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# ADRENAL GLANDULAR LIPIDS AND CIRCULATING CORTICOSTERONE IN SEVERELY DIABETIC RATS

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**Summary.—Young, adult, female Sprague–Dawley rats were fasted for 18 h and then given a single s.c. injection of alloxan (10 mg/100 g body weight) which promptly induced a severe state of diabetes. The animals were killed at frequent time intervals during the 7-day study period in order to record the dynamic changes in their capacity for adrenal steroidogenesis and secretion as measured by fluorometric determination of their circulating corticosterone (Cmpd B) levels as well as by thin layer chromatographic identification of cortical lipid moieties used for steroidogenesis. In addition to severe polydypsia, polyuria and polyphagia, these animals manifested supernormal glucose, triglycerides, free fatty acids and cholesterol in their blood, severe hepatic steatosis, adrenal hyperplasia with lipid depletion from the mineralocorticoid producing z. glomerulosa, thymus gland involution and complete degranulation of their insulin producing islet beta cells. Despite an initial high output of Cmpd B and despite progressive cortical hyperplasia, the serum Cmpd B levels became reduced and many of the animals succumbed suddenly, due most likely to inadequate adrenocortical steroidogenesis. Adrenocortical lipids showed a progressive accumulation of free fatty acids, di- and triglycerides, suggesting that some lipid enzymatic defect could be responsible for the lack of conversion of these lipid entities essential for proper steroidogenesis.**

ALTHOUGH there is considerable evidence accepted by most investigators of definite morphological involvement of the adrenal cortices in diabetic patients and in alloxan diabetic animals (Russi, Blumenthal and Gray, 1945; Becker *et al.*, 1954; Fraley and Totten, 1968; Wexler, 1970, 1971), there is little or no agreement as to whether there is hyper- or hypoactivity of the adrenal cortices of diabetic patients (Miller and Mason, 1945; Wilson *et al.*, 1950; Mortimore *et al.*, 1956; Conn, 1965; Huther and Scholz, 1970). Saba and Hoet (1962) and Devecerski and Frawley (1963) have found increased adrenal steroidogenesis and circulating corticosterone in diabetic animals, while Kraus (1967) has reported decreased pituitary-adrenal responsiveness to acute stress in alloxan diabetic rats.

We have found adrenal hypertrophy accompanied by thymus gland involution (Wexler, 1970, 1971; Wexler, Saroff and Judd, 1970), and an acute rise in circulating corticosterone (Cmpd B) when severe alloxan diabetes was induced, followed by chronic and definitely reduced Cmpd B levels, often associated with the sudden demise of many animals, throughout the course of their severe diabetes. Animals with alloxan diabetes are unable to cope with stressful situations which demand extra adrenocortical steroid production (Kraus, 1967). More recently, Kraus (1973) reported that despite increased activity of the pituitary adrenal

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axis in uncontrolled diabetes, the impaired acute stress response was not due to decreased ACTH synthesis or reserve but to decreased ACTH release.

Because the specific lipid composition of the adrenal cortex is a dynamic reflection of the state of adrenocortical activity, *e.g.*, adrenal cholesterol and triglyceride serve as steroid precursor and as a source of energy in steroidogenesis (Rudman and Garcia, 1966; Macho and Saffran, 1967) we investigated the lipid content of adrenal glands of rats made acutely and severely diabetic (alloxan) using thin layer chromatography (TLC) which is sufficiently sensitive to determine adrenocortical lipid content, both quantitatively and qualitatively.

## MATERIALS AND METHODS

All of the animals used in these experiments were virgin, female rats of the Sprague–Dawley strain (ARS/Sprague–Dawley Farms, Madison, Wisconsin, U.S.A.), 6–8 months of age, weighing 225 ± 5 g, and housed in air conditioned, humidity and light controlled animal quarters. All of the animals were fed a commercial rat chow (Teklad) which has a relatively low fat content (4%) and were given food and tap water to drink *ad libitum*.

