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Some Metabolic Aspects of Tolerance to Bacterial Endotoxin[1]

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ABSTRACT

BERRY, L. JOE (Bryn Mawr College, Bryn Mawr, Pa.), AND DOROTHY S. SMYTHE. Some metabolic aspects of tolerance to bacterial endotoxin. J. Bacteriol. **90**:970-977. 1965.—The tolerance to bacterial endotoxins which is produced in mice given a series of daily injections of heat-killed *Salmonella typhimurium* failed to occur when actinomycin D was administered with the heat-killed cells. Neither ethionine nor 2-thiouracil, when given with endotoxin, altered the development of tolerance. An injection of endotoxin, actinomycin D, or ethionine lowered the activity of the liver enzyme tryptophan pyrrolase more significantly at either 4 or 17 hr postinjection in normal mice than in tolerant mice. Similarly, an injection of either saccharated iron oxide or Thorotrast lowered liver tryptophan pyrrolase activity more extensively in normal than in tolerant animals. Activation of the reticuloendothelial system (RES) of tolerant mice, as determined by an accelerated rate of carbon clearance from the blood, was observed, but this was prevented by the appropriate dose of actinomycin D. Similar results were obtained when saccharated iron oxide, rather than endotoxin, was used to activate the RES, but these animals were not resistant to endotoxin and their tryptophan pyrrolase was normally diminished after an injection of endotoxin. Thus, RES activation may occur without tolerance developing. A more nearly normal level of enzyme activity appears to be characteristic of the tolerant state.

Tolerance to the pyrogenic effects of bacterial endotoxins was first described by Beeson (1947*a*), who also observed (Beeson, 1947*b*) that colloidal blockade of the reticuloendothelial system (RES) of these animals restored them to more or less normal responsiveness. In the intervening years, a number of papers have been published on the role played by the RES in the tolerant state. It is generally agreed that as animals become progressively more tolerant the RES becomes more active, at least as judged by rate of clearance of colloids from the blood stream. There are sufficient exceptions to this generalization, however, to make it unacceptable as an essential cause for tolerance. The human being, according to the results of Greisman et al. (1964), becomes tolerant without apparent RES activation. Conversely, RES activation is known to occur in animals more susceptible, not more resistant, to endotoxin lethality, as shown by mice infected with the BCG strain of *Mycobacterium tuberculosis* (Suter, Ullman, and Hoffman, 1958; Howard et al., 1959). There is also evidence (Watson and Kim, 1963; Freedman, 1960) strongly implicating immune substances as the primary basis for tolerance, but this explanation is not without complications (Freedman and Sultzer, 1964).

The present study was undertaken to determine whether evidence for enzymatic "adaptation" could be detected in mice made tolerant to endotoxin. Interest in such experiments was generated by previous work from this laboratory in which activity of the hormone- and substrate-inducible liver enzyme, tryptophan pyrrolase, was found to be reduced to less than one-half normal only a few hours after injection of an LD_{50} of endotoxin (Berry and Smythe, 1963). Several lines of evidence point to the importance of this enzyme in endotoxin poisoning. First, substances several steps beyond the product of the reaction catalyzed by tryptophan pyrrolase are able to protect against lethality of endotoxin. Second, cortisone, a compound known for a decade to protect against endotoxin (Chedid, 1957) and more recently to induce *de novo* synthesis of tryptophan pyrrolase (Knox and Auerbach, 1955; Feigelson, Feigelson, and Greengard, 1962), maintains a normal activity of the enzyme when given concurrently with endotoxin, but not when cortisone injection is delayed 1 or 2 hr after endotoxin ad-

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ministration (Berry and Smythe, 1964). Cortisone not only fails to maintain tryptophan pyrrolase under these conditions but it also fails to protect against endotoxin death when its injection is delayed. Third, mice exposed to an environmental temperature of 5 C are killed by 1% of the dose of endotoxin lethal for mice at ordinary room temperatures (Previte and Berry, 1962, 1963); cortisone fails to protect them, and it fails to induce tryptophan pyrrolase (Berry, 1964). Fourth, well-established inhibitors of protein synthesis at doses well below the lethal level not only prevent the protective effect of cortisone against endotoxin and prevent the maintenance of tryptophan pyrrolase, but they also potentiate endotoxin and accelerate the reduction of the enzyme (Berry and Smythe, 1964).

