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LIVER TRANSGLUTAMINASE ACTIVITY UNDER VARIOUS EXPERIMENTAL CONDITIONS*

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Abstract—Bacterial endotoxins and *Haemophilus pertussis* vaccine were found to increase the activity of liver transglutaminase in mice, rats, and guinea pigs. Enzyme activity increased also in the spleen of the injected mice, whereas no significant changes were found in the thymus, the lung, and the brain. The enzyme activity in adrenalectomized mice increased in the liver. Pretreatment of the animal with cortisone antagonized the rise in liver transglutaminase after endotoxin administration. The enzyme obtained from the liver of endotoxin-treated mice has the characteristics of transglutaminase obtained from the livers of normal mice. Mice and rats have a low enzyme activity in the liver and show a considerable increase upon administration of *H. pertussis* vaccine and purified endotoxins. Guinea pig liver, which is the richest source of transglutaminase so far found, shows a significant increase only upon the administration of high doses of endotoxins but not of pertussis vaccine.

SOME time ago we described an enzyme system occurring in mammalian tissue which catalyzes the exchange of protein amide groups with primary amines, such as cadaverine, putrescine, histamine, serotonin, glycine amide, and so forth. Hydrazine, ammonia, or hydroxylamine may also serve as replacing amines.[3, 4] Liver, brain, and other tissues contain proteins that may act as acceptors of the amines in this Ca^{2+}-dependent enzymatic reaction; in addition, a number of purified proteins, among them β-lactoglobulin, α- and β-serum globulin, and insulin, are efficient amine acceptors.[5] Further studies with casein, insulin,[6] and synthetic peptides[7] showed that the amide group of protein-bound glutamine, but not of asparagine, was the site of replacement. The enzyme was therefore named transglutaminase. The ϵ-amino group of protein-bound lysine may also act as the replacing amine.[3, 8] Transglutaminase catalyzes not only the exchange of the amide group of protein-bound glutamine by primary amines but also its hydrolysis.[6]

Since the discovery of transglutaminase, considerable efforts have been expended to ascertain whether or not the action of this enzyme could be demonstrated *in vivo*. ^{14}C-labeled cadaverine or putrescine when administered to mice, with and without amine oxidase inhibitors, gave counts in liver proteins that indicated an insignificant

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incorporation only. Equally, liver injury produced by oral administration of carbon tetrachloride or nephrectomy caused no significant increase in the fixation of labeled aliphatic diamines. Similar results, to be presented in detail in a subsequent report,[9] were obtained when ^{14}C-histamine was administered to normal mice.

These results were disappointing since it had been reported that significant incorporation *in vivo* of ^{14}C-mescaline into liver proteins of normal mice can be easily demonstrated.[10] These data will be discussed in more detail in the subsequent paper which deals with the incorporation *in vivo* of histamine into protein. It should be noted that the enzyme system assumed by Block and associates[10] to be responsible for the incorporation into proteins of mescaline and β-phenylethylamine differs from transglutaminase since the former enzyme system is oxygen-dependent, heat- and tyramine-activated, and shows different pH optima and no significant dependence on Ca^{2+}.

Since an incorporation of amines into the liver proteins of mice has not been demonstrated *in vivo* with certainty, preliminary experiments were carried out to ascertain whether or not transglutaminase might catalyze the exchange of free ammonia ($^{15}NH_3$) with protein-bound amide groups. Data obtained so far in cats[11] and mice are equivocal and might be explained by incorporation of the glutamine or asparagine as such in the course of protein turnover. The possibility of a biological function of transglutaminase in the enzymatic formation of a linkage of the ϵ-amino group of protein-bound lysine to the γ-carboxyl of protein-bound glutamic acid[3, 8] has not been explored up to now.

It had been suggested in an earlier communication that proteins into which amine had been incorporated by the action of transglutaminase may have antigenic properties.[12] Since vaccine preparations such as *H. pertussis* vaccine not only increase the sensitivity of mice to histamine but also greatly enhance immune responses,[13] a study of the effect of pertussis vaccine and bacterial endotoxins on transglutaminase activity in mouse organs under various conditions *in vivo* was undertaken. This investigation was followed by a study[9] of the effect of such sensitization on incorporation of histamine into the liver proteins of the mouse *in vivo*.

