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CORTICOSTEROID EFFECTS ON FETAL METABOLISM AND STUDIES

ON STEROID-RECEPTOR COMPLEXES IN PLACENTA OF MICE

by

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ABSTRACT

Teratogenic and lethal effects have been reported from
treatment of pregnant animals with both synthetic and natural
corticosteroids, and were also observed in this investigation.
Injection of dexamethasone into pregnant mice on gestational
day 18 resulted in decreasing net transfer of labeled glucose
from mother to fetus with increasing steroid dosage. Inhibi-
tion was also demonstrable when injections were made into the
fetus in utero. It was concluded that the deleterious effects
of dexamethasone on the survival of mouse fetuses might be
attributable to this action.

All steroid systems studied to date appear to involve a
similar sequence of interactions that precede the different
biochemical effects that are observed in target tissues. Bind-
ing of the steroid to a specific cytoplasmic macromolecule and
translocation of this complex to the nucleus are required in
the mechanism of steroid action. The placenta, being the site
of maternal-fetal transmission of nutrients, was therefore
examined for the presence of intracellular steroid receptors.

Using Sephadex chromatography, the in vitro binding of
radioactive steroids to components in mouse placental nuclei
and cytoplasm was investigated. Specificity was indicated in
competition studies using excess unlabeled competing steroids.
This specificity was confirmed since only the active glucocor-
ticoids formed complexes which demonstrated the ability to
translocate to the nucleus. The binding properties of the
cytoplasmic steroid-receptor interaction were also studied.

From the time course of binding the complex was shown to possess more stability at 0°C than at 37°C, and the distribution of receptors in the cytosol appeared to be homogeneous. The cytoplasmic complex showed lability when heat denaturation and proteolytic digestion were investigated, but did not appear to be affected by nucleases or the sulfhydryl reagents. Kinetic analysis of the binding revealed the presence of high affinity specific binding sites with a dissociation constant of 17.5 nM and a receptor site concentration of 0.26 pmoles/mg protein. Using sucrose density gradient centrifugation, the molecular weight of the cytoplasmic corticosterone-receptor complex was estimated to be approximately 55,800.

This investigation has revealed the existence of glucocorticoid receptors in a target tissue in which the regulation of glucose availability to the fetus may be mediated by corticosteroids. In view of the critical role that glucose plays in the nutritional status of the fetus; and the preeminent influence that glucocorticoids have on glucose uptake in other target tissues, it is then very plausible that the corticosteroids have a regulatory function in fetal growth and viability.

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ABBREVIATIONS USED

AMP	adenosine-5'-monophosphate
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dpm	disintegrations per minute
EDTA	(ethylenedinitrilo)-tetraacetic acid, tetrasodium salt
i.p.	intraperitoneal
KEP	Krebs-Eggleston phosphate buffer (see Appendix I)
POPOP	1,4-Bis(2-(5-phenyloxazolyl))-benzene
PPO	2,5-diphenyloxazole
RNA	ribonucleic acid
RNAse	ribonuclease
s.c.	subcutaneous
TCA	trichloroacetic acid
TLC	thin layer chromatography
TRIS	2-amino-2-hydroxymethyl-1,3-propanediol
uCi	microcurie
UDP	uridine-5'-pyrophosphate
ug	microgram
ul	microliter
uM	micromolar

INTRODUCTION

The metabolic effects of the glucocorticoid hormones have been examined actively over the past forty years. The pioneer work of Britton and Silvette in 1932 arose from the observation that adrenocortical extracts had effects on carbohydrate metabolism and therefore a possible role in the regulation of glucose metabolism (1). Even before this, in 1927, Cori and Cori had demonstrated that adrenalectomized rats and mice failed to maintain adequate glycogen stores (2). However, it was not until the extensive studies of Long, Katzin, and Fry in 1940, characterizing the fall in blood sugar and liver glycogen of fasted, adrenalectomized rats and mice (3) that the adrenocortical hormones were generally recognized as having a profound effect on carbohydrate and protein metabolism as well as on salt and water metabolism. The separation of the extract from the adrenal gland into different components with glucocorticoid and mineralocorticoid activities arose with the identification and synthesis of the different steroids. This in turn has led to the elucidation of the basic aspects of steroid action.

The glucocorticoids are generally considered to be catabolic hormones since their most pronounced metabolic effect in the animal as a whole results in wasting of muscle tissue with an accompanying negative nitrogen balance. On the whole, the actions of glucocorticoids in carbohydrate, protein, and lipid metabolism result in glucose 'sparing', as evident from the

increased catabolism by the peripheral tissues as well as decreased glucose uptake. This in turn leads to increased blood glucose and amino acid levels, thereby increasing gluconeogenesis from the available amino acids mainly by the liver (4) but also by the kidney (5). Adipose tissue undergoes increased lipolysis and decreased lipogenesis (6), thus increasing the blood concentrations of glycerol and fatty acids (7). The glycerol provides an additional substrate for hepatic gluconeogenesis while the free fatty acids supply an alternate energy supply (8) and at the same time their high concentration may inhibit the key glycolytic enzymes of the liver (9), allowing gluconeogenesis to predominate in that organ. In normal animals many of these effects are not evident due to the compensatory actions of insulin which is secreted in response to the hyperglycemia. Only by prolonged or excessive administration of the glucocorticoids can many of these effects be demonstrated in vivo.

Paradoxically, along with these catabolic effects, the glucocorticoids also simultaneously exert an anabolic action. The liver, in contrast to the peripheral tissues, responds to the hormone with dramatic stimulation of several major hepatic metabolic processes. The anabolic effects of glucocorticoids on the liver result in glycogen deposition, increased gluconeogenesis, increased uptake of amino acids, and increased synthesis of RNA and protein. Urea and ketone body production are also increased by the steroids but probably represent secondary changes in the liver (10) and are not directly affected by the

hormones. Glucocorticoids thus appear to have two distinct metabolic effects: (a) an anabolic action on the liver leading to deposition of glycogen as well as formation of RNA and protein; and (b) a decreased glucose and amino acid uptake by extrahepatic tissues, as well as a catabolic breakdown of protein, lipid, RNA, and DNA by these tissues.

Glucocorticoid Effects on Liver Metabolism

The gross hepatic effects of glucocorticoid administration result in hypertrophy of the organ since total weight increases whereas the DNA levels remain essentially constant (10). The general anabolic effect on the liver is most readily manifested by the increase in glycogen deposition, although increased RNA and protein are also evident. These metabolic effects generally require 2 to 4 hours to be demonstrated in vivo with adrenalectomized, fasted animals (11). Well before these effects, about 80 to 100 min after glucocorticoid administration, there is observed an increase in blood glucose (12). This increase may result from a decrease in peripheral glucose utilization or an increase in hepatic glucose production.

Although an increase in the rate of gluconeogenesis in liver slices has been reported (13), in vivo stimulation by glucocorticoids has been difficult to demonstrate using isolated perfused liver (14, 15). However, when 10 mM alanine was used as substrate (20 to 30 times the circulating physiological level), impaired incorporation of alanine into glucose was demonstrated using perfused livers from adrenalectomized rats (16). This was restored to normal by the addition of a

glucocorticoid to the perfusion medium. Since no effect was
seen using physiological concentrations of amino acids (15),
it seems that hepatic gluconeogenesis is unaffected by gluco-
corticoids if the amino acid level is within the range that is
normally handled by the liver. However, the steroids will in-
crease the maximal capacity for hepatic gluconeogenesis under
conditions where the amino acid load is high (13).

An early effect of the glucocorticoids is the rise in
hepatic amino acid levels as a consequence of their release from
the peripheral tissues, especially muscle (13, 17). The uptake
of these amino acids by the liver may also be steroid-enhanced
as suggested by the increased hepatic uptake of the nonmetabo-
lized amino acid, 2-aminoisobutyric acid (18), after glucocor-
ticoid administration. The high concentrations of amino acids
in the liver become ready substrates for steroid-induced gluco-
neogenesis under these conditions. Since alanine comprises
more than 80% of the total amino acid N_2 released by muscle (13),
the rise in hepatic amino acid levels may play more than just a
precursor role. Alanine has been reported to inhibit liver
pyruvate kinase activity (19). This inhibition would prevent
the flow of substrate from phosphoenol-pyruvate to pyruvate,
thus promoting gluconeogenesis.

While the process of gluconeogenesis itself has been dif-
ficult to demonstrate, the activity of any rate-limiting enzyme
in the glucogenic sequence is increased in activity in response
to glucocorticoid treatment (17). This effect is most likely a
result of induction of the gluconeogenic enzymes, a fact borne

out by the observation that the stimulation of gluconeogenesis induced by steroids in liver slices from adrenalectomized rats is blocked by actinomycin D (20). Control of phosphoenolpyruvate carboxykinase synthesis (8) is probably the major mechanism in the longer-term adaptation of gluconeogenesis to glucocorticoid administration. Cortisol has been shown to increase hepatic levels of phosphoenolpyruvate while leaving malate levels unchanged (20). The activities of pyruvate carboxylase (8), glucose-6-phosphatase (21), and fructose-1, 6-diphosphatase (22) have all been reported to be increased after glucocorticoid administration. Both pyruvate carboxylase and glucose-6-phosphatase may be involved in the steroid effect although the stimulation of the latter enzyme may not be due to stimulation of synthesis since the steroid effect is not blocked by actinomycin D (8). The importance of the increase in fructose-1, 6-diphosphatase to the steroid effect is dubious since the enzyme increase follows, not precedes, the increased rate of gluconeogenesis (23). In addition, gluconeogenesis from fructose is not impaired in liver slices from adrenalectomized rats nor are fructose-1, 6-diphosphate levels increased (8).

Along with the early rise in hepatic amino acid levels, there is also an early rise in the free fatty acid levels of the liver (7, 23). This results from the permissive lipolytic action of the glucocorticoids on adipose tissue, causing the release of free fatty acid and glycerol (6, 7). The glycerol supplies additional substrate for gluconeogenesis while the elevated hepatic fatty acid level results in inhibition of glyco-

lytic enzymes, chiefly pyruvate kinase (9). Inhibition of the hexose monophosphate pathway and the Krebs cycle enzymes, isocitrate dehydrogenase and fumarase (23) by the high fatty acid level blocks other pathways of glucose oxidation in the liver, allowing maximum efficiency for the conversion of lactate and pyruvate to glucose. The energy requirements for the liver must now be obtained from other sources, namely, the partial oxidation of long-chain fatty acids which then result in the accumulation of ketone bodies.

In order for gluconeogenesis from amino acids to occur rapidly the enzymes involved in transamination and deamination must also be increased, if they are rate-limiting (17). Glutamic-pyruvic transaminase (24), tyrosine aminotransferase (25), and tryptophan pyrrolase (26) activities are among the many transaminase enzymes increased and provide one of the earliest and most sensitive liver responses to glucocorticoids. The enzymes involved in urea biosynthesis also increase (10) but this is most likely a delayed response to the increased mobilization of amino acids to the liver since their increase occurs some time later. Studies using immunochemical determination of enzyme activity (27), supported by experiments with puromycin (28), have demonstrated that hormonal induction of the transaminase enzymes also involve accelerated rates of synthesis and elevated levels of enzyme protein. The hepatic enzyme induction by the glucocorticoids does not occur in rodents pretreated with actinomycin D (10), again indicating that this is a process also dependent upon continued RNA synthesis.

Even before it was known that glucocorticoid-mediated enzyme induction was sensitive to actinomycin D, it was found that these steroids enhanced the incorporation of inorganic ^{32}P into hepatic RNA (29). This enhancement occurred in all subcellular organelles, and was reflected in an increased specific activity for transfer RNA, ribosomal RNA, and DNA-like RNA. Using ^{14}C-uridine and ^{3}H-guanine as precursors, Feigelson (10) found an increased rate of synthesis of a uracil-rich RNA species within 10 min after the administration of glucocorticoids in vivo, followed by an increased rate of synthesis of a guanine-rich species several hours later. The increased uracil-rich RNA synthesis precedes the induction of tryptophan pyrrolase and tyrosine aminotransferase (10), and may be the template specifying the synthesis of the inducible enzymes. The guanine-rich RNA species synthesized later most likely represents an increased rate of synthesis of ribosomal RNA. The increase in hepatic RNA synthesis after glucocorticoid treatment may result from an increase in RNA polymerase activity. Several groups (30, 31) have reported a rapid rise in hepatic RNA polymerase activity after in vivo administration of glucocorticoids.

Although the RNA and protein content of the liver are elevated approximately 30% and 20%, respectively, within 12 hours of cortisone treatment (10), the 30% increment in hepatic dry weight that occurs in this period is due mainly to the increased glycogen content. Steroid-stimulated livers have a decreased concentration of glucose-6-phosphate and uridine diphosphate glucose (17), suggesting an effect on the activity of glycogen

synthetase by the glucocorticoids. Most likely there is an
activation due to increased precursor supplies as well as a
direct stimulation of the conversion of glycogen synthetase D
(inactive) to I (active). This activation, which may result
in a 200% increase in liver glycogen content within 4 hours (10)
is apparently due to an activation of existing enzyme since
actinomycin D generally has no effect on the process (17). The
stimulatory effect on glycogen synthesis may not be a direct
effect of glucocorticoid but may be mediated by insulin which
is stimulated by the high blood glucose levels. The glucocor-
ticoid-induced hyperglycemia could also result in glucose acti-
vation of glycogen synthetase (32) and inactivation of phospho-
rylase a.

Glucocorticoid Effects on Peripheral Tissue

Interest in the peripheral action of glucocorticoids arose
with the discovery that cortisol injection into fasted, adrenal-
ectomized rats produced an increase in blood glucose well before
demonstrable effects on liver glycogen (12) or gluconeogenesis.
Decreased peripheral glucose uptake would result in this effect
and experiments to locate the sites of this decrease were met
with success using adipose tissue (33, 34), skin (35), and thy-
mus (36, 37). Along with the decreased glucose utilization by
these tissues there is also increased catabolism of protein
from muscle and lipid from adipose tissue, leading to elevated
hepatic levels of amino acids and fatty acids. In general,
nucleic acid metabolism is also depressed by the effect of the
glucocorticoids on the peripheral tissues.

Experiments to demonstrate decreased glucose uptake by
muscle have been contradictory (38). This stems from the low
normal utilization of glucose by muscle (17), especially if
free fatty acid is available as fuel. Several hours after
steroid administration in vivo it is possible to show decreased
glucose uptake by rat hemidiaphragm or heart in vitro (39),
with a specific effect on the initial phosphorylation. This
may not be a direct effect of the glucocorticoid since the rise
in free fatty acid concentration could serve to inhibit the util-
ization of glucose by muscle (17). The increased egress of
amino acids into the circulation from muscle, even in eviscer-
ated preparations (32), is one of the first effects of glucocor-
ticoid administration in vivo. Also demonstrable is decreased
incorporation of labeled amino acids into muscle isolated from
animals pretreated with glucocorticoids. Thus, the effects of
glucocorticoids on muscle are quite likely both catabolic and
anti-anabolic, resulting mainly in an elevated supply of amino
acids to the liver.

The glucocorticoid-induced inhibition of glucose uptake by
adipose tissue results in a decrease in glycerol phosphate and
fatty acid production and may partly explain the anti-lipogenic
effect since there is no convincing evidence that glucocorticoids
directly influence lipogenesis. What has been clearly establish-
ed is that the glucocorticoids have a permissive role in fatty
acid mobilization by lipolytic agents (6) which, if unopposed
by insulin, result in release of free fatty acids and glycerol
from adipose tissue. Fat mobilization by the catecholamines

and lipolytic peptide hormones is believed to involve cyclic
AMP activation of a cellular hormone-sensitive lipase (6). One
attractive possibility for the involvement of glucocorticoids
is that the steroids inhibit cyclic AMP phosphodiesterase (32),
thus allowing the lipolytic effect to be manifested.

The work of Munck and his collaborators showed that thymus
tissue exhibited the same behavior as other peripheral tissues
when exposed to glucocorticoids. After steroid injection there
is decreased incorporation of glucose into thymuses of rats and
decreased glucose uptake by incubated thymus cells (38). An _in_
vitro decrease in both protein synthesis (37) and nucleic acid
metabolism (40) was also observed. The inhibitory effects on
glucose uptake were thought by Munck to be responsible for the
catabolic effects of the glucocorticoids (38). This was indic-
ated by the observation that effects on protein synthesis and
nucleic acid metabolism occur much more slowly than the decrease
in glucose uptake (37), and cytological indications of catabol-
ism take even longer to appear (41). Experimental support for
this hypothesis has come from the work of Young, which showed
an absolute requirement for glucose before the effects of cor-
tisol on protein synthesis and nucleic acid metabolism take
place (42). Further work utilizing lymphosarcoma cells has in-
dicated that the ultimate catabolic action of the glucocorti-
coids leads to inhibition of growth and finally cytolysis.
The general conclusion that emerges is that for lymphoid tis-
sue, and also for adipose tissue and skin, inhibition of glu-
cose uptake is probably an essential first step in the catabol-
ic actions of the glucocorticoids.

Carbohydrate Metabolism in the Fetus and Placenta

The fetus during intrauterine life is dependent upon maternal sources for all of the nutrients and energy supply necessary for growth and differentiation. Measurements of the rate of glucose uptake in fetal lambs suggest that it is fast enough to account for the fetal oxygen consumption (43) and may thus be the principal metabolic fuel of the fetus. Since carbohydrates are also transported, stored, and synthesized by the placenta, each of these functions will have an effect on the availability of carbohydrate to the fetus.

Placental transport of glucose has been characterized as occurring by 'facilitated diffusion' (44) since travel is in the same direction as for a diffusion gradient but the rate exceeds the calculated rate predicted on physicochemical grounds. This transport system is mediated by a carrier --- probably a protein component of the cell membrane --- which can reversibly bind the glucose and carry it across the membrane. The transport is usually down the concentration gradient and can proceed from maternal to fetal or in reverse, depending on the direction of the gradient (45). At very high glucose concentrations it has been possible to saturate the carrier system. Other sugars may also act as competitive inhibitors of the transport although the carrier demonstrates a strong specificity for glucose. This is evident from the observation that fructose, a hexose of similar molecular weight to glucose, crosses the placenta at a tenth of the rate (44). A stereospecificity also exists in the system since the biologically-significant D-sugars are transported much faster than the corresponding L-isomers.