If these observations represent an essential relationship in the response of mice to endotoxin, then the tolerant animal should have a more normal level of liver tryptophan pyrrolase activity than control mice. Moreover, any procedure which interferes with the development of tolerance or its maintenance should be reflected predictably in the level of enzyme activity after an injection of endotoxin.

MATERIALS AND METHODS

Endotoxins. Heat-killed *Salmonella typhimurium* SR-11 suspended in nonpyrogenic isotonic sodium chloride solution (Baxter Laboratories, Morton Grove, Ill.) was used as the basic crude endotoxin for most of the work. Crude washed lyophilized *Serratia marcescens* (generously provided by Merck and Co., Inc., Rahway, N.J.) suspended in nonpyrogenic saline was used as a challenge material. The LD_{50} of this preparation was 1.2 mg in mice housed at 25 C. All endotoxins were administered intraperitoneally as proportional doses of the LD_{50} as calculated by the method of Reed and Muench (1938).

Production of tolerance. Mice were made tolerant to crude *S. typhimurium* endotoxin according to two injection schedules. In one, larger amounts were used on successive days as follows: 0.1, 0.1, 0.2, 0.2, 0.4, 0.4 LD_{50}. These mice are designated Group E. The other schedule involved smaller amounts of endotoxin on successive days as follows: 0.02, 0.02, 0.04, 0.04, 0.08, 0.08 LD_{50}. These mice are designated Group e. These mice were tested experimentally on days 8 to 11, where day 1 corresponds to the day the first injection of endotoxin was given.

Inhibitors. Actinomycin D (kindly supplied by the Institute of Microbiology, Rutgers University, New Brunswick, N.J., through the courtesy of Vernon Bryson) was dissolved in 95% ethyl alcohol (1 mg/ml) and diluted in nonpyrogenic saline, so that the desired dose was contained in 0.5 ml. The dose ranged from 0.1 to 10.0 μg per 22-g mouse (about 4.5 to 450 μg/kg of body weight). The other inhibitors were dissolved in 1.5 N sodium hydroxide, diluted with saline, adjusted to *p*h 8 with hydrochloric acid as judged by use of Hydrion test paper, and finally diluted with nonpyrogenic saline, so that the desired amount was contained in 0.5 ml. Saline at *p*H 8 had no effect on the LD_{50} of endotoxin when injected intraperitoneally. DL-Ethionine (Nutritional Biochemicals Corp., Cleveland, Ohio) was employed in a dose range of 1 to 16 mg per mouse (45.5 to 727 mg/kg of body weight), and 2-thiouracil (Mann Research Laboratories, New York, N.Y.) was used at either 2.0 or 8.0 mg per mouse (91 or 364 mg/kg of body weight).

Reticuloendothelial "blocking" agent. Saccharated iron oxide (Proferrin, Merck Sharpe & Dohme, West Point, Pa.) or Thorotrast (Testagar Co., Detroit, Mich.) was injected intravenously via the tail vein in a volume of either 0.1 or 0.2 ml. Mice were studied experimentally, as indicated, at different times thereafter.

Carbon clearance. The method of Benacerraf, Thorbecke, and Jacoby (1959) was modified slightly to comply with the requirements of the experiments. The carbon suspension (Gunther Wagner, Hannover, Germany), designated C 11/1431a, contained 10% carbon, 4.3% fish glue, 1% carbolic acid, and water. Carbon particles were 200 to 300 A in mean diameter. After the suspension was diluted 1:4 in nonpyrogenic saline, 0.2 ml was injected into the tail vein of mice. After 5 and 15 min, 0.1 ml of blood from the retroorbital plexus was transferred to a Coleman cuvette containing 8 ml of 0.1% sodium carbonate. From a standard curve, the quantity of carbon remaining in the blood was determined after a transmittance reading at 675 mμ in a model 14 Coleman spectrophotometer. The phagocytic index (k) was calculated for each mouse.

Tryptophan pyrrolase assay. Liver tryptophan pyrrolase was measured according to the technique of Knox and Auerbach (1955) as adapted to mice by Berry and Smythe (1963). In all assays, hematin, the cofactor for the enzyme, was added in vitro to convert apoenzyme into holoenzyme (Feigelson and Greengard, 1961). For mouse liver, 20 μg of freshly prepared hematin per reaction vessel were sufficient. All mice were fasted 17 hr prior to sacrifice. This avoids a turbidity commonly seen in homogenized livers of fed mice, possibly due to their glycogen or lipid content.

