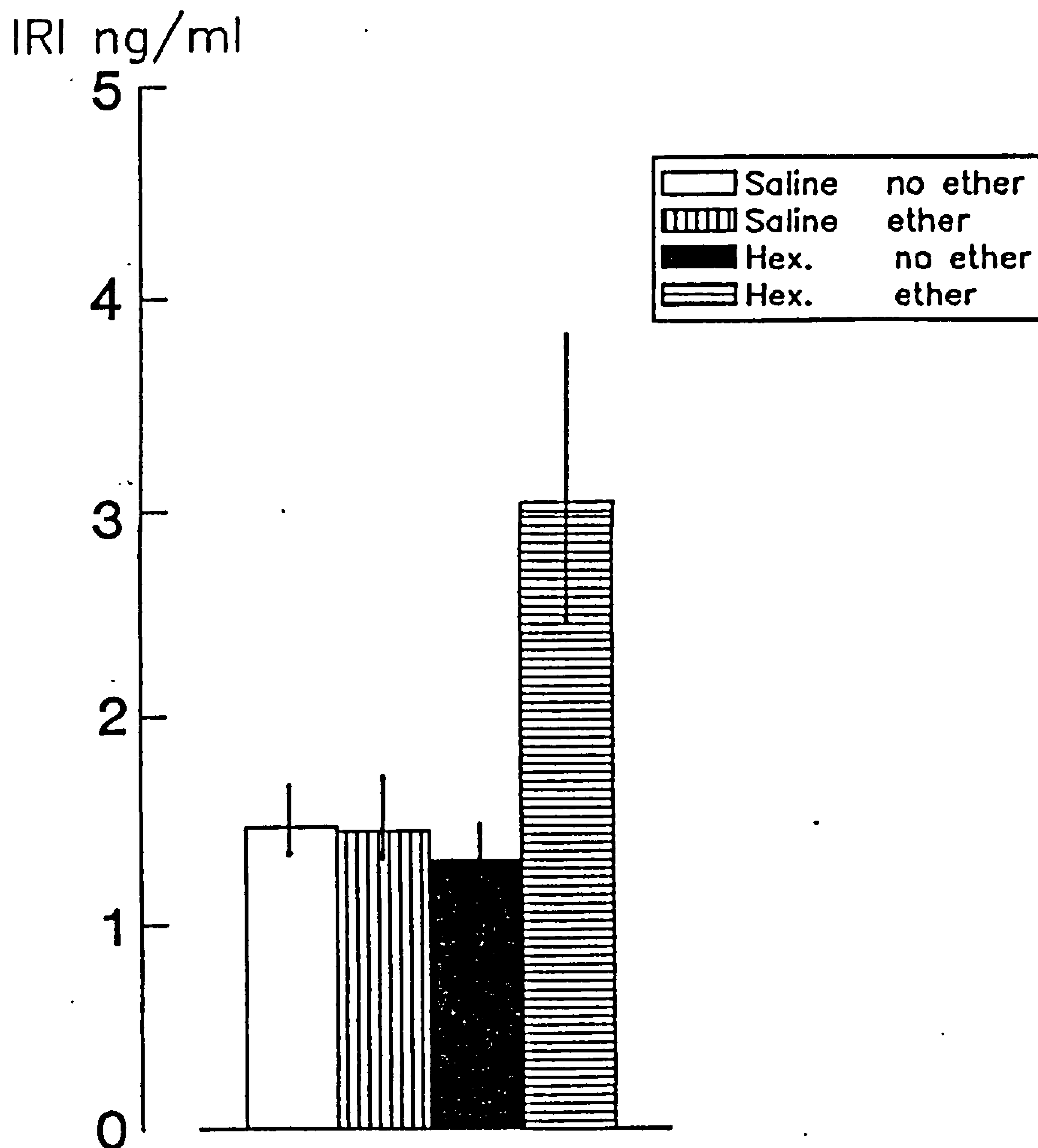


Figure 3.13

Effect of Hexamethonium on the serum immunoreactive insulin concentrations of normal mice.

N=12 for each treatment, values expressed as geometric mean with 95% C.L.

Analysis of variance.

Source	D.F.	F.	P.
Ether	1	7.01	0.05
Hexamethonium	1	3.22	N.S.
Interaction	1	7.42	0.05

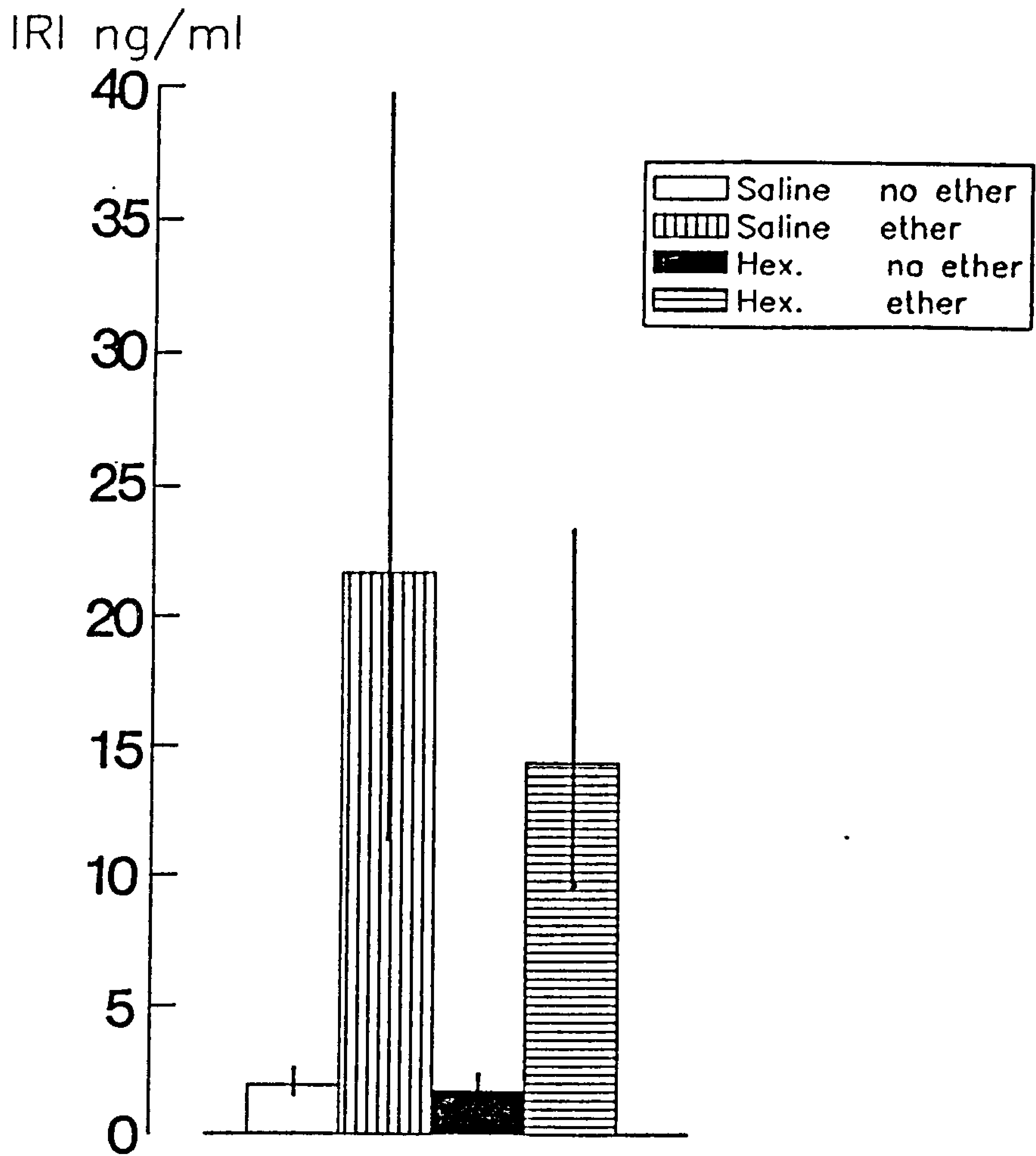


Figure 3.14

Effect of Hexamethonium on the serum immunoreative insulin concentrations of Pertussis Toxin-treated mice.