A single, subcutaneous injection of 10 mg of alloxan (Eastman Kodak) per 100 g body weight was given after an 18-h fast. This 10 mg/100 g dose of alloxan produces severe diabetes along with ketosis and other metabolic derangements (Wexler, 1970; Wexler *et al.*, 1970). Severe polydypsia, polyuria and polyphagia promptly appeared.

No attempt was made to ameliorate the diabetes and mortality was high. The animals were killed by decapitation to avoid the pituitary–adrenal stimulating effects of the stress of anaesthesia. Pertinent organs from each animal were weighed, processed biochemically (see below), or fixed in 10% neutral formalin for histopathological analyses. Blood from each animal was centrifuged (refrigerated) and analysed for circulating levels of corticosterone (Cmpd B) by the fluorometric method of Guillemin *et al.* (1959). In order to determine the concentration and specific kinds of adrenocortical lipids both adrenal glands were removed, weighed, quick frozen and stored at −20°. A lipid extract by the Folch method (Folch, Lees and Sloane-Stanley, 1957) was made of each adrenal gland. The specific lipid components, *e.g.*, phospholipids, mono-, di- and triglycerides, free fatty acids, cholesterol and cholesterol ester, from each adrenal extract were separated using the system described by Freeman and West (1966) using a sandwich-thin layer chromatography developing chamber. Lipid fraction concentrations were determined by means of a Photovolt thin layer chromatography densitometer (Model 530) with an automatic integrator (Model 49). The adrenal lipid extracts were compared with a standard lipid mixture obtained from Applied Science Laboratories Inc., State College, Pennsylvania, U.S.A. Lipid concentration was expressed as μg of lipid/paired adrenal glands and as μg of lipid/g wet weight of adrenal glands. All samples were run in duplicate. Biostatistical analyses of all of the data in these experiments were made using Student's *t*-test as described by Snedecor and Cochran (1967).

## RESULTS

Within 1–3 h after the single injection of alloxan all of the animals were severely hyperglycaemic (600–800 mg/100 ml glucose; normal = 80–100 mg/100 ml) and after 24 h all of the animals manifested greatly elevated levels of cholesterol, triglycerides and free fatty acids, *e.g.*, 1000–1200 mEq/l free fatty acids; normal = 500 mEq/l. On the 3rd and 4th day post-alloxan, 50% of the animals succumbed and in each case the livers were found to be butter yellow in colour due to severe fatty infiltration (Fig. 1) and their adrenal glands cherry red in colour instead of their normal yellow-white coloration concomitant with virtually total involution of the thymus gland. At this time (Day 3) glucose levels had risen to 1100–1200 mg/100 ml in many cases and free fatty acids to as high as 2200 mEq/l. By Day 7, these supernormal levels had receded but the animals remained severely hyperlipidaemic and hyperglycaemic along with other classic signs of severe diabetes. The serum chemical changes in these animals were essentially identical to our previous findings (Wexler *et al.*, 1970). Those animals which survived the acute and severe diabetes all manifested severe hepatic steatosis (Fig. 1), hyperlipaemia, hyperglycaemia, marked adrenocortical hyperplasia (Fig. 2) and complete destruc-