All mammalian placentas studied to date have been shown to take up and utilize glucose. Since the uptake of glucose by other tissues is under hormonal regulation, the rate-limiting passage of glucose through the placental cell membrane may also have similar controls. Changes in the uptake and utilization of glucose by the placenta would allow it to influence the quantity of carbohydrate made available to the fetus.

There have been conflicting observations concerning the effect of insulin on placental glucose uptake. Insulin facilitation of glucose uptake by human placental slices has been reported but has not been confirmed. This may result from differences in tissue preparation, length of gestation, or variation in the amount of insulinase (45), known to be present in placental tissue in different experimental systems. Anaerobiosis has been shown to accelerate placental glucose uptake (46) although it is not known if this is a direct stimulation of the rate-limiting membrane transport step as has been demonstrated for muscle tissue (47). A Pasteur effect has been invoked to explain the increased uptake caused by anoxia (48) since depletion of glucose-6-phosphate will result in increasing the rate of phosphorylation of glucose, thus allowing the transport of more glucose across the membrane.

Inside the placental cell phosphorylation to glucose-6-phosphate is the first step in glucose metabolism. This is apparently accomplished by a nonspecific hexokinase since glucokinase has not been found in human or guinea pig placenta (49). The glucose-6-phosphate then enters glycogen synthesis or glucose

utilization via the hexose monophosphate shunt and glycolysis.
The hexose monophosphate pathway has significant activity early
in gestation (49, 50), presumably to provide pentoses for nucleic
acid production, after which the enzymes of the shunt decrease
in activity towards term (49). The glycolytic pathway, which
is quantitatively the most significant route of glucose utiliz-
ation by the placenta, is accelerated by both anoxia (46) and
maternal diabetes (45). The placental glycogen, which varies
with gestational age, has no clearly established function to
date. It has been suggested that the glycogen could serve as
a reservoir of carbohydrate for the fetus during the period of
rapid growth preceding the termination of gestation (51).

The fetus, although it receives maternal amino acids, pre-
sumably conserves them for protein synthesis since trans-or de-
amination does not occur during fetal life (52) and gluconeo-
genesis from amino acids commences only after birth (53). Thus
the fetus uses glucose as virtually its only energy source and
to some extent its metabolism is analogous to an adult animal
receiving a high-carbohydrate diet. However, not all the glu-
cose which crosses the placenta is oxidized. Some is used for
lipogenesis and a large proportion is stored as liver glycogen.

It is well established that at a particular developmental
stage on and until birth, the fetal liver accumulates large
amounts of glycogen which are rapidly exhausted at birth. This
evidently will provide the newborn with a supply of glucose
after the severance of the maternal supply and before an endo-
genous source is available. In all species studied (52),

the glycogen concentration in the fetal liver and skeletal muscle begins to rise during the last third of gestation. Near term liver glycogen is at least twice the normal adult concentration for most species whereas the skeletal muscle glycogen concentration is 3 to 4 times the corresponding adult level (54). This elevated level drops considerably and liver glycogen reaches 10% or less of its initial value within 2 to 3 hours of birth. Skeletal glycogen also falls to, or below, the adult level within 1 to 3 days after birth.

There is very little known about the factors responsible for the first appearance of glycogen in fetal tissues and the subsequent increase in content. Phosphoglucomutase, UDP-glucose pyrophosphorylase, and glycogen synthetase all appear and become increasingly active in fetal liver tissue just prior to glycogen storage (55). Burton, Greenall, and Turnell (56) have shown a correlation between the increase in liver glycogen levels of fetal mice and the appearance of steroid reductase activity (which increases active corticosteroids) in fetal liver. Stimulation of fetal liver glycogen deposition by glucocorticoids probably results from the rise in blood glucose concentration, both in maternal and fetal plasma. Injection of corticosteroids early in gestation will induce liver glycogen storage to begin earlier in both rats (55) and mice (56).

If decapitated fetuses of adrenalectomized rats are given cortisol the liver glycogen content is increased (55). In rabbits, however, corticosteroids were found to have no effect on glycogen storage even when amounts up to 3 mg were administered.

Only when pituitary prolactin was given together with the corticoid was glycogen deposition evident in the decapitated rabbit fetuses (55). The concept that arose from these observations is that glycogen deposition in the fetal liver is under a dual hormonal control, both by adrenal steroids and a pituitary hormone. With some animals such as the rat a prolactin-like factor is produced by the placenta and therefore in these species corticosteroids become the limiting factor for glycogen deposition in fetal liver.

The phosphorylation of glucose in the fetal liver is accomplished by hexokinase since glucokinase, the specific enzyme of adult liver, is absent (53) and first appears a few days after birth. The glucose-6-phosphate is then channelled into glycogen formation, glycolysis, and the hexose monophosphate shunt. Towards the end of term an increase in the enzymes concerned with glycogenolysis and gluconeogenesis also occurs. Thus phosphorylase activity shows an increase some time after glycogen deposition has commenced (55) while glucose-6-phosphatase activity rises very sharply shortly before birth. Pyruvate carboxylase and fructose diphosphatase are also readily detectable shortly before birth and attain adult levels at term. However, gluconeogenesis may not be operative until after birth since phosphoenolpyruvate carboxykinase has no detectable activity before birth (55).

The Role of Receptors in Glucocorticoid Action

It is clear that there are a multitude of effects attributed to glucocorticoids. As well as initial direct effects upon

specific processes, there are the cellular modifications that occur as a physiological response to long term effects of the hormone. This multiplicity of biochemical responses to glucocorticoids has frustrated the search for the first effect of the hormone within the cell. Nevertheless the basic action of the glucocorticoids on most metabolic processes has been thought to involve modification of the transcriptional or translational aspects of genetic expression.

Karlson (57) elaborated the hypothesis that the hormones, by interactions with the genome, would control the synthesis of proteins in the receptor organ by regulating messenger RNA synthesis. Direct interaction of the steroids with DNA did not seem to be a feasible method for control since it is difficult to see how the steroids themselves could contain enough specificity for binding to specific regions of DNA. Histones have been shown to bind cortisol (58, 59), although the affinity of steroids for this fraction generally appear to be rather low (60). There is also a relative lack of specificity in the structure of the histones, leading to the idea that they interact mainly by non-specifically masking DNA (61). The non-histone or acidic proteins have been thought to be responsible for unmasking specific DNA sequences by counteracting the inhibitory effects of histones (62). Using adrenalectomized rats, Shelton and Allfrey made the observation that both RNA and acidic protein synthesis in liver cells were enhanced within 2 to 3 hours after a single injection of cortisol (63). Whichever component of the nucleus is affected, glucocorticoid treatment has been shown to increase DNA-dependent RNA polymerase activity (30, 31).

These experiments, along with the observation by Feigelson and his collaborators (10, 29) of an enhanced rate of synthesis of DNA-like RNA as well as ribosomal RNA after steroid treatment, indicate that messenger RNA synthesis and with it protein synthesis is accelerated by steroid administration.

By following the subcellular distribution of glucocorticoids in target tissues an understanding of their actions might be obtained. In vivo the liver is the main organ that concentrates glucocorticoids above the blood level since it is an important site of steroid metabolism as well as being a target tissue. After an i.p. injection of labeled glucocorticoid, there is rapid accumulation in the liver with most of the radioactivity present in the cytosol and lesser amounts in the other subcellular fractions. The cytosol radioactivity, of which 6 to 13% is bound to macromolecules, is eluted from columns of DEAE Sephadex A-50 associated with four protein peaks which Litwack (60) has labeled binders I, II, III and IV. Peaks I and III have been found to bind mostly to polar metabolites of cortisol while IV binds most effectively to metabolites of testosterone and progesterone. Binder II is associated with a much higher portion of the unchanged steroid immediately after hormone administration and may represent the cytosol glucocorticoid receptor in the liver. Its binding properties satisfy the basic requirements of a physiological hormone receptor; it is saturated in vivo very rapidly (5 to 10 min) and within a physiological concentration of steroid (0.1 nM).

Due to its relatively poor blood supply the thymus does not concentrate steroids effectively in vivo. However, using in vitro

incubations to circumvent the problems of blood circulation and competition for hormone uptake, thymus cells have been found to also accumulate radioactive glucocorticoids in all subcellular fractions (60, 64). Munck has shown that addition of labeled glucocorticoids of high specific activity to a thymus cell suspension results initially in two types of interactions; nonspecific and specific binding (65). The nonspecific binding is virtually instantaneous (38), and results in distribution of the steroid throughout the cell mainly by a loose adsorption onto various macromolecules and subcellular organelles. This form of binding does not saturate even at high steroid concentrations (10 uM and up) and has no correlation with in vivo glucocorticoid activity.

The physiologically important binding accounts for only a small fraction of the total amount of bound steroid and becomes saturated at about 1 uM (65). If cells treated with radioactive glucocorticoid are diluted into a medium with no steroid, the bound steroid will dissociate at a rate determined by its binding affinity. At $37^{o}C$ most of the steroid dissociates within 1 min and this rapidly dissociating fraction represents nonspecifically bound steroid. A corticosteroid such as cortisone which is not biologically active per se, is bound almost entirely in this manner and exhibits no specific binding (38). For the active glucocorticoids a large portion also dissociates rapidly, but there remains a fraction of higher binding affinity which dissociates much more slowly. The properties of this fraction (66) indicate that it consists of hormone molecules specifically bound to the glucocorticoid receptors.

Cortexolone, a steroid without _in vivo_ glucocorticoid act-
ivity since it lacks an oxygen function at carbon-11, does com-
pete effectively _in vitro_ with cortisol for the specific binding
sites. Munck found that 10 uM cortexolone would completely
abolish the metabolic effects of 1 uM cortisol on thymus cells
(65, 67), thus acting as an anti-glucocorticoid. These experim-
ents with cortexolone, which have been confirmed by other work-
ers (68, 69, 70), indicate that the specific binding sites have
a role in steroid action. Further evidence that the glucocorti-
coid receptors mediate the hormonal effect is provided by studies
on cultured hepatoma cells (71, 72), lymphoid cells (73), and
fibroblasts (74) which showed that absent or decreased sensit-
ivity to steroids is accompanied by a diminished concentration
of specific glucocorticoid receptor sites.

The overall molecular mechanism of glucocorticoid action
on target cells appears quite similar to the theory elucidated
for the other steroid hormones (75, 76, 77). After penetration
of the cell membrane, the steroid is first loosely bound by the
reversible nonspecific binding described earlier. Interaction
then occurs with a limited number of specific binding receptors
present in the cytosol. It is not known at present if these
are freely dispersed throughout the cytoplasm or attached to
membrane structures. After its formation, the steroid-receptor
complex is able to penetrate the nuclear membrane and bind to
specific sites on the chromatin of the cell nucleus. In this
manner the glucocorticoid-receptor complexes are thought to add
a regulatory element which can then modify RNA transcription
or some post-transcriptional event (78).

A current model concerning the initial interaction between glucocorticoids and cytoplasmic receptors postulates that the receptor is an allosteric protein with active and inactive conformations. In the absence of steroids the receptor is present mainly in the inactive form. A biologically active glucocorticoid will bind specifically to the active conformation of the receptor, thereby favoring a shift of the allosteric equilibrium to the active form (71). In this way more complex is formed until either the steroid or the receptor has been depleted. Interaction of these complexes with the nuclear chromatin will result in a maximal glucocorticoid response by the target cell. The inactive corticosteroids display no affinity for either conformation of the protein and therefore have no effect on this system. In contrast, glucocorticoid antagonists will bind preferentially to the inactive conformation of the receptor and form a non-functioning complex. This will shift the equilibrium (72), resulting in depletion of active receptor molecules and prevention of the biologic response even in the presence of active glucocorticoids.

Some experimental evidence to support this model has been obtained using the induction of tyrosine aminotransferase by glucocorticoids in rat hepatoma tissue culture cells (72). The active glucocorticoids were observed to bind to the receptors with an affinity which is directly related to their ability to induce tyrosine aminotransferase. Anti-inducers such as progesterone and 17 α-methyl testosterone bind to the receptors with an affinity predicted from their capacity to inhibit enzyme induction. The kinetics of steroid binding and dissociation

at 37°C are also rapid enough to account for the kinetics of enzyme induction and deinduction.

The Present Problem

The focus of this study concerns an attempt to elucidate the biochemical mechanisms involved in glucocorticoid hormone action. The influence of these hormones in the endocrinology of pregnancy was investigated using several strains of inbred pregnant mice. Differences in the response to corticosteroids of several strains of mice have been attributed to differences in metabolism of the steroids. The A/J strain has been shown to be particularly susceptible to induction of cleft palate and death of a high proportion of fetuses when pregnant mice were treated with glucocorticoids (79). This was attributed to the greater uptake of injected steroid by fetuses of the A/J strain as compared with less sensitive strains (80, 81). Correction of a congenital eye anomaly in mutant mice by administration of cortisone (82) also correlated with higher uptake of labeled steroid (83) by one of the mutant lines.

While the amount of steroid reaching fetal tissues can vary with the strain, factors other than the disposition of the steroid must influence the response of the fetus. The well-documented effects of glucocorticoids on glucose metabolism led to the consideration that the availability of glucose to the fetus might possibly be modified by corticosteroid treatment. Since glucose is the major nutrient which the fetus receives from the mother, this influence of the steroids would have important consequences in both fetal development and survival.

A corticoid effect on glucose transport to the fetus would indicate a steroid influence on placental function, since it is mainly responsible for the transmission of nutrients from mother to fetus (44). The placenta might then be a good target tissue for investigation of the site(s) of specific corticosteroid action.

MATERIALS AND METHODS

Chemicals

The following chemicals were purchased from the Sigma Chemical Company: cortisol, cortisone, progesterone, corticosterone, dithiothreitol, and N-ethyl maleimide. Dexamethasone (Decadron powder) was the gift of Merck and Co. to Dr. M. Darrach in this department. Cortexolone was obtained from Nutritional Biochemicals Corp; 11-epicortisol, bovine serum albumin, and human γ globulin were all from Mann Research Labs; and 11-epicorticosterone was a gift from Dr. J. Babcock of The Upjohn Company, Kalamazoo, Michigan. Pronase (45,000 PUK units/mg), deoxyribonuclease (42,300 Dornase units/mg, RNAse content = 0.102%), ribonuclease (47 Kunitz units/mg), actinomycin D, and p-chloromercuribenzoate were all obtained from Calbiochem. PPO and POPOP (scintillation grade) and Bio-Solv solubilizer formula BBS-3 were all from Beckman Instruments. The Glucostat reagent was obtained from Worthington Biochemical Corp. and Nembutal (sodium pentobarbital) was purchased from Abbott Laboratories Ltd., Montreal. The sesame oil used (U.S.P. grade) was from Bush Boake Allen. All other chemicals used were of reagent grade and were purchased from Fisher Chemical Co., Vancouver.

Solvents

All solvents used were of reagent grade and were purified by distillation before use.

Materials

Scintillation vials (low potassium glass) and plastic caps were from Fraser Medical Supplies, Vancouver. Pharmacia supplied the Sephadex G-25 (coarse grade).

Radiochemicals

The following radiochemicals were purchased from New England Nuclear Corporation, with the indicated specific activities: $4-^{14}C$-cortisol (52 mCi/mmole), $1,2-^{3}H$-cortisone (51.6 Ci/mmole) $4-^{14}C$-progesterone (52.8 mCi/mmole), and ureido-^{14}C-citrulline (4.28 mCi/mmole). Amersham/Searle Corporation supplied the remainder: $1,2-^{3}H$-corticosterone (36 Ci/mmole), $U-^{14}C$-D-glucose (288 mCi/mmole), and $1-^{14}C$-acetic acid (52.9 mCi/mmole). All radiochemicals were used upon receipt and were examined or purified routinely by TLC or paper chromatography.

Animals

The A/J and C57BL/6J inbred strains of mouse were purchased from Jackson Memorial Laboratories, Bar Harbor, Me. Pregnant females usually arrived between day 10-13 of pregnancy. The SWV mice were randomly bred using the facilities of the Cancer Research Centre at this university. All animals were fed a diet of Purina Breeder Chow and water ad lib. For experiments requiring timed pregnancies the date of finding the vaginal plug was designated as day zero of pregnancy and all experiments were performed on the morning of the 18th day.

Methods

1. Steroid injections into pregnant mice

An intraperitoneal injection of the steroid, suspended in

sesame oil, was made into pregnant mice while controls received the same volume of sesame oil only. One hour later 5 uCi of labeled precursor dissolved in 0.1 ml physiological saline (0.15M) was injected subcutaneously into the upper abdomen. After another 15 min each mouse was sacrificed and the fetuses were quickly excised, weighed, and placed on ice.

2. Steroid injections into the fetus

Pregnant SWV mice (gestational day 18, weight 45-50 gm) were anesthetized by injection of 0.1 ml sodium pentobarbital solution (15 mg/ml in physiological saline) subcutaneously and maintained with additional doses when required. The peritoneum was carefully opened to expose the fetuses which were visible through the uterine membrane. Injections of varying doses of dexamethasone suspended in 0.05 ml sesame oil were made into the fetuses in utero. The fetuses were then restored into the abdominal cavity and both the peritoneal membrane and skin were sutured. One-half hour later 5 uCi of U-^{14}C-D-glucose dissolved in 0.1 ml physiological saline was injected subcutaneously into the upper abdomen of the mother. After another 15 min each mouse was killed by cervical dislocation and the fetuses were again removed, weighed and placed on ice.

3. Isolation of acid-soluble fraction of tissues

Either whole fetuses or individual tissues were finely minced and homogenization was accomplished in physiological saline (5 ml/gm wet wt tissue) using a motor-driven Teflon Potter-Elvehjem tissue grinder. The heavy residue was removed by centrifugation at 1200xg for 5 min and 1 ml of 60% (w/v) TCA was added

to every 5 ml of saline supernatant. Small molecules such as monosaccharides, amino acids, and nucleotides remain soluble in the dilute TCA solution but oligopeptides and other components of similar size are completely precipitated (84). After centrifugation at 1200xg for 10 min, the acid-soluble supernatant was decanted into a test tube and the radioactivity determined. The ratio of acid-insoluble to acid-soluble counts recovered did not differ from one experiment to another (Table I).

