FACTORS INFLUENCING CONJUGATION OF STEROIDS BY MOUSE LIVER AND KIDNEY

by

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INTRODUCTION

The steroids of the adrenal cortex are regulators of many physiological processes. The body, in order to maintain homeostasis, has mechanisms that regulate the activity and biological lifespan of these hormones. The peripheral tissues, by oxidation and reduction of the substituted groups of the steroid nucleus at C-3, C-11, C-20 and Δ4-5 can regulate their biological activity (Berliner and Dougherty, 1961).

The liver is the major organ concerned with the inactivation and excretion of these hormones (Berliner and Dougherty, 1961; and Samuels, 1960). The major reactions of the liver are reductive, particularly with respect to ring A. The liver reduces the Δ^4-3 ketone to a 3-hydroxy group (Tomkins and Isselbacher, 1954). Subsequent to this reduction is conjugation with glucuronic acid or sulfate (Robbins and Lipman, 1956; and Isselbacher and Axelrod, 1955). Once the steroid is conjugated it is more water soluble and readily excreted in the bile and urine. The rate of reduction and conjugation controls the biological half-life of corticosteroids.

The control of blood and tissue levels of corticosteroid by the liver is important for maintaining physiological homeostasis. An alteration or defect in this regulatory system could be beneficial or deleterious. An increased or decreased excretion of corticosteroid by the liver could be primary to a pathological condition or a result of a series of pathological events. The alterations in metabolism could, however, be a response to protect the animal from various stresses or pathological events.

This study was undertaken in an attempt to gain new information about the conjugation mechanism and the role it plays in maintaining physiological homeostasis. The experiments were performed in vitro with livers and kidneys of mice. The effect of x-irradiation on conjugation was studied in an attempt to correlate the response of the conjugating mechanism to other physiological alterations caused by irradiation. The biological half-life of steroids seems to be related to their molecular structure (Berliner and Dougherty, 1960b).

The conjugation of 17α-hydroxycorticosteroids was compared to the conjugation of corticosteroids without a hydroxyl group at C-17 in an attempt to elucidate biological half-life steroid structure relationships.

HISTORICAL REVIEW

Detoxification of foreign compounds by conjugation with glucuronic acid was unwittingly discovered by Schmid in 1855. He isolated euxanthic acid which, upon acid hydrolysis, yielded a copper reducing substance. Baeyer in 1870 stated that the reducing substance should have the formula, $C_6H_{10}O_7$, which we know now as the formula of glucuronic acid. Baeyer was of the opinion that the reducing substance was "a kind of saccharic acid" (Williams, 1947). Jaffe in 1874-78 discovered that o-nitrotoluene was conjugated with a hypothetical acid that he considered to be carbohydrate in nature. In 1879 Schmiedberg and Mayer isolated glucurone (lactone of glucuronic acid) from camphorglycuronic acids excreted by dogs that had ingested camphor (Williams, 1947).

The origin of the glucuronic acid was controversial for years until it was demonstrated, in whole animals, that the liver is the major site for glucuronide synthesis. It was also demonstrated that this organ formed glucuronides more efficiently if it contained glycogen or if the animals were fed carbohydrates (Quick, 1926).

Lipschitz and Bueding (1939) postulated the condensation of three carbon fragments to form hexose acids. Mosbach and King (1950) using carbon-14 labeled glucose showed that glucose is converted to glucuronic acid. Free glucuronic acid added to the system was not used to form glucuronides (Douglas and King, 1952; and Sudhof, 1954). Dutton and Storey (1954) isolated and identified uridine diphosphate glucuronic acid (UDPGA) as the glucuronic acid donor.

The first definitive work on the synthesis of glucuronides was performed on whole slices from various organs. It was shown that the liver is the major organ for glucuronidation of foreign compounds. The test substances were borneol, menthol and phenol. It was also shown that the kidney had about 1/6 to 1/12 of the liver's ability to form glucuronides from those compounds (Lipschitz and Bueding, 1939).

Glucuronic acid conjugation of o-aminophenol was demonstrated in liver and kidney slices. The kidney had a capacity 1/10 that of the liver (Storey, 1950). The same study showed that the addition of glucuronate to the incubation medium inhibited glucuronide synthesis. The fact that glucuronate inhibited glucuronide synthesis reinforced the idea that glucuronic acid comes from glycogen through glucose. In order to study this problem in a well-defined system, broken cell preparations were used. It was found that in mouse liver homogenates, glucuronide synthesis did not occur. Glcuronide synthesis would occur, however, if a boiled extract from liver was added to the medium (Dutton and Storey, 1951, 1954). It was found that the active liver factor was a uridine nucleotide containing stable phosphate, labile phosphate and glucuronic acid in the ratio (1:2:1:1). Uridine 5'-phosphate is linked to 1-glucuronic acid phosphate through a pyrophosphate bond. This compound was given the name uridine diphosphate glucuronic acid (UDPGA). The following reaction was postulated: UDPGA + ROH —— UDP + R-O-glucuronic acid. UDPGA also was postulated as having a role in polysaccharide synthesis (Storey and Dutton, 1955). Uridine diphosphate glucose (UDPG) had recently been discovered (Caputto et al., 1950).

Strominger et al. (1954) had shown that UDPGA could be synthesized from UDPG by an enzyme found in particle-free supernatant of animal livers. The oxidation of one mole of UDPG to UDPGA requires two moles of DPN^+ and yields one mole of UDPGA and two moles of DPNH. No intermediates have been found. The glucose moiety is oxidized at C-6.

A method for quantitating glucuronide formation was developed using anthranilic acid for the test material. Recovery is approximately 100% (Shirai and Ohkubo, 1954a). Using this method, various organs of the rat were assayed for glucuronide forming ability. The kidneys, duodenum, ileum, colon and mesenterium, in addition to the liver, were able to form anthranilic-glucuronide. It was found, in agreement with Lipschitz and Bueding (1939), that lactate or pyruvate would augment glucuronide formation and that addition of glucose or fructose showed an inhibiting action. Addition of glycogen and glucose-1-phosphate produced high yields of glucuronide (Shirai and Ohkubo, 1954b).

The first steroid isolated and identified as a glucuronide was pregnandiol-glucuronide from human pregnancy urine (Venning and Browne, 1936). Dutton (1956) using mouse liver homogenate demonstrated the synthesis of pregnane-3β, 20β-diol glucuronide, androsterone glucuronide and allopregnane-3β, 20β-diol glucuronide. The presence of UDPGA was necessary in all cases with the resulting formation of uridine-5'-pyrophosphate and the ester. The naturally occurring glucuronides are beta in structure. The enzyme for this synthesis was located primarily in the microsomes. Enzyme activity in other fractions was considered to be due to contamination from the microsome fraction (Dutton, 1956). Guinea pig, rabbit, mouse and rat liver homogenates all contained the

glucuronide catalyzing enzyme. The amount of enzyme and UDPGA content decreased in order given (Dutton and Greig, 1957). The enzyme was also found in liver homogenates from sheep, pigeon and frog livers (Dutton and Greig, 1957). Dutton (1958) demonstrated glucuronide forming ability in fetal stomach, gastrointestinal tract and kidney using o-aminophenol and (-) menthol as substrates. The fetal rabbit and mouse livers are deficient in glucuronide forming enzyme and probably deficient in UDPGA. In guinea pigs the primary deficiency was UDPGA, and secondarily, the lack of enzyme. Addition of glucose to incubations of fetal guinea pig stomach slices produced a high level of glucuronide formation - higher than fetal liver and kidney.

Glucuronosyl transferase was found in kidney cortex microsomes but was absent in the medulla. The formation of uridine diphosphate glucuronic acid was demonstrated in kidney and gastrointestinal mucosa (Dutton and Stevenson, 1959). They demonstrated that UDPGA was formed from UDPG by the same process as in the liver.

The hepatic conjugation of o-aminophenol with glucuronic acid in rats was not impaired by administration of 12.5 mg of cortisone acetate per day for six days or by administration of 25 mg of cortisone acetate per day for ten days. Cortisone acetate did inhibit glucuronide conjugation in the gastrointestinal mucosa by 50% (Halme, Hartiala and Pekanmaki, 1959).

Hartiala, Nänto and Rinne (1958,1959) demonstrated the effects of x-rays delivered locally to exposed liver and stomach on o-aminophenol glucuronide formation. Using doses of 400 and 1200 r, the liver demonstrated a cyclic decrease in conjugation in response to x-irradiation.

The stomach, on the other hand, decreased and was still below normal on the 21st post-irradiation day. The effect of x-rays on β-glucuronidase was described by Hartiala et al. (1960). There was no real correlation between the effect of x-rays on glucuronide formation and β-glucuronidase. They concluded that β-glucuronidase was not responsible for glucuronide synthesis. There had been some speculation that β-glucuronidase was the enzyme responsible for glucuronide formation and Hartiala's work showed that this was not the case (Hartiala et al., 1961)

Skin strips and homogenates of skin were shown to have glucuronide forming ability using o-aminophenol as substrate (Stevenson and Dutton, 1960).

Stevenson and Dutton (1962) extensively studied the enzymatic catalysis of glucuronide formation in kidneys and gastrointestinal mucosa. Various substrates were used and they concluded there was only one mechanism of glucuronide formation from UDPGA in these tissues. The enzyme, glucuronosyl transferase, was associated with microsomes. UDPGA is formed in kidney and gastrointestinal mucosa by the DPN dependent oxidation of UDPG.

The excretion of steroids as glucuronides has been known since 1936 (Venning and Browne, 1936), but the mechanism had not been elucidated. The injection of progesterone-C^{14} and testosterone-4C^{14} in mice and rats demonstrated that the majority of metabolites were excreted as water soluble compounds which were not hydrolyzed by acid or alkali (Barry et al., 1952). Dorfman and Ungar (1953) showed that a major route of steroid metabolism in the liver was the reduction of

the α,β-unsaturated-3-ketone to tetrahydro form. This reduction produced a hydroxyl group at C-3 which could be readily conjugated with glucuronic acid. The reduction of the Δ^4-3-keto configuration takes place in two steps. The first step is the reduction of the double bond requiring TPNH and is considered to be rate-limiting. The second step is the reduction of the ketone to a hydroxyl group using either TPNH or DPNH as a hydrogen donor. Step one was irreversible and step two was reversible. The two reactions are catalyzed by an enzyme from the particle-free fraction of liver homogenate (Tomkins and Isselbacher, 1954; and Tomkins, 1956a,b).

Fukishima et al. (1955) described the formation of cortols and cortolones, and Caspi, Levy and Hechter (1953) identified four ring A reduced steroids as catabolic products of cortisol.

The formation of steroid glucuronide required an enzyme from mammalian liver microsomes and uridine diphosphate glucuronic acid. The steroid must be reduced at C-3 and coupling takes place at the hydroxyl group at this position and a β-d-glucuronide is formed. This same enzyme catalyzes the conjugation of phenophthalein and thyroxine. This appears to be a general mechanism involving UDPGA and microsomes for the formation of phenolic and alcoholic glucuronides (Isselbacher and Axelrod, 1955).

Berliner and Wiest (1956) demonstrated that in eviscerated animals no polar conjugates of glucuronic acid or sulfate were formed. Five of the seven compounds isolated had the Δ^4-3-keto configuration and no tetrahydro compounds were found. This indicates that the liver is the major organ capable of reducing the Δ^4-3-ketone. Berliner, Grosser and

Dougherty (1958), in eviscerated rats, showed that the liver was the major organ capable of completely reducing ring A of corticosteroids to the tetrahydro form. Fibroblast can produce dihydrocorticosteroids (Berliner and Dougherty, 1958).

Tomkins (1957) showed that a crude enzyme fraction was capable of reducing many different steroids; further purification, however, demonstrated that the enzyme became more specific and would reduce cortisone but not cortisol.

Sandberg, Chang and Slaunwhite (1957) showed that humans converted significant amounts of cortisol to 17-ketosteroids. De Courcy (1957) showed that rat kidney can reduce the ketone at C-20 to a hydroxyl group when a TPNH-generating system is added to medium.

Synthetic steroid analogues are more biologically active than their normal counterparts, because they are metabolized at a slower rate (Glenn et al., 1957).

Female rats reduce the Δ^{4-5} double bond at a greater rate than males. This was due to a Δ^4-5α-reductase isolated from liver microsomes of the male and female rat. The female rat had no Δ^4-5β-reductase while the male did (Forchielli, Brown-Grant, and Dorfman, 1958). Perhaps this explains the more active Δ^4-5α-reductase in the female.

Yates, Urquhart and Herbst (1958) showed that triiodothyronine increased the total hepatic reduction of ring A of cortisone by 38%. Thyroidectomy decreased ring A reduction approximately 50%. They suggested that the rate of ring A reduction of corticosteroids controls

the rate of ACTH excretion in unstressed animals (Urquhart, Yates, and Herbst, 1959).

The liver is the only organ containing steroid sulfokinase as well as phenol sulfokinase. The kidney and intestine contain phenol sulfokinase but no steroid sulfokinase (Nose and Lipmann, 1958).

Gold, Smith and Moore (1959) suggested that once a steroid was conjugated no further reduction or oxidation could take place at C-11 or C-20. Therefore, the conjugation of tetrahydro C-20 ketones would give a Porter-Silber reaction and the conjugated β-cortols or β-cortolones would not (Streeten, 1959; and Fukishima et al., 1955). Beta-cortols and β-cortolones constitute about 30% of the excreted metabolites of cortisol and more tetrahydrocortisone is excreted than tetrahydrocortisol (Streeten, 1959). Delta^{1,4}-3-keto steroids cannot be reduced in ring A by the liver _in vitro_ or _in vivo_ (Streeten, 1959).

Conjugation is known to abolish or reduce biological activity of most steroids (Schneider and Lewbart, 1959). Steroid glucuronides are excreted more rapidly than steroid sulfates (Schneider and Lewbart, 1959).

Mc Guire and Tomkins (1959a) observed that Δ^4-5β-reductases are in the soluble portion of the cell extracts and that Δ^4-5α-reductases are microsomal. These reductases are not one enzyme but a series of enzymes, each capable of discerning small differences in the steroid molecule. Mc Guire and Tomkins (1959b) demonstrated that in thyrotoxicosis there is an increase in TPNH availability and that the intracellular concentration of TPNH regulates the rate of steroid metabolism.

Steroid reduction may act to reoxidize TPNH to TPN and thereby accelerate metabolic processes dependent on oxidized TPN.

Delta^4-3-keto corticosteroids accelerate glucose-6-phosphate oxidation in liver *in vitro* (Yielding and Tomkins, 1960). The major excretory products of cortisone metabolism were tetrahydrosteroids with the dihydroxyacetone side chain.

The rates of inactivation and excretion of various steroids are variable. In man and animals, corticosterone is excreted more rapidly than cortisol (Willoughby, Chen and Freeman, 1959; and Berliner and Dougherty, 1960b). Gold (1960) postulated that the hydroxyl groups at C-11 and C-17 may protect cortisol from metabolic degradation. Berliner et al. (1962) postulated that the 17α-hydroxy group may inhibit cortisol conjugation. The steroid, 11-deoxycortisol, is conjugated five times as fast as cortisol, although the plasma level is about the same.

Peterson (1960) demonstrated in normal men that 40-60% of the administered corticosterone was excreted as glucuronide conjugates within 72 hours. Fukishima et al. (1960) demonstrated that 45-50% of the excretory products of cortisol in men were tetrahydrosteroids with the dihydroxyacetone side chain conjugated with glucuronic acid. Cortols and cortolones accounted for 18-33% and C-19 compounds only 2-12% (17-ketosteroids).

Following oral administration of pure 17-ketosteroids, 3β-hydroxy-17-ketosteroids were excreted as sulfates and the 3α-hydroxy-17-ketosteroids were conjugated as glucuronides (Staib, Teller and Scharf, 1960).

Recently it has been demonstrated that the parenchymal cell of the liver is the primary conjugating cell. The reticuloendothelial cells of the liver can reduce ring A but cannot conjugate steroids. It was also demonstrated that administration of L and D-triiodothyronine would increase the conjugation of unsaturated steroids but not of reduced steroids. ACTH and stress, on the other hand, decreased the conjugation of unsaturated and reduced compounds in vivo. The parenchymal cell can reduce ring A. The Kupffer cell can oxidize and reduce the substituted groups of the steroid molecule (Berliner and Dougherty, 1958: Nabors, Berliner and Dougherty, 1960; and Berliner and Dougherty, 1960a,b).

Berliner, Keller and Dougherty (1961) showed that the ACTH inhibited conjugation, not by affecting glucuronide formation alone, but rather a more general inhibition was evidenced.

Reid (1958) pointed out that two or three weeks following adrenalectomy (when there is a high ACTH level) the concentration of uridine nucleotides was decreased, including UDPGA.

Dog kidneys perfused in vivo with etiocholanolone and androsterone produced the glucuronides of these two compounds. Sixteen per cent of the etiocholanolone was present as glucuronide and 14% of the androsterone was present as glucuronide. No sulfates or phosphates were found (Cohn, Hume, and Bondy, 1960). Stevens, Berliner and Dougherty (1961) demonstrated that the kidney could conjugate C-21 tetrahydrosteroids as glucuronides.