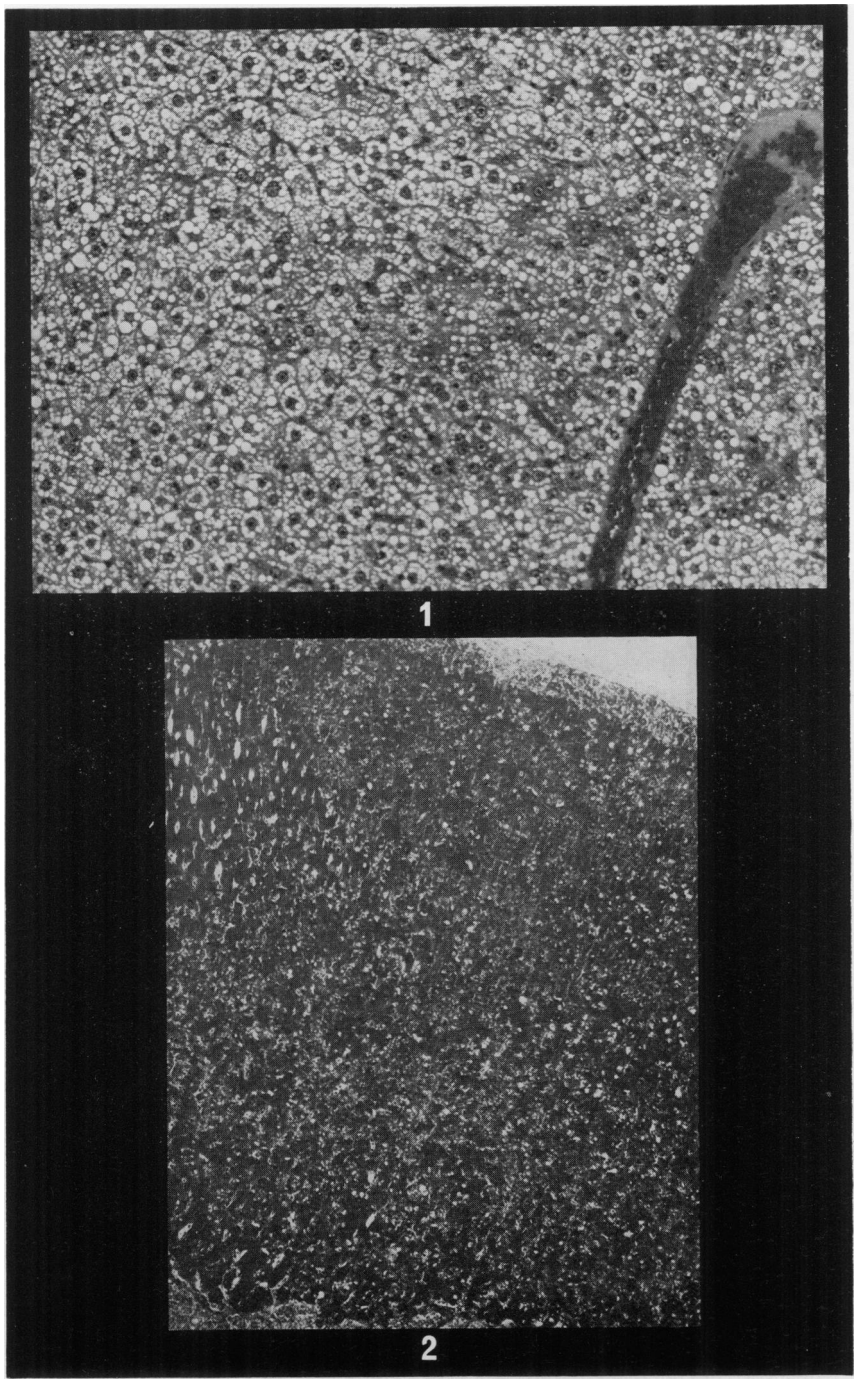


FIG. 1.—Extensive fatty infiltration of the liver which reaches a zenith as early as Day 3, post alloxan induced diabetes. H. and E. × 75.

FIG. 2.—Extensive adrenocortical hyperplasia (Days 1–7) found in severely diabetic rats. The zona glomerulosa (light staining strip in upper right corner of photo) shows extensive lipid depletion indicative of active mineralocorticoid production and release. The greatly hypertrophied zonae fasciculata and reticularis (light staining area in lower left corner of photo is the adrenal medulla) show alternating patches of lipid concentration (dark black areas) and reduced lipid concentration (grey–black patches), indicative of uneven storage, synthesis and release of the glucocorticoid and ketosteroid variety of steroids. Frozen section, Sudan black B. × 30.

tion of their insulin producing beta cells of the islets of Langerhans (Fig. 3).

Despite significant, progressively increasing adrenal weight (Fig. 4) and adrenocortical hyperplasia (Fig. 2), Cmpd B secretion manifested progressive decline (Fig. 4), after an initial surge in production (Day 1), following the acute induction of severe diabetes.

