(5). Extensive studies on the effects of dietary factors on the CF excretion have not been reported. A very interesting aspect of folic acid deficiency in the rat is the diminished response to estradiol induced uterine growth(1). An interference with metabolism of C^{14} labeled estrone in aminopterin-treated rats is reported by Trunnell *et al.*(6).

Summary. Administration of estradiol dipropionate alone or together with PGA enhances the urinary excretion of CF by male rats. Testosterone alone gives a 50% decrease in the CF excretion but is without significant effect when given in combination with PGA. The stimulatory effect of estradiol dipropionate can not be reversed by testosterone in the amounts tested.

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Mechanism of Protective Effect of Hydrocortisone in Staphylococci Infected Adrenalectomized Mice.*† (21999)

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The reduced natural resistance of Addisonian patients as well as of adrenalectomized animals is a general phenomenon not specific for any one type of infection or intoxication. Although the mechanism by which adrenalectomy influences resistance remains relatively obscure, replacement therapy with adrenal cortical hormones has been shown to be beneficial(1,2). On the other hand, adrenal hormones may be detrimental to host resistance(3). It has been suggested, however, that the beneficial effects of cortisone therapy are to be derived only from dosages based on requirement(4,5). In this regard, Robinson, *et al.*(6), have recently pointed out that the detrimental effect of cortisone in resistance to pneumococcal infection in rats is primarily due to overdosage with the hormone, and that an optimal dosage schedule of cortisone can significantly enhance the host resistance of either adrenalectomized or intact infected animals. Hill, *et al.*(7), have described similar findings with systemic moniliasis in adrenalectomized mice treated with cortisone. Germuth, *et al.*(8), found that cortisone-treated rabbits could successfully resist an intravenous inoculum of *Staphylococcus aureus* which was fatal to approximately 50% of the untreated controls. They noted that the bacteria rapidly disappeared from the blood after injection in either treated or untreated animals. Kleiger and Blair(9) have described the acute death of experimental animals resulting from an inoculation of toxigenic staphylococci as an *in vivo* production of toxin by the bacteria.

It has been observed in this laboratory, that a strain of staphylococci which regularly produced a rapidly fatal bacteremia in mice after intraperitoneal inoculation proved to be relatively innocuous if given by the intravenous route. However, adrenalectomy reduced resistance to an intravenous challenge with this microorganism by a factor of at

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least 1000. This deficiency in resistance produced by adrenalectomy is apparently not caused by the impairment of phagocytic or cidal activities of the reticulo-endothelial system, but rather to an increased sensitivity of the host to the noxious effects of the bacterial protoplasm. Hydrocortisone effectively protected the adrenalectomized mice from the toxic effects of an intravenous challenge of either viable staphylococci or a bacterial extract.

Materials and methods. Mice: Eight-weeks-old male mice (CBA) were used throughout this study. Bilateral adrenalectomies were performed by the dorsal approach 18 hours before challenge. These animals were fed *ad libitum* and maintained on 0.9% saline as drinking water. All control mice were sham operated. In order to evaluate the biological effect of hydrocortisone, adrenalectomized mice were used to obviate interference from endogenous adrenal cortical secretion. *Bacteria*: The organism selected for this study was a strain of *Micrococcus pyogenes* var. *aureus*, which had been isolated from a human source. The bacterial cells were prepared for inoculation by growing them for 18 hours on nutrient agar, and then were transplanted to brain-heart infusion broth for an additional three hours of growth. The cells were harvested by centrifugation and washed once, and then resuspended with 0.9% saline. The concentration of bacteria was initially adjusted to a standard turbidity in the Klett-Summerson photocolorimeter, using a 420 mμ blue filter. This was later confirmed by plate counts. *Plate counts*: Determination of viable numbers of bacteria was routinely carried out by serial 10-fold dilution of the sample, of which 0.5 ml aliquots were taken for pour plating (nutrient agar). Three plates were used for each dilution per sample. The average count was obtained after the plates had incubated for 18 hours at 37°C. *Bacterial extract*: Bacteria were grown in brain-heart infusion broth for 18 hours then sedimented by centrifugation and washed 3 times with 0.85% saline. The washed preparation was sonerated in a Raytheon high frequency oscillator at 9.0 to 9.5 kilocycles for 4 hours at 13°C. The opalescent material was removed and centrifuged overnight in the cold. The supernatant was diluted to 1:5 and centrifuged 3 more times to reduce the viable cell count to less than 4/ml. The supernatant was then treated with $(NH_4)_2SO_4$ and the resultant precipitate dialyzed, reprecipitated with ethanol and finally washed with acetone and dried. *Treatment and challenge*: Hydrocortisone acetate (Merck)‡ was given by the intraperitoneal route in the standard volume of 0.1 ml. The hormone was prepared as a saline suspension and given in varying dilutions 2 hours prior to challenge. The challenge inocula of bacteria or bacterial extract were contained in volumes of 0.25 ml and introduced via the tail vein 18 hours after adrenalectomy. The LD_{90} (90% lethal dose = 86.9 ± 3.4%) of viable bacteria for adrenalectomized mice was found to be approximately 3 to 5 million viable cells, while the LD_{90} (90.2 ± 4.1%) dosage of the bacterial extract was found to consist of approximately 2.5 μg of the dried material.

