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ALTERATIONS IN ADENYL CYCLASE ACTIVITY AND GLUCOSE UTILIZATION OF *BORDETELLA PERTUSSIS*-SENSITIZED MOUSE SPLEEN*

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Abstract—The effects of *Bordetella pertussis* sensitization on the cyclic AMP content, adenyl cyclase activity and glucose uptake and utilization of mouse spleen were examined. Injection of *B. pertussis* vaccine into mice resulted in a decrease (36 per cent) in cyclic AMP content of the spleens 4 days after vaccination, while decrease in blood glucose (53 per cent) occurred within 6 hr. Experiments *in vitro* using membrane fractions of spleens have shown that epinephrine, norepinephrine, isoproterenol and sodium fluoride stimulated adenyl cyclase activity to a much less extent in those derived from pertussis-sensitized mice than in those from nonsensitized mice. When nonsensitized mice were injected with propranolol (5–40 mg/kg), a beta-adrenergic blocking agent, cyclic AMP content decreased (58 per cent) to a level equivalent to that obtained by pertussis sensitization. Whereas epinephrine and aminophylline enhanced spleen cyclic AMP content (62 per cent) and blood glucose levels (115 per cent), pretreatment with propranolol blocked this enhancement. These observations are consistent with the hypothesis that adenyl cyclase activity in the spleeen is a function of a beta-adrenergic receptor which is subject to blockade by pertussis sensitization. Uptake and utilization of glucose is markedly enhanced in spleen slices of pertussis-sensitized animals compared with that in nonsensitized, reflecting a probable generalized increase in peripheral uptake which may, in part, explain the hypoglycemia and adjuvancy observed in sensitization.

Bordetella pertussis-sensitized mice display a heightened susceptibility to a wide variety of pharmacological, immunological and pressor agents [1]. Similarities between beta-adrenergic blocking agents and pertussis vaccination with regard to sensitization of these animals to pharmacological mediators such as histamine and serotonin were observed [2, 3]. As a result, the mechanism of pertussis sensitization is regarded as involving beta-adrenergic blockade of receptors of the sympathetic nervous system. However, the blockade of receptors by synthetic adrenergic blockers is only transient although immediate, whereas the blockade by the cell constituents of *B. pertussis* is slow reacting but long lasting [3].

The enhanced histamine response of the sensitized animals represents an acquired hypersensitivity to mediators of the allergic response, independent of classic immunological mechanism. On the other hand, there are several analogies between the physiological and immunological responses of pertussis sensitization and atopic hypersensitivity [4]. Therefore, pertussis sensitization has acquired in recent years increasing popularity as an animal model for the study of atopic abnormalities [5–8].

Pertussis sensitization of mice results in mild hypoglycemia, attenuated hyperglycemic response to stimulation by epinephrine and increased glycogen synthesis [2]. These alterations in carbohydrate metabolism conceivably involve cyclic AMP [9]. Whereas the effects of adrenergic mediators are well known on the adenyl cyclase activity of several tissues [10], little is known about the effects of these agents on the spleen. The study of the effects of pertussis sensitization on the spleen is of particular interest in view of the alterations in adjuvancy, lymphoid circulation and the marked hyperplasia of this organ [1].

It was the aim of this investigation to examine the influence of pertussis sensitization on the synthesis of cyclic AMP in spleen. In addition, the uptake of glucose by spleen slices and the adenyl cyclase activity of spleen membrane preparations were studied *in vitro*, thus obtaining conditions free from the regulatory influences of the autonomous nervous system. The results indicate the equivalence of pertussis sensitization with beta-adrenergic blockade as suggested earlier [5].

MATERIALS AND METHODS

Female CFW mice (20–24 g), a strain recognized for extreme histamine sensitivity upon pertussis vaccination, were obtained from Carworth Animal Farms, New York, N.Y., caged in groups of six to ten animals, and fed *ad lib.*

Sensitization. Merthiolated suspensions of *B. pertussis* (4×10^{10} organisms/ml) were obtained from Eli Lilly & Co. (Indianapolis, Ind.) and diluted 1:2 in sterile, pyrogen-free physiological saline. The diluted vaccine (0·5 ml) was injected intraperitoneally (i.p) in a single dose to sensitize the mice. The animals were

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sacrificed 5 days after vaccination for the use of the spleen unless stated otherwise. The animals were maximally sensitized to histamine by day 5 (LD_{50} = 8 mg/kg). Hyperplasia of the spleen was clearly discernible at this time. In some experiments, propranolol at various doses was injected (i.p.) as a histamine-sensitizing agent.