N=12 for each treatment, values expressed as geometric mean with 95% C.L.

Analysis of variance.

Source	D.F.	F.	P.
Ether	1	101.1	0.01
Hexamethonium	1	0.92	N.S.
Interaction	1	0.717	N.S.

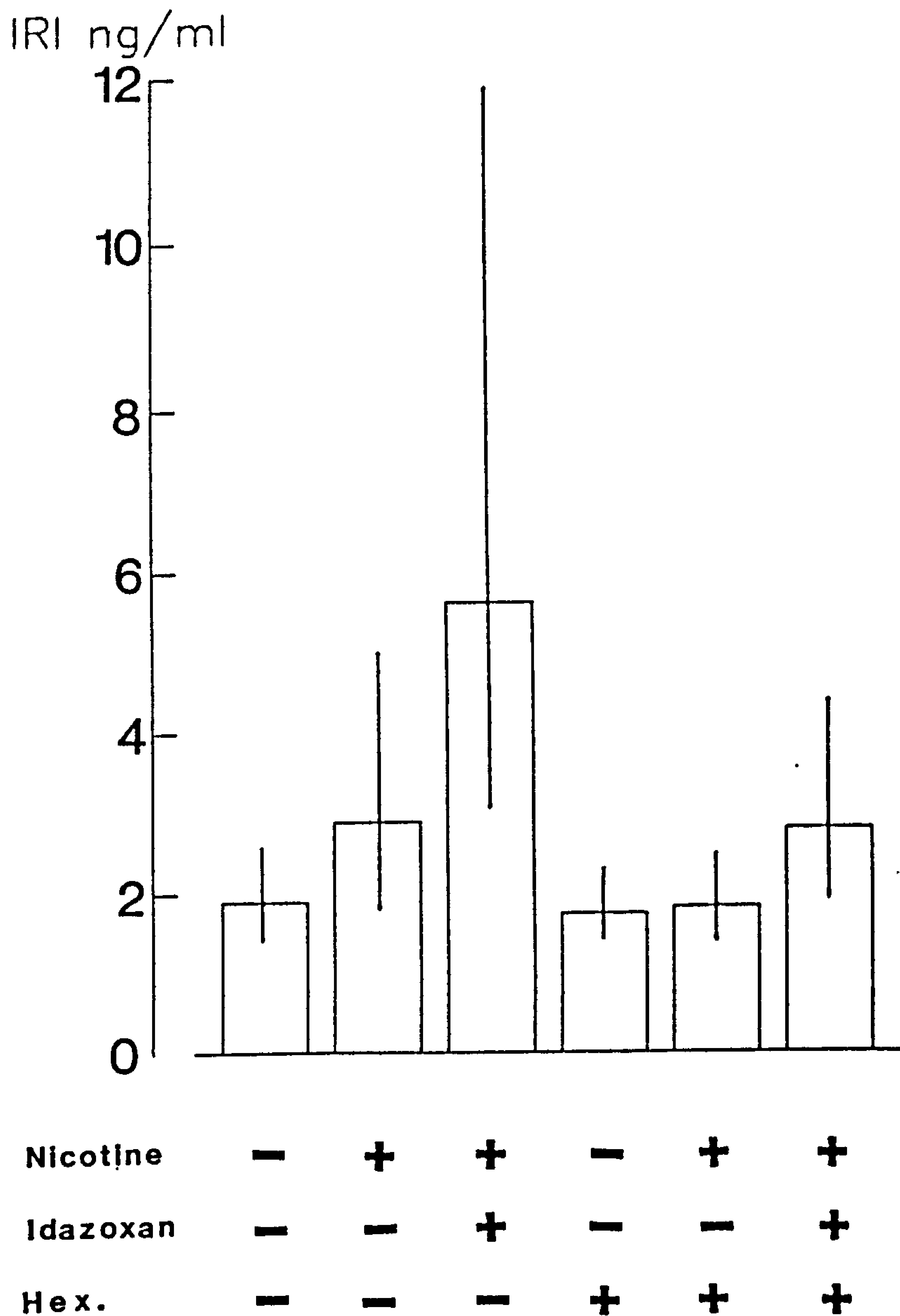


Figure 3.15

Effect of Hexamethonium on serum immunoreactive insulin concentrations after ganglionic stimulation with Nicotine.

N=6 for each treatment, values expressed as geometric mean with 95% C.L.

activation of both sympathetic and parasympathetic post-ganglionic nerves. The increase in insulin concentrations caused by parasympathetic stimulation (with nicotine) was most evident when the α-adrenoceptor mediated inhibitory action of catecholamines was prevented by idazoxan (Figure 3.15). Hexamethonium, at the dose used previously (6 mg kg^{-1}) was effective in preventing the increase in serum IRI caused by nicotine alone, or by nicotine in the presence of idazoxan (Figure 3.15).

3.6 Muscarinic cholinoceptor blockade

Atropine (6 mg kg^{-1}) or saline was given I.P. to B. pertussis infected mice 20 minutes before exposure to ether vapour or air. This dose of atropine was sufficient to prevent carbachol-induced insulin increase (Figure 3.10).

Neither atropine nor ether exposure had a significant effect on serum glucose concentrations (appendix 2n), nor was there an interaction between ether and atropine (F for interaction = 0.18, N.S.).

Atropine did not itself alter serum IRI concentrations (Figure 3.16), nor did it alter the hyperinsulinaemia caused by ether vapour in these infected mice (F for interaction = 0.189, N.S.).

