Adenylate Cyclase Activity During Modulation of Bordetella pertussis

Robert M. Brownlie

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M. DOWNIE
Printers and Bookbinders
(Baltic Chambers)
50 Wellington St. G2 6HJ
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ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Declaration

This thesis is the original work of the author.

Robert M. Brownlie

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List of Abbreviations and Terms

AC	Adenylate cyclase
BDS	Blue dextran-Sepharose
BG	Bordet-Gengou medium
BG-C	BG made from basic ingredients, but with $MgSO_4$ replacing the NaCl (Appendix 1)
BG-X	BG made from basic ingredients (Appendix 1)
C media	Media in which growth induces the C-mode variant
C-mode	Avirulent, "cyanic" phenotype of *B. pertussis*
C modulation	The transition from an X-mode variant to a C-mode variant
CRP	Cyclic AMP-receptor protein
crp	Genetic locus of CRP
CRP-cAMP	CRP bound with cAMP
cya	Genetic locus of AC
DEAE-cellulose	Diethylaminoethyl-cellulose
EDTA	Ethylenediamine-tetraacetic acid
EGTA	Ethyleneglycol-bis-(β-aminoethyl ether)N,N'-tetraacetic acid
F-HA	Filamentous haemagglutinin
HA	Haemagglutinin
H-C	Modified Hornibrook medium with $MgSO_4$ replacing the NaCl (Appendix 1)
HLT	Heat-labile toxin
HSA	Histamine-sensitizing activity
HSF	Histamine-sensitizing factor
HSD_{50}	Dose of heat-killed cells which leads to the death of 50% of animals
H-X	Modified Hornibrook medium (Appendix 1)
IAP	Islets-activating protein
I-mode	Phenotype between that of X- and C-mode

LPF	Lymphocytosis-promoting factor
LPF-HA	Haemagglutinin associated with LPF
LPS	Lipopolysaccharide
MIC	Minimum inhibitory concentration
MPA	Intracerebral mouse-protective activity
NTG	N-methyl-N'-nitro-N-nitroso-guanidine
ou	Opacity units
phase I	Virulent genotype
phase IV	Avirulent genotype
pro-C-mode	Capacity to induce the C-mode variant of B. pertussis
pro-X-mode	Capacity to induce the X-mode variant of B. pertussis
PTS	Phosphotransferase system
RRA	Reactive red-Agarose
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide-gel electrophoresis
SS-NA	Stainer and Scholte medium (Appendix 1) containing 500 $\mu g\ ml^{-1}$ nicotinic acid
SSC buffer	SS-C with the nutrients omitted (Appendix 3)
SS-C	Stainer and Scholte medium with $MgSO_4$ replacing the NaCl (Appendix 1)
$SS\text{-}C_{CAP}$	SS-C containing chloramphenicol (100 $\mu g\ ml^{-1}$)
SSX buffer	SS-X with the nutrients omitted (Appendix 3)
SS-X	Stainer and Scholte medium (Appendix 1)
$SS\text{-}X_{CAP}$	SS-X containing chloramphenicol (100 $\mu g\ ml^{-1}$)
Tris-HCl	Tris(hydroxymethyl) aminomethane-hydrochloride
X bands	Gel bands observed after SDS-PAGE of B. pertussis cell envelopes, which correspond to 28k and 30k polypeptides
X media	Media in which growth induces the X-mode variant
X-mode	Virulent,"xanthic" phenotype of B. pertussis
X polypeptides	The 28k and 30k major cell-envelope polypeptides of B. pertussis

SUMMARY

During the phenotypic and genotypic changes which occur during the processes respectively known as C modulation and phase degradation, several virulence-associated properties of Bordetella pertussis and adenylate cyclase (AC) are lost (Parton and Durham, 1978; Wardlaw and Parton, 1979). The main aim of the present investigation was to assess whether AC plays a causal role in these two distinct processes as this enzyme is known to play a key regulatory role in the Enterobacteriaceae.

Growth of B. pertussis in Stainer and Scholte medium (SS-X) containing high levels of $MgSO_4$, Na_2SO_4, Na butyrate, Na caprylate, Na succinate or nicotinic acid resulted in C modulation as shown by marked reductions in AC activity, histamine-sensitizing activity (HSA) and the 28k and 30k cell-envelope polypeptides (X polypeptides). Although there was some variation between the susceptibility of strains to pro-C-mode salts, there was no instance of components being lost independently of each other. The level of cAMP in the supernate of C-mode cultures was less than 5% of that of X-mode cultures. Magnesium chloride, present at four times the molar concentration of $MgSO_4$ required to induce C modulation, was ineffective at inducing loss of AC activity, HSA, or the X polypeptides.

During $MgSO_4$-induced modulation, AC activity in three compartments (viz. culture supernate, cell-associated but extracytoplasmic, and cytoplasmic) was reduced to the same extent.

Time-course studies on the rate of loss of AC activity, HSA, and the X-polypeptides during $MgSO_4$-induced modulation indicated that these properties were lost simultaneously. Furthermore, losses could be accounted for by complete repression of the synthesis of the components when cells were introduced into C-medium.

The low levels of cAMP in the supernate of C-mode cultures could not be accounted for by the ability of pro-C-mode salts to inhibit in vitro AC activity, or by inhibition of cAMP excretion by

C medium.

Loss of AC activity during C modulation required growth and was not due to prolonged exposure to C medium, as chloramphenicol added to C medium prevented loss of AC activity. Loss of HSA and the X polypeptides also required growth or protein synthesis. C-mode cell lysate did not inhibit AC activity of X-mode cell lysate suggesting that loss of AC activity during C modulation is not due to production of an inactivating or inhibitory factor. Similarly, C-mode cell lysate did not destroy HSA present in X-mode cell lysate.

Sodium fluoride caused marked inhibition of *in vitro* AC activity, but, at the same concentration, had little effect on the synthesis of cAMP during growth. Growth in X medium containing *B. pertussis* AC activator (calmodulin) resulted in a four fold-increase in culture supernate cAMP levels. The critical concentration of $MgSO_4$ required to induce loss of the X polypeptides was 10 - 11 mM. In one culture, containing 10 mM $MgSO_4$ and AC activator, partial loss of the X polypeptides occurred yet cAMP levels in the supernate were twice that which normally occurred in X-mode cultures without activator.

Respiration rates of amino acids by *B. pertussis* variants was investigatec to determine whether cAMP played a role in amino acid catabolism in the organism. The ability of washed suspensions of X- and C-mode and phase IV *B. pertussis* to respire L-glutamate, L-aspartate, L-proline, L-alanine and L-serine was demonstrated. While differences were found between the respiration rates of different amino acids, there were no significant differences between the ability of variants of *B. pertussis* to respire any particular amino acid.

Phosphonomycin resistance has been used as a convenient method to isolate AC mutants of *Escherichia coli*. However, eight independently isolated phosphonomycin resistant mutants of *B. pertussis* possessed the same AC activity as the original strain.

Exogenous cAMP and dibutyryl cAMP, in X and C media, had no effect on the production of AC, the X polypeptides or haemagglutinin.

Attempts were made to determine if a cyclic AMP-receptor protein (CRP) analogous to that in E. coli exists in B. pertussis. [^{3}H] cAMP binding activity was demonstrated in several strains of B. pertussis and was about half that obtained for E. coli. Anti-E. coli CRP gave two precipitin lines with E. coli cell extract but none with B. pertussis cell extract.

In conclusion, the results of this study suggest that AC does not play a causal role in modulation, and that the mechanism responsible for repressing synthesis of X-mode specific components (such as pertussigen) during modulation, also represses synthesis of AC.

INTRODUCTION

PART 1: BORDETELLA PERTUSSIS

1. Pertussis: General Background

Whooping cough (pertussis) is a highly communicable, non-invasive infection of the upper respiratory tract which most commonly affects young infants. The disease is characterised by extremely distressing coughing, lymphocytosis, hypoglycaemia and neurological disorders (Pittman, 1970).

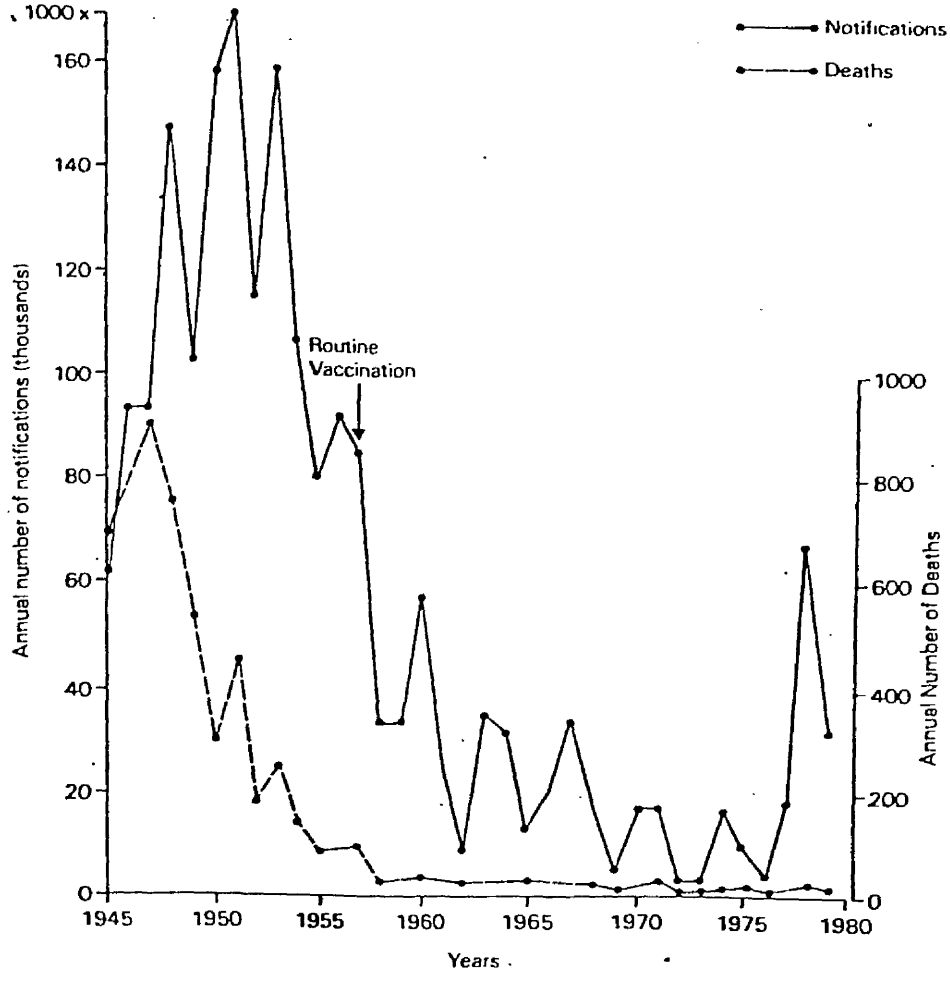
Bordet and Gengou in 1906 first isolated the causative organism, Bordetella pertussis, on a blood-based potato glycerol agar (Bordet-Gengou medium). This is still widely accepted as the best medium for the primary isolation of B. pertussis.

1.1 Epidemiology

Prior to this century, whooping cough was a major cause of child mortality and morbidity, ranking among the most severe infectious diseases of childhood ie. diphtheria, measles, scarlet fever etc. In the developed countries, the incidence of and deaths from whooping cough have dropped dramatically due to the combined effect of improved medical care, improved standards of living and widespread vaccination. Since widespread vaccination began in the UK in 1957, regular epidemics have occurred every 3 - 4 years, but only twice exceeding 35,000 cases per annum up to the year 1980 (Fig. 1). However, pertussis still remains an important childhood disease due to its prolonged and distressing nature. About 10% of cases required hospitalization in a recent epidemic (Miller and Fletcher, 1976). Improved intensive care in hospitals has greatly helped to reduce the fatality rate. Pertussis is a greater problem in the underdeveloped countries which account for 95% of pertussis deaths (World Health Organization, 1977).

Figure 1: Whooping cough notifications and deaths in England and Wales from 1945 - 80

(Taken with permission from Reports, 1981a)


1000 x
160
140
120
100
80
60
40
20
0
Annual number of notifications (thousands)
Notifications
Deaths
Routine
Vaccination
1000
800
600
400
200
0
Annual Number of Deaths
1945
1950
1955
1960
1965
1970
1975
1980
Years

Man is the only known reservoir of B. pertussis. There have been no proven cases of chronic carriers, although clinically unrecognised cases do occur (Linnemann, 1979). Pertussis may appear in adults as an atypical disease, but it occurs with a greater frequency and severity in young infants (Linnemann and Nasenberry, 1977). The fatality rate is highest for infants in the first year of life (Pittman, 1956; Brooks and Buchanan, 1970). Unlike other infectious childhood diseases, there is no maternal transfer of immunity (Reports, 1981a). B. pertussis is transmitted by airborne droplets expelled during coughing. Communicability is very high, ranging from 25 - 50% in schools, to 70 - 100% in household contacts (Linnemann, 1979).

1.2 The disease, diagnosis and treatment

The incubation period of pertussis ranges from 6 - 20 days with an average of 7 days. A catarrhal stage recognizable as a common respiratory infection ensues lasting 1 - 2 weeks. The paroxysmal stage follows during which the patient suffers severe episodes of coughing and usually lasts 4 - 8 weeks but sometimes up to 20. Each attack consists of 10 - 30 forceful coughs and is often so prolonged that anoxia and cyanosis may result. The paroxysms begin without anticipation and continue without interruption until the lungs are almost depleted of air. Finally, with a massive effort, inspiration ensues, and as air rushes into the lungs, the characteristic whoop is often produced. At the termination of the paroxysm, the patient frequently vomits (Pittman, 1970; Olson, 1975). Not all pertussis victims whoop and in the infant, cyanosis and choking are more prominent than the whoop. The greatest danger occurs during the coughing paroxysms during which prolonged hypoxia and apnoea may cause irreversible damage to the central nervous system or death. The most common neurological complications are convulsions, coma, paralysis, deafness, blindness and movement disorders (Pittman, 1970; Linnemann, 1979). The most common causes of death, however, are secondary infections such as pneumonia and broncho-

pneumonia, which may be caused by organisms other than B. pertussis (Reports, 1981a).

B. pertussis may be identified either by direct immunofluorescence or cultural methods (Parker and Linnemann, 1980). Pertussis is often misdiagnosed as: (1) many hospitals do not have the appropriate media or experience in growing B. pertussis, (2) positive cultures decline to 50% after the third week and are negative within a few days if the patient has been given chemotherapy, (3) direct immunofluorescence may produce false positives in inexperienced hands (Linnemann, 1979).

Antibiotic treatment is of little value unless started prior to the paroxysmal stage—a stage which is difficult to anticipate because of problems in diagnosis (Bass et al., 1969). Erythromycin is effective in rendering the patient non-infectious and in preventing secondary infections (Altemeier and Aijoub, 1977). In severe hospitalized cases maintenance of adequate oxygenation, hydration and electrolyte balance is of great importance.

1.3. The organism

B. pertussis is a small, non-motile, Gram-negative cocco-bacillus (0.3 - 0.5 x 0.5 - 1.0 μm), arranged singly, in pairs, and rarely short chains (Pittman, 1974). A capsule has been described (Lawson, 1940), but is not readily detectable in most strains (Munoz and Bergman, 1977). Colonial appearance on Bordet-Gengou medium, is that of small, pearly, glistening, smooth colonies, surrounded by a zone of haemolysis.

B. pertussis is a strict aerobe with an optimum growth temperature of 35° - 37°C. It does not oxidize or ferment carbohydrates but utilizes amino acids as a source of energy, carbon, nitrogen and sulphur. The growth requirements of B. pertussis are relatively simple, but growth is inhibited by several components present in conventional media (Parker, 1976).

The genus Bordetella was proposed by Moreno-Lopez to encompass three serologically-related strains all associated with localized respiratory infections in man and animals (Pittman and Wardlaw, 1981). These are B. pertussis, B. parapertussis and B. bronchiseptica. B. pertussis is the most important to man and causes most cases of whooping cough. B. parapertussis and B. pertussis are strictly human pathogens, whereas B. bronchiseptica is more important in pets and domestic animals, especially piglets. The three species can be differentiated according to Table 1.

The existence of three Bordetella species has recently been questioned (Kloos et al., 1979; 1981) due to their very close genetic relationship as shown by DNA-DNA hybridization. Furthermore, Kumazawa and Yoshikawa (1978) reported the mutagenesis of B. pertussis to an organism biochemically and serologically recognisable as B. parapertussis. Recently, it has been suggested that B. pertussis might be a lysogenic strain of B. parapertussis (Granström and Askelof, 1982).

1.4. Aetiology

MacDonald and MacDonald (1933) verified the causal relationship when they infected two of their own children each with 140 viable organisms of B. pertussis. However, all three species of Bordetella can be isolated from cases with the whooping cough syndrome. Pertussis is generally more severe and prevalent than parapertussis (caused by B. parapertussis) (Linnemann, 1979), but parapertussis may be more significant in some parts of the world (Olson, 1975). B. bronchiseptica is only rarely isolated from cases of whooping cough.

Adenoviruses have been demonstrated in patients with the pertussis syndrome; both in cases where Bordetella has and has not been isolated (Keller et al., 1980). The role of adenovirus infection in pertussis is not known.

Table 1: Characteristics of *Bordetella* species

Property	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*
Flagella	-	-	+
Nitrate reduction	-	-	+
Citrate utilization	-	+	+
Urease activity	-	+	+ (in 4 h)
Oxidase activity	+/-	-	+
Growth on peptone agar	-	+	+
Browning of peptone agar	-	+	-
Specific heat-labile agglutinogen	1	14	12
Heat-labile toxin	+	+	+
Sensitization of mice to histamine	+	-	+
G + C content (moles %)	61, 67-70	61, 68-70	66-69

a, data taken from Pittman and Wardlaw (1981); +, present; -, absent.

2. Vaccination Against Pertussis

Based on the empirical success of vaccines for other diseases, there have been many attempts to develop an effective vaccine against whooping cough. Leslie and Gardner's (1931) observation of phase degradation paved the way by ensuring the selection of strains which would give high levels of protective activity. In addition, problems had to be overcome in developing assays to enable standardization of protective and toxic levels (Pittman, 1956).

During the early 1950's, the Medical Research Council (MRC) conducted a series of randomized double-blind controlled field trials (MRC, 1951). Individual vaccines varied greatly; some giving a high degree of protection even against household exposure to the disease, while others were ineffective. Later trials (MRC,1956) confirmed the existence of potent vaccines. Protection in the field correlated well with the intracerebral mouse protection test (Standfast, 1958; MRC,1959). Thus the intracerebral mouse protection test of Kendrick et al. (1947) was recommended by the World Health Organisation (1964) to standardize the potency of pertussis vaccines .

In the UK, some pertussis vaccination took place during the early 1950's. From 1957 it was promoted nationally as a constituent of the triple vaccine (heat-killed whole cells of B. pertussis combined with diphtheria and tetanus toxoids).

The progressive decline in the incidence and mortality rate of pertussis since the turn of the century and before the introduction of vaccination makes it difficult to assess the impact of vaccination in Britain. Vaccination against pertussis has not been as successful as that against diphtheria and poliomyelitis which have been virtually eradicated from the country (Reports, 1981a). Nevertheless, the decline in the incidence was sustained until 1977 with peaks at intervals of 3 - 4 years only once exceeding 35,000 cases per year.

Eradication of pertussis has been considered as the ultimate goal

(Kendrick, 1975). Anderson and May (1982) suggest that 95% of each year's crop of children must be immunized by the age of two to provide sufficient herd immunity to eradicate the disease. However, the present vaccine does not give 100% protection.

Reports in the UK on the efficacy of vaccines during recent community outbreaks are varied and range from 50 - 90% protection (Jenkinson, 1978; Stuart-Harris, 1979; Church, 1979; Report, 1981b). However, one report from an outbreak in Shetland reported the vaccine to be ineffective (Ditchburn, 1979). These differences may be attributable to differences in efficacy between different batches of vaccine (Griffith, 1978) and differences in other factors such as the nature of exposure.

Pertussis vaccination is associated with a number of mild and severe side reactions. The latter include convulsions, abnormal screaming fits, and brain damage. Brain damage is the most alarming despite its rare occurrence. Assessment of complications is difficult as cases tend to be under-reported and reactions may be attributable to an idiosyncracy on the part of the individual (Reports, 1981a). Reports on the incidence of brain damage vary and have ranged from 1 per 6,000 to 1 in 300,000 injections (Reports, 1981a).

Claims have been made that improved socio-economic conditions has been the main and perhaps sole reason for the decline in the incidence of pertussis and that the risk of brain damage is too high to justify continued use of the vaccine (Bassili and Stewart, 1976; Stewart, 1977; 1979). Doubts over the safety of the vaccine were widely publicized during the mid-1970's when the incidence of the disease was low. This was followed in 1977 - 79 by the largest upsurge of pertussis notifications since 1957. There were 102,500 cases out of which 27 died and about 17 had severe neurological damage (Reports, 1981a). In a study of 98 areas, the incidence of pertussis showed a strong inverse correlation with the vaccine acceptance rate (Stuart-Harris, 1979).

Concern about the reactogenicity of the vaccine led the Joint Committee on Vaccination and Immunization (JCVI) to set up the National

Childhood Encephalopathy Study (NCES). The NCES studied cases from June 1976 - 79 and assessed the rate of brain damage, occurring within 7 days of vaccination, to be 1 in 310,000 injections. In balancing the risks with benefits, the JCVI reaffirmed their advice of 1974 and 1977, to continue recommendation of the vaccine but with due attention to contraindications (Reports, 1981a).

However, with parents confused by the language of statistics and the hysteria of the mass media, the vaccine acceptance rate is still low and we are currently amid another epidemic with notifications as high as those before widespread vaccination(Anderson and May, 1982).

3. Pathophysiologically-Active Components of B. pertussis

Whole cells or products of B. pertussis elicit several pathophysiological effects in animals or animal tissues. Some of these show parallels with the pertussis syndrome in man. A large portion of recent pertussis research has gone towards studying and purifying the components responsible and assessing their role in pathogenesis, immunity and reactogenicity, the aim being to produce a more defined vaccine with greater efficacy and reduced toxicity. However, such knowledge is still limited due to the inadequacy of present animal models, and technical difficulties in purification of components (as shown by the lack of reproducibility between workers).

3.1. Filamentous haemagglutinin

B. pertussis possesses two distinct types of haemagglutinin (HA) activity (Arai and Sato, 1976). One of these is associated with pertussigen (LPF-HA) and has low HA activity, while the other (F-HA) is associated with filamentous molecules with dimensions 2 x 40 nm, and has seven-fold higher HA activity. F-HA has an estimated molecular weight of 100,000. LPF-HA binds specifically to haptoglobin and other sialoproteins, whereas F-HA, is unaffected by sialoproteins but is specifically inhibited

by cholesterol (Irons and MacLennan, 1979). Anti-F-HA, but not anti-LPF-HA, prevented attachment of B. pertussis to mammalian cells in vitro. suggesting a role for F-HA in attachment (Sato et al., 1979; 1981). Further associated properties of LPF-HA are described in section 3.4.

Sato et al. (1979) reported the interaction of specific anti-F-HA with pilus-like structures of B. pertussis. It was widely believed that F-HA was derived from pili and often the term fimbrial haemagglutinin has been used. However, recently published results indicated that pili did not label with anti-F-HA, but were labelled with antibody towards serotype-specific agglutinogen 2 (Ashworth et al., 1982).

3.2. Heat-labile toxin

Heat-labile toxin (HLT) has a cytoplasmic location and is liberated upon cell lysis (Cowell et al., 1979). When administered intraperitoneally, it is lethal to mice, guinea pigs and rabbits (Munoz, 1971). It is dermonecrotic when given subcutaneously and is sometimes referred to as dermonecrotic toxin. HLT is an unstable protein losing all its activity when heated for 56^{o}C for 10 min. It's mode of action is unknown.

The toxin is a poor immunogen. It becomes more immunogenic when converted to a toxoid by treatment with formalin. This may be related to the degeneration of the spleen and possibly lymph nodes which occurs after administration of HLT (Wood, 1940; Munoz, 1971). All three species of Bordetella possess HLT (Olson, 1975).

3.3. Endotoxin

The endotoxin of B. pertussis has the characteristics of the enterobacterial endotoxins. It is toxic, pyrogenic, gives a local Schwartzman reaction and acts as an adjuvant (Ayme et al., 1980). It's chemical composition is unusual in that it possesses two polysaccharide side chains linked to a lipid A moiety. An additional lipid (lipid X) exists within the complex which is also responsible for toxicity, pyrogenicity and the Schwartzman reaction (Le Dur et al., 1978; 1980; Ayme et al., 1980).

3.4. Pertussigen

Purification studies by different workers indicate that several of the pathophysiological activities of B. pertussis are attributes of a single component (Lehrer, 1979; Morse, 1977; Yajima et al., 1978; Munoz et al., 1981a). Munoz (1976) proposed the term "pertussigen" to describe the factor but Pittman (1979) suggested that "pertussis toxin" was more appropriate. Pertussigen is referred to by other names such as histamine-sensitization factor (HSF), lymphocytosis-promoting factor (LPF) and islets-activating protein (IAP), due to it's different manifestations in animals. Although no one has seriously challenged the idea of a single component, few workers have committed themselves to use either "pertussigen" or "pertussis toxin", and still prefer the terms HSF, LPF and IAP. Pertussigen and it's activities are unique to B. pertussis and probably play an important role in the pathogenesis of whooping cough (Pittman, 1979; Wardlaw and Parton, 1983).

Purification procedures for pertussigen are varied and so are the reported physiochemical properties. Independent studies indicate that pertussigen is predominantly protein with a molecular weight between 60k - 100k and consists of 4 - 6 subunits (reviewed by Wardlaw and Parton, 1983). The activities of pertussigen are fairly stable to heat and pH. Little of the histamine-sensitizing or lymphocytosis-promoting activities are lost by heat treatment (30 min at 56^{o}C) during vaccine production.

Pertussigen is associated with the cell envelope of actively growing B. pertussis and is liberated into the medium in older cultures (Munoz and Bergman, 1977).

The following activities have been attributed to pertussigen:-

(i) Histamine sensitization

When mice are given an intraperitoneal injection of pertussis vaccine, they become up to 100-fold more sensitive to a subsequent injection of histamine or serotonin (Parfentjev and Goodline, 1948). This effect has been attributed to HSF and is inseparable from the other activities described below for pertussigen (Munoz, 1976; Lehrer, 1979). HSF sensitizes

mice to other agents or types of shock eg. bradykinin, methacholine, anoxia, cold shock, X-irradiation and endotoxin (Munoz and Bergman, 1968). Sensitization to histamine may persist in the mouse for months (Munoz and Bergman, 1966) and can be detected even when small numbers of live or heat-killed organisms are inoculated intranasally (Geller and Pittman, 1973). Highly purified pertussigen is very potent with an HSD_{50} of 0.5 ng per mouse (Arai and Munoz, 1981).

(ii) Lymphocytosis promotion

In pertussis patients and in experimentally infected animals, there is an increase in the number of circulating leukocytes which is thought to be attributable to pertussigen (Munoz and Bergman, 1977). This activity is not due to a proliferation of new cells (Morse and Riester, 1967), but appears to be due to the inability of circulating lymphocytes to return from the bloodstream to the lymphoid tissues (Morse and Baron, 1970).

(iii) Islets activation

Rats and mice when injected with B. pertussis vaccine, exhibit a marked hyperinsulinaemia in response to insulin secretagogues such as glucose (Sumi and Ui, 1975). The purified component responsible for this effect, IAP, also exhibits histamine-sensitizing and lymphocytosis-promoting activities (Yajima et al., 1978).

(iv) Other activities

Other activities inseparable from histamine-sensitizing and lymphocytosis-promoting activities include: several distinct types of adjuvanticity, induction of hypoproteinaemia and increased vascular permeability in skin and striated muscle, systemic and local toxicity, in vitro mitogenicity for T lymphocytes, and haptoglobin-specific HA activity (Morse, 1976; Munoz and Bergman, 1977; Wardlaw and Parton, 1983).

The mechanism of pertussigen action is not properly understood. It affects a wide range of cell types. Wardlaw and Parton (1983) suggested that pertussigen interacts with these cell types through sialoprotein receptors. Accumulated evidence (Parker and Morse, 1973;

Krzanowski et al., 1976; Ortez, 1977; Lee, 1977) indicates that pertussigen alters the responsiveness of different cells to adrenergic and other agents, and upsets cAMP metabolism. Recently, work by Katada and Ui (1982a,b) and Katada et al., (1982) on the molecular action of IAP on C6 glioma cells, indicated that IAP causes ADP-ribosylation of a membrane protein, which in turn increases receptor-mediated and GTP-induced activation of adenylate cyclase. This mechanism is similar to that of cholera toxin, although it appears that each causes ADP-ribosylation of separate membrane proteins. Cholera toxin blocks hydrolysis of bound GTP whereas pertussigen renders more GTP available for the GTP binding sites (Helmreich and Bakardjieva, 1980; Katada et al., 1982).

3.5. Other active components

A number of other active substances of B. pertussis have been described which are less well characterised. These include a haemolysin (Lautrop, 1960), a polymorphonuclear leukocyte-inhibitory factor which may be another attribute of pertussigen (Utsumi et al., 1978; Imagawa et al., 1980), a neurotoxin (Kuwajima, 1978), a factor which affects macrophage function (Benjamin et al., 1981), and a trachael cytotoxin (Goldman et al., 1982).

4. Role of B. pertussis Components in Disease, Immunity, and Reactogenicity of Vaccines

4.1. Disease

The pathogenic mechanisms of B. pertussis are not well understood. The disease or infection commences when the bacteria attach specifically to the ciliated epithelium of the respiratory tract. The bacteria multiply among the cilia, but do not penetrate any of the tissues (Olson, 1975). Attachment may be mediated via. F-HA (Sato et al., 1979; Pittman, 1979). Pittman (1979) suggests HLT may be involved during the initial stages of infection causing ciliostasis and local inflammatory

lesions. The recently described tracheal cytotoxin by Goldman et al. (1982), which causes ciliostasis in vitro, may also have a prime role in lodgement.

The mechanism of the paroxysmal cough is not understood. Pertussigen has been proposed as the causative agent (Pittman, 1979; Wardlaw and Parton, 1983). However, this does not explain the whooping cough syndrome caused by B. parapertussis or B. bronchiseptica (Olson, 1975). In this respect, HLT, which is common to Bordetella, or the recently reported trachael cytotoxin (Goldman et al., 1982), may play a role. It seems likely that pertussigen is responsible for leukocytosis, hypoglycaemia and other metabolic disturbances of pertussis. The neurological complications of pertussis may result from anoxia or cerebral haemorrage as a direct consequence of the cough. Hypoglycaemia may also be involved (Linnemann, 1979).

4.2. Immunity

Knowledge of the protective factors of B. pertussis is hindered by the inadequacy of current animal models. Pertussis vaccine manufacturers must comply with the conditions of the intracerebral mouse protection test. However, hospital isolates have low mouse intracerebral virulence and serotypes prevalent in child infections do not parallel those isolated from mouse brain infections (Preston, 1976). Other animal models have been proposed to supplement the intracerebral mouse protection test such as pernasal infections in marmosets, rabbits and mice (Stanbridge and Preston, 1974a; Preston et al., 1980; Pittman et al., 1980). However, no infected animal exhibits the paroxysmal cough, which is the essence of the inflicted damage of pertussis in man.

Pittman (1979) has suggested that immunity to pertussis depends on immunity towards the components responsible for attachment and initiation of the disease, and the later toxic effects responsible for the paroxysmal cough. F-HA and pertussigen seem to be the likely candidates.

Sato et al. (1979) and Irons and MacLennan (1979) found purified

F-HA to protect mice against intracerebral and intranasal challenge of B. pertussis. However, LPF—HA was intracerebrally non-protective at 4 µg per mouse (highest, non-toxic dose). In contrast, Munoz et al. (1981b) found purified pertussigen (detoxified by glutaraldehyde treatment) intracerebrally protective at 1.7 µg per mouse, and F-HA (free of histamine-sensitizing activity) intracerebrally non-protective at 12 µg per mouse. Recent published results by Sato and co-workers contradict earlier findings, and indicate that both their LPF and F-HA preparations protected mice from intracerebral challenge (Cowell et al., 1982; Sato et al., 1982).

Watanabe and Nakase (1982) isolated F-HA deficient mutants possessing histamine-sensitizing and lymphocytosis-promoting activities, from strain 18323. These mutants were as protective as the parent strain by the intracerebral mouse protection test, but showed reduced virulence. Thus F-HA is not the sole protective antigen, but may be important in infection.

Preston and co-workers believe the agglutinogens (about eight components which react with corresponding monospecific antisera causing cell agglutination) to play an important role in protection. The relation of the agglutinogens to the other active components is not known, although it appears that agglutinogens 1 and 3 are separable from HSF (Ross and Munoz, 1971) and agglutinogen 2 may be associated with pili (Ashworth et al., 1982). Agglutinogens 1 - 3 were assessed as being the most important for infection in the child (Preston, 1976). Preston and Stanbridge (1976) stressed that the absence of agglutinogens 2 and 3 may go unnoticed in the intracerebral mouse protection test. Preston (1966) proposed that the large pertussis epidemic in the early 1960's was due to a lack of agglutinogen 3 in many commercial vaccines. The World Health Organization (1979) recommended that vaccines contain all three agglutinogens

In summary, it would appear that protectivity is not attributable to a

single protective antigen, but probably relies on several factors. Adjuvanticity of certain components may render others more protective. Immunity towards a toxic component may prevent disease but not infection.

4.3. Reactogenicity of vaccines

The mouse weight-gain test is used to control and minimize toxicity of pertussis vaccines. Several reports suggest this test correlates with reactivity of vaccines in children (reviewed by Wardlaw and Parton, 1983). In present vaccines, at least two toxins, endotoxin and pertussigen, are fully active. Wardlaw and Parton (1983) suggested that these are probably responsible for the reactivity of the vaccine; pertussigen especially, in view of it's potential for causing metabolic and neurological disturbances. Askelof and Bartfai (1979) found that rats injected intraperitoneally with pertussis vaccine exhibited an increase in cerebellar cyclic GMP levels. They suggested that this effect may be related to neurological complications observed in children after vaccination, as some convulsive agents also exhibit such increases in cerebellar cyclic GMP levels. Recently Askelof and Gillenius (1982) found intraperitoneal injection of purified LPF, but not HLT or LPS (lipolysaccharide), to cause this effect in rats.

The recent success in producing a pertussigen toxoid with glutaraldehyde (Munoz et al., 1981a,b) is an encouraging development for an improved vaccine.

5. Growth Requirements and Metabolism of B. pertussis

5.1. Growth requirements

B. pertussis is a fastidious, slow growing organism which will not grow in conventional media. Bordet-Gengou medium (BG) is still acknowledged as the best medium for the primary isolation of B. pertussis and strain maintenance. Several liquid media have been described (Hornibrook, 1939; Cohen and Wheeler, 1946; Wilson, 1963; Stainer and Scholte, 1971), but growth in these media requires a heavy inoculum of actively growing cells.

Paradoxically, B. pertussis has simple growth requirements and will grow in a chemically defined medium containing glutamate, proline, cysteine, glutathione, ascorbic acid, nicotinic acid and various salts (Stainer and Scholte, 1971). The inability of B. pertussis to grow on conventional media, such as nutrient agar, is thought to be due to inhibitory substances in these media. Inhibitory substances include fatty acids, colloidal sulphur and sulphides, organic peroxides and manganous ions (Rowatt, 1957; Parker, 1976). Agar, peptone and other medium constituents contain fatty acids. Agar also contains large amounts of sulphur, and the inhibitory effect of agar may be due to colloidal sulphur compounds formed during autoclaving. Substances such as blood, starch, albumin, charcoal, catalase and anion exchange resins, have been added to media to neutralise inhibitory substances. The inhibitory mechanisms are not known, but are of interest as inhibitory substances select for avirulent forms of B. pertussis, which have greater resistance (Parker, 1976).

Early work indicated that B. pertussis utilized only a few amino acids at a significant rate (Table 2), but no sugars or carbohydrates. Various workers agree that glutamate can be utilized as the principle source of carbon, nitrogen and energy (Jebb and Tomlinson, 1951, 1955; Rowatt, 1955; Goldner et al., 1966; Vajdic et al., 1966; Stainer and Scholte, 1971). Olson (1975) pointed out that it may be pertinent to pathogensis that B. pertussis utilizes amino acids but not carbohydrates, as amino acids are expected to be more abundant in the mucus layer of the respiratory tract and even more so after inflammation and necrosis of the epithelium.

B. pertussis requires an organic sulphur source which is only satisfied by cysteine, cystine or glutathione (Hornibrook, 1939; Jebb and Tomlinson, 1957). Parker (1976) has suggested that this may be due to the neutralization of inhibitors or the requirement for a reduced environment. Oxygenases, which may play a role in amino acid metabolism, require both oxygen and reduced compounds for activity. The organism

Table 2: Amino acids utilized by *B. pertussis*

Workers	Amino acid source in medium	Amino acids utilized (+) or not utilized (-)																			
		glu	pro	asp	ala	ser	gly	thr	tyr	phe	leu	his	gln	met	val	trp	arg	lys	ile	cys	asn
Ungar *et al.* (1950)[b]	Caesin hydrolysate	+	+	+	+	+	+	+	...[a]	...	...	...	...	...	...	...	...	...	...	...	...
Jebb and Tomlinson (1951)[c]	Commercial casamino acids	+	+	+	+	+	-	-	-	-	-	-	...	-	-	-	...	-	-	...	...
Rowatt (1955)[b]	Commercial casamino acids	+	+	+	+	+	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Pusztai *et al.* (1960)[b]	Commercial casamino acids	R[d]	R	R	R	R	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Goldner *et al.* (1966)[b]	Defined amino acid mixture as in Wilson's medium (Wilson, 1963)	R	R	S	R	R	R	-	-	-	-	R	R	-	-	-	...	-	-	...	...
Lane (1970)[b]	Commercial casamino acids	R	R	R	R	R	R	S	-	S	S	S	...	S	-	...	-	-	-	-	...

a, no information (...); b, these workers investigated amino acids utilized during growth; c, these workers investigated respiration of amino acids by washed cells; d, amino acids were utilized rapidly and completely (R) or slightly (S).

also has an absolute requirement for either nicotinic acid or nicotinamide (Hornibrook, 1940; Jebb and Tomlinson, 1955).

5.2. Metabolism

Little is known about the metabolism or physiology of B. pertussis. Jebb and Tomlinson (1951) found that out of 21 amino acids, only L-proline, L-serine, L-alanine, L-aspartate, L-glutamate, and D-glutamate were respired at an appreciable rate by washed cell suspensions. The organic acids α-ketoglutarate, lactate and succinate were also respired. α-Ketoglutarate, was formed during respiration of L-glutamate.

Abe (cited by Fukumi et al., 1953) found B. pertussis able to respire fumarate, malate, succinate, α-ketoglutarate, citrate and acetate, suggesting a functional citric acid cycle. Later studies demonstrated aconitase and isocitrate dehydrogenase activities (Fukumi et al., 1953). Presumably, glutamate enters the citric acid cycle by oxidative deamination to α-ketoglutarate. Other amino acids may be converted to glutamate by interaction with α-ketoglutarate via. transaminases, which have been demonstrated in B. pertussis for various amino acids (Kobayashi and Fukumi, 1954a). Kobayashi and Fukumi (1954b) demonstrated aldolase, but lack of hexokinase activity in B. pertussis cell-free extracts. Thus B. pertussis may not be able to utilize sugars due to the lack of hexokinase or the appropriate permeases. The presence of aldolase suggests that sugar precursors, required for polysaccharide and nucleic acid synthesis, may be synthesized from intermediates of the citric acid cycle via. gluconeogenesis.

Two different electron transport chains have been described for B. pertussis. One of these is dependent on cytochrome oxidase and is cyanide sensitive, while the other is not (Ezzell et al., 1979, 1981a).

