

ADP-ribosylation of BSA by Pertussigen
Follows the same Mechanism as
ADP-ribosylation of Transducine

Submitted in Partial Fulfillment of the Requirements
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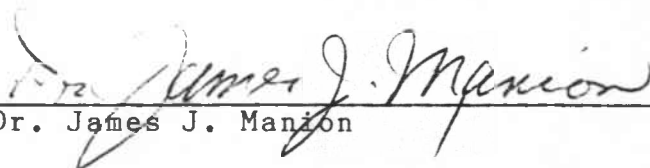
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John F. Cabrera



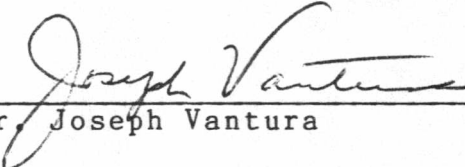
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ABSTRACT

Pertussigen is a toxin responsible for the ADP-ribosylation of the Adenylate cyclase system inhibitory component. It has been verified through polyacrylamide gel electrophoresis that the ADP-ribosylation occurs only if the reaction mixture is supplemented with ATP, NAD and DTT. There is also a linear relationship between time and the amount of protein which is ADP-ribosylated. Pertussigen can also ADP-ribosylize Bovine Serum Albumin (BSA) which offers researchers an inexpensive way to study the action of pertussigen.

Introduction

Bordetella pertussis is a bacterium parasitic to man and is responsible for inducing whooping cough. Past studies show that the disease is caused by a five subunit protein secreted by the bacterium called pertussigen. The bacterium adheres to the ciliated epithelial cells of the respiratory tract and causes irritation of the cells lining the tract. In vitro studies have shown that the protein secreted by this bacterium causes the ADP-ribosylation of transducine.

This paper is concerned with the mechanism of ADP-ribosylation of transducine and BSA by pertussigen. The research attempt to show the effects of certain reactants on ADP-ribosylation and tests the reliability of BSA studies.

Literature Review

Pertussis toxin or pertussigen is an ADP-ribosyltransferase, an enzyme that splits NAD into nicotinamide and transducineribose and transfers the ADP-ribose remnant to a regulatory protein of eukaryotic cells (1). For some time pertussigen and cholera toxin have been known to cause a stimulatory effect on adenylate cyclase. This effect is caused, in both cases, by the interference in the regulatory component of the adenylate cyclase system causing the intracellular cyclic AMP level to rise (1).

The hormone sensitive adenylate cyclase is regulated in both directions. Certain hormones and neurotransmitters stimulate adenylate cyclase and raise the cyclic-AMP level while other hormones and neurotransmitters (cf. Fig. 1) inhibit adenylate cyclase thus lowering the intracellular level of cyclic AMP (cited in [1]). Regulatory proteins, called N_S and N_I , are involved in the stimulation and inhibition of adenylate cyclase. these proteins, along with other coupling factors R_S , R_I are active in the GTP-bound form: i.e. in this configuration, N_S leads to an activation of adenylase cyclase, whereas N_I in the GTP-bound form would lead to an inhibition of adenylase cyclase (cited in [1]).

The two regulatory proteins, N_S and N_I , are the actual points of attack for the cholera toxin and pertussigen. Cholera toxin ADP-ribosylates the regulatory N_S protein. This fixes the N_S protein in the active form and the result is a stimulation of

adenylate cyclase. Pertussigen ADP-ribosylates the inhibitory N_i protein. This prevents activation of the regulatory N_i protein thus regulation of adenylate cyclase is not possible (cited in [1]).

The ADP-ribosylation of the N_i regulatory site by pertussigen has been studied and several significant conclusions