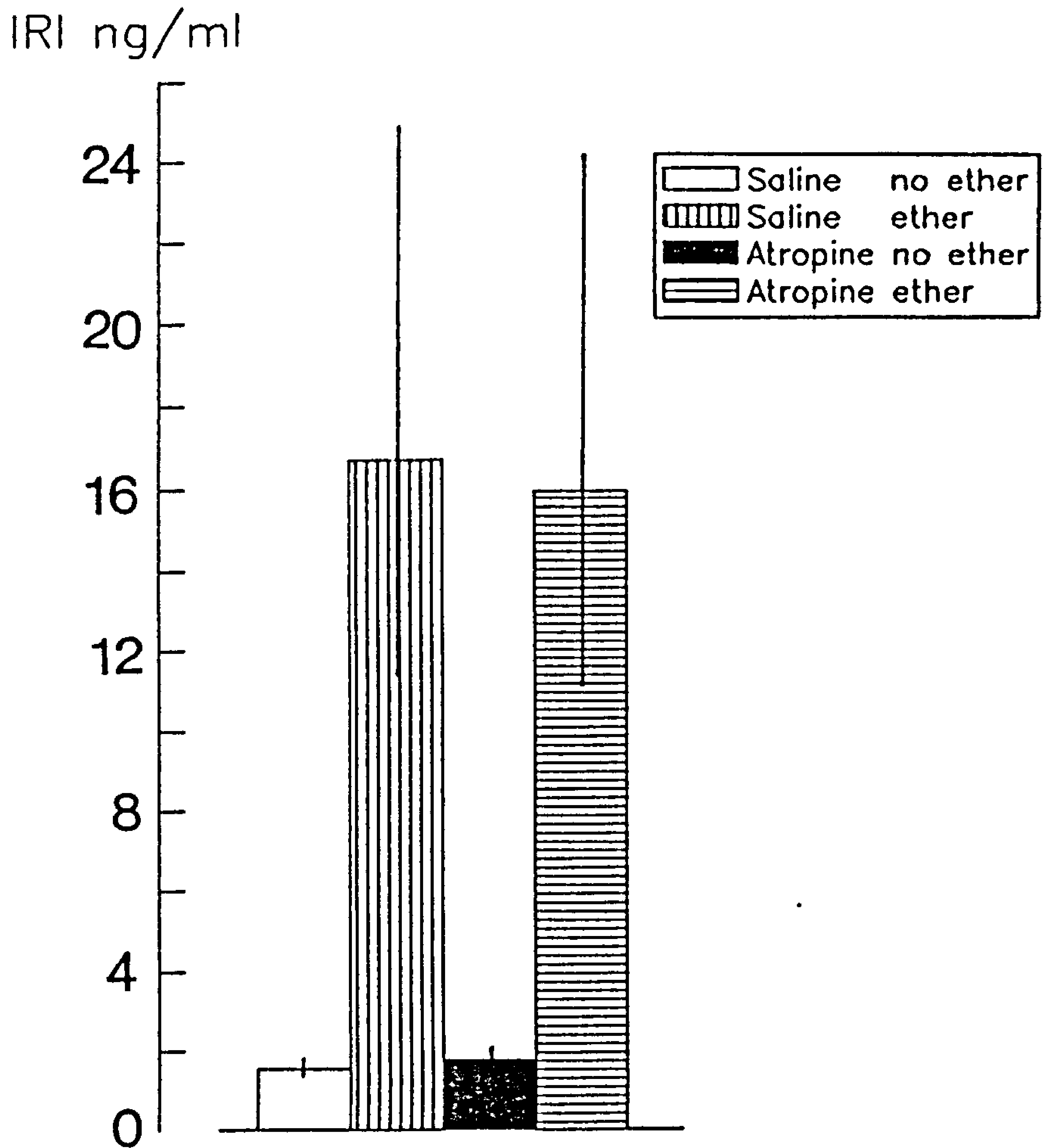


Figure 3.16

Effect of Atropine on the serum immunoreactive insulin concentrations in B. pertussis infected mice.

N=16 for each treatment, values expressed as geometric mean with 95% C.L.

Analysis of variance.

Source	D.F.	F.	P.
Ether	1	129.99	0.01
Atropine	1	0.041	N.S.
Interaction	1	0.189	N.S.

3.7 Opiate receptor blockade using naloxone

Naloxone HCl (4 mg kg^{-1}; Narcan, Dupont) or saline was given I.P. to B. pertussis infected mice 20 minutes before exposure to ether vapour or air. Naloxone caused hyperglycaemia (t test control versus naloxone-treated animals exposed to air = 2.06, $P < 0.05$) (appendix 2o). However, in ether exposed mice naloxone caused a reduction in serum glucose (F for interaction = 4.33, $P < 0.05$).

Exposure to ether produced the expected hyper-insulinaemia in these infected mice (Figure 3.17), but naloxone did not affect this hyperinsulinaemia (F for interaction = 2.34, N.S.).

3.8 Discussion

3.8.1 Changes in serum glucose

The hypoglycaemic effect of pertussis infection, vaccine or toxin is thought to be secondary to raised insulin concentrations (Results Chapter 1). Similarly, the abolition or reversal of adrenaline-induced hyper-glycaemia is attributed to the prevention of catecholamine-mediated inhibition of insulin secretion by pertussis treatment (Gulbenkian et al, 1968; Sumi and Ui, 1975; Furman et al, 1981; Nakamura et al, 1984).

Blockade of α_2 adrenoceptors using phentolamine or idazoxan mimicked the effect of pertussis treatment in causing hypoglycaemia in normal mice (appendix 2, a, b

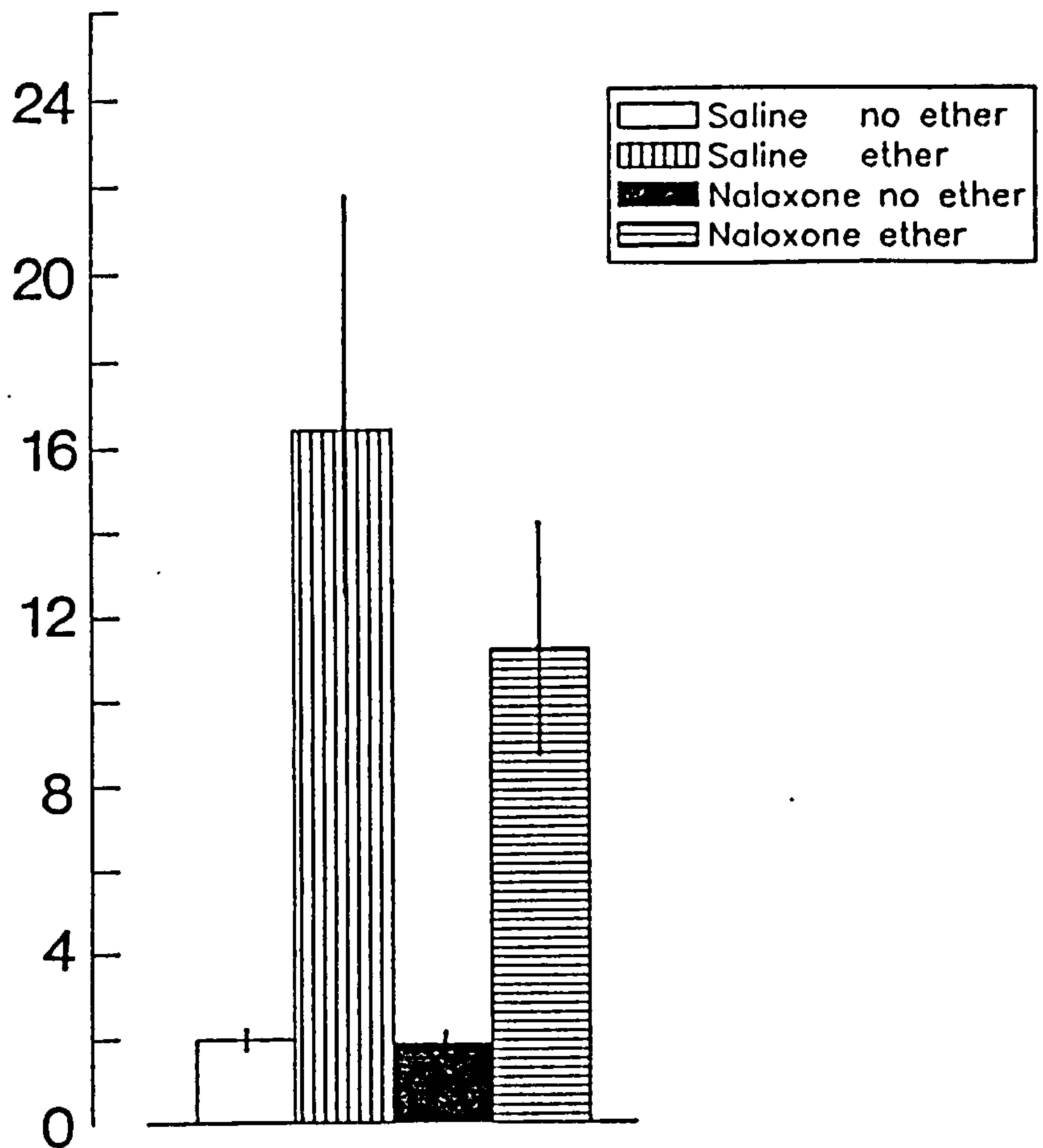


Figure 3.17

Effect of Naloxone on the serum immunoreactive insulin concentrations of B. pertussis infected mice.

