SOME ASPECTS OF
GLUTATHIONE METABOLISM

by

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INTRODUCTION

Sulphur is contained in certain substances of considerable physiological importance. Some of these compounds are methionine, bile salts, heparin, vitamins such as thiamine and protein hormones such as insulin and anterior pituitary hormones.

In 1909 it was found (1) that the adrenal glands had an unusually high sulphur concentration, 3.77% of their dry residue being composed of this element. Such high sulphur concentrations are known to be found only in the epidermis and its horny derivatives. Later Lurie (2) reported that in certain experimental animals the administration of colloidal sulphur resulted in an increased storage of sulphur and cellular hypertrophy of the adrenal cortex and a marked deposition of liver glycogen. The nature of these findings remained obscure at that time.

After the discovery of glutathione by Hopkins in 1921 (3) and its later characterisation as a tripeptide, extensive work was carried out in humans and in experimental animals by different investigators, in an effort to explain the significance of its presence.

The generalised distribution of glutathione in living cells animal, plant or bacteria, is an indication that this substance may be involved in certain functions common to all cells.

A considerable amount of controversial data has been accumulated during the last decade from both clinical and animal investigative work. A possible explanation of this could be the fact that the methods
used for the determination of this tripeptide have been shown to be of a questionable accuracy.

The work of Lazarow (28) on alloxan diabetes during the last few years revived the interest in sulphur and especially in glutathione.

The demonstration in animal (23) and man (22) of a relationship between the pituitary-adrenocortical system and ascorbic acid stimulated a search for a possible relationship of this system to glutathione since both the latter compound and ascorbic acid are powerful reducing agents, occurring in the adrenal cortex in high concentrations (11).

The present work was undertaken in an attempt to accumulate some more information about the effects of certain hormones upon this sulphur-containing tripeptide glutathione, by using a more specific method for its determination, and to provide some evidence for its possible physiological significance.
HISTORICAL REVIEW

1. DEFINITION

Sulphydryl groups seem to be essential in cell metabolism. Their functions are important during all phases of cell life including a "steady state", growth and division.

Two types of thiol compounds exist, the fixed thiol groups of the proteins and the soluble thiol compounds of which glutathione is the most representative example.

Primarily this thesis deals with the soluble thiol compounds and especially with the tripeptide glutathione. In reviewing glutathione metabolism it should be kept in mind that it is impossible to distinguish, especially in certain aspects of intermediary metabolism, between actions referable to the whole molecule of glutathione, or to its SH group as such, or even to the presence of other soluble non-protein SH compounds. It would be more precise to refer to the soluble thiols, or the non-protein SH and use the term glutathione only when specific methods are used, but in this thesis we follow both terms as used in the literature up to now.

2. ISOLATION AND CHEMICAL PROPERTIES

Glutathione was discovered in 1880 by Rey Pailhade (4). It had been forgotten until 1921, when it was isolated from yeast by F.C. Hopkins (3). He showed that this substance can exist in the reduced
form* (GSH) and the oxidised disulfide form (GS-SG). Two molecules of GSH are readily oxidised to GS-SG by oxygen and the disulfide form can rapidly be reduced back to GSH.

At first it was thought that glutathione was a dipeptide but later it was demonstrated to be a tripeptide Y-glutamyl-cysteinyl-glycine (54,55,56).

\[
\begin{align*}
\text{COOH} & \quad \text{CH}_2\text{SH} \\
\mid & \quad \mid \\
\text{NH}_2\text{CHCH}_2\text{CH}_2\text{CO} & - \text{NHCHCO} - \text{NHCH}_2\text{COOH}
\end{align*}
\]

Glutathione (Y-glutamyl-cysteinyl-glycine)

Glutathione contains a sulphydryl group. It seems that this group gives to this tripeptide its biological significance. Some of the properties of the thiol groups, namely those of physiological importance, will be discussed briefly.

Thiols are substances of a high reactive capacity. One of the first properties studied of the thiol compounds was their oxidation by atmospheric oxygen (57). It had been suggested that these substances may be the mediators through which an electron transfer could take place between metabolites and molecular oxygen. Thiols are not, however, easily

* The term glutathione (GSH) usually signifies the reduced form.
oxidised by atmospheric oxygen; a metal catalyst is necessary for their oxidation (52). The catalytic power of the mediators depends upon different factors; the oxidation reduction potential of the catalyst, the pH of the environment, and the position of the SH group in the thiol molecule.

Furthermore, thiols are easily oxidised by a number of oxidising agents such as iron, copper, ferricyanide and halogens (58). On this property a number of quantitative methods have been based. Ionising radiations also rapidly oxidise thiol groups of cells (59). The soluble sulphhydril groups belong to reversible oxidation reduction systems of a very negative potential which seem to regulate energetic processes of the cell (24). Heavy metals react with SH groups to form mercaptides which are easily reversible in the presence of another thiol (58). Dithiols combine with trivalent arsenic to form cyclic compounds of great stability and on this basis BAL was introduced in therapeutics (60).

3. DISTRIBUTION OF GLUTATHIONE

Glutathione seems to be present only in the intracellular fluid as no measurable amounts can be found in extracellular fluids. It occurs in the red blood cells and this has been repeatedly confirmed by different methods (9,7,6,5). Large amounts of glutathione are also found in other tissues particularly in the liver and, according to Binet and co-workers (11) in high concentration in the adrenal gland and in the
lens of the eye (12).

Binet, using iodometric methods, found that the concentration of glutathione in the adrenal gland of the dog was 529 to 593 mg. per 100 gm. of tissue (11).

4. SYNTHESIS OF GLUTATHIONE

Glutathione synthesis represents one of the few studies using isotopic tracers in which the formation of a specific identifiable endproduct has been measured. By incubating rat liver slices in the presence of glycine labeled by N<sup>15</sup>, Bloch (13) and co-workers were able to demonstrate the formation of labeled glutathione. The amount synthesized was 0.1 to 0.2 mg. of glutathione per gm. of liver per hour. The incorporation of glutamic acid as well as glycine was obtained from an experiment (14) in which glutamic acid containing N<sup>15</sup> was incubated along with glycine containing C<sup>14</sup>. The entry of glycine into peptide linkage seems to be associated with energy yielding processes as it was shown in vitro that a stimulation of synthesis occurred upon addition of ATP. Cystine with labeled sulphur was administered by other workers (15), intraperitoneally to rats and then glutathione was isolated from tissue extracts of these animals at different time intervals. The specific activity of the glutathione in the liver was found to be greater in three hours than that of the intestinal mucosa and blood.
5. METABOLISM OF GLUTATHIONE

A. Intermediary Metabolism

Very little is known about the metabolism of glutathione itself. Glutathione seems to be formed in the liver, transported then to the kidney and intestine where it may be broken down by specific enzymes. Binkley in 1948 (17) reported the isolation of two enzymes, glutathionase and cysteinylglycinase responsible for the hydrolysis of glutathione. Cysteinylglycinase was found in all tissues other than nervous tissue, but glutathionase was found only in the kidney and intestine. Glutathionase hydrolyses the Y-glutamyl group and cysteinylglycinase hydrolyses the cysteinylglycine moiety. It was found that glutamin would operate as a coenzyme in the activation of the first step of the hydrolysis of cysteinylglycine (16). Reduced glutathione is readily oxidised by the cytochrome systems of animal tissues (25).

Recently it has been shown that animal tissues contain an enzyme referred to as glutathione reductase capable of reducing the disulfide form of glutathione (18) by means of which GSH levels are maintained constant.

B. Relations between Hormones and Vitamins and Glutathione

The study of the effects of certain hormones upon the glutathione concentration of blood and tissues and the investigations of the relationship of ascorbic acid to glutathione, has further provided some information about the role of glutathione in certain aspects of metabolism.
i) **The effect of adrenocorticotropic hormone**

The investigation of this hormone in relation to glutathione was stimulated by the fact that both seem to play a role in carbohydrate metabolism. A relation between sulphur compounds and carbohydrate metabolism has been established during the last year based upon the following experimental data:

a) the presence of a large quantity of sulphur (3.31%) found as cystine in insulin (27).

b) the selective destruction by alloxan of the beta cells of the islets of Langerhans and the action of glutathione as a protective agent (28).

c) the role of the sulphhydryl groups of certain enzymes especially related to carbohydrate metabolism (29,18).

d) the action of different substances containing sulphur upon carbohydrate metabolism and diabetic patients (30,31,32).

In this review the discussion will be limited only to the relationship of glutathione to carbohydrate metabolism.

In 1943 Dunn and co-workers reported that alloxan produces a selective necrosis of the beta cells in the pancreas (33).

Since it was known that alloxan reacted with sulphhydryl groups of protein (34) and it had been reported that blood glutathione fell when alloxan was administered (35), Lazarow investigated glutathione a naturally occurring SH compound, in the course of alloxan diabetes. In 1945 Lazarow (36,37) first reported that the injection of large doses of
Glutathione (2.0 mEq/Kg.) completely protected rats against a diabetogenic dose of alloxan (40 mgm/Kg.). This protection occurred only when glutathione was injected prior to the alloxan. Other amino acids, phosphate buffers, sodium chloride or ascorbic acid did not exert such a protecting effect, but other SH-bearing substances had an action similar to that of glutathione. Lazarow gave an explanation of these findings, which is based on indirect evidence and is of theoretical interest.

It is probable that certain enzymes of the beta cells require active SH groups (28). It has been previously mentioned that glutathione protects SH groups. Alloxan has been shown to react with thiol groups in two ways - a) by the formation of a new compound identified with an absorption spectra at maximum at 305 m\(\mu\); b) by oxidation to the disulfide form (8). It appears, therefore, that the presence of this tripeptide in the beta cells may affect their susceptibility to toxic substances. Furthermore it has been suggested that insulin, an unusual protein, containing 12% cystine (38), may require for its synthesis glutathione, so that a local utilization of this tripeptide may occur. It has been calculated that if the cysteine contained in glutathione is made available for insulin synthesis, then the synthesis of physiological amounts of insulin could deplete the beta cell glutathione (39,28).

On this basis it was suggested that low glutathione concentration within the beta cells would explain the selectivity of alloxan for the pancreas.

Lazarow extended this hypothesis in an attempt to explain
the types of "exhaustion" diabetes seen in the experimental animal with a progressive beta cell degeneration, occurring in pancreatic remnants after subtotal pancreatectomy or after the administration of anterior pituitary extracts for a prolonged period, or thyroid hormone to partially depancreatized animals.

The possibility that a similar mechanism could be postulated for human diabetes stimulated considerable investigation and speculation. The hypothesis that compounds similar to alloxan and normally present within the body concentration under "toxic" effect was further studied. The work of Griffiths (10) supported somewhat this concept.

He found that the injection of uric acid into a glutathione depleted rabbit produced diabetes. This was in agreement with previous findings that under physiological conditions an enzyme present in dog liver (42,43) can convert uric acid to dialuric acid, which is known to be the first reduction product of alloxan, and which under certain conditions can be oxidised to alloxan in vivo (44). The attempts to find alloxan in the human body have not succeeded (45) and this may be due to the extreme reactiveness of alloxan. However, recently alloxan acid, a metabolic product of alloxan, has been reported as a normal constituent of the urine (46).

Furthermore, the work of Conn introduced a new approach to the role of glutathione in diabetes in man (47,48,49,19). This was based on the following main findings:

a) in normal subjects an insulin-resistant diabetes can be
induced under continued administration of ACTH.

b) during the diabetes produced by ACTH an increase in urinary excretion of uric acid and a decrease in the blood glutathione levels occurred.

c) when glutathione was administered intravenously in an ACTH-induced diabetic state a temporary (one to two hours) decrease in blood and urine sugar levels occurred.

d) in a case of Cushing's syndrome, a diabetic type of intravenous glucose tolerance curve could be converted to normal when glutathione was administered concomitantly with glucose during the test.

These studies were a connecting link between the experimental work on animals and the steroid diabetes in man. The production of an alloxan-like metabolite from the pyrimidine portion of the nucleic acids, which are known to be metabolised in increased amounts as a result of an adrenal steroid effect (lysis of lymphoid tissue, protein catabolism) seemed possible. Lazarow further suggested (8) that under the influence of the adrenal steroids, a possible conversion of potential diabetogenic components into active ones may occur. Alloxan and dehydroascorbic acid, the oxidised form of ascorbic acid, can produce diabetes in experimental animals (50). Dehydroascorbic acid seems to possess many similarities to alloxan (51). Both of these can be reduced to non-diabetogenic derivatives, dialuric acid and ascorbic acid. It was suggested that the decrease of glutathione during the administration of adrenal steroids resulted from an altered oxidation reduction potential within the cell, which
also favors the oxidation of non-diabetogenic compounds (ascorbic acid and dialuric acid) to their diabetogenic derivatives. This hypothesis is highly speculative.

The conclusions of Conn and co-workers have not been confirmed by other workers. Their experiments were carried out in a very small number of subjects and the glutathione values found do not seem consistently or markedly abnormal. Furthermore, some contradictory findings have appeared in the literature in experimental animal work where it was shown that the administration of glutathione in moderate cortisone-diabetic rats intensified the diabetes (8). Several explanations have been offered to account for this effect. The possibility of insulin inactivation or epinephrine release through glutathione has been excluded (8).

The possibility that glutathione could protect the adrenal steroids from destruction by inhibiting the oxidation of the side chain and so prolonging their diabetogenic activity has not been ruled out. Another explanation has been offered by Anderson (8) that the administration of glutathione would promptly supply SH groups to the glycogenolytic enzyme system, previously suppressed by cortisone, causing a sharp entry of the glucose to the already diabetic animal. This mechanism seems possible as sulphhydryl groups are necessary for phosphorylase activity.

The results obtained concerning the effect of ACTH on blood glutathione levels by other investigators are contradictory.

Joiner (52) found no change in blood glutathione in 11 patients
receiving ACTH (iodometric and nitroprusside methods), in spite of a carbohydrate intolerance. Sprague et al (20), found a small change in "blood glutathione" in three patients receiving ACTH. Hess and co-workers (21) obtained in 11 ACTH-treated patients a decrease in glutathione ranging from 5 to 48% of the control value. Goldzieher (61), furthermore, obtained a drop from 3.5 to 43% in the first four hours of intravenous infusion of ACTH given at a rate of 10 I.U. for eight hours. In the intact rat no effect of ACTH (26, 40, 41) upon the blood sulphydryl level was observed.

It has been suggested by Anderson (62) that ACTH may exert many of its metabolic and clinical effects by producing a depletion of sulphydryl groups which are important for the activity of certain enzyme systems like pyruvate oxidase, coenzyme A and others.

Conn postulated that after the intravenous administration of glutathione those effects of ACTH which require free SH groups for their normal function were reversed (49).

These functions are:

a) renal tubular reabsorption of glucose

b) the systems involved in the utilisation of glucose

c) the systems responsible for the production and release of white blood cells.

Shacter (26) suggested that the effect of ACTH in retarding wound healing could be explained on the same basis. The inhibition of the skin spreading action of hyaluronidase by ACTH was thought by Anderson
to be due to a similar mechanism (63).

The pigmentation seen in Addisonian patients has also been ascribed to SH deficiency. It has been suggested that SH groups keep in equilibrium a copper containing enzyme tyrosinase, inhibiting its action on the substrate for pigment production, which occurs only when the SH groups are depleted (64).

ii) The effect of adrenal steroids

The effects obtained with ACTH upon the blood glutathione concentration, studied in relationship to carbohydrate metabolism, concomitantly stimulated the investigation of the adrenal steroids, especially of Compound E, upon the concentration of this tripeptide in blood and tissues. That the adrenal gland is involved in GSH metabolism is suggested by the findings of a high concentration of GSH in the adrenal gland (11) and adrenal vein blood and of a low concentration in the blood of Addisonian patients (65) and of adrenalectomized animals (66).

Hess et al (21) followed the glutathione levels during cortisone administration for 1 to 7 days in 11 patients and obtained a decrease from a pretreatment value of 35 mgm% to 29.1 mgm% which, however, was not significant statistically.

Sprague et al (20) obtained a slight increase in blood glutathione concentration in three patients undergoing cortisone treatment. Recently Conn and coworkers (67) found in one normal individual that the administration of 400 mgm/day of Compound F for three days produced an 11%
decrease of reduced glutathione from the baseline levels.

In the experimental animal the results obtained with the administration of Compound E or F are also conflicting.

Lazarow (8), by using normal or subdiabetic rats (alloxan pretreated rats with normal blood sugar values but decreased glucose tolerance), showed that by injecting subcutaneously Compound E or Compound F in daily doses of 5 or 10 mg, a decrease occurred in the blood glutathione level, which in some cases seemed to parallel the induced glycosuria. However, in certain cases smaller doses of the administered steroid produced a larger decrease in glutathione level without any significant glycosuria.

Shacter et al (26) studied the plasma SH concentration (using the amperometric method) after administering cortisone to intact male rats. No effect was obtained in these animals but when the hormone was administered before a stressing condition was produced (tissue injury), it prevented the fall in plasma SH, which usually follows a surgical trauma. After adrenalectomy a decrease in plasma SH occurred. It appears that the cortical hormones are necessary to maintain the normal level of plasma SH and an excess of the hormones tends to prevent the fall which occurs in stress.

iii) The effect of growth hormone

The effect of growth hormone upon the tripeptide glutathione has interested different investigators since the first crude extract was
available for experimental use.

Gregory and Goss in 1934 (68) administered to rats a growth hormone preparation (obtained by alkaline extraction of beef pituitary) in a prolonged experiment and found a marked increase of glutathione concentration in muscle tissue. Control animals treated with a heat inactivated preparation showed no change.

These authors suggested that glutathione could be used as an index of anabolic activity.