4. Determination of radioactivity

An aliquot of the sample to be counted was placed into a scintillation vial and 10 ml of scintillation fluid consisting of 4 gm PPO and 100 mg POPOP per liter of toluene (85) was added. Bio-Solv solubilizer formula BBS-3 was also added to the samples until the solution appeared clear. The samples were then assayed for radioactivity using a Packard Tri-Carb liquid scintillation spectrometer (Model 3003). Sample quenching was monitored by the channels-ratio method (86) using a commercially available quench set (Amersham/Searle). The counting efficiency for ^{14}C was approximately 78% while the ^{3}H counting efficiency was 32%.

5. Semi-micro determination of blood glucose

Blood samples were obtained from the throat, 0.4 ml blood added to 0.1 ml sodium citrate (28 mg/ml). A 1:40 Somogyi filtrate (87) was then prepared by adding 0.1 ml of the whole blood to 1.9 ml distilled water, mixing, and adding 1.0 ml 1.8% Ba (OH)$_2 \cdot$ 8H$_2$0. After thorough mixing, 1.0 ml 2% ZnSO$_4 \cdot$ 7H$_2$0 was added and the solution was mixed and then centrifuged to remove the precipitate.

TABLE I.   Representative values of radioactivity recovered from fetal extracts

Dexamethasone dose (ug)	Total counts recovered (dpm)		Ratio (a/b)
	Acid-insoluble [a]	Acid-soluble [b]	
Control	10,350	35,400	0.293
	12,210	38,280	0.319
50	14,100	45,800	0.308
	12,320	42,000	0.294
200	12,150	38,800	0.314
	11,200	36,750	0.305

Pregnant mice were injected with various doses of dexamethasone and labeled glucose as described in Methods. The fetuses were removed, and the acid-soluble fraction for each was isolated using the procedure indicated in Methods. The acid-insoluble fraction for each was also retained and solubilized by heating at 100° for 60 min in 1 ml 5N KOH. The alkaline solutions were then neutralized by addition of 5N perchloric acid and radioactivity was determined on aliquots of both fractions.

Glucostat is a prepared reagent for the quantitative, col-
orimetric determination of glucose which makes use of two coupled
reactions catalyzed by glucose oxidase and a peroxidase enzyme
(88). The Glucostat reagent consisting of enzymes and chromogen
was dissolved in 50 ml distilled water and 2 ml of this was added
to 2 ml of each blood filtrate sample or glucose standard. After
a 10 min incubation at room temperature the reaction was stopped
by addition of 1 drop of 4N HCl. After a further 5 min the ab-
sorbance was determined at 420 nm.

6.   Isolation of nuclear steroid-receptor complex

Pregnant SWV mice (gestational day 13 to 18) were killed by
cervical dislocation and the placentas removed and placed on ice.
Extraneous tissue was trimmed away and two placentas per sample
were then lightly homogenized with 2 to 3 strokes of a loose-fit-
ting tissue grinder in 2 ml KEP buffer, pH 7.4, containing 2.8
mM glucose. Radioactive steroid dissolved in ethanol, alone or
with non-radioactive steroids (also dissolved in ethanol), was
added and the samples left on ice for 15 min with occasional
agitation. The samples were then incubated at 37°C for 10 min
in a shaking water bath, put back on ice and diluted by addition
of 50 ml ice cold 1.5 mM $MgCl_2$. This procedure has been used by
Wira and Munck (64) to shatter cell membranes, disrupting cyto-
plasmic material but leaving nuclei intact (Fig. 1). After sit-
ting on ice for 10 to 15 min, the excess supernatant fluid was
decanted off and each sample was centrifuged at 1200xg for 10
min at 6°C. The residue of nuclei was washed twice with 10 ml
ice cold 1.5 mM $MgCl_2$ and resuspended in 0.7 ml of cold buffer

Fig. 1.   Nuclei isolated using hypotonic $MgCl_2$

        Placental tissue was homogenized with a loose-
fitting tissue grinder in 2 ml KEP buffer (pH 7.4, with
2.8 mM glucose), then diluted with 50 ml ice cold 1.5 mM
$MgCl_2$. The prepared nuclei were harvested by centrifu-
gation at 1200xg and resuspended in freshly prepared 5%
glutaraldehyde in 1.5 mM $MgCl_2$. Smears were prepared
and stained with toluidine blue. X 640
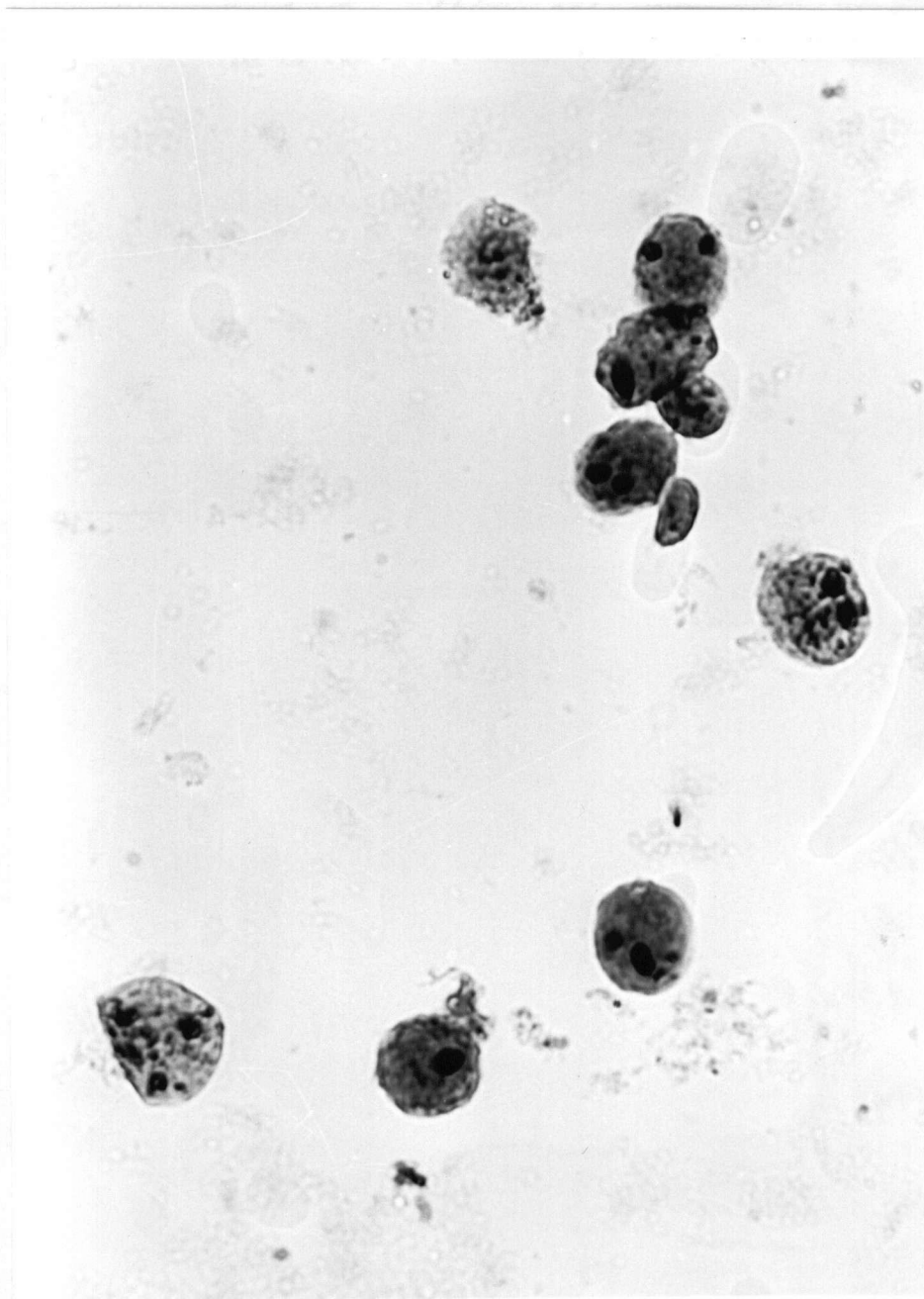
composed of 0.6 M KCl, 10 mM TRIS, and 1.5 mM EDTA, pH 8.0. The
nuclei were then homogenized with 8 to 10 strokes of a tight-
fitting Ten-Broeck ground glass homogenizer and centrifuged at
105,000xg for 10 min at 0 to 4°C in a refrigerated ultracentri-
fuge. An aliquot (usually 0.5 ml) of this nuclear fraction was
then removed and placed on 11 x 180 mm columns of Sephadex G-25
using 0.6 M KCl buffer with 10 mM TRIS and 1.5 mM EDTA, pH 8.0.
The elutions were carried out at 6°C and the peak of bound ma-
terial could be collected within 10 min after application of the
sample. Fractions of approximately 1 ml were collected and the
macromolecular peak was identified by protein determinations
using the method of Lowry, Rosebrough, Farr, and Randall (89).
Radioactive determinations were made on 0.1 ml aliquots in the
same manner as previously stated.

7. Cytosol exchange assay

Pregnant SWV mice (gestational day 13 to 18) were killed and
the placentas removed and trimmed as before. The pooled tissue
(7 to 11 placentas) was finely minced and washed three times
with 10 ml ice cold KEP buffer, pH 7.4, containing 2.8 mM glucose.
The tissue was then resuspended in the KEP buffer (5 ml/gm wet wt
tissue) and homogenized using a Teflon Potter-Elvehjem homoge-
nizer (8 to 10 strokes). The preparation was centrifuged at
1200xg for 10 min at 6°C and the supernatant was recentrifuged
at 105,000xg for 60 min at 0 to 4°C in a refrigerated ultracent-
rifuge to obtain the cytosol fraction. The cytosol exchange as-
say consisted of the incubation of aliquots of the cytosol frac-
tion with ^{3}H-corticosterone (13.8 nM) dissolved in ethanol in

two series. The first series had 200 uM unlabeled corticoste-
rone dissolved in ethanol added while the second set contained
200 uM unlabeled epicorticosterone (also dissolved in ethanol).
After incubation, 0.5 ml aliquots of the samples were placed on
columns of Sephadex G-25 at 6°C using KEP buffer, pH 7.4, with
2.8 mM glucose. The protein and radioactivity determinations
on 1 ml fractions were assayed as before. Specific binding in
this case represented the difference between the binding in the
presence of epicorticosterone and corticosterone (90).

8. Other procedures

Other procedures, used less frequently, will be described
as they are required in the following experiments.

RESULTS

Steroid Effects on Fetal Glucose Metabolism

The glucocorticosteroids have been reported to influence fetal viability, so both natural and synthetic corticosteroids were employed to determine their effects on the fetus. In preliminary experiments the findings of Pinsky and DiGeorge (79) were confirmed. Injection of a sesame oil suspension of dexamethasone (see Appendix II for structural formula) into a pregnant mouse resulted in death of all the fetuses. With the A/J strain this was accomplished using a total dose of steroid as low as 600 ug over three days (Table II). In many cases where a high dosage of dexamethasone had been maintained over a number of days, resorption of the fetuses was evident; they were discolored and emaciated. Only in one instance was there no apparent effect of dexamethasone on fetal viability. In that animal the injections contained a moderate dose (500 ug) but were only given for one day at a very late stage of pregnancy. Using cortisol there was no apparent effect even with a total dose as high as 8 mg which is similar to the data of Pinsky and DiGeorge which reported a much lower incidence of cleft palate using cortisol.

A.   Preliminary experiments on precursor incorporation

The influence of dexamethasone treatment upon the fetal metabolism of two radioactive precursors, acetate and glucose, was examined in order to investigate the lethal effects of the steroid. Using acetate there appeared to be no difference in the counts recovered in a chloroform-methanol extract of the steroid-

TABLE II.   Effect of glucocorticoid treatment on fetal viability

Mouse strain	Dosage per injection	No. of daily injections	Gestational period (day)	Fetuses # dead / total
A/J	500 ug D	2	12 to 15	11/11
	500 ug D	2	14 to 17	8/8
	2 mg D	1	17 to 18	9/9
	100 ug D	2	12 to 15	8/8
	100 ug D	2	14 to 17	7/7
	500 ug D	2	18 to 19	0/9
SWV	2 mg D	1	12 to 15	11/11
	2 mg D	1	14 to 18	7/7
	1 mg D	1	16 to 19	10/10
	2 mg D	1	16 to 19	9/9
	(0.5 ml oil)	2	15 to 18	0/10
	4 mg F	2	16 to 17	0/12
	50 ug F	2	15 to 17	0/9
	100 ug F	2	14 to 17	1/9

Pregnant SWV and A/J mice were treated with i.p. injection(s) of steroid (D=dexamethasone, F=cortisol) dissolved in 0.5 ml sesame oil during the indicated gestational period. On the morning of the last day indicated the mice were killed by cervical dislocation and the fetuses were removed and examined.

treated fetuses when compared with sesame oil-treated controls since recoveries from the individual tissues, although different, did not correlate with treatment (Table III).

When labeled glucose was used, a decrease in incorporation of label into all the tissues examined was evident, resulting in a 53% drop in the total counts recovered from the fetal tissues of the dexamethasone-treated animal compared to the controls. Although the actual counts for the fetal brain tissue were also decreased by dexamethasone injection, the percentage drop was less than for the other tissues. In fact, the fetal brain actually increased in the proportion of recovered total counts from 14% for controls to 23% after dexamethasone administration. The decrease in total radioactivity recovered as well as the change in distribution of the counts, suggested that the steroid was affecting both the carbohydrate metabolism of the fetus itself as well as transport of the labeled glucose from the maternal circulation to the fetus.

B. Dexamethasone effects on glucose incorporation into the acid-soluble fraction of fetal tissues

It was decided that the acid-soluble fraction of fetal tissues would most closely reflect free glucose transported to the fetus (84). The effect of dexamethasone treatment on the counts recovered in this fraction were determined using pregnant A/J mice. Dexamethasone at three concentrations was used (Table IV). The counts incorporated into the fetuses of the steroid-treated animals in all cases were increased over those of the control fetuses since the counts recovered in the individual tissues were generally all higher for the steroid-treated animals. However,

TABLE III.   Effect of dexamethasone upon the incorporation of
              labeled acetate or glucose into a chloroform-
              methanol extract of SWV mouse fetal tissues

Tissue	A c e t a t e		G l u c o s e	
	Control	Dexamethasone	Control	Dexamethasone
Brain	2.6	3.1	14.1	10.9
Liver	9.5	7.2	11.0	4.5
Gut	6.0	5.2	6.7	3.1
Lung	3.5	6.0	11.1	3.0
Carcass	2.1	2.0	6.8	2.9
# Fetuses	9	5	13	9
# Animals	1	1	2	1

Pregnant SWV mice (gestational day 18) were treated
with an i.p. injection of 1 mg dexamethasone suspended in 0.1 ml
sesame oil (controls received 0.1 ml sesame oil) 24 hours before-
hand. A s.c. injection of either labeled acetate or glucose was
made and 1 hour later the animals were killed and the fetuses
were removed, weighed, and placed on ice. The fetal brain, liver,
gut, and lung were excised from the carcass and each tissue was
pooled and weighed. The tissues were homogenized in physiologic-
al saline (5 ml/gm wet wt tissue), then extracted twice with an
equal volume of chloroform-methanol (2:1) and the radioactivity
of the extract was determined. The figures represent counts cor-
rected for the tissue weight (dpm/mg wet wt tissue).

TABLE IV.   Effect of various doses of dexamethasone upon the incorporation of labeled glucose into A/J mouse fetal tissues

Tissue	Dose (ug)			
	0	50	100	200
Brain	16.0	23.3	18.3	17.3
Liver	23.3	33.6	24.2	26.8
Gut	22.3	22.4	25.3	19.3
Lung	22.3	33.2	36.7	27.0
Carcass	20.3	38.3	29.6	24.0
# Fetuses	14	8	9	11
# Animals	2	1	1	1

Pregnant A/J mice were injected with various doses of dexamethasone and with labeled glucose as described in Methods. The fetal brain, liver, gut, and lung were excised from the carcass and each tissue was pooled and weighed. Acid-soluble radioactivity was determined for each tissue and the figures represent counts corrected for the tissue weight (dpm/mg wet wt tissue).

there was a negative correlation with dosage of steroid. The recovered counts were highest in fetuses of mice treated with only 50 ug dexamethasone whereas the 200 ug steroid-treated samples had recovered radioactivies only slightly above the control values.

Various doses of dexamethasone were used to determine the dose-response relationship between the steroid and the incorporation of labeled glucose into mouse fetuses. Fig. 2 shows that the two effects are evident over the concentration range investigated when the A/J strain was used. At the lower steroid doses (50 and 100 ug) there were higher counts recovered in treated fetuses compared to controls. However, the slope of the curve is negative, implying decreasing incorporation with increasing steroid dose, so that at the 300 ug dosage the recovered counts are significantly below control values ($P < 0.001$ using t test). Thus, there is evidence for inhibition of incorporation of labeled glucose into A/J fetuses at every concentration of steroid used. However, at the lower doses the inhibition appears to be masked by an opposing effect which promotes the transfer of the labeled glucose. This opposing effect is most likely due to the hyperglycemia which results when glucocorticoids are administered (12). The elevated blood glucose levels would increase the transfer of glucose from mother to fetus since the gradient difference would be increased (45).