Results. Experimental. Effect of route of infection on resulting bacteremia: In preliminary work it was found that the mouse was much more resistant to infection with this microorganism by the intravenous route than by the intraperitoneal. To investigate this further, mice were divided into 2 groups and injected with 5×10^8 viable bacteria contained in a volume of 0.25 ml. One group of animals received this challenge dose by the intravenous route, while the other group was inoculated intraperitoneally. At intervals of 5, 30, 60, 120, 180 and 240 minutes following injection, 3 mice from each group were lightly anesthetized and blood samples obtained by cardiac puncture with heparinized syringes. These samples were used for estimation of the existing bacteremia (plate counts). Mice not used for bleeding were kept as infected controls. All of the intraperitoneally infected controls were dead (8/8) 24 hours after injection, whereas none (0/8) of the intravenously injected controls exhibited any obvious symptoms of infection following injection with the

‡ The authors wish to thank Dr. Elmer Alpert of Merck and Co., Rahway, New Jersey for the generous supply of hydrocortisone used in these experiments.

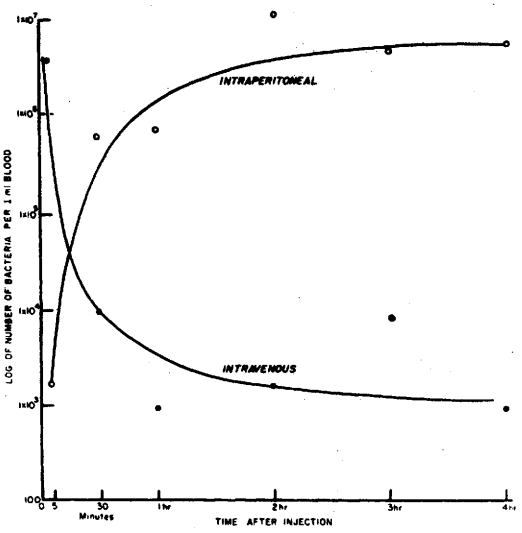

FIG. 1. Comparison of numbers of viable bacteria in blood of mice following infection by intravenous or intraperitoneal routes.

bacteria. It is evident (Fig. 1) that injection of the microorganism by the intraperitoneal route permitted the development of a rapid and overwhelming bacteremia which ultimately resulted in the death of the host. The initial bacteremia (5 minutes) undoubtedly was due only to invasion from the peritoneal cavity which was then maintained at this level by the constant infusion of bacteria produced by multiplication in the cavity. The actual extent of the invasion from the peritoneum cannot be fully appreciated unless the fate of the blood-borne bacteria is considered. The reticulo-endothelial system, which functions as a clearing mechanism for the blood, efficiently removed the inoculum dose of bacteria (Fig. 1), whereas the local response in the peritoneum is apparently overwhelmed allowing a fatal systemic invasion to occur. The two examples illustrate the extremes in bacteremia, which "represents a balance between bacterial multiplication, invasion of the blood stream, and the activity of the clearing mechanism"(10).

