I. OBSERVATIONS ON THE METABOLIC RESPONSE TO SURGICAL TRAUMA.

II. OBSERVATIONS ON THE METABOLISM OF ASCORBIC ACID IN MAN.

by

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INTRODUCTION.

1. GENERAL.

The study of the various aspects of the metabolic response to surgical trauma is one that has interested many workers and, within recent years, a large volume of literature has been devoted to the subject. The rate of advancement has been slow, however, and this may be attributed, initially, to the lack of simple and readily applicable methods of chemical analysis. A great amount of patient and prolonged research has lead to the determination of a pattern for the metabolic changes subsequent to general surgical trauma.

The continuation of this work by a study of operations such as adrenalectomy and hypophysectomy, has been delayed, in this laboratory at least, by the scarcity of patients. Since the number of subjects requiring such drastic and radical treatment is, fortunately, still very small full advantage must be taken of the available cases in the hope that any deductions forthcoming from the results will yield, at least, a pointer to the role of these two glands in the general metabolic responses which follow trauma.

In order to obtain a general outline of the more fully investigated aspects of this subject and, if possible, to try to correlate these findings, a selection of material must be made from the vast
amount of data which is available. Two factors must influence this selection and these are personal choice as regards the bearing of such material on the project in mind as well as the availability of suitable methods of chemical analysis which will allow of a continuation of the work. The topics which will be considered here, therefore, are electrolytes and associated water excretion phenomena, nitrogen metabolism and lastly, the influence of the activity of the adrenal glands on the foregoing aspects.

These various factors will now be considered separately.

2. ELECTROLYTES and WATER EXCRETION.

The observation that there is often a state of oliguria in the period immediately following trauma has been noted for a long time - Pringle (1905), Hawk (1911). However, since there is a period of restriction of water intake at this time, along with relatively large fluid losses at operation, little attention was afforded to the existence of the oliguria as a separate factor.

One result which did follow was the initiation of a regime whereby the patient was infused with what, in many occasions, amounted to very large volumes of isotonic saline in order to supply the supposedly needed water - Coller and colleagues (1936),
Bartlett and co-workers (1938). In so doing the warnings of such workers as Evans (1911), Trout (1913) and later Matas (1924) were completely disregarded. These workers had pointed out that post-operative patients were incapable of tolerating large volumes of infused saline. The practice of infusing saline into such patients, regardless of their need, was subjecting them to the danger of oedema, which could, and often did, lead to fatal results. This work was substantiated by that of Coller et al. (1936), and of Stewart and Rourke (1942), who were able to show that the substitution of a solution of 5% dextrose in distilled water allowed of a normal water exchange without the risk of oedema. In other words, the use of such a solution did not increase the degree of the diminished water excretion which follows trauma.

Jones and his colleagues (1934) found that cats subjected to trauma exhibited a marked retention of both water and sodium and that the phenomena tended to be rather transitory. The existence of marked oedema was noted whenever water retention was evident and, in a few cases, even when there was an excellent diuresis. In the light of more recent work this latter statement probably results from the fact that the periods of urine collection tended to be rather long so that, in some cases, the phase of oliguria was not separated from the ensuing diuresis.

The existence of a low urinary output of sodium
and of chloride had often been noted in patients during the post-operative period - Robineau (1933), Bartlett and co-workers (1938) - but this was thought to be due to the conservation of these ions by the kidney as a result of the diminished intake.

These observations led to the continued use of intravenous saline infusions in the immediate post-operative period and often, in cases where large fluid losses were to be expected during the operation, at the commencement of surgery. However, it was felt that the unrestricted use of saline was not to be recommended since the prolonged administration might lead to oedema, Bartlett et al. (1938), Coller and colleagues (1938), and White and associates (1938). As a result a so-called clinical rule was suggested by Coller et al. (1938) and Bartlett et al. (1938) which gave a rough working guide as to the desired amount of saline to be used. Apart from the volume for volume replacement suggested, the general infusion of one litre on the day of operation was proposed.

The dangerous fallacy of this rule is now seen to lie in the fact that the amount of chloride excreted in the urine was used as the yardstick for measuring salt depletion. Coller and Maddock (1940), have shown that urine chloride content does not necessarily reflect the state of the body content of sodium and of chloride.

It is of interest to note the observation about
this time by Silvette and Britton (1933) concerning the water retention in adrenalectomised rats. It was found also that the administration of adrenocortical extract to such animals resulted in the chloride excretion falling to that of normal rats. This was taken as an indication that the excretion of chloride was controlled by extra-renal means.