Thin layer chromatographic separation and quantification of the cortical lipids of control (Day 0) *vs* severely diabetic rats (Days 3 and 7) demonstrated little or no differences in adrenal lecithin and cholesterol content (Fig. 5). However, there were statistically significant increases in free fatty acids, diglycerides and triglycerides, with each of the latter increasing in concentration with each day's passage of severe diabetes (Fig. 5). The cholesterol ester fraction showed an erratic drop in concentration on Day 3 and a subsequent increase on Day 7. (This pattern of adrenocortical lipid changes applied whether the data were analysed on the basis of micrograms of lipid per pair of adrenal glands (Fig. 4) or lipid per g wet weight of adrenal tissue.)

## DISCUSSION

These experimental findings demonstrate that the acute induction of severe diabetes with alloxan constitutes a severe stressful stimulus to the pituitary–adrenal axis, eliciting prompt and dynamic adrenocortical hyperplasia, increased adrenal weight, Cmpd B secretion and thymus gland involution which attends increased glucocorticoid production. However, despite the initial increased Cmpd B production (Day 1), and despite progressive and persistent adrenocortical hyperplasia (Days 1–7), the adrenal cortices of these diabetic animals were unable to maintain their initial high level of Cmpd B production (Fig. 4). It has been suggested that this initial,

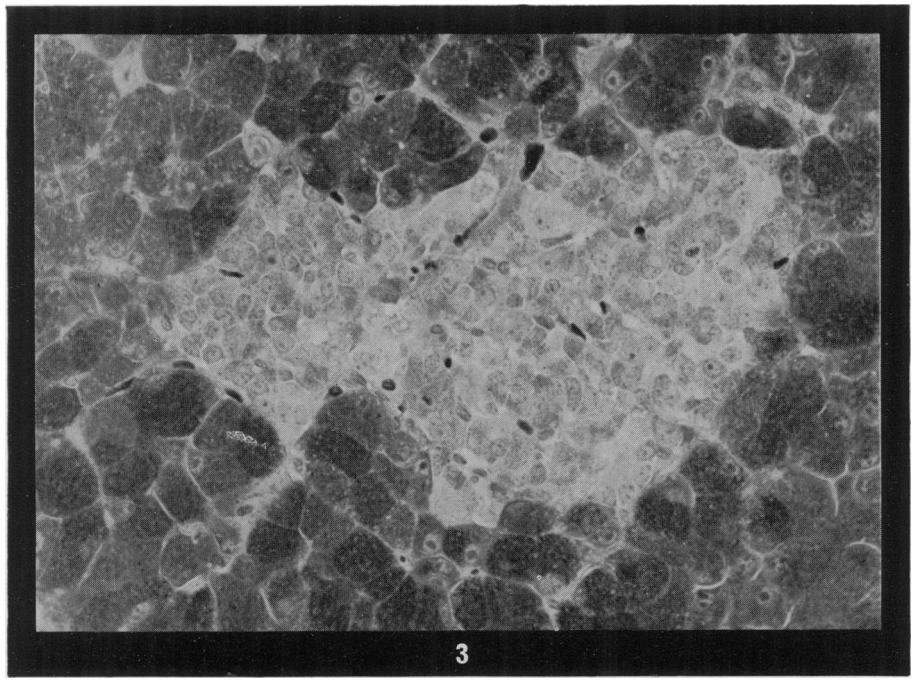


FIG. 3.—Islet of Langerhans of pancreas of an acutely diabetic rat given alloxan and showing the complete degranulation of the normally, richly granulated beta cells. The degranulation of the islet beta cells is synonymous with decreased insulin production. Ald. fuchsin. × 135.

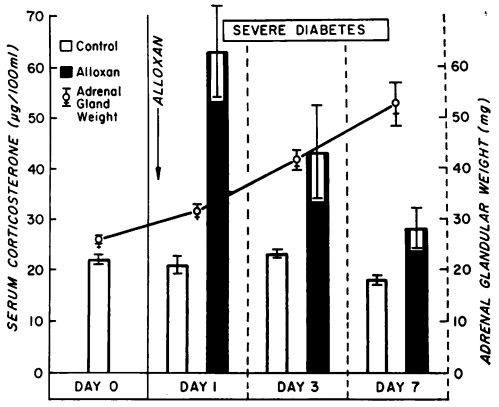


FIG. 4.—Changes in serum corticosterone (Cmpd B) and adrenal glandular weight in control *vs* alloxan treated rats, one, 3 and 7 days after the induction of severe diabetes. Each group consists of 15 animals, the height of each column depicts the mean (± standard error) weight of the left adrenal gland. All of the changes and differences shown were highly significant statistically ($P < 0{\cdot}001$).