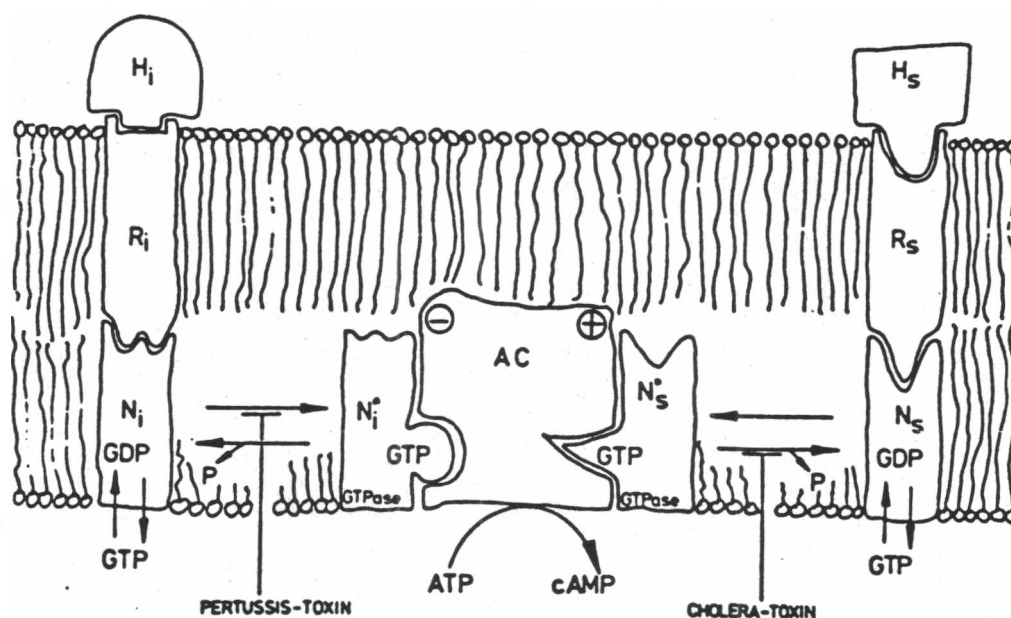


Figure 1

Regulation of hormone-sensitive adenylate cyclase. The adenylate cyclase (AC) activity is increased by the stimulatory N_s protein, and decreased by the inhibitory N_i protein. Both regulatory proteins are active in the GTP-bound form (N_s^* , N_i^*) and become inactive after hydrolysis of the bound GTP by an N_s - or N_i -associated GTPase. Renewed activation of the regulatory proteins, and thus stimulation of inhibition of adenylate cyclase takes place only after a GDP/GTP exchange; this latter is made possible through interaction with the stimulatory ($H_s R_s$) or inhibitory ($H_i R_i$) hormone-receptor complex.

Cholera toxin ADP-ribosylates N_s , and thus blocks the inactivation of N_s^* . This persistently activates the adenylate cyclase.

Pertussis toxin ADP-ribosylates N_i , and thus prevents the activation of N_i by inhibitory hormonal factors. [1]

have been drawn. Katada and Ui demonstrated, *in vitro*, that the activity of adenylate cyclase is enhanced in the presence of pertussigen and speculated that the ADP-ribosylation of a regulatory membrane protein was taking place (4). This was the case when pertussigen was shown to ADP-ribosylate the active (GTP-binding) and inactive conformations of transducine. Experiments with transducine (this protein hydrolyses GTP and c-GMP in retinal photoreceptors) have shown that pertussigen ADP-ribosylates the active and inactive conformations of this protein (6).

The ADP-ribosylation of transducine is dependant on two factors. The rate of ribosylation is known to vary with different concentrations of NAD because the ADP-ribose group for the ribosylation is derived from NAD by the cleavage of the nicotinamide group (3). ATP also promotes pertussigen-catalyzed ADP-ribosylation by interacting with the toxin and not the protein substrate, thus having an allosteric effect on pertussigen (5). The data reported in this paper are concerned with the ADP-ribosylation of transducine and BSA by the action of pertussigen.

Materials and Methods

Materials

Transducine was used in the experiment in its purified form and was stored at -70 degrees C. BSA was prepared from a Bio-Rad supply of the purified protein. A solution of 1 mg/ml of BSA was prepared, the pH was adjusted to 7.5, the solution was then heated to 95 degrees C. for 5 minutes then stored at -70 degrees C. A stock solution of 1.0 M tris buffer was made and adjusted to pH 7.5. Other stock solutions made were 0.1 M DTT, 0.1 M thymidine, 10 mM sonicated DPPC, 10 M GTP, 10 M ATP, and 2% solution of SDS which were all stored at -4 degrees C. NAD used was P labeled and was obtained from Sigma Chemical Co. The purified pertussigen used was donated by J.J. Munoz and was used in 1:1, 1:10, 1:100, and 1:1000 dilutions. TCA was made at 90% and 6% concentrations and stored at 6 degrees C. 12% polyacrylamide gels with SDS were used in the slab form and they were .75 mm in thickness. Nitrocellulose filter paper was used with a vacuum filtration system. A Beckman Geiger counter was used to measure radioactivity and Liquid Scintillation Fluid (LSF), which contained toluene plus PPO and POPOP, was used in conjunction with this machine.