How amino acid metabolism is regulated in B. pertussis is not known but several observations suggest regulatory mechanisms do exist. Some workers have found amino acids not to be utilized until glutamate was depleted from the medium (Rowatt, 1955; Goldner et al., 1966; Vajdic et al.,1966). Pusztai et al. (1960) found proline to be utilized first. On the other hand,

Lane (1970), using an amino acid analyser, reported glutamate, aspartate, alanine, serine, proline, and glycine all to be utilized simultaneously. He claimed previous findings may have been due to the use of insensitive paper chromatographic analysis and the unequal amounts of amino acids in the media used.

Some reports have indicated that varying the concentration of glutamate or proline in media resulted in changes in intracerebral mouse-protective activity (MPA), and HSF (Goldner et al., 1966; Stainer and Scholte, 1969; Lane, 1970). It is not known whether this is due to uptake of these amino acids or changes in the growth rate, but suggests mechanisms by which expression of the pathophysiological activities of B. pertussis may be regulated in response to levels of nutrients.

Both adenylate cyclase and glutamine synthetase have been demonstrated in B. pertussis (Hewlett et al., 1976; Zakharova et al., 1979). These enzymes play a key regulatory role in the acquisition of carbon and nitrogen sources respectively in the enterobacteria (Pastan and Adhya, 1976; Magasanik, 1977). Perhaps these enzymes play a role in the above described regulatory phenomena observed for B. pertussis.

6. Variation of B. pertussis

A notable feature of B. pertussis is it's tendency to undergo antigenic and colonial variation. This can occur by two processes, namely, phase degradation and antigenic modulation. Phase degradation is an irreversible process involving step-wise mutational events and occurs with continous subculture. Antigenic modulation is an environmentally-induced process involving phenotypic changes and is freely reversible.

6.1. Phase variation

Leslie and Gardner (1931) found strains of B. pertussis could be divided into four well defined serological groups, phases I - IV. Phases I and II gave smooth, virulent colonies, while phases III and IV were rough

and avirulent. Subculture of phase I in various media resulted in phase degradation through phases II and III to the stable phase IV, although phase II did not occur as a regular intermediate.

Standfast (1951) found repeated subculture of different hospital isolates to cause independent and random loss of virulence, protective activity, HA, growth requirements and agglutination. In general HA was one of the less stable characters and protective activity one of the more stable characters.

Although the terms phase I and phase IV are still in use to describe virulent and avirulent strains, indentification of the four phases is no longer possible due to the lack of Leslie and Gardner's strains and antisera. Parker (1979) suggested the use of the terms fresh isolate, intermediate strain (laboratory strain) and degraded strain (avirulent strain). Degraded strains grow on nutrient agar presumably because they have developed greater resistance to inhibitory substances than their fresh-isolate ancestors. Parker (1976) suggested that inhibitory substances present in media provide a selective pressure for degraded strains. Peppler (1982) reported the frequency of phase I or II to phase III or IV variation to be between 5×10^{-5} to 5×10^{-6} per culture.

6.2. Antigenic modulation

Lacey (1951) noted that *B. pertussis* grown on BG plates in which the NaCl was replaced by $MgSO_4$, failed to agglutinate with *B. parapertussis* antiserum. He later reported this process to occur uniformly in a whole population and to be completely reversible within one subculture, provided that the inoculum was not too large (Lacey, 1960). The process required 4 - 7 cell divisions and was not due to a direct effect of $MgSO_4$ on the antigens. This was clearly a process involving phenotypic changes not requiring mutation. Growth with NaCl gave X-mode *B. pertussis* (so-called because of the xanthic, ochre appearance of confluent growth) while growth with $MgSO_4$ gave C-mode (cyanic) *B. pertussis*. An I-mode (intermediate) arose when *B. pertussis* was grown on BG with a critical concentration ratio

of NaCl to $MgSO_4$. In addition to antigenic changes, other properties were lost during X to C modulation such as haemolysis, HA, agglutinability by $AuCl_3$ and $HgCl_2$ and intranasal mouse virulence.

Antigenic modulation was also induced by several cations, anions, and organic acids. Some ions were clearly pro-C-mode whereas others were pro-X-mode. Fatty acids were the most potent pro-C-mode substances with activity increasing with chain length. The outcome of any combined mixture of salts depended on the sum of the individual potencies for modulation. Lacey (1960) also reported that growth at 25°C caused similar C modulation. This process was not characteristic of fresh isolates, but required the initial selection of mutants capable of growing at low temperature.

Pusztai and Joó (1967) reported a type of antigenic modulation which is induced by growth in high levels of nicotinic acid (500 $\mu g\ ml^{-1}$). Although these workers did not examine $MgSO_4$-induced modulation, they maintained nicotinic acid-induced modulation was different from that reported by Lacey as: (1) although agglutinability was reduced, specificity was unchanged, (2) no changes occurred in intraperitoneal toxicity. Nicotinic acid-induced modulation has an element of specificity in that nicotinamide, present at the same high level, does not cause variation (Wardlaw et al., 1976)

6.3. Changes in activities and properties during phase variation and modulation

Several activities and properties of B. pertussis are lost during the processes of phase degradation and antigenic modulation along with virulence and protective activity (Table 3). Although smooth to rough variation in the enterobacteria is associated with a loss of the terminal O side chains in their LPS, little work has been done on the effect of phase degradation and modulation on B. pertussis LPS. Aprile and Wardlaw (1973) found heat-stable antigenic determinants to vary between strains, but there was no correlation with phase. Whereas some of the lost properties, such as pertussigen-related activities, F-HA and HLT, may play a role in pathogenesis, the functions of others such as adenylate cyclase and the 28k and 30k cell-envelope polypeptides (X polypeptides), are not known. It appears that these properties are lost in unison during

Table 3: Activities and properties lost during antigenic variation of *B. pertussis*

Property or activity	Reference*		
	Phase degradation	Antigenic modulation	
		$MgSO_4$-induced	Nicotinic acid-induced
MPA	3, 5, 10	9, 12, 18, 21	8, 12
HSF	4, 10	12, 18, 21	8, 12
LPF	23	18, 21	
Adjuvant activity		19	
HA	2, 3, 23	6	
HLT	1	13	22
28k and 30k cell-envelope polypeptides	11, 12, 15	11, 12, 15, 18, 21	12
Agglutinogen 1	7	9	20
Adenylate cyclase	14, 17, 23	14	20
Cytochrome d_{629}	15	15	
Sensitivity to certain antibiotics	15, 16		

* 1, Roberts and Ospeck (1942); 2, Keogh and North (1948); 3, Standfast (1951); 4, Kind (1953); 5, Kasuga *et al.* (1954); 6, Lacey (1960); 7, Eldering *et al.* (1962); 8, Pusztai and Joó (1967); 9, Holt and Spasojevic (1968); 10, Aprile (1972); 11, Parton and Wardlaw (1975); 12, Wardlaw *et al.* (1976); 13, Livey *et al.* (1978); 14, Parton and Durham (1978); 15, Dobrogosz *et al.* (1979); 16, Field and Parker (1979); 17, Hewlett *et al.* (1979a); 18, Wardlaw and Parton (1979); 19, Wardlaw *et al.* (1979); 20, McPheat (1980); 21, Idigbe *et al.* (1981) 22, Livey (1981); 23, Peppler (1982).

antigenic modulation and phase degradation (Wardlaw and Parton, 1979; Peppler, 1982). Some of these components (eg. pertussigen) are located in the cell envelope, others are cytoplasmic (eg. HLT). The common loss of these activities may suggest the perturbation of a common regulatory mechanism by mutational events or the influence of certain ions, being responsible for variation.

Conversely, a few reports, in need of verification, suggest a differential loss of activities. Pusztai and Joó (1967) reported that HSF, but not HLT, was reduced during nicotinic acid-induced modulation. This contrasts with findings of Livey (1981) where HLT was reduced by 60%. Pusztai and Joó also reported the loss of HSF but not agglutinability during nicotinic acid-induced modulation of one strain. Wardlaw et al. (1976) found that succinate-induced modulation caused a 30% loss of HSF but no apparent loss of the X polypeptides.

6.4. Other types of variation

Other forms of antigenic or colonial variation have been reported, but their relation to phase degradation is not known. B. pertussis subcultured from a mouse brain, on occasion, resulted in the segregation of large and small colonies (Andersen, 1952). Small colonies tended to be more intracerebrally virulent and toxic than large colonies. When individual colonies were subcultured, differences in colony morphology disappeared, although their original differences in virulence, toxicity and agglutinogens were retained. Presumed homogenous strains of B. pertussis, when grown on BG and Cohen and Wheeler medium, segregated into many colony types (Cameron, 1967). These differed in terms of colony morphology, haemolysis, agglutinogens, toxicity, HSF and MPA suggesting that variation occurs very readily. Stanbridge and Preston (1974b) reported gradual loss of agglutinogens during serial subculture from type 1,2,3 to types 1,2 or 1,3 to type 1. The process was reversible and agglutinogens could be regained. The rate of mutation varied with strain. Changes in serotype also occurred during the course of per-nasal infections in the marmoset and

rabbit (Stanbridge and Preston, 1974a; Preston et al., 1980). Type 1,2,3 strains could change to types 1,2 or 1,3 but only seldomly and late on in infection to type 1. Moreover, infection with type 1 resulted in mutation to types 1,2, 1,3 and 1,2,3 which were more predominant during infection. However, these changes in serotype do not occur when the mouse brain is infected with type 1. Preston and co-workers stressed the importance of agglutinogens 1, 2 and 3 for infection and protection in the child (Preston, 1976).

7. Adenylate Cyclase of B. pertussis

Adenylate cyclase (AC) activity is demonstrable in all three species of Bordetella (Endoh et al., 1980). Ide (1971) screened 21 species of bacteria for AC activity. When present, AC was either membrane bound, as in Escherichia coli, or cytoplasmic, as in the case of Brevibacterium liquefaciens. Two notable features of B. pertussis AC are it's location and it's activation by calmodulin.

Substantial quantities of AC (about 20% of the total) are found in the supernate of B. pertussis cultures (Hewlett et al ., 1976; Endoh et al., 1980). Hewlett et al.(1976) reported that AC accumulated in culture supernates during exponential growth. This was not due to cell lysis as intracellular markers such as DNA and malate dehydrogenase could not be detected. Of the cell associated AC, about 90% was extracytoplasmic being senstive to trypsin prior to cell disruption. Enzyme activity could be determined by adding ATP to intact cells and assaying for cAMP production. This was referred to as whole-cell AC activity and accounted for a substantial portion of cell-envelope associated AC depending on the concentration of ATP used (Hewlett et al., 1976). It was suggested that AC may have a periplasmic location and is thus easily released into the medium. However, only 1% of AC was lost by sucrose, EDTA treatment and osmotic shock, which would be expected to release periplasmic components (Cowell et al., 1979). Nevertheless, culture supernate AC and

extracytoplasmic cell-associated AC are, sofaras is known, unique to Bordetella (Hewlett et al., 1980; Endoh et al., 1980). Despite the large proportion of extracytoplasmic AC, significant cytoplasmic AC activity probably occurs during growth as cAMP can be detected in culture supernates (20 - 100 pmol ml^{-1}) (Parton and Durham, 1978; Endoh et al., 1980). This is unlikely to be formed by extracytoplasmic enzyme due to lack of the substrate ATP.

Culture supernate AC has been purified and characterized (Hewlett and Wolff, 1976). B. pertussis AC has the smallest molecular weight (70,000) reported so far for this enzyme. Unlike the AC of other prokaryotes and eukaryotes, B. pertussis AC is insensitive to α-ketoacids or GTP. Like other bacterial AC, it has a divalent cation requirement best satisfied by Mg^{++}. It has an optimum pH of 7 - 8 and is sensitive to trypsin and temperature. It is mildly inhibited by sodium salts, but is markedly inhibited by NaF. Despite it's heat sensitivity, significant AC activity is detectable in some commercial vaccines (Hewlett et al., 1977).

The other peculiar feature of B. pertussis AC is it's marked sensitivity to calmodulin. AC activity, for a wide range of 26 other species (pathogenic and non-pathogenic), was unresponsive to calmodulin (Hewlett et al., 1980). Calmodulin, which is synthesized by most eukaryotic cells, has never been demonstrated in prokaryotes and is most probably absent from B. pertussis (Wolff et al., 1980). Calmodulin is a small, heat-stable protein (molecular weight 16,700), which activates a large number of enzymes in a calcium-dependent two-step mechanism (Klee et al., 1980):-

$$\text{calmodulin} + Ca^{++} \longleftrightarrow \text{calmodulin}.Ca^{++}$$

$$\text{enzyme}_{(\text{inactive})} + \text{calmodulin}.Ca^{++} \longleftrightarrow \text{enzyme}.\text{calmodulin}.Ca^{++}{}_{(\text{active})}$$

Hewlett et al. (1978) noted that B. pertussis grown on BG had an AC activity 100-fold greater than when grown in Stainer and Scholte medium. These workers attributed this difference to an activator present in erythrocytes. The activator was very potent and a few subcultures in Stainer and Scholte were required before AC activity reached a basal level.

Activator activity was also found in many commercial protein preparations and mammalian tissues such as liver, muscle and tracheal mucosa (Hewlett et al., 1978; 1979a; Goldhammer et al., 1981). Commercial bovine catalase was a particular good source of activator. The activator was heat and pH stable. While whole-cell AC activity could be stimulated about 240-fold, culture supernate AC was stimulated only 7-fold, and purified AC was insensitive to the activator (Hewlett et al., 1979b). This suggested that an additional factor was required for activation. The activator, from several sources, was eventually identified as calmodulin due to: (1) requirement for Ca ions, (2) sensitivity to ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, a chelator of Ca ions), (3) sensitivity to trifluoperazine, (4) heat and pH stability, (5) similar chromatographic profile to authentic calmodulin (Wolff et al., 1980; Goldhammer et al., 1981). B. pertussis AC is highly sensitive to activation and can be used to trace pico mole quantities of calmodulin. These workers have proposed activation of B. pertussis AC as a convenient model to study the mechanism of action of calmodulin.

Calmodulin activation of B. pertussis AC differs from that of mammalian brain AC as EGTA is ineffective unless added before calmodulin (Wolff et al., 1980). Recently, results from an independent group of workers (Greenlee et al., 1982) conflicted with those of Wolff et al. (1980). Greenlee et al. (1982) found calmodulin activation of B. pertussis AC to be unique in that there was no requirement for Ca^{++} for the majority of the activation observed. Photoaffinity cross-linking experiments with azido [^{125}I] calmodulin suggested that the catalytic subunit of calmodulin-sensitive AC was responsible for rendering culture supernate AC and purified AC insensitive to calmodulin.

Hewlett et al. (1979a) observed a second type of influence on AC activity during growth. When B. pertussis was subcultured from BG to Stainer and Scholte medium there was a marked decline in AC activity (presumably

due to lack of activator). However, during the first few hours of growth in Stainer and Scholte, specific activity gradually increased about 10-fold.

Neither the function of AC in B. pertussis nor the physiological significance of its marked responsiveness to calmodulin is known. AC activity is greatly reduced in C-mode and phase IV B. pertussis. It has been suggested that AC may play a regulatory role in B. pertussis in governing the expression of virulence factors (Parton and Durham, 1978; Hewlett et al., 1979a; Wardlaw and Parton, 1979). This proposal has been further extended by the suggestion that calmodulin may facilitate the parasitic state of the organism in the respiratory tract (Hewlett et al., 1978). However, exogenous cAMP (0.1 - 10 mM) had no effect in inducing the X-mode/phase I phenotype in phase IV and C-mode B. pertussis, although uptake of cAMP was not demonstrated (Dobrogosz et al., 1979; Ezzell et al., 1981b). Alternatively it has been suggested that AC itself may act as a virulence factor (Hewlett et al., 1979a; Confer and Eaton, 1982).

PART 2: ADENOSINE 3',5'-PHOSPHATE IN PROKARYOTES

Adenosine 3',5'-phosphate (cyclic AMP or cAMP) has been demonstrated in a variety of prokaryotes, although it's occurrence is not ubiquitous (Botsford, 1981). Most work on the physiological role of cAMP has been done with Escherichia coli and Salmonella typhimurium. The involvment of cAMP in catabolite repression is well established (Pastan and Perlman, 1970; Rickenberg, 1974; Pastan and Adhya, 1976). However, little is known of the role of cAMP in other bacteria; except that it has been identified, and when added exogenously, altered expression of certain phenotypes. Sections 1 to 5 are restricted to a discussion of cAMP in E. coli and S. typhimurium.

1. Mechanism of Action

The current model of the mechanism of action of cAMP is supported by a variety of physiological, genetic and biochemical evidence (Pastan and Adhya, 1976). The cAMP regulatory model requires two functional proteins, adenylate cyclase (AC) and cAMP-receptor protein (CRP). AC catalyses the hydrolysis of ATP to cAMP and in E. coli and S. typhimurium is a membrane bound enzyme. CRP is a dimer which binds to two molecules of cAMP (one per subunit).

When CRP binds to cAMP, it undergoes a marked allosteric change (Kumar, 1980). In this active conformation, CRP-cAMP preferentially binds to specific sites on the DNA, promoting access of RNA polymerase, and initiation of transcription (Pastan and Adhya, 1976). The CRP-cAMP binding sites have been sequenced for the lac (Dickson et al., 1975), ara (Lee et al., 1981) and gal operons (Taniguchi et al., 1979), and they are similar, but not identical.

CRP-cAMP binds to one site on the lac operon, which in the presence of the inducer, enables transcription of the structural genes. The ara operon has two CRP-cAMP binding sites. Binding to one of these enables

transcription of a gene coding for an *ara* repressor, which blocks the CRP-cAMP binding sites. In the presence of arabinose, the *ara* repressor is ineffective (Lee *et al.*, 1981). The *gal* operon has two promoters. Initiation of transcription at one promoter is inhibited by CRP-cAMP, whereas initiation at the other is stimulated (Adhya and Miller, 1979). Thus optimum transcription of *gal* depends on a critical concentration of cAMP. The proposed rationale for this is that the *gal* operon has a biosynthetic as well as a catabolic role.

Different promoters have different affinities for CRP-cAMP. The *lac*, *ara*, and *tna* (tryptophanase) operons require different concentrations of cAMP for transcription (Lis and Schleif, 1973; Piovant and Lazdunski, 1975). The significance of this is discussed later.

2. Catabolite Repression and Cyclic AMP

It has long been recognized that when glucose was not limited during growth of bacteria, the capacity to ferment various sugars was lost (Gale, 1943). Magasanik (1961) proposed that glucose catabolism, being faster than that of other sugars, resulted in higher levels of catabolic intermediates one of which repressed synthesis of inducible enzymes. Thus the term catabolite repression was proposed.

Several years after Sutherland and co-workers discovered cAMP as an intermediate in the hyperglycaemic effect of adrenalin and glucagon, Makman and Sutherland (1965) observed cAMP in *E. coli*. These workers also noted that the addition of glucose caused a significant decrease in the intracellular cAMP concentration. In later studies, the addition of exogenous cAMP derepressed enzymes and permeases subject to catabolic repression (Pastan and Perlman,1970). Epstein *et al.*, (1975) found good correlation between the effect of carbon source on intracellular cAMP concentrations and β-galactosidase activity.

The strongest evidence for the involvement of cAMP and CRP in catabolite repression came from the isolation of two classes of mutants.

One of these, cya, is defective in AC and the other, crp, is defective in CRP. Both these mutants are pleiotropic and are unable to utilize carbohydrates where metabolism is subject to catabolite repression (Perlman and Pastan, 1969). This ability can be restored in cya, but not in crp mutants, by the addition of cAMP. Sensitivity to various antibiotics and inhibitors is lost in cya and crp mutants, presumably due to the lack of the permeases. This has provided an easy means for their selection (Alper and Ames, 1978; Aono et al., 1979; Swenson, 1979).

Cyclic AMP and CRP are not essential for growth in all conditions as cya and crp deletion mutants have been isolated. However, the growth rate of these mutants is impaired. The reason for this is not known (Pastan and Adhya, 1976).

Criteria which have been used to implicate the involvement of CRP-cAMP in the expression of a particular phenotype include:-

(i) The expression of the phenotype correlates with observed cAMP levels.

(ii)The phenotype is expressed if cAMP is added exogenously.

(iii) An alteration in phenotype occurs by manipulating growth conditions such as to alter cAMP metabolism.

(iv) The phenotype is absent in crp and cya mutants, but can be restored in cya mutants if grown with exogenous cAMP.

Observing the fourth criterion is generally regarded as the most convincing evidence, although the possibility of an alternative regulatory mechanism, itself under control by cAMP, cannot be excluded.

CRP-cAMP is required for the expression of several structural proteins as well as catabolic enzymes and permeases (Table 4). Some of these proteins, though not essential for survival, may aid in nutrient acquisition. A generalization is that cAMP is an indicator of carbon insufficiency and promotes a phenotype which enables exploitation of other available carbon sources. AC may itself be negatively regulated by CRP-cAMP (Botsford and Drexler, 1978; Janeček et al., 1979; Majerfeld et al., 1981).

Table 4: Components and properties controlled by CRP-cAMP in *E. coli*

Component or function	Criterion*	Reference**
Outer membrane proteins	A, B, C	9, 11, 12
Phage receptor proteins	B, C	5, 10
Flagella	B, C	1
Pili	B, C	13
Chemoreception	A, B	3
Extracellular hydrolysis	A, B	8
Cytochromes	B, C	4
Membrane-associated bioenergetic functions	B, C	7
Plasmid replication	A, B, C	2
lac, *ara*, and *gal* operons	A, B, C, D, E	6

* A, glucose effect noted; B, function altered in *cya* and *crp*; C, exogenous cAMP affects function ; D, correlation with cAMP levels and function; E, operon sequenced and CRP-cAMP binding site identified.

** 1, Yokota and Gots (1970); 2, Katz *et al.* (1973); 3, Adler and Epstein (1974); 4, Broman *et al.* (1974); 5, Kumar (1975); 6, Pastan and Adhya (1976); 7, Dills and Dobrogosz (1977); 8, Kier *et al.* (1977); 9, Aono *et al.* (1978); 10, Alderman *et al* (1979); 11, Mallick and Herrlich (1979); 12, Scott and Harwood (1980); 13, Eisenstein *et al.* (1981).

More extensive lists can be found in reviews by Pastan and Perlman (1970), Rickenberg (1974), Pastan and Adhya (1976), Peterkofsky (1976) and Botsford (1981).

3. Other Factors Involved in Catabolite Repression

Accumulating evidence indicates that CRP-cAMP is not solely responsible for catabolite repression:-

1. A mutation causing a defect in the sigma subunit of RNA polymerase, resulted in 15 to 30% of wild-type β-galactosidase activity in a *cya* or *crp* background. This activity is sensitive to catabolite repression (Silverstone *et al.*, 1972).

2. Mutations, which map in *crp*, may suppress *cya* deletions and render the organism sensitive to catabolite repression (Dessein *et al.*, 1978a; Botsford and Drexler, 1978).

3. A low molecular weight, pH stable, and heat-stable factor has been isolated and termed catabolite modulator factor (CMF). CMF plays a negative regulatory role in catabolite repression and acts independently of CRP or cAMP (Dessein *et al.*, 1978a,b).

4. Compounds other than cAMP can activate CRP. Indole acetic acid and imidazole acetic acid can replace cAMP for expression of the *ara* operon (Kline *et al.*, 1980a,b).

5. Mutants with a defective transcription termination factor *rho*, in a *crp* background are able to express *lac*, *mal*, *ara* and *tna* operons which are sensitive to catabolite repression (Guidi-Rontani *et al.*, 1980).

It should be noted that catabolite repression is a feature of many organisms other than the enterobacteria, although glucose is not necessarily the preferred carbon source. *Bacillus megaterium*, *Pseudomonas aeruginosa*, and *Rhizobium meliloti* all exhibit catabolite repression, but cAMP does not appear to be involved (Ullmann, 1974; Ucker and Signer, 1978; Phillips and Mulfinger, 1981).

4. Regulation of Cyclic AMP Levels

An understanding of how intracellular cAMP levels are regulated is central to understanding how sugars effect catabolite repression. There are three possible means for regulating intracellular cAMP concentration: synthesis of cAMP by AC, degradation of cAMP by phosphdiesterase, or excretion of cAMP.

4.1. Synthesis of cAMP

AC is a membrane-bound enzyme and it's activity is regulated by interactions with transport proteins. This may be analagous to regulation of animal cell AC by interactions with hormone receptors (Harwood and Peterkofsky, 1977). AC activity is subject to glucose inhibition in whole cells but not in cell-free extracts (Peterkofsky and Gazdar, 1973). Sugar inhibition of AC depends on sugar transport but not sugar metabolism (Peterkofsky et al., 1975). Glucose and mannitol are transported by the phosphotransferase system (PTS), lactose by a proton symport mechanism and glycerol by facilitated diffusion. All these sugars inhibit cAMP production presumably by different mechanisms.

Peterkofsky and co-workers proposed that sugars transported by PTS inhibit cAMP production by the interaction of AC with enzyme 1 of the PTS (Peterkofsky, 1976; 1977). When enzyme 1 of the PTS is phosphorylated (due to no sugar transport), AC is in the active state. When non-phosphorylated (due to the rapid transfer of phosphate through the PTS during sugar transport), AC is in the non-active state. This model is supported by genetic evidence. An alternative model for PTS mediated AC regulation has been proposed in which AC is activated by allosteric interaction with a phosphorylated crr (catabolite repression resistant) gene product. The crr gene product is phosphorylated by the HPr (heat-stable protein) component of the PTS (Saier and Feucht, 1975; Feucht and Saier, 1980). Sugars transported by proton symport mechanisms may inhibit AC activity by causing collapse of the proton electro-chemical

gradient (Peterkofsky and Gazdar, 1979). It was mentioned previously that AC may be negatively controlled by CRP-cAMP. This may enable the organism to respond rapidly to changes in carbon/energy supplies (Botsford, 1981).

4.2. Degradation of cAMP

The significance of cAMP phosphodiesterase is uncertain. It's specific activity is not affected by the growth medium (Nielsen et al., 1973). Botsford (1981) reported unpublished results which showed that S. typhimurium strains, which lacked phosphodiesterase activity, had the same level of intracellular cAMP as the wild type, although culture supernate levels were three times greater. Thus phosphodiesterase does not appear to play an important role in regulating cAMP levels in response to growth conditions.

4.3. Excretion of cAMP

Cyclic AMP is actively excreted both by cells (Saier et al., 1975) and membrane vesicles (Goldenbaum and Hall, 1979), and substantial quantities of cAMP can be detected in culture supernates of E. coli (Buettner et al., 1973). On the other hand, cAMP uptake occurs by facilitated diffusion (Goldenbaum and Hall, 1979). Efflux of cAMP is stimulated by metabolizable sugars, but not by non-metabolizable analogues such as α-methyl-glucoside, which have the capacity for catabolite repression. During growth, the rate of cAMP excretion is proportional to its synthesis (Epstein et al., 1975). The significance of cAMP excretion is not known.

5. Physiological Strategy of Cyclic AMP Regulation

It has been suggested that the cAMP regulatory system enables the prokaryotic cell to respond to a range of nutritional environments such that the cell preferentially utilizes those carbon sources which are best at promoting growth (Rickenberg, 1974; Alper and Ames, 1978). The cell receives information on the "nutritional status" of the environment through transport and possibly other mechanisms. Some of these have been shown to interact with AC (also located at the cell surface) and alter cAMP production. Intracellular levels of cAMP appear to be inversely related to the efficiency of the available carbon / energy source at promoting growth (Buettner et al., 1973; Botsford, 1975; Epstein et al., 1975; Botsford and Drexler, 1978). CRP and cAMP may exist in equilibrium with CRP-cAMP (Botsford, 1981). Thus CRP-cAMP would be favoured by high levels of cAMP.

It has been suggested that a hierarchy of sensitivity of operons to CRP-cAMP may exist (Rickenberg, 1974; Alper and Ames, 1978). It was previously mentioned that the trn, lac, gal and ara operons all require different concentrations of cAMP for optimum expression. Further evidence for a control hierarchy comes from a series of leaky crp and cya mutants which are defective only in some cAMP sensitive systems (Alper and Ames, 1978).

Thus a wide variety of nutritional environments can be differentiated by receptor-mediated stimuli at the cell surface (transport mechanisms) which are relayed to the transcriptional apparatus via. cAMP. This enables the cell to respond best to nutritional circumstances, and economize on unnecessary protein synthesis.

6. Cyclic AMP in Prokaryotes Other than E. coli

This section serves to illustrate cAMP-associated manifestations in other prokaryotes and to point out the parallels and non-parallels

with the cAMP regulatory system of the model organism E. coli.

Alcaligenes eutrophicus. Synthesis of hydrogenase, in this facultative chemoautotroph, is stimulated by exogenous cAMP when grown heterotrophically. A cAMP-receptor protein has been identified and partially characterized (Tait et al., 1981).

Arthrobacter crystallopoietes. This organism grows rapidly as rods in the presence of succinate or certain amino acids. When the preferred carbon source is exhausted, the organism changes to a spherical form. Cyclic AMP appears to be involved in this transition, but not in catabolite repression (Hamilton and Kolenbrander, 1978).

Benekea harveyi. The enzyme luciferase, responsible for bioluminescence, is subject to the glucose effect and is influenced by cAMP (Ulitzer and Yasphe 1975). This is in contrast to the luminescent system in several species of Photobacterium which is subject to glucose repression, but is not influenced by cAMP (Nealson and Hastings, 1979). An immunologically similar protein to E.coli CRP has been demonstrated in B. harveyi (Nealson and Hastings, 1979).

Caulobacter crescentus. Cyclic AMP and cGMP are involved in the morphogenesis of this dimorphic organism, although they may act indirectly by influencing critical catabolic reactions. Cyclic AMP and cGMP act antagonistically (Kurn et al., 1977). Receptor proteins for cAMP and cGMP have been identified (Shapiro, 1976).

Mycoplasma. Mycoplasma species possess a fully functional PTS for the transport of sugars. Genetic evidence indicates that this interacts with AC. However, in contrast to E. coli, Mycoplasma produce more cAMP during growth with glucose than with fructose (Mugharbil and Cirillo, 1978).

Mycobacteria. Cyclic AMP production has been demonstrated in M. smegmatis (Lee, 1977), M. microti (Lowrie et al., 1975) and M. tuberculosis (Padh and Venkitasubramanian, 1979). In M. tuberculosis and M. smegmatis cAMP production is inhibited by glucose. The significance of this is not known as M. smegmatis lacks any detectable cAMP binding

activity (Padh and Venkitasubramanian, 1980). Lowrie et al. (1975) suggested that M. microti may protect itself within phagocytes by releasing cAMP and thus preventing phagosomal-lysosomal fusion.

Pseudomonas aeruginosa. The role of cAMP in Ps. aeruginosa is not known. Intracellular levels of cAMP do not alter with growth conditions and exogenous cAMP fails to relieve catabolite repression of glucose 6-phosphate dehydrogenase by succinate (the preferred energy/carbon source) (Phillips and Mulfinger, 1981).

Streptomycetes. Cyclic AMP has been reported to be involved in antibiotic production in streptomycetes. In S. griseus, cAMP inhibits production of candicidin, but this is thought to be a non-specific effect and due to phosphate inhibition (Martin and DeMain, 1977). In S. hygroscopius, turimycin production is inhibited by phosphate which can be reversed by exogenous cAMP (Gersch et al., 1978). It has been suggested that cAMP may be involved in the change over from primary metabolism (growth) to secondary metabolism (antibiotic synthesis) (Gersch, 1979).

Vibrio cholerae. V. cholerae mutants lacking AC have been isolated and show many analogies with cya E. coli mutants eg. pleiotropic deficiency in carbohydrate utilization, lack of flagella, and defects in the cell envelope (Yokota and Kuwahara, 1974).

Nitrogen-fixing bacteria. Nitrogen fixation is an energetically expensive process and cAMP appears to play a role in several nitrogen-fixing bacteria in co-ordinating the expression of nitrogen fixation. Exogenous cAMP has been reported to stimulate derepression of nitrogenase in Azotobacter vinelandi (Lepo and Wyss, 1974). In Anabaena variabilis, cAMP levels are lower when the organism grows heterotrophically than when growing autotrophically. When starved of a fixed nitrogen source, cAMP levels rise (Hood et al., 1979). Exogenous cAMP represses formation of glutamine synthetase, glutamate synthase and glutamate dehydrogenase. This in turn probably enhances expression of nitrogen fixation (Upchurch and Elkan, 1978).

As mentioned previously, cAMP is not ubiquitous in bacteria.

Despite intensive searches, cAMP has not been detected in several species of Bacillus (Bernlohr et al., 1974; Sarkar and Paulus, 1975; Setlow and Setlow, 1978), in Bacteriodes fragilus, an obligate anaerobe (Siegel et al., 1977) or in Lactobacillus plantarum (Sahyoun and Durr, 1972). Whether cAMP or it's metabolic enzymes exist in Neisseria gonorrhoeae is disputed by two groups (Morse et al., 1977; Morris and Lawson, 1979).

Ullmann(1974) has questioned whether the observed effects of exogenous cAMP in all organisms are related to underlying in vivo mechanisms. Bacillus megaterium lacks cAMP and it's metabolic enzymes, and yet still exhibits catabolite repression which can be relieved by exogenous cAMP.

PART 3: EXPRESSION OF VIRULENCE

1. Variation in Virulence

Virulence is not a permanent feature of pathogens, but is subject to variation depending on past and present growth conditions. Nor are the responsible components essential for growth under all conditions. It has long been recognised that virulence of many pathogens is lost during subculture *in vitro* and can be regained with animal passage. The smooth to rough transition is a classical feature of several species.

Pathogenic determinants have been studied mainly using *in vitro* grown organisms for the sake of convenience. However, the need to study pathogens during and after *in vivo* growth has been stressed (Smith 1958, 1964). Indeed, several pathogenic determinants only became recognized after the organism had been grown *in vivo*. Examples include the toxin of *Bacillus anthracis*, surface components of *Yersinia pestis* which inhibit phagocytosis, and surface components of *Brucella abortus* which protect against intracellular bacteriocidins of phagocytes (Smith 1958, 1976). This may explain why some attenuated vaccines, such as BCG for tuberculosis and S19 for contagious abortion, are more effective than heat-killed vaccines. Similarly, expression of protective antigens only during growth *in vivo*, may explain why natural infection and recovery affords greater protection than vaccination for many infectious diseases.

Neiseria gonorrhoeae grown in chambers subcutaneously implanted in guinea pigs showed enhanced resistance to human phagocytes and human serum (Penn *et al.*, 1977). Resistance to human serum was a phenotypic change whereas resistance to phagocytes was due to selection for mutants.

Another feature of several pathogens grown *in vivo* is a tendency to become more permeable by changes in the cell wall, or cell membrane. In some cases, the cell wall may largely disappear giving rise to the L-phase variants. Increased leakiness of pathogens may contribute to

in vivo survival by allowing freer entry of nutrients. L-phase variants and "leakiness" may be important properties of intracellular pathogens, and pathogens associated with chronicity and latency (Smith, 1976).

While some of the above may be due to selection of genotypes, others involve phenotypic changes. Smith (1977) has suggested that growth-limiting conditions in the host may contribute to phenotypic changes.

Two main mechanisms exist which enable flexibility of expression of virulence factors in pathogens. One of these is that many pathogenic determinants are plasmid- or phage-encoded (Elwell and Shipley, 1980; Maas, 1981). The other is that regulatory mechanisms exist such that determinants are only expressed under certain circumstances. Other less well-defined mechanisms may exist. Norlander *et al.* (1979) reported that virulent *N. gonorrhoeae* subcultured *in vitro* gave rise to a large number of avirulent colonial variants. This variation occurred after active growth and was sensitive to deoxyribonuclease. These workers suggest that transformation and recombination mechanisms were responsible for the rapid rate of colonial variation. Variation in flagellar antigens in Salmonella (phase variation) involves an invertible DNA segment which undergoes "site-specific" recombination by virtue of inverted repeat sequences (Mäkelä and Stocker, 1981).

2. Regulation of Virulence Factors

It has long been recognized that production of toxins and aggressins is often influenced by the composition of the medium and the stage of growth. However, it is only recently that molecular genetics has entered the field of microbial pathogenicity and little is known of the regulatory mechanisms involved.

Based on studies of toxin production, isolation of mutants defective in toxin production, and sometimes the mapping of mutations, a few transcriptional regulatory models have been proposed. It has long been known

that iron represses synthesis of diphtheria toxin. Murphy *et al.* (1978) proposed that iron acts as a co-repressor which forms a complex with a protein repressor encoded by the chromosome. The iron-repressor complex presumably acts at the corynephage β *tox* operator locus, preventing transcription. Mekalanos and Murphy (1980) suggested a protein repressor mechanism for the regulation of toxin production by *V. cholerae*. A common regulatory mechanism for the synthesis of protein A, coagulase, alpha-toxin and other exoproteins by *Staphylococcus aureus* has been proposed. This involves the activation of a factor, possibly by an intermediate metabolite, which stimulates transcription (Björklind and Arvidson, 1980).

A number of virulence factors or possible virulence factors in different organisms may be regulated in part by cAMP (Table 5). However, there is no evidence to indicate that the regulatory mechanisms involved are the same as those for the *lac*, *gal* and *ara* operons of *E. coli*.

In Part 2 it was mentioned that cAMP was a cytoplasmic indicator of carbon insufficiency. A corollary from this with regard to regulation of virulence factors by cAMP might be that optimum expression of pathogenic determinants occurs when the level of certain nutrients is low, as is likely to occur during the initial stages of infection. This mechanism would enable the pathogen to lodge and invade the host, combat the host defence mechanisms, and thus gain access to better nutritional environments. Certain pathogens such as *Bacillus anthracis* and *Streptococcus pneumoniae* multiply rapidly *in vivo* only during the terminal stages of infection after the host defence mechanisms have been overcome (Smith, 1958).

Table 5: Virulence factors of microbial pathogens possibly controlled by cAMP

Virulence factor	Positive or negative influence	Criterion[a]	Reference
Type 1 pili in *E. coli*	+/-[b]	B, C	Eisenstein *et al.* (1981)
Type 1 pili in *S. typhimurium*	+	B, C	Saier *et al.* (1978)
Heat-stable enterotoxin of *E. coli*	+	A, B, C	Alderete and Robertson (1977) and Martinez-Cadena *et al.* (1981)
Heat-labile enterotoxin of *E. coli*	-	A	Gilligan and Robertson (1979)
Alpha-toxin of *Staphyloccocus aureus*	+	A	Duncan and Cho (1972)
M protein of *Streptoccocus pyogenes*	+	A, C	Pine and Reeves (1978)
Aflatoxin of *Aspergillus parasiticus*	-	D	Applebaum and Buchanan (1979)
Yeast to mycelial transition in *Candida albicans*	+	C[c],	Nimi *et al.* (1980)
Yeast to mycelial transition in *Histoplasma capsulatum*	+	D	Medoff *et al.* (1981)
Pectate lyase in *Erwina carotovora*	+	A, C, D	Tsuyuma (1979)

a, as for Table 4; b, dependent on strain; c, phenotype promoted by exogenous dibutyryl cAMP.

OBJECT OF RESEARCH

B. pertussis produces several pathophysiologically-active components. Some of these have been implicated as virulence factors in the pathogenesis of pertussis, some as protective factors in immunity to pertussis and some as responsible for the reactogenicity of the vaccine. With knowledge of how these various components are regulated in *B. pertussis*, it may be possible to manipulate their production by either cultural or genetic means. This could be used to provide a better starting point in the production of a more acceptable vaccine. Such knowledge might also be useful if *B. pertussis* activities were to be cloned, for example in *E. coli*. It is also of basic importance to understand how a pathogen regulates it's physiology in response to the host environment.

Many of the pathophysiological activities of *B. pertussis* are lost during the phenotypic changes of antigenic modulation and the genotypic changes of phase degradation. This suggests that a common regulatory mechanism lies at the root of these two processes.

In view of the regulatory role that cAMP plays or may play in certain bacterial species in the synthesis of many proteins, including some associated with virulence, it is conceivable that cAMP plays a major regulatory role in the synthesis of virulence factors in *B. pertussis*. The observation that AC activity is extensively reduced in C-mode and phase IV variants, compared to their X-mode and phase I counterparts, has led to the suggestion that AC may be the target site of both modulation and phase degradation (Parton and Durham, 1978; Hewlett *et al.*, 1979a; Wardlaw and Parton, 1979). The main aim of this project was to determine whether the loss of AC activity had a causal or coincidental relationship towards the loss of virulence factors, and to assess the role of cAMP in governing the expression of these factors.