The three mouse strains used were then compared at the 200 ug dexamethasone dose and Fig. 3 shows the recoveries in fetuses relative to controls of the same strain. At this dose the A/J

Fig. 2.   Effect of various doses of dexamethasone upon
           the incorporation of labeled glucose into A/J
           mouse fetuses

           Pregnant A/J mice were injected with various doses
of dexamethasone and with labeled glucose as described
in Methods. Acid-soluble radioactivity was determined
for each fetus to compute the corrected counts (dpm/mg
wet wt fetus) which were then compared to controls ex-
pressed as 100%. Each point represents the mean of at
least 10 fetuses (and at least 3 animals) with the SEM
indicated by the vertical lines.
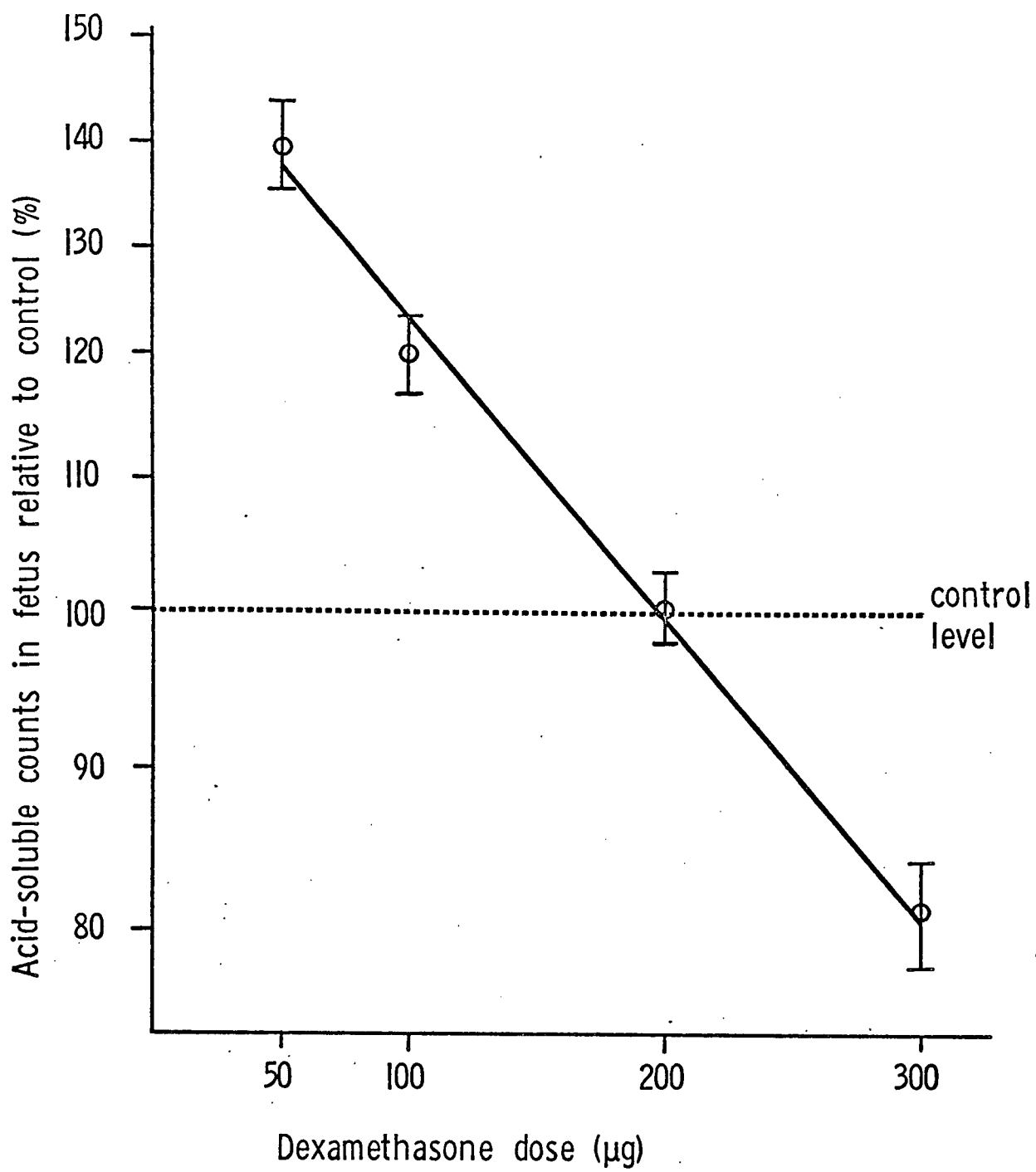
Fig. 3.   Strain differences in the effect of dexamethasone
upon the incorporation of labeled glucose into mouse
fetuses

Pregnant mice were injected with 200 ug dexa-
methasone and with labeled glucose as described in
Methods. Acid-soluble radioactivity was determined
for each fetus to compute the corrected counts (dpm/mg
wet wt fetus) which were then compared to controls of
the same strain expressed as 100%. The vertical lines
indicate SEM and the figures in brackets indicate the
number of determinations.

Acid-soluble counts in fetus relative to control (%)
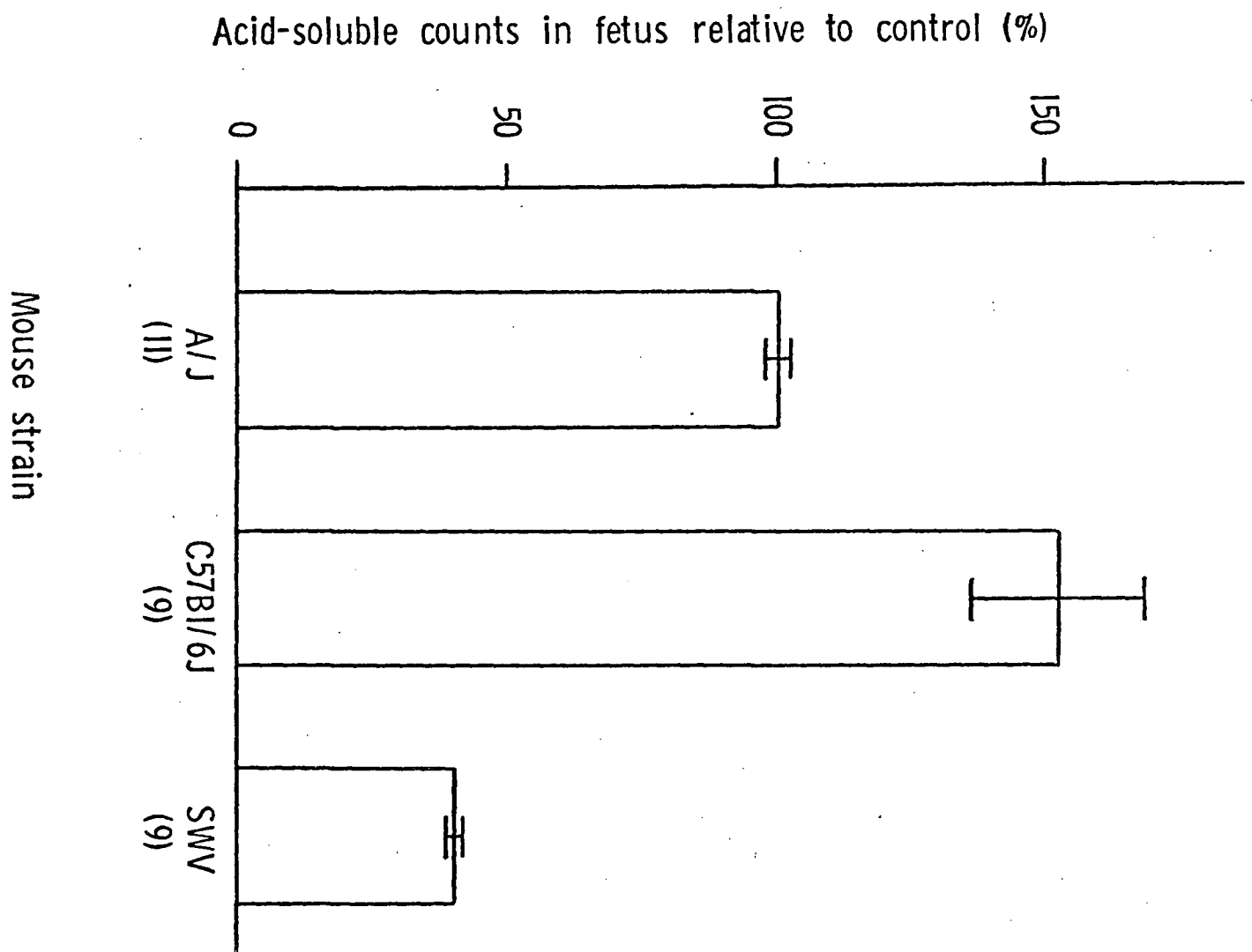
fetuses exhibited an incorporation of label which was equivalent to the control values (as shown in Fig. 2). However, the C57BL/6J fetuses showed a considerable increase in label incorporated relative to controls while conversely, the SWV showed a decrease. Control values among the strains also differed since the SWV fetuses yielded much lower recoveries than either the A/J or C57BL/6J fetuses (Table V). However, the same general dose-response seems to hold for the SWV and C57BL/6J strains as for the A/J since the lower steroid doses resulted in increased incorporation whereas higher doses generally caused a decrease in the amount of label recovered.

C.   Effect of natural glucocorticoids

The naturally-occurring glucocorticoids, cortisol and corticosterone, were used to determine if they would have the same effect on incorporation of labeled glucose as the synthetic steroid. Table VI shows that although an increased incorporation of label is apparent, there is no evidence of inhibition similar to that caused by dexamethasone even when the natural steroids were used at doses as high as 5 mg. Both the natural and synthetic corticosteroids will cause hyperglycemia and in the absence of other factors, this can explain the increased counts recovered in the fetus. In eliciting other effects, however, the natural and synthetic corticosteroids do not have equal activities. Dexamethasone has been reported to produce twenty times as much liver glycogen deposition as does cortisol in the mouse (79). This is similar to the difference in activity encountered when the thymus involution assay is used (Table VII). However, if granuloma inhibition or anti-inflammatory response is used as the index of

TABLE V.   Strain differences in the effect of various doses of
dexamethasone upon the incorporation of labeled
glucose into mouse fetuses

Steroid dose (ug)	A/J	SWV	C57BL/6J
0	34.6±1.2(12,3)	25.8±0.7(8,2)	39.7±1.3(8,2)
50	48.7±1.5(15,4)	28.8±2.3(3,1)	68.4 (2,1)
100	42.3±1.1(11,2)	33.4±2.1(5,1)	---
200	32.8±0.8(8,2)	10.5±0.3(9,2)	61.0±6.4(9,3)
300	28.8±1.2(10,2)	---	26.0±0.6(4,1)

Pregnant mice were injected with various doses of
dexamethasone and labeled glucose as described in Methods. Acid-
soluble radioactivity was determined for each fetus and figures
represent mean of corrected counts (dpm/mg wet wt fetus) + SEM.
The figures in brackets represent the number of fetuses and anim-
als, respectively.

TABLE VI.   Effects of corticosterone and cortisol upon the incorporation of labeled glucose into SWV mouse fetal tissues

Tissue	Control	Corti-costerone (200 ug)	Corti-costerone (1 mg)	Cortisol (5 mg)
Brain	3160	3020	5140	4080
Liver	5460	6350	8560	7200
Gut	3570	3310	3520	3830
Lung	2720	2760	3340	3470
Carcass	12,650	13,820	15,750	19,500
# Fetuses	18	10	7	9
# Animals	2	1	1	1

Pregnant SWV mice were injected with corticosterone or cortisol and labeled glucose as described in Methods. The fetal brain, liver, gut, and lung were excised from the carcass and each tissue was pooled. Acid-soluble radioactivity was determined for each tissue and the figures represent total counts corrected for the number of fetuses. The fetal weights in this experiment were all 950 ± 85 mg.

TABLE VII.   Relative activity of steroids on thymus
               involution

Steroid	Mean thymus weight (mg)	Ratio x 10^3 thymus/body wt
Control	63	4.16 ± 0.16
400 ug corticosterone	44	2.29 ± 0.10
400 ug cortisol	37	2.18 ± 0.08
20 ug dexamethasone	28	1.59 ± 0.12

        The relative activity of steroids was compared using
a thymus involution assay described by Dorfman and Dorfman (91).
Young female SWV mice, 15 to 20 gm each, were injected s.c.
with steroid in olive oil. Two injections of 0.2 ml oil were
made 5 hrs apart. Thymuses were removed and weighed 23 hrs
after the first injection. Each figure represents a mean of
6 to 8 determinations.

potency, the difference in activity is closer to two hundredfold (92). Similarly, the difference in activity of cortisol and dexamethasone in inducing cleft palate in the mouse was reported to be several hundredfold (79).

Therefore, the lack of effect of the natural glucocorticoids on glucose transfer, which correlates with their lack of lethal effects (Table II), may result from a large difference in their relative activity using this parameter. This is brought out by the similarity of the effect caused by the high doses of natural steroid and the lower doses of dexamethasone. (Tables V, VI, VII). There is, therefore, justification from these results in using dexamethasone rather·than the natural glucocorticoids, since acute effects expected to be demonstrated by these experiments might be more easily achieved.

D. Effect of steroid injected directly into the fetus

Since the dose-response for dexamethasone treatment showed two distinct and opposing effects, an experiment was designed to separate these responses. Injections of steroid were performed into the fetuses in utero before treatment of the mouse with labeled glucose, as was previously done. In this manner it was hoped that only the local effects of the steroid on the feto-placental unit would be manifested, without provoking hyperglycemia in the mother. As seen in Fig. 4, the counts recovered in the fetuses were below control values at the lower steroid doses, while at the higher doses (50 and 100 ug) recoveries were similar to control values. Table VIII shows that the effect occurred in all of the fetal tissues examined although again

Fig. 4.   Effect of various doses of dexamethasone injected
directly into the fetus upon the incorporation of
labeled glucose into SWV mouse fetuses

    Pregnant SWV mice were anesthetized and the fetuses
injected with the indicated dose of dexamethasone as
described in Methods. After a s.c. injection of the
labeled glucose into the mother, fetuses were removed
and weighed. Acid-soluble radioactivity was determined
for each fetus to compute the corrected counts (dpm/mg
wet wt fetus) which were then compared to sesame oil-
treated controls expressed as 100%. The vertical lines
indicate SEM for 3 to 4 determinations. Comparison of
the combined high doses (50 and 100 ug) versus the lower
doses combined (5, 12.5, and 25 ug) using the t test
indicates that the difference is significant. (P<0.01).

Figure: Bar chart of Acid-soluble counts in fetus relative to control (%) versus Dexamethasone dose (μg). The x-axis categories are control, 100, 50, 25, 12.5, and 5.
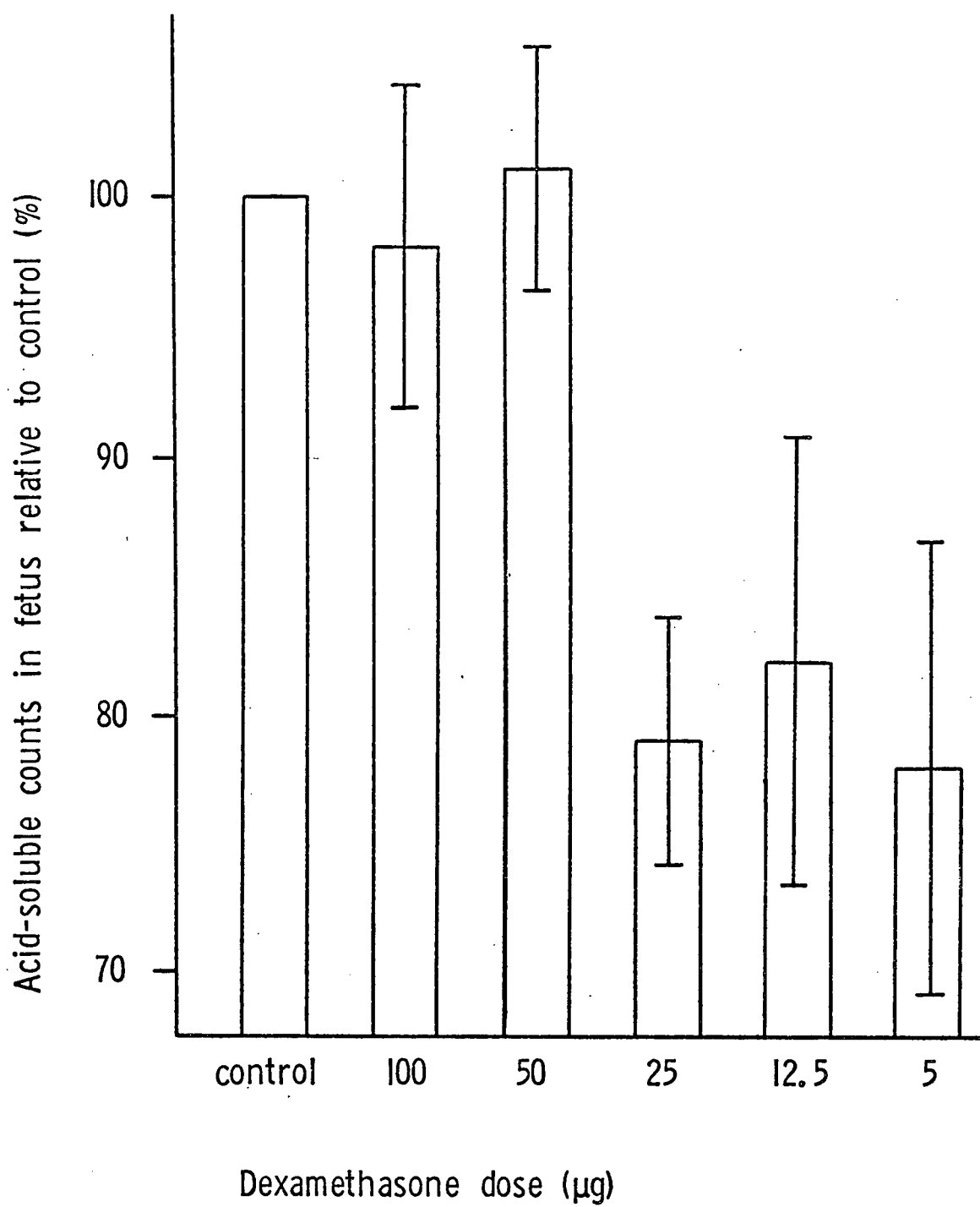
TABLE VIII.   Effect of dexamethasone injected directly into the fetus upon the incorporation of labeled glucose into SWV mouse fetal tissues

Tissue	Dose (ug)		
	0	25	50
Brain	8.6	10.2	9.1
Liver	18.0	10.5	17.7
Gut	9.9	5.8	7.6
Lung	10.8	6.7	9.1
Carcass	13.4	9.3	13.6

A pregnant SWV mouse was anesthetized and the fetuses injected with the indicated dose of dexamethasone as described in Methods. After a s.c. injection of the labeled glucose into the mother; the fetal brain, liver, gut, and lung were excised from the carcass and each tissue was then weighed. Acid-soluble radioactivity was determined for each sample of tissue and figures represent total counts corrected for the tissue weight (dpm/mg wet wt tissue).

the decrease was much less prominent in the fetal brain. This is consistent with the proposal that lower doses exerted their effect only on the feto-placental compartment, whereas higher doses were able to influence the maternal compartment, as if injected into the mothers and were thus able to exert an opposing effect by raising the maternal blood glucose level.