Challenge and collection of the samples. Normal and sensitized animals were challenged with epinephrine hydrochloride (0·5 mg/kg) and aminophylline (50 mg/kg) by injecting them together (i.p.) as a solution in sterile saline 20 min before sacrifice. Control animals received an equivalent volume of sterile saline.

Samples of blood were collected by decapitation of the animals, deproteinized and their glucose concentration was determined by the method described by Nelson [11]. Spleens were removed, immediately frozen in liquid nitrogen and later analyzed for cyclic AMP content.

Measurement of adenyl cyclase activity. Membrane fractions of spleen of normal and pertussis-sensitized (5 days) animals were prepared and adenyl cyclase activity was assayed as outlined by Sutherland *et al.* [12]. The procedure was, briefly, as follows. Spleens were rapidly removed from the mice and chilled in cold physiological saline. All the operations thence were carried out near 4°. Excess blood was removed from the spleens by washing. The latter were then homogenized in sucrose (0·25 M) for 20 sec at low speed in a Waring blender. The debris was removed by centrifugation at 600 *g*. The supernatant containing the membranes was centrifuged at 2000 *g* for 17 min and washed several times in a solution containing glycyl-glycine buffer (pH 7·4, 2 mM) and $MgSO_4$ (1 mM). The membrane fragments were finally suspended in Tris buffer (pH 7·4, 0·4 M) containing ATP (2·0 mM), $MgSO_4$ (3·5 mM) and caffeine (6·7 mM) incubated at 30° for 15 min. Epinephrine, norepinephrine, sodium fluoride (NaF) or isoproterenol were added to the reaction mixture as indicated in the Results section. NaF was used in these experiments to obtain maximum stimulation of adenyl cyclase. The incubation was ended by immersing the test tubes in boiling water for 3 min and then cooling in an ice-cold water bath. The samples were diluted with HEPES buffer (pH 7·4, 10 mM) and assayed for cyclic AMP content. Aliquots containing membrane fractions were removed for protein determination.

Extraction and estimation of cyclic AMP from the tissue samples. Spleens weighing 50–300 mg were rapidly frozen in liquid nitrogen. The frozen tissue samples were homogenized in 20·0 ml of HCl (0·1 N) for 30 sec in a Waring blender at low speed. The procedures outlined by Robison *et al.* [13] were followed for extraction and estimation of cyclic AMP. Aliquots of the homogenates were removed and protein content was determined by the procedure of Lowry *et al.* [14]. The recovery of cyclic AMP from the samples was assayed by the addition of ^{3}H-cyclic AMP (50 µl/25,000 cpm) at the time of homogenization. The radioactivity was assayed in a Packard liquid scintillation spectrometer using a scintillation liquid containing naphthalene (250 g), 2,5-diphenyloxazole (PPO) (17·5 g) and dimethyl POPOP (1,4-bis-[2-(4-methyl-5-phenoloxazolyl)]benzene) (125 mg) in 2000 ml distilled dioxane. The amount of cyclic AMP in the sample was calculated based on a standard curve prepared using various known concentrations of cyclic AMP.

Enzymes. Dog liver phosphorylase, phosphorylase kinase and phosphorylase phosphatase used in the cyclic AMP assay procedure were prepared as outlined by Sutherland *et al.* [12].

Glucose uptake and utilization. The uptake and utilization of glucose were determined by the following procedure. Spleens of the control animals and those of sensitized mice were sliced in the cold using a Stadie–Riggs microtome. The methods of the preparation of tissue slices were outlined earlier [15]. The slices were quickly weighed on a torsion balance and transferred into Krebs–Ringer bicarbonate medium (pH 7·4, 5·0 ml) containing no glucose. Incubation was carried on at 37° in 25-ml flasks on a Dubnoff shaker for 2 hr using a mixture of O_2 and CO_2 (95:5) as the gas phase. This procedure was needed for depletion of endogenous respiratory substrates. After the incubation, the slices were removed and transferred into a medium that was similar but additionally contained glucose (2·0 mM) and a second incubation was carried on for 1 hr. After an equilibration of temperature for 15 min, samples (0·1 ml) were collected at intervals of 15 min for glucose determination by the glucose oxidase method [16] using Glucostat® (Worthington Biochemicals). The decrease in glucose concentration in the medium is interpreted as a composite function of uptake and utilization.