MATERIALS AND METHODS

1. Cultivation, Preparation and Standardization of Bacteria

1.1 Organisms, strains and storage

Organisms and strains used are shown in Table 6. Lyophilized suspensions of *B. pertussis* strains were reconstituted and grown on BG at 35°C for 3 or 4 days. Confluent growth was resuspended to give a turbid suspension in 1% (w/v) casamino acids, pH 7.0 (Appendix 1) containing 10% (v/v) glycerol and was stored either at -70°C or in liquid nitrogen. *E. coli* strains were stored suspended in saline (0.85% (w/v) NaCl) at -70°C.

1.2. Media

BG (Gibco Biocult Ltd.), Nutrient agar (Oxoid), and MacConkey agar (Oxoid) were made up according to the manufacturers instructions. BG was supplemented with 20% (v/v) defibrinated horse blood (Gibco Biocult Ltd.). The procedure for preparation of BG-X and BG-C (X- and C-mode BG made from basic ingredients is given in Appendix 1).

Liquid media were:- 1, SS-X and SS-C (X- and C-mode Stainer and Scholte medium (Stainer and Scholte, 1971)); 2, H-X and H-C (X- and C-mode modified Hornibrook medium (Wardlaw *et al.*, 1976)); 3, Yeast Glucose medium (Pastan *et al.*, 1974). The procedure for preparation of media is given in Appendix 1. SS-X was often modified as described in the Results. Heat-labile additives (viz. bovine catalase, chloramphenicol, phosphonomycin and N-methyl-N'-nitro-N-nitroso-guanidine (NTG)) were filter sterilized before adding aseptically to the autoclaved media (15 psi, 121°C for 15 min). Media containing cAMP and $N^6,O^{2'}$-dibutyryl cAMP were pH adjusted after their addition and filter sterilized. All other ingredients, were added prior to pH adjustment and autoclaving.

Table 6: Organisms and strains

Species	Strain	Obtained from	Further comments
B. pertussis	Taberman	Professor G. T. Stewart Ruchill Hospital, Glasgow	A strain isolated per-nasally prior to the death of an infant.
	18334	Connaught Lab. Ltd. Toronto	A vaccine strain
	18323	Dr. F. Sheffield Nat. Inst. Biological Standards, London.	A mouse intracerebrally-virulent strain.
	D30042 phase I D30042 phase IV	Dr. J. Dolby Clin. Res. Centre, Harrow, London	
E. coli	1100	Dr. C. R. Harwood The Medical School, The University, Newcastle	A "wild type" strain possessing a functional AC and CRP.
	5333	Dr. C. R. Harwood	A crp mutant of strain 1100 lacking a functional CRP.

1.3. Growth and cultivation

Frozen suspensions of *B. pertussis* were allowed to thaw and then spread on BG plates. BG plates were incubated in sealed plastic boxes at 35°C for 48 - 72 h. Bacteria were resuspended in a small volume of sterile liquid medium which was used to inoculate flasks of medium. Except where stated, cultures refer to shake cultures grown in media contained in dimpled Ehrlenmeyer flasks (2 L flasks containing 0.5 L or 1 L of medium, or 250 ml flasks containing 50 ml or 100 ml of medium) which were incubated at 35°C on an orbital shaker at 100 cycles min^{-1}. Static cultures were grown in 250 ml flasks containing 25 ml of medium which were incubated at 35°C. Purity of cultures was checked by Gram-staining and slide agglutination with phase I *B. pertussis* antiserum. Liquid cultures were harvested by centrifugation at 10,000 x g for 15 min at 4°C.

1.4. Standardization of bacterial suspensions

Bacterial suspensions were standardized either by opacity, absorption at 660nm or by protein concentration. Opacity units (ou) were determined by noting the dilution of the suspension which gave the same opacity (as judged by eye) as a 10 ou reference (the International Opacity Standard obtained from W.H.O., International Lab. for Biol. Standards, Nat. Inst for Biological Standardization and Control, Holly Hill, Hampstead, London). Protein concentration was determined by the Lowry method modified for use with whole cells (Herbert *et al.*, 1969) using bovine serum albumin as a standard. For convenience, a standard curve of absorbance at 660 nm versus dry weight and protein concentration was constructed. *B. pertussis* Taberman was grown for 48 h in SS-X and SS-C and harvested. Pellets were resuspended in saline to give a range of absorbance values, and samples were assayed for protein concentration. To determine dry weights, large samples (75 ml) were centrifuged and the pellets resuspended in a small volume of distilled water. Pellets and centrifuge tube rinses were filtered onto previously weighed membrane filters (47 nm diameter, 0.45 μm pore size) and dried to constant

weight. Figure A1 (Appendix 2) is a plot of the combined results from two experiments.

1.5. Cell disruption

Cell lysates were prepared by passing frozen cell suspensions three times through a LKB X-press (Biotech Ltd., Stockholm).

2. Biochemical Methods

2.1. Adenylate cyclase assay

This was based on the method described by Hewlett and Wolff (1976), but with the ATP regenerating system omitted (creatine phosphokinase is now known to be contaminated with *B. pertussis* AC activator). Test-tubes containing 80 µl of 180 mM N-tris-(hydroxymethyl)-methyl glycine (Tricine) pH 8.0, and 40 µl of enzyme, were incubated for 4 min in a 30°C water bath. The reaction was started by the addition of 120 µl 2 mM ATP in 20 mM $MgCl_2$. After 10 min, the reaction was terminated by the addition of 1 ml cAMP assay buffer (Appendix 3) and immersion in a boiling water bath for 10 min. Samples were stored sealed at -20°C and assayed for cAMP at a later date. Up to 100 µg protein of sample was present in assay mixtures. Samples were assayed in duplicate.

Intact cells were often used as an enzyme source and their activity was referred to as intact-cell AC activity. Harvested cells were resuspended in saline and standardized by measuring the absorbance at 660nm. Cell suspensions were assayed as soon as possible and not stored frozen unless cell lysates were to be used as an enzyme source. Specific activity was standardized by protein concentration which was determined using a calibration graph (Appendix 2, Fig. A1). X- and C-mode lysates heated at 100°C for 10 min destroyed all detectable activity when 100 µg protein of sample was assayed for activity (>99% and ≥80% loss of activity for X- and C- mode cell lysates respectively).

When additional compounds were present in the assay mixture, as described in the Results, they were dissolved in 180 mM Tricine buffer, pH 8.0,

at three times their final concentration in the assay mixture and added in place of unmodified Tricine buffer.

2.2. Cyclic AMP assay

Cyclic AMP was assayed by the cAMP binding protein method of Gilman (1970). This assay is based on the competition between unlabelled cAMP and a fixed quantity of [^{3}H] cAMP, for a highly specific binding protein. Unbound cAMP is removed by the addition of activated charcoal followed by centrifugation. Protein-bound [^{3}H] cAMP, in the supernate, is inversely proportional to cAMP in the sample.

The following reagents (preparation given in Appendix 3) were added to 8 x 75 mm test-tubes, which were incubated in an ice bath:- (1) 50 µl of sample or standard, (2) 50 µl of [^{3}H] cAMP reagent, (3) 100 µl of binding-protein reagent. Standards consisted of a doubling dilution series of cAMP ranging from 16 - 0.25 pmol per tube. Cyclic AMP assay buffer was added in place of sample or standard in two tubes ("C_o tubes"). Two "blank" tubes contained 100 µl cAMP assay buffer in place of binding-protein reagent. All tubes were vortex-mixed and incubated on ice for 2 - 3 h. Charcoal reagent (100 µl) was added to 40 tubes at a time. These were vortex-mixed and immediately centrifuged (within 5 min of adding charcoal reagent to the first tube) for 3 min at 3,000rpm (2,300 x g) in a MSE Mistral 6L centrifuge. Supernate (200 µl) was carefully removed into scintillation vials and 3 ml of scintillant fluid (Packard 299) was added. Vials were counted for 5 - 10 min in a Packard Tri-Carb scintillation counter. Vials containing 50 µl of [^{3}H] cAMP reagent plus 3 ml scintillant fluid were also counted to determine the binding efficiency of the binding protein.

The cpm obtained from the "blank" tubes were subtracted from all other counts. C_o was designated as the average blank-subtracted cpm obtained from the "C_o tubes". C_x was designated as the variable blank-subtracted cpm obtained from each sample and standard tube. The C_o/C_x was plotted against cAMP concentration of the standards which

was used to determine the cAMP levels in the unknowns. A typical standard curve is shown in Appendix 2, Fig. 2A.

To test the authenticity of cAMP detected in culture supernate and after enzyme/substrate incubation, some samples were incubated with 10 μg ml^{-1} bovine heart 3',5'-cyclic nucleotide phosphodiesterase and 10 mM $MgCl_2$ for 20 min at 30°C. In testing the validity of cAMP assays, cAMP assay buffer was not added until after the phosphodiesterase treatment as ethylenediamine-tetraacetic acid (EDTA), which is present in the buffer, inhibits phosphodiesterase activity. At least 96% of culture supernate cAMP was destroyed and >99% and >80% of cAMP produced in 10 min by X- and C-mode lysates respectively were destroyed.

2.3. Cyclic AMP binding assay

Cyclic AMP binding activity was determined in extracts of *B. pertussis* and *E. coli* by the method described by Pastan *et al.*(1974). *B. pertussis* strains were grown in SS-X or SS-C for 48 h and *E. coli* strains were grown in Yeast Glucose medium for 18 h. Harvested cells were resuspended in buffer A (Appendix 3) to give a thick suspension. Suspensions were X-pressed and centrifuged at 100,000 x g for 1 h. Supernates were removed and incubated at 4°C overnight with pancreatic deoxyribonuclease (5 μg ml^{-1}) and pancreatic ribonuclease (5 μg ml^{-1}). Samples were adjusted to 20 mg ml^{-1} protein.

Nucleotide reagents were prepared in 10 mM potassium phosphate pH 7.7, with the pH adjusted, if necessary,with NaOH. The assay was performed in an ice bath. To 2 ml centrifuge tubes were added 30 μl of 33 mM 5'-AMP, 3.3 μM [^{3}H] cAMP (specific activity 1.52 μCi $nmol^{-1}$) and 70 μl of sample. Control "blank" tubes, one for each sample tube, contained 30 μl of 33 mM 5'-AMP, 33mM [^{3}H] cAMP (specific activity 0.152 μCi μmol^{-1}) and 70 μl of sample. Control "total" tubes contained 70 μl of sample. Tubes were vortex-mixed and after 5 min incubation, 0.4 ml of cold saturated $(NH_4)_2SO_4$ was added. Vortex-mixed tubes were centrifuged at 12,000 x g, in a Beckman Microfuge, for 5 min. Supernate was carefully removed with

a Pasteur pipette and the remaining moisture was absorbed with a cotton-tipped applicator stick. To "total" tubes was added 30 µl of [^{3}H] cAMP (0.5 µCi ml^{-1}). Pellets were dissolved in 0.5 ml TS-2 tissue solubilizer (Koch-Light) and transferred to a vial containing 10 ml Triton-Toluene scintillant (Appendix 3). The pipette and tube were washed several times with scintillation mixture. Vials were counted for 10 min in a Packard Tri-Carb 300 scintillation counter, after leaving in the dark for several hours until stable counts were obtained. The average cpm of vials containing 10 ml scintillant fluid only, was subtracted from the cpm of the other vials.

Binding activity (pmol [^{3}H] cAMP bound mg(protein)$^{-1}$) was determined using the equation:-

$$(S - B)/(T \times 10) \times A \times 1/P$$

where S is the cpm obtained from assay tube, B is the cpm obtained from "blank" tube, T is the cpm obtained from "total" tube, A is pmol of cAMP per assay tube (ie. 100) and P is the mg protein per tube (ie. 1.4). Samples were assayed in triplicate.

2.4. Cyclic AMP uptake measurements

B. pertussis was grown in SS-X or SS-C for 48 h. Harvested cells were washed once in saline and then resuspended in SSX or SSC buffer (Appendix 3) to give an A_{660nm} of 5.0 (3 mg(dry weight) ml^{-1}). Cell suspensions were incubated at 35^{o}C for 10 min before addition to the reaction mixture. The reaction mixtures consisted of 9 ml SS-X or SS-C with 1 mM [^{3}H] cAMP or 1 mM [^{3}H] N^6,$O^{2'}$-dibutyryl cAMP (specific activity 5 µCi µmol^{-1}) and were preincubated in a 35^{o}C shaking water bath. The reaction was started by the addition of 1 ml cell suspension to the reaction mixture. At timed intervals, 1 ml was rapidly filtered through cellulose acetate membrane filters of 25mm diameter and 0.45 µm pore size (Oxoid) which previously had been washed with SSX buffer. The filters were washed twice with 3 ml SSX buffer and dried in scintillation

vials under an infra-red lamp. Triton-Toluene scintillant (10 ml) was added and vials counted for 10 min. "Blank counts" were obtained by filtering 0.9 ml of reaction mixture prior to the addition of cell suspension. These were washed, dried and counted in the same way as for samples. "Total counts" were obtained by adding 25 µl of reaction mixture to a filter which was dried and counted in 10 ml of scintillant. The background count, of a vial containing 10 ml of scintillant only, was subtracted from these. The following equation was used to obtain nmol mg (dry weight)$^{-1}$:-

$$(X - B)/(400 \times T) \times A \times 1/D$$

where X is the cpm of sample, B is the cpm obtained from "Blank", T is the back ground-subtracted cpm obtained from "Total", A is the nmol of cAMP or dibutyryl cAMP in reaction mixture (ie. 10,000) and D is the mg dry weight of cells filtered (ie. 0.3).

2.5. Measurement of respiration rate

B. pertussis was grown in 100 ml of SS-X or SS-C for 48 h. These cultures were used to inoculated 1 L volumes of SS-X or SS-C (10% (v/v) inoculum) and after 24 h growth, cultures were harvested. Cells were washed twice in SSX to give an A_{660nm} of 25 (15 mg(dry weight) ml^{-1}). Cell suspensions were incubated at 35^{o}C for 15 min to use up endogenous substrates and thereafter at 4^{o}C for up to 1 h while samples were removed to measure respiration rates.

Respiration rates were measured using a Clark oxygen electrode (Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio). SSX buffer (3 ml), and 100 µl cell suspension, were placed into a chamber with a magnetic stirrer, and allowed to equilibrate at 35^{o}C. The oxygen electrode was inserted taking care not to trap any air bubbles, and oxygen uptake was monitored for several minutes to determine the respiration rate due to endogenous substrates (endogenous rate). Substrates were prepared in SSX buffer to give 0.2 M and a pH of 7.3. Substrate (0.3 ml) was injected into the chamber with care taken to ensure no entry of air

bubbles. Oxygen consumption was monitored for several minutes to determine the exogenous rate (details of calculations are given in Appendix 4).

2.6. Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis (SDS-PAGE) was done using a discontinuous buffer system, based on the methods of Ames (1974) and Laemmli(1970). Separating and stacking gels contained 11% and 5% (w/v) acrylamide respectively and were set in 8.0 x 8.0 x 0.3 cm glass moulds. Both gels and electrode buffers contained 0.1% (w/v) SDS. Buffers and gels are described in Appendix 5. Gels were run at a constant current (15 mA per gel) in a Uniscil slab gel electrophoresis unit.

For SDS-PAGE of whole cells, harvested cells were washed once in saline and resuspended to give an A_{660nm} of 5.0 (about 2 mg protein ml^{-1}). Cell suspensions were mixed with an equal volume of solubilizing buffer (Appendix 5) and heated at 100°C for 5 to 10 min. Samples of 25 µl or 50 µl were added to each track of the gel. To estimate molecular weights of separated polypeptides, a solubilized mixture of standard proteins was run in parallel with samples. The standard protein mixture consisted of bovine serum albumin, ovalbumin, chymotrypsinogen, trypsin and cytochrome C (200 µg ml^{-1} of each in distilled water). The protein mixture was solubilized in the same way as samples. A standard curve of distance migrated versis log of molecular weight was used to estimate molecular weights of polypeptides in samples.

3. Miscellaneous

3.1. Histamine-sensitizing activity

Harvested cells were resuspended in saline to 100 ou. Suspensions were heat-killed (56°C for 30 min) and stored at 4°C with merthiolate (0.01% (w/v)). Seven to ten-week old mice (HAM/ICR) were injected intraperitoneally with graded doses of vaccine (0.5 ml per mouse). Mice were challenged 5 days later with 3 mg histamine dihydrochloride in

0.5 ml saline. Survivors were counted 3 h after challenge. The results were analysed by the probit method (Finney, 1971), using a computer programme developed in the Dept. of Epidemiology and Biometrics, School of Hygiene, University of Toronto, Canada.

3.2. Haemagglutinin activity

HA assays were performed using microtitre trays. Saline (50 µl) was added to each well. Sample (50 µl) was added to the first two wells in each row and doubling dilutions were made from the second well along each row. A washed 2% horse red-blood cell suspension (50 µl) was added to each well. The contents of wells were mixed and the trays incubated for several hours at 4^{o}C. The HA titre was recorded as the reciprocal of the highest dilution giving complete haemagglutination. Samples were assayed in duplicate.

3.3 Ouchterlony double diffusion precipitin test.

Gel-diffusion medium (Appendix 3) was poured into glass plates and allowed to set. Wells were cut out (5 mm diameter, 5 mm apart) and a drop of molten medium was added to seal the base of each well. Samples were added to the wells (100 µl) and plates were stored in sealed containers for several days.

3.4. "Crossed-over" immunoelectrophoresis

This was based on the method described by Culliford (1964). Immunoelectrophoresis is used to draw antigens and antibodies together as most antigens,at pH 8.3, migrate towards the anode while antibodies migrate towards the cathode. This results in a more rapid and intense precipitin line compared to a double diffusion system. Molten 0.75% barbitone agar, pH 8.3 (Appendix 3), was pipetted onto clean glass slides (75 x 50 mm) on a level surface and allowed to solidify. Wells (1.5 mm diameter) were cut in the agar and filled with about 10 µl sample. Electrophoresis was done in barbitone buffer, pH 8.3, at 100 V for

30 min using a Shandon electrophoresis apparatus (Kohn type).

To intensify the precipitin lines formed, a staining procedure was done. The slides were immersed in barbitone buffer pH 8.3 overnight to remove unreacted protein, washed twice in distilled water (15 min per wash) and stained with nigrosine solution (saturated in 2% (v/v) acetic acid) for 10 min. Slides were destained by washing in 2% (v/v) acetic acid followed by 2% (v/v) acetic acid, 1% (v/v) glycerol.

3.5. Raising of antiserum

Sample was mixed with an equal volume of Freunds incomplete adjuvant until a stable water-in-oil emulsion was obtained. The emulsion (1 ml) was injected into the muscle of each hind limb of a rabbit. Two further intramuscular injections of the same material (1.5 ml) were administered at two-weekly intervals. Blood samples were taken from the ear before each injection. Six weeks after the first injection, the rabbit was bled out by cardiac puncture. The blood was allowed to clot for 1 h at room temperature and then stored at -20°C.

Goat anti-CRP was a gift from Dr. I. Pastan, Lab. Molec. Biol., Nat. Cancer Inst., NIH., Bethesda, Maryland,U.S.A. U.S. standard *B. pertussis* antiserum (rabbit) was obtained from Dr. C. R. Manclark, Biologics Standards, NIH., Bethesda, Maryland, U.S.A.

3.6. Viable counts

Viable counts were obtained by doing 10-fold serial dilutions of cultures in sterile saline. Appropriate dilutions (0.05 ml) were spread on dry BG plates (three for each dilution) which were incubated for several days until colonies could be counted easily. Confidence limits (95%) were calculated using a weighting method where counts obtained from a large number of observed colonies are given more credibility (A. C. Wardlaw, personal communication).

3.7. Chemicals

Nucleotides, commercial enzymes and proteins, activated charcoal, Reactive-red Agarose and antibiotics were purchased from Sigma Chemical Co. Tritiated compounds were purchased from The Radiochemical Centre, Amersham. Diethylaminoethyl- (DEAE-) cellulose, Sephadex G-200 and Blue-dextran Sepharose were products of Pharmacia, and Aquacide IIA was a product of Calbiochem. NTG was obtained from Aldrich Chemical Co.

RESULTS

1. A Study of Different Possible Modulating Compounds.

Loss of histamine-sensitizing activity (HSA), the 28k and 30k cell-envelope gel bands (X bands) and AC activity occurs when B. pertussis is grown in media where the NaCl is replaced by $MgSO_4$ or in media with higher than normal levels of nicotinic acid (4.1 mM). Lacey (1960) reported that several anions, cations and organic acids could induce C modulation. The effect of several of these, and nicotinic acid, on the production of AC activity, the X bands and HSA was investigated. The simultaneous loss of these would be consistent with the hypothesis of a common regulatory mechanism, possibly involving AC, perturbed by pro-C-mode substances.

B. pertussis was grown for 48 h in SS-X where the NaCl was replaced by various salts at concentrations which Lacey (1960) reported to cause C modulation. NaCl was present at the normal concentration (43 mM) in medium containing Na caprylate or high levels of nicotinic acid, to retain the osmolarity of these media. After 48 h growth, cultures were centrifuged. Culture supernates were assayed for cAMP and AC activity and harvested cells were assayed for HSA, intact-cell AC activity and were analysed by SDS-PAGE.

1.1 Adenylate cyclase activity

Growth in several modifications of SS-X caused a severe reduction in intact-cell AC activity (Table 7). These results are the averages for two experiments. Intact-cell AC activity varied with the strain used after growth in unmodified SS-X, whereas, after growth in medium containing Na_2SO_4, $MgSO_4$ or Na butyrate, all strains showed a marked reduction in AC activity. Growth in these latter media, caused 89 - 98% loss of activity when compared with NaCl-grown cells. Na caprylate-induced loss of AC activity appeared to be strain specific, causing loss for strain 18334 but not for strain 18323. Growth of strain Taberman was inhibited by 0.6 mM Na caprylate. Na lactate-induced loss of AC activity, also appeared to be strain specific, and only occurred in strain Taberman. However, this

Table 7: Intact-cell AC activity of *B. pertussis* strains after growth with various additives

Strains were grown for 48 h in SS-X where the NaCl had been replaced by other additives. Harvested intact cells were assayed for AC activity

Additive and final concentration (mM)		Intact-cell AC activity[a] (and standard deviation)					
		Taberman	% Relative to standard[b]	18334	% Relative to standard[b]	18323	% Relative to standard[b]
NaCl	43	840 (156)	100	425 (25)	100	190 (10)	100
$MgSO_4$	20	16 (7)	1.9	17 (2)	4.0	21 (2)	11
$MgCl_2$	43	915 (140)	109	410 (20)	96	188 (8)	99
Na_2SO_4	43	13 (2)	1.5	13 (3)	3.1	13 (3)	6.8
Na lactate	86	89 (12)	11	410 (30)	96	195 (5)	103
Na succinate	86	27 (5)	3.2	...	...	...	...
Na butyrate	20	14 (2)	1.7	15 (2)	3.5	14 (4)	7.4
Na caprylate[c]	0.6	...[d]	...	17 (2)	4.0	195 (15)	103
Nicotinic acid[c]	4.1	35 (1)	4.1	...	...	...	...

a, pmol cAMP min^{-1} $mg(protein)^{-1}$; b, standard refers to NaCl-grown cells; c, these media also contained NaCl (43 mM); d, not tested (...).

loss of AC activity was less severe than that caused by $MgSO_4$, Na_2SO_4, Na succinate, Na butyrate, or nicotinic acid. Unexpectedly, high levels of $MgCl_2$ caused no significant loss of AC activity for any strain.

Loss of intact-cell AC activity was not due to release of AC as the culture supernate AC activity paralleled the intact-cell AC activity for strain Taberman (Table 8). Results refer to averages of two experiments.

Hewlett et al. (1978) reported B. pertussis AC could be stimulated 240-fold by an activator present in many mammalian tissues and as a contaminant of many commercial protein preparations. Bovine catalase was a particularly good source of activator. This activator has recently been identified as calmodulin (Wolff et al., 1980). It was of interest to determine whether commercial bovine catalase could stimulate intact-cell AC activity after growth in various modulating conditions, since it has been suggested that activator may play a role in facilitating the parasitic state of the organism (Hewlett et al., 1978). Intact-cell AC activity was responsive to activator (present as commercial bovine catalase), whether growth conditions had or had not induced loss of AC activity (Table 9). However, the extent of activation was reduced by 11 - 50% in the latter. Bovine liver catalase was present in the assay mixture at a final concentration of 1 mg ml^{-1}. Results refer to the averages of two determinations.

In proposing AC as the target site for modulation or as one of the causal factors, it was of interest to determine culture supernate cAMP levels after growth in various modifications of SS-X. Culture supernate cAMP is presumably a good indicator of cytoplasmic AC activity during growth, assuming that the substrate ATP is exclusively intracellular. The majority of cAMP produced during growth is excreted into the medium as cAMP levels were the same whether determined from whole cultures (heat treated at 100°C for 10 min and centrifuged at 12,000 x g for 10 min), from culture supernate, or from heat-treated (100°C for 10 min) culture supernate (results not shown). Growth in media which resulted in a marked

Table 8: Culture-supernate AC activity of strain Taberman after growth with various additives

Strain Taberman was grown for 48 h in SS-X where the NaCl had been replaced by other additives. Culture supernate was assayed for AC activity.

Additive and final concentration (mM)		AC activity[a] (and standard deviation)	% Relative to standard[b]
NaCl	43	180 (53)	100
$MgSO_4$	20	16 (2)	9
$MgCl_2$	43	151 (35)	84
Na_2SO_4	43	17 (5)	9
Na lactate	86	52 (23)	29
Na succinate	86	34 (10)	19
Na butyrate	20	15 (2)	8
Nicotinic acid[c]	4.1	19 (5)	11

a, pmol cAMP $min^{-1}ml^{-1}$; b, standard refers to NaCl-grown cultures; c, this medium also contains NaCl (43 mM).

Table 9: Intact-cell AC activity, in the presence and absence of AC activator, after growth with various additives

Strain Taberman was grown for 48 h in SS-X where the NaCl had been replaced by other additives. Harvested intact-cells were assayed for AC activity in the presence and absence of bovine liver catalase (1 mg ml^{-1}).

Addition to medium and final concentration (mM)		AC activity[a] (and standard deviation) With bovine catalase present	AC activity[a] (and standard deviation) With bovine catalase absent	Fold of activation	% Activation relative to standard[b]
NaCl	43	0.84 (0.16)	45 (20)	54	100
$MgSO_4$	20	0.016 (0.007)	0.36 (0.12)	23	42
$MgCl_2$	43	0.915 (0.14)	52 (5)	56	106
Na_2SO_4	43	0.013 (0.002)	0.33(0.03)	25	47
Na lactate	86	0.089 (0.012)	4.1 (0.6)	46	86
Na succinate	86	0.027 (0.005)	0.17 (0.01)	6.3	12
Na butyrate	20	0.014 (0.002)	0.32 (0.03)	23	43
Nicotinic acid[c]	4.1	0.035 (0.001)	0.96 (0.1)	27	51

a, nmol cAMP min^{-1} $mg(protein)^{-1}$; b, standard refers to NaCl-grown cells; c, this medium also contained NaCl (43mM).

reduction in intact-cell AC activity, also had much reduced levels of culture-supernate cAMP compared to NaCl-grown cultures (Table 10). Results refer to averages of two experiments. Growth in medium containing high levels of $MgCl_2$ resulted in about a 1.4-fold increase in cAMP levels compared to standard NaCl cultures. This may be related to the fact that optimum AC activity requires an optimum concentration of Mg^{++} ions (Hewlett and Wolff, 1976).

1.2. Histamine-sensitizing activity

Growth in media which resulted in severe loss of intact-cell AC activity (ie. media containing $MgSO_4$, Na_2SO_4, Na butyrate, Na succinate, and nicotinic acid) caused a similar loss of HSA (Table 11). Results refer to cell suspensions prepared from selected cultures. Data, from which results were calculated, are given in Appendix 6, Tables A1, A2 and A3. Growth of strain Taberman in medium containing Na lactate resulted in a less severe loss of HSA (82%) which was paralleled by a similar loss of AC activity (89%) (Table 7).

1.3. The X bands

Plates 1A, 1B and 1C show the SDS-PAGE analysis of whole cells of B. pertussis after growth in modified SS-X media. Extensive loss of the X bands correlated with extensive loss of AC activity and HSA. Moreover, these bands were detectable, although fainter, after growth of strain Taberman in medium containing Na lactate, where extensive but incomplete loss of AC activity and HSA occurred.

Table 10: Levels of cAMP in supernate of *B. pertussis* cultures after growth with various additives

Strains were grown for 48 h in SS-X where the NaCl had been replaced by other additives. Culture supernate was assayed for cAMP.

Additive and final concentration (mM)		Taberman		18334		18323	
		cAMP level[a]	% relative to standard[b]	cAMP level[a]	% relative to standard	cAMP level[a]	% relative to standard
NaCl	43	112	100	96	100	116	100
$MgSO_4$	20	< 5[c]	< 5	< 5	< 6	< 5	< 5
$MgCl_2$	43	183	163	130	135	182	156
Na_2SO_4	43	< 5	< 5	< 5	< 6	< 5	< 5
Na lactate	86	20	18	85	89	127	109
Na succinate	86	< 5	< 5	...	...	...	...
Na butyrate	20	< 5	< 5	< 5	< 6	< 5	< 5
Na caprylate[e]	0.6	...[d]	...	< 5	< 6	130	112
Nicotinic acid[e]	4.1	< 5	< 5	...	...	...	...

a, pmol cAMP mg(dry weight)$^{-1}$ of cells in culture; b, standard refers to NaCl-grown cultures; c, limit of detection; d, not tested (...); e, these media also contained NaCl (43 mM).

Table 11: HSA of *B. pertussis* after growth with various additives

Strains were grown for 48 h in SS-X where the NaCl had been replaced by other additives. Harvested cells were heat treated (30 min at 56°C) and assayed for HSA.

Additive and final concentration (mM)		Taberman		18334		18323	
		HSD_{50}[a]	% relative potency[b]	HSD_{50}	% relative potency[b]	HSD_{50}	% relative potency[b]
NaCl	43	1.1	100	1.0	100	1.4	100
$MgSO_4$	20	22.7	4.6 (1.4,10)[c]	23.8	4.3 (1.4,10)	15.2	9.3 (4.3,19)
$MgCl_2$	43	1.4	76 (33,170)	1.4	72 (31,160)	1.2	117 (59,238)
Na_2SO_4	43	24.7	4.3 (1.9,8.6)	18.5	5.6 (2.0,12)	38.9	3.6 (1.2,9.0)
Na lactate	86	6.0	18 (9.2,36)	...	...	...	...
Na succinate	86	16.1	6.5 (3.2,13)	...	...	...	...
Na butyrate	20	15.7	7.9[d] (3.9,14)	22.5	4.6 (1.5,10)	14.6	9.7 (4.5,20)
Na caprylate[f]	0.6	...[e]	...	30.1	3.4 (1.0,8.3)	1.2	119 (60,240)
Nicotinic acid[f]	4.1	17.4	6.0 (3.0,11)	...	...	...	...

a, the dose (ou.ml) required to kill 50% of mice, after subsequent challenge with histamine; b, potency relative to NaCl-grown cells; c, 95% confidence limits;d, the relative potency for this sample was calculated using a different NaCl- grown standard; e, not tested (...); f, these media also contained NaCl (43 mM).

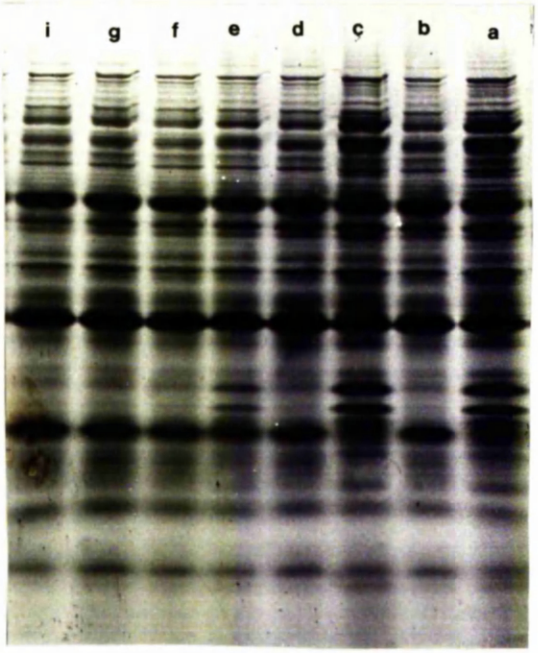
Plate 1: SDS-PAGE profiles of *B. pertussis* cells after growth with various additives

Strains were grown for 48 h in SS-X where the NaCl had been replaced by various additives. Washed cells were analysed by SDS-PAGE. A, strain Taberman; B, strain 18334; C, strain 18323.

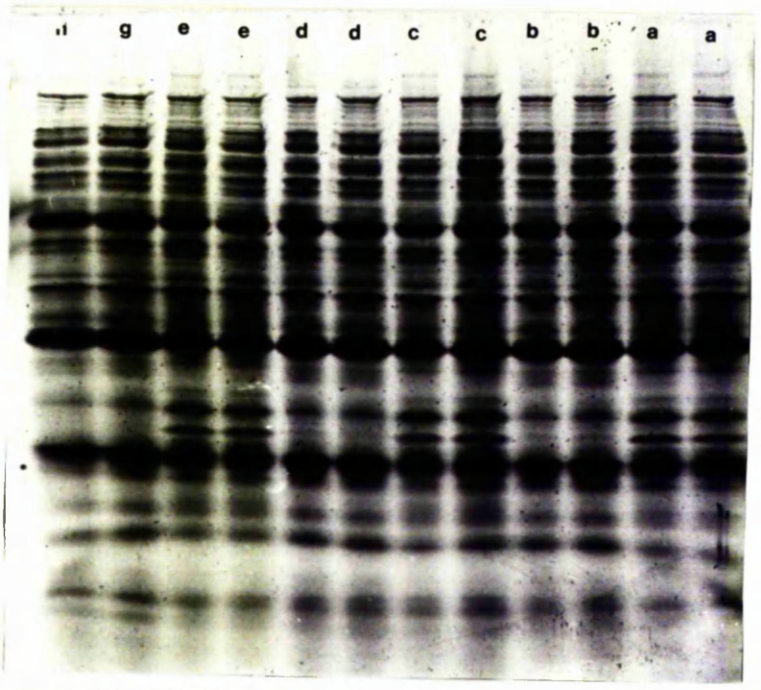
Lane	Additive and final concentration (mM)	
a	NaCl	43
b	$MgSO_4$	20
c	$MgCl_2$	43
d	Na_2SO_4	43
e	Na lactate	86
f	Na succinate	86
g	Na butyrate	20
h	Na caprylate*	0.6
i	Nicotinic acid*	4.1

*, these media also contained 43 mM NaCl.

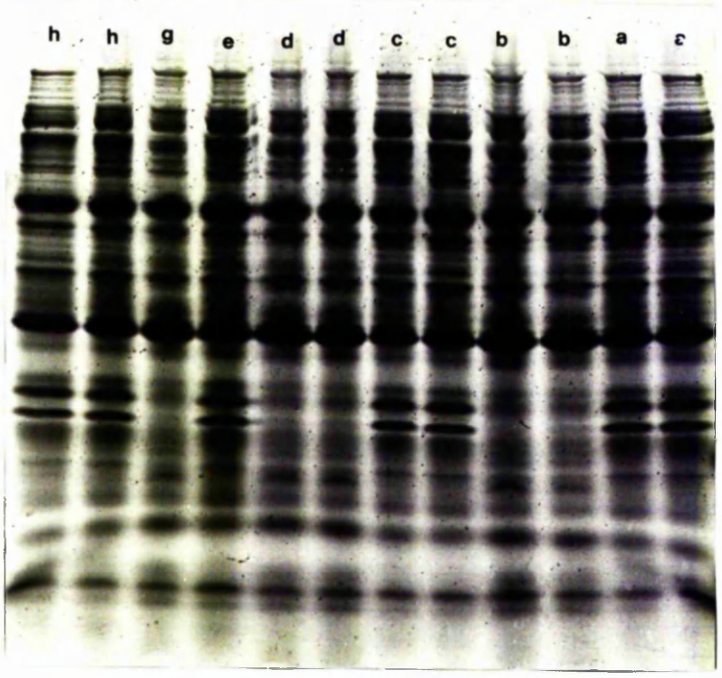
A



B



C



2. Location of Adenylate Cyclase Loss after Growth in C medium

Hewlett et al.(1976) demonstrated at least three compartments of AC activity in cultures of B. pertussis. About 20% was released into the medium, 70% was cell associated, but extracytoplasmic and sensitive to trypsin, and the remaining 10% was insensitive to trypsin. It was therefore of interest to determine which compartments of AC activity were lost during modulation. This would determine if the observed loss of intact-cell AC activity during C modulation was due to the inhibition of translocation of AC to the cell envelope.

Strain Taberman was grown for 48 h in SS-X or SS-C and culture supernates were assayed for AC activity. Cell pellets were resuspended to 20 mg(protein) ml^{-1} and divided into three portions. One portion was treated with trypsin (40 μg ml^{-1}) for 10 min at 37°C, the reaction was terminated by addition of lima bean trypsin inhibitor (40 μg ml^{-1}), and the sample was X-pressed. The second portion was X-pressed and then given the same trypsin treatment. The third portion was X-pressed, not being given any treatment. All samples were assayed for AC activity in duplicate (Table 12). These results show that AC activity is compartmentalized in cultures of both X- and C-mode B. pertussis. The distribution of AC activity compares reasonably with that determined by Hewlett et al. (1976). However, the results presented here suggest a larger portion of AC activity in the supernate. All compartments of AC activity were uniformly lost during growth in SS-C (Table 13).

Table 12: Distribution of AC activity in *B. pertussis* cultures after growth in SS-X and SS-C

Strain Taberman was grown for 48 h in SS-X and SS-C. Fractions of cultures were assayed for AC activity. Results refer to total AC activity per culture

Growth medium	Fraction of culture and treatment	Total AC activity[a]	% of AC activity of culture
SS-X	Supernate	221	46
	Intact cells	157	33
	X-pressed cells	261	54
	Trypsin-treated cells followed by X-pressing	40	8
	Trypsin-treated cell lysate	< 0.2[b]	
SS-C	Supernate	17	65
	Intact cells	4.6	18
	X-pressed cells	8.9	34
	Trypsin-treated cells followed by X-pressing	1.3	5
	Trypsin-treated cell lysate	< 0.2	

a, nmol cAMP min^{-1}; b, limit of detection.

Table 13: The effect of $MgSO_4$-induced modulation on AC activity in various compartments of *B. pertussis* cultures

Location	AC activity[a]		
	X-mode	C-mode	% relative to X-mode
Culture supernate	221	17	7.6
Cell-associated extracytoplasmic[b]	221	7.6	3.4
Cytoplasmic[b]	40	1.3	3.3

a, nmol cAMP $min^{-1}L^{-1}$; b, extracytoplasmic activity is defined as that portion of total cell-associated activity which is sensitive to trypsin treatment prior to X-pressing, while cytoplasmic activity is that portion which is only sensitive to trypsin treatment after X-pressing cells.

3. Studies on the Rate of $MgSO_4$-Induced Modulation

The loss with time of AC activity, HSA and the X bands, were investigated during $MgSO_4$-induced modulation. The purpose was to determine if these properties merely diminished in accordance with the growth rate during modulation. If so, this would indicate that loss of these properties could be accounted for by a complete cessation in the synthesis of the components responsible (ie. AC, pertussigen, and the X polypeptides) when X-mode cells were transferred to SS-C. A slower rate of loss would suggest partial repression of synthesis, whereas, a rapid loss would suggest that inactivation or destructive mechanisms were involved. If AC is a causal factor in modulation, one might expect loss of AC activity to precede loss of other activities.