E. Glucose concentration and relative radioactivity of maternal blood

To determine whether or not the steroid effects could be explained by differences in maternal hyperglycemia, the concentration of glucose and the radioactivity in maternal blood were measured. Using A/J mice, the blood glucose concentration for all the steroid-treated animals was found to be at least twice the control level (Table IX). However, there was no correlation between the blood glucose concentration and the dose of steroid used in the injection.

The relative concentration of glucose to radioactivity in maternal blood was found to be lower for all the steroid-treated animals compared to the A/J controls although there was again no correlation with steroid dose. The SWV and C57BL/6J mice treated with 200 ug dexamethasone exhibited the same blood glucose concentration as the A/J treated at the same dose of steroid although the relative radioactivity of maternal blood for the former strains appeared higher than for the A/J. From these data, there was no indication that the steroid-induced decrease in transfer of labeled glucose from mother to fetus resulted from steroid effects on maternal carbohydrate metabolism.

TABLE IX.   Glucose concentration and relative radioactivity
of maternal blood from steroid-treated mice

Mouse strain	Dexameth- asone dose (ug)	Glucose concentration (mg/100 ml blood)	Relative radioactivity (dpm/microgram glu- cose)
A/J	0	44,35,61	7400,6670,9900
	50	92,136,107	3110,3770,4940
	100	130,74,87	5780,3440,3860
	200	110,221	1160,5420
	300	97,93	2180,3410
SWV	200	130,141,198	5270,5740,7140
C57BL/6J	200	155,173	7100,5110

Pregnant mice (gestational day 18) received an i.p.
dose of dexamethasone suspensed in 0.1 ml sesame oil. They
were treated one hour later with a s.c. injection of labeled
glucose and after another 15 min 0.4 ml blood samples were
obtained from the throat and mixed thoroughly with 0.1 ml sodium
citrate solution. Blood glucose was assayed on prepared Somogyi
filtrates using the semi-micro procedure described in Methods,
while an aliquot of the filtrate was also assayed for radioact-
ivity.

F. Effect of dexamethasone on amino acid incorporation into the
acid-soluble fraction of fetal tissues

Since glucose transport to the fetus appears to be inhibited
by dexamethasone injection into the pregnant mouse, it is pos-
sible that the inhibition resulted from a general effect on pla-
cental transport. To test this, the effect of dexamethasone
upon the incorporation of an amino acid was investigated using
ureido-^{14}C labeled citrulline, an amino acid expected to have a
slow turnover in the fetus. At the two doses of dexamethasone
used (Table X), there was no significant difference in the re-
covery of the label from steroid-treated or control fetuses
(P = 0.6 to 0.7 using t test). Since there was definite inhi-
bition of ^{14}C-glucose transfer at these steroid doses, the effect
appears to be relatively specific for glucose.

Steroid-receptor Complexes in Mouse Placental Tissue

All steroid-sensitive tissues examined to date have been
demonstrated to contain intracellular steroid binding protein
receptor molecules of high affinity. The correlation of receptor
binding with the in vivo activity of the glucocorticoids has
been established for cultured hepatoma cells (71, 72), lymphoid
tissue (73), and fibroblasts (74). Extensive work with thymus
tissue (65, 66, 67), has also indicated that the glucocorticoid-
specific receptors found in these tissues play a physiological
role in mediating all the metabolic effects of the steroid, in-
cluding the inhibition of glucose uptake, which has been pro-
posed as one of the primary corticoid effects (38).

If the decreased incorporation of glucose into fetuses of
glucocorticoid-treated animals were a steroid-mediated effect,

TABLE X.   Effect of dexamethasone upon the incorporation of
            labeled citrulline into SWV mouse fetuses

Dexamethasone dose	Acid-soluble counts (dpm)	Corrected counts (dpm/mg wet wt fetus)
Control	76,500 ± 9,600	25.5 ± 2.1
500 ug	89,000 ± 13,200	27.5 ± 4.4
1 mg	104,200 ± 10,200	33.4 ± 4.0

        Pregnant SWV mice were injected with dexamethasone and
labeled citrulline as described in Methods. Acid-soluble radio-
activity was determined for each fetus and figures represent
mean ± SEM for 4 to 6 fetuses from single animals.

there should be receptors involved in their mode of action. Since the transmission of material from mother to fetus occurs via placental transfer, it seemed feasible that the placenta might contain intracellular molecules which interact specifically with the glucocorticoids. An attempt was therefore made, using labeled steroids of high specific activity, to isolate a steroid-macromolecule complex from within placental cells.

A. Preliminary isolation of nuclear complexes

Homogenates of placental tissue were prepared, incubated with labeled corticosterone and the nuclear fraction isolated as described in Methods. The nuclear fraction was then eluted through Sephadex G-25 to separate the free corticosterone from that bound to macromolecules. A typical elution profile is seen in Fig. 5, with two peaks of radioactivity and a single protein peak. The sharp radioactive peak which coincided with the protein peak, occurred in the void volume, indicating an association of the radioactive corticosterone with a macromolecule. The other peak which trails, is included in the gel volume and represents unbound steroid.

B. Specificity of nuclear receptor binding

Several experiments were performed to determine if the binding in the void volume represented specific binding, possibly of physiological importance. Hormone specificity is one of the prime criteria for distinguishing between specific and nonspecific binding (93). Incubations were performed using labeled cortisol, progesterone, and cortisone to ascertain if these steroids also showed affinity for the receptor. Of the three steroids, only cortisol resembled corticosterone in being an active glucocorti-
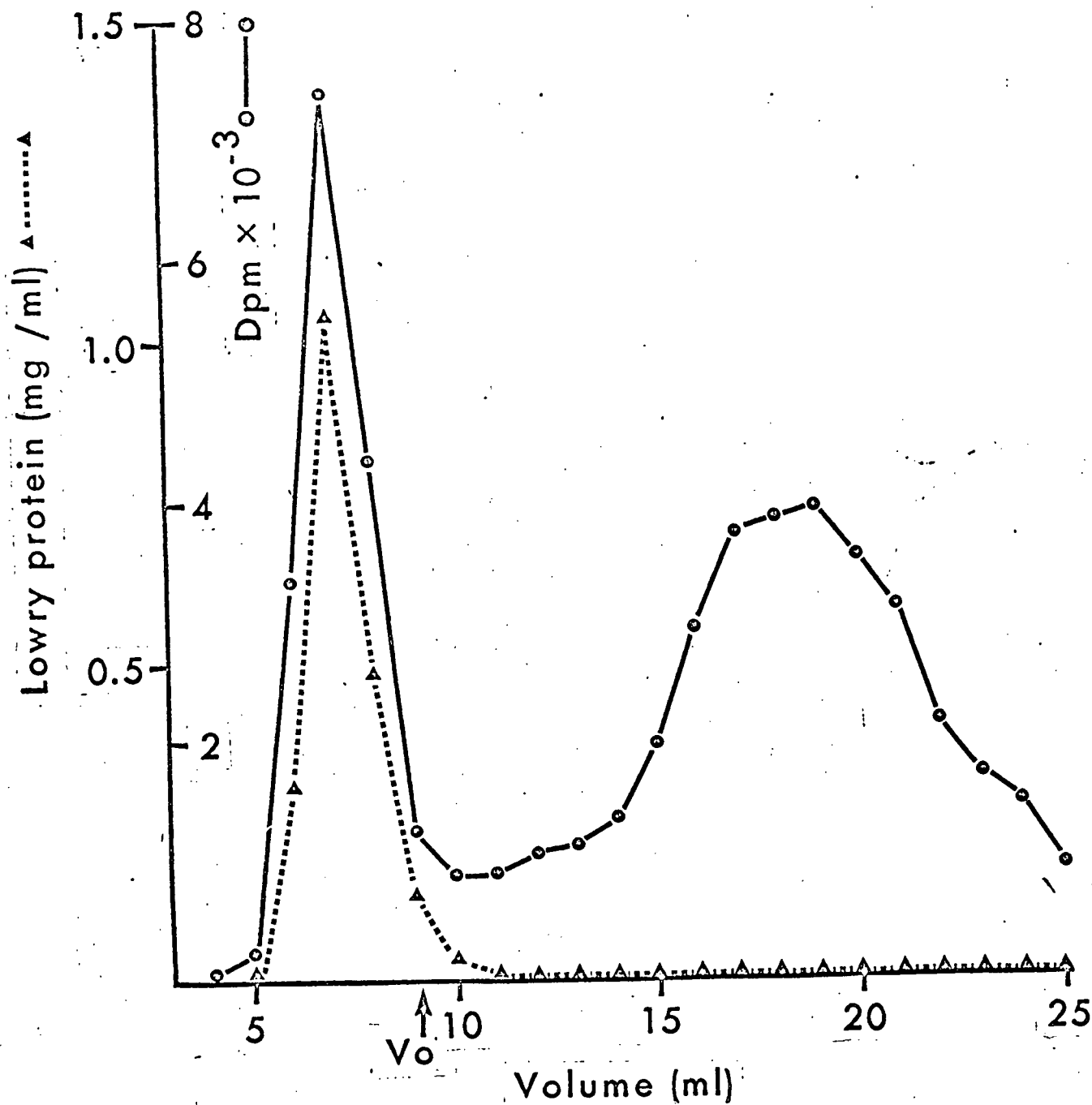
Fig. 5.   Isolation of a ^{3}H-corticosterone-receptor complex on
Sephadex G-25

A placental homogenate was incubated with labeled
corticosterone (1 uM) and the nuclear steroid-receptor
complex was then isolated as described in Methods.

coid _per_ _se_. Progesterone has no glucocorticoid activity and cortisone is only active _in_ _vivo_ by virtue of its conversion to cortisol by the 11 β -hydroxysteroid: NADP oxidoreductase enzyme. The _in_ _vivo_ glucocorticoid activity of these steroids correlated with the binding assay (Fig. 6) since only cortisol gave a radioactive profile on Sephadex similar to corticosterone. When cortisone or progesterone was used, there was no significant radioactive peak occurring in the void volume, only a peak representing the unbound steroid.

Mammalian plasma contains corticosteroid-binding globulin (transcortin or CBG), a protein which will bind corticoids and progesterone with high affinity (94). The possibility that a high concentration of this protein could contaminate the nuclear preparation and account for the specific binding was investigated. A preparation of placental homogenate was washed three times with 2 ml cold buffer before incubating with the labeled steroid. If the binding were due to contamination of the nuclear preparation with serum proteins, then the washed homogenate should show less binding of radioactivity. This was not observed since the washed homogenate, even though it contained much less protein than the control, had a higher relative binding. (Table XI).

In another experiment, 1 ml of maternal blood (which has a high concentration of transcortin) was added to the buffer with which the placental tissue was homogenized. The added transcortin, if it were responsible for the binding, would be expected to increase the specific binding of the sample. However, the sample with maternal blood added had a lower specific binding
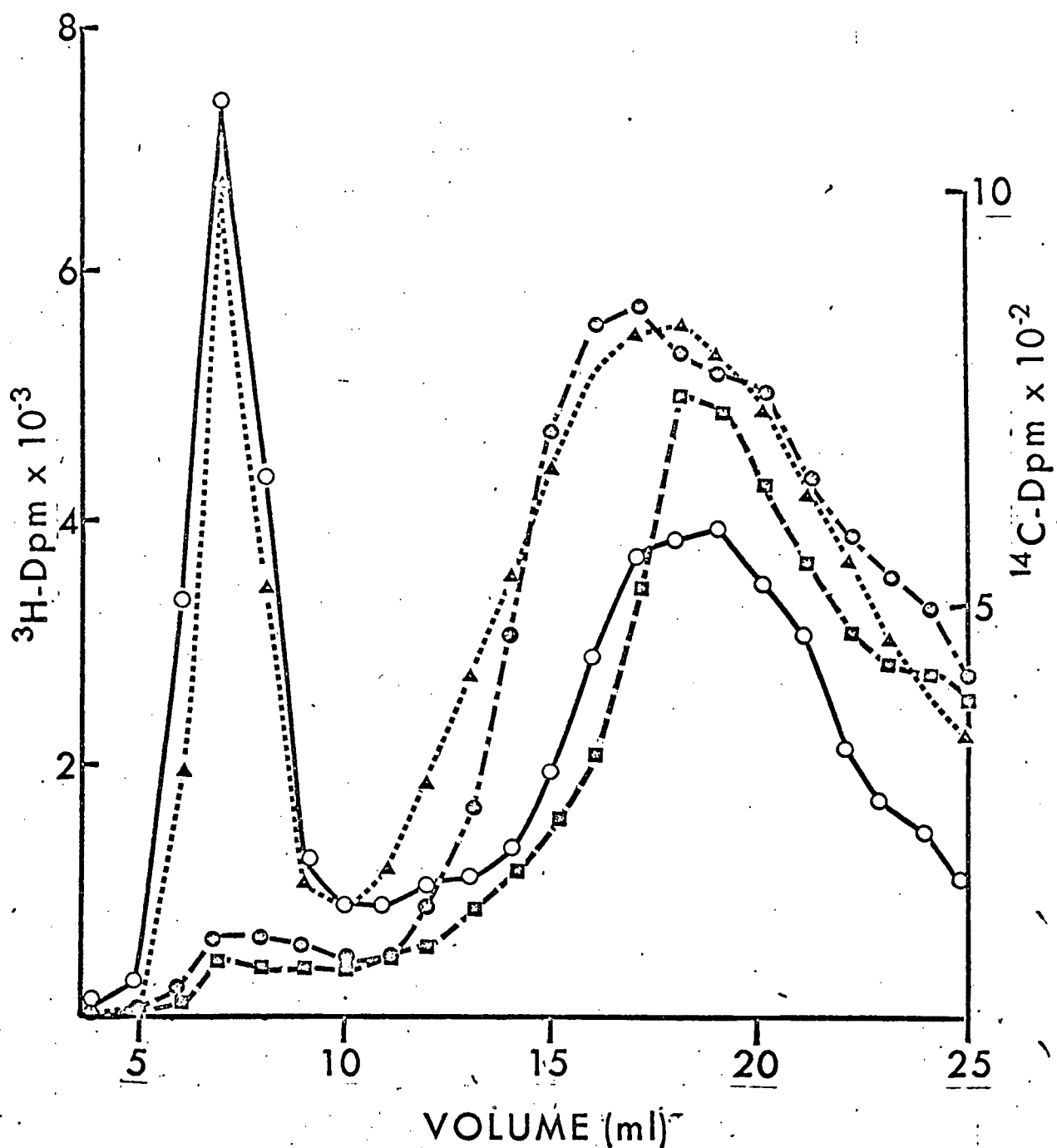
Fig. 6.   Steroid specificity in the isolation of a nuclear
complex

        Placental homogenates were incubated with the indi-
cated labeled steroid (approximately 1 uM) and the
nuclear steroid-receptor complex was then isolated as
described in Methods.

o——o ^{3}H-Corticosterone   ▣—▣ ^{14}C-Progesterone
▲······▲ ^{14}C-Cortisol   ●——● ^{3}H-Cortisone

TABLE XI.   Effect of washing and addition of blood on the
isolation of a labeled steroid complex from nuclei

Sample	Specific binding (dpm)	Protein (mg)	Relative radio-activity (dpm/mg protein)
Control	14,510	2.48	5860
A (washed)	10,670	1.57	6800
Control	8,295	1.68	4940
B (blood added)	5,100	1.63	3130

The placental homogenates were incubated with ^{3}H-corti-costerone (13.8 nM) and the nuclear steroid-receptor complex isolated as described in Methods. Sample A (washed) was washed three times with 2 ml cold KEP buffer before adding labeled steroid. Sample B had 1 ml of whole blood added to the homogenate before addition of the label. Radioactivity associated with the protein peak was used as a measure of specific binding.

relative to control even though the amount of protein isolated was the same as for the control. From this data it appeared that the serum proteins do not account for the specific binding of the nuclear preparation.

Munck and Brinck-Johnsen (65) found that all the metabolic effects of cortisol on thymus cells in vitro could be prevented by addition of the metabolically inactive steroid, cortexolone, which has been shown to compete with active glucocorticoids for the specific binding sites (65). This compound was tested with placental binding and, as Fig. 7 shows, addition of a tenfold excess of unlabeled cortexolone to the placental incubation mixture resulted in the virtual elimination of the specific binding peak of cortisol. The same effect was also observed with labeled corticosterone (Table XII). The abolition of the binding peak by cortexolone is evidence that this peak represents specific binding since cortexolone competes for the specific binding with glucocorticoids in other tissues (64).

The stereospecificity of the corticosterone binding was investigated by testing the effects of addition of unlabeled cortisol and its biologically inactive epimer, 11-epicortisol. Since epicortisol has no metabolic activity as a glucocorticoid and is not an antagonist, it would not be expected to affect the specific binding, whereas cortisol should compete with corticosterone for the specific binding sites. As shown in Fig. 8, addition of the active epimer abolished the specific binding completely whereas 11-epicortisol reduced the total specific binding peak by only 7%.