Haveroff (69) found that in nine hypophysectomized dogs there was a decrease of approximately 10% of the glutathione content of the blood. The injection of crude anterior pituitary saline extracts increased the glutathione in the red cells of normal dogs. Ennor (70), by administering different fractions of anterior pituitary extracts to normal rabbits was unable to obtain constant effects on the blood glutathione concentration.

An interpretation of these early findings based on the use of crude extracts is not permissible. No evidence has been given since to prove the validity of the statement of Gregory and Goss that an increase of tissue glutathione levels would be an index of an anabolic activity. That glutathione may play a role in protein synthesis, however, acting as an intermediate between free amino acids and proteins, has been suggested by Waelsch and Rittenberg (71).

Binkley (17), in the belief that glutathione was concerned with protein synthesis and expecting that synthetic reactions would be
associated with reactions leading to the hydrolysis of glutathione, 
investigated the enzymes responsible for its hydrolysis. However, the 
limited distribution of one of the two hydrolytic enzymes for glutathione 
led him to abandon the concept that hydrolysis of glutathione was a 
specific step for protein synthesis.

Furthermore, during the last few years Hanes and co-workers 
(72) described an enzymatic reaction in which peptides are formed by 
interaction between free amino acids and the tripeptide glutathione without 
hydrolysis taking place (transpeptidation reaction). If this is correct, 
these reactions would be of importance in protein synthesis.

The presence of the γ-glutamyl group in glutathione seems to 
stabilise the cysteinylglycine linkage so that the widespread peptidase 
cannot hydrolyse the cysteinylglycine moiety of glutathione. If the 
γ-glutamyl group is removed (either by transfer or hydrolysis) then the 
cysteinylglycine linkage is susceptible to hydrolysis. This led Hanes 
and co-workers (73) to the concept of glutathione as a "protected" primary 
peptide from which other peptides may arise secondarily. If this is true 
one would expect an increased synthesis of glutathione under the influence 
of an anabolic hormone. Some indirect evidence has been presented recently 
suggesting such an effect of anabolic hormones.

Krahl (74) studied the role of insulin in peptide synthesis 
by following the incorporation of glycine -C14 into glutathione and protein 
by liver slices of diabetic rats.

It was found that diabetes reduces radioactive glycine
incorporation into cuprous glutathione. Addition of glucose alone to the incubation medium in vitro raises the uptake in the diabetic liver slices. Insulin raises still further the incorporation of glycine -C\textsubscript{14} to a value very close to the mean normal level. Whether insulin influences peptide synthesis by a mechanism other than by stimulation of glucose uptake is not known.

The effect of pure growth hormone extracts upon this tripeptide and the possibility of the use of the glutathione concentration as an index of anabolic activity remains to be proven.

iv) The effect of insulin

The possible role of glutathione in carbohydrate metabolism has been discussed previously. A number of workers have been interested in this aspect and further studied the effect of exogenous insulin upon the blood and tissue concentration of glutathione as well as the blood values in patients with impaired carbohydrate metabolism due to relative or absolute insulin insufficiency.

In early work it was found that insulin produces an increase in liver and muscle glutathione (75) and an increase in the blood glutathione concentration of the rabbit (76) and the dog (77) which was independent of the blood sugar level.

In more recent experiments it has been found that the administration of insulin produced a significant decrease in glutathione levels of the livers of rats and an increase of the levels of glutathione in the
blood of rabbits (78).

Binkley (78) attempted to explain the insulin effect upon the liver glutathione concentrations as a stimulation of the transport of the tripeptide from the liver by the red cells. That the liver seems to be the site of glutathione synthesis has been shown by isotope studies and by the low blood glutathione concentration found in hepatectomized animals and patients with acute hepatic disease. The explanation given by Binkley is not in agreement with the in vitro finding of Krahl (74) that insulin stimulated glutathione synthesis in liver slices.

Several reports have been given upon the blood glutathione concentration of diabetic patients.

Caren and co-workers (80) have recently reinvestigated this matter. Fasting blood glutathione determinations were done in 42 diabetic patients (by the iodometric method of Woodward and Fry) and were found to be within the normal range of 25 to 41 mgm% of whole blood. No correlation was observed between blood glutathione and blood sugar levels. There was no increase in the blood glutathione level 3 to 4 hours after the administration of insulin subcutaneously to schizophrenic patients in doses as high as 100 to 580 units.

This is not in agreement with the findings of Henneman and co-workers (81) who found in 3 psychotic patients a slight elevation of blood glutathione 2 to 4 hours after insulin reactions occurred.

The findings of Caren do not support the hypothesis that low glutathione values would explain the impaired pancreatic functions in
Illing and co-workers (82) in 39 diabetic patients, found only in one patient with a severe ketosis a significant decrease in GSH. This reduction may be related to the accumulation of the ketone bodies as it has been shown by Nath (83) and co-workers that the injection of sodium acetoacetate into the rabbit, in increasing doses of 25 to 75 mgm/Kg. of body weight, lowered the blood glutathione on an average of 40-50% in 20 days.

The decrease in glutathione in ketosis may also be explained as a result of the reduction of base (sodium), as Grunert and Phillips have shown low blood glutathione levels in sodium deficient rats. It would be of interest to mention here that Binkley (84), using the method developed by him, by which glutathione and the products of its hydrolysis are measured, found in diabetic patients low blood glutathione values but a high concentration of γ-glutamylcysteine.

It has been known for some time that cysteine and glutathione inactivate insulin in vitro (85). Levine and co-workers (86), in studying insulin sensitivity in connection with glutathione concentration of tissues, found that the livers of animals known to be hypersensitive to insulin had a significantly lower concentration than the normal controls.

However, in a patient with an insulin-resistant diabetes, Caren (80) did not find high glutathione values as expected, so that insulin resistance could not be explained on the basis of an elevated blood glutathione level which inactivated insulin.
v) Relationship between the thyroid gland and glutathione

Certain experimental data which are indirectly correlated give some support to the idea of a relationship between glutathione and the thyroid gland.

It has been shown that thyroidectomy in rats decreases the frequency of alloxan diabetes (87), and that the administration of thiouracil for periods as long as 30 days markedly increases resistance to the diabetogenic action of intravenously injected alloxan (88).

Houssay and co-workers (89) have shown that the administration of thiouracil to normal rats produces an increase of the free SH groups in the liver and kidney of normal rats. This finding probably could explain the increased resistance of the thiouracil-treated animals to alloxan. Certain SH-bearing substances like thioglycerol or B.A.L. have been shown to possess a goitrogenic activity (88).

An effect of SH on the thyroid has been shown in the finding by Lawson (90) that ergothioneine exerts an antithyroid activity comparable to that of thiouracil.

Astwood (91) offered a possible explanation for the action of thiourea-like compounds based on their reducing properties. It is known that thiouracil reacts rapidly with iodine and reduces it to iodide, so that iodination of tyrosine is prevented. It is possible that ergothioneine acts in a similar manner.

No investigation has been carried out upon the blood non-protein SH concentration in thyrotoxic patients. That a diminished
availability of SH groups might permit an increased production of thyroid hormone in thyrotoxicosis seems a possible hypothesis.

vi) Relationship between ascorbic acid and glutathione

Since 1936 when Hopkins and Morgan (92) showed that reduced glutathione prevented the oxidation of ascorbic acid in vitro even in the presence of its oxidase, interest was aroused about the inter-relationship of these two powerful reducing substances in biological processes.

Both substances seem to be similar in many aspects. They are universally distributed in living cells, especially in their reduced state; their concentration is much higher in tissues where synthetic processes are more intense, such as embryonic tissues; and both are sluggish oxidation reduction systems, not oxidised by atmospheric oxygen except in the presence of a catalyst.

It has been shown by Borsook (93) that under certain conditions glutathione reduces dehydroascorbic acid to ascorbic acid but it is not quite clear if this can take place under physiological conditions.

\[
\text{Ascorbic Acid} \xrightarrow{GS-SG} \text{Dehydroascorbic Acid} \\
2\text{GSH}
\]

As the potential of glutathione is strongly negative to that of ascorbic acid, glutathione with this stronger reducing power was considered to have the function of keeping tissue ascorbic acid in the reduced state.

Certain workers tried to base an explanation of the "latent
period" seen before symptoms of scurvy appear in guinea pigs on a scorbutogenic diet, suggesting that tissue glutathione would counterbalance the functioning diminution caused by lack of ascorbic acid.

The problem of a possible peripheral reversible reaction, involving oxidised glutathione and ascorbic acid equilibrium with reduced glutathione and dehydroascorbic acid, was studied by certain workers. Frunty and Vaas (94) administered large doses of ascorbic acid orally and found an 18 to 25% depression of blood glutathione.

Anderson (62) in 5 patients with rheumatoid arthritis administered intravenously 1 to 5 grams of ascorbic acid and failed to find any change in blood glutathione levels.

Santay (95), investigating the relationship of these substances in the guinea pig, administered ascorbic acid parenterally and measured the glutathione concentration of different organs at different time intervals. He found an increase in GSH concentration of the liver, skeletal muscle and adrenal gland of these animals.

Roberts and Spiegl (96) showed that the sulphur-containing amino acids cystine and methionine, when administered to rats caused a large excretion of ascorbic acid in the urine.

The high concentration of glutathione in the adrenals (11) as well as the relation of this substance to ascorbic acid led to the investigation of the pituitary adrenocortical system upon the non-protein SH concentration of blood and adrenal tissue.

The hypothesis that ascorbic acid and glutathione may belong
to a certain oxidation reduction system which may play some part in the processes which elaborate the oxygenated cortical steroids has been attractive to certain investigators.

Goldzieher (97) recently investigated the changes on blood sulphydryl and ascorbic acid during an 8 hour intravenous infusion of 10 I.U. of ACTH (repeated for three successive days) and was unable to detect any inverse relation between these two compounds. A transitory depression of blood SH level was found in the first 4 hours, during which the ascorbic acid levels showed little change. A slight drop in the ascorbic acid blood level occurred on following days in the first 4 hours, but higher depressions on all three days were found after the 8 hours of ACTH infusion.

Interesting has been the recent finding (98) that small doses of ACTH (0.1 mgm) produced in the rat an increase in adrenal SH concentration of 45% in 3 hours, which was considered to be a specific reaction. This effect was not studied for periods of one or two hours after the injection or in hypophysectomized rats, so that any comparison with the effect on the ascorbic acid concentration is not permissible.

C. Some Actions of Glutathione in Certain Metabolic Processes

Despite extensive investigation concerning its functions, little definite is known. A number of these metabolic actions which have been attributed to glutathione are briefly discussed here.
1) Glutathione and enzyme systems

It was at first thought that the function of glutathione was to take part in oxidative processes by acting as a hydrogen carrier (53). All efforts to prove that glutathione is involved in such an oxidation reduction system have ended in failure, probably due to the low oxidation reduction potential of glutathione which is close to that of hydrogen. In spite of this some emphasis has been placed upon the possibility that glutathione may maintain enzymes in the reduced state. There are a number of thiol enzymes taking part in the oxidation of carbohydrate, fats and amino acids, in which the SH groups are essential for activity (99). If these groups are inactivated then the enzymes become inactive. The addition of glutathione restores the activity to normal, either by reducing the oxidised SH group or by reacting with any heavy metal which has been the inactivating agent. Similar is the protective mechanism of glutathione in enzyme systems.

Further, it has been shown by Barron (100) that glutathione regulates the rate of cellular metabolism. If this mechanism is abolished by using different agents to block the SH groups, then reactions proceed at a greater speed and an increase in cellular respiration occurs.

A very specific function of glutathione has been found by Lohmann (101). Glutathione is the co-enzyme of glyoxalase present in muscle and other tissues which transforms methylglyoxal to lactic acid. Jowett and Quastel (102) presented evidence for the combination of glutathione and methylglyoxal to form an addition compound, on which glyoxalase
acts. It has been postulated by a large number of works that methylglyoxal participates in the glycolytic cycle possibly through a secondary pathway, but for two reasons this idea was abandoned: i) lactic acid fermentation proceeds even in the absence of glutathione; ii) the product of the enzymatic transformation of methylglyoxal by means of glyoxalase is D-lactic acid, whereas L-lactic acid is the product of anaerobic glycolysis in muscle (103,104,105).

ii) Glutathione and cell division

It has been found by the experiments of Rapkine (106) that a definite relationship exists between cell division and the glutathione concentration.

Thiols increase before cell division takes place and mitosis can be stimulated by cysteine. In organ regeneration and embryonic developments high SH concentrations have been found by other workers (107) and these processes can be inhibited by heavy metals, known to block the free thiol groups.

Agents such as nitrogen mustards, known to inhibit mitosis, also combine with SH groups.

Interesting have been the findings of the increased sulphydryl concentration of tumor tissues compared to non-tumorous tissue (58). Shacter, by using the amperometric technique, has recently found that when lymphosarcoma was transplanted into rats the plasma glutathione concentration showed a decrease within two days (108).
iii) Glutathione and ionizing radiations

Sulphydryl groups are rapidly oxidised by ionizing radiations. Glutathione has been used successfully as an agent protecting thiol enzymes against the injurious effects of ionizing radiations. It has been shown by several workers (109,110) that pretreatment with glutathione increased the survival rate of mice and inhibited certain changes known to occur with lethal doses of x-ray.

iv) Glutathione and hematopoiesis

It has been suggested that glutathione may be specifically concerned with hematopoiesis.

Cronkite (109) found that mice treated with glutathione before irradiation showed a striking regeneration of the hematopoietic tissue in the post-irradiation period. Further, it has been found that glutathione increases the sickling phenomenon observed in red cells of patients with sickle cell anaemia (111). Ingbar and co-workers (112) have demonstrated an increase in SH values of haemoglobin in patients with sickle cell anaemia.

It has been repeatedly found that blood glutathione concentration increases at high altitudes (113). This high blood content is not due to the increased number of red cells but to increased synthesis of glutathione in the hematopoietic tissue, which may be responsible for the increased production of red cells. It may be mentioned here that in leucemic patients increased blood glutathione concentrations have been found (114).
EXPERIMENTAL WORK

1. GENERAL INTRODUCTION

As is seen from the historical review, glutathione is involved in several aspects of intermediary metabolism.

It has been established that the adrenal steroids, growth hormone, insulin, epinephrine and thyroxin have some influence on carbohydrate metabolism. However, the influence of these hormones on blood glutathione levels in correlation to the effects upon carbohydrate metabolism has not been extensively studied.

The inter-relationship of the two reducing substances glutathione and ascorbic acid in man remains to be clarified.

The recent availability of highly purified growth hormone preparation, the evidence from in vivo and in vitro experiments that this tripeptide may play a role in protein synthesis, further stimulated the investigation of the role of this compound in anabolic processes in the human individual.

The present work was undertaken as an attempt to accumulate more information on the effects of certain of these hormones on the sulphur-containing tripeptide, glutathione, by using a more specific method for its determination. The hormones used in these studies are those known to have an effect upon carbohydrate metabolism and those which have been shown to be anabolic in the human individual. Short-term experiments were chosen as most suitable in some cases in order to
study time response relationships.

2. METHODS: DETERMINATION OF THE SOLUBLE SULPHHYDRYL COMPOUNDS

A. Determination of Glutathione

(1) The manometric method developed by Woodward (115) is the only specific one for the determination of reduced glutathione. It is based upon two facts: a) Glutathione is a specific activator of the enzyme glyoxalase, which catalyses the addition of water to methylglyoxal with the formation of lactic acid. Neither cysteine, thioneine, ascorbic acid nor oxidised glutathione produce any activity. b) The velocity of reaction is a function of the concentration of glutathione as long as this concentration is within certain narrow limits (101,116).

This method is not applicable to clinical investigation owing to the complexity of the apparatus and technique, and also to the difficulty in doing a large number of determinations.

(2) When sodium nitroprusside reacts with sulphhydril groups a purple colour is formed. This colour is stable only for 15 seconds and the reaction is of limited quantitative value for the estimation of glutathione.

Grunert and Phillips (117), however, found that the rapid fading of colour was prevented by a trace of the cyanide ion, which at the same time did not cause any reduction of the oxidised glutathione. They established the optimum concentration of reagents used and confirmed that the intensity of the colour decreased with increasing temperature.
They advised that the estimations be carried out at a standard temperature of 20°C.

The addition of 53.3 µg of glutathione to whole blood, lysed blood or precipitated blood resulted in recoveries ranging from 87 to 108%. When glutathione was added to the medium for homogenising rat liver, the recoveries in six samples ranged from 94 to 104%.

Thompson and Watson (118) modified the procedure by certain changes in the reagents, including the use of ammonium hydroxide to replace sodium carbonate as the final step in colour development.

Disadvantages of the method

a) As mentioned the method is sensitive to changes of room temperature, so that one should carry out the method only under carefully controlled temperature conditions. If not, one should perform standard curves of glutathione solutions for each set of determinations.

b) Certain time factors have to be taken into consideration especially if one uses the modifications of Thompson and Watson:

i) readings have to be done within 30 seconds after the addition of NH₄OH. As the rate of fading of the colour up to 50-60 seconds is sufficiently low, readings in 30 seconds permit reasonable accuracy.

ii) if NH₄OH is not added immediately after the addition of nitroprusside reagent the colour intensity produced is low.

c) Interfering substances: There is a slowly developing nitroprusside reaction given by aceto-acetic acid. Amounts of aceto-
acetic acid equivalent to blood levels of 4 mg/100 c.c. give no measurable colour at 30 seconds, although by 90 seconds a significant degree of colour develops. Amounts much above this level would interfere at 30 seconds. This eliminates the use of this method for glutathione determinations in diabetic patients.