Survival experiments. Mice were challenged with endotoxin and the number of survivors was determined at the end of 48 hr. Injections were given at the same time of day (4:00 PM to 5:00 PM) in an effort to avoid the reported cyclic variations in sensitivity of mice to endotoxin lethality (Halberg and Howard, 1958).

Statistics. Significance of difference in response between treatment groups was calculated by the nonparametric rank order test of White (1952), the rank correlation method of Wilcoxon (1949), or by the chi-square test with Yates' corrected formula (Croxton, 1959).

Mice. Female Swiss-Webster mice weighing 16 to 18 g were purchased from a dealer (Dierolf Farms, Boyertown, Pa.). On arrival, tetracycline antibiotics (Polyotic, American Cyanamid Co., Princeton, N.J.) were added to the drinking water each day for 2 days. Mice were then put on tap water and after 1 or 2 weeks, when body weight reached 22 ± 1 g, they were used experimentally. This treatment was initiated in an effort to improve the reproducibility of results. There is an impression without proof that experimental findings are less variable, and, of equal importance, there is no evidence of undesirable consequence.

Mice were housed 10 per cage with white pine shavings as bedding, and water and pathogen-free mouse food (D and G, The Price-Wilhoite Co., Frederick, Md.) were available *ad libitum*, unless otherwise specified. The animal room and the experimental laboratory were maintained at 25 ± 2 C.

RESULTS

Influence of actinomycin D on development of tolerance to endotoxin. Mice made tolerant with injections of either the larger amount of *S. typhimurium* endotoxin (Group E), or the smaller amount (Group e), were almost equally resistant to the lethal effects of *S. marcescens* endotoxin compared with control mice given six daily injections of saline alone. These relationships are made evident by the data contained in the first three lines of Table 1. Mice receiving the smaller dose of actinomycin D (0.1 μg) combined with the larger dose of endotoxin (line 4, Table 1) and those receiving the reciprocal treatment (line 5, Table 1) were each more susceptible to endotoxin than the corresponding animals made tolerant with the same amount of endotoxin alone. Mice injected with only actinomycin D at three dose levels, 0.1, 1.0, and 2 μg (lines 6, 7, and 8, respectively, Table 1), were not significantly more tolerant than control mice. The important point to emphasize in these results is the ability of actinomycin D to significantly suppress the development of tolerance to endotoxin, even at the low dose level of 0.1 μg per mouse per day. The larger amount of antibiotic, 1 μg, prevents almost completely the development of tolerance. The question as to whether another inhibitor of protein synthesis also prevents tolerance to endotoxin is answered in the sections below.

Influence of ethionine on development of toler-

TABLE 1. *Survival data of mice pretreated with Salmonella typhimurium endotoxin or actinomycin D, or both, and challenged with three times the LD_{50} of Serratia marcescens endotoxin*

Treatment (six daily injections)	Survival		Statistical significance
	No. alive/total injected	Per cent	
1. Control (0.5 ml of saline)	1/95	1	
2. Endotoxin, Group E	27/28	96	
3. Endotoxin, Group e	70/76	92	
4. Group E + actinomycin (0.1 μg)	14/22	63.5	2 vs. 4; $P < 0.01$
5. Group e + actinomycin (1.0 μg)	8/40	20	3 vs. 5; $P < 0.01$
6. Actinomycin (0.1 μg)	3/24	12.5	1 vs. 6; P = NS*
7. Actinomycin (0.1 μg)	3/20	15	1 vs. 7; P = NS
8. Actinomycin (2.0 μg)	1/25	4	1 vs. 8; P = NS

* NS = not significant.

TABLE 2. *Survival data of mice pretreated with Salmonella typhimurium endotoxin or ethionine, or both, and challenged with three times the LD_{50} of Serratia marcescens endotoxin*

Treatment (six daily injections)	Survival		Statistical significance
	No. alive/total injected	Per cent	
1. Control (0.5 ml of saline)	1/20	5	
2. Endotoxin, Group e	20/20	100	1 vs. 2; $P < 0.01$
3. Group e + ethionine (1 mg)	16/19	84	2 vs. 3; P = NS*
4. Group e + ethionine (2 mg)	16/17	94	2 vs. 4; P = NS
5. Group e + ethionine (4 mg)	4/5	80	2 vs. 5; P = NS
6. Ethionine (2 mg)	2/20	10	2 vs. 6; $P < 0.01$

* NS = not significant.