Pasqualini and Jayle (1961) stated that after administering 300 mg of corticosterone or cortisol to normal men, most of these two compounds

were found in urine as ester sulfates. The sulfate was attached at C-21. Roberts and coworkers have demonstrated that following *in vivo* administration of dehydroisoandrosterone-SO_4 this compound was converted to androsterone and etiocholanolone glucuronides. This indicates that the sulfate ester dissociates and can be metabolized to other compounds (Roberts, Vandewiele and Lieberman, 1961).

Hartiala (1961) found that duodenal slices could conjugate as glucuronides steroids having a phenolic A ring. Duodenal mucosa was unable to conjugate progesterone, pregnandiol, androsterone, testosterone or cortisone. Pregnandiol and androsterone have hydroxyl groups at C-3 and they were not conjugated by the duodenum.

Cooke and Taylor (1962) demonstrated that rat livers incubated with progesterone under nitrogen formed no glucuronide. When incubated with air or pure O_2 glucuronides and sulfates were formed.

The incubation of aldosterone with liver slices *in vitro* yielded primarily tetrahydroaldosterone glucuronide. Slices of kidney produced small amounts of tetrahydroaldosterone glucuronide, acid labile aldosterone and free tetrahydroaldosterone. Human kidney slices did form small amounts of acid labile conjugates, some glucuronides and some tetrahydroaldosterone (Sandor and Lantheir, 1962).

Perfusion of human placentas with Tyrode's solution demonstrated that the placenta can produce free and glucuronide conjugated Porter-Silber chromogens *in vitro*. The compounds were identified as cortisol, cortisone, Reichsteins' Substance S, tetrahydrocortisone and tetrahydrocortisol. These compounds accounted for 50-80% of the total Porter-Silber chromogens (Troen, 1961).

Sandberg et al. (1956) showed that terminal patients had elevated plasma 17-hydroxysteroids, had plasma clearance rates slower than normal and excreted less total 17-hydroxysteroids than patients under surgical stress. Englert et al. (1957) pointed out that patients with cirrhosis of the liver had impaired formation of conjugated 17-hydroxycorticosteroids following infusion of cortisol. No impairment of conjugation was seen after infusion of tetrahydrocortisone or dihydrocortisone. They concluded that the delay was in the reduction of the Δ^4-3-ketone to produce tetrahydrocorticosteroids. Tetrahydrocortisone was the principal product in the urine.

Congenital, non-hemolytic, non-obstructive jaundice is a disease in which the excretion of bilirubin in the bile and urobilinogen in feces is greatly impaired. Patients with this disease after infusion of cortisol showed a normal blood clearance rate of the hormone, but the appearance of glucuronide conjugated metabolites in the blood was much slower (Peterson and Schmid, 1957). The fraction of glucuronide metabolites appearing in the urine was half that of normal patients following infusion of cortisol. The half-life of tetrahydrocortisol in patients with this disease was 70 minutes as compared with a half-life of 28 minutes in normal patients. This defect is apparent only after loading patients with ring A reduced steroids (Peterson and Schmid, 1957).

Axelrod, Schmid and Hammaker (1957) demonstrated that the ability to form UDPGA in rats with congenital jaundice is normal. Microsomes from jaundiced rats, incubated with UDPGA, o-aminophenol and bilirubin showed a decreased bilirubin conjugation. Since UDPGA levels are normal

in congenital, non-hemolytic, non-obstructive jaundice, Barniville and Misk (1959) postulated that the defect was in the glucuronosyl transferase. Schmid et al. (1957) showed a markedly decreased glucuronide conjugation of cortisone metabolites, bilirubin, menthol and salicylate in congenital jaundice.

Shocked or stressed rats have a prolonged cortisol turnover when compared to normal rats. This prolongation of cortisol metabolism in shock or stress does not appear to be due to an increased binding of steroids in peripheral tissues. It would appear that the liver's capacity to metabolize steroids in shock or stress is the limiting factor (Firschein et al., 1957).

The livers of stressed mice incubated _in vitro_ with cortisol have a reduced capacity to conjugate the hormone. Livers of normal mice incubated with cortisol and ACTH showed a decreased ability to conjugate steroids. ACTH administered to adrenalectomized mice caused an increase in the cortisol half-life (Dougherty and Berliner, 1958). Eik-Nes and Samuels (1958) stated that changes in cortisol metabolism in life threatening stress conditions are probably a reflection of reduced hepatic activity. They found that stress increased 17-hydroxycorticosteroids in the plasma and this increase was due to a decreased removal rate. Patients with cirrhosis of the liver have a decreased rate of removal of free 17-hydroxycorticosteroids, although plasma levels do not rise. The decreased rate of removal is probably due to a decreased reduction of ring A and not to the conjugation of reduced compounds (Streeten, 1959).

Hsia et al (1960) found an *in vitro* inhibition of o-aminophenol glucuronide formation produced by pregnandiol-3α, 20α, allopregnanetriol and pregnanolone. Progesterone inhibited glucuronide conjugation of o-aminophenol, phenolphthalein and bilirubin. The most effective inhibitors were progesterone, and 17α-ethyl-19-nortestosterone.

Carbon tetrachloride damaged livers had a reduced glucuronide forming ability. Microsomal glucuronosyl transferase was not diminished. Reduction in hepatic glucuronide conjugation induced by carbon tetrachloride is a quantitative change rather than a qualitative change (Isselbacher and McCarthy, 1960).

Herbst et al. (1960) demonstrated that noxious stimuli reduced hepatic capacity to reduce ring A of corticosteroids and also reduced food intake. Hepatic Δ^4-steroid dehydrogenase activity was found to be a nearly linear function of food intake. Noxious stimuli that failed to affect food intake also failed to affect corticosteroid metabolism. It appears that a non-specific decrease in intracellular TPNH was responsible for the loss of Δ^4-steroid dehydrogenase activity.

Adrenal cortical secretions have been known to influence carbohydrate metabolism since 1940. At this time, Long, Katzin and Fry (1940) demonstrated that fed adrenalectomized rats and mice maintained normal carbohydrate levels, but when fasted, these animals lose carbohydrates faster than normal animals and also excrete less nitrogen. The administration of adrenal-cortical extract to fasted normal animals or adrenalectomized animals is followed by a large increase in liver glycogen and a slight hyperglycemia. Muscle glycogen was unaffected.

Concommitantly, there occurred an increase in nitrogen excretion of sufficient magnitude to suggest that the increased protein catabolism is the source of the newly formed carbohydrate.

White and Dougherty (1944) stated that the administration of adrenal-cortical trophic hormone or adrenal-cortical extract caused a decrease in lymphocytes with a simultaneous increase in total serum protein. Thus, the protein needed for gluconeogenesis was made available from the lymphatic tissue. (For reviews on lymphatic tissue and hormones see Dougherty, 1959; and Dougherty, Berliner and Berliner, 1962).

X-irradiation, nitrogen mustards, fasting, and cortisone all enhanced the levels of glutamic-alanine and glutamic-aspartic transaminase in the liver. The increase in these enzymes is probably mediated via the adrenal cortex. Adrenalectomy would greatly decrease these enzyme levels in liver. Glutamic-alanine transaminase was more sensitive to stress than glutamic-aspartic transaminase (Brin and McKee, 1956). These enzymes are important in gluconeogenesis.

Gluconeogenesis is enhanced by x-irradiation. Increased quantities of carbohydrate are made available by increased breakdown of protein and increased transamination (McKee and Brin, 1956). Following x-irradiation of intact fasted animals, liver glycogen drops during the first 8-10 hours. This is followed by a striking increase in liver glycogen which reaches a maximum at 50 hours. A precipitous fall in glycogen values occurs reaching abnormally low values 75-80 hours post-irradiation. Adrenalectomy or hypophysectomy will prevent these changes in liver glycogen in irradiated animals (McKee and Brin, 1956).

Adrenalectomized or hypophysectomized fasted rats are unable to maintain normal fasting blood sugar levels. In normal animals, fasting produces an increase in glucose-6-phosphatase. In adrenalectomized or hypophysectomized animals, fasting does not increase glucose-6-phosphatase activity. In fed animals, adrenalectomy or hypophysectomy caused a significant decrease in liver glucose-6-phosphatase activity (Weber and Cantero, 1957). In cortisol treated animals liver glycogen increases in three hours. Cortisol and its metabolites disappeared from the liver one hour before the increase in liver glycogen occurred and two or more hours before maximum physiological response occurred. Cortisol excites a triggering action on a reaction sequence producing the increase in liver glycogen (Hyde, 1957).

Weber and Cantero (1957) demonstrated that 600 r of total body irradiation had no effect on glucose-6-phosphatase in normal or hypophysectomized rats, but increased hepatic phosphoglucomutase and phosphohexoseisomerase activities. Hypophysectomy abolished this response in irradiated animals. The increased liver glycogen following irradiation is a reflection of the increased phosphohexoseisomerase and phosphoglucomutase activities in the liver.

The incubation of an enzyme from an aqueous extract of liver, together with UDPG and primer glycogen *in vitro* yields approximately equal amounts of UDP and glycogen. This conclusively demonstrates that UDPG is used to synthesize glycogen (Leloir and Cardini, 1957; and Leloir et al., 1959).

The incorporation of carbon-14 labeled glycine into liver glycogen occurs rapidly in stressed intact animals and in adrenalectomized animals

after cortisone administration (Todd and Allen, 1958).

Gallagher (1958) demonstrated that cortisol inhibits oxidative phosphorylation in normal rat liver mitochondria. This effect of cortisol was mediated by increasing the mitochondrial membrane permeability. Cortisol destroyed the semipermeable nature of the mitochondrial membrane.

Arginase activity was decreased following adrenalectomy and could be restored to normal by cortisone. Cortisone also stimulates arginase activity in normal animals (Bach, Carter and Killip, 1958). Cortisol produced an increased trytophan pyrollase activity which was not associated with an increased tryptophan level, or an increase in tryptophan excretory products. This suggests that cortisol is acting as a primary inducer of this enzyme (Civen and Knox, 1959).

Rosen et al. (1959) observed that liver glutamic-pyruvic transaminase (GPT) activity was increased by cortisol, cortisone and several other corticosteroids _in vivo_. Cortisol also increased GPT activity in thymus, pancreas, and, to a lesser extent, in the kidney. Estradiol and thyroxine produced a slight increase in GPT in the liver. Progesterone, estradiol and thyroxine interferred with the GPT response produced by cortisol. This response in GPT may be a secondary response to stress associated with utilization of tissue proteins. Cortisol treatment increases hepatic alanine-α-ketoglutarate transaminase, but had little effect on aspartic-α-ketoglutarate transaminase (Harding et al., 1961).

The physiological maintenance of hepatic fructose-1,6-diphosphate, phosphohexoseisomerase and phosphoglucomutase is partially dependent on

the pituitary-adrenal axis (Weber and Cantero, 1959a). These enzymes are necessary for glycogen synthesis. Glucose-6-phosphatase and fructose-1,6-diphosphatase are necessary for the formation of free glucose. Irradiation causes an increase in phosphogluconate dehydrogenase which shunts glucose into the pentose phosphate pathway. Irradiation does not increase glucose-6-phosphatase. If the irradiated animals are hypophysectomized, these enzyme changes do not occur (Weber and Cantero, 1959b). The shunting of glucose into the pentose pathway will lead to glycogen synthesis (Eisenberg et al., 1959).

UDPGA has been implicated in the synthesis of hyaluronic acid (Markowitz et al., 1959). Hydrolysis of UDPGA *in vivo* and *in vitro* leads to the synthesis of ascorbic acid in goat liver microsomes (Pogell and Leloir, 1961).

Yielding and Tomkins (1959) demonstrated that a large number of steroid hormones inhibit the oxidation of DPNH by mammalian enzymes. In skeletal muscle the site of inhibition was found to be the DPNH-cytochrome c-reductase reaction. (This reductase reaction also leads to the synthesis of two ATP molecules.) This inhibitory effect of steroids could be reversed by α-tocopherol. It is interesting to note, at this point, that oxidized DPN is necessary for the conversion of UDPG to UDPGA. DPN is rate limiting for the conversion of pyruvate to acetyl CoA and CO_2, which allows carbohydrates to enter the Krebs cycle. Steroids can regulate oxidative decarboxylation of pyruvic acid by virtue of their ability to inhibit oxidation of DPNH (Yielding et al., 1960). Using liver slices from cortisone treated animals, Landau et al. (1962) demonstrated an increased incorporation of

pyruvate-C^{14} and $C^{14}O_2$ into glucose and glycogen. The incorporation of CO_2 into pyruvate supports the claim that amino acids can serve as a source of carbohydrate in the presence of glucocorticoids, and that pyruvate or oxaloacetate is an obligate intermediate in the formation of glucose from amino acids.

The primary mechanism by which glucocorticoids increase gluconeogenesis involves stimulation of the pyruvate to hexose pathway and that increases in glucose-6-phosphatase and fructose-diphosphatase are secondary adaptive changes (Kvam and Parks, 1960). Phosphorylase activity can be increased by cortisol, cortisone and progesterone. Adrenalectomy will reduce the level of this enzyme (Willmer, 1960).

Scott in 1937 made the following statement, "The evidence shows that x or γ-rays only influence enzymes when the dose is enormous. Cell division is inhibited by doses which have little or no destructive action on enzymes, and, therefore, the inhibition is produced by a process in which enzymes play no important part," (Dale, 1940).

Purified enzymes in aqueous solutions are radiosensitive only at very low concentrations or very high doses of radiation (Dale, 1940).

White and Dougherty (1945) and Dougherty and White (1946) demonstrated that 200 r within three hours caused a decrease in adrenal cholesterol, a lymphopenia, tissue lymphocyte degeneration and an increase in total serum proteins and gamma globulin. Two hundred rads would produce the same changes one day post-operative in adrenalectomized mice. Ten rads would produce the same result in intact mice, but not in adrenalectomized animals. Irradiation stimulates the pituitary-

adrenal axis (Frank and Dougherty, 1955). Patt et al. (1947) confirmed White and Dougherty and demonstrated that at seven days post-irradiation, adrenal cholesterol greatly increased. The increased cholesterol indicates either increased adrenal activity or adrenal exhaustion. This adrenal response to x-rays (600-900r) was prevented by hypophysectomy, but pituitary abalation did not alter the degree or time course of splenic and thymic involution. Hypophysectomy appeared to potentiate x-ray toxicity (Patt et al., 1948).

Wail and Frenkel (Kretzschmar and Ellis, 1947) in 1925 showed that 4-8 hours after irraidation, changes in mitochondria become apparent when no other morphological change in the cell could be observed. Kretzschmar and Ellis (1947) suggested that the breakdown of mitochondria after irradiation may contribute to a series of metabolic changes incompatable with continuance of the normal function of the cell.

Dogs receiving 300 r exhibited a significant increase in 17-ketosteroids (Zimmerman) some time between the 5th and 12th day post-irradiation. The 17-ketosteroids then fell to subnormal or normal values showing a return to normal on the 30th to 40th days (Lawrence, 1949). Brayer et al. (1954) using pigs showed a cyclic alteration in total neutral adrenal steroid excretion following 750 or 1000 r.

French et al. (1955) demonstrated an increase in plasma 17-hydroxysteroids shortly after doses of 50-800 r, peaking at 4-8 hours and returning to normal within 12 hours. Degree of increase and duration were related to dose. After the first 12 hours steroids remained normal until a terminal increase shortly before death. Terminal response was two to three times that of maximum ACTH response.

Spleen mitochondria from rats given 800 r showed a reduction in oxidative phosphorylation capacity. Normal mitochondria given 2000 r *in vitro* were unaffected. This would indicate a physiological effect rather than a direct effect of x-irradiation (Potter and Betheel, 1952; and Van Bekkum et al., 1954).

Mice given 500 r to the head could be protected with cortisone or deoxycorticosterone given before or after irradiation (Mirand et al., 1952). Santisteban et al. (1954) demonstrated that adrenalectomized irradiated animals die sooner, at a faster rate, and with a higher mortality than intact irradiated animals. Cortisone tends to restore resistance to irradiation in adrenalectomized animals but did not seem to make them more resistant than intact animals. Estrogens and diethylstilbesterol given for a week prior to irradiation will afford some radioprotection. Androgens had no effect. The same amount of protection gained by giving stilbesterol before irradiation was achieved if stilbesterol was given immediately after irradiation (Mirand and Hoffman, 1954).

Oxygen consumption of tissue slices from spleen and thymus given 400 r showed a marked decrease. Maximum inhibition was at 48 hours. Additions of glucose, pyruvate, and Krebs cycle intermediates did not alleviate the radiation induced inhibition of oxygen consumption (Sullivan and Dubois, 1955). Miller et al. (1955) demonstrated that 700 r and 7000 r reduced ATP synthesis by 25% at 72 hours. Animals given 700 r gradually recovered their ATP synthesizing ability. The reduction in ATP synthesis in the 7000 r group was progressive.

Eichel and Sprites (1955) studied the effects of 700 r and 980 r on the liver DPN and DPNH levels. They found that 700 r had very little effect and that 980 r decreased DPNH by 15-32% and had no apparent effect on DPN.

Hypophysectomy reduces the resistance of rats to whole body irradiation. Thyroxine was the only substance found that increased per cent survival. Hypophysectomized, irradiated animals treated with insulin died sooner than hypophysectomized, irradiated controls (Sellers and Barlow, 1955).

Unilaterally adrenalectomized mice given 50 r of x-ray to the head, showed an adrenal hypertrophy ten days after irradiation. Doses greater than 500 r did not cause an adrenal hypertrophy and as dose increased an adrenal atrophy appeared (Mirand and Hoffman, 1955). Adrenal atrophy could be reversed by giving estrogens for seven days.