N=21 for each treatment, values given as geometric mean with 95% C.L.

Analysis of variance.

Source	D.F.	F.	P.
Ether	1	172.82	0.01
Naloxone	1	1.22	N.S.
Interaction	1	2.34	N.S.

and c). Also α_2 adrenoceptor blockade with idazoxan produced further hypoglycaemia in B. pertussis infected mice. However, neither dose of phentolamine caused a further decrease in serum glucose concentrations in infected animals. This may be related to the observation that idazoxan treatment increased serum IRI in non-stressed-infected mice, whereas phentolamine (at a dose of 5 mg kg^{-1}) did not. However, the higher dose of phentolamine increased serum IRI concentrations without causing additional hypoglycaemia.

Neither α_2 adrenoceptor blockade nor pertussis-treatment (toxin or infection) affected ether-induced hyperglycaemia (appendix 2 sections a, b, c, e, g, h and o). Thus, stress-induced hyperglycaemia is not always prevented by pertussis treatment. This is difficult to explain as adrenal medullary catecholamines are thought to mediate stress-induced hyperglycaemia. However, a role for adrenal medullary catecholamines in ether-induced hyperglycaemia is not supported by the failure of propranolol, adrenal demedullation or hexamethonium to modify the ether response.

3.8.2 Changes in serum insulin

Stress-induced hyperinsulinaemia in pertussis-infected, vaccinated or toxin-treated animals may be due to the stimulatory effect of adrenaline (from the adrenal medulla) on insulin secretion, mediated by β adrenoceptors (Figure 3.18). In normal mice adrenaline

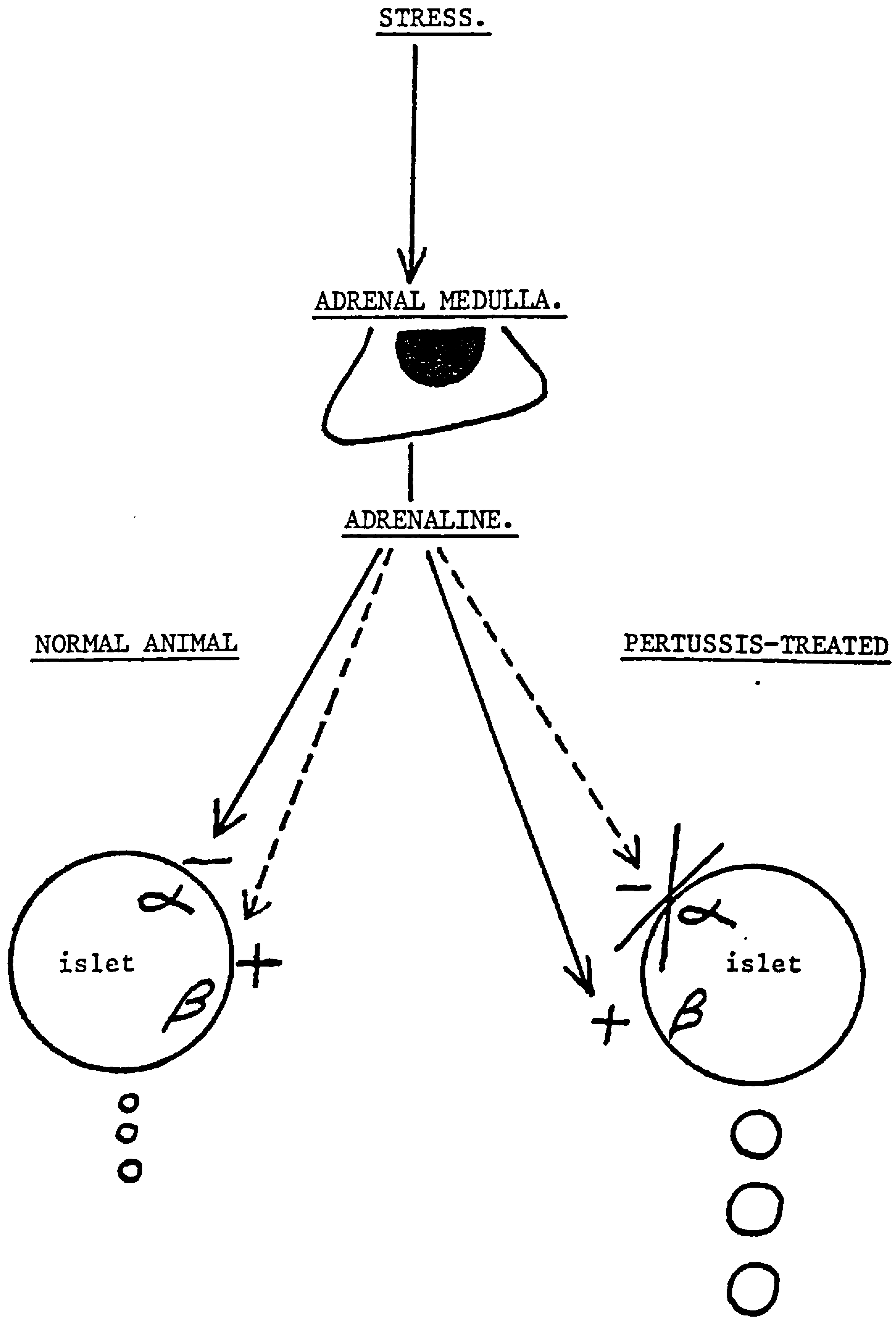


Figure 3.18

Possible mechanism of stress-induced hyperinsulinaemia in pertussis-treated animals (1).

inhibits insulin secretion through an α_2-adrenoceptor mechanism (Nakaki et al, 1981; Yamazaki et al, 1982; Ullrich and Wollheim, 1984). However, in pertussis-infected mice this is prevented by pertussis toxin, thereby allowing the β-adrenoceptor-mediated stimulation of insulin secretion to predominate (Sumi and Ui, 1975).

If the proposed mechanism (Figure 3.18) is correct, then α_2-adrenoceptor blockade in normal mice should mimic the effect of pertussis infection and pertussis toxin. This was indeed the case when the α_2 adrenoceptor blocking drug idazoxan was used in normal mice (Figure 3.3), i.e. ether exposure caused hyperinsulinaemia in idazoxan-treated animals. A similar pattern was seen with phentolamine, although the interaction between phentolamine and ether was not statistically significant (Figure 3.1). The explanation for the difference between phentolamine and idazoxan is unknown.