Other unlabeled steroids were also used in the same manner
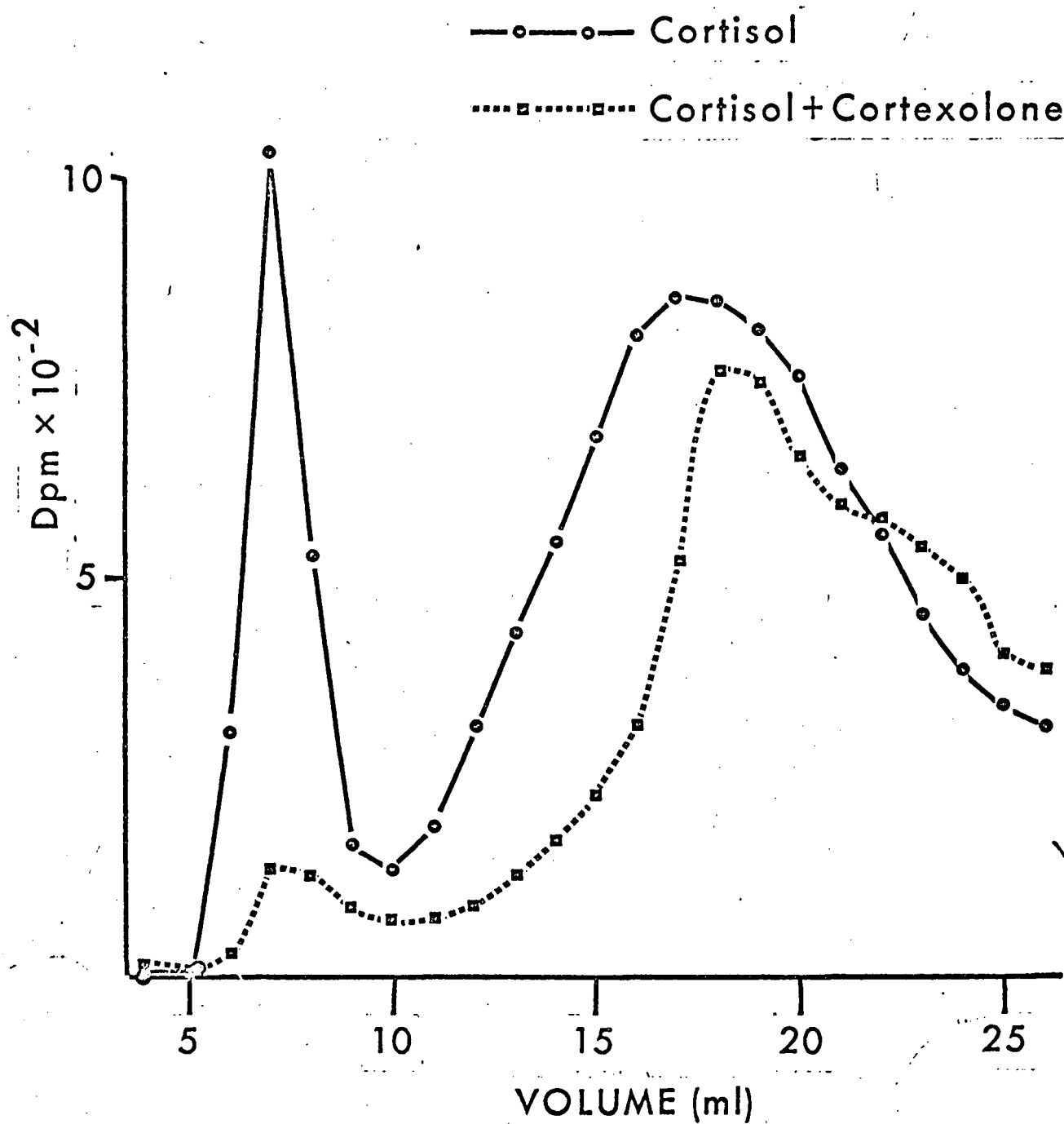
Fig. 7.   Effect of cortexolone on the isolation of a ^{14}C-
cortisol nuclear complex

Homogenates of placental tissue were incubated with
labeled cortisol (0.96 uM) alone or together with 10 uM
unlabeled cortexolone.   The nuclear steroid-receptor
complex was then isolated as described in Methods.

TABLE XII.   Effect of cortexolone on the isolation of a
labeled steroid complex from placental nuclei

Expt. No.	Labeled steroid	Cortexolone added	Specific binding (dpm)	% of control
1	^{14}C-cortisol	0	2,520	100
	(0.96 uM)	10 uM	920	37
2	^{14}C-cortisol	0	1,995	100
	(0.96 uM)	**10 uM**	350	18
3	^{3}H-corticosterone	0	17,100	100
	(1.0 uM)	10 uM	5,690	33

Homogenates of placental tissue were incubated with
labeled steroid, alone or with unlabeled cortexolone added.
The nuclear steroid-receptor complex was then isolated as
described in Methods. Radioactivity in the void volume was
taken to be associated with the macromolecular peak and used
as a measure of specific binding.

TOTAL (tubes 4-10)

	COUNTS(dpm)	PROTEIN(mg)
^{3}H-corticosterone + cortisol	1,250	2.06
^{3}H-corticosterone + epi-cortisol	11,500	2.00
^{3}H-corticosterone alone	12,400	2.10

Fig. 8.   Specificity of ^{3}H-corticosterone binding in placental homogenates

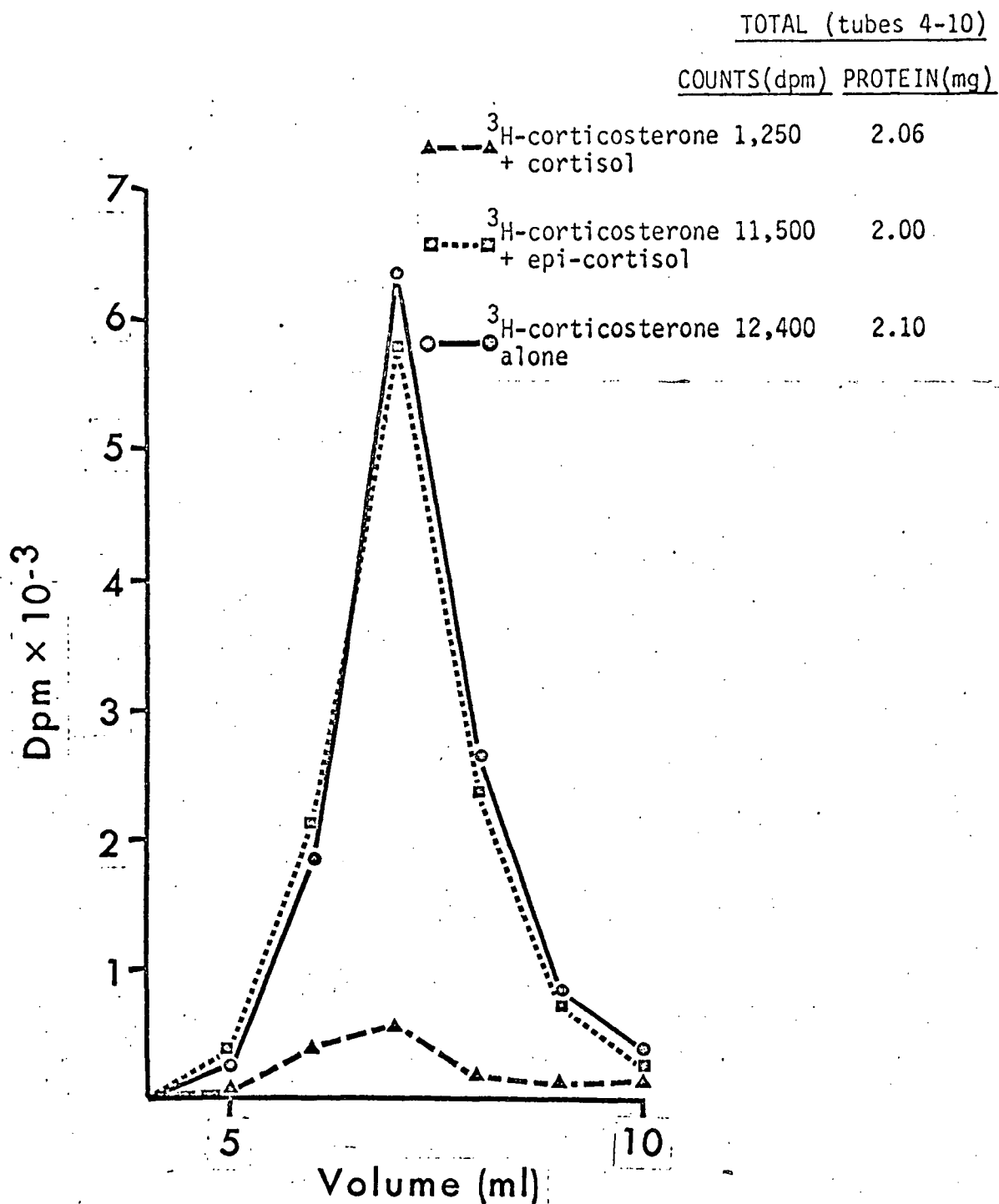Homogenates of placental tissue were incubated with labeled corticosterone (13.8 nM) alone or together with either 11-epicortisol or cortisol at 2 uM concentration. The nuclear steroid-receptor complex was then isolated as described in Methods.

to test their effectiveness in displacing ^{3}H-corticosterone from the specific binding sites (Table XIII). Corticosterone, cortexolone, cortisol, and progesterone were all about equally effective, whereas cortisone was partially effective and 11-epicortisol had no significant effect.

C.   Characterization of the bound steroid

The radioactive steroid specifically bound was analyzed to ascertain if it had been metabolized to any extent. Both ^{3}H-corticosterone and ^{14}C-cortisol were chromatographed as the extracted free steroid, or by first preparing the C-21 acetate derivative. As summarized in Table XIV, greater than 80% of the radioactivity in all cases was present in the UV absorbing zones corresponding to the added authentic carrier compounds. Further characterization of ^{3}H-corticosterone-21-acetate was accomplished by recrystallizing to constant specific activity (Table XV). These data indicate that the radioactivity bound by the nuclear receptor(s) represents the unmetabolized hormone, corticosterone (or cortisol) which was added.

D.   Isolation of cytoplasmic receptor

The isolation of a specific steroid-macromolecule complex from the nuclear fraction of placental cells then prompted the examination of the cytosol for the presence of cytoplasmic binding proteins. The current theory postulates that the nuclear receptor originates from transmission of a cytoplasmic molecule to the nucleus. Placental tissue was fractionated after incubation with the label. As seen in Fig. 9, a large proportion of the counts were in the cytoplasm (fraction C) as well as

TABLE XIII.   Competitive interaction of steroids with ^{3}H-corticosterone binding

Steroid added	Relative radioactivity (dpm/mg protein)	% of control
Control	5120 ± 250	100
Epicortisol	4940 ± 550	97
Cortisone	2190 ± 530	43
Progesterone	820 ± 210	16
Cortisol	650 ± 120	13
Corticosterone	510 ± 110	10
Cortexolone	505 ± 115	10

Homogenates of placental tissue were incubated with ^{3}H-corticosterone (13.8 nM) alone or together with various unlabeled steroids (2 uM), and the nuclear steroid-receptor complex isolated as described in Methods. Radioactivity associated with the protein peak was used as a measure of specific binding and figures represent mean ± SEM for 3 to 4 trials.

TABLE XIV.   Chromatographic characterization of labeled
steroid from isolated nuclear complexes

Compound	% total counts recovered in UV absorbing zone
^{3}H-corticosterone	80.6
^{3}H-corticosterone - 21-acetate	81.0
^{14}C-cortisol - 21-acetate	83.1

Samples of the nuclear steroid-receptor complex from
several experiments were pooled and extracted with 6 volumes
of dichloromethane. The extracts were evaporated under nitro-
gen, and where indicated, acetylation was accomplished using
acetic anhydride (95). Authentic carrier steroids were added,
and the samples were spotted onto Eastman TLC sheets. After
development for 4 hrs in a system composed of toluene: chloro-
form:methanol:water (12:6:2:0.1), the steroid zones were lo-
cated by UV absorbance. Autoradiograms were prepared, and the
UV absorbing zones were then cut out, eluted, and counted.
**The autoradiograms in all cases (except for a faint spot in
the cortisol-21-acetate chromatogram corresponding to cortisol)
showed no trace of radioactivity except for the spot corres-
ponding to the UV absorbing zone.**

TABLE XV.   Identification by crystallization of labeled
              steroid from isolated nuclear complexes

Crystallization	Specific activity (dpm/ug)	
	Mother liquor	Crystals
First	14.4	11.3
Second	19.6	12.1
Third	12.1	11.5
Fourth	11.0	11.7

^{3}H-corticosterone, isolated from pooled samples of
nuclear steroid-receptor complex by extraction with 6 volumes
of dichloromethane, was evaporated under nitrogen and then
acetylated (95). The labeled compound was mixed with a small
amount of carrier corticosterone-21-acetate and then chroma-
tographed as before (Table XIV). The corticosterone zone was
eluted and several mg of freshly recrystallized corticoster-
one-21-acetate were added to the sample in methanol. The com-
pound was crystallized four times by addition of water to the
methanol. The crystals and mother liquor were assayed by UV
absorbance at 240 nm and radioactivity determinations.

MINCED PLACENTAL TISSUE (2 gm)

1. Wash three times with 10 ml cold buffer.
2. Resuspend in 10 ml buffer and incubate
   10 min at 37°C with ^{3}H-corticosterone,
   (1 uCi).
3. Discard buffer and wash three times with
   10 ml cold buffer, then resuspend in
   10 ml buffer and homogenize.
4. Centrifuge at 1200xg for 10 min.

PELLET

1. Wash twice with 5 ml
   cold buffer and resus-
   pend in 10 ml buffer.(A)
2. Centrifuge at 1200xg for
   30 min.

SUPERNATANT

Centrifuge
105,000xg for 60 min.

PELLET   SUPERNATANT (B)   PELLET   SUPERNATANT (C)

Wash twice
with 5 ml
cold buffer.
(D)

Wash twice
with 5 ml
cold buffer.
(E)

WASHED PELLET (F)   WASHED PELLET (G)

Fraction	Total counts (dpm)	% of total recovered
A	57,000	12.0
B	5,800	1.2
C	222,000	46.6
D	3,700	0.8
E	8,600	1.8
F	55,400	11.6
G	124,000	26.0

Fig. 9. Fractionation of placental tissue

All procedures carried out between 0 and 6°C. Buffer
used in all operations is KEP buffer, pH 7.4, containing
2.8 mM glucose.

·the microsomal and mitochondrial fraction (G). When an aliquot
of the cytosol fraction was passed through Sephadex G-25 there
was found again to be a radioactive peak associated with the
protein peak in the void volume which resembled the specific nu-
clear binding. However, epicorticosterone was found to decrease
the amount of radioactivity recovered in this peak, indicating
that there was nonspecific binding present. This was tested
using the cytosol exchange assay as indicated in Methods (Table
XVI)and it was found that 200 uM epicorticosterone decreased the
amount of radioactivity recovered in the area under the protein
peak by 70%. However, the presence of the same concentration
of unlabeled corticosterone decreased it by 96%. It would be
reasonable to suppose that both stereoisomers of corticosterone
would compete almost equally for nonspecific binding sites of
the ^{3}H-corticosterone. But since only 11 β -corticosterone has
glucocorticoid activity, only this isomer should compete with
the labeled steroid for specific binding. Thus, the difference
in displacement caused by these two isomers is an indication of
the specific binding of ^{3}H-corticosterone in the cytosol ex-
change assay (74, 90).

E. Binding properties of the cytoplasmic receptor

    Using the cytosol exchange assay the time course of the
binding interaction was investigated at both 37° and 0°C. At
0° the specific binding reached a maximum by 30 min and then
levelled off. As shown in Fig. 10, the rate of association was
greater at 37°C than at 0°C since the maximum binding was reach-
ed at the higher temperature within 5 min of incubation. How-

TABLE XVI.   Specific and nonspecific binding using the cytosol
               exchange assay

Steroid added	Binding (dpm)	Protein (mg)	Relative radioactivity (dpm/mg protein)
Control	25,500	0.70	36,400
Epicorticosterone	7,700	0.72	10,700
Corticosterone	900	0.66	1,360

The cytosol fraction was prepared as described in Methods and the cytosol exchange assay carried out at $0^{\circ}C$ for 35 min. The total radioactivity associated with the protein peak was used as a measure of binding.
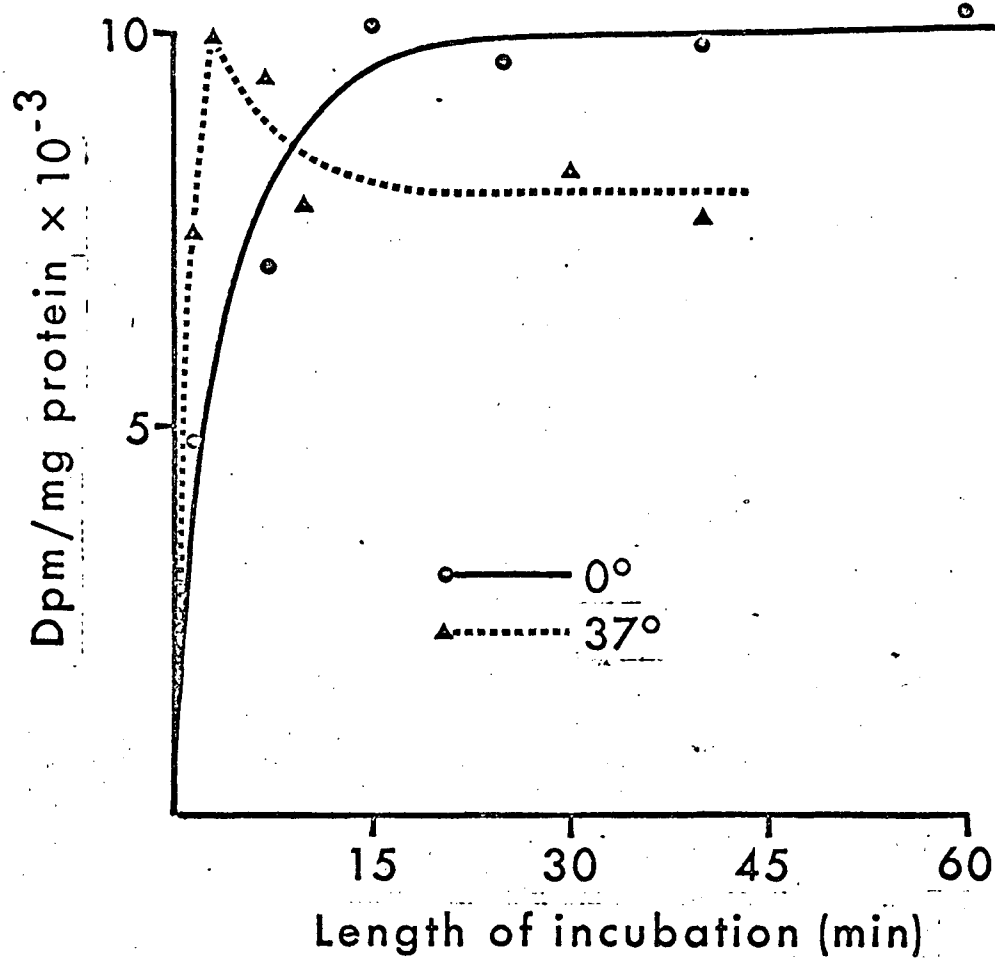
Fig. 10. Time course of binding using the cytosol exchange assay

The cytosol fraction was isolated and the exchange assay carried out at 37°C and 0°C for various times. The amount of specific binding (see Methods) was used to calculate the relative radioactivity (dpm/mg protein).

ever, at the higher temperature, the amount of bound steroid decreased after more than a 5 min incubation, implying a dissociation of the complex. For this reason further experiments using the cytosol exchange assay were performed at 0°C for 35 min to obtain maximum stability of the complex.