Ungar et al. (1955) and Rosenfield et al. (1955) described the effects of 2000 r of γ-irradiation on calf adrenals in vitro. They found a reduction in the amount of cortisol and corticosterone secreted in the irradiated gland versus the contralateral un-irradiated gland. This decrease was ascribed to a significant reduction in 11, 17, and 21-hydroxylation as well as a decrease in the oxidation of Δ^5-3β-hydroxy group to the Δ^4-3-ketone group. Tonkikh (1958) using the muscle work test to evaluate adrenal function following irradiation, found an increase at 24 and 48 hours, and a return to normal by 72 hours. Seven days post-irradiation adrenal output fell below normal and the animals began to die. Administration of ACTH beginning

on the third day post-irradiation did not increase the adrenal output on the seventh, eighth or ninth day. This leads us to suspect that some mechanism which is unresponsive to ACTH is responsible for the terminal decrease in adrenal production. Krebs (1956) states that there are metabolic reactions in the body especially vulnerable to extraneous agents. A decrease in the reaction rates of these "pacemaker" reactions will be mirrored in a diminished overall rate of metabolism. These "pacemaker" reactions are usually reactions involving reduced or oxidized coenzymes. Any change in amount of coenzyme or change in the natural biological equilibrium constant for the amount of reduced or oxidized coenzyme will block one of these "pacemaker" reactions (Yielding and Tomkins, 1959; and Yielding et al., 1960). Berliner, Berliner and Dougherty (1962) demonstrated that dogs with internally deposited radionuclides had a decreased adrenal-cortical biosynthesis. This was due to a lack of 11, 17, and 21-hydroxylation. Adrenal biosynthesis can be restored to control values by adding TPNH.

Glucose metabolism is impaired 24 hours after irradiation and is not a fasting phenomenon. The conversion of glucose-C^{14} into CO_2, fatty acids and glycogen indicated a block in the glycolysis pathway at a point, or points, before the triose level. Fructose metabolism apparently was unimpaired. This block could possibly be an impairment of hexokinase once glucose enters the cell (Hill et al., 1956). Morehouse and Searcy (1957) demonstrated that irradiation increased the incorporation of amino acids into glycogen. It was also observed that

amino acids may serve as a source of carbons for glycogen whether they are glucogenic or ketogenic.

Bacq et al. (1957) observed that adrenals grafted into the anterior chamber of the eye in adrenalectomized rats responded to total body irradiation with the response of adrenals in intact animals. The adrenal ascorbic acid content of hypophysectomized rats with two pituitary grafts in each eye decreased two hours after 850 r of total body irradiation, returned to normal at 24 hours, and did not show the 72 hour drop observed in the controls.

Depression of glycolysis in x-irradiated animals is due to an immediate decrease in DPN rather than to an inhibition of a glycolytic enzyme (Maass and Schubert, 1958). The reaction inhibited by the decreased DPN concentration is the triose phosphate dehydrogenase step in glycolysis. Holzer (1958) observed that modern cytostatic agents are obviously based on a similar mechanism. He found a primary inhibition of DPN metabolism with cytostatic agents. The thymus had a decreased DPN and cytidine diphosphate concentration following 800 r of whole body irradiation. There was also a decrease in nucleotide synthesis as measured by P^{32} uptake into these compounds: DPN, 2'-AMP, 3'-AMP, ATP, ADP, 2' and 3'-CMP, CDP, CTP, UMP and UDPG (Maass and Schubert, 1958).

There is an impaired glucose utilization following 500 r as measured by a glucose tolerance test. This impairment can be correlated with pituitary-adrenal response as well as change in β/α cell ratios in the islets of Langerhans of the pancreas. There is also an

increase in thyroid activity at 24 hours post-irradiation followed by a decrease to below normal values (Allegretti, 1958).

Liver slices from irradiated rats showed a dose dependency for cortisol metabolism. Doses of 428 r and 856 r resulted in a 10-15% decrease in cortisol metabolism at four hours, 24 hours and two weeks post-irradiation. Doses ranging from 1712 r to 8750 r resulted in a 33-35% decrease in liver's ability to metabolize cortisol. Cortisol metabolism was measured with the Porter-Silber reaction which is specific for the 17, 21-hydroxy, 20-ketone side chain (Lott and Pryor, 1959). Berdjis (1959) demonstrated that cortisone reinforced the injurious effects of irradiation. These injurious effects were evident in cortisone treated rats irradiated in both kidneys with 1500 r. The kidneys showed capillary thrombosis and glomerulonephrosclerosis with necrosis and/or hemorrhage.

Kochetov (1959) found, 24 hour post-irradiation, that glucose-6-phosphate and 6-phosphogluconate dehydrogenases undergo an increase, in addition to an increased ribose-5-phosphate breakdown. Fructose diphosphate increased and sedoheptulose-7-phosphate utilization decreased. These changes indicate a shift from the transaldoase reaction to triose condensation into fructose-1,6-diphosphate with subsequent dephosphorylation in irradiation. These enzymatic changes can be correlated with the increase in glycogen and glucose following irradiation.

The effect of ionizing radiation on chromosomes has been known since 1903. Since that time a tremendous amount of work has been done

(Muller, 1958; and Butler, 1959). The effect on cellular DNA synthesis and on mitosis has been elucidated by Lajtha et al. (1958). Cell division can be separated into three time periods: G_1 - long resting period, S - DNA synthesis period, and M - mitosis. Once cells begin DNA synthesis, 200-300 r will not inhibit it. If the cells are not in DNA synthesis but in the G_1 period, 200-300 r will prevent one-half of these cells from entering into DNA synthesis. Therefore, once a cell enters into DNA synthesis and mitosis, large doses of irradiation are necessary to inhibit it. If a cell is in the G_1 period, small doses of irradiation will prolong its resting period, although it may prevent DNA synthesis altogether. There must be a metabolic system present during the resting period which is closely associated with DNA synthesis and which is more radiosensitive than DNA synthesis (Lajtha et al., 1958).

Osawa, Allfrey and Mirsky (1957) showed that calf thymic nuclei will phosphorylate intranuclear adenine, quanine and uridine monophosphates to the corresponding triphosphates aerobically at $0^{o}C$. Creasy and Stocken (1958) found that 100 r of total body irradiation completely suppressed high energy phosphate generation in nuclei; even 25 r led to a 50-80% inhibition (thymus, spleen, lymph nodes, bone marrow and intestinal mucosa). There is recovery following sublethal doses, but not after 1000 r. Recovery commences in the third day post-irradiation and is complete in about 100 hours post-irradiation.

Hogeboom and Schneider (1952) described the synthesis of DPN in cell nuclei. They found that 69-101% of the total enzymatic activity was in the nucleus. The coenzyme is formed in the nucleus and trans-

ferred to the cytoplasm. Hemingway and Carter (1958) demonstrated that cortisone decreased the mitotic rate in regenerating rat liver. This effect was obviated by growth hormone. They suggest that cortisone was blocking the transfer of material between the nucleus and cytoplasm. Hemingway (1959) observed that 200 r caused a considerable reduction of mitosis in regenerating rat liver and moderate to severe nuclear damage in both normal and adrenalectomized rats. In spite of this damage, a high mitotic rate is seen in liver from adrenalectomized rats, mitosis often proceeding in nuclei obviously breaking down. The fact that mitotic rate can be separated from nuclear degeneration indicates that the usually accepted radiation effect comprises at least two factors: (a) a hormonal inhibition mediated by corticosteroids as part of the general stress response, and (b) a breakdown of nuclei due to a block in nucleic acid synthesis. Animals with intact adrenals tolerate radiation better than adrenalectomized animals. This study shows less nuclear degeneration in normal rats than in adrenalectomized rats.

It appears that the primary action of the ionizing radiation is to bring about hyperactivity of the anterior pituitary with a subsequent increase in blood levels of TSH and ACTH. The target glands of these two hormones are stimulated to overproduce their hormones. The excessive amounts of thyroxine and corticosteroids are responsible, in turn, for observed depression in phosphate:oxygen ratios in spleen and liver mitochondria. The effect on the spleen is mediated through the pituitary-thyroid axis and the effect on the liver is mediated through the pituitary-adrenal axis. Head shielding during whole body

irradiation offers complete protection (Benjamin and Yost, 1960).

Irving and Perkinson (1960) observed that I.P. injections of P^{32} inhibited oxygen consumption in rat liver slices. Anaerobic glycolysis was also inhibited. There was an increased liver glycogen. Yokata (1960) demonstrated that 1-3 r would inhibit tissue respiration of the spleen, but did not affect respiration of liver or kidney. Kerppola (1960) observed that rats pretreated with cortisone had decreased oxidative phosphorylation in liver mitochondria. The effect was most pronounced in female rats 12 months old or older. Uncoupling of oxidative phosphorylation by cortisone and inhibition of the associated ATP production seems to provide and explain many of the tissue changes caused by cortisone.

Plasma levels of 17-hydroxysteroids remained within control values following irradiation of rhesus monkeys until shortly before death when a marked increase appeared. The magnitude was greater than that obtainable from ACTH. This effect could be due to a decreased rate of removal of cortisol and its metabolites from the plasma (Wolf, Bowman and Harlow, 1961).

Hilz et al. (1961) observed that the irradiation of Ehrlich ascites tumor cells with 3000 r decreased the intracellular DPN content by 20-50%. The addition of nicotinamide would counteract these effects. When DPN levels were restored, DNA synthesis was restored. Thus, the radiation effect does not appear to be linked to the synthesis of DPN *per se*.

Dose and Dose (1961) showed that the decrease in DPN and ATP in Ehrlich ascites cell was dose-dependent. Potassium transport was also

affected. The addition of inorganic phosphate, ATP and ADP would restore the transport function to normal, but would not restore the DPN levels (dosage in range of kiloroentgens).

Adachi, Chow and Rothman (1962) reported that irradiation of skin caused a decrease in phosphorylase activity by 20%. The synthesis of glycogen from UDPG was increased by 23%. This indicates that UDPG is the main precursor of glycogen in the skin.

The liver is a remarkably radioresistant organ as judged by morphological studies (Ely, Ross and Gay, 1957). The liver usually does not show morphological changes below 12,000 r in ordinary animals (W. D. Claus, 1958). The radiosensitivity of this organ is usually determined by the diet. It has been reported that rats given a low protein diet for 30 days and then x-rayed with 500 r developed nodular atrophic cirrhosis. Rats fed a high protein diet and x-rayed showed no cirrhosis at death (White et al., 1954).

The kidneys are considered to be rather radioresistant, but the statements in the literature are not in accord. Usually doses of several thousand roentgens are necessary to cause renal damage (Warren, 1942). Furth et al. (1954) has reported slight renal changes developing several months after exposure to 500 r in mice. Mouse kidney has been reported to be radiosensitive during compensatory hypertrophy (Rosen and Cole, 1960) following unilateral nephrectomy.

MATERIALS AND METHODS

The tissues used in these studies were taken from male mice of the CBA strain. The average age of the mice at sacrifice was 84 days. The mice were maintained in a controlled environment and were given water and laboratory chow ad libitum.

The control mice and the experimental animals were chosen by random selection. In the irradiation experiments the animals were pooled and then divided into groups prior to irradiation. Five groups were selected 1, 5, 13 and 21 days post-irradiation, and non-irradiated controls. The animals for the rate study were chosen in the same manner The control animals were non-irradiated normal animals. The irradiated animals were irradiated as a group. Five days later they were sacrificed and the kidneys and livers were incubated.

The mice were irradiated under the following conditions: a Westinghouse Quadrocondex x-ray machine was used; the machine settings were 250 kilovolts and 15 milliamps with a 1/2 mm copper + 1 mm aluminum filter; and the irradiation chamber was 50 cm from the tube.

The mice were irradiated in a plexiglass chamber designed to hold 16 animals (Fig. 1). The dose rate was measured in air with a Victoreen thimble type ionization chamber. A total of four measurements of dose rate were made; two at the inner circumference of the chamber and two at the outer circumference. The average of these measurements in roentgens per minute, corrected for standard temperature and pressure, gave a dose rate of 82 r/minute. The animals were given a total of 500 roentgens, which is a LD 50/30 dose for CBA male mice in our laboratory.

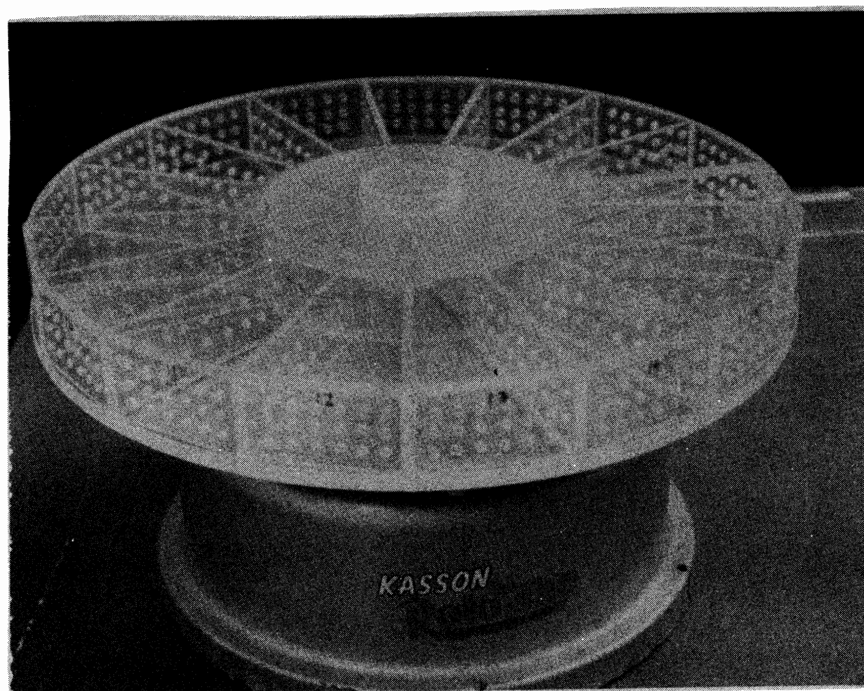

Fig. 1. Mouse Irradiation Chamber

Hormones

Four different steroid hormones were used in this study: corticosterone-1,2-H^3 (4-pregnen-11β,21-diol-3,20-dione), cortisol-4-C^{14} (4-pregnen-11β,17α,21-triol-3,20-dione) - New England Nuclear, Inc., compound X (pregnane-3α-ol-11,20-dione-4-C^{14}), and compound XIII (pregnane-3α,17α-diol-11,20-dione-4-C^{14}) - N.I.H. Endocrinology Study Section (Fig. 2). The stock solutions of steroids were stored in a mixture of benzene:acetone (10:1), at -20°C in a volume large enough to minimize radiochemical decomposition. The steroids were purified by paper chromatography using Zaffaroni* techniques. The pregnane-3α-ol-11,20-dione-4-C^{14} was received as an acetate. In order to obtain the free compound, the acetate moiety was removed by incubating with aqueous K_2CO_3 (2.5%) and methanol for three hours at 37°C. The free steroid was then extracted from the water with chloroform ($CHCl_3$) and purified by paper chromatography. The steroids were diluted with the appropriate non-radioactive steroids to adjust the specific activity to the desired values. The dilution was made with methanol and a very small amount of proplyene glycol. The steroid was pipetted into the flasks and the solvent was removed by evaporation under nitrogen (N_2) leaving the steroid in the propylene glycol.

Incubations

The animals were sacrificed by cervical dislocation. The liver (gallbladder removed) and kidneys were immediately removed and placed in iced buffer.

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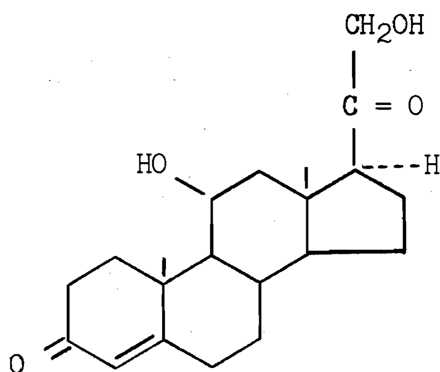
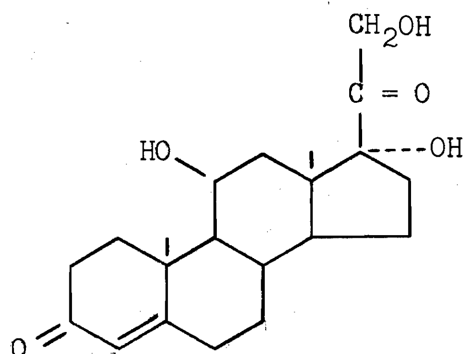
*Recent progress in hormone research, Vol. 8, 1953.

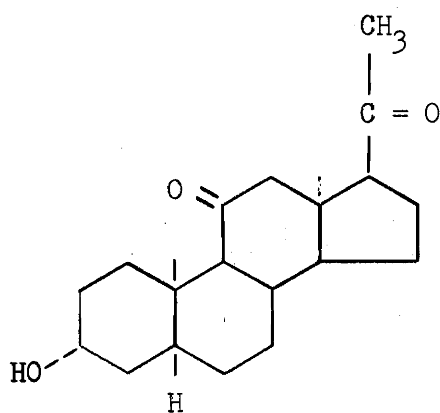
Fig. 2 Structural formulas of steroids used.