Next, if the hyperinsulinaemia caused by ether in pertussis infected mice - and in normal mice treated with an α_2 adrenoceptor blocking drug - is due to the action of adrenaline on pancreatic β adrenoceptors, then propranolol should prevent the response. This was indeed the case in normal animals treated with idazoxan (Figure 3.9). Propranolol may have actions on insulin secretion unrelated to β block (Furman and Tayo, 1974). However, propranolol, in the dose used in these experiments, did not significantly affect carbachol-

induced increases in insulin concentration (Figure 3.10), indicating that the drug was not acting non-specifically. Thus, the prevention by propranolol of ether-induced hyperinsulinaemia in idazoxan-treated mice supports the suggestion that, in these animals, the hyperinsulinaemia is due to the action of catecholamines on β adrenoceptors (Figure 3.18).

However, the response was not blocked by propranolol in *B. pertussis* infected or PT-treated mice, suggesting that the mechanism of the ether-induced hyperinsulinaemia was different in pertussis-treated animals (Figures 3.7 and 3.8). This is at variance with previous work (Furman et al, 1981) which showed propranolol (at a similar dose) to significantly reduce, but not prevent, ether-induced hyperinsulinaemia in B. pertussis infected mice. Also, propranolol completely abolished the *in vitro* enhancement of insulin release caused by adrenaline from perfused pancreas preparations from pertussis-sensitised rats (Katada and Ui, 1977). Both these results would suggest a role for catecholamines, via β adrenoceptors, in stress-induced hyperinsulinaemia in pertussis-treated animals. The reason for the discrepancy between the present findings and earlier observations using propranolol is unclear.

The failure of α adrenoceptor blockade with idazoxan to produce further hyperinsulinaemia in stressed *B. pertussis* infected mice is consistent with α-

adrenoceptor blockade and pertussis sharing a common mechanism i.e. the prevention of the inhibitory action of adrenaline.

Removal of the major peripheral source of adrenaline by adrenal demedullation did not alter the hyperinsulinaemic response to ether in pertussis toxin-treated mice (Figure 3.16), further evidence that the response does not involve stimulation of insulin secretion by adrenaline. The adrenal medullae were removed by adrenal enucleation (Ingle and Higgins, 1938). The contents of the adrenal capsule were removed, leaving a few cortical cells which regenerate during the following 5 weeks (Ingle and Higgins, 1938; Wilkinson et al, 1981). The removal of the adrenal medullary tissue was shown to be successful since the catecholamine content of the glands was drastically reduced by the procedure (Table 3.1). Also the cortical tissue had regenerated, and the hypothalamo-pituitary-adrenal axis appeared to be functioning normally in adrenal demedullated mice, since the serum corticosterone concentrations were not affected by demedullation. Moreover, the increase in serum corticosterone caused by ether exposure was not altered in adrenal demedullated mice (Table 3.1.b).

The lack of effect of adrenal demedullation is supported by the failure of ganglionic blockade with hexamethonium (which would prevent the release of catecholamines from the adrenal medulla and sympathetic

nerves) to prevent the hyperinsulinaemia caused by ether in toxin-treated mice (Figure 3.14). The dose of hexamethonium used (6 mg kg^{-1}) was sufficient to block nicotinic cholinoceptors, since it completely prevented nicotine-induced hyperinsulinaemia in both normal, and idazoxan-treated mice (Figure 3.15).

An unexpected finding was that adrenal demedullation not only failed to prevent stress-induced hyperinsulinaemia in pertussis toxin-treated mice (Figure 3.11) but actually mimicked (qualitatively) the effect of P T in allowing stress-induced hyperinsulinaemia in normal mice (Figure 3.12). Similar findings were made using hexamethonium (Figures 3.13 and 3.14).

It thus appears that the absence of adrenal catecholamines, or the prevention of their inhibitory action using P.T or α_2 adrenoceptor blockade, all result in stress-induced hyperinsulinaemia. Therefore, adrenaline released in response to stress may normally serve to inhibit the insulin secretion which may otherwise occur. This is supported by the recent observation that high intensity exercise increased plasma insulin concentrations to a significantly greater extent in adrenal demedullated rats than in controls (Marker et al, 1986).

3.8.3 Possible mediators of stress-induced hyperinsulinaemia

The previous argument depends upon the release by

stress of some insulin-releasing factor(s) (Fig. 3.19). The ether-induced hyperinsulinaemia in pertussis treated mice is quantitatively much greater than that seen in idazoxan-treated, adrenal demedullated or ganglion-blocked, normal mice (Figures 2.1, 3.5 and 3.8 c.f. Figures 3.3, 3.12 and 3.13). This may imply that the secretion of the substance(s) is increased in pertussis-treated animals. Pertussis toxin treatment enhanced growth hormone releasing factor-induced cAMP synthesis and growth hormone release, and gonadotrophin releasing hormone-evoked LH secretion from anterior pituitary monolayers (Cronin et al, 1983; 1984). Also, ACTH release from AtT-20 (mouse anterior pituitary cell line) was increased after pertussis toxin-treatment (Reisine, 1985), It also increases secretagogue-induced catecholamine release *in vitro* (Tanaka et al, 1987) from an adrenal chromaffin cell line.

Possible candidates for the hyperinsulinaemic substance do not appear to include acetylcholine, since atropine, in a clearly effective dose (Figure 3.10) did not modify stress-induced hyperinsulinaemia (Figure 3.16). Endorphins are released from the anterior pituitary and neurointermediate lobe in response to stress (Berkenbosch et al, 1984; Akil et al, 1985; De Souza and van Loon, 1985; EL-Tayeb et al, 1986) and may stimulate insulin secretion (Ipp et al, 1978; Reid and Yen, 1981; Reid et al, 1984). This action of endorphins can be blocked