METHODS

Enzyme assay. Transglutaminase was assayed in tissues with the fraction of a tryptic casein hydrolysate soluble in 80% ethanol as amine-accepting substrate and hydroxylamine as the replacing amine, according to Mycek and Waelsch.[6] The initial rate of hydroxamic acid formation was measured. A sample of a single liver or pooled pieces from several livers were homogenized with three parts of 0·25 M sucrose solution. The homogenate was centrifuged at 100,000 $\times$ *g* and the supernatant solution used for the enzyme assay. Previous experiments have shown that transglutaminase is present mainly in soluble protein fraction.

To 1 ml of the casein hydrolysate (300 mg/ml) were added 0·1 ml of 0·1 M $CaCl_2$, 0·1 ml of 0·2 M glutathione (pH 7), 0·2 ml of neutralized 0·5 M hydroxylamine, 0·3 ml of Tris buffer (0·2 M, pH 7·8), an aliquot of the supernatant fraction of the homogenate of the organ to be assayed for transglutaminase activity, and water to bring the final volume to 2 ml. After 10 min at 37°, 1·5 ml ferric chloride-trichloroacetic acid reagent[14] was added and the developed color measured at 525 mμ (Coleman Jr. spectrophotometer). Protein was determined by the biuret[15] or the modified Folin

reaction.[16] One unit of enzyme activity corresponds to a change in absorbance of 0·0001/min of incubation time (specific activity in units per mg protein).[6] In the enzyme assay the normal mouse livers with an average activity of 20 units gave an absorbance of 0·1 when 0·2 ml of the supernatant fraction (5 mg of protein) and an incubation time of 10 min were used. Correspondingly, the guinea pig livers with an average activity of 130 units/mg protein gave an absorbance which would be equivalent to 0·7 to 0·8 under the same experimental conditions. For each homogenate two parallel tests were carried out with two aliquots of the enzyme solution (one aliquot being one-half of the other), and all determinations were done in duplicate.

In the experiments with ^{14}C-methylamine the methods described previously were employed.[4]

Animal experiments. Male and female Swiss albino mice of 18 to 22 g body weight, Sprague-Dawley rats (200 to 300 g), and Hartley guinea pigs (400 to 500 g) were used as experimental animals.

Hemophilus pertussis *vaccine and endotoxins.* Mice received 6×10^9, and rats 60×10^9, organisms of phase I of *H. pertussis* vaccine (Lederle) by intraperitoneal injections. Either one or two injections at varying time intervals between the doses were used. The animals were sacrificed between 16 and 216 hr after the last injection.

Lipopolysaccharides (Difco) from *Escherichia coli*, *Salmonella typhosa*, *Serratia marcescens*, and *Staphylococcus aureus* were given intraperitoneally in doses of 2·5 mg and 5·0 mg/kg mouse. Guinea pigs received intraperitoneally *S. typhosa* endotoxin in doses of 800 and 400 μg/kg.

Adrenalectomies. Mice were used for enzyme assay 3 weeks after bilateral adrenalectomy by the retroperitoneal approach. In the postoperative period they were fed normal Purina diet, and drinking water was replaced by 0·9% saline solution.

Cortisone acetate. Cortogen (Schering) was given subcutaneously in daily injections of 2·5 mg/20 g mouse for 4 to 5 days before the enzyme assay.

Body temperature. Changes in rectal temperature of mice were recorded with a thermocouple.

RESULTS

In the first series of experiments the effect of pertussis vaccine on liver transglutaminase in female mice was investigated (Table 1). The enzyme activity in those animals killed 16 hr to 9 days after a single administration of the vaccine is approximately double that of the control mice. After two injections of the vaccine, the second dose being given 2 to 9 days after the first, the enzyme activity was found to be nearly three times that in control animals. Mice were killed 16 hr to 3 days after the second vaccine injection. Since the increase in transglutaminase activity in the liver of female mice is to some degree independent of time (see below) within the limits used, the data are not given separately for each time period. Any error due to the pooling of the experiments would result in a lowering of the mean transglutaminase activity.