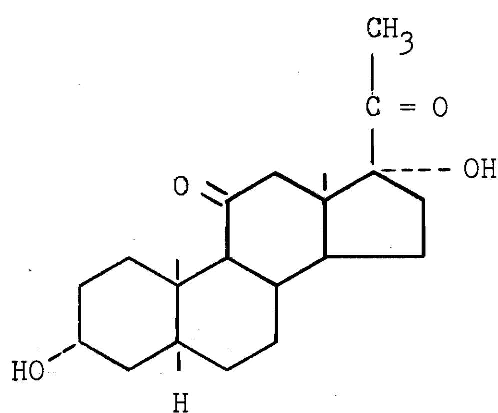
Corticosterone
(4-pregnen-11β,21-diol-3,20-dione)



Cortisol
(4-pregnen-11β,17α,21-triol-3,20-dione)



Compound X
(pregnane-3α-ol-11,20-dione)



Compound XIII
(pregnane-3α,17α-diol-11,20-dione)

The tissues were finely minced with a razor blade. One gram of tissue was placed in a 125 ml Erlenmeyer flask with 20 ml of 0.1M phosphate buffer (pH 7.4). The steroid was already in each flask as mentioned previously. A total of 100 millimicromoles (mμM) of steroid was present in the flask (50 mμM of tritium labeled steroid and 50 mμM of carbon-14 labeled steroid). The standards were prepared from stock solutions in the same manner as the samples except that only one steroid was in each flask.

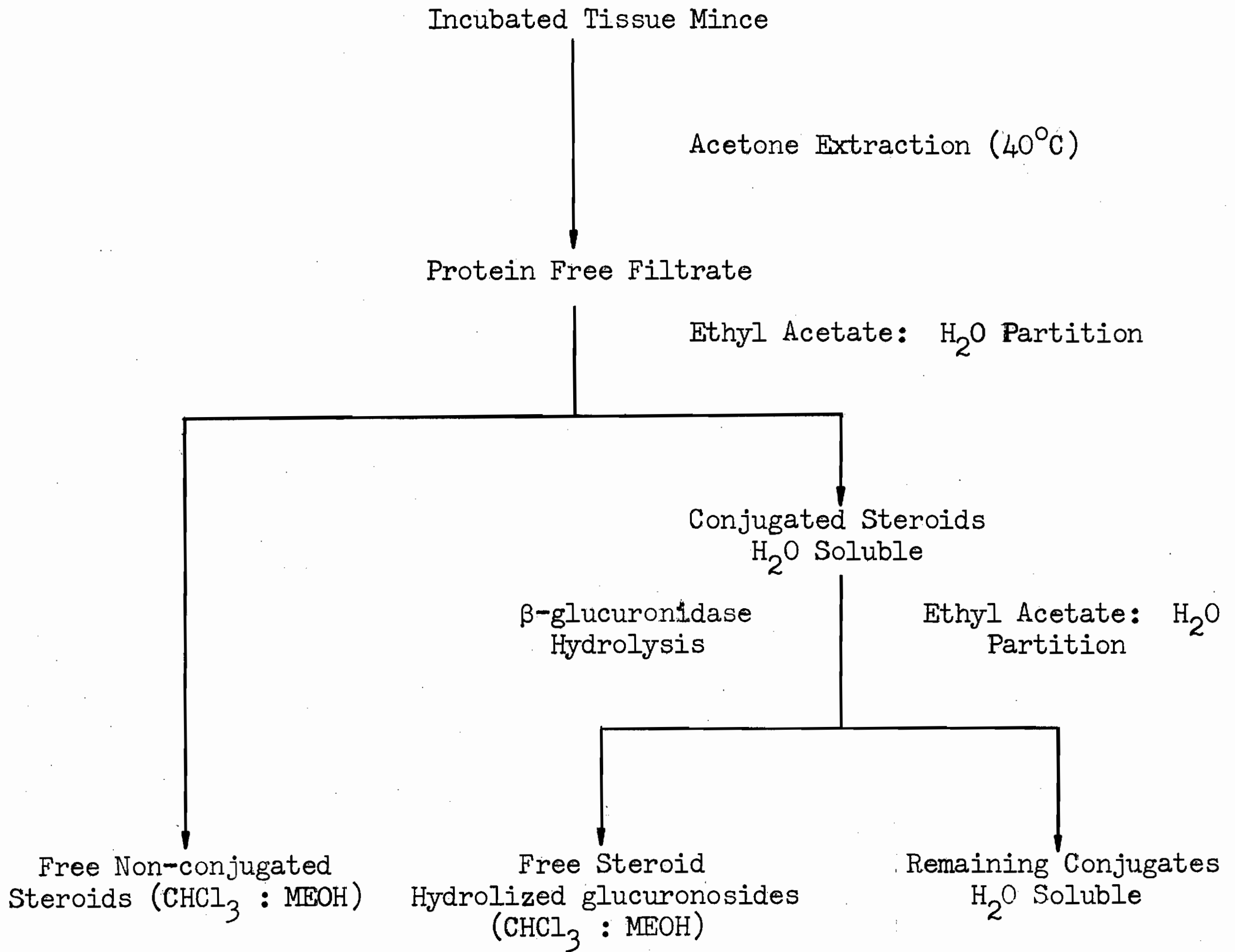
Boiled liver and kidney served as incubation controls. The liver and kidney were boiled for ten minutes and then incubated for three hours with two of the steroids mentioned above.

The ratio of activities of tritium to carbon-14 was 15:1 disintegrations per minute (dpm). The liver was incubated together with corticosterone-1,2-H^3 and cortisol-4-C^{14} or pregnane-3α-ol-11,20-dione-4-C^{14} or pregnane-3α-17α-diol-11,20-dione-4-C^{14}. The same procedure was followed with the kidney. The flasks were incubated at $37^{\circ}C \pm 2^{\circ}C$ in an American Instrument Co. wide range laboratory bath for three hours. The incubations for the rate study were for the following time periods: 5, 15, 30, 60, 120 and 180 minutes for x-irradiated and 15, 60 and 180 minutes for controls. The reaction was stopped by adding 20 ml of acetone to each flask.

Extraction of Products (Fig. 3).

The flasks were extracted three times with equal volumes of warm acetone ($40^{\circ}C$) as soon after incubation as possible. The acetone extract was filtered through "shark skin" (Schleicher and Schuell Co.)

Fig. 3 Flow sheet of extraction procedure.



filter paper to remove particulate matter. The acetone extract was then evaporated *in vacuo* with a flash evaporator in a 40°C water bath. The remaining water residue was exhaustively extracted with equal volumes of ethyl acetate and the extract was evaporated to dryness *in vacuo*. The water fraction was stored at -20°C until a β-glucuronidase hydrolysis could be performed. The ethyl acetate fraction contained the free (non-conjugated) steroids. This fraction was brough to a total volume of 10 ml with chloroform:methanol ($CHCl_3$:MEOH - 1:1) and stored at -20°C.

The aqueous fractions were submitted to a β-glucuronidase hydrolysis by Sigma bacterial β-glucuronidase. The method used was developed by Dr. Charles D. West of the Department of Biological Chemistry at the University of Utah. The water fractions were made up to a standard volume of 20 ml and transferred to a 50 ml Erlenmeyer flask. Fifty mg of ethylenediamine tetraacetic acid (Sigma Chemical Co., disodium salt), 80 mg of cysteine (California Corp. for Biochemical Research), 2 ml of 0.75M phosphate buffer (pH 6.5), 2000 units of β-glucuronidase and one drop of chloroform were added to each flask in the order given. The flasks were incubated for 17 hours at 37 ± 2.0°C.

After hydrolysis, the samples were re-extracted with ethyl acetate as before. The ethyl acetate fraction now contained those steroids that had been conjugated as glucuronides. This fraction was made to a total volume of 10 cc with $CHCl_3$:MEOH (1:1) and stored at -20°C.

Radioisotope Analysis

A Packard automatic liquid scintillation spectrometer series 314A was used for determining the radioactivity in all samples. The advent of this type of instrument makes it possible to count two or more

isotopes simultaneously. Carbon-14 and tritium are ideally suited for this technique of double isotope counting.

In all incubations, with the exception of standards, two different steroids were used, one labeled with tritium and one labeled with carbon-14. Several different methods are available for double isotope work (Okita et al., 1957). We selected the method of simultaneous equations (Mays et al., 1958) because they are readily evaluated by a digital computer.

The simultaneous equation method is based on the use of a carbon-14 standard and a tritium standard. The amount of radioactivity in each sample is represented as a fraction of amount of radioactivity in each standard. The Packard spectrometer is set in the following manner: high voltage 1150 volts, discriminator A = 10 volts, discriminator B = 50 volts, discriminator C = 70 volts. The machine is set to split channel operation, mode 2. Figure 4 gives an idealized representation of the pulse height spectrum of carbon-14 and tritium at the above settings. It also illustrates that most of the H^3 activity occurs in channel I with a small amount in channel II. Carbon-14, on the other hand, has more activity in channel II than in channel I.

Let:

A = count rate of the H^3 standard (a)

B = count rate of the C^{14} standard (b)

K = count rate of the sample

X = the fraction of the standard of (a) in the sample

Y = the fraction of the standard of (b) in the sample

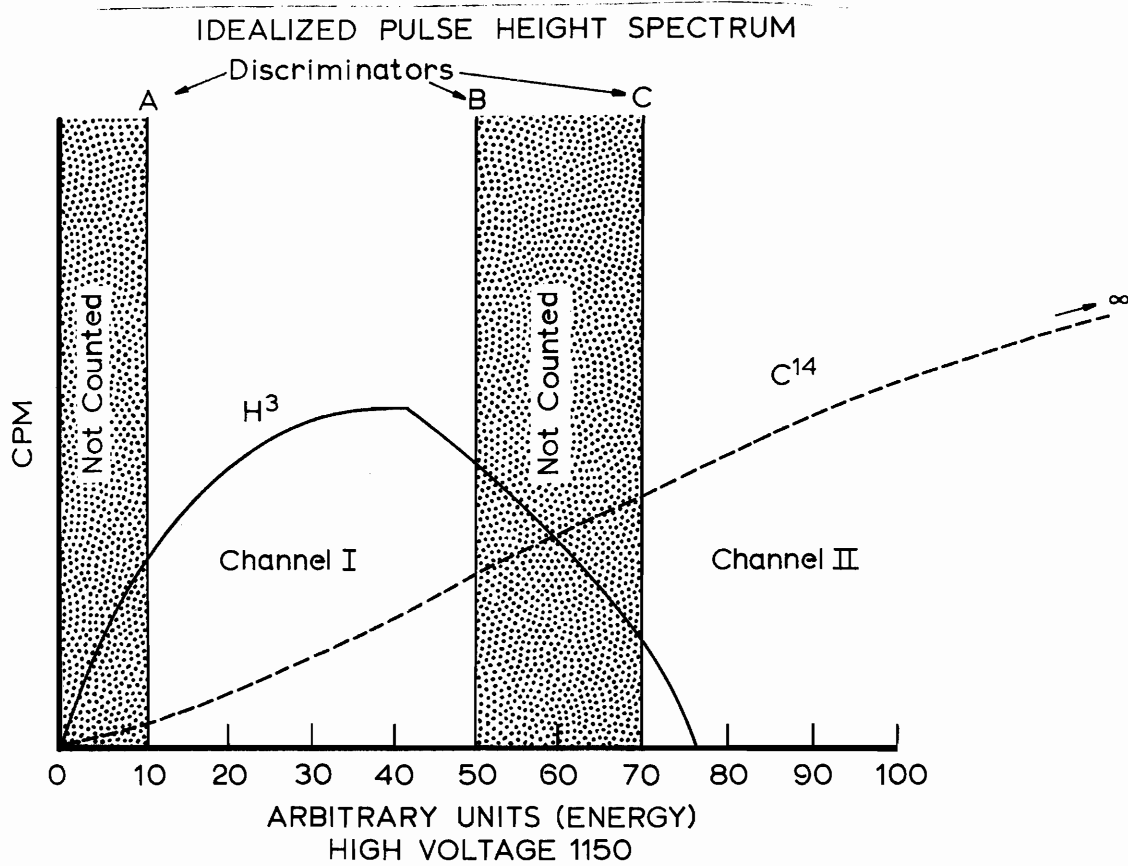


Fig. 4. Idealized Pulse Height Spectrum

In the sample the sum of the individual count rates equals the total count rate:

$$A_1X + B_1Y = K_1 \qquad \text{(channel I)}$$

$$A_2X + B_2Y = K_2 \qquad \text{(channel II)}$$

Therefore,

$$X = \frac{B_2K_1 - B_1K_2}{A_1B_2 - A_2B_1}$$

$$Y = \frac{A_2K_1 - A_1K_2}{B_1A_2 - B_2A_1}$$

The above form of the equation is readily evaluated by digital computers. A Burroughs 205 Datatron computer was used in this study. These equations may also be evaluated by hand, using a desk calculator. The following is the form of equation used when evaluating by hand.

$$X = \frac{K_1 - \left(\frac{B_1}{B_2}\right) K_2}{A_1 - \left(\frac{B_1}{B_2}\right) A_2}$$

$$Y = \frac{K_2 - \left(\frac{A_2}{A_1}\right) K_1}{B_2 - \left(\frac{A_2}{A_1}\right) B_2}$$

The only fault inherent in this method is that mistakes in pipetting or partial loss of sample are greatly magnified.

Radioactive chromatograms were scanned by a Vanguard Model 800 automatic chromatogram scanner. All radioactive samples were counted for a time period sufficient to attain statistical accuracy.

Sample Preparation for Radioactive Analysis

All samples, including standards and zero controls, were made up to a total volume of 10 ml. A 1 ml aliquot was taken and placed in a scintillator vial. The solvent was evaporated under N_2 to dryness. Twenty milliliters of scintillating solution were placed in each vial and cooled to $-8^{o}C$. The samples were then counted.

Statistics

The rate study curves were plotted on logarithmic paper. The logarithm of per cent free steroid was plotted against the logarithm of time in minutes. The plot of observed values gives a straight line. These points can be statistically fit with a line that best fits the observed values. The method of least squares* was used to determine the line of best fit. The resulting empirical equation is a power function of the form:

$$X = at^{b} \qquad (1)$$

where

X = the per cent of steroid remaining free at time t.

a = intercept at $t = 1$.

b = the exponent of t.

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*F. S. Action, Analysis of straight-line data, John Wiley & Sons, 1959.

A logarithmic transformation of the power function yields:

$$\log X = \log a + b \log t \qquad (2)$$

which is analogous to the equation for the straight line:

$$Y = A + BX \qquad (3)$$

In Eq. (2) b becomes the slope of the line and can be treated statistically as a regression coefficient.

Statistical analysis was performed using Student's "t test." The regression coefficients were tested for significant difference from zero and for significant difference between control and x-irradiated groups at the 95% confidence level. A standard table of t was used to determine the range of P.

The original pilot study for this thesis was done using the methods described above with the following exceptions.

1. Only one steroid was incubated per flask.
2. Each flask contained 2.5 μM TPNH and 2.5 μM glucose-6-phosphate.
3. Aliquots of both free steroid and water fractions were counted in the Packard spectrometer. The efficiency was determined for organic and water fractions by using an internal standard of C^{14} toluene. Using these efficiencies, the cpm are converted to dpm, and all subsequent calculations were based on dpm.

4. The per cent of water soluble conjugates was calculated by dividing dpm in water by total dpm (free dpm + H_2O dpm). Per cent glucuronoside was calculated in the same manner by dividing the dpm in ethylacetate after β-glucuronidase by the total sample dpm (free dpm + H_2O dpm).
5. Cofactors (TPNH, glucose-6-phosphate, and UDPGA) were obtained from Sigma Chemical Co.

RESULTS

Conjugation and formation of glucuronides by mouse liver, kidney and small intestine.

A single isotope pilot study. The data in Table I represent the original pilot study made to determine if kidney and small intestine could conjugate, as glucuronosides, Δ^4-3-keto corticosteroids and/or 3-hydroxy corticosteroids. One steroid was incubated per flask.

The data are presented as per cent of the total steroid incubated which is rendered water soluble during incubation and the per cent of that water soluble steroid which is conjugated as a glucuronide. Percentage of total steroid as glucuronoside is also included. Zero controls were subtracted from each value. Figure 5 illustrates the step-by-step conjugation of cortisol.

The small intestine was unable to conjugate any of the three steroids.

The kidney was able to conjugate 32% of the incubated compound X (Fig. 2) and 5% of the incubated corticosterone (Fig. 2) in the presence of TPNH and glucose-6-phosphate. The amount of cortisol conjugated was negligible. In this study, 30% of the compound X was conjugated as glucuronoside by the kidney. The amount of corticosterone present as glucuronoside was not detectable.

