increasing weight this fall is not as great as other studies have shown.

Because of some difference in whole body and large vessel hematocrit the most accurate method of determining total blood volume is separately measuring plasma and red cell volumes, but this requires two different labeling substances to be diluted. The methods using hematocrit and dilution analysis of either plasma or cell volume are subject to the criticism of inaccurate hematocrit and trapped plasma values. Our method circumvents this latter objection with sacrifice of only a minor degree of accuracy. Funkhouser *et al*(12) measured blood volumes in humans both by plasma and red cell labels and their data show that blood volume determined by addition of the plasma volume and red cell volume gave a value which was regularly only 5% lower than values obtained using only RISA and counting whole blood. Thus with only minimal loss of accuracy great simplicity of determinations is achieved.

From a comparison of Fig. 1 and 2 it is apparent that as body weight increases in the rat, as in man, blood volume expands more slowly. Thus in experiments wherein corrections for body weight are important, ratios may give false interpretations of data since blood volume/body weight ratio is not a proportional function. Greater accuracy will be obtained by plotting blood volume *vs* body weight which allows a direct reading of the expected blood volume at any given body weight.

Summary. Total blood volume was determined in 122 male rats using RISA. The values were related to body weight and a least squares regression line was plotted. This regression line enables one to predict total blood volume for any given body weight. The method utilizing RISA and whole blood counting has the advantage of simplicity with minimal loss of accuracy.

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Microcirculatory Effects of Cortisol. Protective Action Against Na_4EDTA Damage.* (29916)

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For the most part, the biological effectiveness of the adrenal corticosteroids has been ascribed either to changes in carbohydrate and protein metabolism, electrolytes, or to some direct influence on cell membranes. These effects become manifest after some hours or even days following administration of the steroid. In contrast, the anti-inflammatory action of corticoids develops quickly.

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Inasmuch as steroids are equally effective locally, they probably act directly on the vascular elements—smooth muscle and endothelium. A possible explanation for this type of action is seen in the recent work of Weissmann and Thomas(1) who suggest that cortisone acts on a subcellular level to stabilize the membranes of lysosomal granules and thereby prevents release of lysosomal hydrolases, products which under normal circumstances could conceivably damage the vessel wall.

It was felt that the use of a circumscribed vascular end-point, instead of more commonly used parameters, such as edema or accumulation of dye-protein complexes, would make it possible to define more precisely the mode of action of steroids in acute situations. Furthermore, by carrying out the experiments on adrenally insufficient animals, it was hoped that the restorative action of steroid replacement would be especially striking. The direct observational technique of Chambers and Zweifach(2) was used to monitor *in vivo* changes in the microcirculation of animals treated with a soluble steroid, cortisol,§ which appears in biologically active form within 5 minutes after intravenous administration.

The end-point selected for this study was the alteration in integrity and permeability of the capillary and venular wall which appears within several minutes after local application of a calcium chelating agent, ethylenediamine-tetra-acetic acid tetra-sodium salt (Na_4EDTA),‖ onto the mesentery. Since a calcium-free state of such a short duration is believed to involve primarily a change in the basement membrane and/or the intercellular binding forces of the endothelial cells(3), such a test reaction would be especially pertinent to the proposed anti-inflammatory action of corticosteroids through an inhibition of the release of lysosomal hydrolases.

In view of the fact that bacterial endotoxins in lethal doses produce a form of shock or circulatory failure which develops in exaggerated form in adrenalectomized animals (4), the restorative action of cortisol in pharmacologic doses was examined in relation to the rapidity with which the soluble form of the steroid could protect adrenalectomized animals from otherwise lethal doses of endotoxin.

Method. Female adult Wistar strain rats (Charles River) weighing 130-180 g were used exclusively. Prior to exposing the mesocecum, the rats were anesthetized with pentobarbital, 3 mg/100 g of body weight, intramuscularly. A mid-line abdominal incision allowed exteriorization of the mesocecum without undue strain on the mesenteric vessels. With the rat on its side, the mesentery can be transilluminated and viewed with the compound microscope, the stage of which has been replaced with a translucent plastic plate mounted on a mechanical manipulator. Warmed Ringer's gelatin or modified (calcium-free) Ringer's gelatin with Na_4EDTA 0.25%, buffered to pH 7.2-7.4, was irrigated over the exposed mesentery maintained at 37°C.

Adrenalectomy was performed under ether anesthesia. Both adrenal glands were removed with the capsules intact to assure that no remnant remained; otherwise, the animal was sacrificed. Postoperatively the animals were maintained on 1% NaCl drinking water, and all experiments were performed on the 7th day following adrenalectomy.

The treated rats received cortisol (Solu-Cortef) 5 mg/100 g of body weight intravenously from 6 hours to 15 minutes prior to the local application of Na_4EDTA (ethylenediamine-tetra-acetic acid tetra-sodium salt).