Bacteremia and splenic uptake of bacteria in adrenalectomized and sham operated mice: Adrenalectomy rendered the mouse highly sensitive to an intravenous inoculum of the bacteria that was relatively innocuous in the sham operated control. To investigate the influence of adrenal cortical secretion on this aspect of resistance, adrenalectomized and sham-operated mice were inoculated intravenously with approximately 3.3×10^6 bacteria. Blood samples were obtained by intracardial puncture at the intervals noted in Fig. 2. The blood samples for each separate period were obtained from at least five mice and pooled. Likewise, the spleens of these animals were rapidly and aseptically removed, weighed, pooled and homogenized with sterile 0.85% saline in glass TenBroeck tissue grinders. Aliquots of the blood and spleen homogenates were taken for plate counting.

The inoculum used would theoretically be sufficient to produce a bacteremia of approximately 10^6 cells per ml of blood, which indicates a precipitous loss of bacteria (Fig. 2) from the blood during the initial period following injection (ca. 90% in initial 5 minutes, ca. 90% of remainder during subsequent 25 minutes). The initial high count of viable bacteria in the spleen is well correlated with the rapid clearance of the bacteria from the blood. Supplemental studies have shown that the reticulo-endothelial activity of the liver (on weight basis) was similarly effective. Since the concentration of viable bacteria in the blood rapidly fell to a relatively insignificant level, the decrease in numbers of viable

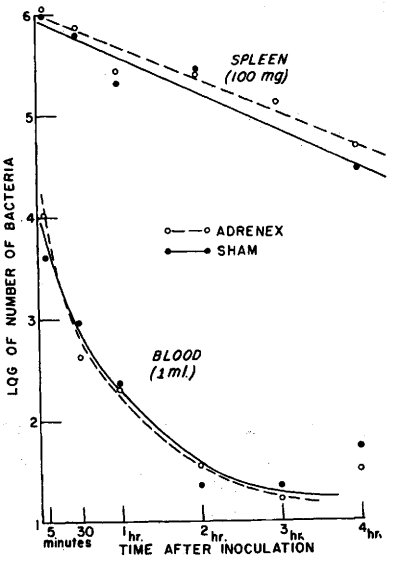

FIG. 2. Comparison of numbers of viable bacteria in blood and spleen of mice, adrenalectomized or sham-operated, following infection by intravenous route.

TABLE I. Independence of Lethal Dose on Numbers of Viable Staphylococci.

Bacterial preparation	No. viable bacteria per inoculum	Mortality	Mean survival time, min.	Probability*
A. Control	4.0×10^5	9/ 9	101.1	
Sonerated	6.3×10^6	10/10	85.9	$P = .01$
B. Control	2.0×10^8	10/10	112.6	
Sonerated	3.2×10^6	10/10	86.1	$P = .01$
Boiled	0.0×10^0	10/10	103.5	$P = .3$–$.4$

* Probability, using Student's "t" test, that the mean survival time of the test group is the same as the mean survival time of the control.

bacteria in the spleen was probably due to the cidal activity of the phagocytic cells. This decrease amounted to approximately 95% in 4 hours. All of the injected control sham-operated mice not used for bacteremia study survived with no evidence of symptoms, whereas all of the injected adrenalectomized mice succumbed. Despite the almost complete disappearance of bacteria from the blood and apparent destruction by the reticulo-endothelial system, symptoms of bacterial intoxication were evident in many of the adrenalectomized mice by the four hour period (ruffled fur, unsteady gait, etc.). The essential difference in the resistance of the adrenalectomized mouse when compared to its control seemed to be in its increased sensitivity to the bacteria *per se*, rather than a deficiency in ability to phagocytize and destroy the pathogen.