The liver was able to conjugate all three steroids. The liver conjugated 19% of the cortisol (Fig. 2), 25% of the corticosterone, and 42% of the compound X. The liver conjugated much more compound X than

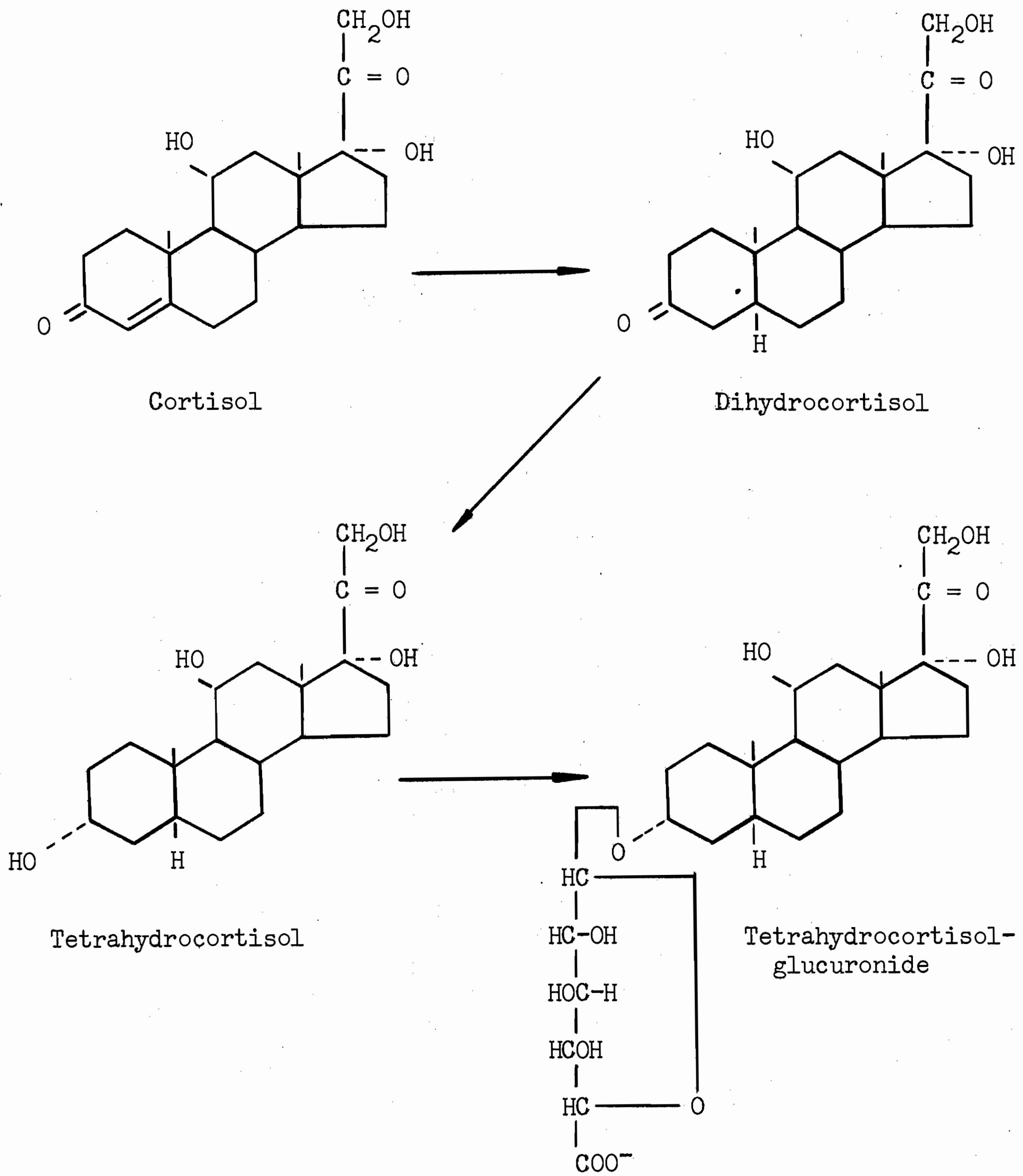


Fig. 5 Example of steps involved in conjugation of cortisol.

TABLE I: *In Vitro* incubation of steroids with various tissue of mice (pilot study).

Tissue	Steroid Incubated*	No. of Incubations	% water soluble Steroids≠	% water soluble as glucuronosides≠
Small Intestine	Cortisol	2	0.0	0.0
	Corticosterone	4	0.0	0.0
	Pregnane-3α-ol-11,20-dione	6	0.0	0.0
Kidney	Cortisol	3	0.0	
	Corticosterone	4	4.96 ± 4.5	Not detectable
	Pregnane-3α-ol-11,20-dione	7	31.8 ± 1.65	29.4 ± 2.03 (9.35 of total steroid)
Liver	Cortisol	2	18.8	Not determined
	Corticosterone	5	25.4 ± 0.81	54.6 ± 0.55 (13.8 of total steroid)
	Pregnane-3α-ol-11,20-dione	8	42.3 ± 2.2	27.2 ± 1.20 (11.5 of total steroid)

* 50 mμ Moles of steroid incubated.

≠ means and standard deviations.

it did either cortisol or corticosterone. The per cent of water soluble corticosterone conjugated as a glucuronoside is double the per cent of water soluble compound X conjugated as glucuronoside. However, the total amount of compound X and corticosterone present as glucuronoside is almost the same. The kidney conjugates compound X at about the same rate as the liver.

Double isotope study. Following the pilot study further work was done to verify the results in Table I. It was decided to incubate two different steroids with the same tissue in the same flask. Corticosterone and compound X were used. The values in Table II are presented slightly different than in Table I. The amount of steroid conjugated becomes per cent of the initially incubated steroid remaining unconjugated after the incubation (free steroid). The per cent water soluble steroid as glucuronoside becomes per cent of the initially incubated steroid rendered free by β-glucuronidase hydrolysis (% steroid as glucuronoside).

After incubation of the kidney, 95% of the corticosterone and 59% of the compound X remained as free steroid. Thirty-eight per cent of the compound X was conjugated as a glucuronoside. The amount of corticosterone conjugated as a glucuronoside was very small. Following incubation of the liver, 72% of the corticosterone and 71% of the compound X remained as free steroid. Six per cent of the corticosterone and 9% of the compound X was conjugated as a glucuronoside.

In this experiment all of the compound X conjugated by the kidney was present as a glucuronoside, in contrast to the liver which formed glucuronosides from 9% of the compound X. On a basis of micromoles of

TABLE II: Conjugation of Steroids by Mouse Liver and Kidney *in vitro* (Two Steroids).

Tissue	Steroid Incubated*	No. of Incubations	% of Initially Incubated Steroid Remaining as Free Compound ≠	% of Initial Steroid Rendered Free by β-Glucuronidase Hydrolysis
Kidney	Corticosterone-1,2-H^3	6	95.19 ± 2.86	-
	Pregnane-3α-ol-11,20-dione-4-C^{14}	6	59.54 ± 5.21	37.59 ± 3.55
Liver	Corticosterone-1,2-H^3	6	72.32 ± 2.61	6.56 ± 1.93
	Pregnane-3α-ol-11,20-dione-4-C^{14}	6	70.91 ± 2.93	8.96 ± 2.28

* 50 mμ Moles Incubated

≠ Mean ± Standard Deviation

compound X glucuronoside formed per gram of tissue, the kidney was more efficient than the liver. The liver conjugated more corticosterone than the kidney because of its ability to reduce ring A. The liver formed slightly more glucuronoside from compound X than it did from corticosterone.

The influence of steroidal structure on conjugation and glucuronide formation.

This experiment was designed to obtain information about the influence of steroidal structure on the glucuronoside forming ability of mouse kidney and liver. Corticosteroids containing the 17α-hydroxy group were compared to compounds without the 17α-hydroxy group. This was the only difference between comparative steroids.

The data in Table III indicate that following incubation of the kidney, 65% of the compound X and 94% of the compound XIII remained as free steroid. The kidney conjugated 30% more compound X than it did compound XIII. The value of P indicates that this is a highly significant difference. The conjugation of cortisol and corticosterone by the kidney is very small and there is no significant difference between them (Fig. 6). The kidney conjugated 18% of the compound X and 3% of the compound XIII as glucuronosides. The difference between compound X glucuronoside and compound XIII glucuronoside is highly significant (Fig. 7). The amount of corticosterone and cortisol glucuronoside is negligible.

After incubation of the liver, 70% of the compound X and 85% of the compound XIII remained as free steroid (Table III, Fig. 6). The

TABLE III: Influence of steroidal structure on conjugation and glucuronoside formation by mouse liver and kidney *in vitro*.

Tissue	Steroid*	No. Incubations	% of initial incubated steroid remaining as non-conjugated steroid≠	P	% of initial steroid rendered free following β-glucuronoidase hydrolysis (% steroid as glucuronosides)≠	P
Kidney	Pregnane-3α-ol-11,20-dione	6	63.05 ± 2.5	P < .01	18.34 ± 6.34	P < .01
	Pregnane-3α-17α-diol-11,20-dione	6	93.8 ± 1.8		3.08 ± 1.09	
	Corticosterone	10	97.72 ± 1.97	.8 < P < .9	1.01 ± 0.50	.6 < P < .7
	Cortisol	2	98.93		0.766	
	Zero incubation control	2	100.20		0	
Liver	Pregnane-3α-ol-11,20-dione	6	70.17 ± 10.6	P < .01	10.90 ± 5.78	.01 < P < .02
	Pregnane-3α,17α-diol-11,20-dione	6	85.36 ± 3.91		3.57 ± 0.20	
	Corticosterone	12	77.58 ± 2.17	P < .01	6.32 ± 1.73	P < .01
	Cortisol	5	87.61 ± 3.60		2.50 ± 0.636	
	Zero incubation control	2	98.2		0	

* 50 mμ Moles incubated.

≠ averages and standard deviations.

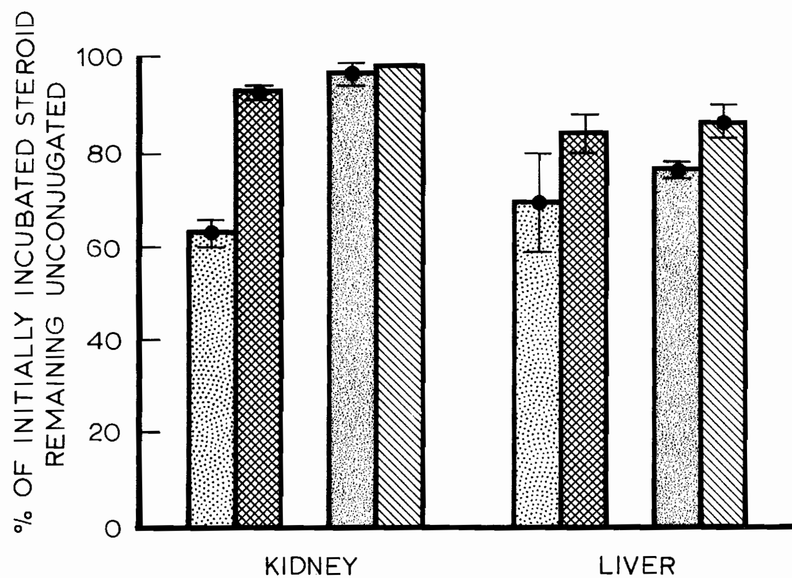


Fig. 6. Conjugation of 17α-hydroxy and non-17α-hydroxy corticosteroids.

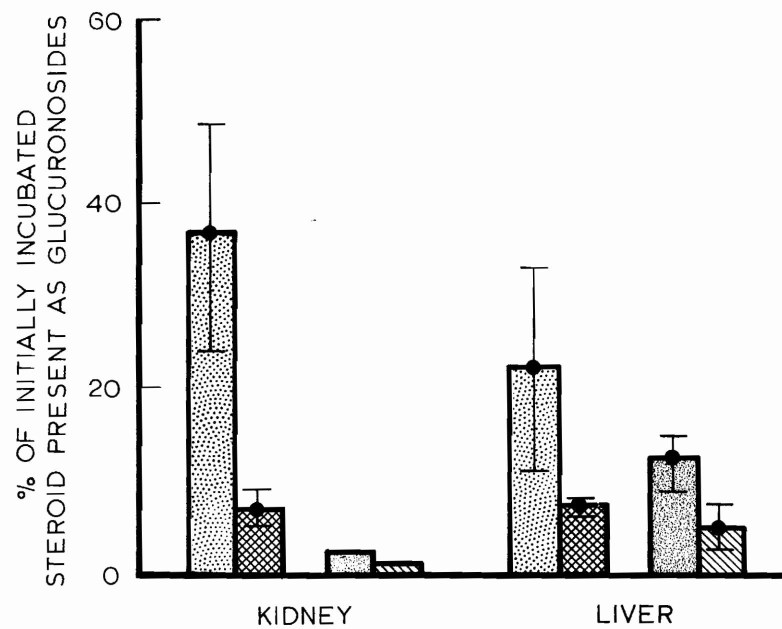


Fig. 7. Glucuronosides formed from 17α-hydroxy and non-17α-hydroxy corticosteroids.

value of P indicates that the difference between compound X and compound XIII is highly significant. The liver conjugated 11% of the compound X and 3.5% of compound XIII as glucuronosides (Fig. 7). This is a highly significant difference. The amount of cortisol remaining as free steroid is about 10% more than the amount of corticosterone remaining as free steroid after incubation with the liver (Fig. 6). This difference is highly significant as is the difference between cortisol glucuronoside and corticosterone glucuronoside (Table III). This study indicates that the presence of the 17α-hydroxy group in cortisol and compound XIII inhibits the conjugation of those two compounds by the liver and kidney.

Effect of total body irradiation on the conjugation of steroids by mouse liver and kidney.

The effect of 500 r total-body irradiation on the conjugation of steroids by mouse liver and kidney was studied at 1, 5, 13 and 21 days post-irradiation. It was found that in most instances irradiation had suppressed conjugation.

The kidney showed a highly significant decrease ($P < .01$) in conjugation at all post-irradiation intervals studied (Table IV, Fig. 8). The decrease in conjugation of compound X was greatest on the first and 13th day, with lesser decreases on the 5th and 21st days. The amount of compound X glucuronoside formed showed almost the same pattern (Table V, Fig. 9). There was an initial decrease on the first post-irradiation day followed by a further decrease on the 5th day. There was a slight return towards normal on the 13th day and a continuation of this return on the 21st day. The amount of corticosterone

Table IV.

Kidney Conjugation of Compound X - 500r

Post Irradiation (days)	Number of Determinations	% of Steroid Unconjugated *	Range of P
Controls	10	61.99 ± 1.64	
1	4	76.43 ± 5.48	$P < .01$
5	4	71.00 ± 3.37	$P < .01$
14	4	72.98 ± 3.08	$P < .01$
21	4	70.30 ± 1.56	$P < .01$

* Mean ± 1 standard deviation.

Table V.

Kidney Compound X-Glucuronosides - 500r

Post Irradiation (days)	Number of Determinations	% Steroid present as a Glucuronoside *	Range of P
Controls	6	37.59 ± 3.55	
1	3	27.79 ± 6.06	$.01 < P < .02$
5	3	22.18 ± 4.07	$P < .01$
13	4	24.58 ± 2.36	$P < .01$
21	4	28.60 ± 3.82	$P < .01$

* Mean ± 1 standard deviation.