Reagents. Epinephrine was obtained from Park Davis & Co., Detroit, Mich.; aminophylline from G. D. Searle & Co., Chicago, Ill.; Glucostat® from Worthington Biochemicals, Freehold, N.J.; cyclic AMP from Sigma Chemical Co., St. Louis, Mo.; ^{3}H-cyclic AMP from Schwarz/Mann, Orangeburg, New York, N.Y.; and the remainder of the chemicals from Fisher Scientific Co., Pittsburgh, Pa. Propranolol was supplied by the courtesy of Ayerst Laboratories, Montreal, Canada.

RESULTS

Inoculation of mice with *B. pertussis* resulted in marked hyperplasia of the spleen and increased sensitivity to low doses of histamine as reported earlier (LD_{50}, 8 mg/kg) [1]. The increase in spleen weight was found to be proportional to the increase in total protein. Thus, the results interpreted on the basis of protein content are directly comparable to those interpreted on the basis of fresh weight.

Since one of the prominent biochemical events that occurs in pertussis sensitization is marked hypoglycemia, the amount of cyclic AMP formed in the spleen was measured at various intervals of time corresponding to the hypoglycemic effect (Table 1). Hypoglycemia was noticeable at 6 hr and persisted as long as 10 days; thereafter, the blood sugar returned to normal. The blood glucose levels of control mice were 121·1 mg/100 ml, while those of the pertussis-sensitized mice decreased in 6 hr to 58·1 mg/100 ml and remained as low as 50·5 mg/100 ml until 10 days. On the other hand, when cyclic AMP of spleens was examined at corresponding periods, there was no change in 6 hr and a slight but significant increase (from 10·34 to 13·94 nmoles/g of protein) was observed in 24 hr. However, between days 4 and 10,

Table 1. Effect of pertussis sensitization on blood glucose and cyclic AMP*

Time after vaccination	Blood glucose (mg/100 ml)	Per cent change	Cyclic AMP (nmoles/g protein)	Per cent change
Control	121·1 ± 6·1		10·34 ± 0·90	
6 hr	58·1 ± 7·1	−53	11·33 ± 1·64	+9 NS
1 day	64·6 ± 12·5	−47	13·94 ± 1·09	+34
2 days	77·9 ± 10·1	−36	11·41 ± 0·97	+10 NS
4 days	48·2 ± 16·7	−61	6·70 ± 1·07	−36
10 days	50·5 ± 17·2	−59	6·21 ± 0·58	−40
16 days	98·0 ± 20·2	−20 NS	9·42 ± 0·87	−9 NS

* Mice (six to ten) were sensitized with pertussis vaccine and killed at indicated times. The control group received sterile saline (0·5 ml) only. Samples of blood were collected and assayed for the content of glucose; spleens were assayed for cyclic AMP. The values were expressed as ± S.E.M. of ten determinations. The values for blood glucose from 6 hr to 10 days are significantly different from the control ($P < 0·001$). The values for cyclic AMP for days 4 and 10 are significantly different from the control ($P < 0·001$). NS = not significantly different from controls.

Table 2. Effect of propranolol on spleen cyclic AMP*

Propranolol (mg/kg)	Cyclic AMP (nmoles/g protein)
0·00	12·46 ± 0·92 (a)
0·18	14·28 ± 1·38 (b)
0·88	17·79 ± 1·49 (c)
1·66	12·15 ± 1·12 (d)
5·00	5·45 ± 0·69 (e)
10·00	5·76 ± 0·96 (f)
20·00	4·79 ± 0·38 (g)
40·00	5·41 ± 0·79 (h)

* Propranolol was injected into mice intraperitoneally, and 20 min later the mice were decapitated and the spleens were removed and assayed for their cyclic AMP content. The values are expressed as nmoles/g of protein ± S.E. of four to eight determinations using six to ten animals in each group. Significance by Student's *t*-test: (a) vs (c), (e), (f), (g) and (h) are $P < 0·001$; (a) vs (b); (b) vs (d); and (e) vs (f), (g) and (h) are not significantly different.

the amount of cyclic AMP in spleens remained low (6·70 to 6·21 nmoles/g of protein). The reasons for this biphasic phenomenon remain unclear.