(3) Several methods have been developed using halogens as oxidising agents. Tunnicliffe (119) introduced the iodometric titration as a quantitative procedure for estimating SH groups. Perlzweig and Delrue (120) introduced the method of back titration of the iodine with thiosulfate after the addition of potassium iodide, followed by iodine solution in known excess. These authors have shown that potassium iodide stabilises the iodine-starch blue colour. These methods and their modifications are the ones which have until now been most widely used in clinical investigation.

Disadvantages of the methods

a) The oxidation of cysteine by means of iodine can proceed by two different pathways as shown by Okunda (121) so that the iodine taken up may vary from 1 to 6 atoms per molecule of cysteine.

Bierich and Kalle (122) described the "dilution effect" (Verdünngs factor) by which they meant that the results depend upon the iodine:glutathione ratio, owing to oxidation beyond the disulfide stage. The same problem was studied by Kuehmnau (123) and Hartner and Schleiss (124) and they pointed out that it is impossible to regulate the
experimental conditions so that any of the reactions represented by the following equations is predominant:

i) \(2R-SH + 2I = R-S-S-R + 2HI\)

ii) \(2R-SH + 6I + 3H_2O = RSO_3H + 6HI\)

iii) \(R-S-S-R + 10I + 6H_2O = 2RSO_3H + 10HI\)

b) These methods are not specific for SH groups as a number of other substances like ascorbic acid are likely to interfere. This fact makes iodine methods useless for the determination of glutathione in tissues, especially the adrenal, or in studies of the inter-relationships of glutathione and ascorbic acid.

Another halogen used is bromine. Hartner and Schleiss (124) found that reduced and oxidised glutathione react quantitatively with nascent bromine to produce the corresponding sulpho acid.

They used as adsorptive precipitating agents of glutathione, cadmium hydroxide and silver chloride.

In this method all the reduced glutathione is oxidised to the disulphide form before it is oxidised by bromine. This method is unsatisfactory because silver lactate, one of the reagents used as the basis of a colorimetric measurement, is extremely sensitive to light (125). Furthermore, cadmium hydroxide precipitation is not specific as it also precipitates uric acid, ascorbic acid and phosphorylated sugars.
B. Determination of Cysteinyl-glycine and Cysteine

Since 1937 Schroder and Woodward (126), and later others, studied the hydrolysis of glutathione by enzymes of animal tissues.

Nakamura and Binkley (127) described a method using hydrolysing enzymes formed in the kidney, followed by the method of Sullivan and Hess (128) for the determination of the hydrolytic products of glutathione.

Disadvantages of the method

This colorimetric method is unsuitable in the presence of ascorbic acid, epinephrine or thiourea as they interfere with colour development. Colorimetric readings must be made as quickly as possible and the method is not very sensitive, the range within which accurate results may be obtained being between 30-120 mg/l.

C. Determination of Ergothioneine

The earlier quantitative methods for ergothioneine were based on the use of its reducing action on the arsenophosphotungstate reagent (129). Hunter (130) reported that, in the presence of strong alkali, the coupled product of ergothioneine with diazotized sulfanilic acid yields a characteristic colour. This reaction, which is sensitive for ergothioneine appears to be the most suitable basis for a quantitative method. Touster (131) proposed a method based on the oxidation of the
sulphur of ergothioneine to sulfate by means of bromine, but this suffers from a lack of specificity. Melville et al. (132) recently devised a quantitative method for blood ergothioneine determination based on the treatment of laked red cells with hydrosulfite and glutathione, precipitations of proteins with trichloroacetic acid, removal of interfering substances by extraction with chloroform and adsorption on an ion exchange resin, and finally applying a modification of the diazotization reaction described by Hunter (130).

The advantages of this method are the sensitivity at low ergothioneine levels, and its usefulness for ergothioneine determinations in tissues (liver, heart, kidney) with greater certainty than with other methods.

D. Determination of Total Non-Protein Sulphydryl Compounds

In 1946 Kolthoff and Harris (133) devised a method of titrating mercaptans in aqueous alcoholic solution with silver nitrate by using a modification of the amperometric titration method. The method is based on the principle that the end-point of the titration is recognised by the production of a diffusion current measured by a galvanometer. In this application during the titration, the SH groups combine with silver and as undissociated silver mercaptide is formed the current is zero until the end-point. After the end-point is reached, there is an excess of silver ions in the solution and the diffusion current of silver is measured by a sensitive galvanometer, this current being
proportional to the concentration of silver ions. When the current readings during the titration are plotted against the volume of reagent added, two straight lines are obtained which intersect at the end-point. The reaction equation is:

\[ \text{Ag}^+ + \text{RSH} = \text{RSAg} + \text{H}^+ \]

or

\[ \text{Ag(NH}_3\text{)}_2^+ + \text{RSH} = \text{RSAg} + \text{NH}_4^+ + \text{NH}_3 \]

Kolthoff and Harris, by using a rotating platinum electrode as the indicator electrode and not applying any electromotive force to the cell, considerably simplified the performance of an amperometric titration.

Benesch and Benesch in 1948 (134) altered this technique introducing it to the determination of SH groups in serum protein, and Weissman-Schoenbach and Armistead (135) used this method in a study of the sulphydryl content of albumin and globulin fractions of normal sera.

Rosenberg, Perrone and Kirk (136), by using a vibrating platinum electrode, were able to determine with reasonable accuracy amounts of sulphydryl as small as about 1 microgram.

Benesch and Benesch (9) further applied the method for the determination of soluble mercapto groups (glutathione) in blood and tissues and in 1952 Bidmead and Watson (138) used the method somewhat altered.

Recently Goldzieher (139,97) introduced several modifications increasing the simplicity and specificity of the amperometric titration.
of SH groups so that it could be easily used for clinical investigation.

This method was chosen as the most suitable for the experimental work planned for the following reasons:

i) the amperometric method being specific for SH groups would permit a study of the inter-relationships of glutathione and ascorbic acid of blood and tissue under the influence of certain hormones. The methods depending upon titrations with oxidising agents were not suitable since these determine both substances.

ii) amperometric titration for non-protein SH groups is not specific for glutathione as such, but other SH-bearing substances like cysteine and ergothioneine which occur in blood and tissue filtrates, are present only in very small quantities and so this method is more specific than any other.

iii) the accuracy of the method according to several workers (9,138,97) is 1-2%.

iv) the procedure is not time consuming, so that it is feasible to carry out a large number of determinations.

v) filtrates prepared for titration are stable for several hours (97), so that one is not limited by certain time factors as in the colorimetric methods.

Apparatus and materials

The apparatus follows the design of Kolthoff and Harris (133) with certain modifications and consists essentially of a rotating electrode
a reference half cell, a salt bridge, a microammeter or galvanometer and a microburette.

**Rotating electrode**

Rotating electrodes were preferred to the stationary platinum wire microelectrodes for two reasons:

i) one is obliged with the stationary electrodes to wait at least 2 minutes after each addition of reagent until a steady state of current is reached.

ii) the diffusion currents measured are much smaller than those measured with the rotating electrodes.

The electrode consisted of soft glass tubing about 15 inches long and 6 mm in diameter, bent to a right angle approximately 1 cm from one end. Into the angle piece a 19-gauge platinum wire was fused, projecting 1/2-inch beyond the glass. The other end of the wire remained inside the glass tube. Dekhotinsky cement was occasionally used for sealing the end of the electrode, but it is not essential. Contact with the platinum wire is made by a column of mercury poured into the glass tube. The glass electrode is fitted into a hollow shaft of a motor.

An ordinary stirring motor was used to rotate the electrode. A simple reduction gear was used to maintain a constant rate of electrode revolution. As this rate is small, 130 r.p.m., and a titration completed within a short time, there is not sufficient variation in speed to affect the output current so that a synchronous motor as originally used by Laitinen and Kolthoff (140) is not necessary. A rheostat to control the
speed of rotation and a constant voltage transformer were also not used, as it was found under the conditions mentioned that the rate of revolution was constant during a single titration or after a considerable number of titrations.

**Reference half-cell**

As the titration is carried out in an ammoniacal medium the potential of the saturated calomel electrode is not negative enough to yield the diffusion current of the amino-silver ions, therefore Kolthoff and Harris (133) used another reference electrode, a mercury mercuric iodide cell, having a potential of -0.23 volt relative to the saturated calomel electrode. A special type of glass container was made up as shown in figure 1. The electrolyte solution is prepared by dissolving 4.2 gm. of potassium iodide and 1.3 gm. of mercuric iodide in 100 ml. of saturated potassium chloride solution. A layer of mercury serves as the electrode and sufficient should be added to give a maximum surface area in order to prevent polarization.

**Salt bridge**

Electrical connection between the supporting electrolyte and the reference electrode is made by a salt bridge consisting of about a two-foot length of transparent plastic tubing (Tygon) 7 mm. outside diameter, filled with saturated potassium chloride solution.

At all connections the outer surfaces of the tubing are greased to prevent "creeping" of the potassium chloride, as this may cause other electrical potentials which would interfere.
The end of the salt bridge which projects into the supporting electrolyte solution consisted of a glass tubing (A) 7.5 inches long and 10 mm. inside diameter (figure 1).

A coarsely sintered glass disk was attached at the lower end of the glass tube and the whole tube filled with a gel of agar 4% and saturated potassium chloride. Great care must be taken in filling this tubing with potassium chloride to avoid inclusion of air bubbles which would be sources of high resistance.

For further protection of the solution from contamination with iodide, the glass tube (A) was enclosed in a larger glass tube (B), 23 mm. in diameter and 6.5 inches long, having a fine sintered glass plug at the lower end and filled with electrolyte solution (C). This electrolyte solution was rinsed out and replaced after several determinations.

Galvanometer

Initially a Beckmann pH meter (model G)* was used as a galvanometer. Later, however, this was replaced by a more sensitive instrument, a Leeds and Northrup D-C galvanometer of a sensitivity of 0.00035 microamps per scale division. A variable shunt was used with this galvanometer to reduce the sensitivity to a constant level of 0.0035 microamps per scale division.

Following Goldzieher's suggestion (139), two variable resistors of 4,000 and 50,000 ohms were included in the circuit (figure 2). These

*As a sensitive galvanometer was not available.
permitted alteration of the critical damping resistance and compensation for variations in the response of the rotating platinum electrode. By this means it was possible to maintain the response of the platinum electrode so that 0.1 ml. of an 0.001 N AgNO₃ solution caused a constant deflection of 15 to 20 galvanometer scale units.

Micro-burette

A Koch microburette of 5 ml. capacity calibrated in 0.01 ml. was used. The glass stoppered reservoir was removed and the free end was connected with an automatic dark glass reservoir. The outlet tip was bent to a slight angle for more convenient use.

As these microburettes deliver small quantities very rapidly, the speed of delivery was decreased so that drops of 0.01 ml. would be delivered when the stopcock was fully open.

This was achieved by controlling the atmospheric pressure at the upper end of the burette as follows: A rubber pressure tubing (figure 3 (A)) connected the upper end of the microburette to a glass "Y" tube, the other limbs of which, thin walled rubber tubes, were attached (B and C). Tube B was freely open to the atmospheric pressure or clamped when in use. Tube C was passed through the inner part of the jaws of a small water bath clamp (D) from which the adjustable screw was removed, (upper part of figure 3 - front view). To the free ends of the jaws a micrometer caliper was attached by means of which the jaws could be compressed, to gradually occlude the lumen of the rubber tube. To the other free end of tube C a Caulfield Pipettor was connected.
Mode of use

During filling of the microburette with the titrant solution from the automatic reservoir tube C was clamped by the micrometer and tube B was open so that there would be a connection with the atmospheric pressure. The burette was filled above zero point. The clamp at B was then closed and the stopcock opened. A small delivery took place of 0.3-0.6 ml. until the pressure in the now closed system was sufficiently reduced to prevent continued flow of the titrant solution. For zero adjustment the stopcock was closed, the piston of the Caulfield pipettor compressed and the micrometer gradually opened. Then on opening the stopcock there was a slow delivery of reagent which could be terminated when zero marking was reached.

A total volume of 0.09 to 0.12 could be delivered for each compression of the piston. For a further delivery one has again, after closing the micrometer, to press the piston and gradually open the micrometer caliper. Readings were made on the scale of the microburette.

Assembly of the apparatus

The motor was mounted on a separate stand connected to the wall, to avoid any vibrations which would be transmitted to the galvanometer. The microburette was fixed on a movable burette holder so that one could adjust the titrant solution to zero at a distance and then approach the titration beaker as closely as possible to the rotating electrode without interfering with its movement. The stationary electrode of the salt bridge was mounted on a movable iron rod to facilitate
By means of a wire making contact with the mercury, the rotating electrode is connected to the positive terminal of the galvanometer and the reference electrode is connected to the negative terminal. The mode of connecting the galvanometer with the variable resistors is given in figure 2.

Reagents

1. Saponin (0.01% in distilled water). This solution is used for producing complete hemolysis. It is known that blood glutathione is contained in the corpuscles and this has been confirmed by Benesch and Benesch by the amperometric method (9).

2. Sulfosalicylic acid (25% in distilled water), as a protein precipitant. For the colorimetric ferricyanide method developed by Mason (137), tungstic acid was used at first. It is well known that glutathione undergoes autoxidation at a neutral or alkaline pH and even at slight acidity. Experiments carried out by Woodward and Fry (141) showed that glutathione is rapidly lost from the slightly acid tungstic acid filtrates by autoxidation. Later trichloracetic acid was used for glutathione determinations by Tunnicliffe (119). Bierich and Kalle (122) found in 10% trichloracetic acid filtrate that there was a loss of 5% in 3 hours. This slow autoxidation caused an appreciable error when the determination was delayed. Other authors found that in trichloracetic acid filtrates of blood there is less sulphhydryl than in tungstic acid filtrates, so that glutathione is incompletely extracted by the former acid. Woodward and Fry (141) introduced sulfosalicylic acid as
a protein precipitant which they found most suitable as it fulfills the following: a) no autoxidation in a 2% sulfosalicylic acid filtrate, except a slight loss after 24 hours; b) a 100% recovery of glutathione added to blood before precipitation of the proteins; c) rapid filtration with a large yield of filtrate.

Sulfosalicylic acid is also suitable for the amperometric method. Benesch and Benesch (9) found that it did not interfere with the amperometric silver titration and furthermore, in the concentrations used, it is soluble in the alcoholic titration mixture. A slight turbidity which occurs sometimes in the sulfosalicylic acid filtrates of some tissues does not interfere with the end results.

3. Sodium ethylenediamine tetraacetate (0.335% in distilled water). This is a synthetic polyamine polycarboxyl salt used as a powerful chelating agent, removing and inactivating trace metals and stabilizing the SH in solution.

4. Silver nitrate 0.001 N. A liter of 0.1 N silver nitrate solution is prepared. This stock solution is kept in a brown bottle in the dark. The 0.001 N solution was prepared before use and placed in the dark automatic reservoir. The 0.1 N silver nitrate solution is standardized against recrystallized sodium chloride according to Mohr's method (142) as follows: about 1 gm. of pure fused sodium chloride was pulverized and spread in a thin layer on a watch glass and dried at 110°-120° for an hour. Amounts of 0.160 g., 0.170 g. and 0.180 g. were weighed out accurately and placed in three 250 c.c. casseroles. To each casserole 125 c.c. of
water and 3 c.c. of 0.1 M potassium chromate solution was added and then titrated with silver nitrate solution which was slowly added as the solution in the casserole was stirred. The end-point of the titration is marked by the change from a lemon yellow to a faint orange colour. The individual results of each titration were calculated and as the average deviation of the series was not more than 2 parts per 1000, the average of the determinations was taken as the value of the silver nitrate solutions.

5. Supporting electrolyte. A 60 Gm. portion of reagent grade ammonium nitrate is added to a 500 ml. volumetric flask containing 250 ml. of concentrated ammonium hydroxide and 20 ml. of water. This is made up to volume at room temperature with distilled water.

5. Results obtained with a Beckmann pH meter

The Beckmann pH meter was standardised against a buffer solution of known pH each time before use. The platinum electrode was connected to the upper terminal jack of the pH meter and the reference half cell to the lower jack. The range switch was set to "+MV". The pH meter was grounded at two points. For the dipping end of the salt bridge we used an 8 cm. length of 7 mm glass tubing as suggested by Weissman and co-workers (135) filled with a 4% gel of agar saturated with potassium chloride. At one end the glass tube was bent at a right angle for convenience. At the other end a tightly rolled coil of Whatman no. 1 filter paper was inserted into the open end which was protected by a
larger tube having an agar plug at the free end, containing electrolyte solution and which was tightly fitted to the 8 cm. length tube. The platinum electrode was standardised daily against a solution of n-dodecyl mercaptan containing 0.0015 mM of SH per ml. as follows: a beaker containing 29 ml. ethanol, 1 ml. of supporting electrolyte and 1 ml. of standard solution was adjusted in position and the rotation of the electrode started. This solution usually gave an initial reading of 30-40 mv. For each addition of 0.1 ml. of silver nitrate a reading was made. Before reaching the end-point the readings remained small. After end-point the deflections correspond to the current of the excess of silver and larger readings are obtained. The end-point is found graphically by plotting the diffusion current against the volume of silver nitrate added. One line can be drawn through the stationary readings and a second straight line through the increasing readings. At the point of intersection the corresponding volume of silver nitrate is read. Type of curves obtained are shown in figure 4.