Strain Taberman was grown in 1 L of SS-X for 48 h. This was used to inoculate SS-C (200 ml of inoculum plus 800 ml of 1.25 x normal strength SS-C). A heavy inoculum was required so that sufficient cells in 100 ml could be removed at early stages of growth and analysed for AC activity, HSA and by SDS-PAGE. SS-X (1.25 x normal strength) was also inoculated (20% (v/v)) as a control. Media were warmed to $35^{o}C$ before inoculation to minimise any lag phase.

3.1. Growth curves

Figures 2 and 3 show growth curves during the X to X and X to C transitions. Biomass was calculated from optical density using a convertion factor derived from a standard curve (Appendix 2, Fig. A1). Growth rates appeared to be consistently greater in SS-C than in SS-X (Table 14). It was a general observation throughout this work that SS-C gave more turbid growth and larger pellets than SS-X.

3.2. Adenylate cyclase activity

During the X to C transition, loss of intact-cell AC activity occurred immediately and continued to decline exponentially thereafter

Figure 2: Changes in biomass during growth of strain Taberman in SS-X and SS-C

SS-X and SS-C (1.25 x normal strength) received a 20% (v/v) inoculum from a 48 h SS-X culture. Biomass was calculated using a convertion factor relating A_{660nm} to dry weight. Biomass was determined for three experiments (○,△,□) during growth in SS-X (A) and SS-C (B).

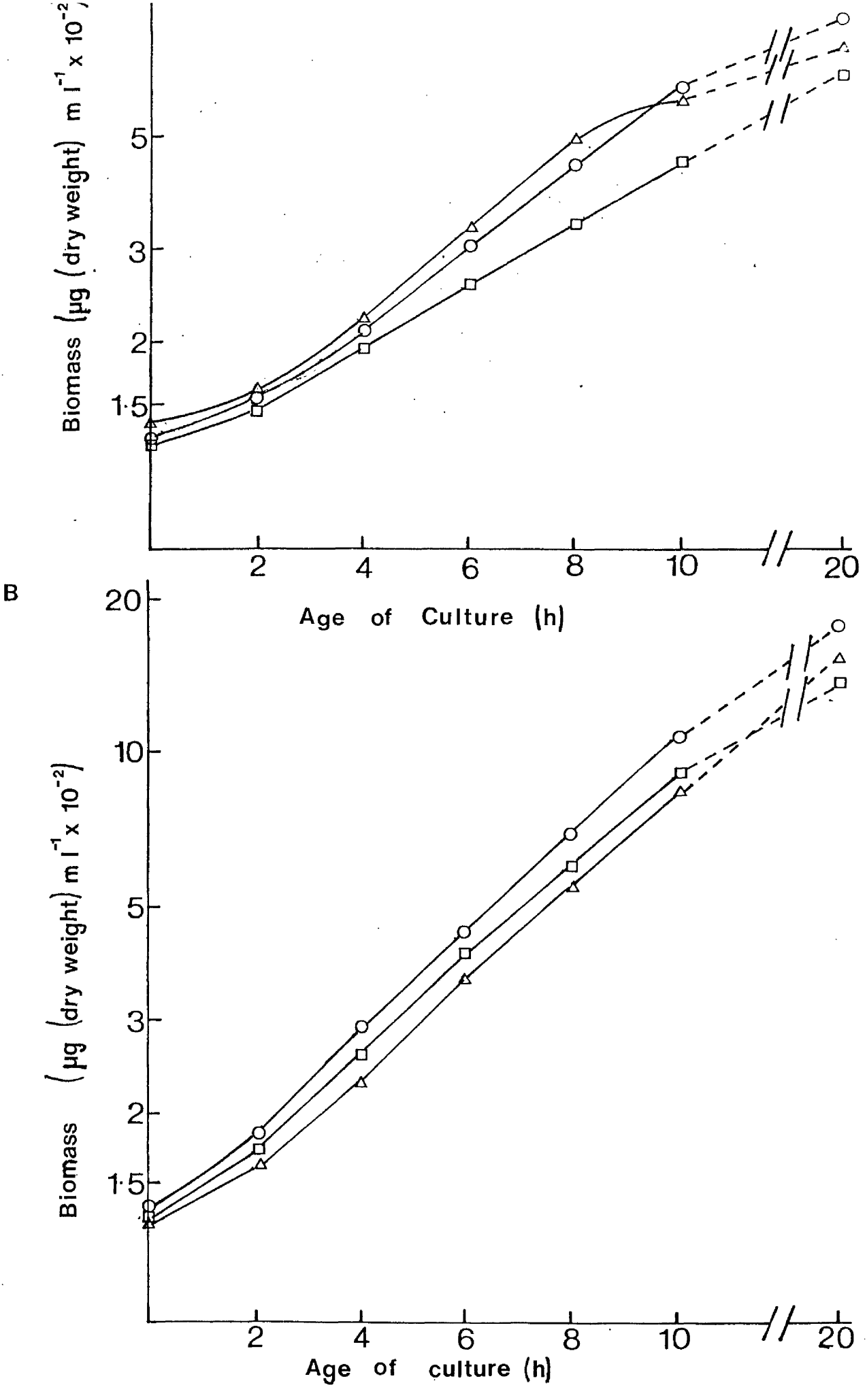

B
Biomass (µg (dry weight) m l^{-1} x 10^{-2})
5
3
2
1·5
2
4
6
8
10
20
Age of Culture (h)
Biomass (µg (dry weight) m l^{-1} x 10^{-2})
20
10
5
3
2
1·5
2
4
6
8
10
20
Age of culture (h)

Figure 3: Changes in viable numbers during growth of strain Taberman in SS-X and SS-C

SS-X and SS-C (1.25 x normal strength) received a 20% (v/v) inoculum from a 48 h SS-X culture. At timed intervals viable counts were determined using BG plates. Viable counts were determined for two experiments (○,□) during growth in SS-X (A) and in SS-C (B). Bars refer to 95% confidence limits.

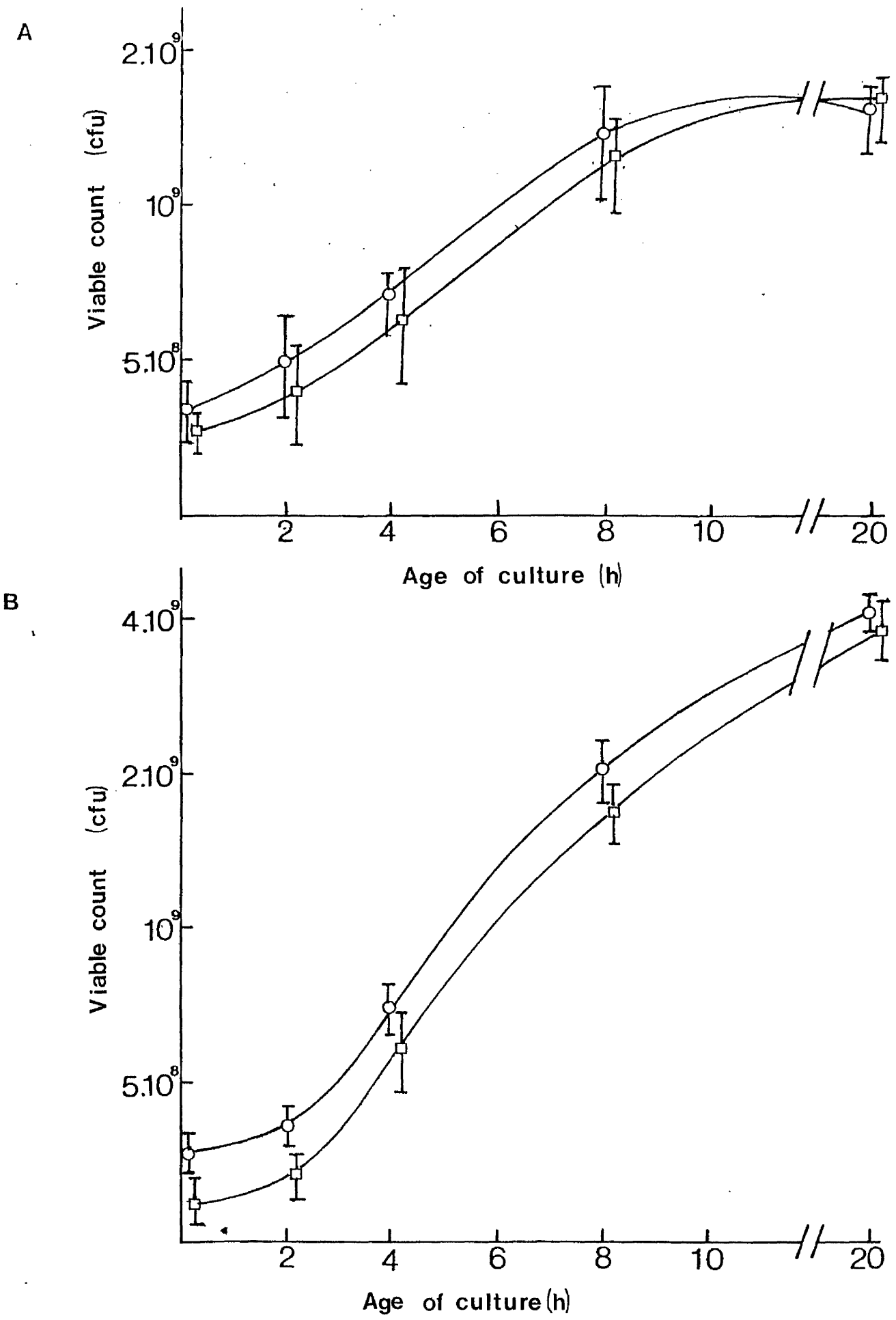

A
2.10⁹
10⁹
5.10⁸
Viable count (cfu)
2
4
6
8
10
20
Age of culture (h)
B
4.10⁹
2.10⁹
10⁹
5.10⁸
Viable count (cfu)
2
4
6
8
10
20
Age of culture (h)

Table 14: Maximum growth rates during growth of strain Taberman in SS-X and SS-C

A 20% (v/v) inoculum from a 48 h SS-X culture was given. Results are derived from Fig. 2 and 3.

Medium	Experiment	Doubling time (h)	
		using optical density	Using viable counts
SS-X	1	3.7	3.9
	2	3.5	3.7
	3	5.0	...
SS-C	1	3.1	2.4
	2	3.2	2.6
	3	3.2	...

... not done

(Fig. 4). Intact-cell activity did not alter greatly during growth in SS-X.

Culture supernate cAMP levels increased gradually during growth in SS-X but appeared to decline slightly during growth in SS-C (Fig. 5) suggesting that SS-C may inhibit cAMP synthesis or activate its destruction.

3.3. Histamine-sensitizing activity

It is difficult to assess the rate of loss of HSA due to the large 95% confidence limits of the HSA results (Fig.6). Nevertheless, these results comply with a gradual loss of HSA during the course of C modulation. Data,from which results were calculated, are given in Appendix 6, Table A4. HSA during growth in SS-X was not tested as this has been previously shown not to alter greatly (Idigbe *et al.*, 1981).

3.4. SDS-PAGE of whole cells

During C modulation, gradual loss of several polypeptides occurred (Plate 2, Fig. 7). A gradual loss of the X bands was observed while the major 37k band remained relatively constant. Other changes were also observed, notably the loss of at least two close bands in the region of 100k. These probably correspond to the 100k band or the 98k and 88k bands reported by other workers to be lost during C modulation of *B. pertussis* (Idigbe, 1979; Dobrogosz *et al.*, 1979). Another minor polypeptide, with a molecular weight of about 20k, may have been derepressed during growth in SS-C. During C modulation, the relative intensity of the X bands declined exponentially when plotted against time (Fig. 8), whereas little changes occurred during growth in SS-X.

Figure 4: Intact-cell AC activity during growth of strain Taberman in SS-X and SS-C

SS-X and SS-C (1.25 x normal strength) received a 20% (v/v) inoculum from a 48 h SS-X culture. At timed intervals samples were removed and harvested intact cells were assayed for AC activity. ○, △, AC activity after growth in SS-X for two determinations; ●, ▲, AC activity after growth in SS-C for two determinations.

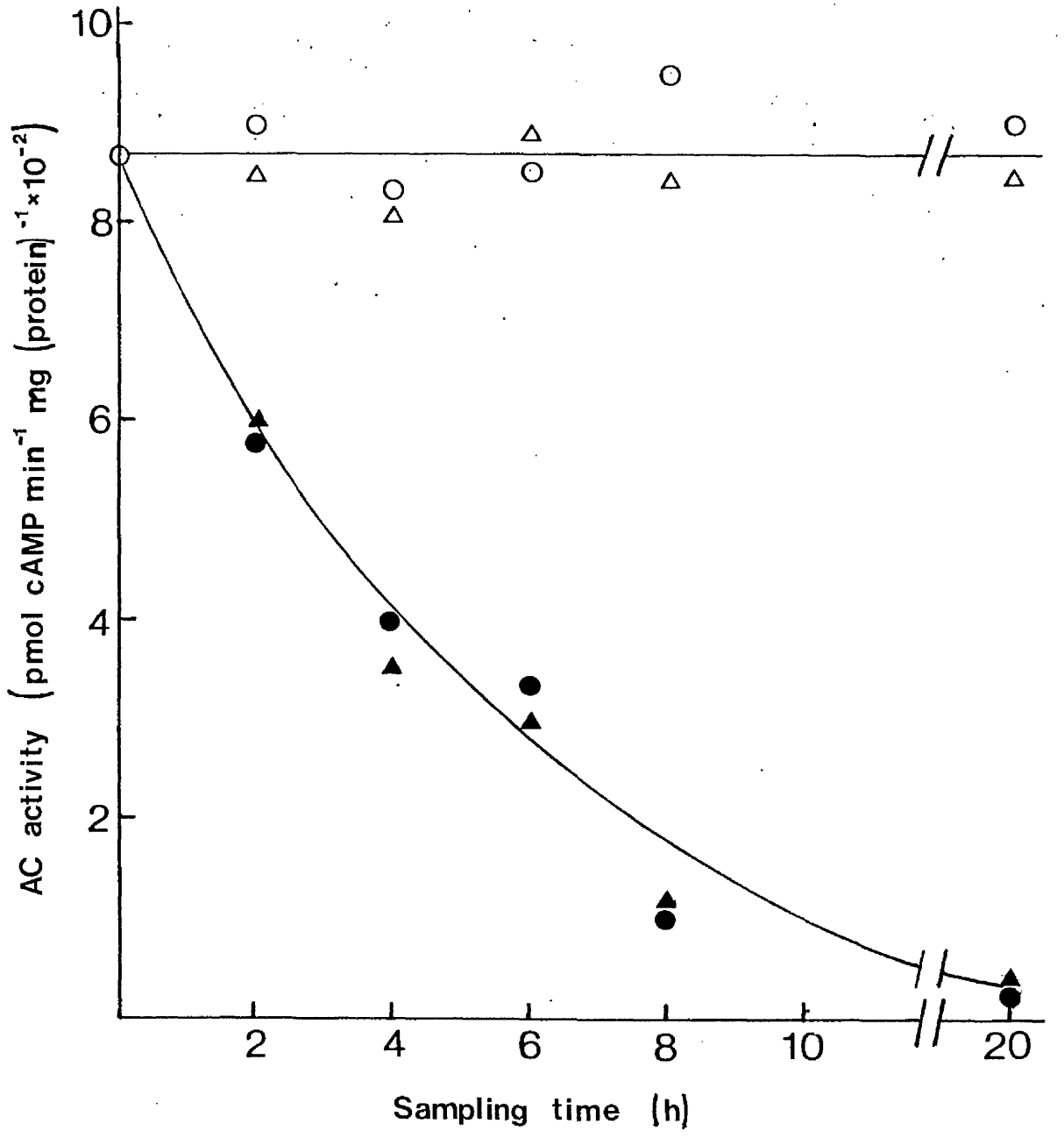

10
8
6
4
2
AC activity (pmol cAMP min^{-1} mg (protein)$^{-1}$ ×10^{-2})
2
4
6
8
10
20
Sampling time (h)

Figure 5: Culture supernate cAMP levels during growth of strain Taberman in SS-X and SS-C

SS-X and SS-C (1.25 x normal strength) received a 20% (v/v) inoculum from a 48 h SS-X culture. At timed intervals samples were removed and supernate was assayed for cAMP. ○, △, cAMP levels during growth in SS-X for two experiments; ●, ▲, cAMP levels during growth in SS-C for two experiments.

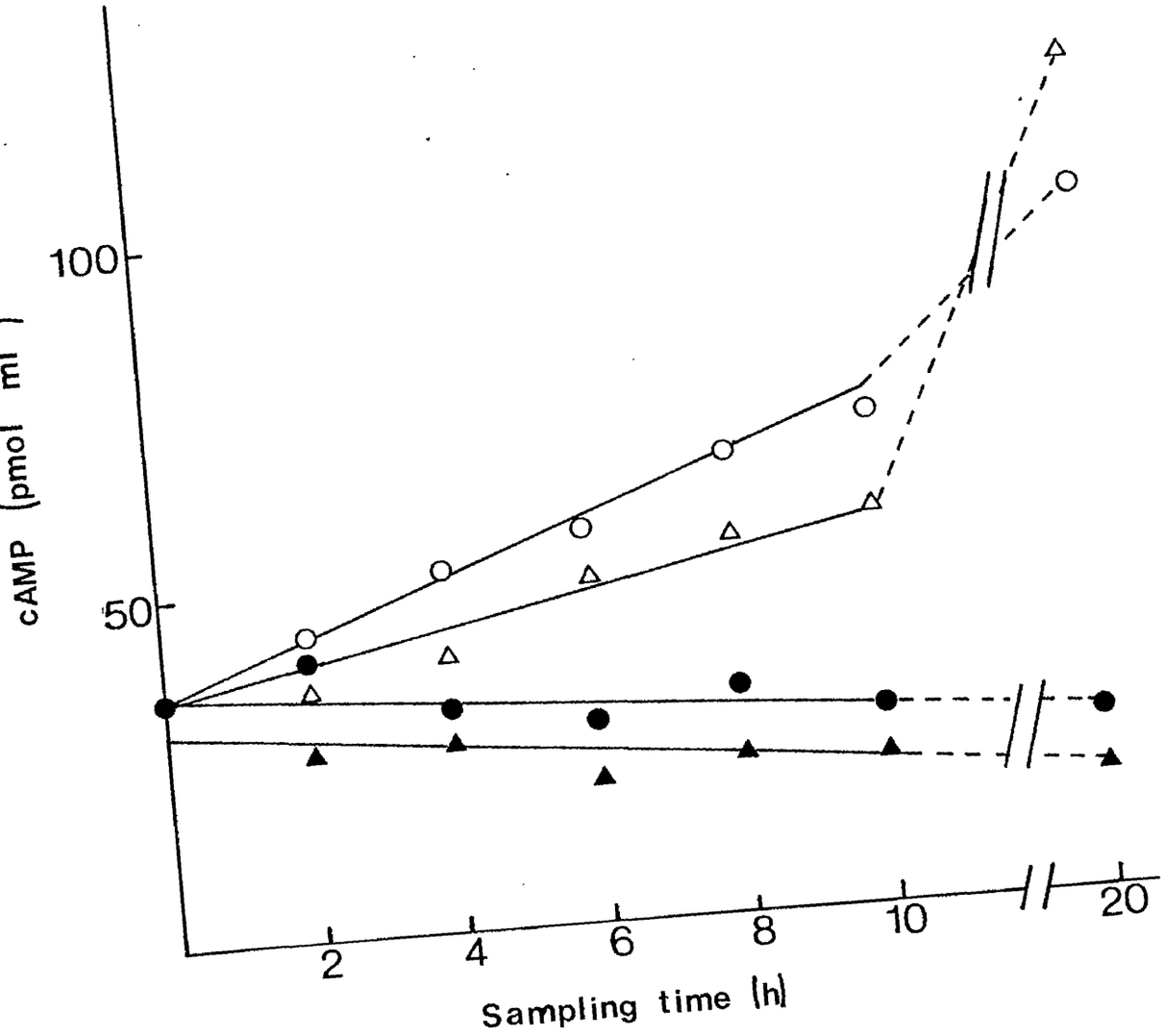

100
50
cAMP (pmol ml^{-1})
2
4
6
8
10
20
Sampling time (h)

Figure 6: Changes in relative potency of HSA of cells during growth of strain Taberman in SS-C

SS-C (1.25 x concentrated)received a 20% (v/v) inoculum from a 48 h SS-X culture. At timed intervals samples were removed and harveste cells (heat treated at 56°C for 30 min) were assayed for HSA. ○, △, HSA potencies relative to inoculum for two experiments; bars indic 95% confidence limits.

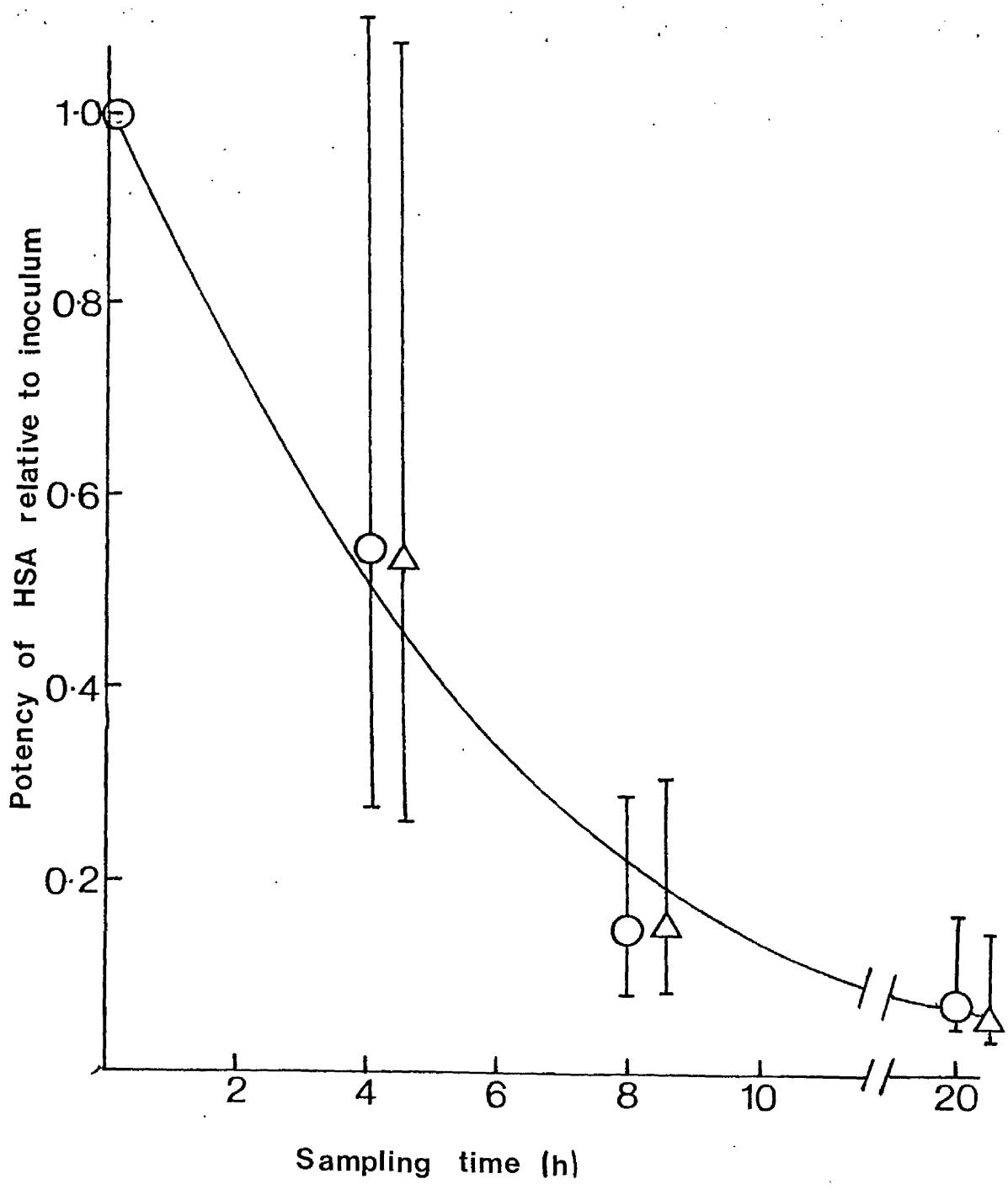

1·0
0·8
0·6
0·4
0·2
Potency of HSA relative to inoculum
2
4
6
8
10
20
Sampling time (h)

Plate 2: SDS-PAGE profiles of *B. pertussis* cells at various stages of C modulation.

A 48 h SS-X culture of strain Taberman was used to inoculate SS-C (20% (v/v) inoculum). At timed intervals cells were harvested, washed and analysed by SDS-PAGE. a, inoculum; b, 2 h; c, 4 h; d, 6 h; e, 8 h; f, 20

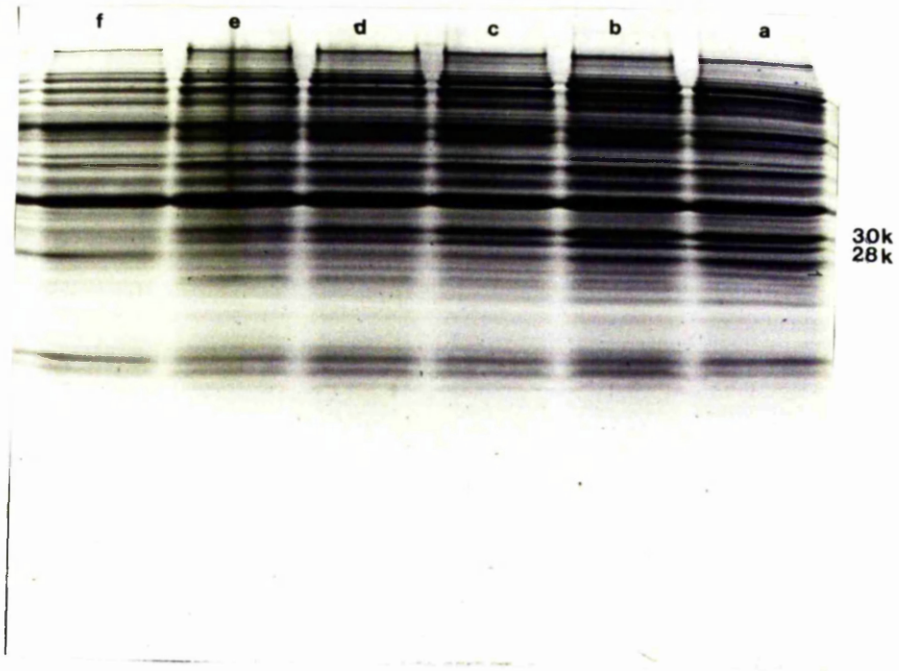

f
e
d
c
b
a
30k
28k

Figure 7: Densitometer traces of SDS-PAGE of whole cells of strain Taberman during C modulation

SS-C (1.25 x normal strength) received a 20% (v/v) inoculum from a 48 h SS-X culture. At timed intervals samples were removed and washed cells were analysed by SDS-PAGE. A, inoculum; B, 2 h; C, 4 h; D, 6 h; E, 8 h; F, 20 h.

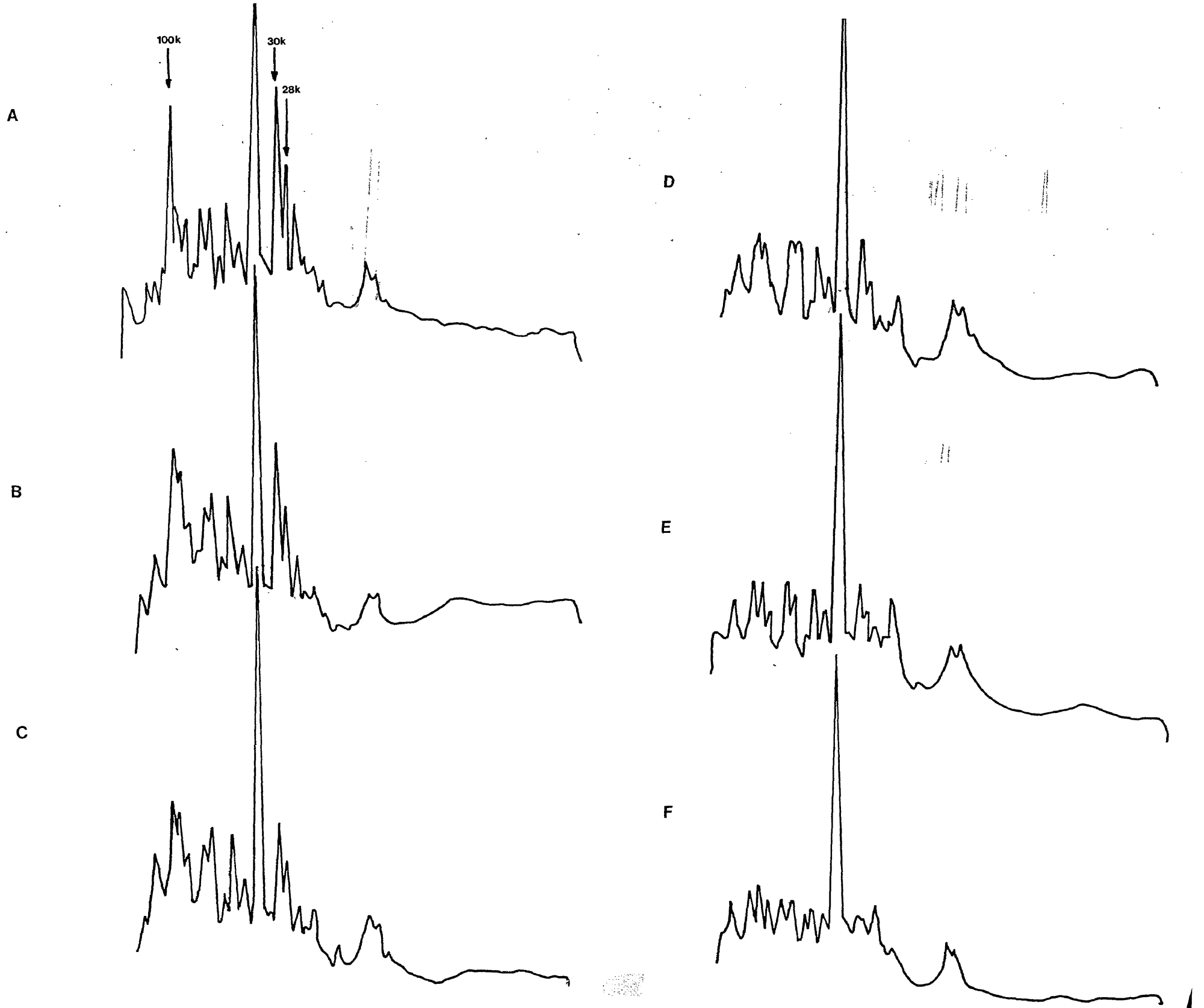
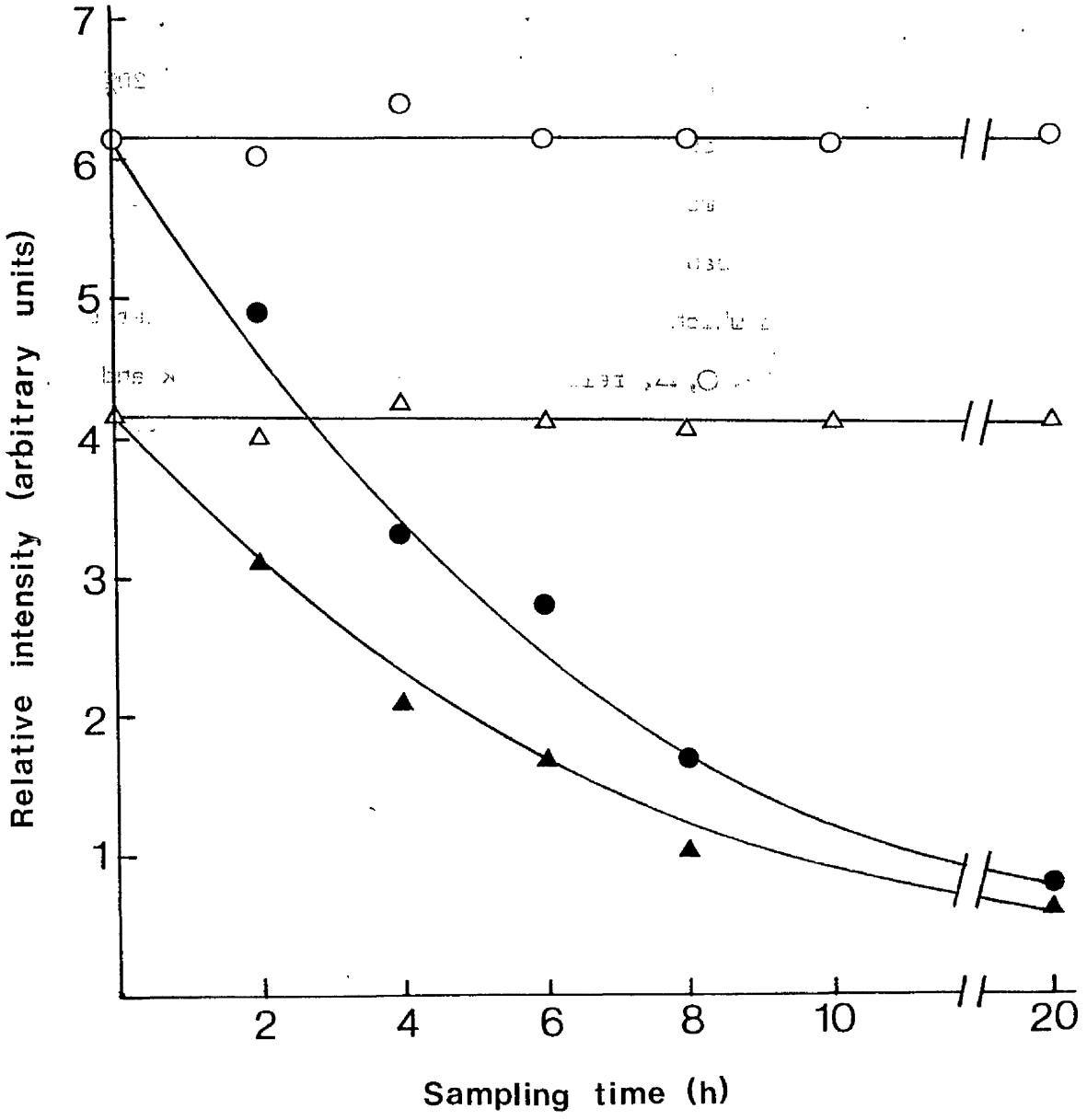

100k
30k
28k
A
B
C
D
E
F

Figure 8: Relative intensities of the X bands during growth of strain Taberman in SS-X and SS-C

SS-X and SS-C (1.25 x normal strength) received a 20% (v/v) inoculum from a 48 h SS-X culture. Samples were taken at various time intervals and washed cells were analysed by SDS-PAGE. The intensities of the 30k and 28k gel bands were standardized against the intensity of the major 37k band which remained relatively constant whether cells were grown in SS-X or SS-C. ○, △, relative intensities of the 30k and 28k gel bands during growth in SS-X; ●, ▲, relative intensities of the 30k and 28k gel bands during growth in SS-C.


Relative intensity (arbitrary units)
7
6
5
4
3
2
1
2
4
6
8
10
20
Sampling time (h)

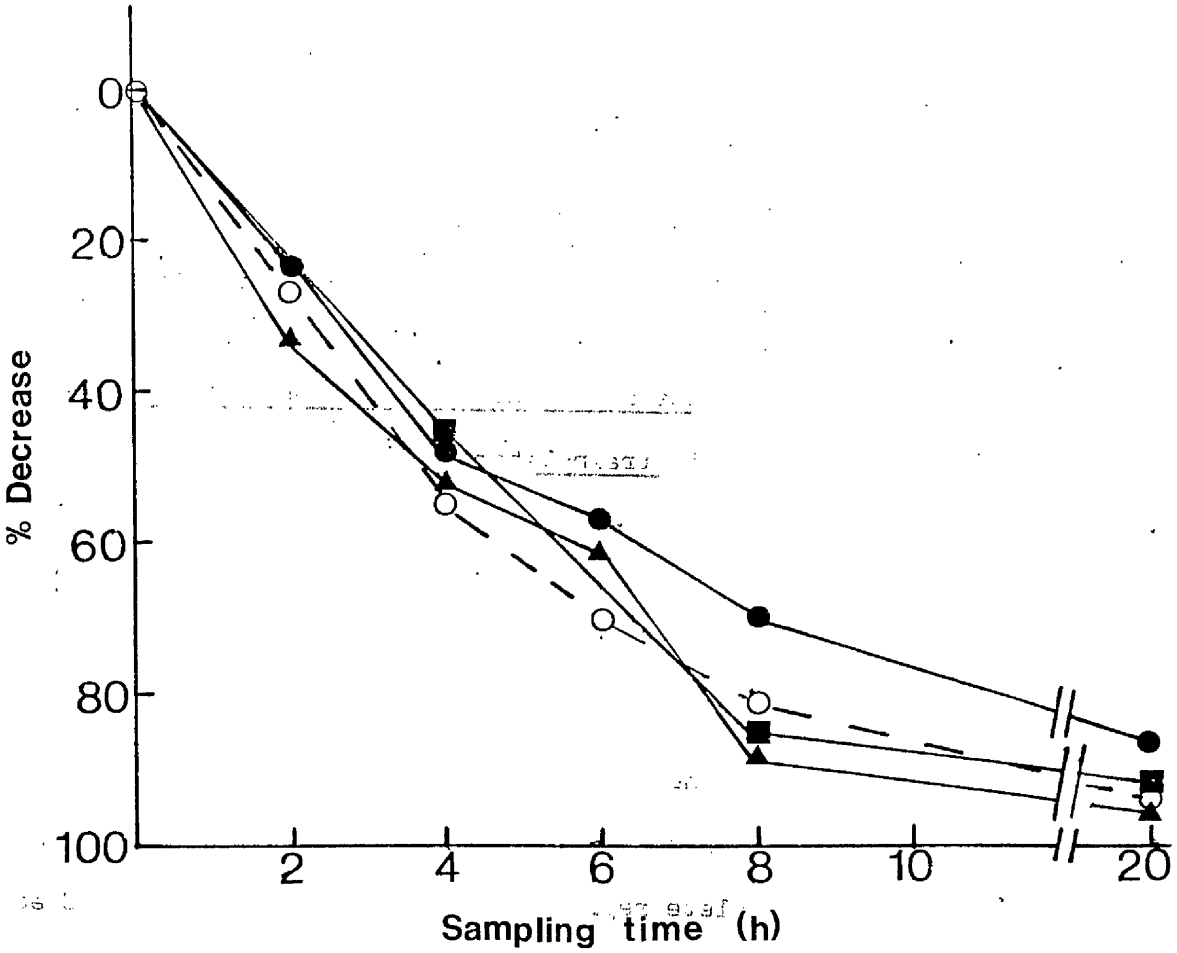
3.5. Expected loss of activities

Fig. 9 shows the percentage loss of intact-cell AC activity, HSA, and the combined intensities of the X bands, which occurred during C modulation. It also contains plots of the expected loss of these properties assuming no de novo synthesis and no inactivation or destruction. These plots were calculated using the growth curves shown in Fig. 2. These results suggest that the loss of AC activity, HSA and the X bands occurs synchronously and that the loss can be accounted for by 100% repression of synthesis of AC, pertussigen and the X polypeptides upon transfer of cells to SS-C.