The increase of enzyme activity after pertussis vaccine was maintained with fluctuations over a period of 2 weeks in the liver of female mice, whereas in the liver of male mice the increased enzyme activity had already begun to return to normal levels after 2 days (Fig. 1).

A number of bacterial lipopolysaccharides was tested in male mice in two concentrations and at two time intervals after a single administration (Table 2). Although all the endotoxins tested increased the transglutaminase levels in mouse liver, the greatest increase was found after the lipopolysaccharide fraction from *S. typhosa*. After the administration of *S. typhosa* endotoxin, changes in body temperature were

TABLE 1. TRANSGLUTAMINASE ACTIVITY IN THE LIVER OF NORMAL MALE AND FEMALE MICE AFTER ADMINISTRATION OF *H. pertussis* VACCINE

Exp. no.	No. of mice	Sex	No. of determinations*	No. of vaccine administrations	Determination, days after last vaccine adm.†	Transglutaminase specific activity‡
1	40	M	15			20·4 ± 1·4
2	59	F	35			24·2 ± 0·9
3	22	F	22	1	0·7 to 9	40·7 ± 2
4	16	F	16	2	0·7 to 3	66·0 ± 3·5
5	3 × 6	F	3	2	0·7	55·3, 70·3, 49·2

* Number of determinations in experiments 1 and 2 refers to determinations partly on single and partly on pooled livers. Experiments 3 and 4 represent determinations on single livers. In experiment 5 each figure represents pooled material from six animals. Enzyme and protein determinations were carried out in duplicate on two aliquots of each homogenate.

† Experiment 3: determination of enzyme 16 to 216 hr after vaccine administration. Experiment 4: Enzyme determination 20 to 72 hr after second vaccine administration; interval between first and second vaccine administration 48 to 216 hr. Experiment 5: interval between first and second vaccine administration 9 days; determination 16 hr after second injection.

‡ (Absorbance at 525 mμ)/(0·0001 × incubation time in min × mg protein per assay) ± SE.

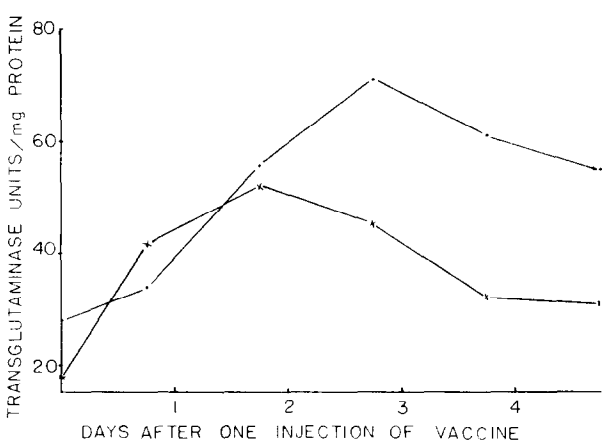

FIG. 1. Time curve of transglutaminase activity in the livers of mice after one injection of *H. pertussis* vaccine; ●———●, female mice, ×———×, male mice. Each point on the curve represents two assays.

measured during the period of the determination of the activity; a latent period of about 6 hr was found after the intraperitoneal injection of lipopolysaccharide before the increase in transglutaminase activity (Fig. 2). During this 6-hr interval the body temperature fell significantly.

In preliminary experiments transglutaminase was measured also in brain, lung, thymus, and spleen of male mice injected either with endotoxin or with pertussis vaccine. The animals were killed 16 hr after the administration of *S. typhosa* lipopolysaccharide, which was given i.p. in doses of 5 mg/kg. Two doses of 6×10^9 organisms of *H. pertussis* vaccine with a time interval of 10 days were administered, and the

TABLE 2. EFFECT OF BACTERIAL ENDOTOXINS ON LIVER TRANSGLUTAMINASE ACTIVITY IN FEMALE MICE*

Organism	Days after administration	Transglutaminase specific activity Endotoxin 2·5 mg/kg	5 mg/kg
E. coli	1	41	51
	2	30	36
S. typhosa	1	46	82
	2	50	53
S. marcescens	1	41	54
S. aureus	1	43	46
Control value			24·2 ± 0·9†

* Two female mice were used for each experiment; duplicate determination of two aliquots of homogenate.