Disadvantages

The modifications of the salt bridge were found unsuitable as the rolled coil of filter paper had to be replaced daily, and due to the handling during the replacement the part of the bridge was frequently cracked. It was also found that the agar plug was unsuitable since a slight leak of the electrolyte solution (C) from (B) would produce a negative pressure in (C), the agar plug would be sucked up leaving a space under it, which would fill with air bubbles difficult to eliminate. As
mentioned, the pH meter was grounded but, in spite of this, the static electricity which was produced during the rotation of the electrode occasionally would interfere. Grounding different parts of the apparatus did not always control these interferences. The main disadvantage in using the pH meter was that, after several titrations, the rotating electrode would start losing its sensitivity as shown by the gradually increasing value (70, 100 or 180 mv.) obtained for the starting reading, and a decreasing value obtained for the addition of 0.1 ml. of silver nitrate after the end point. Cleaning the platinum electrode with nitric acid did not restore the sensitivity to its previous values. It was necessary to prepare a large number of electrodes and, only by trial and error, find one with suitable sensitivity. This was extremely time consuming.

b) Results obtained using a sensitive galvanometer.

For the reasons mentioned above, a more sensitive galvanometer was substituted for the pH meter and changes made in the salt bridge, as described in pages 38-40.

The modifications of Goldzieher (97) were adopted by means of which the sensitivity of the platinum electrode could be controlled. The procedure was as follows: the protective glass tube (C) of the dipping end of the salt bridge was filled with supporting electrolyte and slipped into place. This outer shell was not filled with saturated potassium chloride as some workers have done since this occasionally caused the platinum electrode to respond erratically. This may be due to a leak of
potassium chloride into the supporting electrolyte (which would take place if the rubber stopper between the two parts were not tightly fitted together) where it would react with silver nitrate or precipitate in the solution and on the platinum wire, thereby changing the response characteristics. Next in a 250 c.c. beaker were placed 29 ml. of absolute ethanol together with 1 ml. of the supporting electrolyte and 1 ml. of SH solution. The beaker and electrodes are placed in position.

Any air bubbles under the dipping end of the salt bridge were removed by slight shaking of the glass electrode. Between titrations the electrodes were washed off with distilled water or supporting electrolyte and dried with a filter paper. After using an indicator electrode for considerable time (after several hundred titrations) it occasionally became insensitive as the platinum wire became slightly coated with silver. The electrode was then cleaned with concentrated nitric acid. When a new or freshly cleaned electrode was placed in the ammoniacal solution a large current of 20 to 30 microamps was observed. After waiting 5-10 minutes this current decreased practically to zero.

Titration: A trial run to test sensitivity was made at the beginning of each series of determinations by adding 0.1 ml. of silver nitrate to the electrolyte beaker. This caused a 15 scale division deflection per 0.1 ml. silver nitrate. The accurate response of the platinum electrode was indicated by obtaining a straight line passing through zero (figure 5A,B) for the blank titration. A standardisation was done using a freshly prepared solution of glutathione (46.5 mg. in
500 ml.) in water with a small amount of sodium-ethylene diamine tetraacetate. This was stable for 48 hours. This solution contained 1.0 mg. sulphhydril per 100 c.c. The proportion of the reagents were as previously described; in addition 0.2 ml. of sodium ethylene diamine tetraacetate were added. The beaker was set in place and the rotation of the platinum electrode started. After waiting a few minutes the galvanometer stabilised at zero. Galvanometer readings were then made for each addition of 0.1 ml. of silver nitrate until a deflection of 80 scale units was obtained.

The galvanometer readings were plotted on rectangular coordinate paper as shown in figure 5, against the volume of silver nitrate as abscissa. The straight line of the curve was then extrapolated down to the zero ordinate and the volume of silver nitrate read. Blood blank titrations were done according to Goldzieder's modification using sodium-p-chloromercuribenzoate 0.001 N (346 mg. per liter) as a specific removing agent of SH compounds.

Titration values obtained at this stage were due to non-specific silver removing substances.

After p-chloromercuribenzoate has been used the electrodes are run for a minute or two in supporting electrolyte containing some cysteine which neutralises any excess of this reagent.

Preparation of the blood filtrate

4.0 ml. oxalated blood were added to 14.0 ml. of saponin solution and allowed to stand, with occasional shaking, for five minutes.
2.0 ml. of the 25% sulfosalicylic acid were added with shaking and the solution left for another five to ten minutes and then filtered through harden filter paper (Whatman no. 50). This solution is stable for several hours.

**Calculations**

For the blood determinations one must correct for the dilution of the filtrate (5x), for the electrode standardisation factor found with glutathione and for the hematocrit of the blood. The final value may be expressed either as milligrams of sulphhydryl per 100 c.c. of packed red cells or as milligrams of sulphhydryl per 100 ml. of blood corrected to a hematocrit of 50%.

**Recovery experiment**

To test the accuracy of the method, the ability to recover glutathione added to blood was determined.

A solution of glutathione in a concentration of 136 mgm/ was prepared and aliquots containing 0.93 mgm, 1.86 mgm and 2.79 mgm of GSH were added to three samples of 4 c.c. blood each and then treated according to the usual procedure for preparing blood filtrates mentioned on page using the same dilution ratio (1:15). Two recovery experiments were done by using 20 c.c. of oxalated blood from two normal individuals.

In table 1 the normal blood values found, the added values and the recovered amounts are given, expressed in glutathione mgm/.
TABLE 1

GLUTATHIONE RECOVERY BY THE ALPEROMETRIC METHOD

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Glutathione mg. per 100 ml. blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Original</td>
</tr>
<tr>
<td></td>
<td>Amount</td>
</tr>
<tr>
<td>N.K.</td>
<td>32.5</td>
</tr>
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<td></td>
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<td></td>
<td>46.5</td>
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<td></td>
<td>69.75</td>
</tr>
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<td>J.A.</td>
<td>30.0</td>
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<tr>
<td></td>
<td>23.25</td>
</tr>
<tr>
<td></td>
<td>46.5</td>
</tr>
<tr>
<td></td>
<td>69.75</td>
</tr>
</tbody>
</table>

It thus appears that the method as described above gave satisfactory recovery of glutathione in blood with a mean value of 96.2% and standard deviation of ± 2.7

Discussion of the method

Advantages:

1) Simplicity. The apparatus is easy to assemble and use, provided that the variable resistors and a sensitive galvanometer are available. Under these conditions variations of the sensitivity of the
platinum electrode can be controlled. The performance of each titration is simple and the time required is not greater than 3 minutes. This permits a large number of determinations.

ii) Specificity of the method. The method is specific for non-protein SH groups. The use of a blank titration with p-chloromercuribenzoate gives complete specificity for non-protein SH groups. In practice, however, it is not found necessary to include this step (page 58). According to Bidmead and Watson (138), with the amperometric titration method no oxidised glutathione is measured. Under the conditions of this technique it seems that no hydrolytic decomposition of oxidised glutathione occurs. Amino acids in concentrations much greater than glutathione do not react with ammoniacal silver nitrate (9). Uric acid and ascorbic acid, the latter in a six-fold molecular excess over glutathione, were found not to interfere. Within the group of non-protein SH compounds (glutathione-ergothioneine-cysteine) there is discussion as to the specificity of the method. Bidmead and Watson (138) were unable by this method to recover ergothioneine added to blood before protein precipitation.

This finding has been confirmed by Melville and Lubschez (132) by using their method for quantitative determination of ergothioneine. By using different acids for protein precipitation in preparing blood filtrates, they found that with sulfosalicylic and trichloroacetic acid no ergothioneine was detectable. They further discovered that by adding various reducing agents to the laked cells, before the precipitation of
proteins with trichloroacetic acid, they obtained satisfactory recoveries for ergothioneine. This suggests that ergothioneine may be bound to red cell protein (in a linkage which is split by a reducing agent) and therefore precipitated by these acids. Assuming that ergothioneine is not precipitated and that by the amperometric method it is also titrated, the amount of non-protein SH contributed by ergothioneine values given by recently developed methods - that 2.34 mg. for the method of Hunter (130); 2.12 mgm% for the method of Lawson et al (143) and 2.22 mgm% according to the method of Melville and Lubschez (132), the amount of ergothioneine contributed to the total value of 3.5 mgm of non-protein SH measured by the amperometric method will be 0.27 mgm%, which would account for 1/13 of the total.

Cysteine is present in still smaller concentrations and so its interference would be of no practical importance.

From the above discussion it is obvious that by this method one probably determines only glutathione, but it is preferable to express the values as non-protein SH corrected for hematocrit values.

iii) Sensitivity of the method. Amounts of 0.0030 mgm per 1 ml. of filtrate can be accurately measured and this would respond to a concentration of glutathione of 15 mg. per 100 ml. of blood, which is only half of the normal blood GSH level. Such low values are usually not expected but still smaller amounts could be titrated after changing the dilution factor in preparing the protein-free filtrate.

iv) Reproducibility. The reproducibility was satisfactory
when the experimental conditions were controlled. In the physiological range of concentration, the amperometric titrations were reproducible within 2%.

v) According to Bidmead and Watson (138) the mean recovery of added GSH to blood by using the amperometric method was 98.4% (S.D. ±4.3%). Similar results were obtained by Benesch and Benesch (9) for blood and tissue recovery experiments. The recovery obtained in the two experiments carried out for six blood samples was 96.2% (S.D. ±2.7).

**Disadvantages**

i) the method is not absolutely specific for glutathione comparing it to the manometric method.

ii) the rotating platinum electrode is the most sensitive part of the apparatus and should be carefully handled. Slight cracks, poor sealing and long use may significantly change its sensitivity.

A new standard curve must be made whenever the electrode is changed. One is not obliged to do an electrode standardisation each day before use, but a trial run to test sensitivity should be done.

3. **PRELIMINARY STUDY**

In order to study the effects of certain hormones upon the non-protein SH blood levels in normal individuals and patients by using a more specific method, it was necessary to establish the physiological blood values obtained by this method. Furthermore, because of the nature of the experiment planned it was necessary to know the differences between
fasting and non-fasting blood values.

Non-protein sulphydryl determinations were undertaken in a group of 16 normal individuals. Variations between fasting and non-fasting states in the same subjects were studied and also variations between non-fasting samples taken several days apart, from the same individuals. In a group of 10 male and female subjects from 20 to 50 years of age, blood samples were taken in the morning in a post-absorptive condition and a second sample 2 hours later after a light breakfast. In 4 subjects repeat fasting samples were obtained and in 6 repeat non-fasting samples.

Hematocrit values were determined in all these subjects according to the method of Wintrobe (144).

Results

The results obtained are presented in detail in table 11. The values are expressed as mg. of non-protein sulphydryl per 100 ml. of blood corrected for hematocrit values of 50% and as glutathione mg. per 100 ml. of blood corrected for the same hematocrit factor. The mean fasting values for 12 subjects are 3.45 (S.D. ± 0.4) mg.% of SH, and 32.08 (S.D. ± 3.7) mg.% of glutathione per 100 ml. of blood. In the second group of 10 subjects the values found are 3.5 (S.D. ± 0.34) mg.% of SH, and 32.55 (S.D. ± 3.1) mg.% glutathione. In 6 of these subjects the third determination in a non-fasting state gave the following: 3.41 (S.D. ± 0.21) mg.% of SH, and 31.71 (S.D. ± 1.95) mg.% for glutathione.
TABLE II
BLOOD NON-PROTEIN SH VALUES OF NORMAL INDIVIDUALS IN FASTING AND NON-FASTING CONDITIONS

<table>
<thead>
<tr>
<th></th>
<th>Fasting</th>
<th>Non-Fasting</th>
<th>Non-Fasting</th>
</tr>
</thead>
<tbody>
<tr>
<td>SH mg%</td>
<td>GSH mg%</td>
<td>SH mg%</td>
<td>GSH mg%</td>
</tr>
<tr>
<td>J.A.</td>
<td>3.2</td>
<td>29.7</td>
<td>3.4</td>
</tr>
<tr>
<td>*</td>
<td>3.3</td>
<td>30.6</td>
<td>3.3</td>
</tr>
<tr>
<td>M.S.</td>
<td>3.9</td>
<td>36.2</td>
<td>4.0</td>
</tr>
<tr>
<td>N.K.</td>
<td>3.4</td>
<td>31.52</td>
<td>3.5</td>
</tr>
<tr>
<td>M.K.</td>
<td></td>
<td></td>
<td>3.4</td>
</tr>
<tr>
<td>J.M.</td>
<td></td>
<td></td>
<td>3.4</td>
</tr>
<tr>
<td>D.H.</td>
<td>4.0</td>
<td>37.2</td>
<td></td>
</tr>
<tr>
<td>*</td>
<td>3.9</td>
<td>36.2</td>
<td></td>
</tr>
<tr>
<td>*</td>
<td>3.9</td>
<td>36.2</td>
<td></td>
</tr>
<tr>
<td>C.G.</td>
<td>3.0</td>
<td>27.9</td>
<td>3.2</td>
</tr>
<tr>
<td>C.C.</td>
<td>3.3</td>
<td>30.6</td>
<td>3.2</td>
</tr>
<tr>
<td>L.N.</td>
<td></td>
<td></td>
<td>3.1</td>
</tr>
<tr>
<td>E.K.</td>
<td>3.2</td>
<td>29.7</td>
<td></td>
</tr>
<tr>
<td>*</td>
<td>3.3</td>
<td>30.6</td>
<td></td>
</tr>
<tr>
<td>*</td>
<td>3.3</td>
<td>30.6</td>
<td></td>
</tr>
<tr>
<td>L.B.</td>
<td>4.0</td>
<td>37.2</td>
<td>4.0</td>
</tr>
<tr>
<td>*</td>
<td>4.0</td>
<td>37.2</td>
<td>3.9</td>
</tr>
<tr>
<td>M.M.</td>
<td>3.1</td>
<td>28.8</td>
<td>3.1</td>
</tr>
<tr>
<td>A.C.</td>
<td>4.0</td>
<td>37.2</td>
<td>3.9</td>
</tr>
<tr>
<td>L.M.</td>
<td>3.1</td>
<td>28.8</td>
<td>3.3</td>
</tr>
<tr>
<td>B.F.</td>
<td></td>
<td></td>
<td>3.5</td>
</tr>
<tr>
<td>L.B.</td>
<td>3.2</td>
<td>29.7</td>
<td>3.4</td>
</tr>
</tbody>
</table>

*In these subjects determinations were repeated on another fasting day.

The values presented in this table are corrected for a 50% hematocrit.
The blank titration with p-chloromercuribenzoate was used in eight determinations. As the differences between the two titrations in all these determinations was not greater than 0.01 ml. silver nitrate, which would give a difference of only 0.1 mg. of SH per 100 ml. of blood, the blank titration was not used as a routine procedure either in the determination of the normal blood levels or in the later experimental work.

Discussion

The normal values obtained by other workers using the amperometric titration method have been reported as 3.44 (S.D. ± 0.54) mg.% for SH and 32.0 (S.D. ± 5.1) mg.% for glutathione in 46 determinations, (138) and 2.70 mg. per 100 ml. of blood (25.1 mg.% of glutathione) in an unspecified number of determinations (97).

The values obtained above are in agreement with the normal levels obtained by Bidmead and Watson (138) and with those obtained by the specific manometric method (115). The normal values given by Goldzieher are considerably lower; the explanation for this is not apparent. The normal glutathione values obtained by the amperometric titration method are lower than those obtained by the less specific iodometric methods; the mean value for the method of Woodward and Fry (141) is 35.5 mg.% (S.D. ± 5.1). The results obtained by the modifications of Thompson and Watson of the colorimetric nitroprusside method (118) are in good agreement with the results of the amperometric method.
The differences obtained between the fasting and non-fasting samples for the same individuals were not significant \( (p > 0.1)^* \). This is in agreement with the findings of Henneman and Altshule (81) who found no changes after meals. Differences of a small range were obtained between the two non-fasting values or repeated fasting values in the same subjects.

Though there was considerable variation in values from one subject to another, the day to day variation in any one individual was somewhat smaller.

4. THE EFFECT OF ADRENAL STEROIDS ON BLOOD SULPHHYDYL LEVELS

The results published in the literature by different investigators upon the effect of the adrenal steroids on the blood glutathione levels are not in agreement (page 14). These discrepancies may be due not only to the non-specific methods used but also to the fact that the obtained values were not corrected for a hematocrit factor. As all the non-protein SH is in the red cells the influence of haemodilution or haemoconcentration is of significance especially when the adrenal cortical hormones are used and blood volume changes are expected. Another explanation for these discrepancies may be that early responses may have been missed after the administration of the hormones, as the time factor has usually been neglected.

By eliminating the above sources of possible error, an attempt

\[ t = \frac{\bar{d}}{\sqrt{\frac{\Sigma (d - \bar{d})^2}{\eta - 1}}} \]

*The following formula was used in determining the t-value for paired groups:
was made to study the effect of Compound E and Compound F upon the non-protein SH blood level of normal individuals.

In order to investigate a possible inter-relationship between non-protein SH and ascorbic acid and especially to detect a reversible reaction (page 23) if any, of these reducing substances in blood, the subjects were previously saturated with ascorbic acid.

The finding that ACTH administered intravenously had a temporary effect upon the glutathione level for the first 4 hours (97) and that despite the continuing infusion these values were restored to normal, stimulated the study of Compound F administered intravenously with an attempt to determine if this effect of ACTH is mediated through the adrenal gland by the elaboration of Compound F.

In comparison to these studies where an excess of certain adrenal steroids was administered, a further investigation was undertaken to determine the blood non-protein SH in patients with an insufficiency of adrenal steroids.

Plan of study

The studies were carried out in 6 normal subjects and 4 Addisonian patients.

The first group* (cases no. 1,2,3) consisted of three normal subjects, two male and one female (no.3), aged 20-30, who were given 225 mg. of ascorbic acid daily for a control period of 5-10 days. Cortisone acetate was then administered in a dose of 225 mg. daily orally in

*These three subjects belonged to a larger group under investigation in the McGill University Clinic, in which the effect of cortisone acetate upon the ascorbic acid excretion was studied. No data are given in this thesis upon the ascorbic acid values obtained for this group—only a brief comment is made.
three divided doses for three days during which time the ascorbic acid was continued.