Fig. 8: Kidney; Conjugation of Compound X - 500r

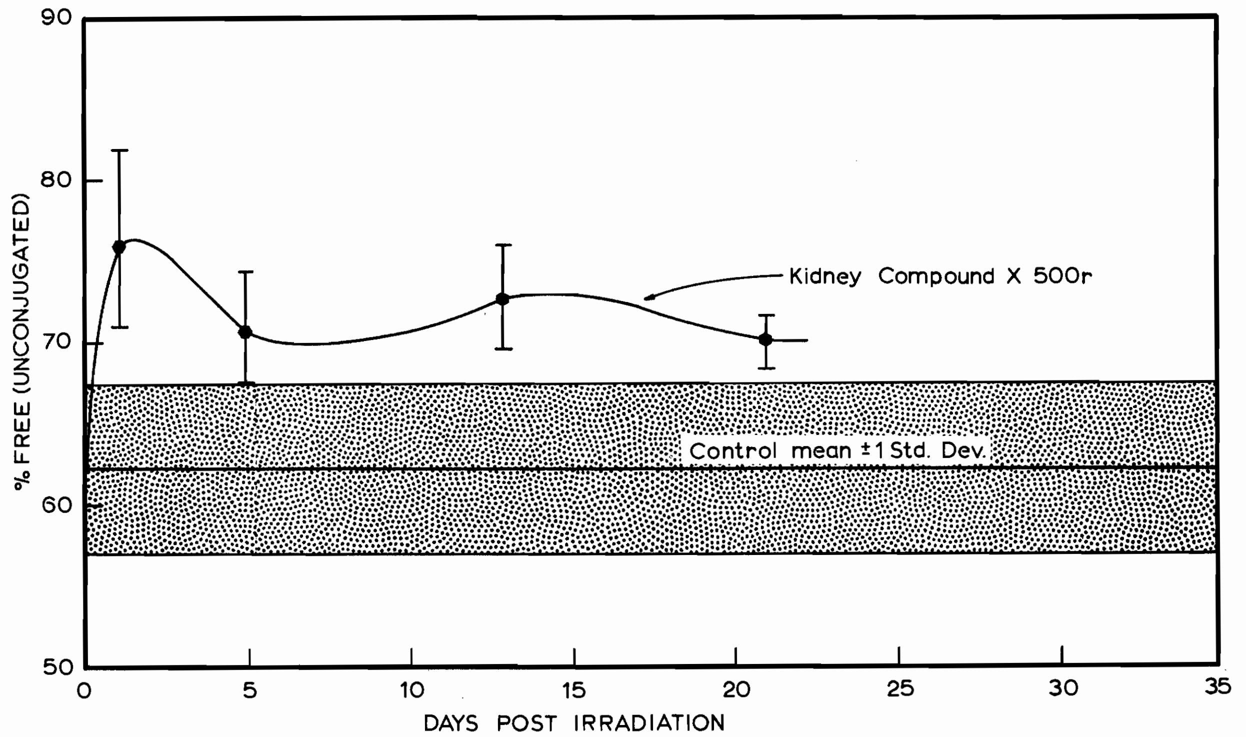
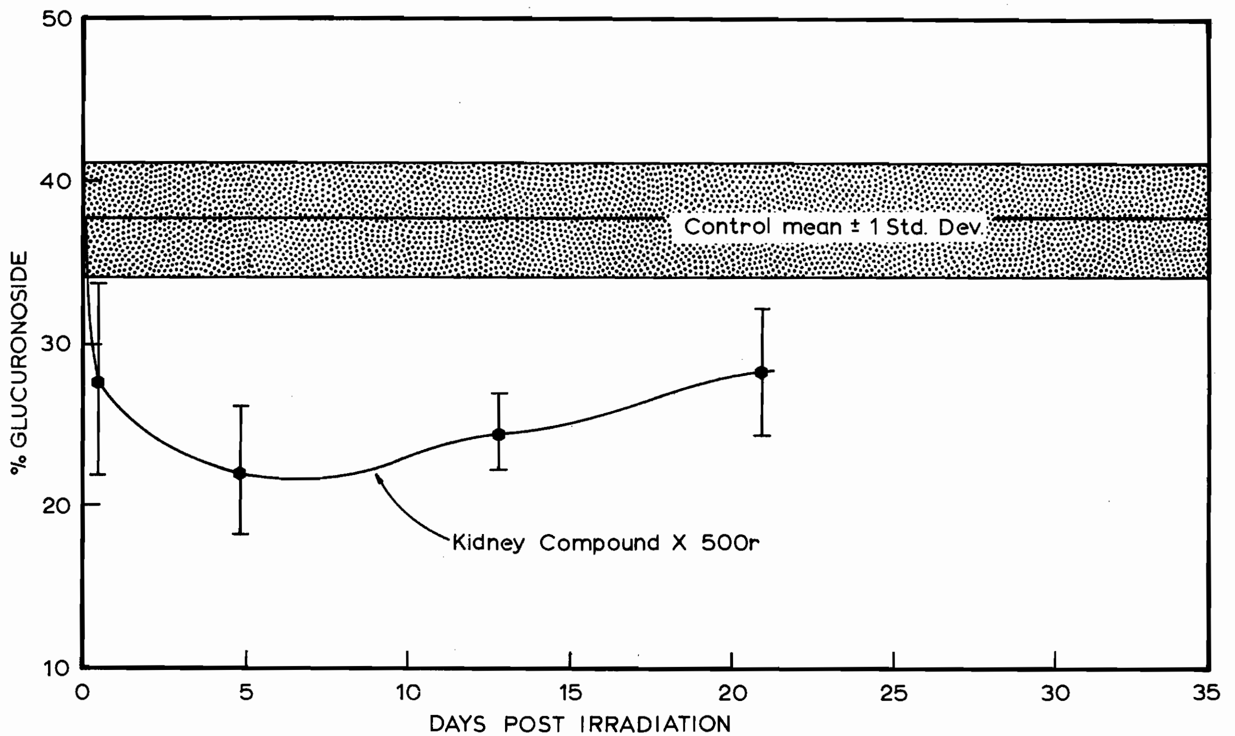


Fig. 9: Kidney; Compound X-Glucuronoside - 500r



conjugated by the kidney was so small that it was not followed during the experiment.

The conjugation of compound X by the liver following 500 r of total-body irradiation was similar to that of the kidney (Table VI). The liver, however, exhibited a more cyclic response and the time sequence was different. The liver exhibited the greatest decrease in conjugation of compound X on the 5th and 21st days post-irradiation with the greatest decrease on the 21st day (Fig. 10). The decrease on the 5th and 21st days are highly significant ($P < .01$). The decrease on the first day post-irradiation is not significant. The decrease in conjugation on the 13th day has a P value range of ($0.05 < P < .1$). This indicates a trend towards the significant decrease on the 21st day. The graph representing the amount of compound X glucuronoside formed (Fig. 11) is reciprocal to the graph of the conjugation of compound X by the liver. The greatest decrease in glucuronide formation occurs on the 5th ($P < .01$) and 21st days (Table VII) with the greatest decrease on the 21st day ($P < 0.01$). There are slight decreases from the normal mean on the first ($0.05 < P < 0.10$) post-irradiation (Fig. 11, Table VII).

The effect of x-irradiation on the conjugation of corticosterone by the liver is less well delineated than with compound X (Table VIII). The only highly significant decrease occurs on the 21st post-irradiation day ($P < .01$). Figure 12 illustrates the effect of x-rays on the conjugation of corticosterone. The slight decreases on the 5th and 13th days follow the same cyclic pattern as for compound X, although the P values for these two points indicate they are insignificant ($0.05 < P < .1$).

Table VI.

Liver Conjugation of Compound X 500r

Post Irradiation (days)	Number of Determinations	% of Steroid Unconjugated *	Range of P
Controls	10	68.19 ± 5.24	
1	4	73.05 ± 10.3	.20 < P < .30
5	4	79.65 ± 3.24	.P < .01
13	4	73.32 ± 2.92	.05 < P < .10
21	4	82.85 ± 1.62	P < .01

* Mean ± 1 standard deviation.

Table VII.

Liver Compound X-Glucuronosides - 500r

Post Irradiation (days)	Number of Determinations	% Steroid present as a Glucuronoside *	Range of P
Controls	9	8.90 ± 2.33	
1	4	6.22 ± 0.91	.05 < P < .10
5	4	4.59 ± 0.40	P < .01
13	4	6.56 ± 1.06	.05 < P < .10
21	4	3.54 ± 1.28	P < .01

* Mean ± 1 standard deviation.

Fig. 10: Liver; Conjugation of Compound X - 500r

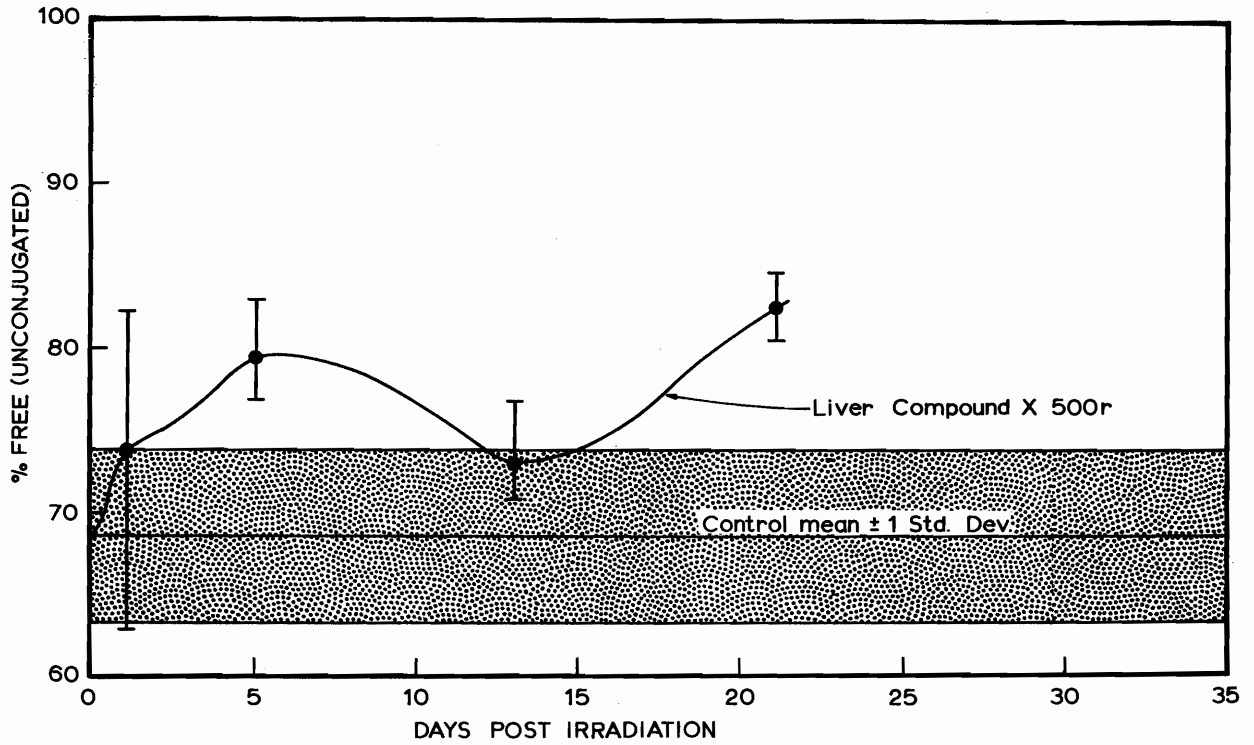


Fig. 11: Liver; Compound X-Glucuronoside - 500r

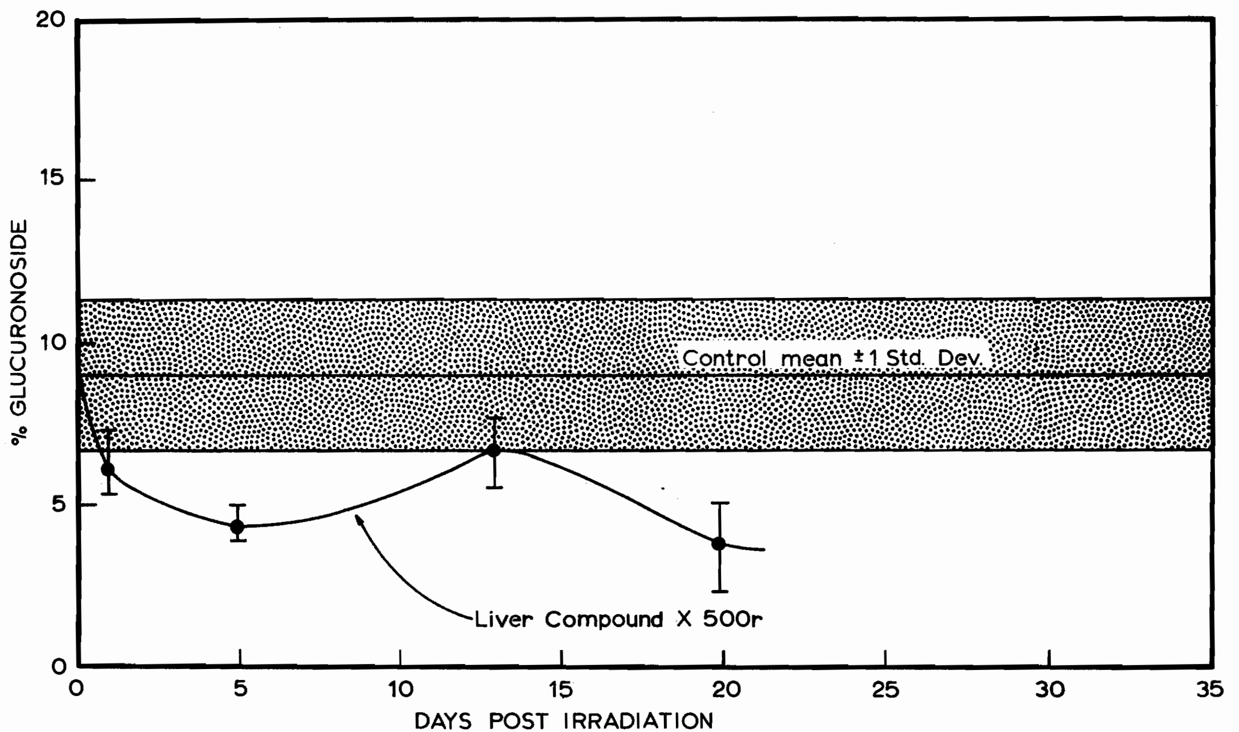


Table VIII.

Liver - Conjugation of Corticosterone - 500r

Post Irradiation (days)	Number of Determinations	% of Steroid Unconjugated	Range of P
		*	
Controls	10	75.03 ± 4.61	
1	7	74.84 ± 6.3	$.90 < P$
5	7	79.78 ± 2.38	$.05 < P < .10$
13	7	78.54 ± 2.38	$.05 < P < .10$
21	7	86.27 ± 3.06	$P < .01$

* Mean ± 1 standard deviation.

Table IX.

Liver - Corticosterone-Glucuronoside - 500r

Post Irradiation (days)	Number of Determinations	% Steroid present as a Glucuronoside	Range of P
		*	
Controls	10	6.42 ± 1.84	
1	7	5.78 ± 2.10	$.50 < P < .70$
5	6	4.22 ± 0.77	$.01 < P < .02$
13	7	4.34 ± 1.09	$.01 < P < .02$
21	7	4.27 ± 1.24	$.01 < P < .02$

* Mean ± 1 standard deviation.

Fig. 12: Liver; Conjugation of Corticosterone - 500r

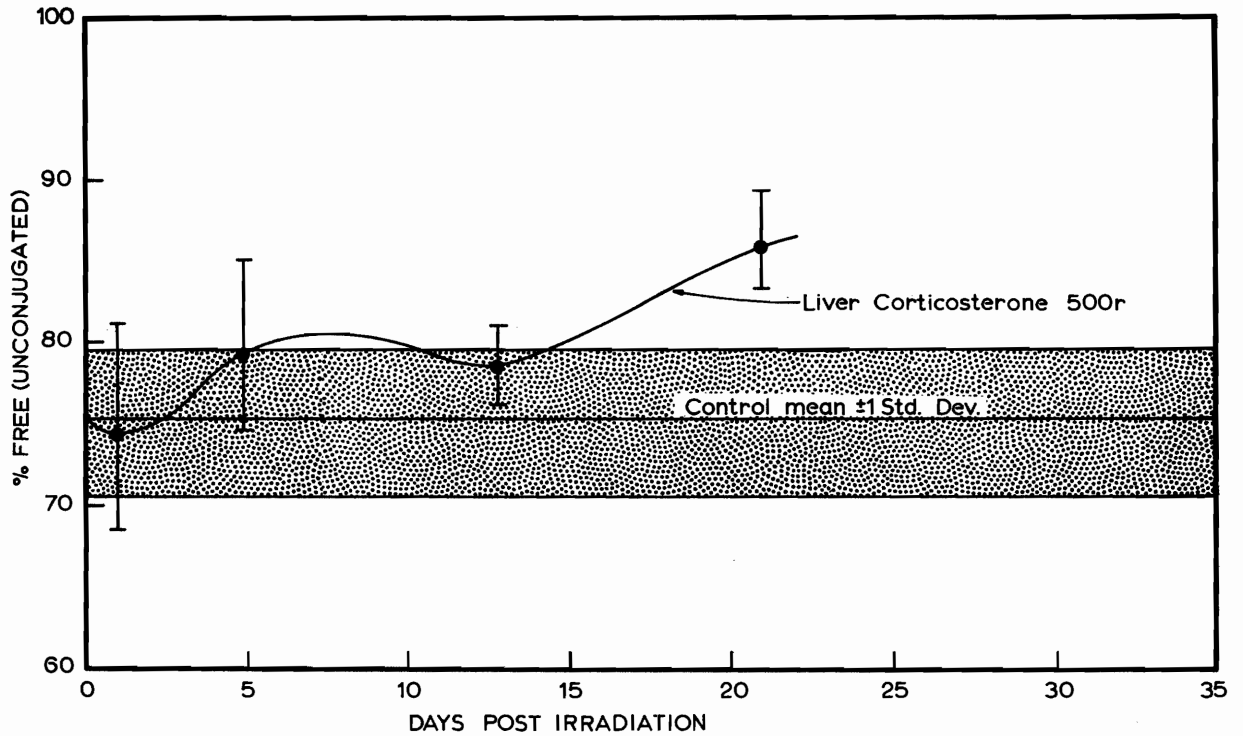
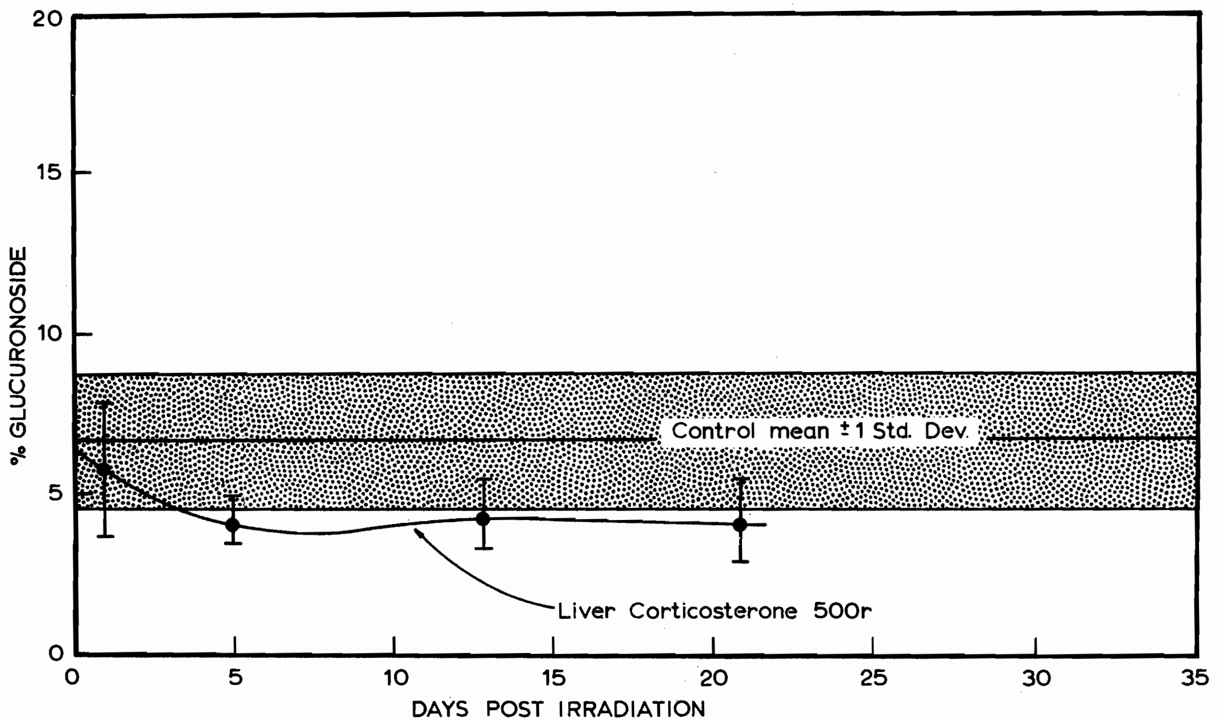


Fig. 13: Liver; Corticosterone-Glucuronoside - 500r



The amount of corticosterone conjugated as a glucuronoside is shown in Fig. 13. Table IX shows that there are significant decreases from normal on the 5th, 13th and 21st post-irradiation days. There is an insignificant decrease on the first post-irradiation day. To summarize this experiment it can be said that x-irradiation does decrease conjugation and glucuronoside synthesis in liver and kidney.