Various doses of propranolol, a beta-adrenergic blocking agent, were administered (i.p.) to normal mice, and the effect on cyclic AMP content of the spleens was examined after 20 min (Table 2). A marked increase from 12·46 to 17·79 nmoles/g of protein) was noted at a dose of 0·88 mg/kg. At doses in excess of 5 mg/kg, there was a marked decrease (from 12·46 to 5·45 nmoles/g of protein) in cyclic AMP levels of spleens, the amounts being equivalent to those obtained by sensitization by pertussis.

The effects of aminophylline, a phosphodiesterase inhibitor, in combination with epinephrine, were examined on cyclic AMP content of spleen and the blood glucose concentration of normal and propranolol-treated mice (Table 3). The concentration of blood glucose nearly doubled (from 121·1 to 260·5 mg/100 ml). In addition, a significant increase ($P < 0·05$) was noted in the steady state level of cyclic AMP after the treatment with aminophylline and epinephrine for 20 min.

When sensitized with propranolol (20 mg/kg) prior to the administration of a mixture of aminophylline and epinephrine, the expected increases in the levels of blood glucose and cyclic AMP were very much attenuated, if the samples were collected at 30 and 60 min. However, if propranolol was administered 180 min before the sacrifice, there was no change in blood glucose levels maintained by epinephrine and aminophylline. On the other hand, there was significant diminution of cyclic AMP level of spleens, if propranolol was administered up to 340 min before the collection of samples, although the values tend to return to control levels.

The measurements of cyclic AMP from the tissue would only give the net amount of the nucleotide which is subject to the action of endogenous physiological mediators. In order to assess the degree of activation of adenyl cyclase free from autonomic control, cell-free preparations of spleen were made and enzyme activity was measured in the presence of epinephrine, isoproterenol, norepinephrine and NaF. The results show (Table 4) that the rate of synthesis of cyclic AMP by the preparations from normal and

Table 3. Effect of epinephrine and aminophylline on blood glucose and spleen cyclic AMP levels in propranolol-sensitized mice*

Treatment time					
Propranolol (min)	Epinephrine +aminophylline (min)	Blood glucose (mg/100 ml)	Per cent decrease	Spleen cyclic AMP (nmoles/g protein)	Per cent decrease
	20	260·5 ± 14·0		16·80 ± 1·12	
30	20	156·6 ± 24·1	40	6·17 ± 1·10	64
60	20	155·1 ± 17·0	41	6·42 ± 0·91	62
100	20	176·3 ± 15·6	33	5·22 ± 0·72	69
180	20	260·1 ± 18·0	1 NS	7·39 ± 0·92	57
340	20	248·2 ± 20·1	5 NS	10·78 ± 1·77	36
		121·1 ± 6·1	54	10·34 ± 0·90	39

* Aminophylline (50 mg/kg) and epinephrine (0·5 mg/kg) were injected (i.p.) in all cases 20 min prior to the killing of the mice. Propranolol was injected (i.p.) at indicated times before the killing of the mice. Samples of blood were assayed for the content of glucose and spleens for cyclic AMP. The values are expressed as ± S.E. The values for propranolol treatment significantly differ ($P < 0·01$) from the control. NS = not significantly different from aminophylline–epinephrine treatment. The values represent four to six determinations using six to ten animals in each group.

Table 4. Activation of adenyl cyclase of normal and pertussis spleen*

Additions	Concn (M)	Cyclic AMP (nmoles/g protein) Normal	Sensitized
None		177 ± 92 (a)	150 ± 48 (A)
Epinephrine	10^{-6}	347 ± 177 (b)	201 ± 72 (B)
	10^{-5}	749 ± 172 (c)	487 ± 81 (C)
	10^{-4}	1123 ± 111 (d)	589 ± 104 (D)
Isoproterenol	10^{-6}	192 ± 58 (e)	153 ± 42 (E)
	10^{-5}	721 ± 169 (f)	472 ± 52 (F)
	10^{-4}	1108 ± 128 (g)	604 ± 113 (G)
Norepinephrine	10^{-5}	343 ± 67 (h)	113 ± 82 (H)
	10^{-4}	509 ± 88 (i)	284 ± 88 (I)
Sodium fluoride	10^{-2}	2392 ± 258 (j)	738 ± 42 (J)