Transducine assay

The transducine assay was performed by making a reaction mixture containing 25 ul of 1 M tris solution at pH 7.5, 12.5 ul of 0.1 M DTT, 12.5 ul of 0.1 M thymiline, 12.5 ul of 10mM DPPC,

25 ul of 1mM ATP, 10 ML of NAD P, and 27.5 ML of H₂O to bring the total volume of the reaction mixture to 125 ul. A second reaction mixture was made with 25 ul of 1mM GTP instead of ATP.

The reaction vessel contained 40 ul of transducine and 10 ul of pertussigen in a 1:1 dilution. The reaction was started by adding 50 ul of the reaction mixture to the reaction vessel. The reaction mixture was tested for the presence of ADP-ribosylation of transducine at 0, 5, 10, 30, 60, and 120 minutes after reaction was started. The reaction was stopped at each time interval by adding 10 ul of the reaction into 1 ml of a 2% SDS solution. After reaction was stopped 125 ul of a 95% TCA solution was added to the vessel containing the 10 ul of the reaction and the 1 ml of SDS. The resulting solution was then filtered by vacuum filtration and .45 u nitrocellulose filter paper was used in the system. The filter papers were washed 10x with 10 ml of 6% TCA solution and a final wash was done with 90% ethanol. The filter papers were then dried in an incubator at 60 degrees C. The radioactivity of the paper was then measured in a Beckman geiger counter with the aid of LSF. The data were recorded and graphed.

BSA assay

For the BSA assay the reaction mixture was the same as described for the transducine assay. ATP and GTP were interchanged in this reaction mixture as it was for the transducine reaction mixture. The reaction vessel contained 20 ul of a 1 mg/ml solution of BSA, and 10 ul of pertussigen in a

1:1 dilution. The reaction was started by adding 50 ul of the reaction mixture into the reaction vessel and the reaction was tested ADP-ribosylation of BSA using the same procedure followed for transducine. Samples that were tested were removed at the same time intervals as transducine assay samples were removed.

Different concentrations of BSA were tested by diluting the amount of BSA in the reaction vessel. Again the same reaction mixture was used and ATP and GTP were also interchanged. Four reaction vessels were used and each contained 2 ul, 12.5 ul, 20 ul, and 40 ul of BSA respectively. Each reaction vessel received 10 ul of a 1:1 dilution of pertussigen and a respective amount of H₂O to bring the volume of the vessels to 50 ul. The reaction was started by introducing 50 ul of the reaction mixture into the reaction vessels and 10 ul samples were tested for ADP-ribosylation of BSA at the same time intervals as the transducine assay.

Different concentrations of pertussigen were tested in each assay. The concentrations tested were prepared by making dilutions of 1:1, 1:10, 1:100, and 1:1000 and introducing 10 ul of each dilution into a reaction vessel. These tests were conducted with ATP and then compared to similar tests with GTP.

Polyacrylimide gels

The reaction mixture used contained 50 ul of 1 M tris, 25 ul of DTT, 25 ul of 0.1 M thymidine, 30 ul of NAD ³²P, 50 ul of 10 mM ATP or 10 mM GTP, and 65 ul of H₂O. The reaction vessels contained 20 ul of BSA, 20 ul of H₂O and 10 ul of pertussigen. 50 ul of the reaction mixture was added to the vessel to start

the reaction. Samples were taken out at the same time intervals as in the transducine assay and the reaction was stopped by adding 10 ul into 1 ml of 2% SDS. Some reactions were allowed to run uninterrupted for the full 2 hours before being stopped. Once stopped the reaction was then loaded into a 12% polyacrylimide gel with SDS and voltage was applied to run the gel. After the tracking dye of the gel had run through, the gel was removed from its apparatus, stained with camasi blue, and dried at 90 degrees C. on a gel dryer. After the gel was dry it was exposed on x-ray film for 24 hours and the film was analyzed.

In the reaction vessels used here different concentrations of pertussigen (1:1, 1:10, 1:100, and 1:1000 dilutions) were tested. The reaction was also tested with ATP and with GTP in place of ATP. Pertussigen was also tested in a heat-inactivated form.

Results

The radioactivities of the filtrates from the transducine assay and BSA assay were recorded and are graphed in the following figures.