TABLE 3. *Survival data of mice pretreated with Salmonella typhimurium endotoxin or 2-thiouracil, or both, and challenged with three times the LD_{50} of Serratia marcescens endotoxin*

Treatment (six daily injections)	Survival		Statistical significance
	No. alive/total injected	Per cent	
1. Control (0.5 ml of saline)	0/30	0	
2. Endotoxin, Group e	16/19	84	1 vs. 2; $P < 0.01$
3. Group e + 2-thiouracil (2.0 mg)	31/40	77	2 vs. 3; P = NS*

* NS = not significant.

TABLE 4. *Liver tryptophan pyrrolase activity in control and tolerant mice given cortisone, endotoxin, or inhibitor*

Treatment*	Liver tryptophan pyrrolase activity†	
	Control mice	Tolerant mice
1. No treatment	25.1 ± 1.7 (15)‡	26.5 ± 1.4 (16)
2. 17 hr after LD_{50} of *Serratia marcescens* LPS	9.9 ± 1.8 (7)	17.1 ± 1.3 (10)
3. 17 hr after LD_{50} of *Salmonella typhimurium* endotoxin	5.8 ± 0.9 (10)	19.4 ± 1.2 (10)
4. 4 hr after actinomycin D (10 μg)	13.5 ± 1.4 (8)	22.7 ± 2.1 (9)
5. 4 hr after ethionine (16 mg)	10.7 ± 0.6 (14)	19.3 ± 0.9 (14)
6. 17 hr after ethionine (8 mg)	1.9 ± 0.3 (8)	4.5 ± 0.6 (9)

* All mice fasted 17 to 21 hr.

† Micromoles of kynurenine per gram (dry weight) of liver per hour.

‡ Each value is the mean plus or minus the standard error of the mean for the number of determinations shown in parentheses.

ance to endotoxin. Ethionine is known from previous work to potentiate endotoxin and to prevent the protective effect of cortisone in endointoxication (Berry and Smythe, 1964). With this in mind, the experiments now to be described were undertaken. The results (Table 2) clearly establish that ethionine given concurrently with endotoxin at three dose levels failed to impair the development of tolerance. Even the largest dose, 4 mg, which killed 15 of 20 mice prior to the time of challenge on day 8, did not appear to alter the increased resistance to endotoxin lethality as the data (line 5, Table 2) suggest. Injections of ethionine alone had no effect on susceptibility to endotoxin (last line, Table 2).

Influence of 2-thiouracil on development of tolerance to endotoxin. Another inhibitor of protein synthesis, 2-thiouracil, which is known to potentiate endotoxin and to act in this respect similarly to actinomycin and ethionine (Berry and Smythe, 1964), was given with each daily injection of endotoxin, and tolerance was as marked in these animals as when endotoxin alone was administered (Table 3, lines 2 and 3). Therefore, both this inhibitor and ethionine had no effect on the sequence of changes responsible for the development of tolerance. One must presume, in light of this evidence, that actinomycin, but not all inhibitors of protein synthesis, interferes with the onset of tolerance to endotoxin.

Comparison of liver tryptophan pyrrolase activity in normal and tolerant mice. Since the enzyme tryptophan pyrrolase is known to be depressed in endotoxin poisoning (Berry and Smythe, 1963), its response to endotoxin in tolerant mice was determined (Table 4). The level of enzyme was the same in tolerant and in control animals (line 1, Table 4), the latter having received an injection of saline each time the tolerant mice were given an injection of endotoxin. At 17 hr after the injection of an LD_{50} of each of two endotoxins, a markedly greater decrease in enzyme activity was observed in livers of control mice than was observed in tolerant mice (lines 2 and 3, Table 4). There was also a significantly smaller decrease in tryptophan pyrrolase activity in livers of tolerant mice compared with that in controls 4 hr after the injection of either actinomycin D or ethionine (lines 4 and 5, Table 4). Even 17 hr after an injection of ethionine, the activity of enzyme was greater in tolerant mice than in normal animals (P = 0.01; line 6, Table 4). There is no doubt, therefore, that the enzyme tryptophan pyrrolase is more active in tolerant mice than in control animals, not only after endotoxin but after actinomycin D and ethionine as well.