Blood non-protein SH and ascorbic acid were determined at frequent intervals and total urine ascorbic acid excretion was measured daily.

The second group (cases no. 3, 4, 6) consisted of three normal male subjects (aged 20-33) in whom hydrocortisone (free alcohol) was administered. Cases no. 3 and 4 received 75 mg. of ascorbic acid three times daily throughout the experiments, which lasted from 13 to 17 days.

During the control period 24-hour urine collections were made using glacial acetic acid as a preservative. Total ascorbic acid excretion and blood levels for ascorbic acid and non-protein SH were determined daily. On the second to last day hydrocortisone 100 mg. contained in 500 ml. of 5% dextrose solution in 1% alcohol was administered intravenously for a six-hour period starting at 8 a.m. The subject fasted, following a 6 p.m. meal on the preceding day, and was allowed no food until the end of the experiment. One hour before starting the infusion the subject was permitted to lie in bed and rest. Water (250 c.c.) was given orally every two hours during the infusion period starting at 8 a.m. in order to ensure adequate urine volumes. During the day of the infusion the ascorbic acid was taken precisely at 8 a.m., 2 p.m., and 8 p.m. At the end of the infusion period a glucose tolerance test was done by injecting 50 c.c. of 50% dextrose in 10 minutes. The subject was allowed to have a meal after the glucose tolerance test was ended.
Two days before the hydrocortisone infusion, a control infusion was performed under the same conditions. The fluid given intravenously was 500 c.c. of a 5% dextrose solution to which 1% alcohol was added. The day between the two infusions was a rest period for the subject during which the regular amounts of ascorbic acid were taken.

During the infusion experiments blood samples obtained before starting the infusion and at 30-minute intervals for the first hour, and then hourly samples for the rest of the period. After the glucose was administered, samples at 30 minutes, 1 hour and 2 hours were taken. At 7 a.m. of the day of the infusion the bladder was emptied and thereafter hourly specimens were collected.

The third group (cases no. 7, 8, 9, 10) consisted of 4 known Addisonian patients followed for several years at the Endocrine Clinic of the Royal Victoria Hospital. These patients were being treated with one or more of cortisone, DCA or salt. In order to study the effect upon the non-protein SH in a state of insufficiency of the adrenal hormones, the treatment was withdrawn for 3 to 8 days and blood samples were taken before, during and after this period. The following determinations were carried out: non-protein SH blood levels, hematocrits, serum, sodium, potassium and chloride.

Materials and methods

Cortisone acetate (Compound E) in tablet form, 25 mgm each, prepared by Merck and Co. Inc., was administered orally.
Hydrocortisone (Compound F - free alcohol), prepared by
Merck and Co. Inc., 100 mg in 500 c.c. of 5% dextrose in 1% alcohol
was administered intravenously.

The ascorbic acid was prepared by Hoffman-La Roche Ltd.,
Montreal, and was administered orally in tablet form.

Blood and urinary ascorbic acid determinations were done
according to the technique of Roe and Ruther (145) based on the estim-
ation of the 2-4 dinitrophenylhydrazine derivative of dehydroascorbic
acid.

Urinary creatinine was determined by a modification of Folin’s
method (146) as a rough guide to the accuracy of urine collections (22).

Blood sugar determinations were carried out by the method of
Folin and Wu, modified for the photoelectric colorimeter (147).

Total blood eosinophil counts were made using the phloxine
propylene glycol diluting fluid, described by Randolph (148) and a Levy
counting chamber.

Blood pressure and temperatures were taken at hourly intervals
during the infusion period.

Urinary formaldehydogenic (total and free) steroids were deter-
mimed during the control period in 24- or 48-hour pooled urine specimens.
For the total amount extraction was carried out after hydrolysis with
spleen-b-glucuronidase; for the free fraction direct extraction of the
acidified urine was performed.

In both cases the corticoids were determined by periodic acid.
oxidation according to a modification of the method of Daughaday, Jeffe and Williams (149).  

17-ketosteroids were determined according to the method of Callow, Callow and Emmens (150) with a correction factor applied eliminating urinary chromogens.  

During the control and hydrocortisone experiments these determinations were carried out in six-hourly pooled specimens before, during and after the infusion.  

Serum potassium was determined by a modified cobaltinitrate procedure of the Breh and Gaebler method (151).  

Serum sodium was determined by a modified method of Barber and Kolthoff (152).  

Serum chlorine was determined by the method of Wilson and Ball (153).  

Results  

First group (cases 1, 2, 3) The values obtained after the administration of Compound E for blood non-protein SH are given with the hematocrit values in table III. In these subjects no change was detectable in the blood non-protein SH concentration during cortisone administration. In case no. 3 (No. 3) the hematocrit value dropped from 45% to 41% but returned to its previous level after cortisone was stopped. In the same subject the readings obtained for non-protein SH on the 7th and 8th days of the experiment were slightly lower, but when corrected for the hematocrit values no detectable change was shown.
<table>
<thead>
<tr>
<th>Days of study</th>
<th>Case no. 1 (N.K.)</th>
<th>Case no. 2 (K.H.)</th>
<th>Case no. 3 (M.C.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortisone acetate - SH - mg%</td>
<td>Non-protein Hematocrit (%)</td>
<td>Cortisone acetate - SH - mg%</td>
</tr>
<tr>
<td>1</td>
<td>3.3</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.4</td>
<td>49</td>
<td>3.4</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.3</td>
<td>49</td>
<td>3.3</td>
</tr>
<tr>
<td>5</td>
<td>3.3</td>
<td>49</td>
<td>3.4</td>
</tr>
<tr>
<td>6</td>
<td>3.5</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>3.4</td>
<td>50</td>
<td>225</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>225</td>
<td>3.2</td>
</tr>
<tr>
<td>9</td>
<td>3.2</td>
<td>48</td>
<td>225</td>
</tr>
<tr>
<td>10</td>
<td>225</td>
<td>3.4</td>
<td>48</td>
</tr>
<tr>
<td>11</td>
<td>225</td>
<td>3.3</td>
<td>49</td>
</tr>
<tr>
<td>12</td>
<td>225</td>
<td>3.3</td>
<td>49</td>
</tr>
<tr>
<td>13</td>
<td>3.4</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>
The effects of cortisone on the blood and urine ascorbic acid were inconsistent. Case no. 1 (N.K.) showed no change in the ascorbic acid blood level and no increase in the urinary output of ascorbic acid during cortisone acetate administration. The second subject (case no. 2, K.N.) showed a prompt increase in excretion of ascorbic acid during cortisone acetate administration which fell to values lower than those of the control period when the hormone was stopped. Blood ascorbic acid determinations were not carried out for the whole period of the experiment but the values obtained when cortisone was administered showed no difference from those of the control period. The third case (McN) showed no change in ascorbic acid output when under the influence of the hormone, but with the withdrawal of cortisone there was a prompt decrease in ascorbic acid excretion.

Second group (cases no. 4,5,6) The results of the second group, receiving Compound F intravenously, will be presented separately for each subject.

Case no. 4 (J.A. - table V, figure 6): The saturation period with ascorbic acid lasted 13 days. Blood non-protein SH values during this period ranged between 3.3 and 3.6 mgm%. Blood ascorbic acid showed a constant stepwise increase from 0.9 mgm% to 1.5 mgm%. There was a gradual increase of ascorbic acid output until equilibrium was reached. During the control infusion period the non-protein SH varied between 3.4 and 3.2 mgm%. During the hydrocortisone infusion experiment the non-protein SH values ranged between 3.2 mgm% and 3.5 mgm%.
The blood ascorbic acid showed values ranging between 1.24 and 1.47 mgm% during the control infusion. There was a decrease after the 4th hour of the hydrocortisone infusion to levels of 0.96 to 1.19 mgm% until the end of the experiment so that 2½ hours after the infusion was stopped the blood ascorbic acid level was still lower than that of the control.

The excretion of urinary ascorbic acid in the hydrocortisone experiment rose to a total of 61.7 mg. in 12 hours (infusion and post-infusion period) above the control level.

In table IV the amounts of ascorbic acid excreted for the 6-hour infusion period and for 6 post-infusion hours are compared, for the control and hydrocortisone experiment.

**TABLE IV**

<table>
<thead>
<tr>
<th>Hours</th>
<th>CONTROL Total amount excreted mg/vol. urine</th>
<th>HYDROCORTISONE Total amount excreted mg/vol. urine</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-2 a.m.</td>
<td>85.7</td>
<td>125.3</td>
<td>40.1</td>
</tr>
<tr>
<td>infusion period (6 hr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-8 p.m.</td>
<td>46.1</td>
<td>67.7</td>
<td>21.6</td>
</tr>
<tr>
<td>post-infusion period (6 hr)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE V  (Case No. 4 - J.A.)

<table>
<thead>
<tr>
<th>Days of study</th>
<th>Hour</th>
<th>Infusion</th>
<th>Ascorbic acid intake*</th>
<th>Urinary Creatinine *</th>
<th>Urinary Ascorbic acid*</th>
<th>Blood Ascorbic acid</th>
<th>Non-protein SH - mg/24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>225</td>
<td>1.38</td>
<td>70.5</td>
<td>0.94</td>
<td>3.25</td>
</tr>
<tr>
<td>2</td>
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<td></td>
<td>1.64</td>
<td>99.6</td>
<td>1.10</td>
<td>3.32</td>
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<td>110.0</td>
<td>1.25</td>
<td>3.34</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td>1.79</td>
<td>134.2</td>
<td>1.31</td>
<td>3.41</td>
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<tr>
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<td>1.79</td>
<td>260.0</td>
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<td>1.65</td>
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<td></td>
<td></td>
<td>1.95</td>
<td>179.6</td>
<td>1.43</td>
<td>3.21</td>
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<td>8</td>
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<td></td>
<td></td>
<td>1.76</td>
<td>202.5</td>
<td>1.38</td>
<td>3.20</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
<td>1.84</td>
<td>196.6</td>
<td>1.38</td>
<td>3.24</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td>1.79</td>
<td>170.0</td>
<td>1.36</td>
<td>3.45</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td>1.45</td>
<td>130.0</td>
<td>1.45</td>
<td>3.30</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td>1.59</td>
<td>177.5</td>
<td>1.36</td>
<td>3.23</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td>2.09</td>
<td>220.7</td>
<td>1.36</td>
<td>3.35</td>
</tr>
</tbody>
</table>

*The values are given for Ascorbic acid as mg/24 hrs. and Creatinine as gr./24 hrs. Where hours are indicated ascorbic acid is expressed as mg/1 hour and creatinine as gr./1 hour.
<table>
<thead>
<tr>
<th>Days of study</th>
<th>Hour</th>
<th>Infusion</th>
<th>Ascorbic acid intake</th>
<th>Urinary Creatinine</th>
<th>Urinary Ascorbic acid</th>
<th>Blood Ascorbic acid</th>
<th>Non-protein SH - mgm%</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>8 a.m.</td>
<td>75</td>
<td>0.07</td>
<td>11.7</td>
<td>1.35</td>
<td>3.42</td>
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</tr>
<tr>
<td>8,30</td>
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<td>3.32</td>
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TABLE V (continued)

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<th>Days of study</th>
<th>Hour</th>
<th>Infusion</th>
<th>Ascorbic acid intake</th>
<th>Urinary Creatinine</th>
<th>Urinary Ascorbic acid</th>
<th>Blood Ascorbic acid</th>
<th>Non-protein SH - mgm%</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>1 p.m.</td>
<td></td>
<td>0.10</td>
<td>27.2</td>
<td>1.12</td>
<td>3.55</td>
<td></td>
</tr>
<tr>
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<td>2.30</td>
<td>Glucose</td>
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<td>0.03</td>
<td>19.1</td>
<td>1.12</td>
<td>3.46</td>
</tr>
<tr>
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<td>Tolerance</td>
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<td>1.04</td>
<td>3.49</td>
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</tr>
<tr>
<td>4</td>
<td>Tret</td>
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<td>6.7</td>
<td>1.19</td>
<td>3.63</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td>225</td>
<td>1.60</td>
<td>122.3</td>
<td>1.33</td>
<td>3.60</td>
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</table>
SUBJECT (J.A.)

<table>
<thead>
<tr>
<th>TIME IN HOURS</th>
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<tbody>
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</tbody>
</table>

**INFUSION**
- CONTROL
- HYDROCORTISONE
- ASCORBIC ACID (75 MG)

**ORAL**
- ASCORBIC ACID

**EOSINOPHILS % FALL**

**HEMATOCRIT (%)**
- 48
- 46
- 44
- 42

**NON-PROTEIN SH (MG/%)**
- 3.7
- 3.5
- 3.3
- 3.1

**BLOOD ASCORBIC ACID (MG%)**
- 1.3
- 1.1
- 0.9

**URINARY ASCORBIC ACID (MG/HR)**

**CREATININE (GM/HR)**
- 0.1
- 0.05

**GLUCOSE TOLERANCE TEST**

Figure 6
From table IV it is seen that the largest amount is excreted during the hydrocortisone infusion and that this amount is almost double that excreted for the 6 hours following the infusion. The hematocrit showed a drop from 45.5 to 42.5 during the first 3 hours of the hydrocortisone infusion and then rose to 47.5. Furthermore, during the 3rd and 4th hours there was an increase in blood pressure from 120/80 to 139/112 with no change in the pulse rate. The eosinophiles showed a decrease in this experiment, starting after the 3rd hour and dropping to zero at the end of the infusion.

Case no. 5 - (L.B. - table VII, figure 7): showed the following. The saturation period lasted 10 days. Initially the ascorbic acid excretion was below 15 mg./24 hours and only after 6 days was there a gradual increase in the output. It should be mentioned that when the control infusion was done the patient was not fully saturated.

Blood non-protein SH values ranged during this period from 3.9 to 4.1 mg/m3. Blood ascorbic acid showed an increase from 0.50 mg/m3 to 1.60 mg/m3 during the control period. During the control infusion the non-protein SH showed a decrease for the 1st hour from 4.0 mg/m3 to 3.7 mg/m3 and 3.3 mg/m3, after which the values ranged between 4.0 and 4.1 mg/m3. A similar drop was obtained during the hydrocortisone infusion which was maintained until the 2nd hour.

The blood ascorbic acid level rose slightly at the 4th hour of infusion, then fell somewhat below the initial level in both the control and hydrocortisone infusion experiments. The ascorbic acid excreted
during the hydrocortisone infusion period rose above the control level by 140 mg./6 hours and 77 mg. for the following 12 hours.

In table VI the values obtained for the ascorbic acid output are given for the first 6-hour infusion period and for the following 12 hours of the post-infusion period.

**TABLE VI**

**URINARY ASCORBIC ACID EXCRETION DURING THE CONTROL AND HYDROCORTISONE EXPERIMENT (Case no. 5 - I.R.)**

<table>
<thead>
<tr>
<th>Hours</th>
<th>CONTROL</th>
<th>HYDROCORTISONE</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total amount excreted</td>
<td>Total amount excreted</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mg./vol. urine</td>
<td>mg./vol. urine</td>
<td></td>
</tr>
<tr>
<td>8-2 a.m. infusion period (6 hours)</td>
<td>52.71</td>
<td>199.4</td>
<td>146.69</td>
</tr>
<tr>
<td>2-8 a.m. post-infusion period (12 hrs)</td>
<td>115.52</td>
<td>193.3</td>
<td>77.76</td>
</tr>
</tbody>
</table>

From this table it is seen that the amount excreted during the 6 hours of hydrocortisone infusion is almost three times above that excreted during the control infusion, and almost twice as much above the amount excreted for the following 12 hours.

The hematocrit showed, during the hydrocortisone infusion, an early slight decrease and then a rise between the 5th and 6th hours from 41 to 43%. During this period a rise of blood pressure occurred from
**TABLE VII (Case no. 5 - L.B.)**

<table>
<thead>
<tr>
<th>Days of study</th>
<th>Hour</th>
<th>Infusion</th>
<th>Ascorbic acid intake*</th>
<th>Urinary Creatinine *</th>
<th>Urinary Ascorbic acid*</th>
<th>Blood Ascorbic acid-mg.%</th>
<th>Non-protein SH - mg.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>1.67</td>
<td>10.2</td>
<td>0.50</td>
<td>4.0</td>
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<td></td>
<td>225</td>
<td>1.67</td>
<td>11.9</td>
<td>0.54</td>
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<td>1.72</td>
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<tr>
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<td>1.73</td>
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<tr>
<td>5</td>
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<td>1.77</td>
<td>11.8</td>
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<td>-</td>
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</tr>
<tr>
<td>6</td>
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<td></td>
<td>1.99</td>
<td>12.5</td>
<td>1.07</td>
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<tr>
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<td>21.2</td>
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<td>8</td>
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<td>34.8</td>
<td>1.60</td>
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<td>69.3</td>
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<td></td>
<td>135.2</td>
<td>1.51</td>
<td>4.1</td>
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</tr>
</tbody>
</table>

*The values are given for ascorbic acid as mg/24 hrs. and creatinine as gr./24 hrs. Where hours are indicated ascorbic acid is expressed as mg/1 hour and creatinine as gr./1 hour.
<table>
<thead>
<tr>
<th>Days of study</th>
<th>Hour</th>
<th>Infusion</th>
<th>Ascorbic acid intake</th>
<th>Urinary Creatinine</th>
<th>Urinary Ascorbic acid</th>
<th>Blood Ascorbic acid</th>
<th>Non-protein SH - mgm%</th>
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<td></td>
<td></td>
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<tr>
<td>8 a.m.</td>
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<td>11 Infusion</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
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</tr>
<tr>
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</table>
### TABLE VII (continued)

<table>
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<th>Ascorbic acid intake</th>
<th>Urinary Creatinine</th>
<th>Urinary Ascorbic acid</th>
<th>Blood Ascorbic acid</th>
<th>Non-protein SH - mgm%</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
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<td>0.07</td>
<td>5.86</td>
<td>1.51</td>
<td>4.10</td>
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</tr>
<tr>
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<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.52</td>
<td>3.85</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>0.10</td>
<td>5.86</td>
<td>1.58</td>
<td>3.70</td>
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<td>6.12</td>
<td>1.65</td>
<td>3.82</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td>0.08</td>
<td>16.9</td>
<td>1.09</td>
<td>4.0</td>
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<td>Infusion</td>
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<td>22.6</td>
<td>1.68</td>
<td>4.20</td>
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</tr>
<tr>
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<td>0.09</td>
<td>20.8</td>
<td>1.52</td>
<td>4.20</td>
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</tr>
<tr>
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<td></td>
<td></td>
<td>0.08</td>
<td>19.3</td>
<td>1.52</td>
<td>4.12</td>
<td></td>
</tr>
<tr>
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<td>11.8</td>
<td>1.62</td>
<td>4.25</td>
<td></td>
</tr>
</tbody>
</table>
Figure 7
115/75 to 142/92 without any change in the pulse rate. In this experiment the eosinophiles showed a decrease greater than 50% after the 4th hour of infusion.