high Cmpd B production is associated with extensive sugar absorption and water movement from the gut (Axelrod, Lawrence and Hazelwood, 1970). Adrenal cholesterol, and especially the triglyceride and cholesterol ester fraction, have been shown to be directly involved in adrenocortical steroidogenesis. Adrenocortical lipolytic enzymes, activated by ACTH, will cause free fatty acid release from triglyceride. These free fatty acids are then oxidized to yield acetyl co-enzyme A or reduced NADP used for oxidation during adrenocortical steroidogenesis (Rudman and Garcia, 1966; Macho and Saffran, 1967). Therefore, the progressive increase in free fatty acids, diglycerides and triglycerides in the adrenal cortices of these severely diabetic rats (Days 3 and 7) attests to the availability of essential lipid moieties which, judging from their progressive accumulation, are not being utilized for the conversion of cholesterol ester precursors into definitive adrenal steroid. We have encountered very similar conditions in repeatedly bred male and female rats which develop a Cushing's disease-like spectrum of hyper-

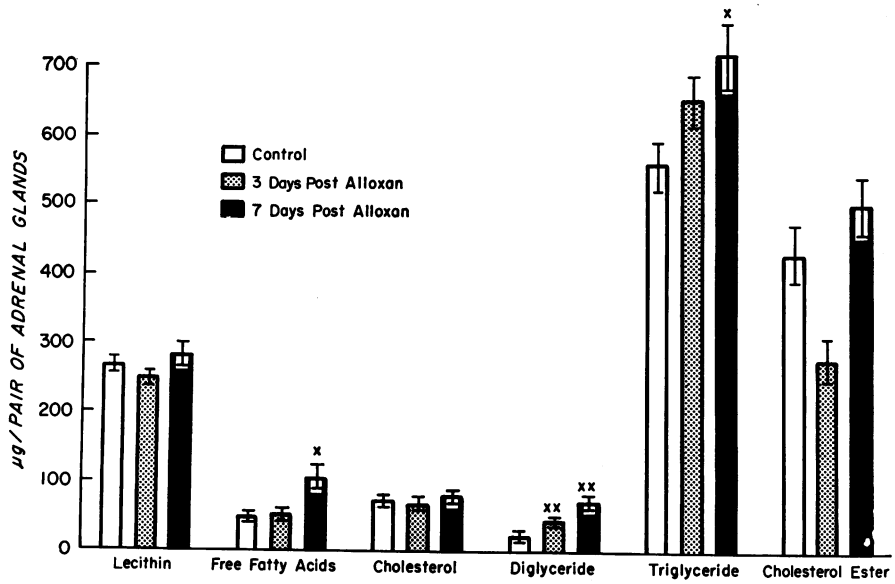


FIG. 5.—Adrenocortical lipids (expressed as μg per pair of adrenal glands) separated by thin layer chromatography, illustrating the differences between the adrenocortical lipids of control *vs* alloxan treated, (3 and 7 days post alloxan) rats having severe diabetes. Each column, consisting of 15 samples, represents the mean and standard error for each lipid component, $x$ = statistical significance ($P < 0{\cdot}05$); $xx$ = statistical significance of higher degree ($P < 0{\cdot}01$).