Rate study.

In this study the effect of irradiation on the rate of conjugation was studied. The lines were calculated using a least square fit. The rate equations (Table X) represent the per cent of steroid remaining free at time t (unconjugated). The intercept at t = one minute is represented by (a), and (-b) is the exponent of the t or the regression coefficient of the line. The hypothesis was established that the regression coefficient or (-b) was equal to zero (horizontal line). In all cases this hypothesis was rejected ($P < .01$). The regression coefficients of the lines are significantly different from zero.

The regression coefficients of the control line and of the irradiated line were compared statistically. No significant difference in regression was found between any of the control and irradiated lines.

The conjugation of compound X by the kidney demonstrated the greatest effect of x-ray. The rate was not altered by x-irradiation, but the line was displaced from the control (Fig. 14). If we examine the intercepts at t = 1, we can see that there is a difference between control and x-irradiated of 28%. The two lines are parallel, but the total amount of steroid conjugated at the end of three hours in the control group is greater than in the x-irradiated group.

TABLE X: Effect of x-irradiation on rate of conjugation

Tissue	Steroid	Equation of Line ($x = at^{-b}$)
Kidney	Compound X	Control $x = 95.3\ t^{-.09754}$
		Irradiated $x = 123\ t^{-.1180}$
Liver	Corticosterone	Control $x = 106\ t^{-.06736}$
		Irradiated $x = 111\ t^{-.07132}$
	Compound XIII	Control $x = 109\ t^{-.05050}$
		Irradiated $x = 108\ t^{-.03823}$

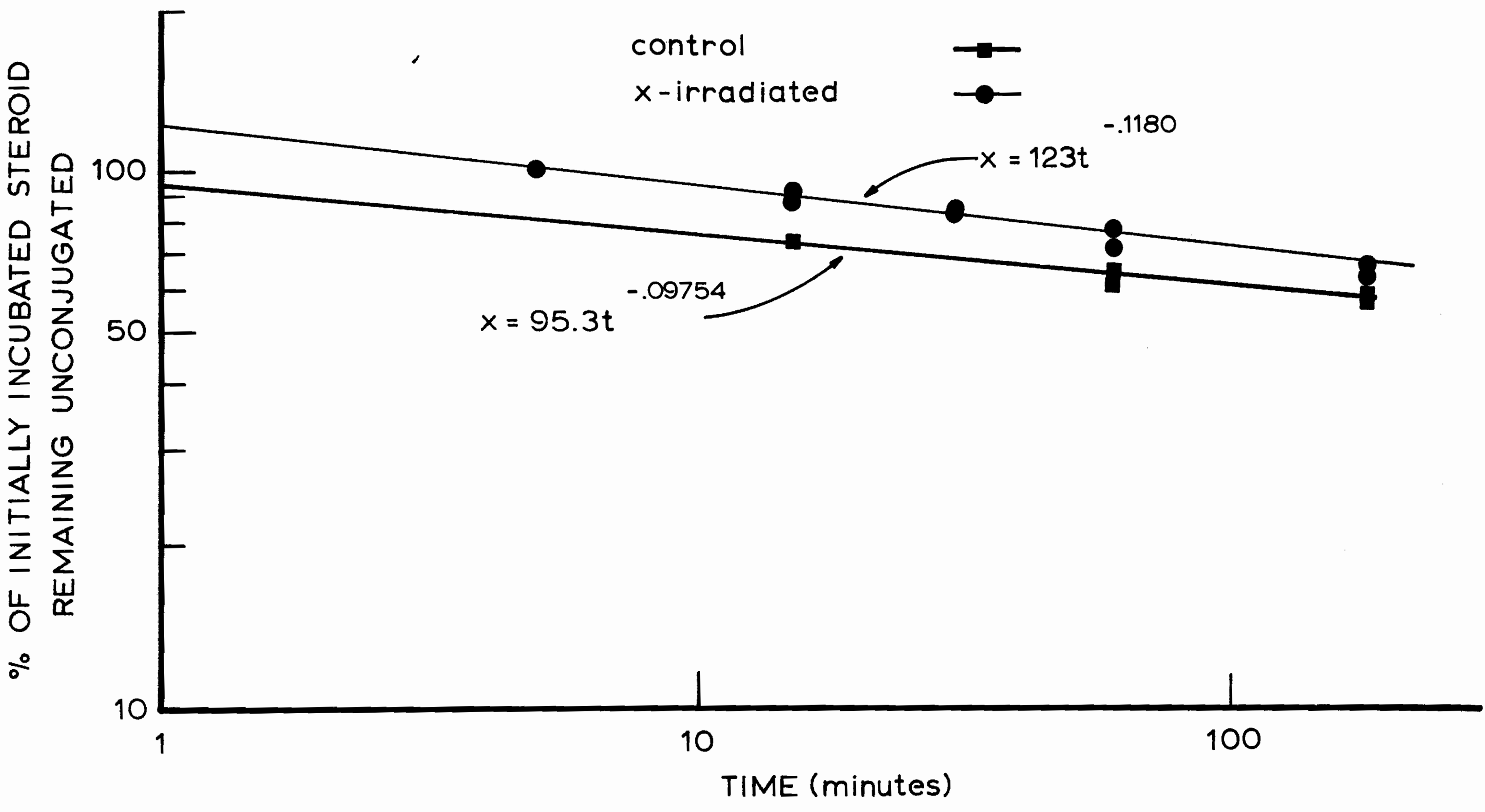


FIG. 14 KIDNEY RATE STUDY
Conjugation of Compound X

FIG. 15 LIVER RATE STUDY
Conjugation of Compound XIII

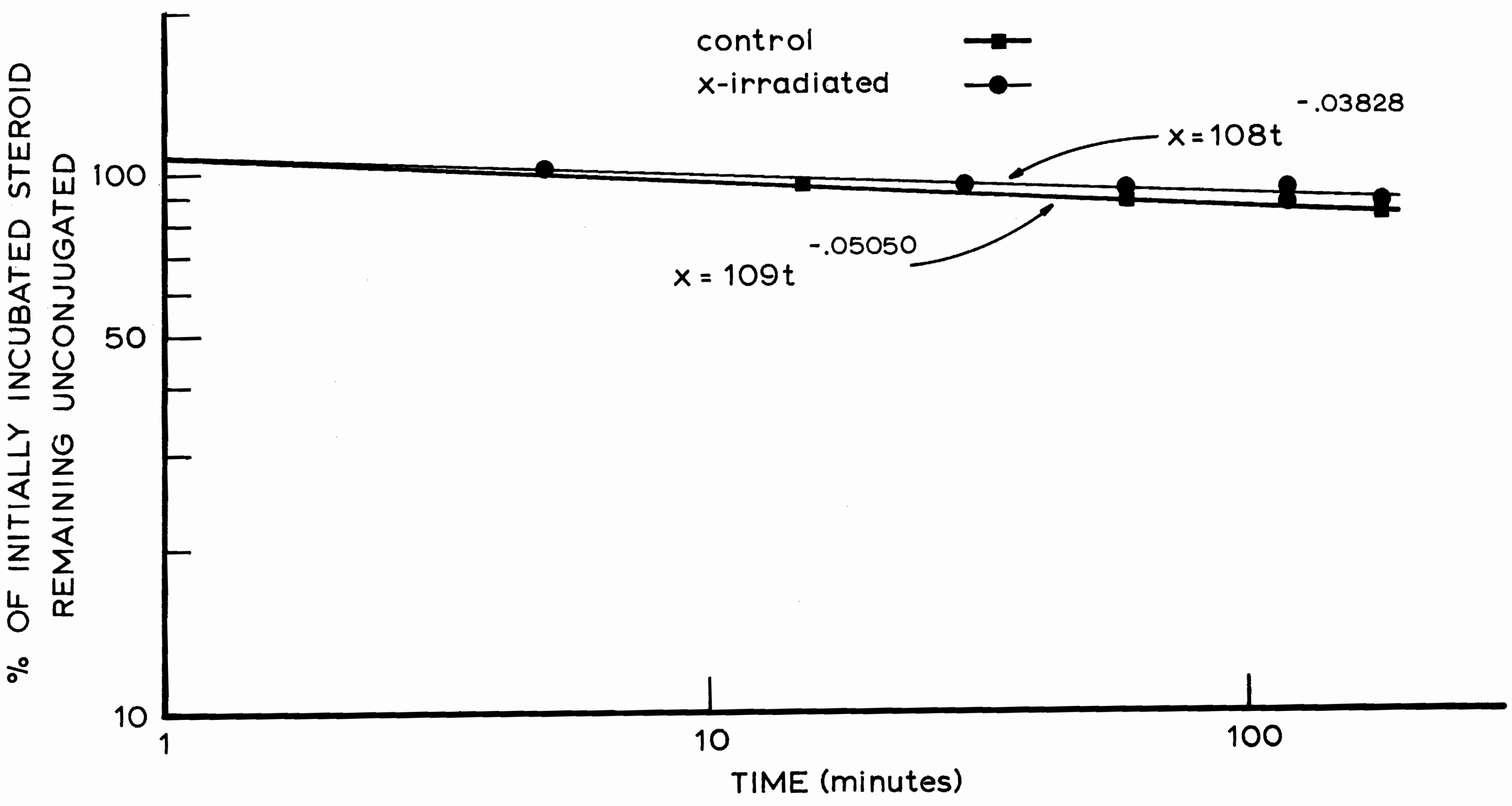
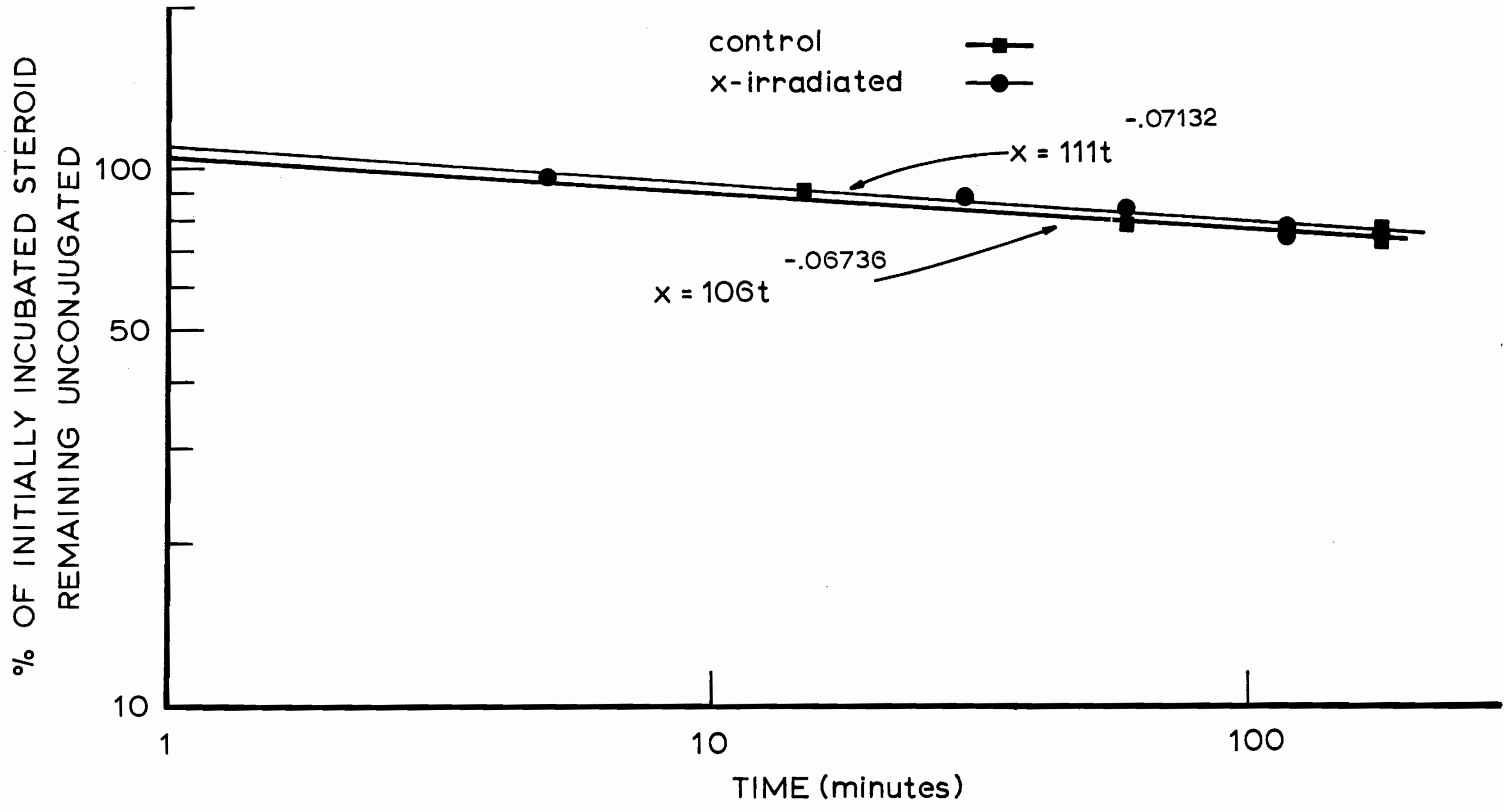


FIG. 16 LIVER RATE STUDY
Conjugation of Corticosterone



The rate study of the conjugation of compound XIII by the liver demonstrates that the control and x-irradiated lines are parallel and that there is very little difference between the amounts conjugated in three hours (Fig. 15).

X-irradiation did not affect the rate of conjugation of corticosterone nor did it alter the amount conjugated in three hours (Fig. 16). The results of the β-glucuronidase hydrolysis were erratic, and, therefore, are not included.

Effect of UDPGA on conjugation.

The data of Table XI represents an attempt to restore conjugation to normal following 500 r of whole-body x-irradiation. Kidneys and livers of mice irradiated with 500 r were taken at one day post-irradiation and incubated with uridine diphosphate glucuronic acid (UDPGA). The only apparent effect of the UDPGA on conjugation was in the kidney. Incubation of the kidney minces with UDPGA increased the amount of compound X conjugated as a glucuronoside. UDPGA had no effect on the conjugation of corticosterone by the kidney.

Conjugation by the liver showed no response to the addition of UDPGA to the incubation medium.

TABLE XI: Effect of UDPGA on Glucuronide Conjugation - 500r

Tissue	Cofactor	% Compound X Free	% Compound X Glucuronoside	% Corticosterone Free	% Corticosterone Glucuronide (average)
Kidney	UDPGA	61.60	31.30	92.94*	2.40
	None	68.91	26.77	93.46	2.23
Liver	UDPGA	58.22	Lost	65.77	Lost
	None	56.03	22.11	61.91	26.45

* Average of two measurements.

DISCUSSION

In order for a corticosteroid to be conjugated as a glucuronide it must be reduced in ring A and have a hydroxyl group at C-3 (Dorfman and Ungar, 1953; and Berliner and Dougherty, 1961). This reduction is a two-step process and requires TPNH and DPNH. The reduction of the double bond at C-4 Δ^{4-5} is a rate limiting reaction (Tomkins and Isselbacher, 1952) and requires TPNH. The reduction of the ketone at C-3 can utilize either TPNH or DPNH and is not rate limiting. Once the hydroxyl group is present at C-3, the steroid is immediately conjugated as a glucuronide or sulfate (Berliner and Dougherty, 1961). It appears that the glucuronide is the preferred form, since it is cleared from plasma by the kidney faster than the sulfate conjugate (Schneider and Lewbart, 1959). Once the steroid is conjugated it is now water soluble and can easily be excreted in the bile or urine.