Case no. 6 (M.G.) was not placed on a supplementary intake of ascorbic acid.

Urine specimens were collected every two hours during the infusion period. Blood non-protein SH determinations were done as previously at 1-hour intervals, but blood ascorbic acid estimations were performed every two hours.

The results are given in tables VIII and IX.

**TABLE VIII**

**URINARY ASCORBIC ACID EXCRETION AND ASCORBIC ACID BLOOD LEVELS DURING THE CONTROL AND HYDROCORTISONE EXPERIMENT (Case no. 6 - M.G.)**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm./2 hrs.</td>
<td>mg/2hrs</td>
</tr>
<tr>
<td>6-8</td>
<td>0.14</td>
<td>0.84</td>
</tr>
<tr>
<td>8-10</td>
<td>0.13</td>
<td>0.81</td>
</tr>
<tr>
<td>10-12</td>
<td>0.12</td>
<td>0.41</td>
</tr>
<tr>
<td>12-2</td>
<td>0.13</td>
<td>0.82</td>
</tr>
<tr>
<td>2-4</td>
<td>0.16</td>
<td>2.82</td>
</tr>
</tbody>
</table>

In this table it is seen that a slight decrease in the urinary excretion of ascorbic acid occurred during the infusion of hydrocortisone.
Furthermore, in the first 2-hour sample after the end of the hydrocortisone infusion, a five-fold increase of ascorbic acid excretion occurred compared to that of the control.

In table IX the results obtained for blood non-protein SH and hematocrit values are given.

**TABLE IX**

**BLOOD NON-PROTEIN SH AND HEMATOCRIT VALUES DURING THE CONTROL AND HYDROCORTISONE EXPERIMENT (Case no.6 - M.G.)**

| Hour | CONTROL | | HYDROCORTISONE |
|------|----------||----------------|
|      | Non-protein SH - mgm% | Hematocrit | Non-protein SH - mgm% | Hematocrit |
| 0    | 3.8      | 48.5 | 3.88 | 47.5 |
| 30'   | 3.8      | 47  | 4.1  | 45.5 |
| 1    | 3.9      | 47.5 | 3.6  | 45.5 |
| 2    | 3.9      | 47  | 3.9  | 45  |
| 3    | 3.9      | 47.5 | 3.88 | 45.5 |
| 4    | 3.99     | 47.5 | 3.9  | 46.5 |
| 5    | 4.1      | 47.5 | 3.93 | 47.5 |
| 6    | 4.1      | 47.5 | 4.1  | 47  |
| 6.30 | 3.97     | 47.5 | 4.0  | 47  |
| 7    | 4.0      | 47  | 3.9  | 47  |
| 8    | 3.93     | 47.5 | 3.9  | 47  |
In this subject no change in the non-protein SH values occurred. The hematocrit dropped during the hydrocortisone infusion from 47.5% to 45%. After the 4th hour of hydrocortisone infusion there was an increase in the blood pressure from 106/70 to 122/76. No change in the pulse rate occurred.

After the 2nd hour of hydrocortisone infusion in this subject a progressive decrease in the eosinophiles occurred.

The values obtained for the formaldehydogenic (free and total) steroid excretion as well as those for the 17-ketosteroid excretion are given in tables X and XI. During the control period (8 to 13 days) no changes were obtained. The stressful situation of the control infusion increased both the formaldehydogenic steroid (free and total) and the 17-ketosteroid excretion. During the hydrocortisone infusion the excretion of the formaldehydogenic steroids further increased, but the 17-ketosteroids showed a decrease, both during the infusion and post-infusion period compared to that of the control.

Third group (cases no. 7,8,9,10). The results obtained in this group are given in table XII. Three patients showed a decrease in blood non-protein SH after the withdrawal of treatment from levels of 3.45 mgm% to 2.9 mgm%, 3.8 to 3.4 mgm% and 4.5 to 3.9 mgm%. A decrease in blood pressure was found in three cases when the patients were not on treatment and in two patients an unexpected drop in hematocrit values was observed. The changes in serum electrolyte values were not consistent. In two patients (nos. 7,10) a decrease in serum chlorine and sodium occurred and in cases
DISCUSSION

First and second groups (Corticoid excess)

Although no definite conclusions can be drawn from the small number of subjects used in the present series, the results obtained with Compound E and Compound F indicate that these hormones have no specific effect upon the non-protein sulphydryl blood levels.

Most of the changes were within ± 0.4 mgm% which is within the range of variation under normal physiological conditions. In case no. 5 (L.B.) a decrease was obtained with hydrocortisone from a control value of 4.0 mgm% to a level of 3.5 mgm% in the first hour of infusion. However, a similar drop was obtained in the control infusion.

The prompt but transitory effect of ACTH upon the non-protein SH blood levels when administered intravenously (97) and the failure to obtain any effect with Compound F when administered intravenously suggests that the effect of ACTH is not mediated through the adrenal by the elaboration of Compound F.

An attempt was made in this experiment to study the possible relationship between ascorbic acid and glutathione.

A failure of previous workers (94,92) in studying this aspect was that the subjects used were not saturated with ascorbic acid. It is known that in species (man, monkey, guinea pig) which do not synthesize ascorbic acid but depend on food as the source of the vitamin, the tissue
### TABLE X
**Urinary Steroid Excretion During the Ascorbic Acid Saturation Period**

<table>
<thead>
<tr>
<th>Days of study</th>
<th>Case No. 4 (J. A.)</th>
<th>Case No. 5 (L. B.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free F.S. mg/24hrs</td>
<td>Total F.S. mg/24hrs</td>
</tr>
<tr>
<td>1 + 2</td>
<td>.638</td>
<td>18.51</td>
</tr>
<tr>
<td>3 + 4</td>
<td>.662</td>
<td>12.1</td>
</tr>
<tr>
<td>5</td>
<td>.912</td>
<td>36.0</td>
</tr>
<tr>
<td>6 + 7</td>
<td>.570</td>
<td>19.9</td>
</tr>
<tr>
<td>8 + 9</td>
<td>.300</td>
<td>26.2</td>
</tr>
<tr>
<td>10 + 11</td>
<td>.669</td>
<td>16.8</td>
</tr>
<tr>
<td>12</td>
<td>.364</td>
<td>12.2</td>
</tr>
<tr>
<td>13</td>
<td>.771</td>
<td>16.65</td>
</tr>
<tr>
<td>14 (C)</td>
<td>1.149</td>
<td>18.8</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>16 (F)</td>
<td>2.132</td>
<td>38.7</td>
</tr>
<tr>
<td>17</td>
<td>.213</td>
<td>10.6</td>
</tr>
</tbody>
</table>

C = Day of control infusion  
F = Day of Compound F infusion
### TABLE XI

**URINARY STEROID EXCRETION DURING HYDROCORTISONE (COMPOUND F) ADMINISTRATION**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Experiment</th>
<th>FREE F.S. (mgm/6 hrs) Pre-Infusion</th>
<th>Post-Infusion</th>
<th>TOTAL F.S. (mgm/6 hrs) Pre-Infusion</th>
<th>Post-Infusion</th>
<th>17-KETOSTEROIDS (mgm/6 hrs) Pre-Infusion</th>
<th>Post-Infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 4</td>
<td>C</td>
<td>.162</td>
<td>.650</td>
<td>.194</td>
<td></td>
<td>2.67</td>
<td>8.22</td>
</tr>
<tr>
<td></td>
<td>Comp. F</td>
<td>.152</td>
<td>1.200</td>
<td>.687</td>
<td></td>
<td>2.87</td>
<td>17.75</td>
</tr>
<tr>
<td>Case 5</td>
<td>C</td>
<td>.407</td>
<td>.282*</td>
<td>8.12</td>
<td>5.9*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Comp. F</td>
<td>.950</td>
<td>.421*</td>
<td>22.1</td>
<td>14.7*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 6</td>
<td>C</td>
<td>.211</td>
<td>.275</td>
<td>.605</td>
<td></td>
<td>2.43</td>
<td>6.77</td>
</tr>
<tr>
<td></td>
<td>Comp. F</td>
<td>.104</td>
<td>2.087</td>
<td>1.625</td>
<td></td>
<td>2.75</td>
<td>19.27</td>
</tr>
</tbody>
</table>

- **Pre-infusion period** = urine collection from 8 p.m. until 8 a.m. (values calculated per 6 hours)
- **Infusion period** = 8 a.m. until 2 p.m.
- **Post-infusion period** = From 2 p.m. until 8 p.m. (*urine collections for these samples from 2 until 8 a.m., calculated for 6 hours).

- C = Control; Comp. F = Compound F
TABLE XII

BLOOD CHANGES IN ADDISONIAN PATIENTS AFTER THE WITHDRAWAL OF TREATMENT

| Subject | Period | Day of determination | Non-protein Hematocrit (SH) - mgm/100 ml | Hematocrit (%) | Blood Pressure | SERUM ELECTROLYTES | | | |
|---------|--------|----------------------|------------------------------------------|----------------|---------------|-------------------|-----------------|---|
|         |        |                      |                                          |                |              | Chloride (mgm/100 ml) | Sodium (mgm/100 ml) | Potassium (mgm/100 ml) |
|         | B       | 6                    | 3.3                                       | 53.5           | 102/80        | 341               | 292              | 25.9 |
|         | C       | 1                    | 2.9                                       | 50             | 115/80        | -                 | -                | -    |
|         |         | 8                    | 3.3                                       | 52             | 120/80        | -                 | -                | -    |
| Case 8  (L.Y.) | A       | -                    | 3.8                                       | 53             | -             | -                 | -                | -    |
|         | B       | 3                    | 3.4                                       | 49             | 90/70         | 367               | 318              | 17   |
|         |         | 7                    | 3.4                                       | 40             | 110/80        | 382               | 328              | 16.6 |
|         | C       | 14                   | 4.2                                       | 42             | 102/80        | -                 | -                | -    |
| Case 9  (A.A.) | A       | -                    | 3.3                                       | 49             | 140/100       | 369               | 312              | -    |
|         | B       | 3                    | 3.8                                       | 42             | 110/70        | 385               | 313              | 17.4 |
|         | C       | 4                    | -                                         | -              | 140/90        | 377               | 311              | 20.2 |
| Case 10 (L.C.) | A       | -                    | 4.5                                       | 43             | 90/60         | -                 | -                | -    |
|         |         | 8                    | 3.9                                       | 45             | 90/60         | 360               | 314              | 25   |

A = period under treatment; B = period during which no treatment was administered; C = period during which treatment was reinstituted.
concentration is somewhat lower (154,155).

In order to study a reversible reaction between two substances in a body fluid one should attempt to eliminate the withdrawal of one or other of the substances from the site of reaction to other tissues. In the case of ascorbic acid which shows a concentration gradient from blood to tissues, possible movement from the former to the latter can be minimized by saturation of all tissues prior to the experiment. This was accomplished by administering large doses of ascorbic acid for 7-10 days.

In subjects so prepared, no change in the blood non-protein SH concentration was obtained in spite of the gradual increase of the ascorbic acid concentration in the blood from levels of 0.9 mgm% to 1.50 mgm% during the control period. No changes were obtained during the intravenous infusion of Compound F and no reversible reaction of these substances was seen, even when a decrease of blood ascorbic acid occurred after the 3rd hour of infusion (case no. 4, J.A.).

It was also observed, as previously found in this Clinic, that the saturation with ascorbic acid was reached within 12 days and that after saturation the output of ascorbic acid was lower than the intake, not withstanding the fact that the ascorbic acid content of the diet was not included in the balance.

In the two subjects saturated with ascorbic acid the increased excretion during the 6 hours of infusion was much greater than in the following 6 hours. The third subject, who was not on a constant ascorbic
acid intake, showed an increased output only for the first two hours after the end of the infusion. The changes in blood ascorbic acid values did not parallel the changes which occurred in the urinary ascorbic acid output. The first subject showed a consistent decrease of 0.4 mgm% in the blood ascorbic acid level during the hydrocortisone infusion after the 3rd hour, lasting for the following 5 hours until the end of the experiment.

It has been found previously in this Clinic (22) that when ACTH was administered intramuscularly to 15 patients saturated with ascorbic acid, 12 of these showed a definite increase in the urinary output of ascorbic acid.

Of nine patients receiving cortisone intramuscularly only one showed an increased output of ascorbic acid, while when cortisone was administered orally a marked excretion of ascorbic acid occurred. An attempt was made to explain this high output of ascorbic acid and several alternatives were suggested:

  a) an increase in renal clearance associated with a lowering of the threshold

  b) an increased and continued release of ascorbic acid from the adrenal

  c) a release of ascorbic acid from other tissues.

From the present work, in spite of the fact that only two subjects were studied, further information can be obtained regarding the mechanisms involved.
In both subjects (case no. 4 - J.A. and case no. 5 - L.B.) saturated with ascorbic acid, the rate of renal clearance was calculated and the values obtained are given in the following table:

**TABLE XIII**

**CLEARANCE RATE OF ASCORBIC ACID ml/hours.**

<table>
<thead>
<tr>
<th>Hours</th>
<th>Case no. 4 (J.A.)</th>
<th>Case no. 5 (L.B.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control Hydrocortisone</td>
<td>Control Hydrocortisone</td>
</tr>
<tr>
<td>8 - 9</td>
<td>78 79</td>
<td>44 38</td>
</tr>
<tr>
<td>9-10</td>
<td>76 144</td>
<td>48 38</td>
</tr>
<tr>
<td>10-11</td>
<td>153 146</td>
<td>72 101</td>
</tr>
<tr>
<td>11-12</td>
<td>127 209</td>
<td>74 134</td>
</tr>
<tr>
<td>12-1</td>
<td>68 236</td>
<td>75 136</td>
</tr>
<tr>
<td>1 - 2</td>
<td>78 133</td>
<td>69 131</td>
</tr>
<tr>
<td>2 - 3</td>
<td>109 287</td>
<td>136 231</td>
</tr>
<tr>
<td>3 - 4</td>
<td>75 60</td>
<td>27 77</td>
</tr>
</tbody>
</table>

In the control the clearance rate is relatively constant while in the hydrocortisone infusion period the clearance rate increased considerably. This increased clearance as well as the higher total excretion obtained during the hormonal infusion, compared to that of the control, would indicate a specific renal effect of Compound F upon the ascorbic acid excretion.

The fall in blood level in case no. 4 (J.A.) indicates, on the
basis of a rough calculation, a loss of 52.50 mg. from the blood and extracellular fluid. This is not accounted for by the excess excretion of ascorbic acid after the 4th hour when the decrease in blood ascorbic acid level occurred. This would indicate either an increased uptake by the tissues or increased utilization. That increased utilization of ascorbic acid can occur under the effect of a steroid has been indirectly shown by in vivo experiments (156).

It should be stressed that the hypothesis presented is highly speculative in view of the fact that the above findings were obtained in only one of the two subjects under investigation.

The results obtained in the experimental animal are in partial disagreement with those obtained in the human. Booker and co-workers (157), in experiments on dogs to which ascorbic acid was administered and the effect of adrenocortical extract, desoxycorticosterone and Compound E studied, found that these hormones reduced the urinary excretion of ascorbic acid and caused an increase of plasma and cell ascorbic acid.

In discussing the steroid excretion in their experiments, it must be mentioned that no control period was carried out when the patient was not on ascorbic acid supplement. However, in spite of the gradual increase in the blood ascorbic acid levels and the increase in ascorbic acid output, no change was detectable in the formaldehydogenic corticoids and 17-ketosteroids.

It has been found by other workers that the neutral reducing corticoid excretion has been diminished when patients were given large
doses of ascorbic acid (1 Gm./day) for two weeks (61).

That a possible relationship exists between ascorbic acid and the activity of the adrenal gland has been shown not only in the experimental animal but in humans also. In scorbutic patients with low 11-oxycorticoid excretion, the administration of ascorbic acid produced a further temporary decrease of these (158). It has also been shown that a rise in urinary 11-oxycorticoid excretion is less when ACTH is given in combination with ascorbic acid (159). During the infusion period of hydrocortisone there was an increased excretion in the free and conjugated formaldehydogenic corticoids which continued for the second 6-hour period after the end of the infusion. No increase in the 17-ketosteroid excretion was seen, but rather a slight decrease as compared to that of the control period.

The findings are in agreement with the work of Conn (160) who found that after intramuscular administration of 400 mgm per day for 4 days of Compound F (free alcohol), a mild rise in the formaldehydogenic steroids and a fall in the 17-ketosteroids occurred, whereas a steep rise in both excretory products was found when the material was given by the oral route. The explanation given for this was that in the latter case this steroid would be carried through the portal circulation in a high concentration to the liver and there metabolized.