TABLE 5. *Liver tryptophan pyrrolase activity in control and tolerant mice after injection of RES blocking agent and agent plus endotoxin*

Treatment*	Liver tryptophan pyrrolase activity†	
	Control mice	Tolerant mice
1. 6 hr after iv saline (0.2 ml)	27.1 ± 1.8 (10)	26.5 ± 1.4‡ (16)
2. 6 hr after iv saccharated iron oxide (0.2 ml)	14.8 ± 1.3 (10)	27.4 ± 2.0 (10)
3. 6 hr after iv saccharated iron oxide (0.2 ml) and 4 hr after ip endotoxin (0.1 LD_{50})	9.2 ± 0.6 (12)	15.2 ± 2.2 (12)

* All mice fasted 17 to 21 hr; iv = intravenous, and ip = intraperitoneal.

† Micromoles of kynurenine per gram (dry weight) of liver per hour. Each value is the mean plus or minus the standard error of the mean for the number of determinations shown in parentheses.

‡ No saline injected.

TABLE 6. *RES activation in tolerant mice as measured by carbon clearance*

Treatment (six daily injections)	Carbon clearance (K value)	Statistical significance
1. Control (0.5 ml saline)	0.044 (18)*	
2. Endotoxin, Group E	0.065 (10)	2 vs. 1; $P < .05$
3. Endotoxin, Group e	0.062 (20)	3 vs. 1; $P < .01$
4. Group E + actinomycin (0.1 μg)	0.064 (11)	4 vs. 1; $P < .01$
5. Group e + actinomycin (1.0 μg)	0.051 (18)	5 vs. 1; NS†
6. Actinomycin (0.1 μg)	0.050 (12)	6 vs. 1; NS
7. Actinomycin (1.0 μg)	0.036 (12)	7 vs. 1; NS
8. Group e + 2-thiouracil (2 mg)	0.059 (17)	8 vs. 1; $P < .01$
9. 2-Thiouracil (2 mg)	0.043 (12)	9 vs. 1; NS

* Each value is the mean for the number of determinations shown in parentheses.

† NS = not significant.

Influence of RES blocking agents on liver tryptophan pyrrolase activity. Since RES blocking agents are known to restore the tolerant animal to normal responsiveness, the effect of such an agent on liver tryptophan pyrrolase was determined. Values for the enzyme in normal and in tolerant mice 6 hr after intravenous saline were identical (Table 5, line 1). At 6 hr after an intravenous injection of 0.2 ml of saccharated iron oxide, the enzyme level in control mice was reduced to about one-half normal, whereas that in tolerant mice was unaltered (line 2, Table 5). The combination of saccharated iron oxide and endotoxin (0.1 LD_{50}) sensitized both groups of animals to enzymatic suppression, but more significantly in control mice than in tolerant mice (line 3, Table 5). When Thorotrast was substituted for saccharated iron oxide, a comparable reduction in tryptophan pyrrolase (not shown in the table) was observed in normal animals (no tolerant mice tested). It appears that the RES blockade exerts an inhibitory effect on at least this one inducible liver enzyme, similar to that described after an injection of endotoxin. This raises the question as to whether the suppression of enzyme activity under these conditions is the consequence of RES activity, or whether it must be presumed that the colloids capable of producing this effect were contaminated with endotoxin. Although no definitive answer can be given to this question (no unique assay for endotoxin exists at the present time), no gram-negative organisms could be cultured from the colloid, animals failed to develop tolerance after a series of injections, and the urinary nitrogen-excretion assay for endotoxin (Berry and Smythe, 1961) was negative for saccharated iron oxide and Thorotrast. All of these facts suggest that endotoxin was present, if at all, in minute amounts. Whatever the answer, it is of interest to note that the agents commonly used to show that RES blockade sensitizes to endotoxin, a well-confirmed fact, also suppress tryptophan pyrrolase.

Comparison of carbon clearance in mice subjected to the different treatments. Groups of mice given treatments similar to those described in the preceding sections of this paper were used to determine RES activity by use of the carbon-clearance technique (Table 6). The three groups showing the greatest degree of tolerance as judged by survival (see Tables 1 and 2) had rates of carbon clearance as high as any observed

TABLE 7. *RES activation in mice treated with saccharated iron oxide, as measured by carbon clearance*

Treatment (five daily injections)	Carbon clearance (K value)	Statistical significance
1. Control, saline alone	0.044 (18)*	
2. Saccharated iron oxide, 0.8 mg daily	0.078 (16)	2 vs. 1; $P < .01$
3. Saccharated iron oxide, 0.8 mg daily, + actinomycin, 1 μg daily	0.043 (12)	3 vs. 1; NS†

* Each value is the mean for the number of determinations shown in parentheses.
† NS = not significant.