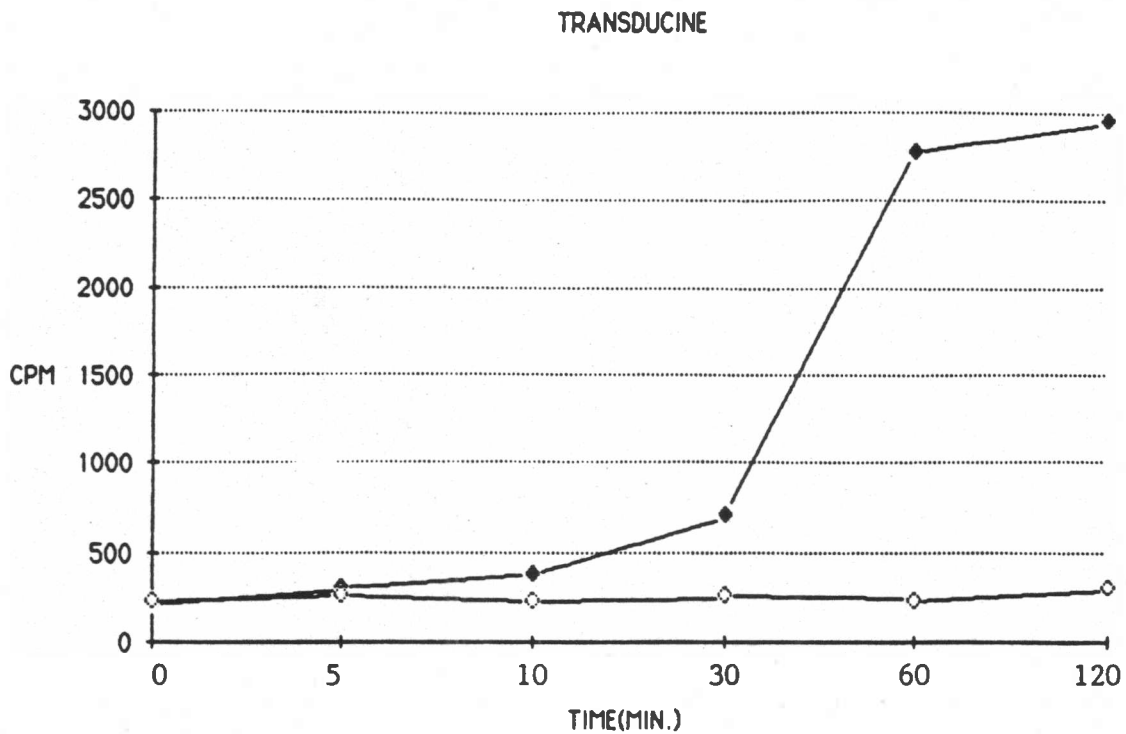


Figure 2.

The effects of different concentrations of pertussigen toxin on ADP-ribosylation of transducine with GTP. \diamond represent counts per minute of a reaction containing a 1:10 dilution of pertussigen and \blacklozenge represents a 1:1 dilution of pertussigen. The activity measured at the various time intervals suggests a strong correlation between the amount of ADP-ribosylized transducine and the concentration of pertussigen present in the reaction mixture.