Since the protein concentration of the isolated cytosol fraction can vary according to variation in placental size, it was necessary to demonstrate that the amount of specifically bound steroid is related linearly to the concentration of cytoplasmic extract. This was investigated by making dilutions of the isolated high speed cytosol and then performing the cytosol exchange assay on the aliquots. The linear relationship which results (Fig. 11), shows that there is no artifact in the binding due, for example, to aggregation of protein molecules at high concentrations (72).

The effect of various treatments on this initial cytoplasmic interaction between steroid and binding molecule was examined using the cytosol exchange assay. Along with the labeled steroid, hydrolytic enzymes or sulfhydryl reagents were added and the samples incubated at 37°C for 10 min (except for the heat-treated sample). As shown in Table XVII, pronase at a concentration of 1.5 mg/ml decreased the specific binding by 90% whereas deoxyribonuclease and ribonuclease had no effect. Heat treatment also had a drastic effect on the stability of the complex but the sulfhydryl reagents had only a minor effect.

F. Kinetic examination of the binding

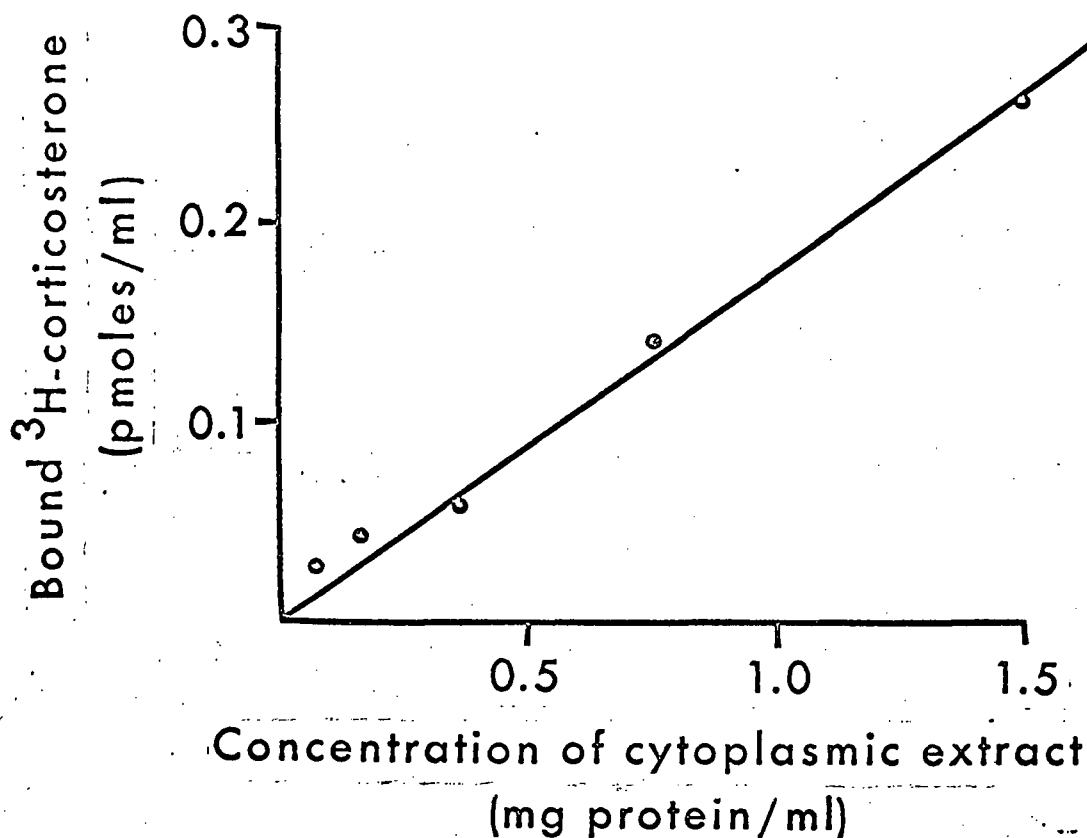A study of the kinetics of corticosterone binding to the

Fig. 11.   Homogeniety of cytosol receptor distribution

The cytosol fraction was isolated as before and various dilutions were prepared using KEP buffer (pH 7.4, with 2.8 mM glucose). The cytosol exchange assay was then carried out at 0°C for 35 min. The radioactivity recovered in the specific binding fraction was used to calculate the amount of bound corticosterone.

TABLE XVII.   Sensitivity of the cytosol steroid-receptor
               complex to heat, hydrolytic enzymes, and
               sulfhydryl reagents

Treatment	Relative activity (dpm/mg protein)	% of control
Control	8950 ± 230[a]	100
Heat (65°C, 10 min)	1010[b]	11
Pronase	890[b]	10
DNAse	9500[b]	106
RNAse	9200[b]	103
N-ethyl maleimide	8260[c]	92
p-chloromercuribenzoate	8460[c]	95
Dithiothreitol	8210[c]	92

The cytosol fraction was prepared and 50 ul hydrolytic enzyme or sulfhydryl reagent was added to 0.45 ml aliquots. After addition of the labeled corticosterone and the unlabeled competing steroids, the exchange assay was carried out at 0°C for 35 min (except for the heat-treated sample) and then eluted through Sephadex. The difference in radioactivity associated with the protein peak for the two series (see Methods) represents specific binding. The pronase concentration was 1.5 mg/ml; DNAse and RNAse, 1.0 mg/ml; and the sulfhydryl reagents were all used at 1 mM concentration.

[a] mean $\pm$ SEM of 6 samples
[b] average of 2 determinations
[c] mean of 3 samples

cytosol receptors was performed in order to estimate the total receptor content of the tissue as well as the affinity of the hormone for the receptor. This was done by preparing aliquots of the cytosol fraction and incubating them with varying concentrations of ^{3}H-corticosterone. If the amount of specific binding is determined as before using the cytosol exchange assay, then this represents the amount of bound corticosterone. The method of Scatchard (96) was used for plotting this data since a more accurate analysis is permitted by this method (97).

The results are plotted as the ratio of the bound to unbound ^{3}H-corticosterone against the amount of bound ^{3}H-corticosterone. From such a plot the number of high affinity binding sites as well as the affinity of corticosterone for these sites can be obtained. In the equation:

$$B/u = 1/K_d \quad (n - B) \qquad (i)$$

B represents bound ^{3}H-corticosterone, u is unbound ^{3}H-corticosterone, K_d is the dissociation constant for the steroid-receptor complex, and n represents the total number of high affinity binding sites specific for ^{3}H-corticosterone. The reciprocal of the slope will therefore represent the dissociation constant ($-K_d$) and extrapolation of the slope to the abscissa will give the value for n directly.

The Scatchard analysis of the binding assay using ^{3}H-corticosterone revealed a plot with two components (Fig. 12). Slope II, which has low affinity and an almost infinite number of binding sites, represents nonspecific binding which results when high

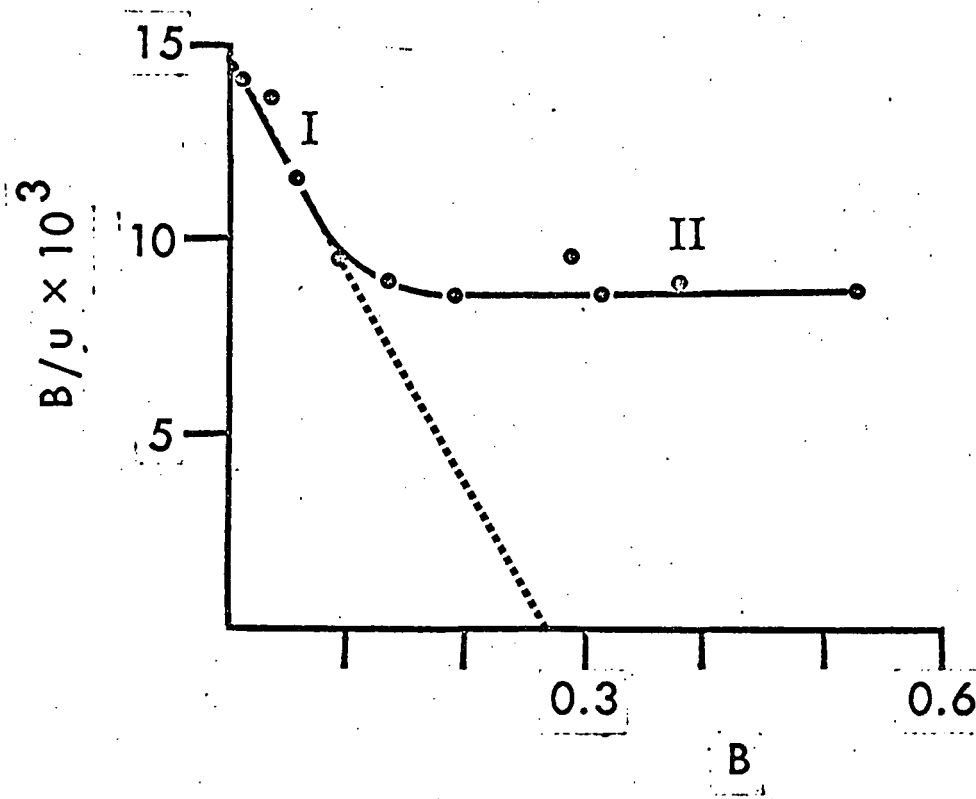Fig. 12.   Scatchard plot of ^{3}H-corticosterone binding in SWV
            mouse placental cytosol

      The cytosol fraction was isolated and increasing
concentrations of labeled corticosterone were added to
aliquots. The exchange assay was then carried out at
0°C for 35 min. The radioactivity recovered in the
specific binding fraction was used to calculate the
amount of bound corticosterone (B).

concentrations of ^{3}H-corticosterone are used. The steeper slope (I) most likely represents high affinity specific binding with a limited number of receptor sites. Using the extrapolated slope (I), the calculated Kd = 17.5 nM and n = 0.26 pmoles/mg protein. These values compare favorably with those obtained by other workers using similar systems. Rousseau, Baxter, and Tomkins (72) using cortisol and cultured hepatoma cells obtained values of Kd = 11.0 nM and n = 0.63 pmoles/mg protein. Ballard and Ballard (98) using dexamethasone with rabbit placental tissue reported Kd = 3.7 nM and n = 0.26 pmoles/mg protein. The latter report, which appeared while this work was in progress, is the only reference in the literature pertaining to the isolation of receptors from placental tissue.

G. Sucrose density gradient analysis

A sucrose density centrifugation of the cytosol steroid-receptor complex was done to identify the number of binding components present in the specific binding fraction from Sephadex. A crude estimate of the molecular weight for the receptor can be made using the method of Martin and Ames (99). The gradients were prepared using a method modified by Nagy (100) in which the linearity of density of the sucrose gradient was checked by determining the refractive index of each fraction. If the gradient is linear, then there will be linear migration by the molecules and for molecules of roughly the same partial specific volume, the following equation will be true:

$$\frac{d_{unknown}}{d_{standard}} = \left[\frac{MW_{unknown}}{MW_{standard}}\right]^{2/3} \qquad (ii)$$

In the above equation, d is the distance the molecule has migrated from the top of the gradient and MW represents the molecular weight (99). Using this equation and the radioactivity profile for the sedimentation as shown in Fig. 13, the molecular weight of the cytoplasmic receptor was estimated to be approximately 55,800.

The sucrose density centrifugation also demonstrated the presence of a single binding peak which was abolished by the addition of excess unlabeled dexamethasone or corticosterone. The fact that dexamethasone will compete with ^{3}H-corticosterone for binding on the receptor is significant. Corticosteroid-binding globulin has been shown to have no affinity for this synthetic corticosteroid (72). The complete abolition of the binding peak by dexamethasone, therefore, provides further evidence that these binding components are clearly different from plasma transcortin.

H. Relating the presence of placental receptors to the inhibition of glucose transfer

An attempt was made to interrelate the two areas of experimental work in this study. Transport across a tissue can be considered as an extension of uptake by cells of the particular tissue (101). Therefore, inhibition of uptake by placental cells could lead to inhibition of transport of the molecule under study. If glucose uptake by placental tissue is inhibited due to glucocorticoid interaction with receptors resulting in a response from the nucleus, then inhibition of this response will abolish the steroid effect. However, if only the maternal hyperglycemia is inhibited and not the effects on fetal uptake of glucose, then the recovered counts in the treated fetuses would be expected to

Fig. 13.   Sucrose density centrifugation of the cytosol steroid-receptor complex isolated from Sephadex chromatography

Sucrose gradients of 5 to 20% in KEP buffer (pH 7.4, with 2.8 mM glucose) were prepared with a Beckman gradient former (100). The cytosol fraction was prepared and incubated with labeled corticosterone (13.8 nM) alone or with unlabeled steroids (see below), for 35 min at 0°C. The samples were then run through Sephadex G-25 at 6°C to obtain the fractions eluting in the void volume. Samples of these fractions (0.2 ml) were layered onto pre-cooled sucrose gradients. The gradients were centrifuged at 4°C for 29 hrs at 284,000xg in a Beckman L2-65B ultra-centrifuge using a SW 40 Ti swinging bucket rotor.

Marker proteins, 1.5 mg in 0.2 ml of KEP buffer (pH 7.4, with 2.8 mM glucose), were layered onto sucrose gradients and centrifuged along with the samples. The markers used were human γ globulin (γ G) and bovine serum albumin (BSA) with molecular weights of 160,000 and 67,000.

After centrifugation, fractions were collected by piercing the bottom of the cellulose nitrate tube containing the gradient with a Beckman manual fraction collector. A flow rate of 1 to 2 drops per second was maintained and a total of 20 fractions, each containing 22 drops, was collected into scintillation vials or test tubes. Determinations for radioactivity and Lowry protein were assayed as indicated in Methods.

Epicorticosterone,   200 uM   o——o

Dexamethasone,   200 uM   ▲······▲

Corticosterone,   200 uM   ■——■
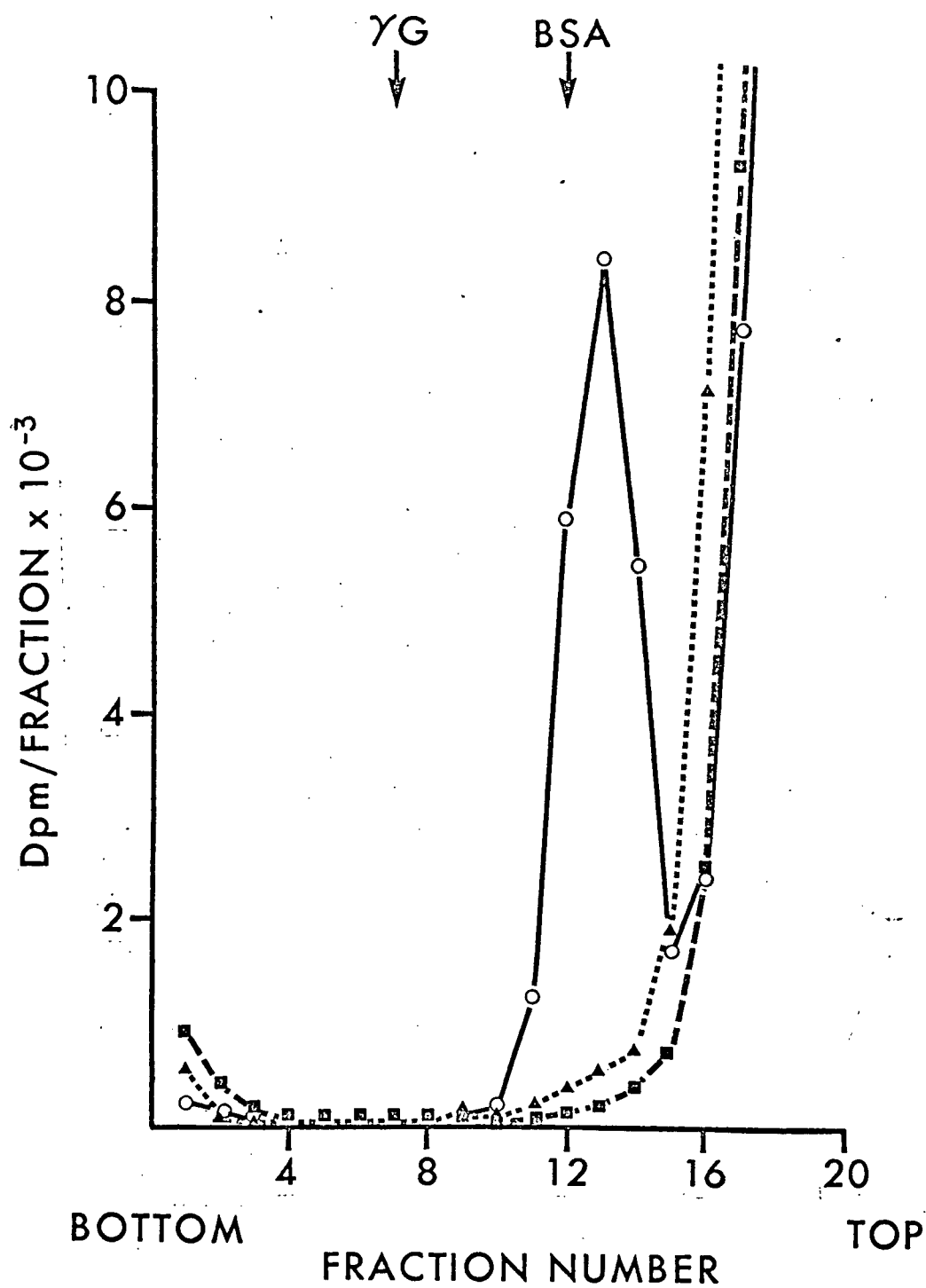
be much lower (see Fig. 2). Injection of actinomycin D just be-
fore administration of dexamethasone resulted in recovery of
counts in the treated fetuses equivalent to untreated controls
(Table XVIII). This indicates that all effects of the steroid
were prevented by the actinomycin D and suggests that the steroid-
induced inhibition of glucose uptake requires a nuclear response
which is sensitive to metabolic inhibitors.