In the present experiments the results are similar to those obtained by the intramuscular route.

The slight decrease in the 17-ketosteroid excretion was explained
by Conn (160) as an inhibition of endogenous ACTH production. A similar response was obtained during the first 6 hours of the intravenous infusion of hydrocortisone, compared to the control infusion, suggesting a quite rapid inhibition of the release of endogenous ACTH.

**Third group (Corticoid deficiency)**

In the third group, three Addisonian patients showed a decrease in the non-protein SH from 10 to 18% of the control value. One of these patients showed the lowest value one day after treatment was again started. One patient showed an increase of 0.5 mgm% during the period of withdrawal of treatment.

Levels of blood glutathione have been determined in patients with Addison's disease (by the iodate method) and Binet and Pautonnet (11) reported that low glutathione levels were found in such patients and that DCA treatment resulted in a return to normal. Grunert and Phillips (161) tried to explain this finding as a result of sodium depletion. They found that sodium deficient rats had low glutathione blood concentrations and suggested that this fact would explain the increased sensitivity to alloxan usually found in these animals.

In the patients studied above, the sodium decrease was not high in spite of early physical and clinical signs of adrenal insufficiency present. No close relationship was observed between the blood non-protein SH values and the serum chloride concentration.
5. THE EFFECT OF GROWTH HORMONE UPON THE NON-PROTEIN SULPHHYDRL LEVEL OF BLOOD AND LIVER TISSUE

a. The Effect on the Blood Non-protein SH

The interpretation of an increase or decrease in blood glutathione concentration as an index of anabolism or catabolism is not permissible, as studies of tissue and blood glutathione concentrations under such conditions have not been carried out up to now.

It has been shown only in animal experiments that when amino acids are administered orally or intraperitoneally an increase in the non-protein SH of the liver and adrenal tissue occurs (162). In man, recent experiments have suggested that some anabolic effects are produced by infusions of purified growth hormone along with an amino acid mixture (163).

An attempt was made to study the effect of an amino acid infusion upon the blood non-protein SH concentration and to compare this effect, if any, to that of an anabolic hormone-growth hormone administered concomitantly during the amino acid administration. Comparison was made under similar conditions when ACTH was administered.

Kinsell and co-workers (164), in studying the fate of $^{35}$ labelled methionine under different conditions, found that under testosterone propionate therapy a protein anabolic effect was obtained, with diminished excretion of nitrogen and sulphur. A study was undertaken to follow the non-protein SH blood levels in a patient receiving another anabolic
hormone - that is, testosterone.

Plan of study

Two normal young men were used as experimental subjects (case no. 11 (D.H.) age 24 and case no. 12 (E.K.) age 29). Three separate experiments were carried out on alternate days. On the first day, with the subject in a fasting condition, a control infusion was done with 350 ml. amino acid solution over 30 minutes. Venous blood samples were taken without stasis at 15-minute intervals for the 1st hour and continued at hourly intervals for 5 hours more.

The second experiment was performed on the third day under the same conditions and 80 mgm of growth hormone was administered during the half-hour of the infusion of the amino acid solution.

After a second rest day the same experiment was repeated by administering 40 mgm of ACTH during the amino acid infusion period.

In the second case, no. 12 (E.K.) 300 ml. of amino acid solution was administered for the same time interval and 40 mgm of growth hormone was administered instead of 80 mgm. The dose of ACTH remained the same. The growth hormone was diluted in distilled water (20 mgm. in 10 ml.) and administered through the rubber tubing of the intravenous set.

The third case, receiving testosterone as an anabolic hormone, was a male patient, case no. 13 (R.M.) 24-years-old with a history of rheumatoid arthritis involving both knee joints. The patient was without treatment during a control period of 7 days. He then received testosterone in aqueous suspension intramuscularly, 50 mgm daily for 11 days.
During both periods morning fasting blood samples were taken daily before the testosterone was administered and non-protein sulphydryl levels were determined. Twenty-four hour urine collections were carried out and total nitrogen determined.

**Materials and methods**

Growth hormone was prepared by the Armour Laboratories from porcine pituitaries by the Raben-Westermeyer method (Lot no. R 491082 P). According to the assays carried out by the Armour Laboratories, the contamination of this preparation with other pituitary hormones is negligible. However, the contamination with ACTH is relatively high as each vial contains 0.03 I.U. per mg. of solids, which would account for 8.25 I.U. of ACTH in the subject receiving 80 mg. of growth hormone and 4.1 I.U. of ACTH in the subject receiving 40 mg. of growth hormone. The pH of the powder preparation is 3.9 and it is easily dissolved in water.

Adrenocorticotropic preparation used was prepared by the Armour Laboratories (Lot no. L 59009).

The amino acid solution was prepared by Merck and Co. by recombination of amino acids derived from casein by complete acid hydrolysis and contained, according to the microbiological assay, the ten essential amino acids plus glycine and traces of glutamic acid. (No SH-bearing amino acids were contained in this solution). All the amino acids were in the L-form with the exception of d,l-tryptophane.

Urinary nitrogen was determined by a semi-micro modification of the standard Kjeldahl procedure (164).
Results

The results obtained in these experiments are given in figure 8 and are expressed as a percentage change from the control level for the sake of uniformity.

Non-protein SH values obtained during the growth hormone and ACTH infusion are given for comparison in the same figure.

At the upper part of the figure the values obtained during the control infusion of the amino acid solution alone are presented. A considerable variation was found during the control amino acid infusions in both subjects. During the growth hormone experiment both subjects showed a decrease of 12 to 18% during the first hours of infusion. The lowest value was obtained in case no. 12 (E.K.) 30 minutes after the start of the infusion. There was no correlation with the dose of growth hormone administered. At 2 hours after the infusion the values were back to normal. In case no. 11 (D.H.) a 5 to 10% increase was obtained during the 3rd, 4th and 5th hours after the infusion.

In the ACTH infusion experiment one subject (case no. 12, E.K.) showed a decrease less than 10% while the other (case no. 11, D.H.) showed a decrease of 23%. Both had returned to baseline levels after 3 hours.

In the second subject (D.H.) the ACTH infusion period was interrupted for technical difficulties and then prolonged for another 15 minutes until the total amount of 300 ml. of amino acid fluid was injected.

No significant changes in hematocrit values occurred with one exception, case no. 11 (D.H.) who showed a drop at the second hour of the
Figure 8
ACTH experiment from 44% to a level of 38.5%. The hematocrit values thereafter ranged between 40% and 42%.

The patient R.M. (case no. 13) with rheumatoid arthritis who was receiving testosterone in aqueous suspension intramuscularly, showed a range of 3.8 to 4.0 mgm% in blood non-protein SH during the control period, and a range of 3.7 to 4.0 mgm% during the hormonal treatment.

Though there was a nitrogen retention of 7-8 gr./24 hours, there was no change in non-protein SH.

b. The Effect of Growth Hormone upon the Non-protein Sulphydryl of Liver Tissue

It has been previously pointed out that the liver is one of the main sites of glutathione synthesis. The livers of rats contain enzymes capable of synthesizing but not hydrolysing glutathione.

Gregory and Goes in 1935 (165) showed that a single injection of growth hormone (prepared according to the method of Evans) administered into a mature female rat produced a 30% decrease of liver glutathione concentration in 8 hours and a 55% decrease in 12 hours after the injection. They suggested that this effect could furnish a method for the estimation of the potency of growth hormone.

In 1938 this was reinvestigated by Lee (166) but the results obtained were not satisfactory as the glutathione method was not accurate. Binkley (78), in studying the effects of insulin upon the liver glutathione concentration, found that 5 units of insulin caused a steady fall in the
content of liver glutathione, reaching the lowest value in 2 hours after
the administration of the hormone. Furthermore, the magnitude of the
effect was dependent upon the dosage, suggesting the use of this effect
for the assay of insulin preparations.

An experiment was undertaken to investigate the effect of a
purified preparation of growth hormone on the non-protein sulphhydryl
groups of rat liver tissues in an attempt to study the time and dose re­
response relationship.

Materials and methods

Male rats of an inbred hooded strain with a body weight of 90
to 100 grams were used. Each group contained 5 to 6 animals. They were
fasted for 18 hours before use, but allowed to take water ad libitum
during this period.

The growth hormone was administered in 1 ml. of distilled water
intraperitoneally; control animals received 1 ml. normal saline. After
varying intervals of time the animals were anesthetised with nembutal and
liver samples obtained. These were taken as quickly as possible, blotted
with filter paper to remove any excess blood, and weighed on a torsion
balance. They were then ground in an all glass tissue grinder which was
immersed in an ice bath. The grinding was carried out in 2.5% sulfo­
salicylic acid containing 0.5% sodium ethylene diamine tetracetate using
2 ml. of this solution for each 200 mgm of tissue. The emulsions were
centrifuged for 20 minutes at 3,000 r.p.m. and the supernatant used for
titration.
In the calculations one must correct for the electrode standardisation and for the dilution involved in preparation of the filtrate. The results are expressed as mgm of non-protein SH per 100 gm of tissue. Not more than six determinations were carried out at a time. This number allowed ease of handling so that titrations could be carried out within 30 minutes. The growth hormone preparation used was the same as that administered to human subjects (page 93).

Experiment no. 1

A preliminary experiment was done to determine the normal non-protein SH values obtained in non-fasting rats. At the same time sections of two different lobes of the liver were taken in order to determine possible variations in different sections of the liver. In table XIV the values obtained are given as SH mgm per 100 gm of tissue. The mean differences between two sections was found to be 0.5 ± 0.24*. This difference is not statistically significant (t-value = 2.08; P > 0.05).

**TABLE XIV**

LIVER NON-PROTEIN SH OF NORMAL NON-FASTED RATS

<table>
<thead>
<tr>
<th>Number of animals</th>
<th>Non-protein SH mg, per 100 gr. of liver tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Section A</td>
</tr>
<tr>
<td>1</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>23.4</td>
</tr>
<tr>
<td>3</td>
<td>23.4</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
</tr>
<tr>
<td>5</td>
<td>26.4</td>
</tr>
<tr>
<td>6</td>
<td>23.0</td>
</tr>
</tbody>
</table>

Mean values: 24.03 (S.D. ± 1.2) 24.52 (S.D. ± 1.4)

* (X ± S.E.)
Experiment no. 2

As it was originally planned to study the effect of growth hormone on the non-protein SH of liver tissue in hypophysectomized animals, determinations were done in a group of 8 hypophysectomized rats 24 hours after hypophysectomy, without prior fasting. The completeness of hypophysectomy was confirmed at autopsy. Unfortunately this work was not continued because of technical difficulties which occurred later in preparing the hypophysectomized animals. The values obtained are given in table XV. Determinations of non-protein SH in this experiment were carried out in two separate samples obtained from different lobes of the liver. The mean value of liver non-protein SH for 7 animals was 12.05 (S.D. ± 1.2) and 12.98 (S.D. ± 1.8) for the duplicate liver samples. The mean of differences between two sections (1.5 ± 0.23)

**TABLE XV**

<table>
<thead>
<tr>
<th>Number of animals</th>
<th>Non-protein SH per 100 gr. of liver tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Section A</td>
</tr>
<tr>
<td>1</td>
<td>12.2 mg.</td>
</tr>
<tr>
<td>2</td>
<td>12.6</td>
</tr>
<tr>
<td>3</td>
<td>11.2</td>
</tr>
<tr>
<td>4*</td>
<td>21.6</td>
</tr>
<tr>
<td>5</td>
<td>12.2</td>
</tr>
<tr>
<td>6</td>
<td>11.0</td>
</tr>
<tr>
<td>7</td>
<td>14.3</td>
</tr>
<tr>
<td>8</td>
<td>10.3</td>
</tr>
</tbody>
</table>

Mean value: 12.05 (S.D. ± 1.2) 12.98 (S.D. ± 1.8)

*At autopsy this animal showed incomplete hypophysectomy. There was a loss of 10-20 grams of body weight for each animal 24 hrs. after hypophysectomy. Animal 4 had a body weight of 15 gr. above the highest body weight in the rest of the group. Animal 4 was excluded from the statistical calculation characterised as a non-hypophysectomized rat.
was found to be statistically significant (t value = 6.5; P < 0.001).

The values obtained for liver non-protein SH are of the same order as those obtained by other workers applying the amperometric titration method. These values are given in table XVI.

**TABLE XVI**

NORMAL VALUES OF NON-PROTEIN SH CONCENTRATION OBTAINED IN LIVER TISSUE OBTAINED BY THE AMPEROMETRIC TITRATION METHOD

<table>
<thead>
<tr>
<th>Authors</th>
<th>Animal</th>
<th>Number of animals</th>
<th>Condition</th>
<th>Non-protein SH per 100 gr. tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goldzieher (98)</td>
<td>rat</td>
<td>4</td>
<td>overnight fast</td>
<td>31.3 ± 3.5 mg.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>fasting</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&quot; (162)</td>
<td>4</td>
<td>fasting</td>
<td>23.4 ± 3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 hrs.</td>
<td></td>
</tr>
<tr>
<td>Beck (167)</td>
<td>mouse</td>
<td>7</td>
<td>-</td>
<td>22.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>-</td>
<td>21.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>-</td>
<td>25.9</td>
</tr>
<tr>
<td>Ingbar (40)</td>
<td>rat</td>
<td>12</td>
<td>fasting</td>
<td>21.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4-10 hrs.</td>
<td></td>
</tr>
</tbody>
</table>

**Experiment no. 3**

A preliminary experiment was carried out in order to determine the suitable doses by which a measurable change could be obtained.

All animals were fasted for 18 hours, injected, and samples taken after a further 2 hours during which the fasting was continued. The duration of fasting and the time of injection were those previously used by Binkley (78) by which he obtained the highest effect in the insulin
experiments.

Three groups of 4 animals each were used. The first group was kept as a control, receiving 1 ml. of normal saline intraperitoneally. Group 2 received 20Y of growth hormone and the third group 40Y.

From the values obtained it is seen (table XVII) that 40Y increased the liver non-protein SH 5 mgm above the control value. A smaller increase was obtained with the lower dose.

A further experiment to confirm the results obtained with 40Y was carried out in a larger group of animals and the obtained values are given in table XVIII.

**TABLE XVII**

LIVER NON-PROTEIN SH VALUES AFTER THE ADMINISTRATION OF GROWTH HORMONE

<table>
<thead>
<tr>
<th>Number of animals</th>
<th>Treatment</th>
<th>Non-protein SH mg. per 100 gr. tissue</th>
<th>Mean values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>16.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>15.9</td>
<td></td>
<td>15.6 (S.D.±0.6)</td>
</tr>
<tr>
<td>4</td>
<td>14.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>17.5</td>
<td></td>
<td>18.2 (S.D.±0.609)</td>
</tr>
<tr>
<td>6</td>
<td>18.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>19.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>18.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>21.2</td>
<td></td>
<td>20.07 (S.D.±0.99)</td>
</tr>
<tr>
<td>10</td>
<td>20.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>19.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>19.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE XVIII

LIVER NON-PROTEIN SH VALUES AFTER THE ADMINISTRATION OF

40Y OF GROWTH HORMONE

<table>
<thead>
<tr>
<th>Number of animal</th>
<th>Treatment</th>
<th>Non-protein SH mg. per 100 gr. tissue</th>
<th>Mean values</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>14·0</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>18·0</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Control (normal saline)</td>
<td>15·6</td>
<td>15·3</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>14·6</td>
<td>(S.D.±1·47)</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>14·2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>15·6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>growth hormone (40Y)</td>
<td>21·4</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>19·0</td>
<td>20·2</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>21·4</td>
<td>(S.D.±1·45)</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>18·2</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>21·0</td>
<td></td>
</tr>
</tbody>
</table>

Experiment no. 4

In order to study the time response relationship to the dose of 40Y selected as suitable, an experiment was carried out by keeping the experimental conditions constant, changing only the time interval between the injection and sampling.

Intervals of 1, 2, 4 and 6 hours were studied during which the animals were still fasted. Furthermore, as Gregory and Goss (165) obtained an effect after longer intervals, samples were taken at 20, 24, 26 and 30 hours. For the same time intervals control experiments were performed. Each group contained 4 to 6 animals. The average weight of
the rats was 90-100 grams. The growth hormone solution was prepared frequently and kept under refrigeration. It was discarded after 24 hours and a new solution prepared. The results obtained are given in table XIX.

Discussion

From the experiments performed in human subjects and during the control amino acid infusion alone, no consistent changes in the blood non-protein SH were obtained.

When growth hormone was administered non-protein SH decreased 12 to 18% for only one hour. A dose response relationship was not obtained in these two subjects when 80 and 40 mg. of growth hormone were administered.

As in the ACTH experiment, an almost identical decrease was obtained to that found after the administration of growth hormone, no conclusion can be drawn. The question that possibly a growth hormone effect could have been overcome by an ACTH contamination cannot be answered at the present time, but in view of the small doses of ACTH used in order to produce a decreasing effect according to recent work (97), this appears to be probable.

In the patient with rheumatoid arthritis under testosterone treatment, no changes were obtained in the non-protein blood SH level in spite of a positive anabolic effect of this hormone.