† From Table 1.

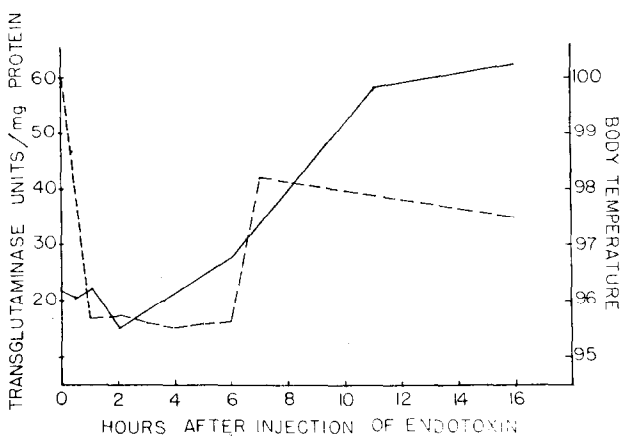

FIG. 2. Time curve of transglutaminase activity and body temperature in male mice. Each point on the temperature curve represents the average value from four animals. Enzyme levels were measured in pooled livers from two mice. *S. typhosa* endotoxin (5 mg/kg) was administered by the intraperitoneal route; — — —, body temperature; ———, transglutaminase activity.

mice were killed 16 hr after the second dose of vaccine. After endotoxin administration transglutaminase activity was increased in the spleen, whereas the enzyme levels did not change in lung, thymus, and brain.

Animals other than mice were also tested for changes in transglutaminase activity after pertussis vaccine or endotoxin adminstrations. In both male and female rats an increase in liver enzyme was found, but this increase was not so pronounced as in

mice and was not maintained for more than 3 or 4 days after one injection. In guinea pigs, in which the normal concentration of enzyme is approximately five times that of the mouse liver (specific activity approximately 130), transglutaminase activity increased only slightly after pertussis vacine administration. On the other hand, administration of the *S. typhosa* preparation led to doubling the enzyme concentration in the guinea pig liver (130 to 260).

The effect of agents other than vaccines and endotoxins on transglutaminase activity in the livers of mice was tested. Reversible degeneration of hepatic cells and hypoglycemia, together with decrease in hepatic glycogen, is known to occur in animals after administration of hydrazine, which was therefore given to mice by the intraperitoneal route in doses of 50 mg/kg. Transglutaminase activity in the liver 16 to 20 hr later did not differ from that in normal animals. Thioacetamide has been reported[17] to increase calcium levels in rat livers 65-fold when given subcutaneously in doses up to 200 mg/kg. Since transglutaminase is a Ca^{2+}-dependent enzyme, the effect of thioacetamide on the liver enzyme was tested. Mice were injected with 200 and 400 mg/kg of thioacetamide, and 24 hr later the enzyme activity was recorded; no increase was found in repeated experiments. Epinephrine in oil, in doses of 1 and 2 mg/kg, was given intramuscularly to mice; two animals were used for each dose. Liver transglutaminase activity recorded 16 hr later was found to be the same as simultaneously tested enzyme levels in the livers of normal animals. A mixture of two enzymes, streptokinase-streptodornase, marketed under the name of Varidase, is known to have antigenic properties.[18] Streptokinase was reported also to increase local vascular permeability and release histamine when given intradermally to rats.[19] The intraperitoneal administration of Varidase in 50,000 and 100,000 units/kg, in single and repeated doses (8 to 10 days) did not increase the transglutaminase levels in the liver. Similar results were obtained after subcutaneous injections of β-lactoglobulin in doses of 500 mg/kg. In some experiments subcutaneous doses of β-lactoglobulin were followed by intraperitoneal injection of the β-lactoglobulin after a period of 10 days. Transglutaminase activity was not increased in any of those experiments.

Adrenalectomized mice are 100 to 1,000 times more sensitive to lethal doses of bacterial endotoxins;[20] the levels of transglutaminase in such animals were therefore determined (Table 3).