Salmonella enteritidis endotoxin (Difco) was used. The LD_{75} was established at 1 mg/50 g body weight intravenously with a group of 25 normal rats. With adrenalectomized rats maintained on 1% NaCl drinking water but no steroid replacement, the LD_{100} was established at 1.7 μg of endotoxin/50 g body weight (0.8 μg/50 g body weight was lethal for 11/18 or 59% of the adrenalectomized rats).

Results. As a test for the efficacy of the technique of adrenalectomy, 8 adrenalectomized rats were maintained postoperatively without steroid replacement and were not supplied with supplementary sodium in the drinking water. Within a week 7 of these

§Solu-Cortef—Upjohn.

‖ Versene®—Dow Chemical Co.

TABLE I. Effect of Cortisol on Vascular Damage by Na_4EDTA* in Mesentery.

	Total	Degree of microcirculatory damage		
		Minimal	Moderate	Extensive
		(%)	(%)	(%)
Normal rats	19	13 (68)	6 (32)	0 (0)
Adrenalectomy, no cortisol	19	2 (10)	6 (32)	11 (58)
Adrenalectomy, cortisol† 1-6 hr prior to Na_4EDTA	31	23 (74)	7 (23)	1 (3)
Adrenalectomy, cortisol† 30 min prior to Na_4EDTA	19	7 (37)	9 (47)	3 (16)
Adrenalectomy, cortisol† 15 min prior to Na_4EDTA	18	1 (5)	7 (39)	10 (56)

* Na_4EDTA 0.25% in calcium-free Ringer's gelatin.
† Cortisol (Solu-Cortef) 5 mg/100 g of body weight.

animals had died; one animal was finally sacrificed at the end of 2 weeks. Presumably the survivor had adequate accessory adrenal function despite the adrenalectomy.

The microvascular bed of 20 adrenalectomized rats was examined under standard conditions with the usual drip solution of warmed Ringer's gelatin. Initially, the vessels appeared normal in most of the animals. The constrictor response of the arterioles and precapillaries to topical application of epinephrine (0.05 μg) was essentially the same as in normal animals. However, within about 15-20 minutes after exposure, the vessels became hypotonic and less responsive to epinephrine; hyperemia was apparent, and stasis and petechiae developed in numerous areas. These findings are consistent with the previous report of Zweifach(5) on a Wistar strain of rats.

Na_4EDTA in normal rats. In the normal unoperated animal, significant vascular damage appeared after irrigation of the mesentery with Na_4EDTA only when solutions of 1% or higher were used. The lower concentration of Na_4EDTA (0.25%) which was used in the adrenalectomy experiments, represented a minimally effective dose with only 32% of the animals (6/19) showing only traces of stasis or petechiae after a standard 10-minute period of application (Table I).

Na_4EDTA in salt-maintained adrenalectomized rats (no cortisol). In marked contrast to the normal rats, 0.25% Na_4EDTA resulted in rapid deterioration of the capillary and venular vessels. Within 10 minutes red cell aggregation was already marked, stasis was sufficiently extensive to interrupt capillary and venular flow, and discrete capillary and venular hemorrhages developed. The earliest changes were seen in the venules where the prominent outer sheath appeared to swell and collars of red blood cells formed around the vessels. In about 58% of the adrenalectomized rats (11/19) extensive damage of this type occurred throughout the vascular bed following application of Na_4EDTA, and in an additional 31% (6/19) moderate changes were noted with fewer vessels affected. Only 10% of the adrenalectomized rats which received no steroid replacement (2/19) showed no significant damage from the application of Na_4EDTA, in line with the 12% which apparently have accessory adrenal tissue. It is noteworthy that vascular injury following the application of Na_4EDTA to the mesentery of the adrenalectomized rats was not associated with sticking of leucocytes or platelets, the characteristic feature of the classic inflammatory response.

Na_4EDTA in salt-maintained adrenalectomized rats following cortisol therapy. When cortisol in pharmacologic doses of 5 mg/100 g of body weight was administered intravenously to the adrenalectomized rats at intervals of from 1-6 hours prior to the application of Na_4EDTA, the propensity to Na_4EDTA damage was reduced to essentially that of the normal animals. In experiments where the same dose of cortisol was administered 30 minutes before application of the Na_4EDTA, only 16% of the animals (3/19) showed extensive capillary and venular damage, 47% (9/19) showed a moderate degree

TABLE II. Protective Action of Cortisol Against Lethal Endotoxemia.

	Endotoxin (i.v.)	Total	Died	Survived	% survival
Normal rats	3.0 mg	25	19	6	24
Adrenalectomy, no steroid	2.5 μg	18	11	7	39
Idem.	5.0 "	18	18	0	0
Adrenalectomy, cortisol* 30 min prior to endotoxin	5.0 "	12	4	8	66
Adrenalectomy, cortisol* 15 min prior to endotoxin	5.0 "	10	9	1	10

* Cortisol (Solu-Cortef) 5 mg/100 g of body weight.

of vascular stasis and hemorrhage, while 37% (7/19) showed no adverse effects from Na_4EDTA (Table I). On the other hand, when cortisol was administered only 15 minutes prior to the application of Na_4EDTA, 92% of the animals (17/18) developed moderate to extensive capillary and venular stasis and extravasation of red blood cells with petechiae formation.