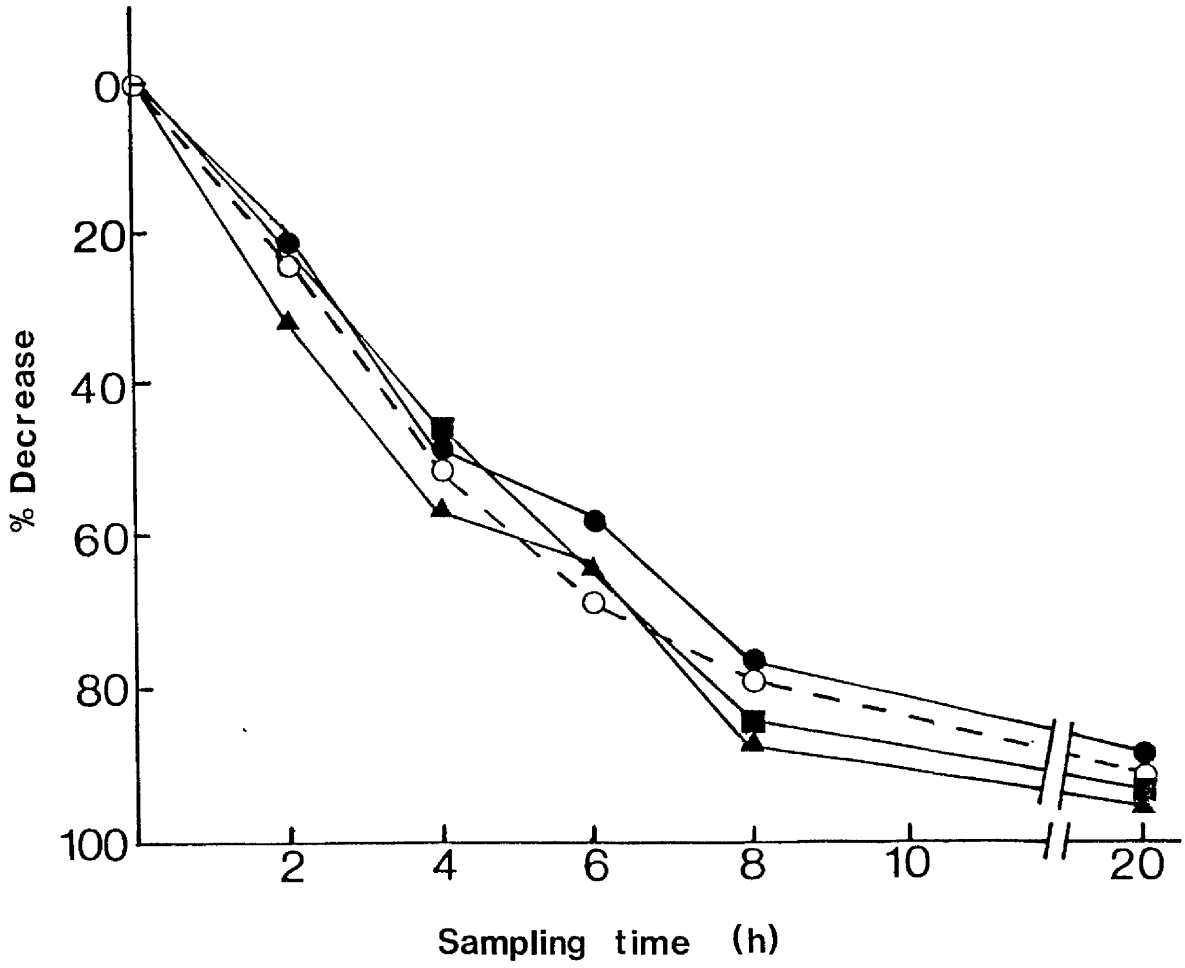
Figure 9: Loss of AC activity, HSA and the X bands during C modulatio of strain Taberman

SS-C (1.25 x normal strength) received a 20% (v/v) inoculum from a 48 h culture. At timed intervals, harvested cells were assayed for AC activity, HSA and analysed by SDS-PAGE. The theoretical loss assuming no de novo synthesis was calculated using the growth curves shown in Fig. 2. A, experiment 1; B, experiment 2; ○- -○, theoretical loss assuming complete repression; ▲——▲, intact-cell AC activity; ●——●, combined relative intensities of the X bands; ■—■, HSA.

A


0
20
40
60
80
100
% Decrease
2
4
6
8
10
20
Sampling time (h)

B


0
20
40
60
80
100
% Decrease
2
4
6
8
10
20
Sampling time (h)

4. A Study of Possible Inhibition Mechanisms Involved in Modulation

4.1. The effect of pro-X- and pro-C-mode compounds on adenylate cyclase activity

If cAMP plays a major role in modulation, it is possible that pro-C-mode substances alter cAMP metabolism by directly inhibiting AC activity. It was therefore of interest to determine whether these substances could inhibit AC activity when present in the assay mixture. The effect of nicotinamide was also investigated as, unlike nicotinic acid, this compound does not induce modulation at 500 mg L^{-1} (4.1 mM) (Wardlaw et al., 1976). Intact cells as well as cell lysates were used as enzyme sources, as the cell surface is known to have an affinity for some ionic species, notably divalent cations. Results for one experiment, samples assayed in duplicate, are shown in Table 15. Both nicotinamide and nicotinic acid caused substantial loss of activity (about 60%). However, the other pro-C-mode substances caused no greater loss of activity than did NaCl.

4.2. The effect of chloramphenicol on modulation

Three possible explanations for the loss of AC activity during growth in SS-C are:- (1) AC is permanently inactivated by a direct, prolonge exposure to the medium constituents of SS-C, (2) AC is inactivated or destroyed by some factor synthesized or activated during modulation, (3) AC synthesis is repressed. To test the first possibility, B. pertussis was incubated in SS-X and SS-C with and without chloramphenicol added. Chloramphenicol was present at a final concentration of 100 μg ml^{-1}. This concentration completely inhibited growth and incorporation of tritiated methionine into acid-insoluble material (results not shown). Flasks received a 10% (v/v) inoculum of a 48 h culture of strain Taberman grown in SS-X. Flasks were incubated as for normal growth conditions and harvestec after 24 h and 48 h.

Intact-cell AC activities are shown in Table 16. Results refer to the averages of duplicate cultures, each of which was assayed in duplicate.

Table 15: The effect of salts and organic acids on AC activity of strain Taberman

Strain Taberman was grown for 48 h in SS-X. Harvested intact cells and X-pressed cells were assayed for AC activity with various additives present in the assay mixture.

Addition to assay mixture and final concentration (mM)		Intact-cell AC activity[a]	% relative to standard[b]	Cell-lysate AC activity[a]	% relative to standard[b]
Tricine buffer alone		960	100	1540	100
NaCl	43	755	79	1225	80
$MgSO_4$	20	995	104	1630	106
Na_2SO_4	43	695	72	1165	76
Na lactate	86	610	64	1185	77
Na succinate	86	675	70	1155	75
Na butyrate	20	825	86	1530	99
Na caprylate	0.6	935	97	1585	103
Nicotinic acid	4.1	360	38	605	39
Nicotinamide	4.1	340	35	610	40

a, pmol cAMP $min^{-1} mg(protein)^{-1}$; b, standard refers to activity in tricine buffer alone.

Table 16: The effect of chloramphenicol on $MgSO_4$-induced loss of AC activity

A 48 h culture of strain Taberman was used to inoculate media (10% (v/v) inoculum). After 24 h and 48 h incubation, culture supernate was assayed for cAMP and harvested intact-cells were assayed for AC activity.

Medium	24 h incubation		48 h incubation	
	AC activity[a]	cAMP level[b]	AC activity	cAMP level
SS-X	945	120	990	165
$SS\text{-}X_{CAP}$	760	25	660	35
SS-C	25	15	15	15
$SS\text{-}C_{CAP}$	690	15	625	15

a, pmol cAMP $min^{-1}mg(protein)^{-1}$; b, pmol cAMP ml^{-1}.

Prolonged exposure to SS-C_{CAP} (SS-C with chloramphenicol (100 μg ml^{-1})) resulted in 20 - 40% loss of activity and was similar to the loss observed by exposure to SS-X_{CAP}. Growth in SS-C resulted in substantial loss of activity (95 - 99% loss). Cyclic AMP levels did not increase greatly during non-growing conditions or during growth in SS-C although levels appeared to be slightly higher during exposure to SS-X_{CAP}.

HSA for selected samples are shown in Table 17 (further data, from which results were calculated, are shown in Appendix 6, Table A5). Prolonged exposure to SS-X_{CAP} or SS-C_{CAP} caused no significant loss of HSA compared to growth in SS-C where HSA was reduced by about 94%. Similarly, $MgSO_4$-induced loss of the X-bands was inhibited by chloramphenicol indicating that protein synthesis or growth was required for this effect (Plate 3).

The effect of chloramphenicol on C to X modulation was also investigated Expression of AC activity, HSA and the X bands during these conditions required growth or protein synthesis (Table 18, 19 and Plate 4). Further data, from which HSA results were calculated, are given in Appendix 6 Table A6. Culture supernate cAMP was detectable only after growth in SS-X.

4.3. The effect of $MgSO_4$ on the production of extracellular cAMP

Certain experiments suggested that SS-C may inhibit production of cAMP by an X-mode inoculum. When SS-C was inoculated with a heavy inoculum of X-mode B. pertussis, there appeared to be no net gain of cAMP in the culture supernate (Fig. 5) although active AC exists in the inoculum initially. Also, after growth of B. pertussis in SS-C containing AC activator, using an X-mode inoculum, there were still no detectable levels of cAMP in the supernate (Section 6.2). To test whether SS-C did inhibit production of culture supernate cAMP, the following experiment was carried out. A 48 h SS-X culture of strain Taberman was harvested and cells were washed once in saline. Cells were resuspended in SS-X_{CAP}

Table 17: The effect of chloramphenicol on $MgSO_4$-induced loss of HSA

A 48 h culture of strain Taberman was used to inoculate media. After 24 h and 48 h incubation, harvested cells were heat killed (56°C for 30 min) and assayed for HSA.

Medium and time of incubation (h)		HSD_{50}	% relative potency[b] (and 95% confidence limits)
SS-X	48	1.7	100
$SS\text{-}X_{CAP}$	48	1.9	93 (50, 176)
SS-C	48	29.1	6 (2, 13)
$SS\text{-}C_{CAP}$	24	2.2	79 (47, 149)
$SS\text{-}C_{CAP}$	48	1.7	100 (53, 189)

a, the dose (ou.ml per mouse) required to kill 50% of the mice, after subsequent challenge with histamine; b, relative to activity of cells after growth in SS-X.

Plate 3: SDS-PAGE profiles of *B. pertussis* cells after incubation in X and C medium with and without chloramphenicol

A 48 h SS-X culture of strain Taberman was used to incoulate media (10% (v/v) inoculum). After 48 h incubation, harvested and washed cells were analysed by SDS-PAGE. Media were: a, SS-X; b, $SS\text{-}X_{CAP}$; c, SS-C; d, $SS\text{-}C_{CAP}$

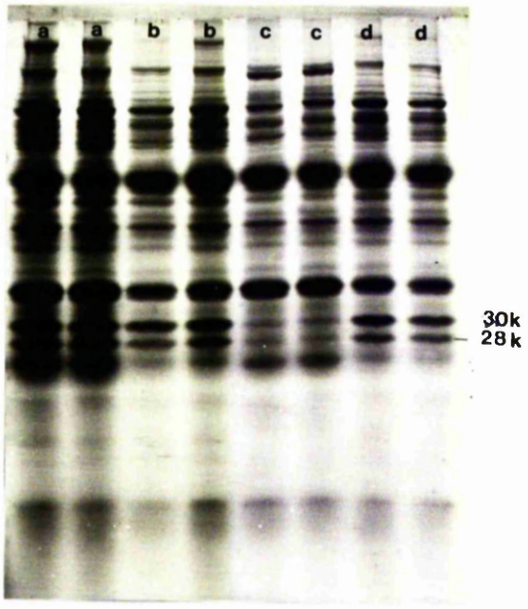

a
a
b
b
c
c
d
d
30k
28k

Table 18: The effect of chloramphenicol on the regain of AC activity during C to X modulation

A 48 h SS-C culture of strain Taberman was used to inoculate media. After 24 h and 48 h incubation, harvested intact cells were assayed for AC activity and culture supernate was assayed for cAMP.

Medium	24 h incubation		48 h incubation	
	AC activity[a]	cAMP level[b]	AC activity	cAMP level
SS-X	885	55	915	115
$SS\text{-}X_{CAP}$	15	< 5	10	< 5
SS-C	< 5[c]	< 5	< 5	< 5
$SS\text{-}C_{CAP}$	15	< 5	15	< 5

a, pmol cAMP $min^{-1} mg(protein)^{-1}$; b, pmol cAMP ml^{-1}; c, limit of detection.

Table 19: The effect of chloramphenicol on the regain of HSA during C to X modulation

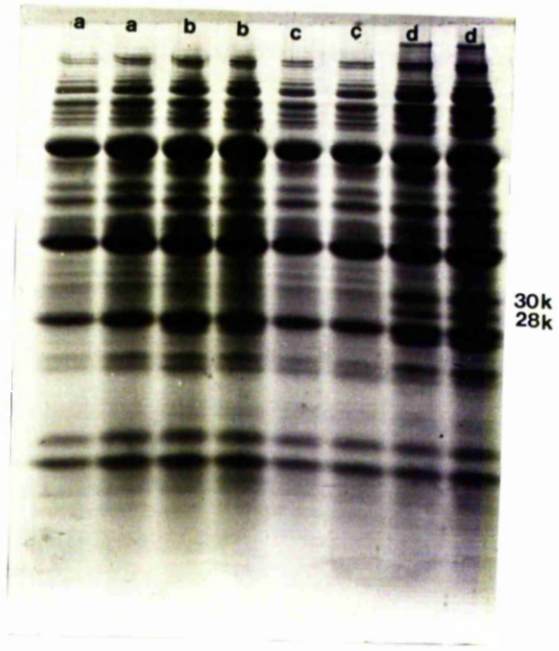
A 48h SS-C culture of strain Taberman was used to inoculate media. After 48 h incubation, harvested cells were heat killed (56°C for 30 min) and assayed for HSA.

Medium	HSD_{50} [a]	% relative potency [b] (and 95% confidence limits)
SS-X	0.8	100
$SS\text{-}X_{CAP}$	43.1	1.9 (0.0, 6.9)
SS-C	> 20 [c]	< 4.2
$SS\text{-}C_{CAP}$	66.0	1.3 (0.0, 5.5)

a, the dose (ou.ml per mouse) required to kill 50% of the mice after subsequent challenge with histamine; b, relative to activity of cells after growth in SS-X; c, no animals died using 20 ou.ml per mouse as highest dose.

Plate 4: SDS-PAGE profiles of *B. pertussis* cells after incubation of C-mode cells in X and C medium with and without chloramphenicol

A 48 h SS-C culture of strain Taberman was used to inoculate media (10% (v/v)inoculum). After 48 h incubation, washed cells were analysed by SDS-PAGE. Media were: a, SS-C_{CAP}; b, SS-C; c, SS-X_{CAP}; d, SS-X.


a
a
b
b
c
c
d
d
30k
28k

and SS-C_{CAP} to give an A_{660nm} of 25. Suspensions (10 ml) were incubated in 150 ml flasks on an orbital shaker at 35°C. At timed intervals 1 ml was removed and supernate was assayed for cAMP. One series of cell suspensions contained AC activator (100 µg bovine catalase ml^{-1}). Results (Fig. 10) were similar for two experiments. Production of extracellular cAMP was the same for cells incubated in SS-X or in SS-C. These results indicate that SS-C does not inhibit production of extracellular cAMP.

4.4. Possible inactivating factors in C-mode cell lysates

The possibility that a factor, which inactivated AC, was produced during growth in SS-C was investigated by mixing X-mode and C-mode cell lysates of strain Taberman at 35°C and 4°C and assaying for AC activity at timed intervals. Strain Taberman was grown in SS-X and SS-C for 48 h, harvested, and resuspended to 2.5 mg(protein) ml^{-1} prior to X- pressing and mixing. X- and C-mode cell lysates were mixed in equal volumes and incubated statically. Chloramphenicol was added (100 µg ml^{-1} final concentration) to prevent possible contamination. Results (Table 20) show that no inactivation appeared to occur when X-mode cell lysate was incubated with C-mode cell lysate. Similar results were obtained for strain 18334 (results not shown).

Idigbe et al.(1981) found HSA was lost at a greater rate than could be accounted for by repression of synthesis during C modulation of strain 18334 in Hornibrook medium. This suggested destructive or inactivation mechanisms were involved. C-mode cell lysates were therefore investigated to see if they contained destructive or inactivation factors for X-mode HSA. Strain Taberman was grown for 48 h in SS-X and SS-C. Harvested cells were resuspended to 100 ou in culture supernate (in case destructive factors were released into the medium) and X-pressed. X- and C- mode cell lysates were mixed in equal volumes and incubated for

Figure 10: The effect of $MgSO_4$ and AC activator on the production of extracellular cAMP by resuspended X-mode cells

Harvested cells of strain Taberman from a 48 h SS-X culture were washed once and resuspended in media containing chloramphenicol (100 μg ml^{-1}) to give an A_{660nm} of 25. Suspensions (10 ml) were incubated in 150 ml flasks in an orbital shaker at 35°C. At timed intervals samples were removed and supernate was assayed for cAMP. Media were $SS\text{-}X_{CAP}$ (□), $SS\text{-}X_{CAP}$ with AC activator (100 μg ml^{-1} bovine catalase) (△), $SS\text{-}C_{CAP}$ (■), and $SS\text{-}C_{CAP}$ with AC activator (▲).

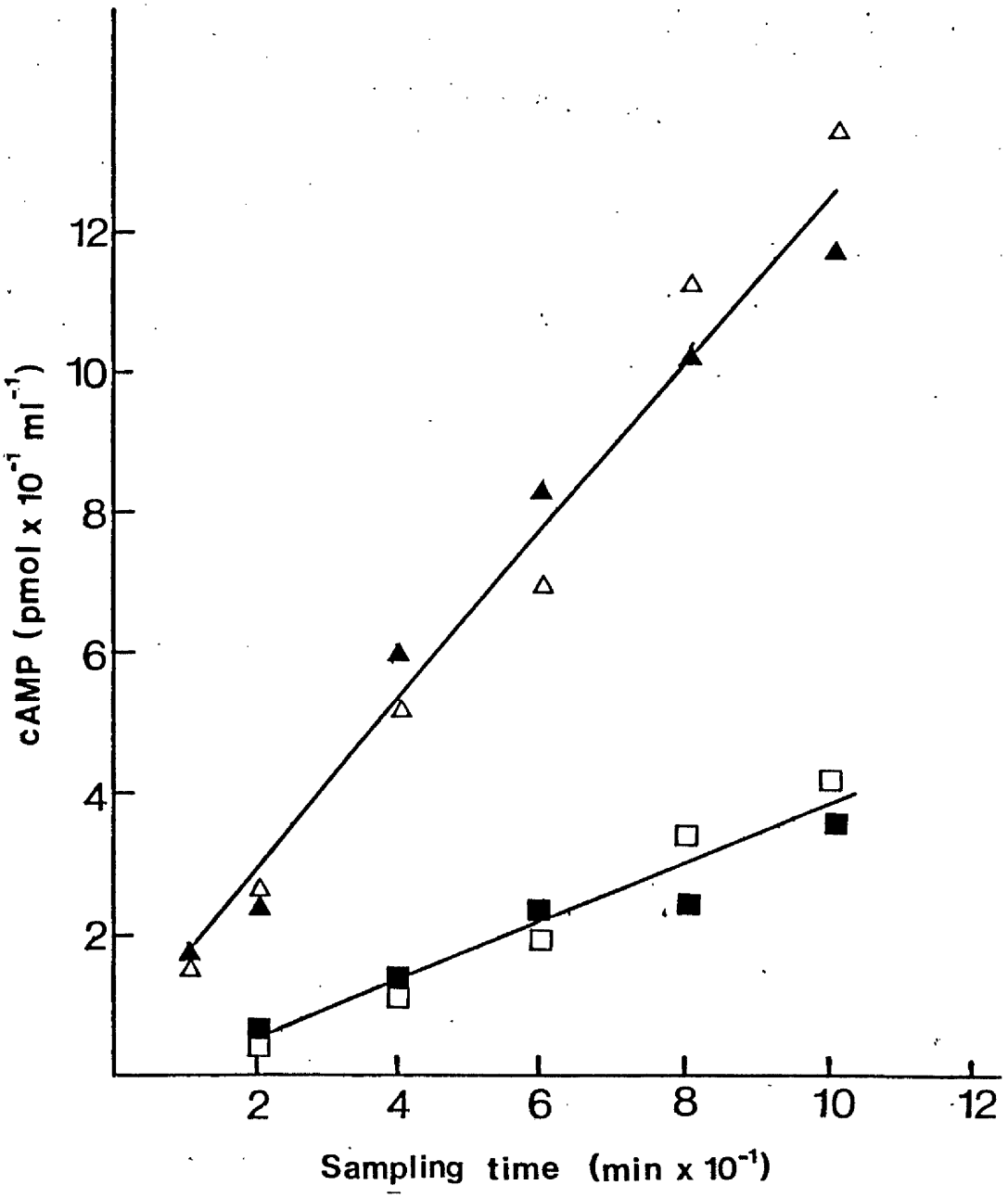

cAMP (pmol x 10⁻¹ ml⁻¹)
12
10
8
6
4
2
2
4
6
8
10
12
Sampling time (min x 10⁻¹)

Table 20: AC activity in X- and C-mode cell lysates amd mixtures of cell lysate

Strain Taberman was grown for 48 h in SS-X or SS-C. Harvested cells were X-pressed and lysate mixtures were assayed for AC activity after various incubation conditions.

Incubation conditions prior to AC assay	AC activity[a]			
	X-mode cell lysate	C-mode cell lysate	X/C-mode cell lysate[b]	Expected[c]
None	1390	55	815	720
24 h at 4°C	1350	70	740	710
0.5 h at 35°C	1280	50	630	665
4 h at 35°C	1055	40	555	555
24 h at 35°C	630	15	365	325

a, pmol cAMP $min^{-1}mg(protein)^{-1}$; b, X- and C-mode cell lysates mixed in equal volumes; c, expected activity of cell lysate mixture assuming no loss of activity by destructive factors.

24 h at 35°C with chloramphenicol (100 μg ml^{-1} final concentration). X- and C-mode cell lysates were also incubated alone. Samples were heat treated at 56°C for 30 min and assayed for HSA (Table 21). Further data, from which results were calculated, are given in Appendix 6, Table A7. No destructive factors were demonstrated in C-mode cell lysate, since the activity in the mixture was approximately half the activity of the X-mode cell lysate. However, small changes in activity would not have been detected in such an experiment because of the large confidence limits obtained. A similar experiment was done with strain 18334 grown for 48 h in X- and C-mode Hornibrook medium, as this was the strain and medium used by Idigbe et al. (1981). Results are shown in Table 22 and further data, from which results were calculated, are given in Appendix 6, Table A8. Again, no destructive factors were clearly evident in C- mode cell lysates as X-mode/C-mode cell-lysate mixtures had approximately half the activity of an X-mode cell lysate.

Table 21: The effect of incubating C-mode lysate with X-mode lysate on HSA of strain Taberman

Strain Taberman was grown in SS-X and SS-C for 48 h. Harvested cells were X-pressed. Cell lysates and lysate mixtures were incubated statically for 24 h at 35°C. Samples were heat treated (56°C for 30 min) and assayed for HSA.

Sample	HSD_{50} [a]	% relative potency[b] (and 95% confidence limits)
X-mode cell lysate	1.5	100
C-mode cell lysate	> 20[c]	< 7.5
X/C-mode cell lysate mixture[d]	4.0	37 (18, 75)
Expected[e]	3.0	50

a, the dose (ou.ml per mouse) required to kill 50% of the mice after subsequent challenge with histamine; b, relative to HSA of X-mode cell lysate; c, no animals died using 20 ou.ml per mouse as highest dose; d, X- and C-mode cell lysates were mixed in equal volumes before incubation at 35°C; e, expected HSD_{50} of the X/C-mode cell lysate mixture assuming no inactivation (ie. twice the HSD_{50} of the X-mode cell lysate).

Table 22: The effect of incubating C-mode lysate with X-mode lysate on HSA of strain 18334

Strain 18334 was grown in H-X and H-C for 48 h. Harvested cells were X-pressed. Cell lysates and lysate mixtures were incubated statically for 24 h at 35°C. Samples were heat treated (56°C for 30 min) and assayed for HSA

Sample (and incubation conditions)	HSD_{50}[a]	% relative potency[b] (and 95% confidence limits)
X-mode cell lysate (none)	1.1	100
X-mode cell lysate (24 h at 35°C)	1.4	74 (40, 136)
C-mode cell lysate (24 h at 35°C)	17.0	6.2 (3.1, 12)
X/C-mode cell lysate mixture[c] (24 h at 35°C), sample 1	1.6	68 (37, 125)
X/C-mode cell lysate mixture[c] (24 h at 35°C), sample 2	2.0	52 (28, 96)
Expected[d]	2.6	54

a, the dose (ou.ml per mouse) required to kill 50% of the mice after subsequent challenge with histamine; b, relative to HSA of X-mode cell lysate not previously incubated at 35°C; c, X- and C-mode cell lysates were mixed in equal volumes before incubation at 35°C; d, expected HSD_{50} of X/C-mode cell lysate mixture assuming no inactivation (calculated from HSD_{50}'s of X- and C-mode cell lysates incubated alone).

5. Purification of Adenylate Cyclase and the Effect of Antisera on Adenylate Cyclase Activity

Concomitant loss of AC activity with loss of the other properties during C modulation may be due to either a coincidental or causal relationship If AC is the target site of phase degradation or modulation, it would be expected that the loss of AC is due to mutation, in the case of phase degradation, or by some inactivating mechanism operating during C modulation. Attempts were made to demonstrate whether AC was synthesised to the same level in X-mode, C-mode and phase IV B. pertussis by immunological means. The first stage involved purification of AC.

Blue dextran-Sepharose (BDS) was used with considerable success in the purification of bovine brain AC (Stellwagen and Baker, 1976). BDS binds to many proteins possessing a dinucleotide fold. Bovine brain AC was purified by mixing brain homogenate with BDS and AC was eluted with 1 mM ATP. This gave 50% yield and 150-fold purification in one step. Reactive red-Agarose (RRA) also binds to proteins with dinucleotide folds which can be specifically eluted by nucleotides. It was thought BDS or RRA might be useful in the purification of B. pertussis AC since B. pertussis AC may be structurally similar to brain AC as both respond to calmodulin. A 48 h SS-X culture of strain 18334 was harvested and X-pressed in 10 mM Tris-HCl, pH 8.0. Supernate (100,000 x g for 60 min) was diluted in an equal volume of 10 mM Tris-HCl, pH 8.0, to give 4.2 mg(protein) ml^{-1}. Crude cytoplasmic fraction (3 ml) was added to 0.5 ml of packed swollen gel of BDS or RRA previously equilibrated with 10 mM Tris-HCl, pH 8.0, and mixed frequently on ice for 1 h. The tubes were centrifuged and supernates collected (unabsorbed fractions). The gels were washed three times with 8 ml of buffer, Buffer containing 10 mM ATP (3 ml) was mixed with the gel, and after 30 min incubation on ice, tubes were centrifuged and eluants were collected. AC activities for the different fractions are shown in Table 23. BDS and RRA appeared to

Table 23: Affinity chromatography of *B. pertussis* AC with Blue dextran-Sepharose and Reactive red-Agarose

Strain 18334 cytoplasmic fraction (12.6 mg protein) was added to 0.5 ml packed swollen gel of BDS or RRA. After 1 h incubation on ice, supernates were collected (unabsorbed fractions). Gels were washed three times. Buffer containing 10 mM ATP was added (3 ml) and supernate was collected after 30 min incubation on ice (ATP-eluted samples).

Sample	AC activity[a]
Cytoplasmic fraction	2.35
Supernate after absorption with BDS	2.35
Supernate after absorption with RRA	2.30
ATP elution from BDS	< 0.016[b]
ATP elution from RRA	< 0.016

a, nmol cAMP $min^{-1}ml^{-1}$; b, limit of detection.

have little affinity for B. pertussis AC under the conditions used. SDS-PAGE of samples showed that most polypeptides remained in the unabsorbed fractions and were not detectable in the ATP eluted samples (results not shown).

Subsequent purification was based on a method described by Hewlett and Wolff (1976) whose starting material was culture supernate. Culture supernate AC activity did not vary greatly between four strains (results not shown), although strain Taberman appeared to produce the highest levels and was chosen for purification of AC. Maximum levels of culture supernate AC activity occurred after 48 h growth in SS-X (results not shown). Strain Taberman was grown in 500 ml amounts of SS-X for 48 h. Culture supernate (5 L) was pooled and adjusted to pH 7.4. The supernate was pumped through a DEAE-column (2.5 x 25 cm) previously equilibrated with 10 mM Tris-HCl, pH 7.4, at approximately 90 ml h^{-1}. The column was washed with 1 L 10 mM Tris-HCl, pH 7.4, at the same flow rate. A 100 - 300 mM NaCl gradient was used to elute AC (flow rate 9 ml h^{-1}) and eluate was collected in 4.5 ml fractions. Fig. 11 shows a typical elution profile which was similar for two other experiments. DEAE-cellulose chromatography gave good separation of AC activity from the major protein peak. The main peak of AC activity was pooled for further purification (in this case fraction numbers 37 - 43) and concentrated to 5 ml by dialysis against Aquacide IIA. Sample was pumped through a G-200 Sephadex column (2.5 x 50 cm) previously equilibrated with 10 mM Tris-HCl, pH 7.4 (flow rate of 9 ml h^{-1}) and 4.5 ml fractions were collected. A broad peak of AC activity was observed for eight fractions, but no protein peaks were observed (results not shown). Fractions containing AC activity were concentrated by dialysis against Aquacide IIA to 2.5 ml. A summary of measurements during the purification procedure is given in Table 24. Specific activity of the final preparation was about 72% of that of Hewlett and Wolff (1976) whose preparation which gave a single gel band after SDS-PAGE. However, due to the poor yield and the low protein content of the purified material achieved in the present study, no gel bands were apparent when analysed by SDS-PAGE. SDS-PAGE of material after

Figure 11: DEAE-cellulose chromatography of *B. pertussis* AC

Culture supernate (5 L), from 48 h SS-X cultures of strain Taberman, was adjusted to pH 7.4 and pumped through a DEAE column at a flow rate of 90 ml h^{-1}. The column was washed with 1 L 10 mM Tris-HCl, pH 8.0. A 100 - 300 mM NaCl gradient was used to elute AC (flow rate 9 ml h^{-1}). Eluate was collected in 4.5 ml amounts. ◇,protein concentration; ◆, AC activity; ----, NaCl concentration.

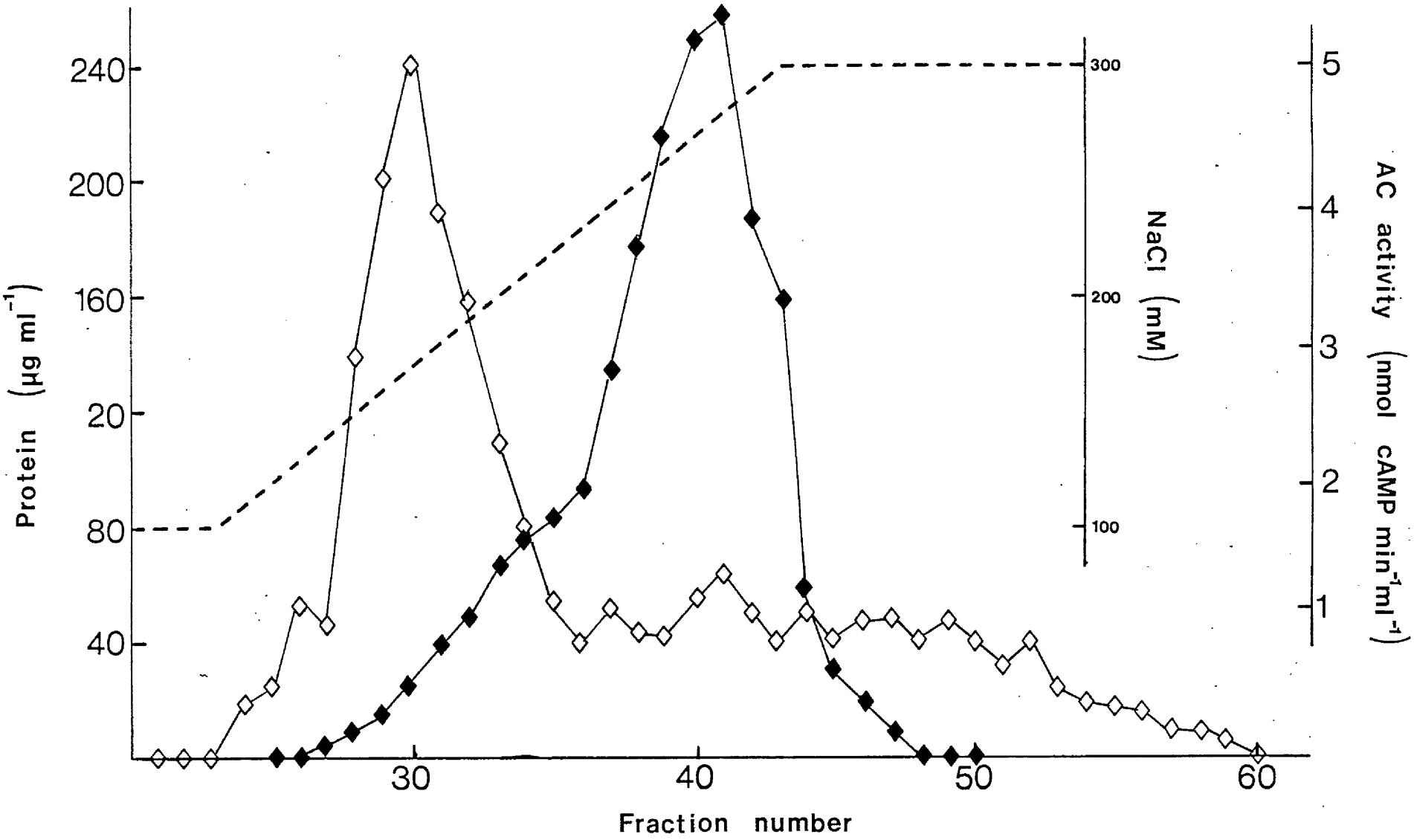

240
200
160
20
80
40
Protein (µg ml⁻¹)
300
200
100
NaCl (mM)
5
4
3
2
1
AC activity (nmol cAMP min⁻¹ml⁻¹)
30
40
50
60
Fraction number

Table 24: Purification of AC

Purification step	Protein concentration ($\mu g\ ml^{-1}$)	Volume (ml)	Total protein (mg)	Total AC activity[a]	Specific AC activity[b]	Recovery (%)
Culture supernate	153	5000	765	384	0.50	100
DEAE-cellulose chromatography	517	5	2.6	186	72	48
Sephadex G-200	51	2.5	0.128	13	102	3.4

a, nmol cAMP min^{-1}; b, pmol cAMP $min^{-1} mg(protein)^{-1}$.

DEAE-cellulose chromatography revealed 5 faint bands, the most dense of which corresponded to a polypeptide with a molecular weight of about 70,000 (results not shown).

The pooled material after Sephadex G-200 chromatography was used to raise antiserum in a rabbit. Unfortunately, after injecting the rabbit it was discovered that the pre-immune serum gave a strong reaction with an X-mode B. pertussis cell lysate. Antiserum, raised six weeks after the first injection of material was tested for AC inhibitory activity as a final resort. X-mode cell lysate (100 μg(protein) ml^{-1}), present in saline or saline and 250 mM EGTA (the highest concentration of EGTA which would dissolve), was mixed with an equal volume of serum and incubated at $4^{o}C$ overnight before assaying for AC activity. EGTA was included to eliminate the effect of calmodulin present in serum which would activate B. pertussis AC and possibly mask any inhibitory effect due to antibody.

Neither antiserum raised against the AC preparation nor the U.S. standard antiserum showed any significant inhibitory activity with or without EGTA, when compared to the effect of normal serum (Table 25). Raised antiserum concentrated 20-fold by dialysis against Aquacide IIA, also showed no inhibitory activity, but increased activity relative to other sera presumably because of the higher concentration of calmodulin. EGTA present at 125 mM greatly reduced activator activity, but not totally.

Table 25: The effect of antisera and EGTA on cell-lysate AC activity

Sera was mixed with an equal volume of X-mode cell lysate (strain Taberman) and incubated at $4^{o}C$ overnight before assaying for AC activity.

Antiserum	AC activity[a]	
	Without EGTA	With EGTA (125 mM)
None	1.36	1.38
Normal serum[b]	49.8	2.13
Antiserum raised against AC preparation	48.9	2.17
Antiserum raised against AC preparation (20 x concentrated)	77.6	2.40
U.S. standard antiserum	28.9	2.15

a, nmol cAMP $min^{-1}mg(protein)^{-1}$; b, serum obtained from an unimmunized rabbit which gave no precipitin reaction with a B. pertussis extract.

6. Manipulation of Cyclic AMP Production During Growth

6.1. The effect of NaF on adenylate cyclase activity

Hewlett and Wolff (1976) reported that NaF strongly inhibited activity of purified AC. This was confirmed in this study using cell lysate as an enzyme source (Fig. 12). NaF at 40 mM, and greater, caused 90% loss of activity. It was thought NaF might be a useful tool in answering the question whether cAMP is involved in modulation, if B. pertussis could grow in media containing NaF. Strain Taberman was grown in SS-X for 48 h with varying concentrations of NaF added aseptically after filter sterilization. However, NaF present at concentrations up to 40 mM, did not inhibit production of cAMP during growth (Table 26) although growth was largely inhibited at 40 mM NaF and completely inhibited at 80 mM. The X bands were still present after growth in all media (results not shown).

6.2. The effect of commercial bovine catalase during growth in X and C medium

Commercial bovine catalase is a good source of B. pertussis AC activator (Hewlett et al., 1978) which has now been identified as calmodulin (Goldhammer, 1981). It was thought that this might be a useful tool in determining whether cAMP plays a role in modulation. Strain Taberman was grown in SS-X and SS-C containing bovine catalase for 48 h. Before addition to the medium, a suspension of bovine catalase in distilled water (10 mg ml^{-1}) was heated at 100°C for 5 min (calmodulin is heat-stable (Hewlett et al., 1979b)). The precipitate was removed, and the supernate was filter sterilized and added to the medium (1% (v/v) final concentration). Culture supernates were assayed for cAMP, and cells for AC activity, HSA and the X bands (Table 27). Further data of HSA tests, which were used to calculate results, are given in Appendix 6, Table A1. Bovine catalase appeared to have little or no influence on production of the X bands or HSA. Intact-cell AC activity was higher after growth with catalase and this may be due to the irreversible nature of activation. Culture supernate cAMP was undetectable after growth in SS-C even with bovine

Figure 12: The effect of NaF on cell-lysate AC activity

Harvested cells from a 48 h SS-X culture of strain Taberman were X-pressed in saline and assayed for AC activity with NaF present in the assay mixture.