TABLE 8. *Liver tryptophan pyrrolase activity in mice with RES activated by five daily injections of 0.8 mg of saccharated iron oxide*

Treatment*	Liver tryptophan pyrrolase activity†
1. Controls	19.4 ± 2.0 (12)
2. 6 hr after 4 mg of iv saccharated iron oxide	5.5 ± 2.0 (8)
3. 17 hr after ip $LD_{50/8}$ of endotoxin	5.0 ± 1.4 (13)

* All mice fasted 17 hr; iv = intravenous, and ip = intraperitoneal.
† Micromoles of kynurenine per gram (dry weight) per hour. Each value is the mean plus or minus the standard error of the mean for the number of determinations shown in parentheses.

(Table 6, lines 2, 3, 8). Mice also showing high RES activation were those represented by the results summarized in line 4. These animals were significantly less tolerant, however, than the groups to which reference was just made, but they were also more tolerant than control animals. The remaining groups showed no statistically significant increase in RES activity. Among these groups are the mice that yielded the data presented in line 5. These animals are of interest because actinomycin was able to prevent not only the development of tolerance but RES activation as well. Under the conditions of these experiments, tolerance was always associated with an activated RES. The data show, however, that full activation may occur without full tolerance (line 4).

Carbon clearance in mice given a series of injections of saccharated iron oxide. Previous data indicated that RES activation was an essential part of tolerance. To compare the data achieved with endotoxin with the response of mice with RES activated by a colloid other than endotoxin, the experiments described in this section were undertaken. Five daily intravenous injections of saccharated iron oxide were administered to mice, and the rate at which they cleared carbon is shown in Table 7, line 2. This rate is faster numerically than in any of the groups represented by the data of Table 6, and it is close to double that of control animals (Table 7, line 1). A concurrent intraperitoneal injection of actinomycin with each injection of saccharated iron oxide prevented the RES activation (Table 7, line 3) just as it did with endotoxin (Table 6, line 7). Apparently, the enhanced uptake of carbon by the phagocytic cells of the mouse after daily injections of RES activating agents is subject to inhibition by actinomycin.

Liver tryptophan pyrrolase in mice with RES activated by saccharated iron oxide. The extent to which an activated RES can influence the inhibitory effect of endotoxin on tryptophan pyrrolase is shown in Table 8. As the value in line 1 shows, the series of five injections of 0.8 mg of saccharated iron oxide alone failed to alter the level of tryptophan pyrrolase. The value of 19.4 units of enzyme activity is similar to values reported elsewhere (Berry and Smythe, 1964) for mice given no prior injections. When a large dose of saccharated iron oxide (4.0 mg) was given to these mice, a decrease in tryptophan pyrrolase within 6 hr to a value less than one-third that for controls was found (Table 8, line 2). Similarly, an injection of one-eighth the LD_{50} of endotoxin results, after 17 hr, in an enzyme level about one-fourth the control value (Table 8, line 3). Augmentation in the rate of carbon clearance is not sufficient in itself, therefore, to explain the activity of tryptophan pyrrolase observed in tolerant animals (see Table 4). Moreover, the mice treated with saccharated iron oxide with activated RES were not more resistant to endotoxin lethality than were untreated control mice. Of 15 controls, 5 survived endotoxin at a dose level that killed all of 15 animals treated with saccharated iron oxide, but 100% of tolerant animals survived this same dose.

DISCUSSION

These experiments make evident previously unreported characteristics of the endotoxin-tolerant animal and permit certain inferences in regard to the metabolic events that might be

required for the development of the tolerant state. Actinomycin is believed to prevent deoxyribonucleic acid-dependent ribonucleic acid (RNA) synthesis and, consequently, protein synthesis (Reich et al., 1961; Reich, 1964). One must tentatively assume this to be the most probable mechanism with which it prevents tolerance and RES activation when administered concurrently with an amount of endotoxin that, given alone, is effective in both respects. The amount administered is sufficiently small to raise more than a reasonable doubt that it could act in the manner recently described by Revel and Hiatt (1964). With large, milligram doses in rats, protein synthesis was found to be impaired without apparent involvement of RNA synthesis. Our findings appear to differ from those of Rosen et al. (1964), who found, with rats, that a single injection of 50 μg of actinomycin D per 100 g of body weight elevated tryptophan pyrrolase at 48 hr but not at 24 hr postinjection. They reported no lowering of enzyme activity at times prior to 24 hr. Their dose level was approximately the same, per unit of body weight, as that used for the results presented in Table 4, but the LD_{50} for mice is approximately twice (about 100 μg/100 g of body weight) the amount reported for rats. Until more is known about the biochemical action of actinomycin, its site of attack, as described here, remains uncertain.