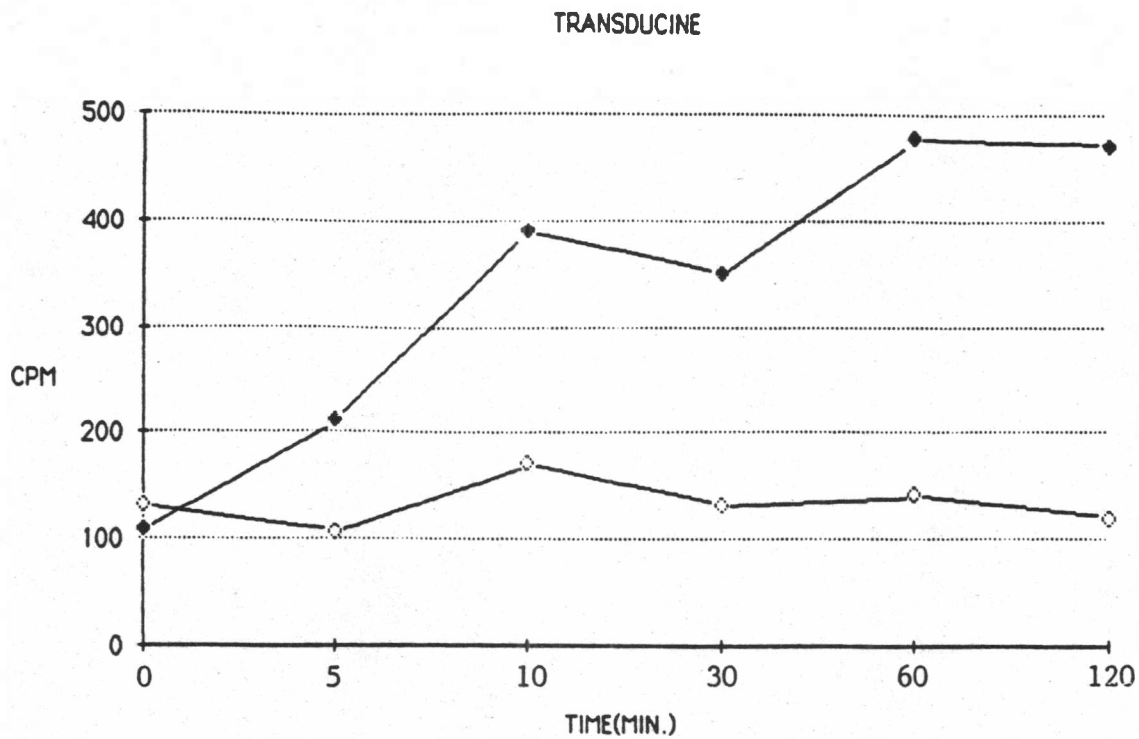


Figure 3.

The effects of different concentrations of pertussigen toxins on ADP-ribosylation of transducine with ATP. \diamond represent counts per minute of a reaction containing a 1:10 dilution of pertussigen and \blacklozenge represents a 1:1 dilution of pertussigen. The activity measured at the various time intervals suggest a strong correlation between the amount of ADP-ribosylized transducine and the concentration of pertussigen present in the reaction mixture.