* Membrane fragments were prepared from the spleens of sensitized and control mice and assayed for the activity of adenyl cyclase in the presence of epinephrine, isoproterenol, norepinephrine and sodium fluoride. The amount of cyclic AMP synthesized was assayed. Statistical significance by the Student *t*-test: (a) vs (e); (A) vs (B); (a) vs (A); (A) vs (E); (A) vs (H); (b) vs (B); and (c) vs (C) are not significantly different ($P > 0.05$). The rest of the values of the sensitized mice are significantly different from the normal ($P < 0.01$), so also with controls. The values represent four to six determinations using six to ten animals in each group.

pertussis-sensitized animals was similar. Epinephrine stimulated the activity of both preparations severalfold, but the increase obtained in normal spleen preparations was nearly twice that of the pertussis-sensitized preparations. Furthermore, stimulation obtained with isoproterenol at identical concentrations was not significantly different from that with epinephrine ($P > 0.05$). Again, as with blood glucose levels, the values obtained from the measurement of enzymatic activity were generally lower in preparations from sensitized animals than in those from the controls.

Norepinephrine, at a molar concentration equivalent to epinephrine (10^{-5} M), stimulated adenyl cyclase to a lesser extent, with no increase in cyclic AMP formation in sensitized preparations. Furthermore, in the presence of 10 mM NaF, adenyl cyclase activity of the pertussis-treated spleens was only one third of that of the non-treated spleens.

In order to relate the alterations of cyclic AMP to glucose utilization by sensitized spleen, studies *in vitro* were done by incubating the tissue slices in Krebs–Ringer bicarbonate medium. No distinction was made between uptake and utilization, the disappearance of glucose being taken to represent the cumulative effect of both processes. Preliminary experiments have revealed that the uptake of glucose was poor, presumably due to abundance of endogenous substrates. Transfer of the slices into a second incubation medium containing glucose, after a preincubation in a similar medium free of glucose, resulted in active uptake. The results in Fig. 1 show that glucose was depleted from the medium at rates linear with time after an initial lag of 15 min. The rate of glucose utilization was much higher in spleen slices of sensitized animals than in those of the controls.

DISCUSSION

The metabolism of cyclic nucleotides has been extensively studied in several tissues and under different physiological conditions [17–21]. In recent years especially, cyclic AMP has been implicated in the mechanism of pathogenicity in a number of bacterial infections [22–24]. However, in all of these infections thus far examined, the synthesis of cyclic AMP was found to be augmented. On the contrary, the results of this investigation show that *B. pertussis* injected into mice causes a decrease in cyclic AMP of the spleens.

In conformity with the earlier studies, hypoglycemia was observed in pertussis-sensitized mice. Furthermore, hypoglycemia is obtained within 6 hr after the administration of pertussis vaccine, whereas the decreases in cyclic AMP results after 4 days when the mice are maximally sensitive to histamine anaphylaxis [2]. Thus, the histamine sensitivity closely corresponds to changes in the content of cyclic AMP. However, after 16 days the levels of blood glucose as well as spleen cyclic AMP return to normal (Table 1). This is the time at which histamine tolerance is restored in *B. pertussis*-sensitized mice [2]. Thus, histamine sensitivity, hypoglycemia and the decrease of cyclic AMP are temporally interrelated. In agreement with this view, Fishel *et al.* [25] observed that the administration of large doses of epinephrine protected the normal mouse against the lethal effect of a mixture of histamine and serotonin [25]. In their experiments, the protective effect of epinephrine was correlated with an increase in blood glucose.

Studies based on systemic interruption of ganglionic transmission by surgery together with those using dichloroisoproterenol, a beta-adrenergic blocking agent, indicate that hypoglycemia in pertussis mice is a consequence of imbalance of adrenergic receptors [5]. In the present investigation, propranolol, another beta-receptor blocking agent, was used