Toxic factor and resistance: From the results of the previous experiment it seemed possible that the digestive processes of the phagocytic cells of the reticulo-endothelial system might liberate toxic substances from the bacterial cell to the circulation which, in the adrenalectomized mouse, could attain a lethal concentration due to the greater sensitivity of this animal to noxious material. Preliminary observations showed that the lethality of various preparations of bacteria for adrenalectomized mice (measured as mean survival time) was more directly related to the concentration of bacterial substance than the numbers of viable bacteria in the inoculum. Bacteria formalinized and washed, heated to 58°C, exposed to boiling temperatures or incubated in distilled water or with antibiotics, were not less toxic than control bacterial suspensions. To simulate the result of digestive action by phagocytic cells on the ingested microorganisms, bacteria were "solubilized" by disruption in a high frequency oscillator (sonerated) for 4 hours at 13°C.

A comparison of the toxicity of sonerated and control preparations was made following intravenous injection of the materials into adrenalectomized mice. In part A of Table I it can be seen that "solubilization" of the bacteria significantly increased the toxicity of this preparation, even though the numbers of viable bacteria were markedly reduced. The experiment was repeated (B) using a 1 to 2 dilution of the control which had been immersed in a boiling water bath for 30 minutes. Analysis of the toxicity of the control vs. the heated preparation showed no significant difference, although the latter inoculum was completely sterilized by the heat treatment. On the other hand, "solubilization" of the bacteria significantly enhanced the toxicity of the preparation. The results suggest that since the intravenously injected bacteria are rapidly localized in the tissues of the reticulo-endothelial system and therein destroyed (Fig. 2), the lethal effect of the bacteria may depend on solubilization of the ingested bacteria by the phagocytic cells and subsequent release of the toxic material to the circulation of the sensitive host.

Protective effect of hydrocortisone: The observation has been made that staphylococci administered by the i.v. route are rapidly cleared from the blood and destroyed by the cells of the R. E. system. If the digestion of these phagocytized bacteria liberates toxic bacterial substance to the circulation and thereby fatally intoxicates the adrenalectomized mouse, replacement therapy should be effective in protecting mice against a lethal

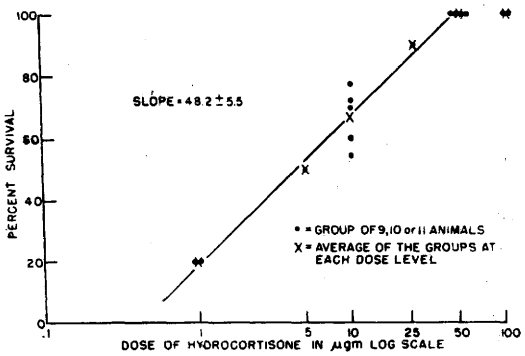

FIG. 3. Protective effect of hydrocortisone in adrenalectomized mice against 1 LD_{50} (i.v.) of a toxic bacterial extract.

dose of either soluble bacterial extract or viable bacteria. Adrenalectomized mice were treated with graded doses of hydrocortisone two hours prior to an i.v. injection of the cell-free bacterial extract. Observations on mouse survival were routinely carried out over a period of 72 hours.

Fig. 3 illustrates the various degrees of protection, relative to hormone dosage, provided to mice by hydrocortisone against a dose of bacterial extract that was lethal to 90.2 ± 4.1% of the untreated controls (40 mice). The straight line in Fig. 3 was obtained by the method of least squares. Hormone-treated mice surviving for 24 hours following the challenge died at a rate no greater than non-challenged adrenalectomized controls.

The animals used to test the effect of hydrocortisone in mice challenged with an i.v. dose of viable staphylococci were prepared, treated and observed in essentially the same manner as in the previous experiment. In the dosage range from 0.25 to 25 γ of

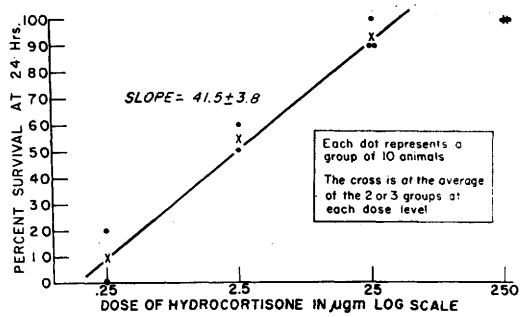

FIG. 4. Protective effect of hydrocortisone in adrenalectomized mice against 1 LD_{50} (i.v.) of viable *M. pyogenes* var. *aureus*.

hydrocortisone, a straight line relationship of log dose to response was obtained by the method of least squares (Fig. 4). It is interesting to note that a 10-fold increase in hormone dosage did not diminish the protective response (*i.e.*, 250 γ). Since non-moribund animals surviving 24 hours after injection died at a rate no greater than non-infected controls, it was evident that the hormone effect was not limited to a mere prolongation of survival time. Bacteremia in survivors, if present, was extremely light and the spleens contained few to no detectable organisms.