Ethionine, now known to have effects on liver more complex than that likely to result merely as an analogue of methionine (Villa-Trevino, Shull, and Farber, 1957), fails to alter the development of tolerance. This is also true of 2-thiouracil. The timing of the inhibition, its duration, and, indeed, its site, may, among other things, contribute to the differences obtained with the inhibitors employed.

Another interesting aspect of these data is the resistance of liver tryptophan pyrrolase to endotoxin poisoning in tolerant mice. This could readily be explained as being due to an activated RES capable of sequestering or detoxifying the endotoxin, thereby preventing it from exerting its full influence. Smith et al. (1963) have published evidence for an enhanced endotoxin detoxifying capability in spleen homogenates derived from tolerant dogs, compared with controls. Such an action would not likely account for the resistance of the enzyme in tolerant mice to inhibition by both actinomycin and ethionine. Moreover, the results obtained in mice where the RES was activated by injections of saccharated iron oxide make it unlikely that sequestration alone is the crucial variable in level of tryptophan pyrrolase activity in tolerant animals. Despite the fact that an explanation is not readily available for why the liver enzyme in tolerant mice fails to become as readily inhibited as in control animals, it serves just as effectively in adding one more reason for believing that enzyme maintenance is a crucial problem in the animal poisoned with endotoxin. To be able to predict that tryptophan pyrrolase should behave as it was found to do in the tolerant mouse may be fortuitious, but it is nevertheless an essential requirement for a valid working hypothesis.

If tolerance depends on immune phenomena, as suggested by Watson and Kim (1963), Greisman et al. (1964), and others, actinomycin would have to act to prevent it. This substance has been reported to suppress antibody synthesis (Svehag, 1964), but only within limited periods of time and under conditions not obviously comparable with those employed for the production of the tolerant state. This could be interpreted, however, as evidence for a role in tolerance of a type of humoral response not evaluated by the techniques employed in the studies of Svehag. In any event, it is difficult to understand how the immune status of an animal would alter its response to inhibitors such as actinomycin and ethionine.

Although the phenomenon of tolerance remains incompletely understood, this report adds one new concept, i.e., the resistance to endotoxin and known inhibitors of protein synthesis of at least one liver enzyme, tryptophan pyrrolase. The basis for this enzymatic change remains to be elucidated in further investigations.

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LITERATURE CITED

BEESON, P. B. 1947*a*. Tolerance to bacterial pyrogens. I. Factors influencing its development. J. Exptl. Med. **86**:29–38.

BEESON, P. B. 1947*b*. Tolerance to bacterial pyrogens. II. Role of the reticuloendothelial system. J. Exptl. Med. **86**:39–44.

BENACERRAF, B., G. J. THORBECKE, AND D. JACOBY. 1959. Effect of zymosan on endotoxin toxicity in mice. Proc. Soc. Exptl. Biol. Med. **100**:796–799.

BERRY, L. J. 1964. Endotoxin lethality and tryptophan pyrrolase induction in cold-exposed mice. Am. J. Physiol. **207**:1058–1062.

BERRY, L. J., AND D. S. SMYTHE. 1961. Effects of

bacterial endotoxins on metabolism. III. Nitrogen excretion after ACTH as an assay for endotoxin. J. Exptl. Med. **113**:83–94.

BERRY, L. J., AND D. S. SMYTHE. 1963. Effects of bacterial endotoxins on metabolism. VI. The role of tryptophan pyrrolase in response of mice to endotoxin. J. Exptl. Med. **118**:587–603.

BERRY, L. J., AND D. S. SMYTHE. 1964. Effects of bacterial endotoxins on metabolism. VII. Enzyme induction and cortisone protection. J. Exptl. Med. **120**:721–732.

CHEDID, L. 1957. Hormones et infection, des effets opposes de la cortisone sur la resistance de l'hote au cours d'une salmonellase experimentale. Supplements, Bull. Biol. de France et de Belgique. **42**:1–151.