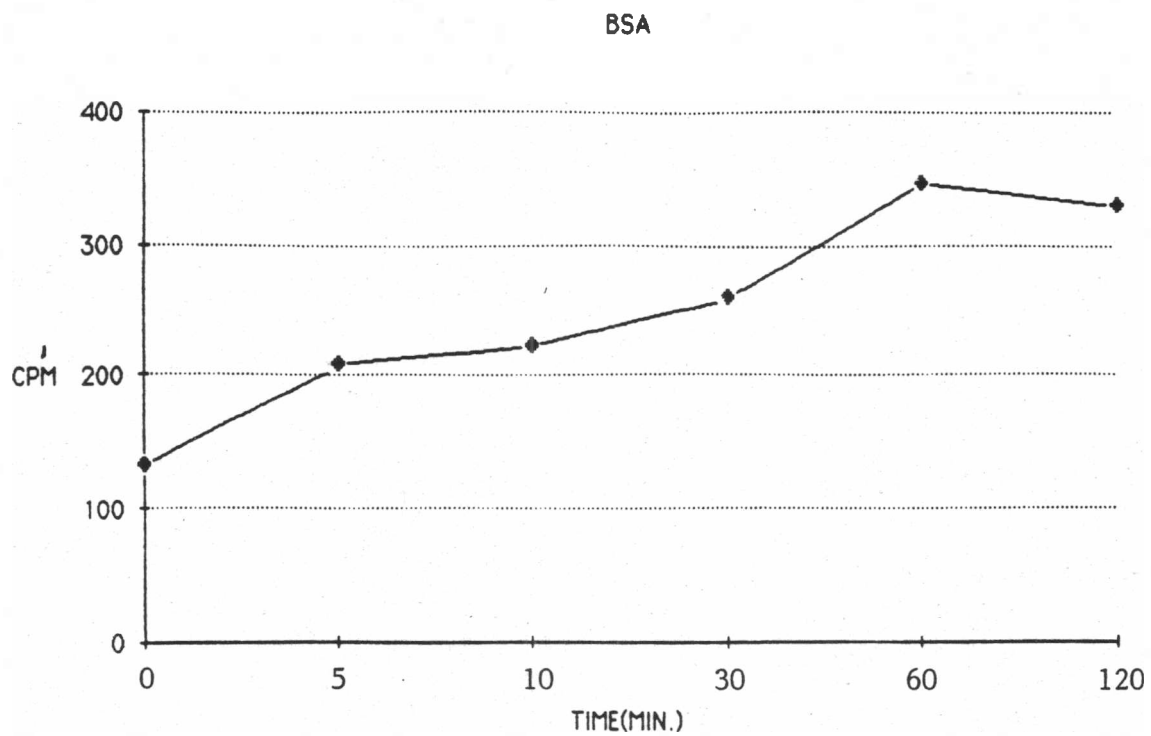


Figure 4

ADP-ribosylation of BSA in the presence of ATP. The graph shows a linear relationship between time and counts pre minute (cpm) found in the reaction mixture due to ADP-ribosylation. Pertussigen concentration used here was 1:1.

BSA

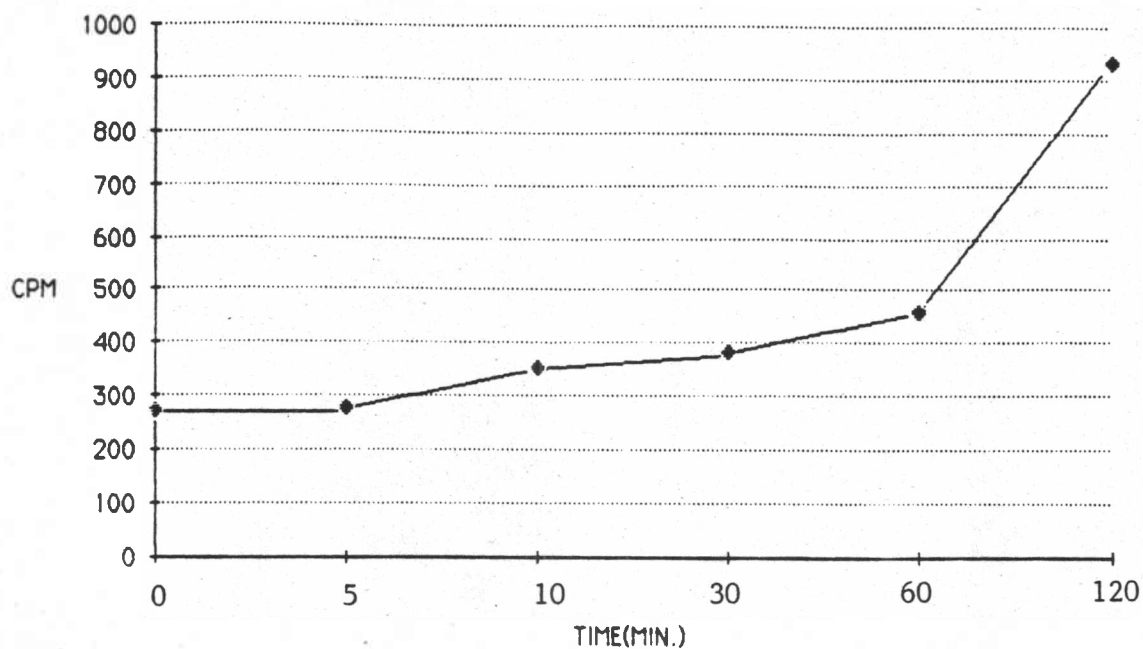


Figure 5

ADP-ribosylation of BSA in the presence of ATP. The dilution of pertussigen used was 1:1 and the graph shows a linear relationship between time and counts per minutes (cpm). Note the increase of ADP-ribosylation between 60 and 120 minutes. This suggests that a period of pre-incubation is required before BSA is ADP-ribosyloized. This is a characteristic which showed up in other graphs.