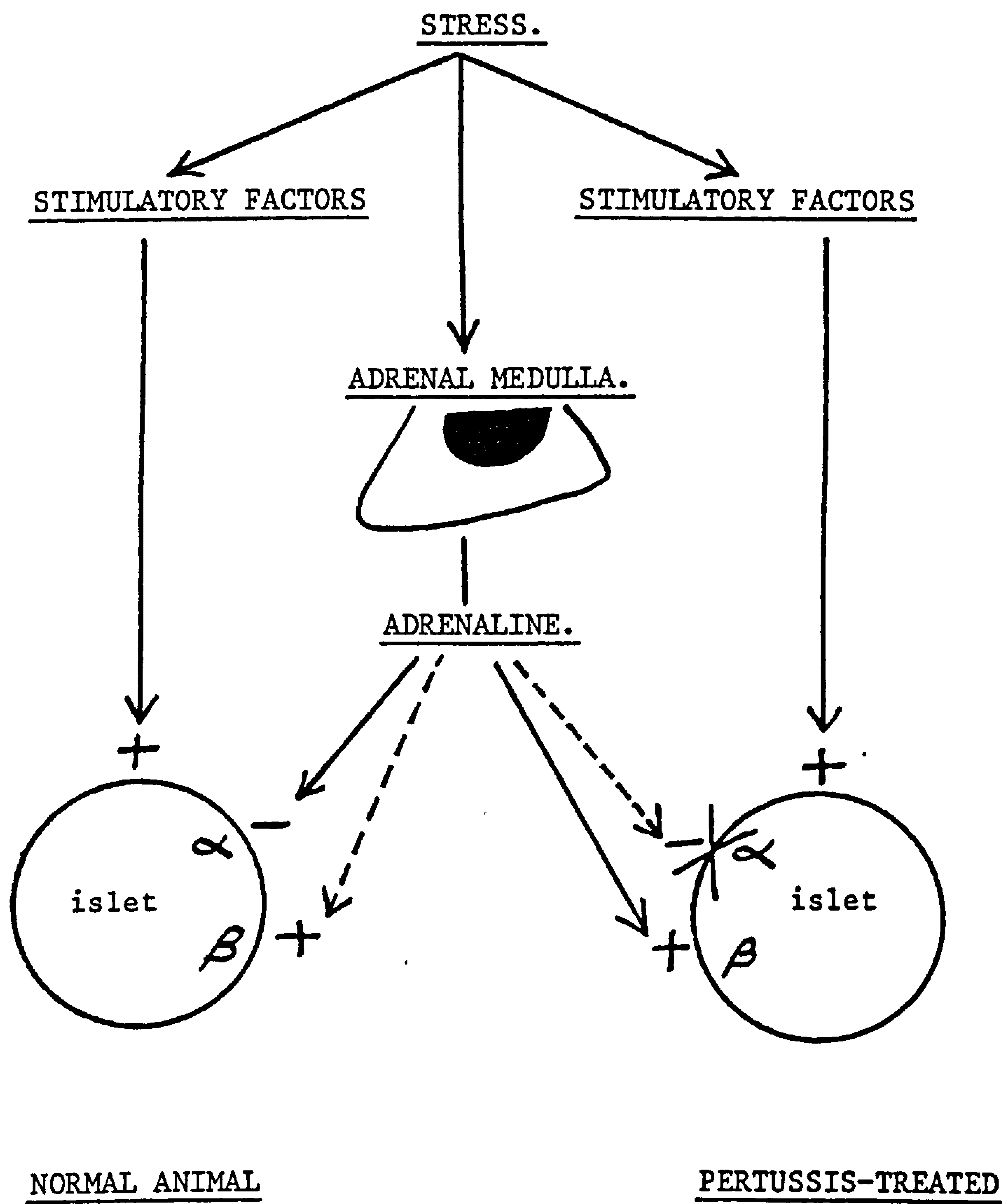


Figure 3.19

Possible mechanism of stress-induced hyperinsulinaemia in pertussis-treated animals (11).

by naloxone (Ipp et al, 1978). However, the failure of naloxone to modify stress-induced hyperinsulinaemia in pertussis-infected mice argues against a role for endorphins in mediating this response (Figure 3.17).

Admittedly, no direct evidence was obtained that naloxone was blocking opiate receptors in the present experiments, but the dose used was adequate to precipitate a withdrawal syndrome in morphine-dependent mice (Furman, personal communication). However, the possibility remains that the dose was inadequate to block opiate receptors on the pancreatic islets.

The islets possess autonomic VIP, gastrin and cholecystokinin (CCK)-containing nerves (Larsson and Rehfeld, 1979; Bishop et al, 1980; Rehfeld et al, 1980) that may have a stimulatory effect on insulin secretion (Raptis et al, 1975; Ahren and Lundquist, 1981). However, the failure of hexamethonium to modify the stress-induced hyperinsulinaemia suggests the lack of involvement of stimulatory autonomic nerves (Figure 3.14).

Stress releases other substances such as glucagon and corticotrophin which can stimulate insulin secretion (Engeland et al, 1977; Kikkawa et al, 1981). Pilot experiments with these substances (data not shown) revealed no consistent hyperinsulinaemic effect. Moreover, although Lundquist and Rerup (1967) showed synthetic ACTH to be insulinotropic in the mouse, the

doses required appeared to be much greater than those needed for steroidogenesis, therefore casting doubt on the physiological significance of ACTH as an insulinotropic agent.

Various ACTH-related peptides such as corticotrophin like intermediate lobe peptide (CLIP, 18-39 ACTH) or β cell-tropin (22-39 ACTH), are also secreted from the pars intermedia of the pituitary (Billingham et al, 1982). Of these peptides, β-cell-tropin showed marked insulin secretagogue activity (Beloff-Chain et al, 1979) and has been shown to be present in human plasma (Salvatoni et al, 1986). These substances may well be co-released with ACTH in response to stress, and may mediate the stress-induced hyperinsulinaemia seen in pertussis-treated mice. Similarly, the stress-induced hyperinsulinaemia seen in normal mice treated with α_2 adrenoceptor blocking drugs, hexamethonium or in adrenal demedullated animals may also involve these peptides. However, there is no information on the release of these potent insulinotropic agents in response to stress, and their involvement remains highly speculative.

Results Chapter 4

Metabolic alterations in infants with clinical pertussis

4.1 Introduction

4.2 Study design

4.3 Effect of anticoagulants in glucose and IRI determinations

4.4 Miscellaneous data

4.5 Plasma glucose and IRI concentrations

4.6 Plasma calcium, magnesium and phosphate concentrations

4.7 Discussion

4.1 Pertussis vaccine and B. pertussis respiratory infection produce hypoglycaemia and hyperinsulinaemia in experimental animals (Gulbenkian et al, 1968; Pittman et al, 1980; Furman et al, 1981). This has been attributed to pertussis toxin present in the vaccine, or elaborated during the course of infection with B. pertussis (Pittman, 1984a).

However, there have been few reports of metabolic changes during human pertussis. In 1936 Regan and Tolstoouhov reported hypoglycaemia in children with pertussis. In light of the data presented in section one, this may have been due to an excessive secretion of insulin caused by pertussis toxin. Similarly, PT-mediated hyperinsulinaemia could be responsible for the attenuation of adrenaline-induced hyperglycaemia observed in pertussis cases (Badr-El-Din et al, 1976). However, no measurement of serum insulin concentrations have been made in infants with pertussis. Only one report has been made of plasma insulin concentrations in response to B. pertussis components in the human. Hannik and Cohen (1979) showed a slight but significant increase in plasma insulin, without hypoglycaemia in healthy children given pertussis vaccine.

Of the other activities attributed to PT, lymphocytosis is often present in human pertussis, and is indeed used as a diagnostic sign of the disease (Walker et al, 1981). Histamine sensitising activity

has been reported (Sanyal, 1960), but also denied (Pieroni et al, 1971) in children with pertussis.

The object of the present study was to determine whether hyperinsulinaemia and/or hypoglycaemia are present in hospitalised cases of pertussis. Hypoglycaemia may contribute to the rare neurological complications which can accompany the disease. Plasma calcium, magnesium and phosphate measurements were also performed on the samples, since a previous study suggested that blood constituents other than glucose might be altered in whooping cough (Regan and Tolstoouhov, 1936).