The parenchymal cells of the liver comprise 60.5% of the total cells in the liver. The reticuloendothelial cells comprise 33.4% of the total cells (Daoust, 1958). Recently it has been shown that the parenchymal cell controls the conjugation of steroids, and that the reticuloendothelial cell has a large capacity to reduce ring A, but cannot form water soluble conjugates. The parenchymal cell has the ability to reduce ring A and is the only cell in the liver which can form water soluble conjugates (Berliner and Dougherty, 1960).

Histologically, the kidney has very little, if any, reticuloendothelial cells. Since the kidney has no reticuloendothelial cells, it cannot reduce significant amounts of Δ^4-3-ketosteroid and, thus,

cannot conjugate them. The kidney does contain UDPGA and glucuronosyl transferase (Stevenson and Dutton, 1962), and so when incubated with a steroid reduced in ring A, it can form steroid glucuronides. The physiological significance of this finding has not been elucidated and could bear further study. It is interesting to note that the kidney can conjugate as much or more compound X as the liver on a per gram basis.

In the liver the reticuloendothelial cell probably absorbs the steroid from the medium by pinocytosis and reduces the ring A to the tetrahydro form. At this point the reduced steroid is passed to parenchymal cells where conjugation takes place, followed by excretion into the bile or back into the blood for excretion through the kidney.

The data from the first two experiments indicate that the liver can conjugate more corticosterone than it does cortisol. It should be noted, also, that in the liver (double isotope study), the presence of corticosterone and compound X together appear to have inhibited, to a small degree, the conjugation of compound X. If we look at the single steroid study, we see that the liver conjugated 42% of the compound X and in the double steroid experiment the liver only conjugated (100 - 70.9 = 29.1) 29% of the compound X. This indicates a competitive relationship between compound X and tetrahydrocorticosterone for the available enzyme and UDPGA. It has been demonstrated that the mechanism of glucuronide formation is a general one for phenols and alcohols, and that only one enzyme is implicated (glucuronosyl transferase) (Isselbacher and Tomkins, 1955). The kidney, on the other hand, does not show this competitive inhibition of glucuronide formation, since the

amount of corticosterone reduced is so small that the amount available to be glucuronidated is insignificant and, therefore, it does not compete with compound X.

Conjugation of compound X cannot occur at any other site in the molecule other than C-3 because compound X does not have a hydroxyl group at C-21. Corticosterone, however, could be conjugated as a sulfate or glucuronide at C-21. It has been reported that large quantities of corticosterone C-21 sulfate have been found following administration of corticosterone to men (Pasqualini and Jayle, 1961). The liver contains steroid sulfokinase and, therefore, is capable of forming steroid sulfate conjugates (Nose and Lipman, 1958). Berliner et al. (1962) demonstrated that about 5-6% of the total conjugates of corticosterone formed by the perfused rat liver were sulfates. The kidney does not contain steroid sulfokinase and, therefore, it only produces glucuronide conjugates, as is indicated by the present data.

The β-glucuronidase hydrolysis does not always give reproducible results. This is illustrated by comparing the results of the pilot study to the results from the double steroid study. The total amount of compound X present as glucuronoside from the kidney in the pilot study is only 9.35%, while in the double steroid study 37.59% of the total compound X was present as a glucuronoside. The glucuronide formed from compound X by the kidney represents 92.9% of total water soluble conjugates. Further studies have substantiated this.

Although the small intestine can form glucuronides from phenolic steroids and other phenolic compounds (Hartiala, 1961), it was unable to conjugate cortisol, corticosterone or compound X.

Approximately one-half of the water soluble conjugates formed by the liver are neither sulfates nor glucuronides. The identity of these compounds is unknown. It would be of interest and, I feel, of great importance to identify these compounds.

The biological half-life of sterols and steroids seems to be dependent on the number of carbons and the number of oxygen functions in the molecule (Berliner and Dougherty, 1960). Gold (1960) postulated that the presence of hydroxyl groups at C-11 and C-17 may partially protect ring A of cortisol from metabolic degradation. Berliner et al. (1962) postulated that the presence of the 17α-hydroxy group of cortisol was hindering the conjugation of that corticosteroid. The data obtained in this laboratory have conclusively shown that the presence or absence of the 17α-hydroxy group does influence the conjugation of C-21 steroids. The liver conjugates compounds without a 17α-hydroxy group (Corticosterone and compound X) to a greater extent than it does compounds containing the 17α-hydroxy group (cortisol and compound XIII). It appears that the presence of the 17α-hydroxy group hinders the glucuronide conjugation of the C-21 steroids containing this chemical group. This hinderance of conjugation is present with reduced and Δ^4-3-ketosteroids. The kidney also forms less glucuronosides from 17α-hydroxysteroids than it does from non-17α-hydroxysteroids. This reduction in conjugation is reflected in the amount of glucuronoside formed from these compounds in the liver and in the kidney.

The inhibition of conjugation by the 17α-hydroxy group is probably a steric hinderance of glucuronosyl transferase. It is not an interference with reduction of ring A, since reduced and non-reduced

17α-hydroxysteroids are conjugated as glucuronosides to about the same extent in liver.

The fact that the mouse is primarily a corticosterone producer could explain why the mouse has the capacity to synthesize more glucuronosides from corticosterone than it does from cortisol. Also, in humans, corticosterone has a shorter biological half-life than cortisol, although humans are primarily cortisol producers (Peterson, 1959). The fact that less cortisol is inactivated per unit time than corticosterone could partially explain why cortisol has more biological activity *in vivo* than corticosterone.

X-rays have been shown to alter glucuronidation of o-aminophenol in the liver and stomach (Hartiala et al., 1958). The data presented in this paper indicate that whole-body x-irradiation affects the conjugation of steroids by liver and kidneys *in vitro* in much the same way. The reduction in conjugation could be a secondary response to the stress of irradiation. The mechanism involved is probably mediated via the pituitary-adrenal axis.

The response of the glucuronide forming system is probably due to a cyclic lack of UDPGA. The possible lack of UDPGA is evidenced by the decreased conjugation of reduced steroids as well as the decreased conjugation of Δ^4-3-ketosteroids. If the defect were in the reduction of the ring A, there would be no decrease in conjugation of reduced steroids.

The decrease in available UDPGA could be a secondary result of several different effects of ionizing radiation. It is well known that irradiation is a "stressor" and elicits a response in the pituitary-

adrenal axis (Dougherty and White, 1946). This would result in an increase in ACTH and an increase in plasma 17-hydroxy steroids (French et al., 1955). Stressed animals have been shown to have a decreased ability to conjugate steroids (Dougherty and Berliner, 1958). Berliner, Keller and Dougherty (1961) showed that administration of ACTH caused a decrease in cortisol conjugation. The increase in plasma 17-hydroxy-steroids leads to an increased gluconeogenesis followed by an increased glycogen storage in the liver (Long et al., 1940; and McKee and Brin, 1956). Uridine diphosphate glucose is a branch point in carbohydrate metabolism. Uridine diphosphate glucose is an obligatory intermediate in the synthesis of glycogen (Leloir et al., 1959), and is implicated in the synthesis of hyaluronic acid. Also, the hydrolysis of UDPGA leads to ascorbic acid in goat liver (Pogell and Leloir, 1961).

Irradiation causes an increased activity in the enzymes essential for glycogen synthesis. These enzymes are fructose-1,6-diphosphatase, phosphohexoseisomerase and phosphoglucomutase (Weber and Cantero, 1959a). Glucose metabolism is impaired following irradiation and is not a fasting phenomenon (Hill et al., 1956). The depression of glycolysis is due to a decrease in available DPN rather than to an inhbition of a glycolytic enzyme (Maass and Schubert, 1958). This block in glycolysis is caused by a decrease in oxidized DPN, which blocks the triose phosphate dehydrogenase reaction. This block of glycolysis and impairment of glucose utilization in glycolysis leads to a slight hyperglycemia. Irradiation also causes an increase in the pentose phosphate pathway which is due to increased glucose-6-phosphate and 6-phosphogluconate dehydrogenase activity (Weber and Cantero, 1959b; and Kochetov, 1959).

The increase in glycogen synthesis and the increased activity of the pentose phosphate pathway following irradiation could lead to a decreased production of UDPGA.

This point is well demonstrated in the study with corticosterone in liver. Whole-body x-irradiation did not cause a significant decrease in conjugation of corticosterone by the liver at 1, 5 and 13 days post-irradiation. It did, however, cause a significant decrease in the amount of corticosterone-glucuronoside formed at 5, 13 and 21 days post-irradiation. This apparent difference can be attributed to a decreased availability of UDPGA. The non-significant decrease in conjugation is possibly due to the conjugation of corticosterone as a sulfate or other conjugate.

Krebs (1958) has pointed out that certain pacemaker reactions in the body are influenced markedly by availability of coenzymes. The oxidation of one mole of UDPG to UDPGA requires two moles of oxidized DPN. The availability of oxidized DPN could very well be rate limiting in this reaction. Cortisol is known to inhibit oxidative phosphorylation (Gallagher, 1958). Cortisol also limits the oxidation of DPNH by inhibiting the DPNH-cytochrome-C reductase reaction, producing a deficiency in oxidized DPN. This lack of oxidized DPN explains the inhibitory effect of steroids on glycolysis. The depression of glycolysis in Ehrlich ascites cells following irradiation is due to lack of DPN (Maass and Schubert, 1958). It should be noted that DPN controls the conversion of pyruvate to acetyl CoA and CO_2 (Yielding et al., 1960).

The reduction in oxidative phosphorylation is well known and is caused by relatively small doses (Potter and Betheel, 1952; and Van

Bekkum et al., 1954). Maximum inhibition occurred in spleen and thymus at 48 hours following irradiation and could not be alleviated by addition of glucose, pyruvate or Krebs cycle intermediates (Sullivan and Dubois, 1955). Benjamin and Yost (1960) demonstrated that the reduction in liver oxidative phosphorylation was mediated by the pituitary-adrenal axis and the uncoupling in the spleen was due to increased thyroid activity.

The reduction in oxidative phosphorylation leads to a decrease in ATP production which prevents the oxidation of reduced coenzymes.

Thus, the reduction in glucuronidation, biochemically speaking, can be due to (1) increased utilization of UDPG to glycogen and, therefore, a decrease in the amount available to be oxidized to UDPGA; and (2) a reduction in the amount of oxidized DPN available to oxidize the UDPG to UDPGA.

The addition of UDPGA to the kidney incubation medium caused an increase in the conjugation of compound X. This increase was reflected in the amount of compound X glucuronoside formed. This evidence would indicate that in the kidney the decrease in conjugation caused by 500 r of whole-body x-irradiation is mediated by a lack of UDPGA. This is supporting evidence for the hypothesis presented here. In the liver, however, UDPGA apparently had no effect on conjugation or glucuronoside synthesis. This is attributed to the presence of alternate pathways of conjugation in the liver which at one day post-irradiation are able to compensate for a lack of UDPGA.

If we examine the reduction of glucuronide formation in terms of general homeostatic mechanisms, we can relate the cyclic variations of

the responses to changes in adrenal-liver-pituitary responses.

The muscle work test has been used to evaluate adrenal function following irradiation. It was found that the adrenal output increased at 24 and 48 hours following irradiation and returned to normal by 72 hours. Seven days after irradiation, adrenal output fell below normal and the animals began to die. ACTH could not restore adrenal function to normal (Tonkikh, 1958). Irradiation to exposed calf adrenals caused a decrease in amount of corticosteroid produced. This decrease was ascribed to lack of hydroxylation at C-11, C-17 and C-21. These hydroxylations are necessary for production of cortisol and corticosterone (Ungar et al., 1955; and Rosenfeld et al., 1955). In dogs with internally deposited radionuclides, Berliner et al. (1961) found that the adrenal cortisol biosynthesis was decreased and apparently there was a lack of 11, 17 and 21-hydroxylations. The decrease in cortisol output by the adrenal could be returned to control values by the addition of TPNH, which indicates that irradiation does not affect the enzyme systems but decreases the amount or availability of reudced coenzymes essential for hydroxylation.

The liver has been postulated to control the blood level of ACTH and corticosteroids by feedback mechanism. The reduction of ring A and conjugation of steroids decrease the blood levels which, in turn, causes an increase in ACTH production. The increased ACTH level stimulates the adrenal to produce more steroids. The increased corticosteroids feedsback to the pituitary and decreases ACTH level (Urquhart et al., 1958). The liver controls the level of steroids in the blood so it can indirectly control ACTH production by increasing or decreasing

the rate of corticosteroid inactivation. Reid (Brook Lodge Symposium) showed that increased levels of ACTH caused a decrease in uridine nucleotides including UDPGA. Perhaps the cyclic decrease in conjugation is due to a decreased steroid production which leads to an elevated ACTH level. This increased ACTH level would decrease available UDPGA and thus produce the decreased glucuronide formation. The cyclic response curve could be explained by the time lag between ACTH production and the response of the adrenal and vice versa.

The rate study indicates that the rates of conjugation are the same but that the total amount of steroid inactivated at the end of three hours in the kidney is greater in the control animals than in the x-irradiated animals. The fact that the regression coefficients are the same would indicate that x-irradiation does not affect the enzyme itself and, therefore, is not rate limiting, but rather that the availability of the substrates (steroid and UDPGA) is rate limiting. Since the same amount of steroid is present in both control and experimental animals, the only other variable is availability of UDPGA.

The liver did not demonstrate a difference in rate of conjugation of corticosterone or compound XIII. There was, also, very little difference in per cent conjugated per unit time, although the control groups are consistently below the x-irradiated groups. In the previous study, the amount of corticosterone conjugated five days following irradiation was not significantly different from the controls. Compound XIII is not conjugated by the liver as well as compound X. Compound X was used in the previous irradiation study and was significantly different from the controls at five days post-irradiation. We were unable to use compound X for the rate study because the amount

we had available was limited and was used to better advantage with the kidney. We were unable to obtain any more compound X from the National Institutes of Health or from any commercial source. This study should be extended and rates of conjugation re-examined on the 21st day post-irradiation where there are large differences between the irradiated group and the controls. The data obtained in the rate study should be analyzed kinetically to determine reaction order and rate constants.

The conjugation of steroids by the liver, kidney and possibly other organs may not be a simple detoxification process to eliminate the steroid from the body. Reduction of the steroids in ring A, reduction at C-20 and oxidation of C-11 decrease the biological activity of the steroid. Berliner and Dougherty (1961) call these oxidations and reductions "biotransformations." These biotransformations may very well be associated with the steroid's mechanism of action. For example, the reduction of ring A generates oxidized TPN and DPN (Tomkins, 1959b). The reduction of the ketone at C-20 also generates oxidized coenzymes. The production of oxidized coenzymes will increase reaction rates dependent on oxidized coenzymes (Yielding and Tomkins, 1960). The inactivation of cortisol involves the oxidation of the hydroxyl group at C-11 to a ketone generating reduced TPNH (Sweat and Bryson, 1960). The reduction of the double bond in deoxycorticosterone produces a steroid anesthetic which is sold commercially (P'an et al., 1955). The reduction of androgens produces the 5α-isomer, androsterone, and 5β-isomer etiocholanolone. Androsterone is a biologically active blood cholesterol lowering agent (Hellman et al., 1959). Etiocholanolone is a pyrogenic steroid causing an elevation in body temperature

(Kappas et al., 1960). Pregnandiol, the major metabolite of progesterone is also pyrogenic (Berliner and Dougherty, 1961).

The conjugation and inactivation of hormones needs to be re-examined in terms of what these alterations mean biologically.

SUMMARY

Kidney slices from male CBA mice are able to conjugate ring A reduced 3-hydroxy steroids as glucuronides. Glucuronides are formed from pregnane-3α-ol-11,20-dione and pregnane-3α,17α-diol-11,20-dione. The kidney did not form glucuronides from cortisol or corticosterone since it was unable to reduce ring A. Most of the water soluble conjugates formed by the kidney are glucuronides.

The liver can reduce ring A and can form glucuronide conjugates from cortisol and corticosterone. The liver can form glucuronides from pregnane-3α-ol-11,20-dione and pregnane-3α,17α-diol-11,20-dione. Glucuronides comprise about one-half of the total water soluble conjugates.

The presence of the 17α-hydroxy group hinders the conjugation of C-21 steroids containing this chemical grouping in liver and kidney. This was shown to be an interference with glucuronosyl transferase causing a decreased glucuronide formation with 17-hydroxycorticosteroids. This effect was exhibited in the liver and the kidney.

In vitro conjugation of compound X as a glucuronide by the kidney was significantly decreased on the 1st, 5th, 13th and 21st days following 500 r of whole-body irradiation. Irradiation significantly decreased the *in vitro* conjugation of compound X by the liver on the 5th and 21st days post-irradiation. The decrease in conjugation on the 1st day post-irradiation was non-significant and the decrease on the 13th day post-irradiation has a P value range of $(0.05 < P < .1)$. This decrease in conjugation was reflected in the amount of steroid released

by β-glucuronidase hydrolysis. The *in vitro* conjugation of corticosterone by the liver was significantly decreased on the 21st day after irradiation with 500 r. The amount of glucuronide formed from corticosterone on the 21st day was also decreased. There was no significant decrease in conjugation or glucuronide formation with corticosterone on the 1st, 5th or 13th day post-irradiation.

X-irradiation did not affect the rate of conjugation of compound X in the kidney. The control animals conjugated more compound X at the end of three hours than irradiated animals. The rates of conjugation of compound XIII and corticosterone in the liver were not altered by irradiation.

The total amount of compound X and corticosterone conjugated by the irradiated animals' livers was slightly less than the total amount conjugated by the control animals' livers.

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