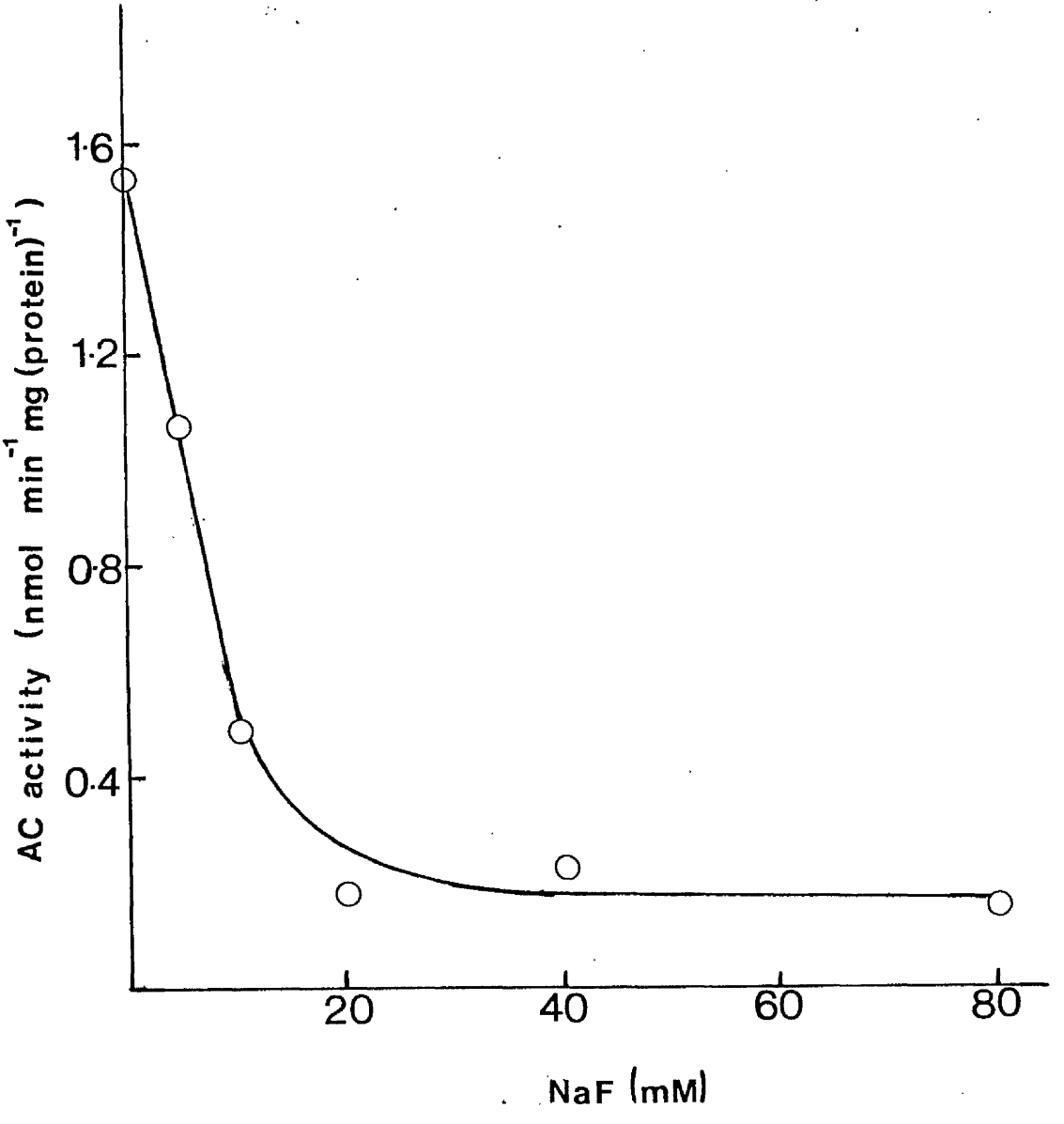

1·6
1·2
0·8
0·4
AC activity (nmol min^{-1} mg (protein)$^{-1}$)
20
40
60
80
NaF (mM)

Table 26: The effect of NaF on culture supernate cAMP levels

Strain Taberman was grown for 48 h in SS-X containing various concentrations of NaF. Culture supernate was assayed for cAMP.

NaF (mM)	cAMP[a]	A_{660nm}
0	105	1.48
5	107	1.68
10	115	1.27
20	95	0.89
40	125	0.29

a, pmol mg(dry weight of cells)$^{-1}$

Table 27: The effect of AC activator, present in the growth medium, on the production of X-mode associated properties

Strain Taberman was grown for 48 h in media. Supernate of a heat-treated (100°C for 5 min) solution of bovine catalase (10 mg ml^{-1}) was added to media (1% (v/v)) as a source of activator. Culture supernate was assayed for cAMP, harvested cells were assayed for AC activity and the X bands, and heat-treated cells (56°C for 30 min) were assayed for HSA.

Medium	Bovine catalase[a]	Culture supernate cAMP level[b]	Intact-cell AC activity[c]	% relative HSA[d] (and 95% confidence limits)	X bands[a]
SS-X	-	110	840	100	+
SS-X	+	565	3280	65 (34, 121)	+
SS-C	-	< 5[e]	15	11 (6, 21)	-
SS-C	+	< 5	25	7 (3, 13)	-

a, present (+) or absent (-); b, pmol cAMP ml^{-1}; c, pmol cAMP $min^{-1}mg(protein)^{-1}$; d, relative to HSA of cells grown in SS-X without bovine catalase; e, limit of detection.

catalase.

It appeared that modulating conditions were too severe to permit cAMP production even with activator in the growth medium. Lacey (1960) reported that an intermediate- (I-) mode arose when B. pertussis was grown with a critical ratio of pro-X-mode salt to pro-C-mode salt. It was thought that if cAMP plays a role in modulation, activator present in the medium might shift the critical concentration of $MgSO_4$ required to induce modulation. Strain Taberman was grown for 48 h in a series of modified SS-X media where the NaCl was proportionally replaced by $MgSO_4$, and in a parallel series containing bovine catalase (added as described previously). The critical concentration of $MgSO_4$ required to induce modulation, as judged by loss of the X bands was about 11 mM (Table 28) and was not affected by the presence of activator. The concentration of $MgSO_4$ required to induce an I-mode variant was fairly critical and an I-mode variant, showing partial loss of the X bands, was obtained only once in three experiments. Culture supernate levels of cAMP were only detectable in those cultures where the X bands were present. In cultures containing activator, cAMP levels ranged from 490 - 580 pmol ml^{-1}, while in cultures without activator, levels ranged from 80 - 140 pmol ml^{-1}. The one case where an I-mode variant was obtained (ie. where the X-bands were partially lost), was in a culture containing activator. Culture supernate cAMP in this culture was 210 pmol ml^{-1}. This was about twice as high as that which occurred in X-mode cultures without activator.

Table 28: The critical concentration of $MgSO_4$ required to induce loss of the X bands

Strain Taberman was grown for 48 h in SS-X where the NaCl was proportionally replaced by $MgSO_4$. Supernate of a heat-treated (100°C for 5 min) bovine catalase solution (10 mg ml^{-1}) was added (1% (v/v)) as a source of AC activator. Harvested cells were analysed by SDS-PAGE for the presence of the X bands.

Final concentration of $MgSO_4$ (mM)		0	4	6	8	9	10	11	12	14	16	18	20
Final concentration of NaCl (mM)		43	34	30	26	24	22	19	17	13	8.6	4.3	0
Bovine catalase absent	Experiment 1	X	X	X	X	...	X	...	C	C	C	C	C
	2	X	...	...	X	X	X	C	C	...	...	...	C
	3	X	...	...	X	X	X	C	C	...	...	...	C
Bovine catalase present	Experiment 1	X	X	X	X	...	I	...	C	C	C	C	C
	2	X	...	...	X	X	X	C	C	...	...	...	C
	3	X	...	...	X	X	X	C	C	...	...	...	C

X, the X bands were present; C, the X bands were absent; I, the X bands were partially absent; ..., not tested.

7. The Effect of Exogenous Cyclic Nucleotides

The observed influence of exogenous cAMP on a phenotype has often been used as a criterion for establishing a regulatory role for cAMP. Dibutyryl cAMP is ineffective in overcoming catabolite repression in the enterobacteria, but has been used in other microorganisms, eg. streptomycetes and eukaryotic microorganisms, to illustrate regulatory phenomena (Ring et al.,1977; Nimi et al., 1980). Dibutyryl cAMP is often more effective than cAMP due to the butyryl group rendering the molecule more lipophilic and hence more permeable. Dibutyryl cAMP is also more resistant to phosphodiesterase (Posternak et al., 1962).

The effect of exogenous 5 mM cAMP and dibutyryl cAMP on the X bands, intact-cell AC activity and HA activity was investigated. Strain Taberman was grown for 48 h in SS-X, SS-C, and SS-NA (SS-X with nicotinic acid present at 4.1 mM), all with and without cAMP or dibutyryl cAMP. Cultures were harvested and washed three times with saline (to remove contaminating nucleotides) and assayed for intact-cell AC activity and the presence of the X bands. Exogenous cAMP and dibutyryl cAMP had little, if any, effect on intact-cell AC activity after growth either in SS-X, SS-C, or SS-NA (Table 29). Similarly, neither of these nucleotides had any noticable effect on SDS-PAGE profiles after growth in these media (Plate 5).

To investigate the effect of these nucleotides on production of HA activity, strain Taberman and D30042 I were grown in H-X and H-C, with and without nucleotide, statically for 5 days. Hornibrook medium was used as B. pertussis does not grow well statically in Stainer and Scholte medium (K. A. Burns, personal communication). Harvested cells were resuspended to 50 ou and assayed for HA activity. HA activity was investigated as at the time of this work it was believed that most HA activity in B. pertussis was due to pili, which in E. coli and S. typhimurium has been shown to be regulated by cAMP (Saier et al., 1978; Eisenstein et al., 1981). However, growth of B. pertussis with either nucleotide, had no

Table 29: The effect of exogenous cyclic nucleotides during growth on intact-cell AC activity

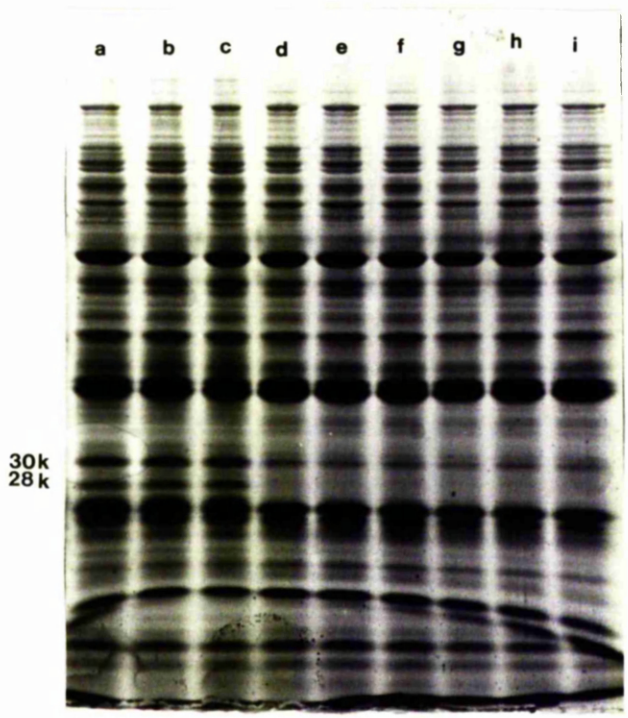
Harvested intact cells, of 48 h cultures of strain Taberman, were assayed for AC activity. Cyclic nucleotides were present in media at 5 mM.

Medium	Cyclic nucleotide	AC activity[a]
SS-X	none	380
SS-X	cAMP	470
SS-X	dibutyryl cAMP	420
SS-C	none	15
SS-C	cAMP	15
SS-C	dibutyryl cAMP	20
SS-NA	none	20
SS-NA	cAMP	20
SS-NA	dibutyryl cAMP	25

a, pmol cAMP $min^{-1} mg(protein)^{-1}$

Plate 5: SDS-PAGE profiles of *B. pertussis* cells after growth in media containing exogenous cyclic nucleotides

Strain Taberman was grown for 48 h in media. Cyclic nucleotides were present at 5 mM. Harvested, washed cells were analysed by SDS-PAGE. Media were: a, SS-X; b, SS-X with cAMP; c, SS-X with dibutyryl cAMP; d, SS-C; e, SS-C with cAMP; f,SS-C with dibutyryl cAMP; g, SS-NA; h, SS-NA with cAMP; i, SS-NA with dibutyryl cAMP.


a
b
c
d
e
f
g
h
i
30k
28k

effect on HA activity (Table 30).

There existed the possiblity that B. pertussis may be impermeable to the nucleotides. The ability of B. pertussis to excrete cAMP into the medium, at levels comparable to those found in E. coli cultures, suggested that transport proteins existed. The ability of X- and C-mode strain Taberman to take up cAMP and dibutyryl cAMP in SS-X and SS-C was investigated. Results for single experiments are shown in Fig. 13 - 15. "Uptake" of cAMP was demonstrated for both X-mode and C-mode cells in SS-X and SS-C, and dibutyryl cAMP for X-mode cells in SS-X and SS-C. However, it is not known whether the results are due to nucleotide binding to the cell wall or their entry into the cytoplasm. Nor is it known how much of the nucleotide which entered the cell, if any, was subsequently metabolized . The rate of cAMP "uptake" by cells grown previously under X-mode conditions appeared to be higher than for cells grown previously under C-mode conditions, but it is not known if this is significant.

Table 30: The effect of exogenous cyclic nucleotides during growth on HA activity

Strains were grown statically for 5 days. Cyclic nucleotides were present in media at 5 mM. Harvested cells were resuspended to 50 ou. and assayed for HA activity.

Medium	Cyclic nucleotide	Haemagglutination titre	
		Taberman	D30042 I
H-X	none	16	4
H-X	cAMP	16	4
H-X	dibutyryl cAMP	16	4
H-C	none	< 2	< 2
H-C	cAMP	< 2	< 2
H-C	dibutyryl cAMP	< 2	< 2

Figure 13: Time course of uptake of [^{3}H] cAMP by X-mode *B. pertussis* cells

Harvested and washed cells from a 48 h SS-X culture of strain Taberman was used. The reaction mixture consisted of cells (0.3 mg(dry weight) ml^{-1}), 1 mM [^{3}H] cAMP (specific activity 5 $\mu Ci\ \mu mol^{-1}$) in SS-X, ○, or SS-C, ●. At timed intervals, the reaction was stopped by rapidly filtering 1 ml of cell suspension.

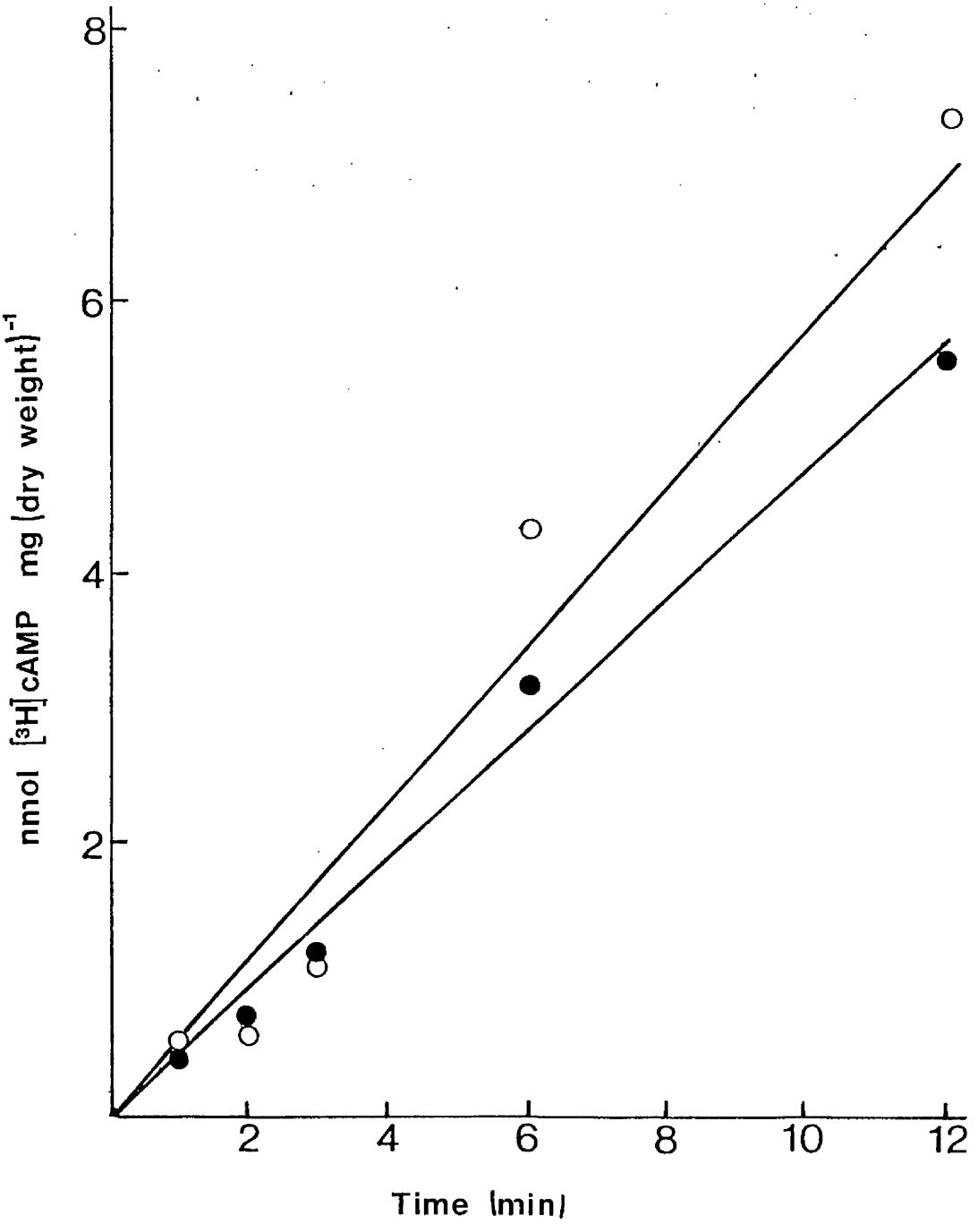

8
6
4
2
nmol [3H]cAMP mg (dry weight)-1
2
4
6
8
10
12
Time (min)

Figure 14: Time course uptake of [^{3}H] cAMP by C-mode *B. pertussis* cells

Harvested and washed cells from a 48 h SS-C culture of strain Taberman was used. The reaction mixture consisted of cells (0.3 mg(dry weight) ml^{-1}), 1 mM [^{3}H] cAMP (specific activity 5 $\mu Ci\ \mu mol^{-1}$ in SS-X, ○, or SS-C, ●. At timed intervals, the reaction mixture was sto by rapidly filtering 1 ml of cell suspension.

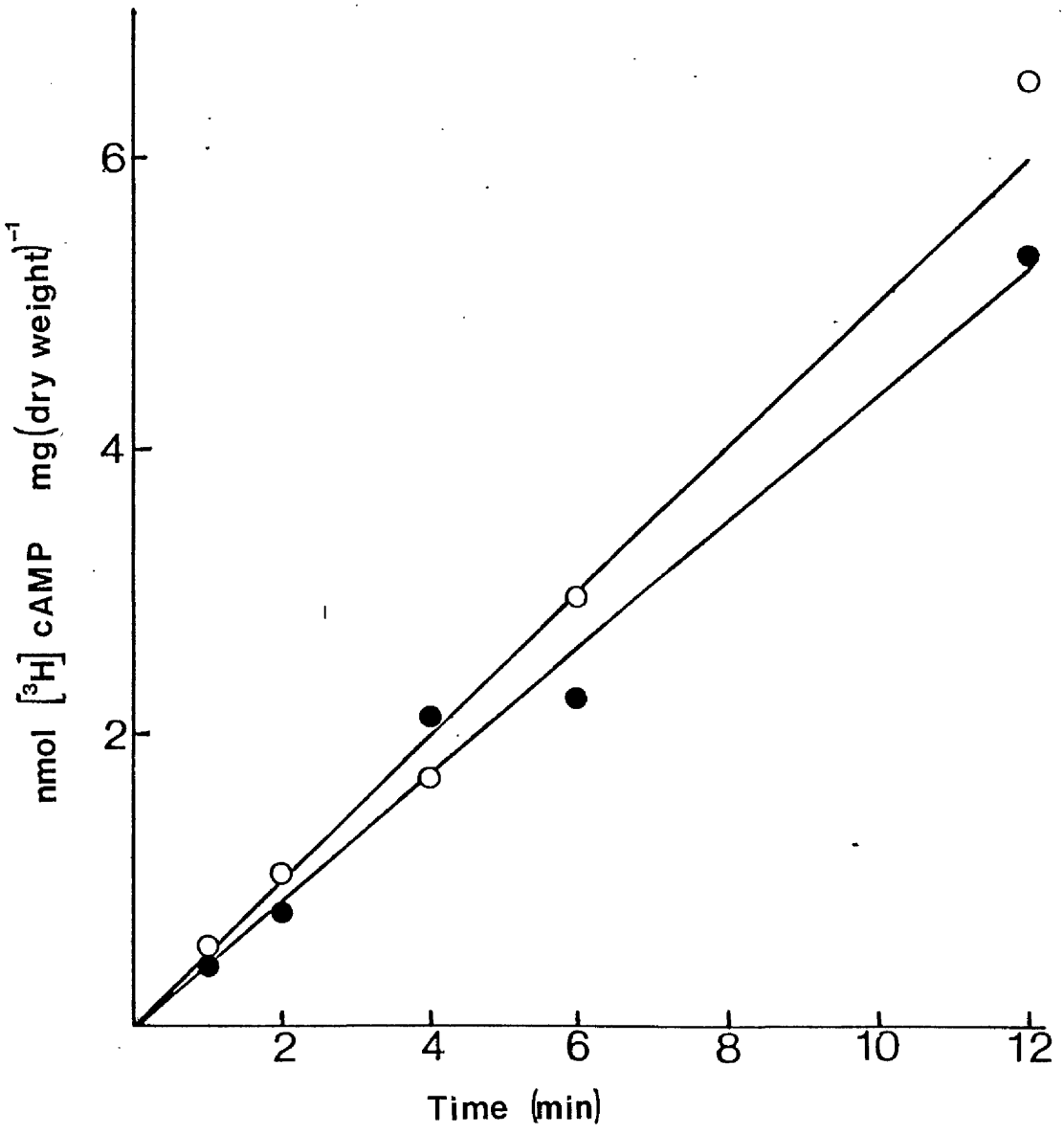
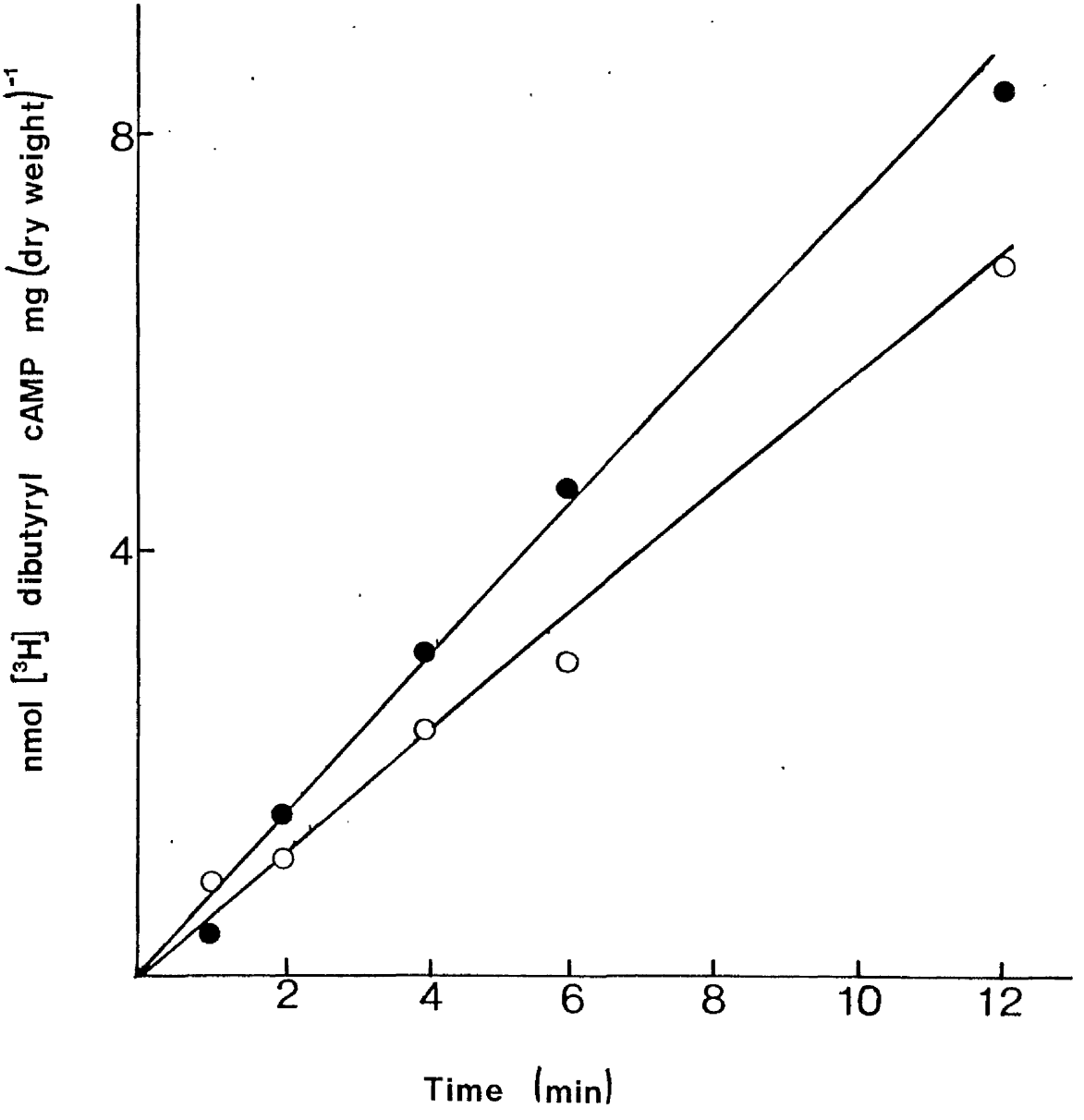

nmol [3H] cAMP mg(dry weight)-1
6
4
2
2
4
6
8
10
12
Time (min)

Figure 15: Time course of uptake of [^{3}H] dibutyryl cAMP by X-mode *B. pertussis* cells

Harvested and washed cells from a 48 h SS-X culture of strain Taberman was used. The reaction mixture consisted of cells (0.3 mg(dry weight) ml^{-1}), 1 mM [^{3}H] dibutyryl cAMP (specific activity 5 $\mu Ci\ \mu mol^{-1}$) in either SS-X, ○, or SS-C, ●. At times intervals, the reaction was stopped by rapidly filtering 1 ml of cell suspension.


nmol [³H] dibutyryl cAMP mg (dry weight)⁻¹
8
4
2
4
6
8
10
12
Time (min)

8. Respiration of Substrates by Variants of *B. pertussis*

The *cya* and *crp* mutants of *E. coli* and *S. typhimurium* are pleiotropic and are unable to ferment the various sugars whose catabolic enzymes are subject to catabolite repression. In addition, they lack the ability to catabolize certain amino acids such as tryptophan and histidine (Pastan and Adhya, 1976). This is because expression of the operons for the above catabolic activities requires CRP-cAMP. Work by Ring *et al.* (1977) suggests that cAMP may play a regulatory role in the uptake of several amino acids by *Streptomyces hydrogenans*. It was thought possible that cAMP could play a major regulatory role in *B. pertussis* by enabling the organism to select for preferred carbon/energy sources (amino acids in the case of this organism). Indeed, it has been reported that glutamate is the preferred carbon source (Introduction, Part 1, 5.2.). It was therefore of interest to compare the respiration rates between X- and C-mode, and phase IV variants, for different amino acids, since the latter two variants have greatly reduced AC activity.

Table 31 shows respiration rates of several amino acids by X- and C-mode and phase IV variants for three experiments. C-mode variants were obtained by subculturing twice in SS-C, and X-mode and phase IV variants were obtained by subculturing twice in SS-X. Respiration was not detected with the sugars D-ribose, D-glucose, or sucrose or with the amino acids glycine, L-glutamine, L-arginine, L-lysine, L-threonine or L-histidine (< 0.3 µl $O_2 min^{-1} mg(\text{dry weight})^{-1}$) (results not shown). The data appeared to be reasonably normally distributed when assessed by Rankit numbers (results not shown). The results were analysed by analysis of variance. No significant differences could be found between the ability of variants to respire any particular amino acid. When analysed by two factor analysis of variance, differences between the respiration rates for different amino acids were significant at the 0.1% probability level while differences between variants were

Table 31: Respiration rate for amino acids by variants of B. pertussis

Strain D30042 I was grown twice for 24 h in either SS-X or SS-C and strain D30042 IV was grown twice for 24 h in SS-X. Respiration rates were determined for twice washed cells using a Clark oxygen electrode. Cells were present at 0.5 mg(dry weight) ml^{-1} and substrate at 18 mM.

Amino acid	Respiration rate[a] (and standard deviation)		
	X-mode	C-mode	Phase IV
L-glutamate	6.3 (0.9)	6.5 (0.6)	6.5 (0.3)
L-aspartate	5.6 (0.8)	7.2 (1.3)	6.5 (0.2)
L-proline	3.5 (0.6)	3.9 (0.2)	3.4 (0.2)
L-serine	2.5 (0.3)	2.9 (0.2)	2.5 (0.3)
L-alanine	2.2 (0.3)	2.4 (0.1)	2.7 (0.3)

a, $\mu l\ O_2\ min^{-1}\ mg(dry\ weight)^{-1}$

insignificant at the 5% level. The greatest respiration rates were observed for L-glutamate and L-aspartate followed by L-proline, then L-alanine and L-serine. SSX buffer, replaced by SSC buffer, did not alter the respiration rates of an C-mode cell suspension for the above amino acids (results not shown). Therefore, $MgSO_4$ does not directly alter the respiration rate.

In a single experiment, the respiration rates of X- and C-mode variants for various metabolic intermediates were compared (Table 32). Significant respiration was detected for the substrates acetate, pyruvate, lactate, succinate, and formate. Succinate was respired at the greatest rate. However, there were no big differences observed between the ability of the two variants to respire any particular substrate. Citrate was not respired by either variant.

Table 32: Respiration rates for metabolic intermediates by X- and C-mode variants of *B. pertussis*

Strain D30042 I was grown twice for 24 h in SS-X or SS-C. Respiration rates were determined for twice-washed cells using a Clark oxygen electrode. Cells were present at 0.5 mg(dry weight) ml^{-1} and substrate at 18 mM.

Substrate	Respiration rate[a]	
	X-mode	C-mode
Acetate	2.1	1.7
Pyruvate	1.5	1.9
Lactate	1.6	1.2
Succinate	7.8	6.8
Formate	1.7	1.9

a, $\mu l\ O_2\ min^{-1}\ mg(dry\ weight)^{-1}$

9. Isolation of Phosphonomycin Resistant Mutants

The absence or presence of a particular phenotype in *crp* and *cya* mutants of *E. coli* and *S. typhimurium* has been used as strong evidence for a regulatory role of cAMP in the expression of such phenotypes (Introduction, Part 2, 2). The difficulty in isolating AC mutants of *B. pertussis* is that it's function is unknown. In the enterobacteria, antibiotic resistance has been used as a basis to select for *crp* and *cya* mutants which lack the appropriate permeases. Phosphonomycin resistant mutants isolated in *S. typhimurium* were almost all unable to ferment lactose or ribose. Isolated colonies were 50% *cya* and 50% *crp* (Alper and Ames, 1978). It was thought that phosphonomycin might be useful in isolating AC mutants of *B. pertussis*.

Phosphonomycin resistance of a phase IV strain and X- and C-mode variants was compared (Table 33). To test the minimum inhibitory concentration for C-mode variants, strains were first grown on BG-C before transfer to BG-C containing phosphonomycin. Phosphonomycin was included in BG media to give a doubling dilution series. A degraded avirulent strain (D30042 IV) was significantly more resistant than it's virulent counterpart (D30042 I). However, C-mode variants were as sensitive as X-mode variants. In addition, C-mode inocula would not grow on nutrient agar containing 20 mM or 40 mM $MgSO_4$ whereas strain D30042 IV would (results not shown). This suggests that there may be differences between the C-mode and phase IV phenotypes in terms of sensitivity to inhibitory agents.

Attempts were made to isolate AC mutants by mutagenesis with NTG and selection for phosphonomycin resistance. Isolating a mutant with reduced AC activity and a phase IV phenotype would not verify a virulence regulatory role for cAMP as phophonomycin may select for degraded strains. On the other hand, isolating a mutant lacking AC activity but with a phase I phenotype, would strongly suggest that AC plays no role in the

Table 33: Minimum-inhibitory concentration of phosphonomycin for *B. pertussis* variants

Three day BG-X and BG-C cultures were used to inoculate BG-X and BG-C respectively, containing two-fold increasing concentrations of phosphonomycin. After three days incubation the minimum-bacteriostatic concentration was recorded.

Strain	MIC of phosphonomycin ($\mu g\ ml^{-1}$)	
	Medium	
	BG-X	BG-C
Taberman	3.12	3.12
18334	6.25	6.25
D30042 phase I	3.12	3.12
D30042 phase IV	> 200	> 200

regulation of properties lost during C modulation or phase degradation.

To determine the optimal concentration of NTG and sampling time, strain Taberman was grown in SS-X for 40 h. Different concentrations of NTG were then added to three cultures which were sampled at various times to determine the viable count (Fig. 16). NTG present at 50 μg ml^{-1} for 20 min gave about 50 % killing and these conditions were used to isolate phosphonomycin mutants. It has been reported previously that 50% killing gives optimal mutagenesis for auxotrophs (Adelberg et al., 1965) as more severe conditions lead to double mutations.

To isolate phosphonomycin resistant mutants, samples (1 ml), of NTG-treated cultures, were centrifuged in sterile disposable centrifuge tubes in a Beckman microfuge (12,000 x g for 1 min). Pellets were resuspended in 1% (w/v) casamino acids (Appendix 1) and spread on BG plates containing phosphonomycin (100 μg ml^{-1}). This resulted in a large number of phosphonomycin resistant colonies (about 50 per plate). Colonies were subcultured onto BG containing phosphonomycin (100 μg ml^{-1}). to give single colonies. Further subcultures from these single colonies were used for subsequent analysis. Control cultures (ie not treated with NTG), gave no phosphonomycin resistant colonies.

Eight phosphonomycin resistant mutants, each isolated from a different NTG-treated culture, were grown for 48 h in SS-X and analysed for AC activity, culture supernate cAMP levels, and the X bands. All eight mutants, designated Tab 1 to Tab 8, possessed the same levels of intact-cell AC activity as the wild type and produced similar amounts of cAMP in the culture supernate (Table 34).

Figure 16: The effect of exposure to NTG on viability of strain Taberman

Strain Taberman was grown in SS-X for 40 h. NTG was then added to give final concentrations of 100 μg ml^{-1} (○), 50 μg ml^{-1}(△), and 25 μg ml^{-1} (□). At times intervals dilutions were plated out on BG plates to determine the viable count.

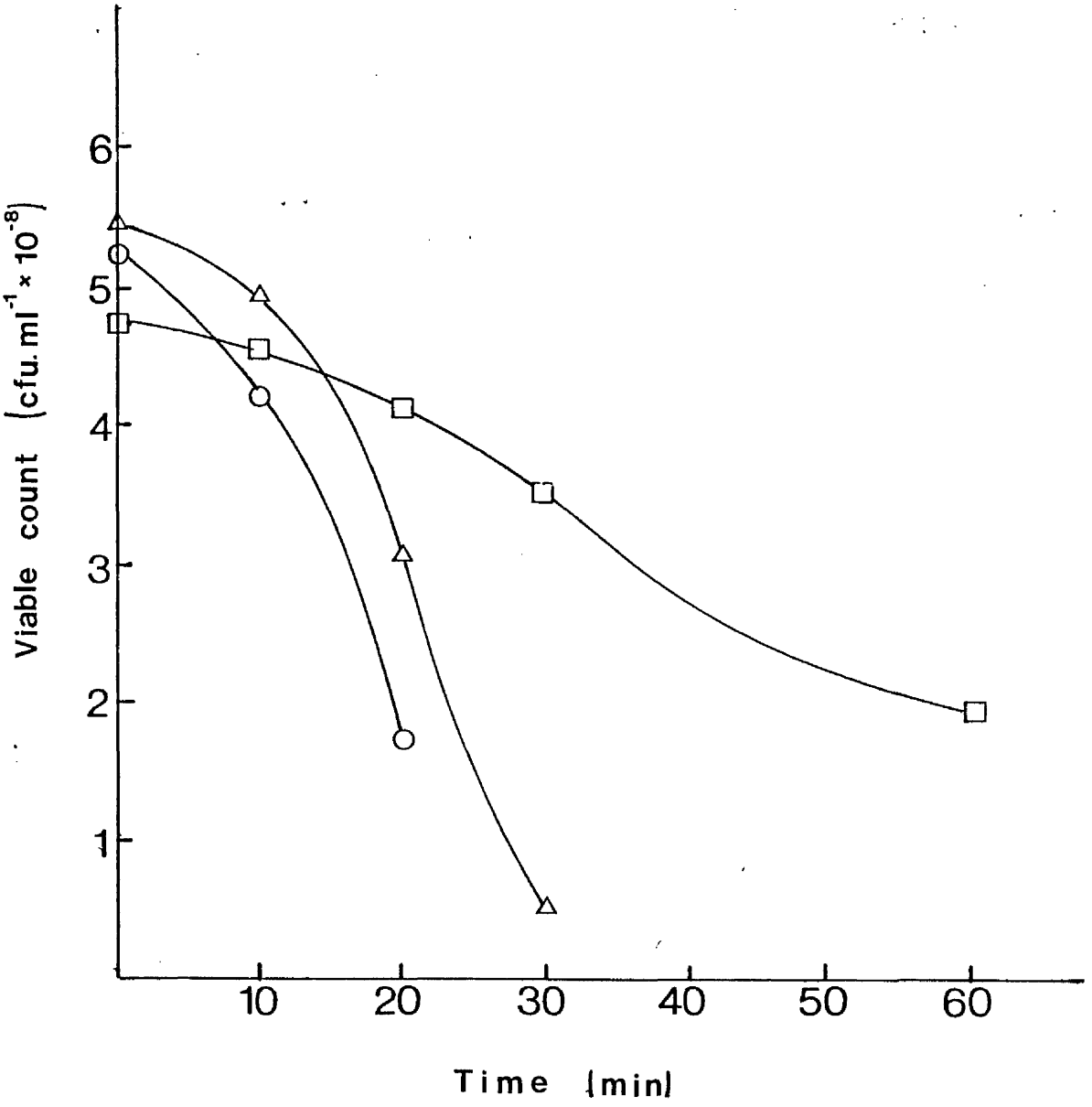

Viable count (cfu.ml^{-1} × 10^{-8})
6
5
4
3
2
1
10
20
30
40
50
60
Time (min)

Table 34: Intact-cell AC activity and supernate cAMP levels of cultures of phosphonomycin resistant mutants of *B. pertussis*

Strains were grown for 48 h in SS-X. Intact cells were assayed for AC activity and culture supernate for cAMP.

Strain	Intact-cell AC activity[a]	cAMP level[b]
Taberman (original strain)	835	120
Tab 1	855	135
Tab 2	925	110
Tab 3	780	125
Tab 4	960	115
Tab 5	775	125
Tab 6	825	140
Tab 7	840	110
Tab 8	855	140

a, pmol cAMP $min^{-1} mg(protein)^{-1}$; b, pmol cAMP ml^{-1}

10. Cyclic AMP Binding Activity

In *E. coli* and *S. typhimurium*, the cAMP regulatory model is dependent on a functional CRP. Little is known about the role of cAMP in other prokaryotes. However, in some organisms where cAMP related phenomena have been observed, cAMP-receptor proteins have been identified. Some of these have been shown to be immunologically similar to CRP (Anderson and Pastan, 1973).

If cAMP plays a regulatory role in *B. pertussis* it is most likely that it possesses a cAMP binding protein analogous to CRP. Cyclic AMP binding activity was measured in cytoplasmic fractions of *B. pertussis* strains and *E. coli* crp^+ and crp^- strains (Table 35). Cyclic AMP binding activity was higher for *B. pertussis* strains than for *E. coli* crp^-, although not as high as that for *E. coli* crp^+. There appeared to be no great differences between X- and C-mode variants or between phase I and phase IV variants.

The presence in *B. pertussis* of an immunlogically similar protein to *E. coli* CRP was tested by Ouchterlony double diffusion precipitin test and by "crossed-over" immunoelectrophoresis using goat anti-*E.coli* CRP serum (4 x concentrated). One strong and one faint precipitin line were observed against an *E. coli* 1100 cytoplasmic fraction, but nothing against a *B. pertussis* Taberman cytoplasmic fraction even at twice the protein concentration of the *E. coli* cytoplasmic fraction (Fig. 17). In addition, no reaction was observed against a cytoplasmic fraction of *B. pertussis* 18334. Similarly, "crossed-over" immunoelectrophoresis gave two precipitin lines with the *E. coli* 1100 cytoplasmic fraction but nothing with the *B. pertussis* Taberman cytoplasmic fraction (Fig. 18)

Table 35: [^{3}H] cAMP binding activity in extracts of *E. coli* and *B. pertussis*

E. coli strains were grown for 18 h in Yeast Glucose medium and *B. pertussis* strains were grown for 48 h in SS-X. Supernate of X-pressed cells was assayed for cAMP binding activity.