adrenocorticism, hyperglycaemia, hyperlipidaemia and arteriosclerosis, eventually culminating with a condition of impaired steroidogenic potential and deranged cortical lipids (also measured by thin layer chromatography), essentially identical to those described in these diabetic rats (Wexler and Lutmer, 1972). These diabetic and arteriosclerotic breeder rats also manifest hyperlipidaemia and severe fatty infiltration of the liver. It is well established that hepatic synthesis of free fatty acids and cholesterol is impaired in experimental diabetes (Corder and Kalkhoff, 1969). This impaired lipid synthesis in diabetic rats can be corrected by administration of insulin. This would suggest that some hepatic enzymatic defect, responsive to insulin, is responsible for the impaired lipid synthesis in the liver. A similar situation may be applicable in the case of the adrenal cortex of diabetic animals (see below). The severe hepatic steatosis, therefore, is probably due to the decreased lipoprotein carrier synthesis which occurs in experimental diabetes. Thus, the observed supernormal serum lipid levels must come from some source other than liver, probably impaired peripheral tissue utilization of triglyceride, since dietary lipid would not account for the severe degree of hyperlipidaemia observed.

The superabundance of circulating and hepatic lipid in these severely diabetic rats would suggest that the adrenal cortex would be provided with more than sufficient lipid as potential steroid precursor material. However, the progressively decreasing steroidogenic capacity of these adrenal glands, *i.e.*, especially when expressed as circulating Cmpd B/mg adrenal tissue, despite plentiful lipid, suggests that there may be an adrenocortical lipid enzymatic defect in diabetic animals as in the case of the liver (see above). Thus, the findings of Kraus (1967, 1973) would have some applicability to the conditions observed here. This author found that pituitary ACTH concentration is normal in alloxan diabetic rats but there appears to be a block to stressful stimuli at the hypothalamic–hypophyseal level. Further, Kraus found that although the ability to synthesize and release ACTH in response to acute stress is impaired in alloxan diabetic rats, they, nonetheless, are capable of synthesizing and releasing large amounts of ACTH in the diabetic state *per se*. Although we did not measure plasma or pituitary ACTH levels, utilizing the findings of Kraus (1967, 1973), our data would best fit the hypothesis that these severely diabetic rats are capable of synthesizing and releasing ACTH but some defect, intrinsic to their adrenal cortices, interferes with proper steroidogenesis despite plentiful stores of cortical lipid. The fact that the adrenal cortices in our subjects are so greatly hypertrophied and hyperplastic would tend to confirm that there was, indeed, adrenocortical vascular or trophic stimulation. The chromatographic evidence of an accumulation of cortical lipid moieties essential to steroidogenesis and the histopathological evidence of dispersed clumps of lipid would reinforce our contention that the primary defect in adrenocortical steroidogenesis in severely diabetic rats probably resides within the adrenal gland itself and might be a lipid enzyme system. We have found (unpublished observations) that when these severely diabetic rats are given exogenous ACTH their diabetes is greatly ameliorated. Insulin also improves adrenocortical steroidogenesis and reduces the extent of adrenocortical hyperplasia (Cahill, Vance and Urrutia, 1962). It is possible, therefore, that the exogenous ACTH improves the diabetic animal's capacity to survive the severe metabolic stress by causing the synthesis and release of adrenal glandular steroids through correction of a lipid converting enzyme defect. As mentioned earlier, these severely diabetic rats die suddenly and in great numbers. We believe that their acute demise is due to a combined lack of insulin, impaired hepatic metabolism and acute adrenal insufficiency.

Finally, there is an additional possible mechanism of action which is of special interest. Many of our surviving alloxan diabetic rats develop hypertension. The spectrum of circulating steroids becomes altered so that steroids other than Cmpd B, *e.g.*, aldosterone, 18-OH-B, 18-OH-DOC and DOC, are produced by the deranged adrenal glands concomitant with the severity of their diabetes (to be published). The lipid depletion of the zona glomerulosa in these animals (Fig. 2) is indicative of some derangement of mineralocorticoid production and release. Abnormal mineralocorticoid production, because of its potassium wasting effect, could aggravate the existing impaired carbohydrate metabolism (Conn, 1965; Slaton and Biglieri, 1965), by interfering with the insulin releasing effectiveness of the few surviving beta cells not destroyed by alloxan. This possibility is now under investigation by us. Any one of the aforementioned steroids could be responsible for the hypertension in these alloxan diabetic rats. Thus, the measurement of Cmpd B alone could be a false representation of the real secretory status of the adrenal cortex and of the true spectrum of steroids produced by these animals.

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