From the experiments performed in rats the normal non-protein
### TABLE XIX

**LIVER NON-PROTEIN SH VALUES IN VARYING TIME INTERVALS AFTER THE ADMINISTRATION OF 40 µ OF GROWTH HORMONE**

<table>
<thead>
<tr>
<th>Time interval after injection and sampling (hour)</th>
<th>No. of animals</th>
<th>CONTROL Non-protein SH mg./100gr. tissue</th>
<th>GROWTH HORMONE Non-protein SH mg./100gr tissue</th>
<th>Significance (t value)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>17.5 (S.D.+2.17)</td>
<td>16.4 (S.D.+1.41)</td>
<td>-.97</td>
<td>&gt;.3</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>15.4 (S.D.+1.16)</td>
<td>20.1 (S.D.+1.22)</td>
<td>8.55</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>17.7 (S.D.+2.55)</td>
<td>16.3 (S.D.+1.45)</td>
<td>1.17</td>
<td>&gt;.2</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>18.1 (S.D.+2.6)</td>
<td>18.8 (S.D.+1.55)</td>
<td>1.58</td>
<td>&gt;.5</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>18.6 (S.D.+1.13)</td>
<td>18.2 (S.D.+1.55)</td>
<td>1.51</td>
<td>&gt;.6</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>18.3 (S.D.+1.30)</td>
<td>21.0 (S.D.+2.44)</td>
<td>1.40</td>
<td>&gt;.1</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>20.2 (S.D.+1.79)</td>
<td>21.8 (S.D.+1.45)</td>
<td>1.55</td>
<td>&gt;.1</td>
</tr>
<tr>
<td>24</td>
<td>5</td>
<td>29.1 (S.D.+2.4)</td>
<td>27.5 (S.D.+2.14)</td>
<td>1.16</td>
<td>&gt;.2</td>
</tr>
<tr>
<td>26</td>
<td>5</td>
<td>23.2 (S.D.+1.84)</td>
<td>21.0 (S.D.+1.45)</td>
<td>2.08</td>
<td>&gt;.5</td>
</tr>
<tr>
<td>30</td>
<td>6</td>
<td>30.5 (S.D.+1.95)</td>
<td>28.5 (S.D.+2.47)</td>
<td>1.55</td>
<td>&gt;.1</td>
</tr>
</tbody>
</table>
SH values obtained for the liver tissue are in agreement with those found by other workers using the amperometric method, with the exception of Goldzieher who obtained somewhat higher values in a group of 4 normal animals. By the specific manometric method similar values have been obtained for liver non-protein SH and this, according to Ingbar (40), indicates that glutathione is the principal soluble non-protein SH in the liver tissue. No explanation is offered for the significant differences found in the two liver sections of the hypophysectomized animals. A significant decrease was found in the liver non-protein SH values (t value 20.6; P= <0.001) of the hypophysectomized animals compared to the normal non-fasting rats. This is not in agreement with the findings of Binkley (168).

It should be mentioned that the rats used by Binkley in his experiments were maintained for 3 weeks after hypophysectomy and then fasted for 16 hours before liver samples were taken. The control animals fasted for varying periods after the injection showed an initial fall in liver SH concentration to a low level at 2 hours, then a slow rise to or above initial levels by 20-30 hours. Growth hormone had no significant effect except for the 2-hour samples which, as in experiment 3, showed a higher concentration of SH after the injection of the hormone. The explanation for these variable results is not clear since little is known of the intermediary metabolism of glutathione. The increase occurring after prolonged fasting might be due to increased synthesis (as only enzymes capable of synthesizing but not hydrolyzing glutathione have been found in the liver) or increased transportation to the liver of the constituent amino acids of glutathione produced either by increased hydrolysis in the kidney (which is known to contain hydrolytic enzymes) or by a catabolic phase of protein metabolism. That
the results are not related to the stress of handling or injection was shown by injecting 4 non-fasted animals with 1 ml. of normal saline and measuring liver non-protein SH two hours later. The average value obtained was 25.25 mgm per 100 grams of tissue, which is in the normal range of the non-injected rats. That the decrease is not due to endogenous ACTH release is also indicated by the work of Goldzieher (98) who showed no change in the liver non-protein SH three hours after the injection of 0.1 mgm of ACTH.

However, the variability of the results and the relatively small effect of 40Y of hormone indicate that this action of growth hormone does not constitute a good basis for bioassay. Possibly hypophysectomized animals with lower non-protein SH liver values may be more sensitive to such small doses of growth hormone preparations.

6. THE EFFECT OF INSULIN UPON THE BLOOD NON-PROTEIN SULPHHYDRL LEVEL

The inconsistent results obtained by several workers in studying the effect of insulin on the blood non-protein SH may be explained by the variety of conditions and treatments of the diabetic patients used.

Furthermore, the possibility that a prompt and not prolonged effect may have been missed after insulin administration suggested the study of non-protein SH values after an intravenous administration of insulin to a normal subject. A patient with diabetes mellitus with insulin resistance was also studied in order to detect whether the resistance was a result of increased blood glutathione values (see page 20).
Plan of study

A normal male subject aged 22 (case no. 14 - H.W.), in a fasting condition, received 350 ml. of an amino acid infusion* over 30 minutes. In the first 15 minutes the insulin was administered intravenously in a dose of 0.1 units/Kg. of body weight (total dose 7.5 units).

Blood samples for non-protein SH were taken every 10 minutes for the first ½-hour, every 15 minutes for the second ½-hour and hourly thereafter for a period of 6 hours, during which the patient continued fasting. Blood sugar determinations were simultaneously carried out. The insulin used was an aqueous solution of crystalline zinc insulin prepared by the Connaught Laboratories. A 36-years-old woman (case no. 15 - E.R.) known as a diabetic for the last 10 years, was admitted to the metabolic ward of the Royal Victoria Hospital. This patient showed an insulin resistance and required large amounts of insulin to control glycosuria and hyperglycemia. Dosage as high as 1,100 units per day had been administered. The nature of the resistance was not determined. In this patient non-protein sulphhydril determinations were carried out during a period in which the patient was not under insulin or other treatment. Blood samples were taken in the morning in a fasting condition.

Results

The values obtained during the intravenous administration of insulin are given in table XX. No change was obtained in the non-protein

*In order to compare the results with those obtained with growth hormone and ACTH, the amino acid infusion was concomitantly administered.
SH in spite of the significant decrease of the blood sugar values* in the 30 and 45 minute samples. The range of non-protein SH during the whole infusion period was 3.3 to 3.6 mgm%.

In the diabetic patient (case no. 15 - E.R.), during a period of 23 days during which no treatment was administered, the blood fasting sugar values ranged between 220 and 385 mgm%, the highest values obtained during the end of this period. No ketosis was detected. During this time the non-protein sulphhydril estimations did not give values higher than 3.7 mgm% which is in the upper limit of the normal range and corresponds to a glutathione value of 34.4 mgm%. This patient showed in certain instances considerable variation in non-protein SH blood values (between 2.8 and 3.7 mgm%). The patient gave values around 3.9 mgm% for non-protein SH when under insulin treatment.

**TABLE XX**

**BLOOD NON-PROTEIN SH LEVELS AFTER INTRAVENOUS INSULIN ADMINISTRATION**

<table>
<thead>
<tr>
<th>Time</th>
<th>Non-protein SH mgm%</th>
<th>Hematocrit %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.36</td>
<td>49</td>
</tr>
<tr>
<td>10'</td>
<td>3.50</td>
<td>48.5</td>
</tr>
<tr>
<td>20'</td>
<td>3.49</td>
<td>47</td>
</tr>
<tr>
<td>30'</td>
<td>3.46</td>
<td>47.5</td>
</tr>
<tr>
<td>45'</td>
<td>3.39</td>
<td>48.5</td>
</tr>
<tr>
<td>1</td>
<td>3.31</td>
<td>49</td>
</tr>
<tr>
<td>2</td>
<td>3.46</td>
<td>49</td>
</tr>
<tr>
<td>3</td>
<td>3.53</td>
<td>48</td>
</tr>
<tr>
<td>4</td>
<td>3.51</td>
<td>49</td>
</tr>
<tr>
<td>5</td>
<td>3.62</td>
<td>49</td>
</tr>
</tbody>
</table>

*See thesis of Dr. A. Carballeira.*
Discussion

The results obtained in case no. 15 (E.R.) are in agreement with the findings of Caren (80) in a similar patient with insulin resistance. Lazarow (8) injected glutathione intraperitoneally (2.5 mgm per Kg.) in rats simultaneously with varying doses of regular insulin (2.5 to 20 units per Kg.) subcutaneously. Determinations of blood sugar were made ½, 1, 2 and 4 hours after injection and it was found that glutathione modified only slightly the effect of insulin in lowering the blood sugar. These results suggest that it is unlikely that insulin resistance could be explained on the basis of high glutathione concentration.

No changes in glutathione were obtained in the subject to whom insulin was administered intravenously, in spite of the significant changes in blood sugar values. An effect similar to that obtained by Krah (7) in in vivo experiments where insulin in the presence of carbohydrate increased glutathione synthesis in liver tissue was not detected in the human blood SH concentration. Changes in the products of hydrolysis of glutathione as found by Binkley (84) in diabetic patients cannot be detected by the amperometric method.

As previously reported by other workers, no relationship was observed between blood sugar concentration and blood SH concentration.

In this discussion it is relevant to bring up from the first group of experiments done with hydrocortisone and ACTH some of the findings related to carbohydrate metabolism. Conn (19) showed low glutathione values in a patient with Cushing's syndrome with an abnormal intravenous
glucose tolerance test. When a glucose tolerance test was carried out in the same patient with the addition of 2 grams of glutathione administered intravenously, the curve of the test was normal. In the same patient after a 4-month period during which over 90% of the adrenal tissue was removed, a normal glucose tolerance test was obtained and the blood glutathione value had increased to normal. It seemed from this study that some relationship existed between glutathione and the glucose tolerance.

The blood sugar method of Folin and Wu is known to measure a considerable amount of non-fermentable reducing material of which glutathione is the most important quantitatively.

Mosenthal (169) has shown that when values of blood sugar obtained by this method were compared to those obtained for the true blood sugar during a glucose tolerance test, a considerable variation in the non-glucose reducing substances occurred during the test. A much larger variation was obtained when this test was performed in diabetic patients; this was attributed to variations in glutathione concentration. According to the findings of Mosenthal in a large number of glucose tolerance tests, the non-glucose reducing substances were affected and in 23 of the curves (46%) at least one determination exceeded the upper normal limit accepted as 30 mgm per 100 ml. Because of these two pieces of evidence, linking blood glutathione to glucose tolerance test, an attempt was made in the normal subjects receiving hydrocortisone and in one subject receiving growth hormone and ACTH, to study the non-protein SH during a
Figure 9

GLUCOSE TOLERANCE TEST

CONTROL

HYDROCORTISONE

ACTH

BLOOD SUGAR (MGL)

BLOOD NON-PROTEIN SH (MGL)

TIME IN MINUTES

Figure 9
glucose tolerance test and compare the curve obtained under the control infusion to that of the hormonal infusion where an impaired carbohydrate metabolism was expected. The glucose tolerance test was performed at the end of the 6-hour infusion period for the hydrocortisone experiments and the technique used is given in the plan of study for that experiment. In figure 9 the values obtained during the glucose tolerance test for the non-protein SH are given. In the lower part of this figure the blood sugar values are given for the glucose tolerance test**. The variations of the non-protein SH during the test and after the control infusion is not higher than ± 5%. After the hormone infusion there was no consistent effect on the SH though the glucose tolerance was impaired in all cases. The range of values was somewhat greater than in the control tests but was still ± 10% of the starting level. Furthermore, in a thyrotoxic patient studied* with very low non-protein SH blood values, a glucose tolerance test was carried out and was normal.

In summary the results seem to indicate:

a) that in spite of a definite effect of hydrocortisone on carbohydrate metabolism seen in the decreased tolerance during a glucose test, no consistent change in the blood non-protein SH levels was obtained.

b) in spite of a moderate and transitory decrease of the non-protein SH after the ACTH infusion, the blood glucose tolerance values appeared to be only slightly higher compared to the control.

c) in one patient with significantly low value of non-protein SH, a normal glucose tolerance was obtained.

* The experimental data of this patient are given in the last section of the experimental work.
**These values have been made available through the courtesy of Dr. A. Carballeira.
From this study it was not possible to detect any parallelism between glutathione blood values and impaired carbohydrate metabolism.

7. **BLOOD NON-PROTEIN SULPHHYDRYL DETERMINATIONS IN THYROTOXIC PATIENTS**

In order to investigate the possible relationship of the non-protein SH groups in connection with the thyroid gland, blood non-protein SH group determinations were carried out in two thyrotoxic patients before and after treatment was started.

**Plan of study**

Two thyrotoxic patients were studied before and during treatment.

Case no. 16 (B.B.), a 51-years-old woman with uncomplicated exophthalmic goitre, the diagnosis being established by radio iodine uptake studies. Liver function tests were normal. On treatment with 400 mgm of propylthiouracil daily there was subjective and objective improvement.

Case no. 17 (Y.B.), a 29-years-old female with thyrotoxicosis and complicating impairment of liver function as shown by the usual tests. On treatment with Tapazol 30 mgm daily and Lugol's solution 30 min. daily, there was improvement in both the thyroid and hepatic conditions.

In both patients blood samples for non-protein SH were taken before and during treatment in a non-fasting condition. In case no. 17 (Y.B.) an intravenous glucose tolerance test was performed by administering 50 c.c. of a 50% dextrose solution in 2 minutes. Blood samples for sugar determinations were taken at 30 minutes, one hour and two
<table>
<thead>
<tr>
<th>Day of study</th>
<th>Case no. 16 (B.B.)</th>
<th>Case no. 17 (Y.B.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood non- protein SH mgm%</td>
<td>Hematocrit %</td>
</tr>
<tr>
<td>1</td>
<td>3.7</td>
<td>45</td>
</tr>
<tr>
<td>2</td>
<td>4.0</td>
<td>43</td>
</tr>
<tr>
<td>3</td>
<td>3.8</td>
<td>46</td>
</tr>
<tr>
<td>4</td>
<td>3.8</td>
<td>45.5</td>
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<tr>
<td>5</td>
<td>4.1</td>
<td>45</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>4.1</td>
<td>46</td>
</tr>
<tr>
<td>8</td>
<td>3.9</td>
<td>46</td>
</tr>
<tr>
<td>9</td>
<td>3.8</td>
<td>45.5</td>
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<tr>
<td>10</td>
<td>4.0</td>
<td>44.5</td>
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<td>11</td>
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<tr>
<td>12</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>3.9</td>
<td>46</td>
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<tr>
<td>14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15</td>
<td>3.7</td>
<td>45</td>
</tr>
<tr>
<td>16</td>
<td>4.0</td>
<td>45.5</td>
</tr>
<tr>
<td>17</td>
<td>3.9</td>
<td>45.5</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
hours after the end of the injection.

Results

The results obtained are given in table XXI.

In case no. 16 (B.B.) blood non-protein SH varied from 3.7 to 4.1 mgm\%. Patient Y.B. showed a range in non-protein SH from 2.3 to 3.0 mgm\%. During the glucose tolerance test performed on this patient the following values were obtained:

<table>
<thead>
<tr>
<th>Time (mins)</th>
<th>Blood sugar (mgm%)</th>
<th>Blood non-protein SH (mgm%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>75</td>
<td>3.0</td>
</tr>
<tr>
<td>30</td>
<td>108</td>
<td>3.1</td>
</tr>
<tr>
<td>60</td>
<td>83</td>
<td>3.09</td>
</tr>
<tr>
<td>120</td>
<td>69</td>
<td>2.84</td>
</tr>
</tbody>
</table>

Discussion

Patient B.B. showed no change during the period under treatment in spite of clinical improvement. The second patient, Y.B., showed extremely low values which correspond to blood glutathione values of 21.3 mgm\%. On no other occasion were such low values obtained in a human individual during this work. It is not permissible to conclude that the low non-protein SH values obtained in this patient were due to the thyrotoxic state as this patient had an impaired liver function. It has been found that low glutathione values are obtained in liver disease. During
treatment no change was seen in the non-protein SH. Patient Y.B. (no. 17) consistently showed values somewhat higher after 14 days of treatment. The results obtained during the glucose tolerance test are discussed on page 109-113.

These two experiments do not give evidence for a possible low blood value of non-protein sulphydryl in hyperthyroid patients. A larger group should be tested and for a prolonged period of time before, during and after treatment.
SUMMARY

1. The role of glutathione in metabolism has been reviewed. Emphasis was placed upon the relationship between different hormones and vitamins and this tripeptide in intermediary metabolism.

2. The available methods for the determination of glutathione in blood and tissue have been critically reviewed. A method for the determination of total non-protein sulphhydryl groups has been presented and advantages and disadvantages discussed. The specificity, reproducibility and simplicity of the method have been found satisfactory for clinical investigative use.

3. When an excess of adrenal cortical steroids (Compounds E and F) were used by different routes of administration, no effect upon the non-protein SH concentration of normal individuals was obtained but low values were found in patients with adrenal steroid insufficiency. The prompt and transitory effect of ACTH upon glutathione levels seems not to act through the adrenal gland.

4. In studying inter-relationships of the two powerful reducing agents in human blood, ascorbic acid and glutathione, no reverse or parallel reactions in blood were seen under the influence of adrenal steroid hormones. Some data are given favouring a renal effect in explaining the high excretion of ascorbic acid under the influence of an intravenous administration of 100 mgm of Compound F.

5. In administering a purified growth hormone preparation intravenously, concomitantly with an amino acid infusion, the produced changes do not
support the early concept that glutathione could be used as an index of anabolic activity.

No consistent changes were found with small doses of growth hormone on the non-protein SH of liver tissue in normal rats. Under the conditions used it was felt that this could not be applied as the basis for a bioassay of growth hormone. The mechanism of the biphasic response found in the liver non-protein SH under prolonged fasting is not clearly understood.

6. In studying different aspects of carbohydrate metabolism in relation to glutathione blood concentrations, no constant relationship was observed. Earlier work relating adrenal steroids, blood sugar and blood glutathione could not be confirmed.

7. In two patients no evidence could be obtained that non-protein sulphydryl values are related to thyroid activity.
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