CROXTON, F. E. 1959. Elementary statistics with applications in medicine and the biological sciences, p. 267–283. Dover Publications, Inc., New York.

FEIGELSON, P., AND O. GREENGARD. 1961. A microsomal iron-porphyrin activator of rat liver tryptophan pyrrolase. J. Biol. Chem. **236**:153–157.

FEIGELSON, P., M. FEIGELSON, AND O. GREENgard. 1962. Comparison of the mechanisms of hormonal and substrate induction of rat liver tryptophan pyrrolase. Recent Progr. Hormone Res. **18**:491–507.

FREEDMAN, H. H. 1960. Passive transfer of tolerance to pyrogenicity of bacterial endotoxin. J. Exptl. Med. **111**:453–463.

FREEDMAN, H. H., AND B. M. SULTZER. 1964. Role of humoral mediator in tolerance to the pyrogenicity of bacterial endotoxin. Proc. Soc. Exptl. Biol. Med. **115**:607–610.

GREISMAN, S. E., H. N. WAGNER, JR., M. IIO, AND R. B. HORNICK. 1964. Mechanisms of endotoxin tolerance. II. Relationship between endotoxin tolerance and reticuloendothelial system phagocytic activity in man. J. Exptl. Med. **119**:241–264.

HALBERG, F., AND R. B. HOWARD. 1958. Twenty-four hour periodicity and experimental medicine. Postgrad. Med. **24**:349–358.

HOWARD, J. G., G. BIOZZI, B. N. HALPERN, C. STIFFEL, AND D. MOUTAN. 1959. The effect of *Mycobacterium tuberculosis* (BCG) infection on the resistance of mice to bacterial endotoxin and *Salmonella enteritidis* infection. Brit. J. Exptl. Pathol. **40**:281–290.

KNOX, W. E., AND V. H. AUERBACH. 1955. The hormonal control of tryptophan peroxidase in the rat. J. Biol. Chem. **214**:307–313.

PREVITE, J. J., AND L. J. BERRY. 1962. The effect of environmental temperature on the host-parasite relationship in mice. J. Infect. Diseases **110**:201–209.

PREVITE, J. J., AND L. J. BERRY. 1963. Studies on the potentiation of endotoxin in mice by exposure to cold. J. Infect. Diseases **113**:43–51.

REED, L. J., AND H. MUENCH. 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**:493–499.

REICH, E. 1964. Actinomycin: correlation of structure and function of its complexes with purines and DNA. Science **143**:684–689.

REICH, E., R. M. FRANKLIN, A. J. SHATKIN, AND E. L. TATUM. 1961. Effect of actinomycin D on cellular nucleic acid synthesis and virus production. Science **134**:556–557.

REVEL, M., AND H. H. HIATT. 1964. Actinomycin D: an effect on rat liver homogenates unrelated to its action on RNA synthesis. Science **146**:1311–1313.

ROSEN, F., P. N. RAINA, R. J. MILHOLLAND, AND C. A. NICHOL. 1964. Induction of several adaptive enzymes by actinomycin D. Science **146**: 661–663.

SMITH, E. E., S. H. RUTENBERG, A. M. RUTENBERG, AND J. FINE. 1963. Detoxification of endotoxin by splenic extracts. Proc. Soc. Exptl. Biol. Med. **113**:781–784.

SUTER, E., G. E. ULLMAN, AND R. G. HOFFMAN. 1958. Sensitivity of mice to endotoxin after vaccination with BCG (Bacillus-Calmette-Guerin). Proc. Soc. Exptl. Biol. Med. **99**:167–169.

SVEHAG, S. E. 1964. Antibody formation *in vitro* by separated spleen cells: inhibition by actinomycin or chloramphenicol. Science **146**:659–661.

VILLA-TREVINO, S., K. H. SHULL, AND E. FARBER. 1963. The role of adenosine triphosphate deficiency in ethionine-induced inhibition of protein synthesis. J. Biol. Chem. **238**:1757–1763.

WATSON, D. W., AND Y. B. KIM. 1963. Modification of host responses to bacterial endotoxin. I. Specificity of pyrogenic tolerance and the role of hypersensitivity in pyrogenicity, lethality and skin reactivity. J. Exptl. Med. **118**:425–446.

WHITE, C. 1952. The use of ranks in a test for significance for comparing two treatments. Biometrics **8**:33–41.

WILCOXON, F. 1949. Some rapid approximate statistical procedures. American Cyanamid Co., New York.