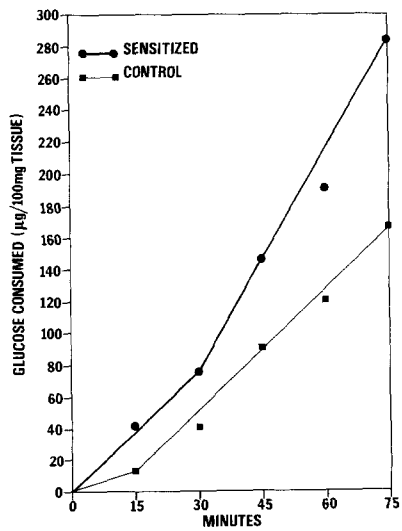

Fig. 1. Glucose uptake and utilization in pertussis-sensitized mouse spleen slices. Mice were injected with pertussis vaccine and sacrificed after 5 days. Spleen slices of control and pertussis-sensitized mice were incubated in Krebs Ringer bicarbonate medium (5 ml) containing glucose (2 mM) for 90 min. After temperature equilibration of 15 min, samples were withdrawn from the medium and assayed for depletion of glucose. Each point represents an average of four to six determinations; the values of sensitized mice differ significantly from the controls ($P < 0.01$).

as a sensitizing agent in order to evaluate the nature of the adrenergic receptor in spleen and a comparison could be made with pertussis sensitization. Propranolol decreased the cyclic AMP content of the spleen when administered in doses in excess of 5 mg/kg, though stimulation was observed at a small dose (0·88 mg/kg) (Table 2). This stimulation is not explicable from the data presented here. However, it is conceivable that some catecholamine release may occur at low doses of propranolol causing an increase in cyclic AMP. It is well known that propranolol exerts both sympathomimetic as well as sympatholytic action depending on the tissue and dose [26]. None the less, the stimulation of adenyl cyclase by epinephrine and isoproterenol to a far greater extent than by norepinephrine (Table 4), together with the propranolol blockade of epinephrine-stimulated cyclic AMP (Table 3) is evidence that the activation of adenyl cyclase in the spleen is a function of beta-adrenergic receptors.

The blood glucose and spleen cyclic AMP levels are lowered transiently by propranolol. It is this transient action of propranolol that differs from that of pertussis. In the latter, the onset of blockade is slow but lasts longer. That pertussis blockade is similar to propranolol blockade is also demonstrated by the work of Fishel *et al.* [25] who observed comparable degrees of lethal sensitivities to mixtures of serotonin and histamine in both cases. However, the persistence of beta-receptor blockade by *B. pertussis* components makes it a more convenient experimental model for the study of atopic abnormality.

The pool of cyclic AMP in spleens, as measured in experiments *in vivo*, is the result of its degradation and synthesis, both processes being subject to the control of the autonomic nervous system. Also, the data obtained after pretreatment *in vivo* is dependent upon the rate of absorption, distribution and metabolism of the drug in question. On the other hand, results obtained in studies *in vitro* (Table 4 and Fig. 1) are derived free from such interactions. The experiments *in vitro* thus show that the activity of adenyl cyclase in the absence of stimulators is similar in controls and in the preparations from sensitized mice. On the other hand, there is a marked attenuation of the stimulatory response of pertussis preparations to epinephrine, isoproterenol and NaF. Pertussis components seem to exercise similar action on cyclic AMP levels of cultured human lymphocytes [7]. However, the constellation of the pharmacological changes to pertussis sensitization is limited to very few strains of mice and rats [1].

On the basis of dualistic concept of adrenergic-receptor balance [27], when beta-receptor blockade occurs, there could be adrenergic responses corresponding to alpha-receptor activation. The net effect of this phenomenon appears to be an increased uptake of glucose in sensitized spleens (Fig. 1). Preliminary studies showed that the disappearance of glucose from the incubation medium was due to its increased oxidation to CO_2 [28]. A similar increase in uptake and utilization of glucose was noted in peripheral lymphocytes by alpha-receptor stimulation [29]. This increased uptake could be associated with and lead to lymphocytosis observed as an adjuvant effect in pertussis sensitization [5, 9]. It would be interesting to study whether a similar increase in glucose uptake and utilization would occur in the spleen in the presence of alpha-receptor agonists.

Biochemically the increased uptake of glucose may be explained as a result of an alteration in the control of transport of glucose which, in the skeletal muscle, is regulated by glucose 6-phosphate [9]. Whether a similar control operates in the sugar uptake in spleen and other immuno-competent systems is largely conjectural at present.

It is inconceivable that any causative relation exists between blood glucose level and cyclic AMP content in the spleen. The correlation observed between them reflect a conjoint and collateral relationship of adenyl cyclase function to generalized beta-adrenergic blockade and consequent increase in peripheral uptake of glucose as postulated earlier [5].

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