Significant increase of enzyme activity was found in adrenalectomized mice: however, large individual variations were always encountered. Sham-operated mice did not differ from normals. Cortisone administration antagonized the increase in transglutaminase activity due to *S. typhosa* endotoxin treatment. Mice treated with cortisone alone did not differ from normals in their transglutaminase activity.

Since the test for enzyme activity was routinely carried out with hydroxylamine as the replacing amine, it appeared desirable to ascertain that the increased activity was due to an enzyme with the same properties as those established for transglutaminase in the preceding studies. The supernatant fluid (after centrifugation of $100{,}000 \times g$) of the sucrose homogenate of mice livers after the second pertussis administration was therefore tested for transglutaminase activity with hydroxylamine and with ^{14}C-methylamine as the replacing amine (Table 4). As may be seen, the ratios of enzyme activity tested with two replacing agents did not differ greatly, a fact which indicates that we are dealing with an increase of transglutaminase with the properties of the enzyme present in untreated animals.

It may be asked whether the increase of transglutaminase could be due to the accumulation of an activator and not to an increase of the enzyme itself. Therefore, 24 hr after the second pertussis injection, which was given 6 days after the first, the pooled livers of four mice were subjected to the enzyme purification procedure developed in this laboratory.[5] Transglutaminase simultaneously was purified from the

TABLE 3. EFFECT OF ADRENOCORTICAL HORMONES ON TRANSGLUTAMINASE LEVELS IN THE LIVERS OF MALE MICE*

Exp. no.	Treatment	No. of animals	Transglutaminase specific activity
1	Normal	8	18·0 ± 2·0†
2	Adrenalectomy	18	25·3 ± 2·1
3	Sham-operated	12	18·7 ± 0·8
4	Cortisone	8	17·9 ± 2·0
5	*S. typhosa* LPS‡	6	50·4 ± 6·2
6	*S. typhosa* LPS and cortisone	10	24·9 ± 2·3

* The number of animals in each experiment represents the number of enzyme assays. Cortisone, in doses of 2·5 mg/mouse, was injected subcutaneously for 4 consecutive days before administration of endotoxin. The enzyme was assayed 16 hr after i.p. injection of endotoxin, which was given simultaneously with the last dose of cortisone.

† SE of the mean; $0{\cdot}05 > P > 0{\cdot}025$ for experiments 1 and 2; $P < 0{\cdot}001$ for experiments 5 and 6.

‡ *Salmonella typhosa* lipopolysaccharide.

TABLE 4. TRANSGLUTAMINASE ACTIVITY TESTED WITH TWO AMINES IN THE LIVER OF PERTUSSIS-TREATED AND CONTROL ANIMALS

	Enzyme activity	*Pertussis* Control	Counts per min/ mg protein	*Pertussis* Control
Replacing amine	NH_2OH		$^{14}CH_3NH_2$*	
Normal (2)	15·7	3·9	760	3·3
After 2nd pertussis injection (2)	61·5		2,500	

* Two ml contained 0·1 ml of 0·2 M Tris buffer (pH 7·8), 0·2 ml of 0·1 N $CaCl_2$, 10 mg β-lactoglobulin, 0·1 ml of 0·2 M glutathione (pH 7), and 10 μmole of ^{14}C-methylamine (0·05 μc/μmole). Two aliquots of the enzyme solution were tested and the determinations carried out in duplicate.

livers of four untreated control animals. The ratios of the activities of the approximately tenfold purified enzyme of the pertussis-treated to that of the control animals, when tested with hydroxylamine and with methylamine, were 2·4 and 2·6 respectively. This result appears to exclude an increase of an easily dissociable activator owing to the pertussis treatment. The results taken together support the view that the administration of the vaccine causes an increase of the same enzyme, transglutaminase, present in the liver of the normal animal.

It seemed of interest to ascertain whether, concomitant with increase of this enzyme activity, other enzymes increase as a result of pertussis administration, particularly since it had been shown that histidine decarboxylase activity increased in various stress situations[21] and also after pertussis vaccine.[22] In preliminary experiments glutamine synthetase, measured as glutamotransferase,[23] and proteinase, estimated by cleavage of hemoglobin, did not show any increases when transglutaminase had increased significantly.