4.2 Study Design

The study was approved by the Glasgow Northern District Ethical Committee, and involved 52 children aged between 1 month and 3 years who were admitted to the Infectious Diseases Unit at Ruchill Hospital, Glasgow, between January 1984 and March 1986. Of these children, 25 were diagnosed as having pertussis by the criteria of Walker et al (1981). These criteria were the presence of a typical paroxysomal cough and at least one of the following: i) a whoop and a duration of 21 days or more; ii) a whoop and a pronounced lymphocytosis; iii) a positive pernasal swab for *B. pertussis*. The remaining 27 children served as controls and were diagnosed as having an infectious disease other than pertussis. Patients with gastro-enteritis were excluded from the

study, since glucose and insulin homeostasis may be disturbed both by the condition and its treatment. The diarrhoea and vomiting of gastro-enteritis can cause severe dehydration, disturbed electrolyte balance and exhaustion. Nitzan et al (1968) showed that hyper-natraemia caused an increase in fasting glucose concentrations, and a decreased ability to dispose of an exogenous glucose load. Malabsorption of nutrients caused by vomiting would also affect metabolic homeostasis. In young children, treatment of diarrhoea often involves the frequent use of balanced electrolyte solutions, which contain high concentrations of glucose, sucrose or dextrose.

Ethical considerations allowed only a single specimen of blood to be taken from each patient. This was collected when a routine blood sample was taken for medical reasons, usually at admission. Blood was distributed between heparin-fluoride, lithium-heparin and EDTA containing tubes. All plasmas were separated within 2 hours, and stored at -20°C before assay.

4.3 Effect of anticoagulants on glucose and IRI determinations

Initially all blood samples were collected in fluoride-heparin anticoagulant tubes which are used routinely in hospital for the assay of blood glucose, since the fluoride prevents glycolysis by inhibiting

glycolytic enzymes. To determine whether fluoride-heparin interfered with either the glucose determination or the IRI assay, blood from two volunteers was collected in both anticoagulants and was assayed in duplicate. The results are given in Table 4.1, and show that there was no significant difference between measured glucose in the two anticoagulants, but that the serum IRI concentrations were consistently lower in the heparin-fluoride samples. To investigate this further, human insulin standards from the Amersham insulin R.I.A. kit was used to give 10 μU, 20 μU and 80 μU solutions. These were added to both anticoagulants and assayed against the rat insulin standards in the normal assay. The results are given in Table 4.2, and show that fluoride-heparin gives lower IRI values than the lithium-heparin tubes. Because of this, all human samples were collected in both anticoagulants.

4.4 Miscellaneous data

The pertussis and control groups were similar in both sex distribution and age (pertussis 12 m, 13 f; control 16 m, 11 f: median age and range - pertussis 7 (1-36); control 9 (2-30)). The median time of blood sampling in relation to previous feeding was similar (pertussis 115 minutes, control 119 minutes), as was the range of times after food (pertussis 3-315, control 10-450). Of the 25 pertussis cases, 10 had lymphocytosis as defined by Walker et al (1981), and 15 yielded B.

Table 4.1

Effect of fluoride-heparin anticoagulant on serum glucose and IRI concentrations from two persons.

	Lithium-heparin		Fluoride-heparin	
	IRI	Glucose	IRI	Glucose
Sample 1.1	1.41 1.43	4.9	1.22 1.18	4.9
Sample 1.2	1.38 1.45	5.2	1.28 1.28	5.0
Sample 2.1	1.71 1.75	4.5	1.36 1.12	4.6
Sample 2.2	1.77 1.72	4.7	0.79 0.99	4.6

Note: Each volunteer gave two samples which were assayed for glucose (mmol/l) and in duplicate for insulin (ng/ml), using the standard insulin assay.

Table 4.2

Effect of fluoride-heparin anticoagulant on the determined IRI concentrations of standard human insulin solutions, determined against rat insulin standards.

Standard (µU/ml)	Fluoride-heparin (ng/ml)	Lithium-heparin (ng/ml)
10	0.766 (0.53,1.14)	0.67 (0.56,0.82)
20	1.99 (1.82,2.19)	2.38 (0.48,12.02)
80	6.11 (4.89,7.64)	7.48 (7.29,7.67)

Note: Results expressed as geometric mean (95% C.L.), N=6

pertussis from pernasal swabs. The two groups did differ in their duration of stay in hospital, the pertussis cases having a median of 11 days (range 0-24), and the control a median of 6 days (range 2-13). This was taken to be a measure of duration of illness. Also, whereas none of the pertussis cases had been vaccinated against pertussis, 11 of the control patients were known to have been vaccinated (14 not vaccinated, two unknown).

4.5 Plasma glucose and IRI concentrations

Table 4.3 shows the results of the glucose and IRI analyses of the human specimens. With both anticoagulants the mean IRI concentration in pertussis plasmas was higher than that of controls, but only in the heparin-fluoride plasmas was this difference statistically significant ($P < 0.05$). Comparing the two anticoagulants, the average IRI of lithium heparin plasmas was 24% higher than that of the heparin-fluoride plasmas. This difference was highly significant when analysed by paired-sample t-test ($P \ll 0.01$).

There was no significant difference in the plasma glucose concentrations between pertussis and control samples (Table 4.3), and the anticoagulant used did not affect glucose concentration.

4.6 Plasma calcium, magensium and phosphate concentrations

The mean concentrations of calcium, magnesium and phosphate are given in Table 4.4, and show no significant

Table 4.3

Mean levels, 95% confidence limits and t-tests of the plasma insulin and glucose in pertussis and control patients.

	IRI (μU/ml)		Glucose (mmol/l)	
	heparin-fluoride plasmas	lithium-heparin plasmas	heparin-fluoride plasmas	lithium-heparin plasmas
Pertussis	13.3 10.2-17.3 n=24	15.5 11.8-20.5 n=24	5.19 4.8-5.6 n=24	5.00 4.58-5.41 n=24
Control	8.9 7.2-11.0 n=27	11.4 8.6-15.2 n=21	4.85 4.50-5.20 n=27	4.78 4.33-5.24 n=21
P values (<)	0.05	N.S.	N.S.	N.S.

Note: plasma glucose values shown as arithmetic mean, plasma insulin values shown as geometric mean.

Table 4.4

Mean levels, 95% confidence limits and t-tests on calcium, magnesium and phosphate levels in plasmas from pertussis and control patients.

	calcium	magnesium	phosphate
Pertussis	2.3 2.17,2.43 n=20	0.83 0.79,0.88 n=16	0.042 0.035,0.049 n=14
Control	2.31 2.11,2.5 n=21	0.87 0.64,1.09 n=16	0.036 0.032,0.040 n=18
P values (<)	N.S.	N.S.	N.S.

Note: values given as mmol/l

difference between pertussis and control plasmas.

4.7 Discussion

This study suggests that plasma insulin concentrations may be slightly elevated in pertussis, but that hypoglycaemia, if it occurs at all, is uncommon. This is in contrast to results obtained in B. pertussis infected mice, which consistently show both hyperinsulinaemia and hypoglycaemia (Pittman et al, 1980; Furman et al, 1981). It also contradicts the findings of Regan and Tolstoouhov (1936) who clearly showed hypoglycaemia in infants with whooping cough. The results also differ in that a lower plasma phosphate in pertussis cases was not shown. These authors, however, measured whole blood sugar by a method which estimates total reducing sugar, rather than glucose specifically. However, the children studied by Regan and Tolstoouhov were older than those studied here (78% were more than 36 months old). This may be relevant since we have found that the metabolic effects of pertussis toxin are developed fully only in older mice (Figures 2.13 and 2.14).

The hyperinsulinaemia seen in the B. pertussis infected mouse can be attributed to an exaggerated serum insulin response to feeding, and a qualitatively abnormal serum insulin response to stress (see results sections 1 and 2). It is possible that a small proportion of

pertussis patients may experience a transient but marked increase in insulin which was not detected in this study. The violent coughing spasms which occur during the paroxysmal stage of the disease are prolonged, and can be accompanied by apnoea with resultant hypoxia and cyanosis. By analogy with the animal work presented in section two, these stressful episodes may well give rise to marked hyperinsulinaemia, with resultant hypoglycaemia, in susceptible infants.