Organism and strain	[^{3}H] cAMP binding activity[a]		
	Experiment		
	1	2	3
E. coli 1100 (*crp*$^+$)	6.9	4.7	2.9
E. coli 5336 (*crp*$^-$)	0.4	0	1.1
B. pertussis Taberman	3.1	1.8	3.8
B. pertussis Taberman[b] (C-mode)	2.2		
B. pertussis 18323	2.7		
B. pertussis D30042 I	1.8		
B. pertussis D30042 IV	1.6		

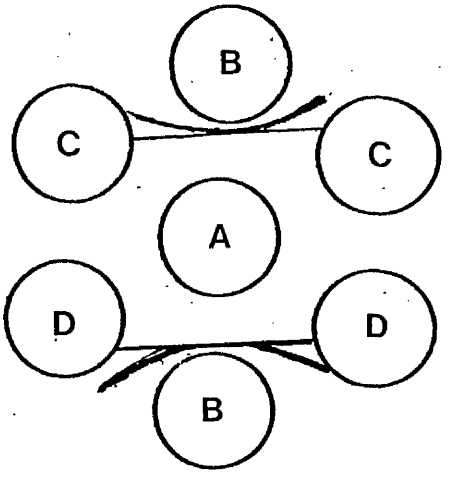
a, pmol [^{3}H] cAMP bound mg(protein)$^{-1}$; b, grown in SS-C for 48 h.

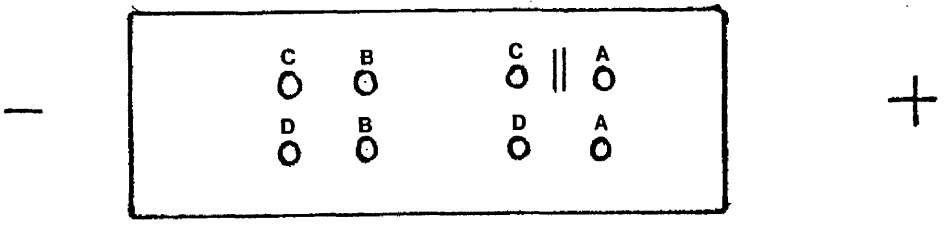
Figure 17: Ouchterlony double diffusion precipitin test of anti-*E. coli* CRP against *B. pertussis* and *E. coli* cytoplasmic fractions

A, goat anti-*E. coli* CRP serum; B, *E. coli* 1100 cytoplasmic fraction (15 mg(protein) ml^{-1}); C, *B. pertussis* Taberman cytoplasmic fraction (15 mg(protein) ml^{-1}); D, *B. pertussis* Taberman cytoplasmic fraction (30 mg(protein) ml^{-1}).

Figure 18: "Crossed-over" immunoelectrophoresis of anti- *E. coli* CRP against *B. pertussis* and *E. coli* cytoplasmic fractions

A, goat anti- *E. coli* CRP; B, normal rabbit serum; C, *E. coli* 1100 cytoplasmic fraction (15 mg(protein) ml^{-1}); D, *B. pertussis* Taberman cytoplasmic fraction (15 mg(protein) ml^{-1}).


B
C
C
A
D
D
B


C
B
C
A
D
B
D
A
−
+

DISCUSSION

1. The Modulating Effect of Different Compounds

Lacey (1960) found that marked antigenic changes occurred when B. pertussis was grown on BG where the NaCl had been replaced by one of several salts or organic acids. Since then, the study of modulation has been restricted mainly to $MgSO_4$- or nicotinic acid-induced modulation. The present study showed that several compounds, other than $MgSO_4$, induced modulation as judged by the loss of the X bands, HSA and AC activity. Growth in Stainer and Scholte media containing high levels of $MgSO_4$, Na_2SO_4, or Na butytate, consistently caused loss of these properties for three strains of B. pertussis: Taberman, a hospital isolate; 18334, a recognized laboratory strain; and 18323, a mouse intracerebrally-virulent strain.

It has been reported that strain 18323 is resistant to $MgSO_4$- and nicotinic acid-induced antigenic changes and loss of virulence (Pusztai and Joó, 1967; Adams, 1970). However, the 18323 strain used in these studies, reproducibly lost three X-mode specific properties when grown with 20 mM $MgSO_4$; but there was some evidence to suggest that strain 18323 was more resistant to modulation than strain 18334. Sodium caprylate (0.6 mM) caused modulation for strain 18334 but not for strain 18323; strain Taberman would not grow with 0.6 mM Na caprylate. Modulation of all three strains occurred when grown with 20 mM Na butyrate; but when grown with 10 mM Na butyrate, modulation occurred for strains Taberman and 18334 but not for strain 18323 (results not shown). Growth with 86 mM Na lactate caused partial modulation of strain Taberman but not of strains 18334 or 18323 suggesting that strain Taberman may be the most sensitive to modulation of the three strains. The function of modulation in nature is not known but perhaps continuous subculture in "unnatural" conditions (eg. in vitro, or the mouse brain), selects for strains which are less able to modulate. The possibility that low-intracerebral virulence of most strains is due to their tendency to modulate in the brain seems unlikely (Adams, 1970): such strains

removed from the mouse brain during the decline phase of infection, and transferred to another mouse brain, did not continue to decrease in numbers, but initially increased in numbers following the normal course of infection for low-intracerebrally-virulent strains. Nevertheless, intracerebral-virulence might be due to a virulence factor which is permanently expressed in intracerebrally-virulent strains, but which is expressed only during certain growth conditions (ie. not during growth in vitro or the mouse brain) in intracerebrally- avirulent strains. The ability to regulate expression of this virulence factor might be linked to the ability to modulate.

Growth in media containing 43 mM $MgCl_2$ did not cause modulation for any of the strains. Lacey (1960) reported this concentration of $MgCl_2$ and other Mg salts, present in BG, to cause modulation in terms of antigenic changes; although he reported $MgCl_2$ to be a weak pro-C-mode salt compared to $MgSO_4$. In the present study, the critical concentration of $MgSO_4$ required to induce loss of the X bands for strain Taberman was 10 - 11 mM. This suggests that the important component of $MgSO_4$ for inducing modulation is the SO_4^{--} anion. Whether higher concentrations of $MgCl_2$ could induce modulation was not investigated. It is possible that $MgCl_2$ may induce antigenic changes without causing loss of other properties.

Lacey (1960) reported that various organic acids were pro-C-mode substances. In this study, Na butyrate, Na succinate, Na lactate, Na caprylate, and nicotinic acid all induced modulation although some strain specificity was observed. Sodium caprylate was a potent pro-C-mode compound for strain 18334. When present at 0.6 mM, Na caprylate caused substantial loss of AC activity, HSA and the X bands. This is consistent with Lacey's (1960) findings that fatty acids were potent pro-C-mode compounds with potency increasing with chain length. Compared with the effect of other pro-C-mode substances, Na lactate (86 mM) caused a less severe loss of AC activity, HSA and the X bands of strain Taberman; but had no effect on the expression of these activities for strains 18334 or 18323. These conditions may have

induced the I-mode state, described by Lacey (1960), in strain Taberman.

After growth in media where cells underwent a marked reduction in intact-cell AC activity, activity still responded to activator but activation was reduced by 50%. This contrasts with Hewlett et al. (1980) who reported that AC activity was unresponsive to activator after growth in medium containing high levels of Mg^{++} (presumably high levels of $MgSO_4$). Hewlett et al. (1979b) reported that during purification of AC, responsiveness to activator was lost suggesting the requirement for an additional factor for activation. It is possible that during modulation, this factor, as well as AC activity, is reduced. On the other hand, Greenlee et al. (1982) suggested that partial proteolysis was responsible for rendering purified AC insensitive to calmodulin. If this is true, it is possible that partial proteolysis of AC during modulation may explain loss of responsiveness to calmodulin and even possibly loss of AC activity.

Molecular weight determinations suggest that pertussigen, the X polypeptides and AC are separate entities (Hewlett and Wolff, 1976; Irons and MacLennan, 1979). Furthermore, pertussigen is unique to B. pertussis whereas the X polypeptides have been demonstrated in B. pertussis and B. bronchiseptica and AC activity has been demonstrated in all three Bordetella species (Endoh et al., 1980; Ezzell et al., 1981b). The simultaneous loss of these components during growth in different modifications of SS-X is consistent with the hypothesis of a common regulatory mechanism grossly perturbed during modulation. As yet, there is no genetic evidence to support such a hypothesis but the suggestion that alteration of a regulatory mechanism lies at the root of modulation and phase degradation has been expressed elsewhere (Wardlaw and Parton, 1979; Peppler, 1982). The three components used as markers for modulation are all associated to a large extent with the cell envelope. For this reason it would be of interest to know whether HLT, which has a predominantly cytoplasmic location, is also lost

after growth in various modulating media. It has been reported that HLT is greatly reduced during growth in medium containing high levels of $MgSO_4$ (Livey et al., 1978). From this study, there was no evidence to suggest that different mechanisms were involved during modulation induced by different salts.

A few reports have suggested that some modulating conditions result in a differential loss of activities. Pusztai and Joó (1976) reported that loss of HSA but not HLT occurred during nicotinic acid-induced modulation. However, Livey (1981) and recently Schneider and Parker (1982) reported that nicotinic acid-grown B. pertussis contained greatly reduced levels of HLT. Wardlaw et al.(1976) found that succinate-induced modulation of strain 18334 caused 70% loss of HSA but no apparent loss of the X bands. It is possible that these conditions induced an I-mode variant. It may be that within the common regulatory mechanism there exists a hierarchy of sensitivities which enables differential expression of different components. Differential expression would only be observed during conditions where the regulatory mechanism was moderately perturbed (ie. conditions which induced the I-mode variant). Perhaps this might explain discrepancies between different workers In the present study, 86 mM Na lactate caused less severe loss of AC activity, HSA and the X bands compared to other pro-C-mode compounds such as $MgSO_4$; but differential expression of these properties was not obvious. It would be interesting to know whether levels of HLT were also reduced after such growth conditions.

2. Adenylate Cyclase Activity in *B. pertussis*

2.1. Levels of cAMP in cultures of *B. pertussis* variants

Although C-mode and phase IV variants have reduced AC activity compared to their X-mode and phase I counterparts, determinations of their intracellular levels of cAMP have not been reported. This is relevant when proposing a regulatory role for AC as cAMP, and not AC, is the regulatory molecule in *E. coli*. When *E. coli* is grown in media containing glucose, many proteins are repressed due to a low level of intracellular cAMP. This low level of cAMP arises through direct inhibition of AC activity. However, harvested *E. coli* cell suspensions, broken and intact, have higher AC activity after growth in media containing glucose than in media containing glycerol as the carbon source (Janeček *et al.*, 1979). Recent results suggest that this is due to direct inactivation of AC by CRP-cAMP (Bankaitis and Bassford, 1982). This illustrates that reduced levels of *in vitro* AC activity does not necessarily implicate involvement of cAMP in any process. Determining the intracellular concentration of cAMP in bacteria is technically difficult due to the very low levels present. This has led to variable results between different workers, even for *E. coli* (Pastan and Adhya, 1976; Botsford, 1981). Attempts to determine the intracellular concentration of cAMP in *B. pertussis* after 48 h growth in SS-X and SS-C were unsuccessful. The charcoal concentrating method of Epstein *et al.*(1975) was used. This may suggest that the intracellular concentration of cAMP in *B. pertussis* is very low (< 0.2 pmol mg(dry weight)$^{-1}$). Pastan and Perlman (1970) reported the intracellular levels of cAMP in *E. coli* to vary from 0.5 - 1.0 pmol mg(wet weight)$^{-1}$, depending on the carbon source.

In view of the difficulties encountered in measuring intracellular cAMP levels in *B. pertussis*, the levels in culture supernate or whole culture were determined. It seems probable that culture supernate cAMP orginates from the cytoplasm as ATP is likely to be exclusively

intracellular; cAMP is exceptional among the nucleotides in being excreted from bacterial cells (Lichenstein et al., 1960). In E. coli cAMP is excreted at a rate proportional to it's synthesis (Epstein et al., 1975). Cyclic AMP levels in B. pertussis SS-X or SS-C cultures were the same whether determined from culture supernate or whole cultures. This suggests that the majority of cAMP produced during growth is excreted into the medium. Supernate levels of cAMP in 48 h X-mode cultures of B. pertussis ranged from 0.1 - 0.2 nM.
Supernate levels of cAMP in cultures of E. coli after exponential growth vary from 0.1 - 0.5 nM (Buettner et al., 1973). After growth in media where substantial loss of AC activity, HSA and the X bands occurred, extracellular levels of cAMP were less than 5% of that of X-mode cultures. It is likely that levels of intracellular cAMP were similarly reduced as later experiments showed that SS-C did not inhibit excretion of cAMP from B. pertussis cells. If cAMP plays a regulatory role in B. pertussis, the large changes in cAMP levels which occur during modulation, would have the potential to cause phenotypic changes.

2.2. Locations of adenylate cyclase activity

Hewlett et al. (1978) reported that four locations of AC activity existed in B. pertussis cultures: one associated with the cytoplasm, two associated with the cell envelope, and one with the culture supernate. Results presented here confirm this. However, a substantially higher portion of AC activity (50%) was found in the culture supernate than reported by Hewlett et al. (20%). This is perhaps due to the differences in assay procedures. Hewlett et al. included creatine phosphokinase in the assay mixture to regenerate ATP. Several-fold more cAMP is produced in such assays as commercial creatine phophokinase is now known to be contaminated with calmodulin (Goldhammer et al., 1981).

In all three main compartments (culture supernate, extracytoplasmic but cell associated, and cytoplasmic), AC activity was substantially lost after $MgSO_4$-induced modulation. This would suggest that the observed loss of intact-cell AC activity, was not due to inhibition of translocation from one compartment to another. It is not certain whether "trypsin insensitive" AC activity is due to free cytoplasmic AC or AC associated with the cytoplasmic membrane, although this could of been determined by including a centrifugation step. Furthermore, it is not certain which compartment of AC is responsible for cAMP produced during growth. The active site must be exposed to the cytoplasm where ATP is available. The finding that culture supernate levels of cAMP were 3.5 - 7.5 -fold higher after growth in SS-X containing activator (which has a molecular weight of 16,700 and therefore unlikely to enter the cell) than after growth in normal SS-X, suggests that at least some extracellular cAMP is produced by AC associated with the cytoplasmic membrane. The finding that in vivo AC activity is responsive to extracellular activator, indicates that the AC of B. pertussis has the potential for a regulatory role in relaying cell surface mediated events to the genome by the production of cAMP. Cell surface mediated stimuli may be in the form of nutrient transport as in the Enterobacteriaceae or possibly in the form of changes in the ionic environment as in modulation of B. pertussis

2.3. The nature of the loss of adenylate cyclase activity during modulation

If AC is the target site of modulation, it would be expected that the observed loss of AC activity during modulation would be due to inactivation as opposed to repression of AC synthesis. Observing repression of AC synthesis would indicate that the target site of modulation must be another regulatory mechanism which governs expression of AC. Time-course studies on $MgSO_4$-induced modulation revealed that loss of specific intact-cell AC activity paralleled gain in biomass and thus

could be accounted for by complete inhibition of AC synthesis. Furthermore, loss of AC activity did not precede, but coincided with and paralleled the loss of the X bands and HSA. These results indicate that loss of these activities during modulation can be accounted for by the synchronous and complete repression of synthesis of the responsible components (ie. AC, pertussigen and the X polypeptides). This is consistent with recently reported results by Hall et al. (1982) who investigated the rate of loss of AC activity and the X bands during modulation. However, the results do not negate the possibility that in vivo cAMP metabolism is immediately altered upon transfer of X-mode cells to SS-C, and that cAMP regulates the expression of AC as well as other X-mode specific components.

Specific intact-cell AC activity did not change greatly during growth in SS-X suggesting that AC is synthesized at a constant rate during growth in such media. This contrasts with observations by Hewlett et al. (1979a) who reported that specific intact-cell AC activity increased by about 10-fold during the first few hours of growth. Perhaps this is related to the fact that these workers probably used a smaller inoculum of cells which had been grown on BG. In the present study a 20% (v/v) inoculum from a 48 h SS-X culture was used.

After incubation of X-mode B. pertussis in $SS\text{-}X_{CAP}$ and $SS\text{-}C_{CAP}$, specific intact-cell AC activity was about 60 - 80% of that after growth in SS-X; whereas after growth in SS-C, activity was about 1.0 - 2.5% of that after growth in SS-X. This observation suggests that AC is a fairly stable enzyme and that the loss of AC activity observed during modulation is not due to direct inactivation by prolonged exposure to the medium, but requires growth or protein synthesis. Similarly, protein synthesis or growth was required for the regain of AC activity during C to X modulation. This is presumably because the enzyme has to be synthesized de novo as opposed to being reactivated.

Time-course studies of modulation suggested that synthesis of AC

was repressed upon introduction of cells to C medium. To verify that AC was repressed during modulation attempts were made to purify AC and raise antiserum towards it so as to be able to detect the enzyme by immunological means. Sofaras is known, this is the first time attempts have been made to raise antiserum towards a prokaryotic AC. However, this was unsuccessful probably because of the poor yield of the purification method and the small amount of material injected into the rabbit. Excessive loss of activity (93%) occurred during Sephadex G-200 chromatography. Perhaps this step could be refined to give a yield comparable to the 50% yield achieved by Hewlett and Wolff (1976). Dye-ligand chromatography has been used successfully for the purification of bovine brain AC, but B. pertussis AC had little, if any, affinity for either BDS or RRA. Presumably, the structures of the active sites of B. pertussis AC and bovine brain AC are different, despite their similar responsiveness to calmodulin.

When investigating the effect of sera on AC activity, EGTA was included in order to inhibit the effect of AC activator present in serum which might have masked any inhibitory effect of the antiserum. EGTA (125 mM) greatly reduced AC activator activity present in sera, but not totally. This may perhaps relate to the observation by Greenlee et al. (1982) that EGTA does not totally eliminate responsiveness of B. pertussis AC to calmodulin. Neither antiserum raised towards purified AC nor the U.S. standard antiserum showed any significant inhibitory activity, with or without EGTA, for B. pertussis AC activity when compared to the effect of normal serum. This was presumably due to the lack of AC inhibitory antibody in these sera.

The low levels of extracellular cAMP which occurred after growth in media where X-mode components were lost, cannot be accounted for by direct inactivation of AC by pro-C-mode compounds. Most of these, when included in the assay mixture, caused no more inhibition of intact-cell AC activity than did the pro-X-mode salt, NaCl. The moderate inhibition caused by NaCl, Na_2SO_4, Na succinate, and Na butyrate was

presumably due to Na^+ ions which Hewlett and Wolff (1976) reported to cause mild inhibition of AC activity. The inhibition caused by nicotinic acid and nicotinamide was more marked (about 60% loss of activity). Nicotinic acid has been reported to decrease cAMP levels in adipocyte cells of several mammalian species (Aktories et al., 1980). Lee (1978) reported that 1 mM nicotine adenine dinucleotide (NAD) caused 65% loss of AC activity in Mycobacterium smegmatis. It is thus possible that the inhibition caused by nicotinic acid and nicotinamide was due to a metabolized product such as NAD.

These studies on the direct effect of salts on AC activity are limited in that AC activity in vitro (ie. under the conditions of the assay) does not necessarily reflect that of AC activity in vivo (ie. during growth). This is further complicated in B. pertussis by the unusual compartmentation of AC. Cytoplasmic AC, which is presumably responsible for intracellular cAMP produced during growth, may be subject to different regulatory mechanisms than intact-cell AC activity. On the other hand, inhibition mechanisms may require the integrity of intact cells and would thus not be apparent when using cell lysate as an enzyme source. In E. coli, glucose inhibition of AC requires glucose transport and is only observed when toluene-treated cells at $30^{o}C$ are used as an enzyme source (Harwood and Peterkofsky, 1975). Similarly, mammalian AC is a part of a membranous multicomponent system requiring membrane integrity for regulation (Helmreich and Bakardjieva, 1980). In vivo AC activity of E. coli is also influenced by factors such as growth rate (Wright et al., 1979; Calcott, 1982).

Growth in SS-X containing 4.1 mM nicotinic acid resulted in culture supernate levels of cAMP less than 5% of that after growth in normal SS-X. It is unlikely that the observed 60% in vitro inhibition of AC by nicotinic acid explains this fully. It is even more unlikely that this inhibition by nicotinic acid explains nicotinic acid-modulation as nicotinamide, which also caused 60% in vitro inhibition of AC, does not induce modulation (Wardlaw et al., 1976). Differences in the levels

of extracellular cAMP in X- and C-mode cultures cannot be attributed to differences in energy charge as intracellular pools of ATP are similar for X- and C-mode cells (Ezzell et al., 1981a).

Workers who have suggested that AC may play a role in modulation and phase degradation have assumed that cAMP plays a positive regulatory role in the synthesis of pertussigen, and the X polypeptides etc. However, the possibility existed that cAMP may in fact play a negative regulatory role. SS-C,or other modulating media, may inhibit excretion of cAMP and hence allow it to accumulate inside cells. Cyclic AMP may in turn inhibit synthesis of pertussigen, the X polypeptides and AC etc. This seemed equally plausible especially as it has been reported that CRP-cAMP may repress AC synthesis in E. coli (Botsford, 1981). This possibility now seems unlikely as extracellular cAMP was produced at the same rate whether large quantities of cells were incubated in $SS\text{-}X_{CAP}$ or $SS\text{-}C_{CAP}$. These results also suggest that SS-C does not directly alter in vivo cAMP metabolism unless for some reason growth is required for such alteration. Responsiveness to activator was the same whether X-mode cells were incubated in $SS\text{-}X_{CAP}$ or $SS\text{-}C_{CAP}$.

Presumably the apparent non-synthesis of cAMP which was observed when SS-C received a 20% (v/v) inoculum of X-mode cells was due to an inadequate level of cells. During growth in SS-X, AC is probably produced exponentially. Thus a large proportion of cAMP present in culture supernate after 48 h growth probably appears during the late exponential phase of growth when maximum levels of enzyme are present.

Chloramphenicol present in SS-C prevented loss of intact-cell AC activity, but this did not negate the possibility that AC is inactivated during modulation by the synthesis of a specific destructive or inactivating protein, or a protein which synthesizes a chemical inhibitor. Specific proteolysis of AC has been mentioned already as a possible explanation for the responsiveness to calmodulin which occurred after modulation. There was no evidence for AC-inactivation or destructive factors in

C-mode cell lysates when X- and C-mode cell lysates were mixed and incubated for various periods of time. These results also eliminate the possibility that the observed low specific AC activity in C-mode cells is due to a greater phosphodiesterase activity. As most AC is associated with the cell envelope, inactivation or destructive mechanisms may require the integrity of intact cells. This could be tested by adding chloramphenicol to the medium at various stages of growth during modulation such as to allow initial expression of a possible inactivating mechanism.

2.4. Manipulation of cAMP levels in *B. pertussis* cultures

It was thought that NaF, a strong inhibitor of AC activity, and bovine catalase containing AC activator (calmodulin), might be useful in determining whether cAMP plays a role in modulation.

NaF caused marked inhibition of *in vitro* AC activity. However, it had little effect on the production of extracellular cAMP during growth, although it inhibited growth at high concentrations. A possible explanation is that *B. pertussis* is impermeable to NaF. Alternatively, medium constituents may alter the responsiveness of AC to NaF. Calcott (1982) reported phencyclidine to be effective in reducing intracellular levels of cAMP in *E. coli*. It would be of interest to determine if this drug has the same effect with *B. pertussis*

Attempts to shift the critical concentration of $MgSO_4$ required to induce modulation with AC activator were unsuccessful. Cyclic AMP could not be detected in supernates of C-mode cultures (ie. cultures in which the X bands were lost) whether bovine catalase was present or absent. This is presumably because of the lack of active enzyme in C-mode cultures. X-mode cultures had the same levels of HSA and the X bands (as judged by eye) whether activator was present or absent. However, culture supernate levels of cAMP were 3.5 - 7.5 -fold higher in cultures containing bovine catalase. This observation does not necessarily eliminate a causal role for cAMP as maximum synthesis

of the X polypeptides and pertussigen may already occur in X-mode cultures and cannot be increased by higher levels of cAMP.

With one exception, loss of the X bands always correlated with low levels of extracellular cAMP (< 5 pmol ml^{-1}). In the one exception, partial loss of the X bands occurred after growth in medium containing 10 mM $MgSO_4$ and bovine catalase. However, the culture supernate level of cAMP was about twice as high as that which normally occurred in SS-X-grown cultures. This observation suggests that cAMP does not play a causal role in modulation. It is possible, however, that most of this cAMP was produced during the initial period of growth and during this period normal synthesis of the X polypeptides occurred; and that the synthesis of the X polypeptides ceased later during growth when AC activity was reduced. This possibility seems unlikely as time-course studies of modulation revealed that loss of *in vitro* AC activity did not precede but paralleled the loss of the X bands suggesting that if cAMP is involved in modulation, direct and immediate inactivation of *in vivo* AC activity could only account for modulation. An investigation of a time-course study of *in vivo* AC activity during growth in pro-I-mode medium containing calmodulin, may shed light on the above discussion.

Exogenous cAMP and dibutyryl cAMP (5 mM) had no effect in counteracting $MgSO_4$- or nicotinic acid-induced loss of the X bands or AC activity, or $MgSO_4$-induced loss of HA activity. Nor did either nucleotide alter synthesis of these components during growth in SS-X or H-X. This supports findings by Dobrogosz *et al.* (1979) and Ezzell *et al.* (1981a) who reported that exogenous cAMP at concentrations from 0.1 to 10 mM failed to relieve $MgSO_4$-induced loss of the X polypeptides or cytochrome d_{629}. This failure to relieve modulation does not appear to be due to the inability of the organism to take up nucleotide as present experiments demonstrated the incorporation of [^{3}H] cAMP and [^{3}H] dibutyryl cAMP into X-mode *B. pertussis* cells after incubation in SS-X and SS-C; and the incorporation of [^{3}H] cAMP into C-mode *B. pertussis* cells after incubation in SS-X and SS-C. Differences in "uptake" rates between

different conditions were slight and further experiments would be required to determine if differences were significant. At 1 mM [^{3}H] cAMP, "uptake" rates varied between 0.4 - 0.6 nmol min^{-1} $mg(dry\ weight)^{-1}$. This is slightly higher than that reported for E. coli (about 0.3 nmol $min^{-1}mg(protein)^{-1}$) when [^{3}H] cAMP was present at 1 mM (Goldenbaum and Hall, 1979). The greater rate of "uptake" observed for [^{3}H] dibutyryl cAMP, in the present study, may be related to the generally greater permeability of cells to this analogue. These preliminary results do not demonstrate conclusively the uptake of these nucleotides as binding of nucleotide to the cell wall is possible. Cells are generally impermeable to phosphorylated compounds such as nucleotides. Therefore, it is likely that if cAMP is taken up, uptake occurs by facilitated diffusion as in E. coli or possibly by active transport. If this is the case, uptake will presumably be a temperature dependent process and will exhibit saturation kinetics. Further studies would be required to substantiate uptake of these nucleotides. The extent at which these nucleotides are metabolised, if at all, once they enter the cell is not known. Paper chromatography of cells after uptake of [^{3}H] cAMP may be useful in assessing the extent at which cAMP metabolism occurs after uptake. The inability to detect intracellular cAMP in B. pertussis may perhaps be due to phosphodiesterase activity.

2.5. Phosphonomycin resistant mutants of B. pertussis

The isolation of cya and crp mutants of E. coli provides the most convincing evidence for many of the regulatory roles of cAMP in this organism. A rational basis for the isolation of B. pertussis AC mutants is difficult to deduce as none of the functions of AC in B. pertussis are known. Isolated phosphonomycin resistant mutants of S. typhimurium were reported to be almost all either cya or crp in equal proportions (Alper and Ames, 1978). Eight phosphonomycin resistant mutants of B. pertussis each possessed the same level of intact-cell AC activity

and produced the same levels of extracellular cAMP during growth as the wild type strain.

Phosphonomycin (L-cis-1,2-enolpropylphosphoric acid) is a structural analogue of phosphoenolpyruvate and specifically inhibits the first step of peptidoglycan synthesis, namely, the transfer of the enolpyruvate from phosphoenolpyruvate to UDP-N-acetylglucosamine. This inhibition is irreversible. Phosphonomycin has little effect on other enzymes such as pyruvate kinase where phophoenolpyruvate is the substrate (Rogers et al., 1980). In E. coli and S. typhimurium, phosphonomycin is actively transported by two independent transport systems; that for L-α-glycerophosphat and that for hexose phosphates (Chopra and Ball, 1982). Both these transport systems are subject to regulation by cAMP (Koch et al., 1964; Winkler, 1970). B. pertussis utilizes only amino acids as sources of energy, although it is possible that the organism possesses permeases for sugars which might be used as precursors for biopolymers. Perhaps phosphonomycin is transported by a different mechanism in B. pertussis. A third mechanism for phosphonomycin transport has been suggested which is related to the transport of inorganic phosphate (Kahan et al., 1974). Although the majority of phosphonomycin resistant mutants of bacterial species are defective in the two main transport mechanisms, it is possible that all or some B. pertussis mutants are defect in enolpyruvate transferase. There has only been one case reported where resistance in a bacterial species was due to a decreased affinity of the enolpyruvate transferase for the antibiotic (Venkateswaran and Wu, 1972) and this was a temperature sensitive mutant.

The observation that a phase IV strain of B. pertussis was much more resistant to phosphonomycin than it's phase I counterpart suggests that the transport protein(s) required for phosphonomycin sensitivity are lost during phase degradation. Dobrogosz et al. (1979) and Field and Parker (1979) reported that degraded strains were more resistant to certain antibiotics (viz. penicillin, ampicillin, streptomycin,

erythromycin and tetracycline). In this study C-mode variants of B. pertussis were as sensitive to phophonomycin as X-mode variants. In addition C-mode variants of B. pertussis would not grow on nutrient agar containing $MgSO_4$ while the phase IV variant would. Dobrogosz et al. (1979) reported that while phase IV variants were more resistant to the previously mentioned antibiotics, sensitivity was often enhanced in C-mode variants compared to X-mode variants. This suggests that phenotypic differences do exist between the C-mode and the phase IV phenotype. This perhaps relates to the observation of Leslie and Gardner (1931) that phase degradation was a step-wise process. Field and Parker (1979) reported that during serial passage of fresh isolates of B. pertussis, the ability to grow on solid medium without blood occurred before the loss of pertussigen and HLT. Perhaps the intial stage of phase degradation involves loss of sensitivity to various inhibitors. Sensitivity to certain antibiotics may require the same transport proteins which are responsible for sensitivity to the inhibitors present in convential media such as fatty acids, colloidal sulphur and sulphides, organic peroxides and manganous ions. However, none of the eight isolated phosphonomycin resistant mutants grew on nutrient agar. This suggests that phosphonomycin sensitivity and "nutrient-agar sensitivity" might be lost separately during phase degradation. The second stage of phase degradation might involve the loss of the same components lost during modulation, due to further mutations.

2.6. Respiration of substrates by variants of B. pertussis

Out of the eleven amino acids tested, washed suspensions of B. pertussis respired only L-glutamate, L-aspartate, L-proline, L-serine and L-alanine at detectable rates. This is consistent with findings by Jebb and Tomlinson (1951) who tested 21 amino acids. However, these workers reported the respiration rate for glutamate to be about 10-fold higher than reported here; the respiration rates they reported for other amino acids varied

considerably with strain. In the present study, glutamate, aspartate, and proline were respired at the greatest rates. This perhaps explains why these are the only amino acids which have been reported to support growth when present as the principle carbon source (Jebb and Tomlinson, 1955; Stainer and Scholte, 1971).

Various workers have reported that glutamate is the preferred carbon source and is utilized before other amino acids. In the present study, B. pertussis was grown in either SS-X or SS-C which contain only glutamate and proline as principle carbon sources. Cells were harvested during the mid-exponential phase (after 24 h growth) and were shown to possess the potential to respire aspartate, serine and alanine. This supports the observation of Lane (1970) that glutamate is not utilized preferentially unless perhaps glutamate directly antagonizes uptake of other amino acids. This would be entirely distinct from the mechanism in E. coli where glucose is preferentially utilized because the permeases and catabolic enzymes for other sugars are repressed.

While significant differences existed between respiration rates for different amino acids, there were no significant differences between the respiration rates for the different variants for any particular amino acid. As the C-mode and phase IV variants produce much lower levels of cAMP during growth than the X-mode variant, this suggests that cAMP is not involved in the regulation of the permeases or enzymes required for amino acid utilization. This also indicates that the mechanisms of modulation and phase degradation, whatever they may be, do not affect the respiration of amino acids. Similarly C modulation did not affect the ability of cells to respire acetate, pyruvate, lactate, succinate, or formate.

Phase IV and C-mode variants lack cytochrome d_{629}, but larger quantities of other cytochromes are synthesized (Ezzell et al., 1981a). This presumably compensates for loss of cytochrome d_{629} and accounts for respiration rates being similar between variants. Alternatively, amino acid transport may be the rate limiting step in B. pertussis .

2.7. Cyclic AMP binding activity in B. pertussis

So far as is known, all cAMP regulatory systems in bacteria require a functional CRP. Although a cAMP-binding protein has only been identified in a few bacteria, eg. E. coli, S. typhimurium, Caulobacter crescentus, Myxococcus xanthus, Benejea harveyi, and Alcaligenes eutrophicus, (Pastan and Adhya, 1976; Shapiro, 1976; Orlowski, 1980; Nealson and Hastings, 1979; Tait et al., 1981), there has never been a case reported where a cAMP-binding protein has not been found where cAMP clearly plays a regulatory role. In this study, [^{3}H] cAMP binding activity was detected in several strains of B. pertussis and, on average, was about half that obtained for E. coli crp^{+} and about four times that obtained for E. coli crp^{-}. It is not known whether this binding activity is attributable to a protein with a real physiological function. A more satisfactory way to detect cAMP binding proteins is to use 8-azido-[^{32}p] cAMP, a photoaffinity analogue of cAMP in conjunction with SDS-PAGE. This is a more sensitive method and yields information as to how many cAMP binding proteins exist and their molecular weight. This analogue competes with cAMP for CRP in E. coli (Aiba and Krakow, 1980) and has been used to detect a cAMP binding protein in M. xanthus (Orlowski, 1980).

While anti-E. coli CRP gave a precipitin reaction with a cytoplasmic E. coli fraction, it gave no reaction with B. pertussis cytoplasmic fractions even at twice the protein concentration of the E. coli cytoplasmic fraction. Using this same antiserum, Ouchterlony double-diffusion studies showed cross-reactivity between purified E. coli CRP and cell extracts of E. coli, Proteus mirabilis, Photobacterium fisheri, Klebsiella aerogenes, S. typhimurium, and Caulobacter crescentus (Anderson and Pastan, 1973). The cAMP receptor protein of Benekea harveyi is immunologically homologous with CRP from E. coli (Nealson and Hastings, 1979). Whether the cAMP receptor proteins of Alcaligenes eutrophicus or Myxococcus xanthus are immunologically related to that

cAMP receptor proteins exist which do not react with anti-*E. coli* CRP. The G + C ratio of *B. pertussis* lies at the fringe of that of organisms which possess a CRP immunologically homologous to *E. coli* (Table 36). Thus during evolution, an immunologically distinct CRP may have arisen in *B. pertussis*. On the other hand, AC may have evolved different functions in prokaryotes; in some it may have adopted a regulatory role requiring a functional CRP, whereas in others it may have adopted different roles not requiring CRP (eg. a role in virulence possibly altering cAMP-related functions in the host).

2.8. The possible role of adenylate cyclase in *B. pertussis*

Pastan and Perlman (1971) proposed four criteria for implicating cAMP in the mediation of cell phenomena, namely:- (1) cAMP levels must be correlated with observed cell function, (2) exogenous cAMP must alter the function in a predictable manner, (3) inhibitors or stimulators of cAMP metabolism must also alter the function, (4) the activities of the enzymes which synthesize or catabolize cAMP should be altered by the conditions which alter the cell function. With regard to the possible role cAMP plays in modulation in *B. pertussis*, only the first criterion has been satisfied and this is only insofaras culture supernate levels of cAMP correlated with production of the X bands and HSA after growth in various modulating media.

Several lines of evidence indicate that AC is not the target site for modulation and that low levels of cAMP are due to repression of AC synthesis, itself subject to the regulatory mechanism perturbed by the modulating environment:-

(1) Loss of intact-cell AC activity during $MgSO_4$-induced modulation could be accounted for by complete repression of AC synthesis.

(2) All searches for direct or indirect inhibition or inactivation mechanisms of AC activity, which might occur during modulation were negative.

Table 36: DNA relatedness of bacteria possessing a protein which cross reacts with anti-*E. coli* CRP

Bacterial species	G + C content[a] (moles %)	Cross reaction with anti-*E.coli* CRP (+) or no reaction (-)[b]	Regulatory role for cAMP
Clostridium sticklandii	26	-	NK[c]
Staphylococcus aureus	30-39	-	NK
Proteus mirabilis	38-41	+	NK
Methanosarcinia barkeri	44	-	NK
Photobacterium fisheri	40-46	+	NK
Benekea harveyi	45-48	+[d]	Yes[d]
Escherichia coli	50-51	+	Yes[e]
Salmonella typhimurium	50-53	+	Yes[e]
Klebsiella aerogenes	52-59	+	Yes[e]
Caulobacter crescentus	62-67	+	Yes[f]
Bordetella pertussis	61-70	-	NK

a, data from Starr *et al.* (1981); b, data from Anderson and Pastan (1973); c, not known (NK); d, Nealson and Hastings (1979); e, Botsford (1981); f, Shapiro (1976).

This study also suggests that it is unlikely that cAMP plays a secondary role in modulation in repressing synthesis of pertussigen and the X polypeptides as:-

(1) Loss of intact-cell AC activity during $MgSO_4$-induced modulation did not precede, but paralleled the loss of HSA and the X bands.

(2) After the growth and the transition of an X-mode variant to an I-mode variant, in medium containing calmodulin, culture supernate levels of cAMP were twice as high as for normal X-mode cultures, yet the X-bands were considerably diminished.

(3) Exogenous cAMP or dibutyryl cAMP in the growth medium,had no observable effect on intact-cell AC activity, HA activity or the presence of the X bands after growth of B. pertussis in X and C media.

Three observations suggest that during the course of evolution, AC may have adopted different physiological roles in E. coli and B. pertussis (1) 50% of AC activity in B. pertussis cultures was found in the culture supernate and 90% was extracytoplasmic, (2) the marked responsiveness of B. pertussis AC to calmodulin, (3) the lack of a CRP in B. pertussis immunologically similar to E. coli CRP. This may be related to the different physiologies of the two organisms: E. coli is a facultative anaerobe which utilizes sugars preferentially as sources of energy, B. pertussis is a strict aerobe which utilizes only amino acids as sources of energy.

Several of the components lost during modulation and phase degradation, such as pertussigen, HLT and F-HA, are strong or plausible candidates for pathogenic determinants. In view of this, perhaps AC is itself a virulence factor. Indeed several bacterial toxins act by penetrating target cells and activating the host-cell AC. Examples include cholera toxin, a heat-labile toxin of certain E. coli strains, and possibly pertussigen of B. pertussis. The former two toxins possess a subunit which enables attachment to specific receptors on the host cells and subsequent penetration of the other subunit which activates the eukaryotic AC. The mechanism of pertussigen is less well understood, but as pertussigen

consists of several subunits, some are presumably responsible for attachment to the target cells while others are presumably responsible for activation of host cell AC.

B. pertussis AC is a soluble enzyme consisting only of one polypeptide with a molecular weight of about 70,000 (Hewlett and Wolff, 1976). It is thus difficult to explain how AC gains access to the host cell cytoplasm unless it acts in conjunction with another factor. Leppla (1982) recently reported that one of three components of anthrax toxin, edema factor (EF), is in fact an AC. Purified EF required either boiled eukaryote cell lysate or calmodulin for detectable activity, but specific activity in their presence was very high (20 μmol $min^{-1}mg(protein)^{-1}$). EF requires another factor, protective antigen (PA), for it's toxic action (edema after intradermal injection in rabbits or guinea pigs). EF added with PA to Chinese hamster cells increased intracellular cAMP levels by 50-fold. Leppla (1982) suggests that PA interacts with specific receptors enabling EF to gain access to the cytoplasm.