DISCUSSION

The administration of pertussis vaccine and of endotoxins leads to an increase in transglutaminase activity in the liver of the rodents tested, (*i.e.* mice, rats, and guinea pigs). The extent of this rise appears to be related to the normal level of enzyme activity of the liver in the particular species. Mice and rats have a low enzyme activity in the liver and show a considerable increase upon the administration of pertussis vaccine, whereas the liver of guinea pigs, which is the richest source of transglutaminase so far found, shows a significant increase only upon the administration of *S. typhosa* endotoxin, an agent also more effective than pertussis vaccine in mice. In contrast to mice and rats, guinea pigs show a higher sensitivity not only to histamine but also to endotoxins.

Among the experimental conditions tested, only adrenalectomy led to an increase in transglutaminase activity in the mouse liver. Cortisone, on the other hand, suppressed the increase of transglutaminase activity found after endotoxin administration. It appears that the control of the synthesis of transglutaminase is removed by the absence of adrenocortical hormones. It is known that some of the adrenal hormones influence protein synthesis and suppress the formation of antibodies. In order to assess the specificity of the effect of the adrenal insufficiency and of adrenal hormones on transglutaminase and its increase by endotoxins, other enzymes will have to be included in the investigation. In preliminary experiments, neither glutamine synthetase nor proteinase activities increased in the liver of pertussis-treated mice when a significant rise in transglutaminase activity could be shown.

Because of a very large spread of individual variations in transglutaminase, a considerable number of animals was included in the group of adrenalectomized mice. In spite of many experiments, individual variations remained rather persistent. This observation may find an explanation in the suggestion that bacterial endotoxins derived from the intestinal flora remain in the circulation and produce toxic effects in the host tissue when the defense mechanisms against these endotoxins are changed.[24]

The effect of bacterial endotoxins and pertussis vaccine on various other enzymes has been reported; a decrease in succinic dehydrogenase[25] as well as in histaminase,[26] respectively, has been claimed. In conjunction with our findings of the increase of transglutaminase activity as a result of the administration of these agents, the observations of Schayer and Ganley[21, 22] are of particular interest. These investigators found that stress situations, among them the administration of vaccine, endotoxin, and epinephrine, or exposure to cold, led to a significant rise in the activity of histidine decarboxylase in various organs of mice. It should be noted that, in contrast to transglutaminase, this enzyme occurs in high concentrations in lung and skin, whereas liver shows a very low activity only. Decarboxylase increased in all organs studied in

situations of stress,[21, 22] but significant increases of transglutaminase were found only in liver, and a small increase in spleen, upon the administration of pertussis vaccine and endotoxins. Furthermore, epinephrine administration led to an increase of decarboxylase activity but was without effect on the level of transglutaminase. The increases of transglutaminase levels are of considerably longer duration than those of decarboxylase. Whereas it may be attractive to consider the possibility that transglutaminase increases adaptively in response to the increased intracellular concentration of histamine, which would be a result of the stimulation of histidine decarboxylase, the differences enumerated above in the distribution and in the response of the enzymes to various agents do not lend support to such a hypothesis.

The effect of the administration of histamine and histidine with and without various amine oxidase inhibitors was tested on the enzyme level with negative results, which do not exclude the possibility that endotoxins supply or liberate in tissues an unknown agent to which transglutaminase shows an adaptive increase. It appears more likely that in response to the endotoxin administration there may be a cellular reaction, resulting in the proliferation of cell types common to liver (and possibly spleen) which contain a high enzyme level. Some support for such an interpretation of our findings may be seen in the observation of the proliferation of histiocytes in the liver of mice as a result of vaccine administration.[26]

A preliminary histological study of the spleen, thymus, lymph nodes, and liver after one injection of 100 μg/20 g mouse of *S. typhosa* endotoxin was attempted. None of those organs showed any abnormal increase in large lymphocytes or plasma cells, except for the liver where infiltration in the portal sinuses with small and large lymphocytes was observed.

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