The competing steroid, cortexolone, was also administered
in an attempt to block the effect of dexamethasone in vivo. How-
ever, treatment with cortexolone had no effect on the dexametha-
sone-induced inhibition (Table XIX). It is not known, however,
to what extent cortexolone can compete with dexamethasone and
displace the latter from specific binding sites. Thus, although
these experiments are not conclusive, there is some indication
that the processes involved show similarities to those in other
tissues where steroid-receptor interactions have been studied.

TABLE XVIII.   Influence of actinomycin D on dexamethasone-
                induced effects in pregnant mice

Mouse strain	Control	Dexamethasone alone	Dexamethasone & actinomycin D
C57BL/6J	36.0 ± 3.2(9,3)	53.9 ± 5.7(9,3)	29.5 ± 2.0(9,3)
A/J	32.3 ± 2.2(8,3)	31.9 ± 0.4(6,2)	31.9 ± 1.6(9,3)

     Pregnant mice (gestational day 18) received an i.p. injection of 20 ug actinomycin D in 0.1 ml physiological saline or saline alone. They were injected one min later with an i.p. dose of 200 ug dexamethasone suspended in 0.1 ml sesame oil. One hour later a s.c. injection of labeled glucose was made and after another 15 min the fetuses were removed, weighed, and placed on ice. Acid-soluble radioactivity was determined for each fetus and figures represent mean of corrected counts (dpm/ mg wet wt fetus) ± SEM. The figures in brackets represent the number of fetuses and animals, respectively.

TABLE XIX.   Effect of cortexolone on dexamethasone inhibition
of the incorporation of labeled glucose into SWV
mouse fetuses

Expt. No.	Dexamethasone alone	Dexamethasone & cortexolone	Cortexolone alone
1	11.3+0.4	10.8+0.4	---
2	11.2+0.5	11.0+0.5	12.2+0.4

Pregnant SWV mice received an i.p. dose of 6 mg cortexolone in sesame oil or 0.6 ml sesame oil spread over 6 injections 15 min apart. They were then treated with a single i.p. dose of 200 ug dexamethasone 20 min after the first cortexolone injection. One hour after dexamethasone treatment a s.c. injection of labeled glucose was made and after another 15 min the fetuses were removed and weighed. Acid-soluble radioactivity was determined for each fetus and figures represent mean of corrected counts (dpm/mg wet wt fetus) + SEM for 4 to 6 fetuses from single animals.

DISCUSSION

The metabolic effects of glucocorticoid hormones on liver, adipose, and muscle tissue have been studied extensively. However, very little is known concerning the influence of these hormones on fetal development or their possible role during gestation. Corticosteroid hormone levels in the mouse decrease during the first half of pregnancy but increase about thirteen-fold by the late stages of gestation (102). The passage of corticosteroids across the mouse placenta has been demonstrated (80, 81, 103), although the fetus may be protected from high concentrations of corticosteroids.

Pregnancy in the mouse, as in most other mammals, markedly increases the transcortin level (102). The binding of the corticoids by transcortin would effectively lower the concentration of free steroid, and only the free unbound corticosteroids have biological activity (102).

Also, the 11 β-hydroxysteroid: NADP oxidoreductase enzyme, present in both placenta and in fetal tissues (56), will convert glucocorticoids to their inactive 11-dehydro metabolites (103). This could explain the failure of the natural corticosteroids to elicit _in vivo_ responses except with supraphysiological doses (56, 104). By using dexamethasone, a synthetic corticoid which does not undergo extensive metabolism and is not bound by transcortin (94), acute effects upon the feto-placental unit might be more easily demonstrated.

Maternal corticosteroids normally do not affect fetal growth directly, whereas glucose is the most critical factor (105).

There is clinical evidence that maternal glucose loading has favorable effects on management of fetal distress due to placental "insufficiency or dysfunction" (106, 107). Animal experiments have also shown that hypertonic glucose can increase both fetal and neonatal survival and may also have favorable effects on the fetus under conditions of hypoxia (106).

In an attempt to explain the toxic and teratogenic effects induced by dexamethasone (Table II), the transfer of important precursors from mother to fetus was examined. From the known effects of glucocorticoids on peripheral utilization of glucose, it was expected that dexamethasone administration would result in an elevation of the maternal blood glucose level. Since under most circumstances the placental transport of glucose is a passive process dependent only on the concentration gradient, an increase in the maternal blood glucose level should result in an increased flow of glucose to the fetus. What was unexpected, however, was that inhibition of glucose transfer also occurred when the animals were treated with dexamethasone (see Fig. 2 and Table III). This inhibition, which required a higher level of dexamethasone than the induction of maternal hyperglycemia, counteracted the increased levels of maternal blood glucose at the higher doses.

Separation of these two effects was evident in the experiments where the dexamethasone was injected directly into the fetus in utero (Fig. 4 and Table VIII). At the low doses, only inhibition was evident whereas above a critical level (between 25 and 50 ug dexamethasone) the steroid presumably reached mater-

nal tissues at a sufficient level to induce hyperglycemia, just
as if the steroid had been injected into the mother.

The deleterious effects of excess corticoids which have been
observed (79), can be attributed to the reduction of glucose
available to the fetus. The inhibition by corticosteroids of
glucose uptake by peripheral tissue has been well documented.
However, steroid-mediated effects on feto-placental uptake or
transport of glucose have not previously been demonstrated. It
is possible, if corticosteroids inhibit feto-placental uptake of
glucose, to reconcile the paradoxical observations of Hoar (108)
that cleft palate in guinea pigs can be induced by adrenalectomy
of mothers (109) as well as by administration of excess corticos-
teroid. The pattern of abnormalities manifested by embryos sub-
jected to these opposite levels of adrenal activity was found to
be strikingly similar (108). In the light of the present work,
however, this could be explained as follows: adrenalectomy re-
sults in low maternal glucose levels,thereby lowering the amount
available to the fetus. An excessive level of corticosteroid,
although it would induce maternal hyperglycemia, would also re-
sult in inhibition of uptake of glucose by the fetus. Thus the
same net effect is produced on the fetus when the animals are
subjected to either hypo- or hyperadrenocorticalism: in both
cases it is the glucose which is critical.

There is a correlation between fetal and placental size in
almost every species examined (110). However, fetal growth and
placental growth do not always go hand in hand. In these cases
it may be that an increase in placental weight would not be

accompanied by any corresponding increase in its capacity to transfer nutrients to the fetus. Other factors, such as the pressure at which maternal blood arrives at the placenta, may affect both placental and fetal growth, although independently (110). Wigglesworth (111) has stated that in many cases of fetal growth retardation the primary placental abnormality has been impairment of the blood supply, followed by reduced growth of the placenta. Reduction of the blood flow in the placenta would be detrimental to the supply of many of the vital nutrients required by the fetus. Corticosteroids have been shown to reduce placental blood flow (112) which could influence placental transfer function, although no work has been done to ascertain the critical nutrients affected.

The biological role of this steroid-induced inhibition could involve control over fetal growth and development. The glucocorticoids cause increased maternal blood glucose levels which will be reflected in fetal hyperglycemia (105). Conditions of stress cause increased secretion of adrenal steroids and increased glucose being available to the fetus. Since glucose is the critical factor for fetal growth; if its entry were unrestricted, then the result could be uncontrolled growth of the fetus. This is believed to be the case in "heavy" infants of diabetics where the elevated glucose level results in accelerated fetal growth (105). If uncontrolled, this may have deleterious effects on fetal development and survival. The fetal death which resulted from dexamethasone treatment (Table II) probably represents severe inhibition since the synthetic steroid would escape the

biological controls (i.e., binding and metabolism) which normally function.

A possible mechanism whereby corticosteroids could inhibit the transfer of glucose from mother to fetus is indicated by the presence of receptors in placental tissue. The hormone specificity of the placental nuclear binding fulfils one of the criteria for receptor binding and also provides information concerning the nature of the cytoplasmic interaction. Cortisol and corticosterone interact with the active conformation of the receptor, forming complexes which are translocated into the nucleus. Progesterone, cortisone, and cortexolone in this system behave as competitors with labeled glucocorticoids for binding. However, their initial interactions must differ since cortisone and progesterone do not form complexes that can be isolated from the nucleus (Fig. 6); whereas cortexolone will apparently form a nuclear complex (38). This complex, however, has no glucocorticoid activity since it is not apparently recognized by the nuclear acceptor site(s) (113). The cortexolone therefore possesses sufficient structural specificity to bind to specific sites but cannot interact with the receptor to produce the "active complex" necessary to initiate metabolic effects. Epicortisol does not compete with specific glucocorticoid binding and therefore has no affinity for any of the conformations of this receptor system.

Corticosteroid-binding globulins have been isolated from virtually all vertebrate species examined, including the mouse (94). These proteins are present in low concentration (less than 1 uM) but exhibit a high binding affinity for the corticoids

($K_a = 10^8$ to 10^9 M^{-1}) as well as progesterone. Since progesterone does not form a nuclear complex in placenta this was one indication that the binding was not due to transcortin. The experiments with washed homogenate and with addition of maternal blood represent further checks on this point. Transcortin will compete with the receptors for binding to the labeled steroid but very little of the isolated nuclear binding could represent a steroid-transcortin complex. This data also indicates that the isolated nuclear binding represented binding to a receptor which may have physiological significance.

The presence of receptor molecules in the nucleus arises from translocation of these molecules from the cytoplasm, where the initial interaction occurs. Therefore, examination of the cytosol for the presence of these receptors is a fundamental criteria for establishing a physiological role for the nuclear binding. The binding properties of the cytoplasmic receptor indicates that the complex was unstable during long incubations at 37°C. This instability has also been reported for crude extracts from thymocytes, even at 4°C (114). Many factors such as low protein concentration, the absence of stabilizing compounds which might be present in intact cells, or the presence of proteolytic enzymes could influence the stabilization (115).

The nature of the corticosterone-receptor complex cannot be analyzed in great detail with a crude extract. However, the sensitivity of the binding to heat denaturation and proteolytic digestion suggests that the integrity of the protein is important for the binding. The fact that neither deoxyribonuclease nor

ribonuclease will hydrolyze the corticosterone-receptor complex suggests that nucleic acid has no role in the. cytoplasmic binding. The sulfhydryl reagents also seem to have only a minor effect on the cytosol binding, which is different from the effects reported for the thymocyte receptor (115).

The kinetic analysis of the binding reveals that the specific receptors can be saturated but there are also present nonspecific sites which are not saturated even at the highest concentrations of corticosterone used. This pattern of steroid binding has also been reported for thymus tissue, in which cytoplasm and nuclei both show two classes of receptors (116). The non-saturable fraction most likely represents the nonspecific binding that Munck has reported (38, 65), and is found with all steroids if used at high concentrations (62).

This was substantiated by the sucrose density analysis of the specific binding fraction from Sephadex (Fig. 13). The lower steroid concentration used, along with the lengthy centrifugation time, would virtually eliminate any nonspecific binding. The single radioactive peak, which sedimented at approximately 4S, has a similar elution profile on sucrose gradients to the glucocorticoid-binding macromolecule that Kaiser, Milholland, and Rosen have isolated from rat thymocytes (117).

Plasma transcortin appears to be ruled out as one of the glucocorticoid receptors in mouse placenta. In contrast to the tight binding of cortisol and corticosterone, transcortin does not bind dexamethasone (72, 94). Therefore, dexamethasone would not be expected to compete with labeled corticosterone for binding if

the binding component were transcortin. Since excess unlabeled dexamethasone prevents the binding of ^{3}H-corticosterone by the cytosol receptor (Fig. 13), this indicates that transcortin (as in the case with the receptor from nuclei) does not account for any of the cytosol receptor binding.

Many of the properties of this receptor from the placenta are similar to those properties reported for glucocorticoid-binding macromolecules from other tissues. Thus, there is good correlation between the in vivo hormone activity and the binding properties of steroids in this system. The sensitivity of the cytosol complex to heat and pronase dissociation and the lack of effect of deoxyribonuclease and ribonuclease also demonstrates that this cytosol receptor, as with all receptors examined to date, is at least in part protein. A notable difference in the properties, however, is the apparent lack of effect by the sulfhydryl reagents on the binding. Whether or not this has any significance in the physiological function of this receptor remains to be established.

If the glucocorticoid-induced inhibition of glucose uptake by mouse fetuses is related to the presence of cytoplasmic and nuclear receptors in placental tissue, then the two processes should respond to similar treatments. The apparent abolition of both the maternal hyperglycemia and the inhibition of fetal uptake by actinomycin D indicates that these processes require de novo nucleic acid synthesis. The interaction of glucocorticoids with target tissues has been shown to be sensitive to metabolic inhibitors. To establish this link conclusively would require

further investigation using specific metabolic inhibitors to
demonstrate that the inhibition of glucose uptake by steroids
proceeds along some chain of metabolic events similar to
other glucocorticoid-induced effects (63, 118, 119).

Since the fluorinated corticosteroids bind with much grea-
ter affinity to glucocorticoid receptors than the natural ster-
oids (117); examining the competing effects of, for example,
a 9α-fluoro derivative of cortexolone may indicate more con-
clusively the physiological significance of this receptor.
This might be accomplished by using such a compound to block
the dexamethasone-induced inhibition in vivo. The ultimate
proof to link the two processes, however, would involve isola-
tion of a specific factor induced in placental tissue via the
receptor system in a manner analogous to the induction of
specific enzymes in adult liver. If this factor then played
a role in the transport or uptake of sugars by placenta, the
role of the receptors in this action of the glucocorticoids
could be established.

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APPENDIX I

Preparation of Krebs-Eggleston Phosphate Buffer, pH 7.4

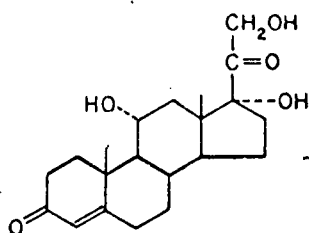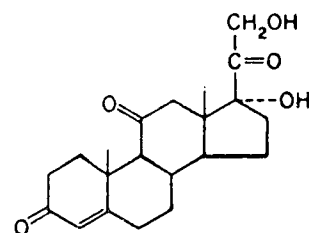| Stock solutions	Concentration (M)	Relative volume used
NaCl	0.154	100
KCl	0.154	4
$CaCl_2$	0.110	3
KH_2PO_4	0.154	1
$MgSO_4 \cdot 7H_2O$	0.154	1
$Na_2HPO_4 \cdot 2H_2O$*	0.100	12

*adjusted to pH 7.4 with 1N HCl

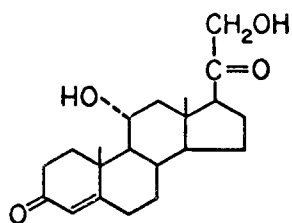# APPENDIX II.

Structures of the Steroids Used
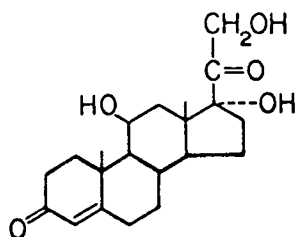
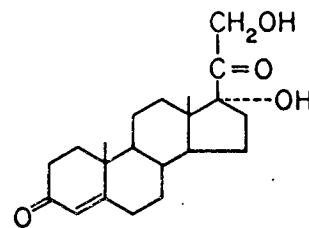

IIβ-cortisol

IIα-cortisol
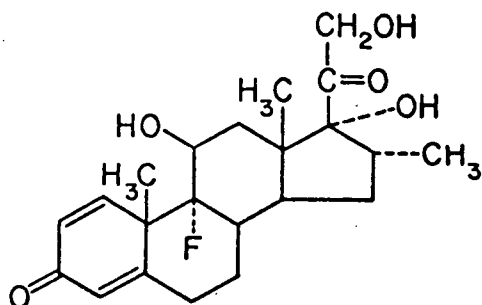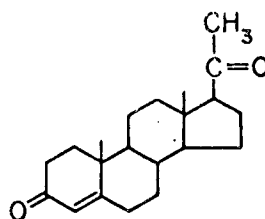

cortisone
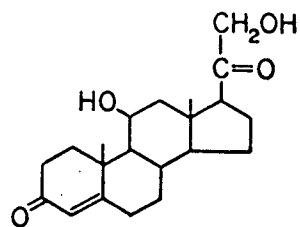
IIβ-corticosterone

IIα-corticosterone

cortexolone

dexamethasone

progesterone

APPENDIX III

Effect of Stress on Pregnancy in A/J and C57BL/6J Mice

Although there is no documented evidence for a strain difference in the susceptibility to stress between the A/J and C57BL/6J mice, there are indications that a difference exists. The "Handbook on Genetically Standardized Jax Mice", issued by The Jackson Laboratory, notes that there is a 10% difference in the number of fertile matings between the two strains (C57BL/6J higher). When pregnant mice are ordered there is also a difference in the percentage of pregnant animals in each strain. The C57BL/6J had greater than 90% pregnancy whereas the A/J occasionally arrived with less than 30% of the animals pregnant. Even though the two strains did not arrive together, there were indications that a difference in their response to stress during shipping did exist.

To test this, pregnant animals of both strains were ordered at the same time. These animals were then treated from gestational day 13 to 17, inclusive, by placing them in a shaker for 3 hrs each day and shaking them at a frequency of 180 cycles per min. The animals were then examined on day 17 for evidence of pregnancy. Out of 14 animals each, the A/J had two definite pregnancies with viable fetuses and two animals with definite indications of resorption. The C57BL/6J had five definite animals pregnant and two others with evidence of resorption. Therefore, out of 14 animals each, the A/J had a total of four possible pregnancies; and the C57BL/6J had seven possible.