BSA

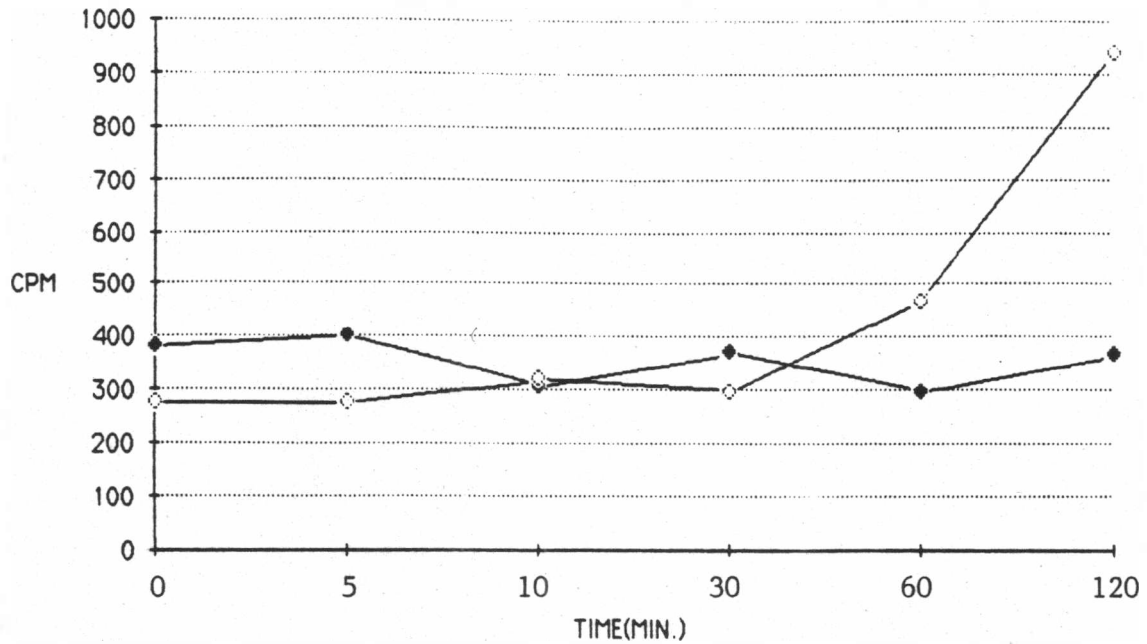


Figure 6

The effect of different dilutions of pertussigen on ADP-ribosylation of BSA. The dilutions of pertussigen tested were 1:1 (◇) and 1:10 (◆). The reaction was carried out in the presence of ATP and data shows a strong correlation between ability to ADP-ribosylate BSA and concentration of pertussigen present.

The polyacrylimide gels revealed that there are certain components of the reaction that had to be present in a certain concentration. ATP had to be present in order for ribosylation to occur. DTT also had to be present for reaction to take place. ADP-ribosylation did not occur if ATP was replaced by GTP. Pertussigen had to be present in the 1:1 dilution for any ribosylation to occur. When samples were taken at time intervals there was a noticeable relationship between time and amount of activity present in the gel. These results obtained from the polyacrylimide gel electrophoresis are represented in Figures 7-9.

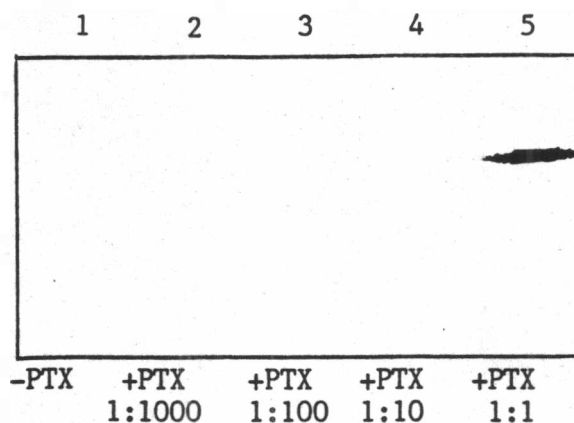


Figure 7

The effect of different concentrations of pertussigen (PTX) on ADP-ribosylation of BSA. The reaction mixture contained ATP, NAD P, and it was allowed to run for 2 hours. The reaction was then put in a 2% SDS solution to stop all activity and loaded into 12% Polyacrylimide gels. The electrophoresis was executed, the gels were dried, then exposed on x-ray film for 24 hours. The results of the x-ray exposure is what is represented in the figure. The only dilution of pertussigen which showed ADP-ribosylation of BSA was a 1:1 dilution.