Discussion. The remarkable sensitivity of the adrenalectomized animal to noxious substances has provided a unique opportunity to study the pathogenesis of a controlled bacteremia. It has been found that after the introduction of bacteria into the blood of either adrenalectomized or sham-operated mice, the pathogens are rapidly removed from the circulation. Both the spleen and liver participated in the removal, and, on a weight basis, were similarly active. Following the uptake by the spleen, a sterilization of the bacteria ensued, and it is postulated that the digestion of the bacteria by the reticulo-endothelial cells resulted in a release of toxic (or irritating) bacterial substances. Harris and Ehrich (11) have reported that soluble antigens of locally injected washed Shigella bacteria or foreign erythrocytes appear at the site of injection, draining lymph node and in the efferent lymphatics, at times which suggest solubilization and release of these antigens from the phagocytic cells of the local area. It has been found that "solubilization" (by soneration) of the bacteria decreases the survival time of i.v. injected adrenalectomized animals even though the numbers of viable bacteria are greatly reduced. Inocula of bacteria sterilized by exposure to boiling water bath for 30 minutes were as toxic as viable control material.

Since the intact mouse is markedly resistant to a given dose of either the bacteria or the bacterial extract given by the intravenous route, it is likely that the release of soluble bacterial substances by the reticulo-endothelial system in the intact mouse is accomplished without any untoward effects. However, in

the adrenalectomized mouse, which is notoriously sensitive to bacterial toxins, etc., this destruction of the bacteria does not provide suitable protection. Perhaps in the intact mouse, which has been given an intraperitoneal inoculation of the bacteria, the resulting bacteremia (Fig. 1) assumes such proportions that the R. E. system accumulation of bacterial material produced during the period of infection may be sufficient to constitute a lethal dose within a relatively short time after injection (5 to 7 hours). Smith and Keppie (12) have shown the irreversible nature of bacterial intoxication with anthrax bacillus despite control of the bacteremia by antibiotics.

Single administrations of graded doses of hydrocortisone were shown to furnish protective responses relative to hormone dosage in adrenalectomized mice injected intravenously with an LD_{100} of either viable bacteria or bacterial extract. Since this protection did not deteriorate with time, the pathogenic activity of this organism after an intravenous injection must be essentially due to the release (solubilization) of its cellular components, rather than to multiplication and concomitant toxigenic activity. The apparent enhancement of the detoxification mechanisms of the sensitive host by hydrocortisone would require no new property not already generally ascribed to this hormone. The antiphlogogenic activity of hydrocortisone, by limiting the chain-like sequence of events due to a noxious stimulus, can so markedly reduce the systemic reaction to the stimulus as to completely inhibit the appearance of symptoms(4).

Summary. 1. The increased tolerance of mice to an i.v. challenge of staphylococci as compared to an injection of the bacteria by the i.p. route was found to be related to the rapid clearance of bacteria from the blood following the i.v. injection. However, adrenalectomy markedly reduced the resistance to an i.v. challenge without altering the rates of clearance or bacterial destruction by the R. E. system. 2. It was postulated that digestion of the bacteria by the R. E. system liberated bacterial material which was noxious to the adrenalectomized mouse. "Solubilization" of the bacterial preparation increased its toxicity as compared to control viable or heat-sterilized preparations, indicating the independence of this toxicity to numbers of viable bacteria. 3. Hydrocortisone pretreatment of adrenalectomized mice was found to be protective, relevant to dosage, in minute amounts, against i.v. challenge with either viable staphylococci or extracts of this organism.

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