It should be noted that the control group consisted of children suffering from various infectious diseases, and therefore were not ideal.

Since both groups were similar in sex and age distribution, had similar food and the samples were obtained at similar times after food, it can be suggested that the slight increase in plasma insulin is due to the pertussis. In animal studies, pertussis toxin is the component of pertussis vaccine and B. pertussis infection which causes hyperinsulinaemia (Katada and Ui, 1980). Thus, the modest hyperinsulinaemia reported here may be another manifestation of pertussis toxin activity in the human, in addition to lymphocytosis.

The reason for the difference in measured insulin concentrations between the two anticoagulants is unknown. It does, however, appear to be due to direct interference by heparin-fluoride with the insulin assay rather than a reaction within the plasma sample, since the measured

concentration of insulin in a solution of insulin in buffer was lower in the heparin fluoride tubes (Table 4.2).

Concluding Remarks

The work in this thesis showed clearly that there is an abnormal increase in insulin in response to feeding or to stress in *B. pertussis* infected or pertussis-toxin-treated mice. Although hypoglycaemia was not detected in the clinical study, the slight hyperinsulinaemia suggests that the metabolic activities of P.T. are manifest in human patients. Indeed the previously reported lymphocytosis (Walker et al, 1981) and attenuation of adrenaline-induced hyperglycaemia (Badr-el-Din et al, 1976) are also measures of pertussis toxin activity.

It is therefore possible that hyperinsulinaemia, with resultant hypoglycaemia, may contribute to the rare neurological sequelae which follow exposure of human infants to pertussis toxin (either during the course of the disease, or following vaccination). Some patients may be at particular risk, perhaps because of age, some genetic susceptibility to the action of pertussis toxin, or because of exposure to some additional stress (e.g. concurrent viral or bacterial infection, coughing spasms or even altered routine) which could result in hyper-insulinaemia. Alternatively, sensitivity to, or elaboration of the unknown hyperinsulinaemic factor(s) could vary between patients.

Appendix 1

1. <u>Buffer A</u>

Bovine Serum Albumin (Sigma RIA Grade)	5.1 g
Thiomeresal (BDH)	0.25 g
Sodium dihydrogen orthophosphate (BDH)	6.2 g

Made to 900 mls with distilled water, then 0.1N NaOH added to pH 7.4. Made to 1 litre with distilled water. Stored at 2-4°C.

2. <u>Buffer B</u>

Buffer A + 0.9% NaCl, stored at 2-4°C.

3. <u>Buffer C</u>

Buffer A	50%
Defibrinated Horse Serum (Flow)	50%

Mix gently with 0.1 g diatomaceous earth (Sigma), then filter through grade 1 qualitative filter paper (Whatman). Filtrate kept cold until use (2-4°C).

Appendix 2

Serum glucose concentrations for results section 2. Values given as mmol/l, expressed as arithmetic mean (S.E.).

(a) Phentolamine 40 mg/kg n = 6, normal mice

Saline, no ether	11.03	(0.47)
Saline, ether	12.58	(0.44)
Phentolamine, no ether	7.40	(0.51)
Phentolamine, ether	8.03	(0.44)

(b) Phentolamine 5 mg/kg n = 6, normal mice

Saline, no ether	13.7	(0.59)
Saline, ether	15.25	(0.44)
Phentolamine, no ether	9.65	(0.39)
Phentolamine, ether	10.53	(0.58)

(c) Idazoxan 5 mg/kg n = 14, normal mice

Saline, no ether	11.16	(0.49)
Saline, ether	12.04	(0.36)
Idazoxan, no ether	7.70	(0.33)
Idazoxan, ether	9.65	(0.20)

(d) Phentolamine 40 mg/kg n = 18, infected mice

Saline, no ether	5.67	(0.33)
Saline, ether	6.11	(0.34)
Phentolamine, no ether	5.60	(0.21)
Phentolamine, ether	5.52	(0.23)

(e) Phentolamine 5 mg/kg n = 5, toxin-treated mice

Saline, no ether	7.64	(0.37)
Saline, ether	9.54	(0.80)
Phentolamine, no ether	6.40	(0.19)
Phentolamine, ether	9.38	(0.78)

(f) Idazoxan 5 mg/kg n = 14, infected mice

Saline, no ether	5.99	(0.36)
Saline, ether	6.95	(0.52)
Idazoxan, no ether	4.39	(0.31)
Idazoxan, ether	5.86	(0.41)

(g) Propranolol 20 mg/kg n = 18, infected mice

Saline, no ether	5.34	(0.26)
Saline, ether	6.51	(0.41)
Propranolol, no ether	5.16	(0.31)
Propranolol, ether	5.49	(0.25)

(h) Propranolol 20 mg/kg n = 5, toxin-treated mice

Saline, no ether	8.76	(0.53)
Saline, ether	10.14	(0.39)
Propranolol, no ether	8.54	(0.15)
Propranolol, ether	11.30	(0.36)

(i) Propranolol 20 mg/kg plus Idazoxan 5 mg/kg
n = 14, normal mice

Saline, no ether	10.37	(0.29)
Saline, ether	11.71	(0.30)
Idazoxan plus propranolol, no ether	9.13	(0.25)
Idazoxan plus propranolol, ether	9.46	(0.32)

(j) Adrenal demedullation n = 7, normal and toxin-treated mice

i) Normal mice

Sham, no ether	9.93	(0.19)
Sham, ether	11.94	(0.53)
Adrenal demedullated, no ether	10.21	(0.24)
Adrenal demedullated, ether	11.43	(0.53)

ii) Toxin-treated mice

Sham, no ether	6.77	(0.25)
Sham, ether	8.03	(0.28)
Adrenal demedullated, no ether	7.10	(0.16)
Adrenal demedullated, ether	7.40	(0.37)

(k)	Adrenal demedullation n = 8, normal mice		
	Sham, no ether	9.79	(0.23)
	Sham, ether	11.01	(0.25)
	Adrenal demedullation, no ether	9.24	(0.34)
	Adrenal demedullation, ether	10.04	(0.23)
(1)	Hexamethonium 6 mg/kg n = 12, normal mice		
	Saline, no ether	10.86	(0.41)
	Saline, ether	12.23	(0.25)
	Hexamethonium, no ether	10.04	(0.53)
	Hexamethonium, ether	10.83	(0.42)
(m)	Hexamethonium 6 mg/kg n = 12, toxin-treated mice		
	Saline, no ether	8.98	(0.46)
	Saline, ether	9.85	(0.46)
	Hexamethonium, no ether	8.26	(0.33)
	Hexamethonium, ether	8.80	(0.52)
(n)	Atropine 6 mg/kg n = 16, infected mice		
	Saline, no ether	6.25	(0.33)
	Saline, ether	6.33	(0.31)
	Atropine, no ether	6.66	(0.33)
	Atropine, ether	6.96	(0.26)
(o)	Naloxone 4 mg/kg n = 21, infected mice		
	Saline, no ether	5.51	(0.20)
	Saline, ether	6.44	(0.20)
	Naloxone, no ether	6.30	(0.32)
	Naloxone, ether	6.05	(0.36)

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