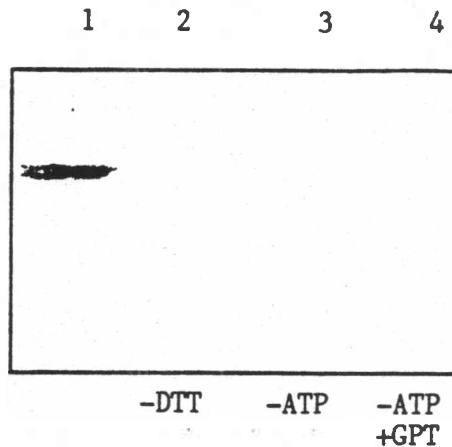


Figure 8

The effects of ATP, DTT, and GTP on ADP-ribosylation. The results represented in this figure are obtained from exposing the polyacrylimide gel on x-ray film for 24 hours. In lane 1 a reaction mixture with all reactants present was used and served as the control. Lanes 2 and 3 had DTT and ATP absent from the reaction mixture and no ADP-rybosilation was observed. Lane 4 received GTP instead of ATP and repeated experiments showed no ADP-rybosilation occurred in this situation.

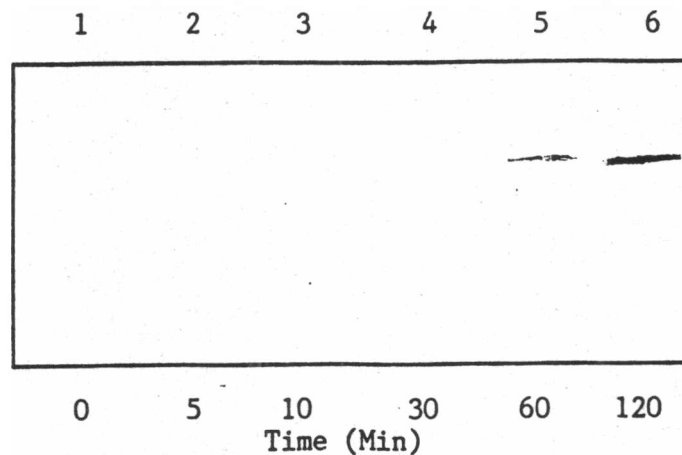


Figure 9

The amount of ADP-ribosylated BSA vs time. Lanes 1-6 represent samples taken from the reaction at various times. The results represented in this figure are those obtained from an x-ray film after being exposed to the gel for 24 hours. One can see a linear correlation between the time and amount of radioactivity present in the gel.

Discussion and Conclusion

Early in our research the transducine assay was very difficult to interpret because the data was so inconclusive. The quantification of data proved to be difficult and unreliable. The standard deviation varied so much from one experiment to the next that conclusions could not be drawn. The BSA and transducine assay seemed to be working with both GTP and ATP (Fig. 2-6). Later this data was contradicted using polyacrylimide gel electrophoresis. We believe the data that was obtained from the geiger counter is unreliable because our reaction volume was too small and the instrument was not sensitive enough to detect the small quantities of radioactivity we were looking for.

By exposing the gels on x-ray film we demonstrated that ATP was necessary in order for ADP-ribosylation to occur thus contradicting earlier data which suggested that the reaction could occur with GTP as well as ATP. We believe ATP must interact with the pertussigen before any ADP-ribosylation takes place thus pertussigen must have a site specific for ATP binding.

The polyacrylinimide gels revealed that ADP-ribosylation only occurred with a 1:1 dilution of pertussigen. Little or no ADP-ribosylation was observed with concentrations that were less than 1:1. The amount of ADP-ribosylized BSA was found to vary linearly with time (Fig). DTT was necessary for ADP-ribosylation. DTT is a reducing agent and it appears the pertussigen must be reduced in order to activate its action.

The conclusion of this paper is that ADP-ribosylation is occurring on both BSA and transducine by a similar mechanism. This mechanism requires the presence of ATP and NAD. ATP is apparently reacting with pertussigen in some way to promote the completion of the ADP-ribosylation. NAD is essential in this reaction because it supplies the ADP-ribose group which is attached to the adenylate cyclase inhibitory component. DTT is also essential to the reaction because of its role in reducing the pertussigen which is a requirement for activation. Thymidine and DPPC were only present in the reaction mixture because of their catalytic effect on the reaction. Since transducine is a membrane-bound protein, the DPPC provided a hydrophobic environment for the protein to orient itself and therefore created an environment similar to that of a membrane. It is not known why thymidine has a catalytic effect on the reaction but it has been demonstrated in previous research (6). Thymidine and DPPC are not essential in the reaction mixture and DPPC was left out in the reaction mixture which was analyzed by gel electrophoresis. The fact that BSA gets ADP-ribosylation by pertussigen is important. The commercial availability of this protein and its low cost makes it ideal to study the mechanism for ADP-ribosylation.

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