The fact that B. pertussis AC also has marked responsiveness to calmodulin suggests that it may have evolved along similar lines and may be another virulence factor. Perhaps B. pertussis AC acts in conjunction with pertussigen. If pertussigen activates eukaryotic AC, it may activate B. pertussis AC. This may account for the large losses of activity which occurred during purification of AC in this study and by Hewlett and Wolff (1976). This could easily be tested by assaying AC activity in mixtures of purified AC and pertussigen. Adenylate cyclase activity is considerably higher in toxinogenic strains of V. cholerae than non-toxinogenic strains. Furthermore, AC activity increases dramatically during the stationary phase of growth when toxin production is highest suggesting that cholera toxin activates AC of V. cholerae (Chakrabarti and Ganguly, 1980). However, there was no evidence for pertussigen activating

B. pertussis AC in the present study, as no activation was apparent when C-mode cell lysate (which contains reduced levels of pertussigen when compared to X-mode cell lysate) was mixed with X-mode cell lysate and assayed for AC activity.

Padh and Venkitasubramanian (1980) could not detect cAMP binding activity in Mycobacterium smegmatis by several assay methods. These workers suggest that during evolution, mycobacteria have lost the capacity to synthesize a functional CRP and this may explain why mycobacteria have lost the ability to utilize many sugars (Padh and Venkitasubramanian, 1980). AC may have evolved to play a role in virulence in some mycobacteria. Observations by Lowrie et al. (1975) suggest that M. microti may protect themselves in phagocytes, by releasing cAMP into phagosomes at concentrations which would inhibit phagosomal and lysosomal fusion. Muse et al. (1979) reported that with nonimmune serum, phagocytosis by alveolar macrophages was greater for phase IV variants of B. pertussis, than for phase I variants. Furthermore, bactericidal activity of phagocytized phase IV variants was considerably greater than for phase I variants. This may perhaps be attributable to high AC activity of phagocytized phase I B. pertussis. Calmodulin present in phagocytes would further enhance AC activity. Cyclic AMP inhibits a number of functions in neutrophils and macrophages such as lysosomal discharge, phagocytosis, chemotactic motility and antibody-dependent cellular cytotoxicity (Ignarro, 1977).

Recently, Confer and Eaton (1982) reported that, after incubating neutrophils with B. pertussis extract, the intracellular level of cAMP in the neutrophils was higher than ever reported before. In addition, these neutrophils had high AC activity which these workers attributed to B. pertussis AC due to it's heat stability (100°C for 5 min). However, in the present study, 100°C for 10 min destroyed all detectable B. pertussis AC activity. Hewlett and Wolff (1976) reported that purified B. pertussis AC lost 90% of activity after 20 min at 56°C. There have been no previous reports of B. pertussis AC being heat stable at 100°C for 5 min.

Furthermore, Confer and Eaton (1982) did not report having tested the heat sensitivity of B. pertussis AC themselves. Heat-stable AC activity in neutrophils after ingestion of B. pertussis cells is a puzzling observation which as yet cannot be explained by ingestion of B. pertussis AC or any of the other known biologically active components of B. pertussis.

Bemis and Kennedy (1981) suggested that Bordetella AC may play a role in ciliostasis by depleting epithelial cells of ATP. This could only be explained, however, if AC was able to penetrate host cells. Ciliostasis by B. pertussis is more likely explained by the recently reported tracheal cytotoxin (Goldman et al., 1982).

3. Possible Mechanisms of Modulation

Time-course studies of $MgSO_4$-induced modulation indicated that synthesis of AC, pertussigen and the X polypeptides was completely inhibited at an early stage and pre-existing X-mode components were merely diluted out with growth. This is consistent with recently published results which indicated that the X polypeptides and AC were diluted out in accordance with the growth rate (Hall et al., 1982). The observed rate of loss of pertussigen was less convincing due to the insensitivity of the HSA test. Idigbe et al. (1981) reported that strain 18334, grown in modified Hornibrook medium containing 20 mM $MgSO_4$, underwent a rapid loss of HSF, LPF, HLT, MPA and the X bands: much more so than could be accounted for by a simple growth-dilution effect. This was concluded from an analysis of six independent experiments. These workers suggested that selective destruction took place during modulation. Idigbe et al. (1981) observed a much slower growth rate; the mean generation time during exponential growth was 6 - 7 h while that observed in the present study was about 3.2 h. Differences in results may be due to differences in either growth rate, the strain used or the medium used.

In the present study, growth rates were consistently slightly

higher during growth in SS-C than in SS-X, whether determined by total counting or viable counting methods. A possible explanation is that the energy which is conserved when synthesis of X-mode components ceases, is used to synthesize biopolymers required for growth. However, in other studies (Idigbe et al., 1981) growth rates were the same whether cells were grown in X or C medium.

Studies with chloramphenicol indicated that the loss of HSA, AC activity and the X bands during modulation was not due to a direct prolonged exposure to C medium, but required growth or protein synthesis. There was no evidence for destructive or inactivation factors for pertussigen, which might have been synthesized during growth in SS-C, when X-mode and C-mode cell lysates were mixed together and incubated for 24 h at 35°C. This was the case for strain Taberman grown in SS-X and SS-C and for strain 18334 grown in H-X and H-C. However, moderate destruction or inactivation of pertussigen would not be noticable due to the wide 95% confidence limits obtained for the HSA tests. It is also possible that destructive mechanisms require the integrity of intact cells or are extremely heat-labile. Synthesis of selectively destructive or inactivation factors would be a very inefficient way to control an organism's physiology and is not generally observed in bacteria. However, it is not known if modulation has a real physiological counterpart in nature. The reason why the toxin of Bacillus anthracis was only discovered after in vivo studies, was because of an inactivating substance produced during growth in vitro (Harris-Smith et al., 1957). Similarly, Elliott (1945) reported that the M protein of certain strains of group A streptoccoci was destroyed during certain growth conditions due to synthesis and activation of a specific protease.

The critical concentration of $MgSO_4$ required to repress synthesis of the X polypeptides was around 10 mM (using increments of 1 mM). In six series of concentrations, an I-mode variant which exhibited partial loss of the X bands was only observed once. Thus the concentration of M required to induce the I-mode variant is extremely critical,

lying between 10 - 11 mM. Lacey (1960) reported that 6.5 - 8.5 mM $MgSO_4$ in BG, induced the I-mode variant as judged by moderate antigenic changes. Presumably these growth conditions cause only partial repression of X-mode specific components.

So far, all reported pro-C-mode organic compounds contain a carboxyl group. That nicotinic acid can induce modulation, but not nicotinamide, suggests that the carboxyl group may be important in inducing modulation. It seems unlikely that nicotinic acid-induced modulation is related to nicotinic acid transport as nicotinic acid is present in such excess in normal SS-X that during growth, nicotinic acid uptake would always be saturated and at maximum velocity (W. McCPheat, personal communication). Not all organic acids induce modulation. Na glutamate is present in SS-X at 57 mM. However, Lane (1970) found that growth in Cohen and Wheeler medium, where the casein hydrolysate was replaced by high levels of Na glutamate, resulted in loss of HSA and MPA. Presumably, other factors are involved such as molecular size and conformation, dissociation constants, pH etc.

Recently Schneider and Parker (1982) reported that two analogues of nicotinic acid, 6 chloronicotinic acid and quinaldic acid, both having a pyridine ring substituted with a carboxyl group in positions 2 or 3, were also able to induce modulation of B. pertussis. Two analogues of nicotinamide, isoniazid and 2-chloronicotinamide, did not induce modulation but inhibited nicotinic acid-induced loss of X-mode antigens. Isoniazid also prevented nicotinic acid-induced loss of the X-bands whereas 2 chloronicotinamide only prevented loss of X-mode antigens. Perhaps these nicotinamide analogues are able to compete with nicotinic acid for the target site of modulation, but lack the carboxyl group required to induce modulation. Modification of the carboxyl group by methylation or acetylation abolished the modulating activity of nicotinic acid stressing the importance of the carboxyl group in inducing modulation. The ability of 2 chloronicotinamide to inhibit nicotinic acid-induced loss of X-mode antigens, but not the X bands, may be of practical importance in

the production an improved vaccine and requires further investigation.

As yet, there is very little information on which to base a satisfactory explanation of modulation. The control of X-mode and phase I specific components by plasmids seems unlikely as Kloos et al. (1979) showed that there was no correlation between variation and presence of extra-chromosomal material. The majority of components lost during modulation are associated with the cell surface or released into the medium. Thus there are three stages at which modulation could act: transcription, translation, or post-translation modification and translocation into the cell envelope. The third seems unlikely as HLT, which has a cytoplasmic location, is lost during $MgSO_4$-induced modulation. It has been suggested, however, that post-translation modification may be involved in the synthesis of active HLT (Pittman, 1979). In the present study, "cytoplasmic" levels of AC were also reduced during $MgSO_4$-induced modulation. Of the former two possibilities, regulation at the level of transcription is by far the most common and efficient mechanism in prokaryotes.

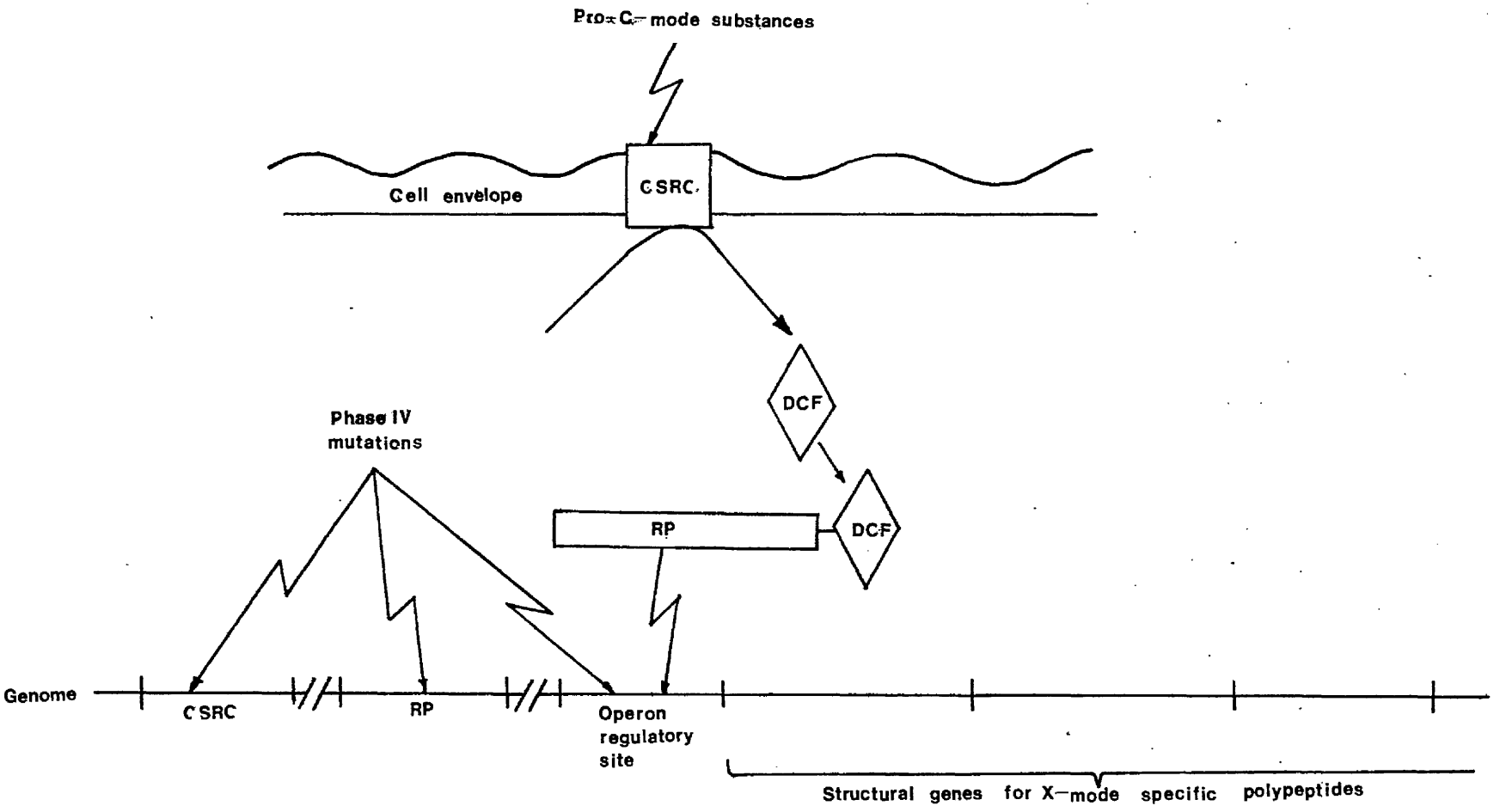
McPheat (1981) found that nicotinamide (which does not induce modulation as does nicotinic acid), upon entering B. pertussis cells, was very rapidly metabolized to nicotinic acid. This suggests that nicotinic acid-induced modulation may begin at the cell surface.

A possible mechanism for modulation is given in Fig. 19. This model is highly speculative and is based on the assumptions that the first events of modulation (or at least nicotinic acid-induced modulation) commence at the cell surface, modulation acts at the stage of transcription, and that most transcription regulatory mechanisms require the involvement of a cytoplasmic protein.

Pro-C-mode compounds may interfere with a regulatory component located at the cell surface. Interference with this component may affect the synthesis of a diffusable cytoplasmic factor (DCF) such as a co-repressor, co-inducer or regulatory nucleotide. DCF may either selectively activate or inactivate gene expression. X-mode specific proteins may be encoded on the same or different operons, all sensitive to DCF.

Figure 19: A possible mechanism for modulation

Pro-C-mode substances interact with a cell surface regulatory component (CSRC). This in turns alters the production of a diffusable cytoplasmic factor (DCF). DCF acts in conjunction with a cytoplasmic regulatory protein (RP) which together, either activate or inactivate X-mode specific gene expression. Phase IV mutations may occur at genes encoding CSRC or RP, or at the operon regulatory site (only if one operon is involved).


Pro-C-mode substances
Cell envelope
CSRC
DCF
Phase IV
mutations
RP
DCF
Genome
CSRC
RP
Operon
regulatory
site
Structural genes for X-mode specific polypeptides

DCF would presumably act in conjunction with a regulatory protein such as a repressor, or a protein analogous to CRP in E. coli, or a RNA polymerase component. Some pro-C-mode substances may act as co-repressors themselves similar to Fe^{++} in the regulation of diphtheria toxin (Murphy et al., 1978). Phase IV mutations could exist either in the RP, the cell surface regulatory component, or the regulatory site of the operon (only if the X-mode specific components are all linked on the one operon).

Glutamine synthetase has been demonstrated in B. pertussis cell extracts by Zakharova et al.(1979). This enzyme plays a key regulatory role in controlling the utilization of nitrogen sources in the enterobacteria and several other bacterial species (Magasanik, 1977). As B. pertussis utilizes only amino acids as carbon or energy sources, it is conceivable that glutamine synthetase may play a key regulatory role in this organism. However, it is difficult to apply the regulatory model of glutamine synthetase in the enterobacteria to the physiology of B. pertussis. As B. pertussis utilizes only amino acids as energy sources, the organism is never faced with a situation where growth is limited by a lack of nitrogen source before being limited by a lack of energy source. In fact, cultures of B. pertussis contain high levels of NH_4^+. In a preliminary experiment (results not shown), low glutamine synthetase activity (about 0.3 µmol γ-glutamyl hydroxamate h^{-1}) was detected in X-mode, but not C-mode, cell lysates of strains Taberman and 18334 after assay mixtures were left overnight at 35°C (the γ-glutamyl hydroxamate transferase assay described by Shapiro and Stadtman (1970) was used). The significance of this observation is not known. Glutamine synthetase and it's modifying enzymes (modification of glutamine synthetase is an important aspect of it's regulatory mechanism) have a cytoplasmic location in the enterobacteria (Ginsburg and Stadtman,1973). Presumably, if glutamine synthetase is involved in modulation , it is involved at a cytoplasmic stage.

Understanding the mechanism of modulation will probably depend on a greater knowledge of the genetics of B. pertussis about which nothing is known at present. Perhaps the recent report on the ability to transfer chromosomal genes via. plasmids in B. pertussis (Weiss and Falkow, 1982) is the starting point of a new line of research in pertussis. Most of the phathophysiological activities of B. pertussis are difficult to assay. In view of this, gene fusion studies may be useful in studying the regulation of virulence factors in B. pertussis. Currently, lac Z fusion studies are being used to study the regulation of membrane proteins in E. coli. A more extensive survey of pro-C-mode substances and anti-modulating substances (such as isoniqzid and 2 chloronicotinamide which Schneider and Parker (1982) reported to inhibit nicotinic acid-induced loss of X-mode antigens) on the physiology of B. pertussis may also shed light on the mechanism of modulation.

Out of the sera of fifty convalescent pertussis patients, forty two possessed X-mode antibodies and three possessed C-mode antibodies (Lacey, 1960). This observation suggests that modulation is not merely a laboratory phenomenon, but occurs in nature. While only two of the forty two sera with X-mode antibodies were from adults, all three sera with C-mode antibodies were from persons over the age of 19 who had only a mild cough. Thus perhaps the C-mode variant is a pathogenically dormant state associated with adults who act as carriers. The environment of the upper respiratory tract of the adult host may trigger the C-mode state. The substances which induce the C-mode state in nature may be totally different from those in the laboratory.

Modulation is not unique to B. pertussis; non-genetic variation of surface antigens occurs in several microorganisms (Lacey, 1961). Yersinia pestis is similar to B. pertussis in that it also undergoes phenotypic changes when grown at different temperatures. In the gut of the flea vector, virulence of Y. pestis is masked by growth at 25^{o}C. When such bacilli are phagocytized at 37^{o}C by monocytes (in the mammalian

host) they multiply intracellularly, they then emerge as virulent organisms possessing the antiphagocytic factors F1 (a capsular protein) and the VW antigens (Sonnwirth and Swartz, 1980).

The ability of B. pertussis to modulate in nature requires further investigation. Vaccines prepared from X-mode cells may not protect persons from infection with C-mode B. pertussis. Thus C-mode carriers could exist which trigger epidemics when the population of susceptible infants reaches the threshold level.

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APPENDICES

Appendix 1: Media and Diluents

1. 1% (w/v) Casamino acids

	$g \cdot L^{-1}$
Casamino acids (Difico)	10
$MgCl_2 \cdot 6H_2O$	0.1
$CaCl_2$	0.016
NaCl	5

The above ingredients were dissolved in distilled water, the pH was adjusted to 7.1 with 1.0 N NaOH, and the mixture was autoclaved (15 psi, 121°C for 15 min).

2. BG-X and BG-C

Washed, peeled and diced potatoes (312 g) were boiled in 2.5 L of distilled water for about 30 min until soft. The mixture was filtered through gauze muslin to give a potato extract infusion.

	BG-X	BG-C
Potato extract infusion	2 L	2 L
NaCl	5 g	--
$MgSO_4 \cdot 7H_2O$	-	10 g
Glycerol	10 ml	10 ml
Agar (Oxoid No. 3)	20 g	20 g

The pH was adjusted to 7, and the mixtures were kept in a Koch-steamer for 15 min and autoclaved in 50 or 250 ml amounts.

3. Stainer and Scholte medium

This is the "16G + 1P" medium originally described by Stainer and Scholte (1971), but with cysteine replacing cystine.

		g L^{-1}
1.	L-glutamate (monosodium salt)	10.72
2.	L-proline	0.24
3.	NaCl	2.5
4.	KH_2PO_4	0.5
5.	KCl	0.2
6.	$MgCl_2.6H_2O$	0.1
7.	$CaCl_2$	0.02
8.	Tris	6.075
9.	L-cysteine	0.04
10.	$Fe_2SO_4.7H_2O$	0.01
11.	Ascorbic acid	0.02
12.	Nicotinic acid	0.004
13.	Glutathione	0.1

Ingredients 1 - 8 were dissolved in distilled water, the pH was adjusted to 7.6 with HCl and the medium was autoclaved. Ingredients 9 - 13 were sterilized by membrane filtration and were separately added to the medium just before use. This medium is referred to as SS-X. SS-C was prepared in the same way, but with NaCl replaced by 5 g L^{-1} $MgSO_4.7H_2O$

4. Modified Hornibrook medium

	g L^{-1}
1. Casamino acids (Difco Technical)	10
2. $CaCl_2$	0.002
3. KCl	0.2
4. K_2HPO_4	0.25
5. $MgCl_2.6H_2O$	0.025
6. NaCl	5
7. Nicotinamide	0.001
8. Soluble starch	1
9. Glutathione	0.01

Components 1 - 7 were dissolved in distilled water. Starch was dissolved in boiling water before adding to the rest of the medium. The pH was adjusted to 7.0 with Na_2CO_3 and the medium was autoclaved. Glutathione was filter sterilized and added to the medium just before use.

5. Yeast Glucose medium

	g L^{-1}
1. Yeast Extract (Difco)	10
2. KH_2PO_4	5.6
3. K_2HPO_4	28.9
4. Glucose	10
5. Thiamine	0.01

Ingredients 1 - 3 were dissolved in 900 ml distilled water. Ingredients 4 and 5 were filter sterilized in 100 ml distilled water and added separately to the rest of the medium.

Appendix 2: Standard curves

Figure A1: Standard curve relating A_{660nm} to protein concentration and dry weight

△, ▲, protein concentration for X- and C-mode cell suspensions respectivel

□, ■, dry weights for X- and C-mode cell suspensions respectively.

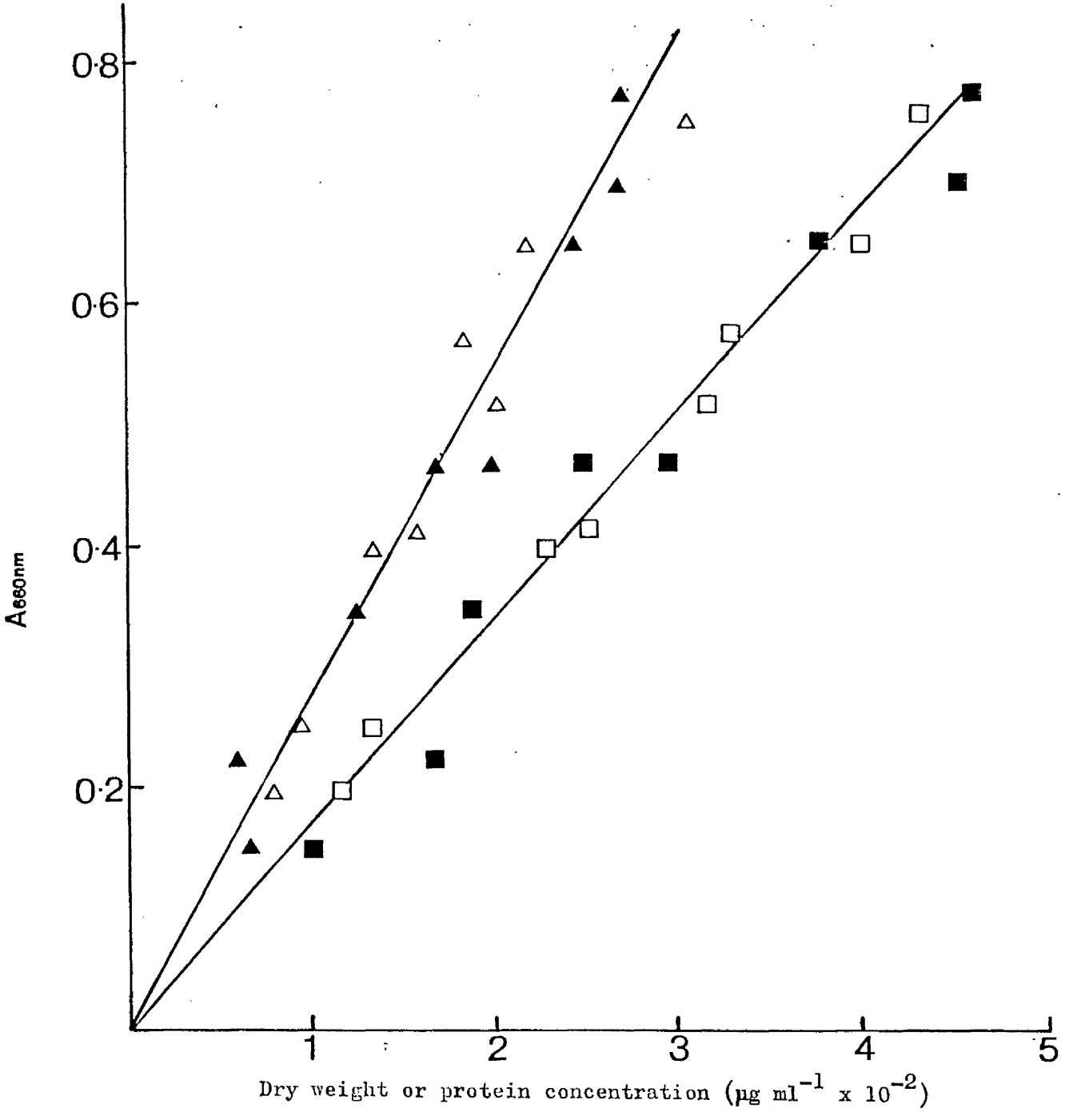
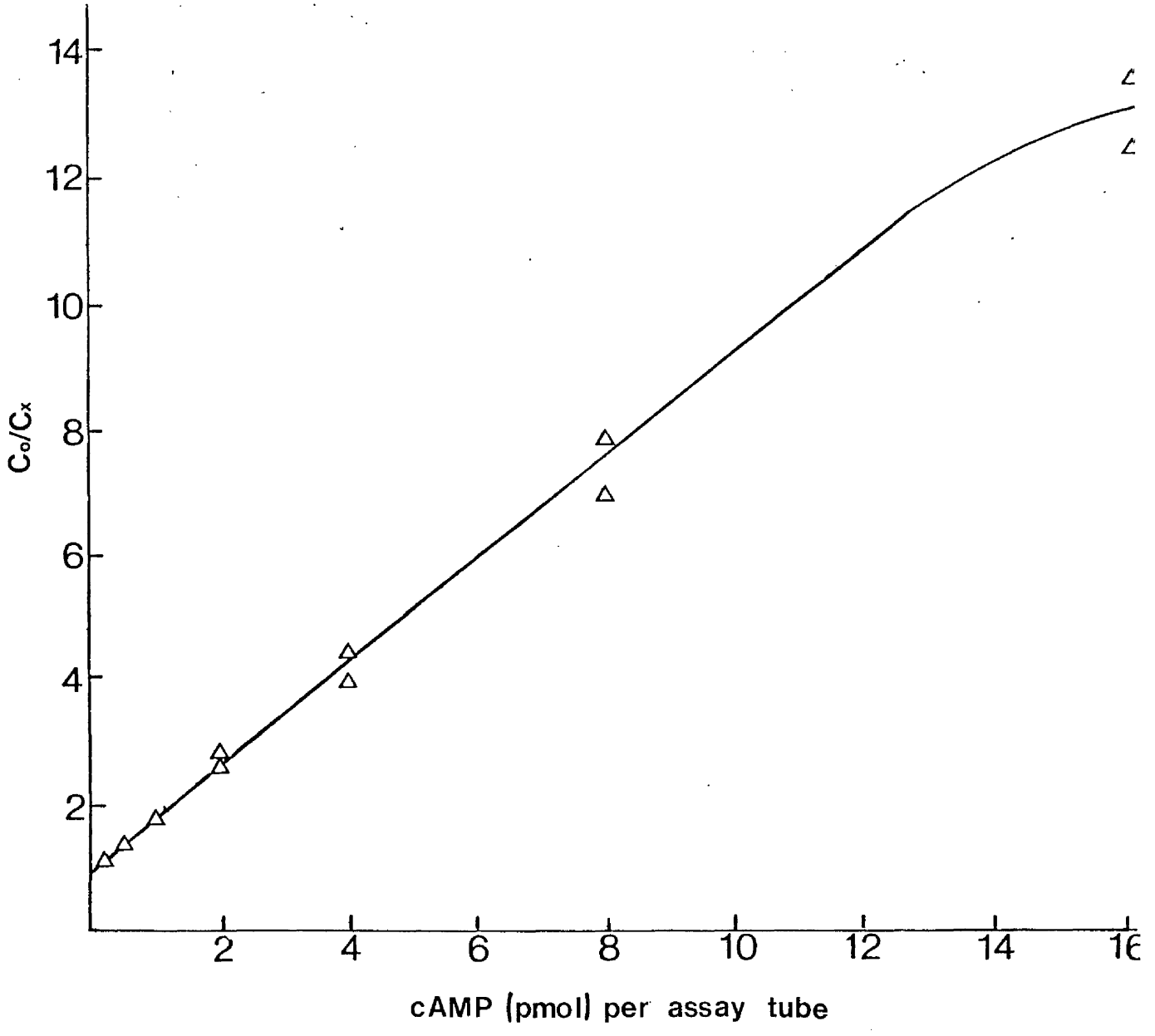

0·8
0·6
0·4
0·2
A660nm
1
2
3
4
5
Dry weight or protein concentration (µg ml^{-1} x 10^{-2})

Figure A2: Typical standard curve obtained from a cAMP assay


14
12
10
8
6
4
2
C_o/C_x
2
4
6
8
10
12
14
16
cAMP (pmol) per assay tube

Appendix 3: Assay Buffers and Reagents

Cyclic AMP assay buffer

	$g\ L^{-1}$
Tris	6.06
Na_2 EDTA	0.149

The above ingredients were dissolved in distilled water and the pH was adjusted to 7.5 with HCl.

Cyclic AMP standards

Cyclic AMP was dissolved in cAMP assay buffer to give 320 pmol ml^{-1}. Doubling dilutions were done in assay buffer to give a series of dilutions from 5 pmol ml^{-1} to 320 pmol ml^{-1}. These were used as standards in the cAMP assay.

[^{3}H] cAMP reagent for cAMP assay

[^{3}H] cAMP was diluted in cAMP assay buffer to give 0.5 µCi ml^{-1} (specific activity 25 Ci $mmol^{-1}$) and stored at - 20°C.

Cyclic AMP binding protein reagent

Binding protein, prepared from rabbit muscle, was donated by Dr J. P. Durham, Dept. of Clinical Onocology, Glasgow University. Stock suspensions were stored in 50% (v/v) ethylene glycol at -20°C Cyclic AMP binding protein buffer was prepared as follows:-

Tris	1.2 g
Na_2 EDTA	297 mg
Bovine Serum Albumin	200 mg
β-mercaptoethanol	0.278 ml

The above ingredients were made up to 200 ml with distilled water and the pH was adjusted to 7.5 with HCl. Sufficient binding protein was

dissolved in buffer to bind 55% of label in the absence of unlabelled cAMP under the conditions of the cAMP assay.

Charcoal reagent

Activated Charcoal	2.5 g
Bovine Serum Albumin	250 mg

These were dissolved in 100 ml cAMP assay buffer and stored at -20°C. During the assay, the charcoal reagent was stirred continuously for 15 min before adding to the assay tubes and stirring was continued throughout the assay.

Buffer A (for cAMP binding activity assay)

Tris	242 mg
K acetate	118 mg
Dithiothreitol	15.4 mg
Na_2 EDTA	7.4 mg

These were made up to 200 ml with distilled water and the pH was adjusted to 8.2 with glacial acetic acid.

Triton-Toluene scintillant

Triton X-100	1 L
Toluene	2 L
2,5-Diphenyloxazole	8 g
1,4-Di-2-(4-methyl-5 phenyl-oxazolyl benzene	200 mg

This scintillant was not used within 10 h of preparation.

SSX and SSC buffers

These buffers were prepared in the same way as SS-X and SS-C media (Appendix 1) except that ingredients 1,2 and 9 - 13 were ommitted.

Barbitone buffer, pH 8.3

Barbitone sodium (8.5 g) was dissolved in 450 ml distilled water, the pH was adjusted to 8.3 with HCl, and the volume was made up to 500 ml with distilled water.

0.75% Barbitone agar, pH 8.3

Ionagar was made up to 0.75% (w/v) in barbitone buffer, pH 8.3, and placed in a Koch-steamer for 30 min.

Appendix 4: Calculation of Respiration Rates

The percentage decline in O_2 saturation was determined both before and after the addition of substrate. The former was due to respiration of endogenous reserves.

The endogenous respiration rate ($\mu l\ O_2\ min^{-1}$) was calculated from the formula:-

$$A/100 \times B/C$$

where A is the percent O_2 consumed before addition of substrate, B is the volume (μl) of O_2 in saturated buffer at $35^{o}C$ (approximately 17 μl) and C is the time (min) O_2 uptake was monitored for (usually 3 or 4 min). The respiration rate after the addition of substrate was calculated in the same way . The endogenous rate was subtracted from this to determine the respiration rate of the substrate.

Appendix 5: Gels and Buffers for SDS-PAGE

Stock solutions

1. 30% Acrylamide

Acrylamide	30 g
NN'methylenebisacrylamide	0.8 g

This was made up to 100 ml with water and filtered through Whatman No. 1 filter paper.

2. Running buffer, pH 8.8

Glycine	72.06 g
Sodium dodecyl sulphate (SDS)	5.0 g
Tris	15.14 g

This was made up to 5 L with water.

3. Staining solution

Commassie blue R250 (Sigma)	1.25 g
Methanol (50% (v/v))	454 ml
Glacial acetic acid	46 ml

4. Destaining solution

Methanol (50% (v/v))	100 ml
Gacial acetic acid	150 ml
Water	1750 ml

5. Solubilizing buffer

Tris	242 mg
EDTA	58 mg
β-mercaptoethanol	10 ml
Glycerol	20 ml
Bromophenol blue (0.1%(w/v))	4 ml
SDS (20%(w/v))	20 ml

This was made up to 100 ml with water and the pH was adjusted to 8.0.

Preparation of gels

1. Separating gel

30% Acrylamide	27.5 ml
1 M Tris-HCl, pH 8.8	28.1 ml
SDS (20%(w/v))	375 µl
N,N,N',N',-Tetramethyl-ethylenediamine (TEMED)	19 µl
Ammonium persulphate (1%(w/v))	7.5 ml
Water	11.5 ml

2. Stacking gel

30% Acrylamide	3.4 ml
0.5 M Tris-HCl, pH 6.8	5.0 ml
SDS (20% (w/v))	100 µl
TEMED	5 ml
Ammonium persulphate (1% (w/v))	2 ml
Water	9.5 ml

Gels were prepared in 8.0 x 8.0 x 0.3 cm glass moulds. The bottom 6 cm consisted of separating gel whilst the remaining 2 cm consisted of stacking gel.

Appendix 6: Data of HSA Tests

Table A1: The HSA results used to calculate the data presented in Tables 11 and 27 for strain Taberman

Addition to the medium	Deaths/challenged								HSD_{50}
	Dose per mouse (ou.ml)								
	20	10	5	2.5	4	2	1	0.5	
NaCl					5/5	4/5	2/5	1/5	1.05
$MgSO_4$	2/5	1/5	0/5	0/5					22.70
$MgCl_2$					5/5	3/5	1/5	1/5	1.35
Na_2SO_4	1/5	1/5	0/5	0/5					24.72
Na lactate	5/5	4/5	2/5	0/5					6.02
Na succinate	2/5	1/5	1/5	0/5					16.12
Nicotinic acid	3/5	1/5	1/5	0/5					17.40
NaCl and bovine catalase					5/5	3/5	1/5	0/5	1.62
$MgSO_4$ and bovine catalase	2/5	1/5	1/5	0/5					16.12
NaCl[a]					5/5	4/5	2/5	0/5	1.24
$MgCl_2$					4/5	3/5	0/5	0/5	2.20
Na lactate	5/5	3/5	1/5	0/5					
Na butyrate	2/5	1/5	0/5	0/5					

a, these results are from a second independent HSA test. Only those for the "Na butyrate" sample were used for Table 10 and were compared using the "NaCl" sample as the standard.

Table A2: The HSA results used to calculate the data presented in Table 11 for strain 18334

Addition to the medium	Deaths/challenged								HSD_{50}
	Dose per mouse (ou.ml)								
	20	10	5	2.5	4	2	1	0.5	
NaCl					5/5	4/5	2/5	1/5	1.03
$MgSO_4$	1/3	1/5	0/5	0/5					23.81
$MgCl_2$					5/5	3/5	2/5	0/5	1.41
Na_2SO_4	3/5	0/5	1/5	0/5					18.49
Na butyrate	2/5	0/5	1/5	0/5					22.49
Na caprylate	1/5	1/5	0/5	0/5					30.11

Table A3: The HSA results used to calculate the data presented in Table 11 for strain 18323

Addition to the medium	Deaths/challenged Dose per mouse (ou.ml)								HSD_{50}
	20	10	5	2.5	4	2	1	0.5	
NaCl					5/5	3/5	2/5	0/5	1.41
$MgCl_2$					5/5	3/5	2/5	1/5	1.20
$MgSO_4$	3/5	2/5	0/5	0/5					15.17
Na_2SO_4	1/5	0/5	0/5	0/5					38.90
Na butyrate	4/5	0/5	1/5	0/5					14.57
Na caprylate					5/5	4/5	1/5	1/5	1.19

Table A4: The HSA results used to calculate the data presented in Figure 6

Sample	Deaths/challenged									HSD_{50}
	Dose per mouse (ou.ml)									
	20	10	5	2.5	1.25	4	2	1	0.5	
Inoculum						5/5	4/5	2/5	0/5	1.23
Exp. 1 4 h		5/5	5/5	2/5	1/5					2.23
8 h	5/5	3/5	1/5	0/5						8.15
20 h	3/5	2/5	0/5	0/5						15.15
Exp. 2 4 h		5/5	4/5	3/5	1/5					2.29
8 h	5/5	2/5	1/5	1/5						7.76
20 h	2/5	1/5	1/5	0/5						16.79

Table A5: The HSA results used to calculate the data presented in Table 17

Growth conditions and sampling time		Deaths/challenged								HSD_{50}
		Dose per mouse (ou.ml)								
		20	10	5	2.5	4	2	1	0.5	
SS-X	48 h					5/5	3/5	1/5	0/5	1.72
$SS\text{-}X_{CAP}$	48 h					5/5	2/5	1/5	0/5	1.85
SS-C	48 h	0/4	1/5	0/5	0/5					29.12
$SS\text{-}C_{CAP}$	24 h					5/5	2/5	0/5	0/5	2.18
$SS\text{-}C_{CAP}$	48 h					5/5	1/5	1/5	1/5	1.72

Table A6: The HSA results used to calculate the data presented in Table 19

Growth conditions	Deaths/challenged Dose per mouse (ou.ml) 20	10	5	2.5	1.25	0.625	HSD_{50}
SS-X			5/5	4/5	3/5	2/5	0.83
$SS\text{-}X_{CAP}$	1/5	1/5	0/5	0/5			43.09
SS-C	0/5	1/5	0/5	0/5			65.95

Table A7: The HSA results used to calculate the data presented in Table 21

Sample	Deaths/challenged Dose per mouse (ou.ml)						HSD_{50}
	20	10	5	2.5	1.25	0.625	
X-mode cell lysate			5/5	3/5	2/5	1/5	1.50
C-mode cell lysate	0/4	0/4	0/4	0/3			> 20
X/C-mode cell lysate mixture	5/5	5/5	3/5	1/5	0/5		4.03

Table A8: The HSA results used to calculate the data presented in Table 22

Sample	Deaths/challenged										HSD_{50}
	Dose per mouse (ou.ml)										
	20	10	5	2.5	1.25	0.63	4	2	1	0.5	
X-mode cell lysate (no incubation)							5/5	4/5	2/5	1/5	1.1
X-mode cell lysate (24 h at 35°C)							5/5	3/5	2/5	0/5	1.4
X/C-mode mixture sample 1		5/5	5/5	4/5	2/5	0/5					1.6
X/C-mode mixture sample 2		5/5	5/5	3/5	1/5	0/5					2.0
C-mode cell lysate	3/5	1/5	0/5	0/5							17.0