Lethal endotoxemia in salt-maintained adrenalectomized rats. A dose of 5 μg of endotoxin intravenously in a 150-180 g rat was lethal to 100% of 18 adrenalectomized rats within 48 hours (normal rats require 3000 μg/rat). When a comparable group of adrenalectomized rats were treated with cortisol (5 mg/100 g of body weight) 30 minutes prior to intravenous injection of 5 μg of endotoxin, 66% (8/12) survived beyond 48 hours (Table II). The same dosage of cortisol, administered just 15 minutes prior to the endotoxin, was not effective and 90% (9/10) died within 48 hours.

Discussion. Only meager information exists concerning the possible mechanism by which the acute biologic effects of corticosteroids are mediated. The absorption and metabolic disposal of water soluble cortisol administered intravenously was examined by Melby and St. Cyr(6) who found that biologically active steroid appears within the blood stream 5 minutes after injection. No additional activity appeared after 30 minutes from administration.

Metabolic studies on the effects of biologically active steroids do not demonstrate any effects in less than 2 hours on the glucose tolerance curve(7,8). The temporal relationship between metabolic changes of this kind and the anti-inflammatory action of cortisol has not been determined. Evidence which bears indirectly on this point is given by Janoff *et al*(9), who showed that plasma acid phosphatase levels in rats pretreated with cortisone acetate intraperitoneally 30 minutes prior to endotoxemia were significantly lower than in untreated controls. In the light of previous *in vitro* work of Weissmann and Thomas(1), this effect can be interpreted as evidence that the capacity of cortisone to stabilize the lysosomal membrane is a rapid event probably unrelated to the more delayed metabolic sequelae.

The vascular effects of comparatively low concentrations of Na_4EDTA are essentially the same in normal and in adrenally-insufficient rats. The difference between the two groups lies in the smaller dose required to produce an equivalent damage after adrenalectomy and the rapidity with which these effects develop in the adrenally-insufficient rats. It should be emphasized that the damage inflicted by Na_4EDTA differs sharply from that observed with noxious or phlogenic stimuli. The classical signs of inflammation, leucocyte and platelet adhesion, endothelial swelling, and thrombosis, are completely lacking. Instead, the earliest changes are a tendency for red cells to be slowed during their passage along the capillaries and venules. Complete stasis then ensues, followed shortly by extravasation of red cells into the tissue proper. It should also be pointed out that these effects occur primarily in the capillary and venular vessels; only after the damage is extensive are the arterial vessels affected.

The nature of the changes induced by the Na_4EDTA in the adrenally-insufficient rat suggests that the intercellular bond and/or

the basement membrane have been weakened to the point that plasma and red cells leak into the surrounding tissues. The conspicuous absence of endothelial swelling and leucocyte sticking would seem to argue strongly against the endothelial cell itself being affected sufficiently to account for the changes observed.

The predilection of the capillaries and venules to the disruptive action of Na_4EDTA in adrenally-insufficient rats can be reversed by soluble cortisol in as short a time as 30 minutes. It is difficult to conceive how a direct action on the endothelial cell proper could be reflected on the properties of the basement membrane in such a relatively short period. Inasmuch as there is no evidence that Na_4EDTA acts on the endothelial cell, the blunting of the effects of Na_4EDTA by the cortisol represents further proof for this explanation. In line with the action of Na_4EDTA on the intercellular junction and/or the basement membrane cortisol would appear to have a direct restorative action on these extracellular structures.

Since the topical application of Na_4EDTA results in a localized effect on the mesenteric vessels, a tissue which is relatively devoid of lysosome-rich cells such as leucocytes, macrophages, or reticuloendothelial elements, the present data on the restorative action of cortisol cannot be used as evidence for a stabilizing effect on the lysosomal membrane.

The experiments on lethal endotoxemia were included because this condition is associated with a profound derangement of smooth muscle reactivity(4), and the ability of the cortisol to counteract the lethal action of bacterial endotoxin within as short a period as 30 minutes demonstrates another apparently direct action of corticoids through an action on the cell membrane of these vascular components.

Conclusions. The above experiments have led us to conclude that corticosteroids have a direct chemical action on the extracellular basement membrane or intercellular binding forces which are most probably responsible for the loss of vascular integrity in the adrenally-insufficient rat. It is suggested that a stabilizing action of cortisol on the extracellular basement membrane and intercellular cement of capillaries and venules constitutes a hitherto unreported property of this hormone in relation to its anti-inflammatory action.

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Amniotic Fluid Measurement in Cortisone Treated and X-Irradiated Mice.* (29917)

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Cleft palate resulting from puncturing the amniotic sac with a needle has been attributed to leakage of amniotic fluid and consequent constriction of the embryo by surrounding membranes(1). The question can be raised as to whether some other cleft pal-

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