The continued administration of saline following Coller's rule was uninterrupted, with few exceptions, until a strong plea by Coller and his colleagues (1944) brought the more salient facts concerning 'salt intolerance' to the notice of surgeons and called for the retraction of the clinical rule. One of the main reasons for the administration of the saline had been to raise the lowered plasma chloride levels but Coller et al. (1944) were able to prove that these were little affected by the infusion and did not regain the so-called normal level. Later, Coller and his co-workers (1945), demonstrated that, on the average, a retention of 53% of the dose of sodium and 19% of the water was evident following the injection of isotonic saline. Such a retention of salt indicates the withdrawal of approximately two litres of fluid from the intracellular compartment in order to maintain isotonicity. In order to cause an increased retention of water with a lowered retention of sodium chloride, it was suggested that hypotonic saline might be substituted. The increased retention would thus allow
of an extra amount of water available for excretory functions.

That the primary oliguria was not due to the effects of the anaesthetic was shown by Coller and associates(1943), and by Moyer(1950). As a result of an extensive study of the various mechanisms of the kidney in a person who had been anaesthetised but not subjected to operative processes, they were able to demonstrate that there was no deleterious action of the anaesthetic upon the kidney parenchyma. Post-operatively, however, there is a fall in the glomerular filtration rate as well as a fall in the renal blood flow and therefore the cause must be the operation itself. Since there was no evidence of either albumen or red cells in any of the post-operative urines it was stated that the possibility of increased glomerular capillary permeability was eliminated. It was suggested, therefore, that the primary causes of the post-operative oliguria were the changes in body water, ionic distribution and ionic concentration as well as changes in electrolytes and acid-base balance, the latter being partially due to hyper-ventilation.

An interesting point was brought to light by Limbert and co-workers(1945) and by Cooper, Iob and Coller(1949), who discovered that the rate of sodium excretion was greater in the oliguric phase than it was in the period when a very dilute urine was being
excreted. Here we have the first indication that the excretion of sodium is not related to that of water in the post-traumatic state and that we are dealing with two separate, if inter-related, phenomena. The more severe the operation the more marked and prolonged is the period of oliguria. In some cases complete anuria has been known to follow operation. In order to eliminate the possibility that the more severe traumas involve greater fluid loss with resultant diminution in body water transfusions of blood were given, during the time of surgery, in amounts calculated to replace that which was lost. Even under these conditions marked oliguria was still evident and also it was observed that the specific gravities of the urines were of the same order as those from cases in which no attempt had been made to replace the fluid lost - Cooper et al.(1949). Hence the oliguria did not appear to be due entirely to the state of hydration of the patient.

Evidence that the retention of water did not result from the retention of sodium, per se, was forthcoming from the work of Holland and Stead(1951) who studied the effect of pitressin injections on the excretion of water, sodium, potassium and chloride in normal persons. Whilst there was a marked oliguria, due to the increased tubular re-absorption without alteration in the glomerular filtration rate or renal blood flow, following the injection there
was no demonstrable effect on the rate of excretion of sodium, potassium or chloride.

Habif and co-workers(1951) pointed out that the output of sodium, potassium and chloride decreased more than the filtration in the glomeruli and that these changes indicate a relative increase in tubular re-absorption of both water and electrolytes. This is contrary to the previous work and it is taken as an indication that there must be more than one process operating in the post-traumatic state. It is possible that the diminished flow of filtrate along the tubule permits a more effective re-absorption of water and solutes, regardless of the needs of the body and the state of endocrine activity, and thus produces both the oliguria and diminution in the excretion of the electrolytes as long as filtration is low. Here we have agreement with Ariel and Miller(1950) that the changes are not due to any intrinsic renal dysfunction or to kidney damage. Ariel and Miller suggested that the probable cause was a hormonal imbalance resulting from the surgery and reflected in the diminished glomerular filtration rate and renal blood flow. That there was a decrease in these processes had been suggested by Elman and his colleagues(1949) who, while observing the existence of oliguria post-operatively, had noted a fall in creatinine output also. This fall in G.F.R. will reduce the excretion of sodium, as shown by Chalmers and associates(1952).
The transitory nature of the oliguric phase has been demonstrated frequently. As a result of the work of Wilkinson and his colleagues (1949) the fact was established that chloride is excreted in the same manner as sodium in the post-operative period. Many other investigators have verified these findings. In 1953 Le Quesne and Lewis observed that there was a period of diminished water excretion, or primary water retention, which followed the operation and which coincided with a period of reduced sodium output in the urine. In many cases the initial sodium 'retention' was followed by a period of increased sodium excretion but this phase was not always discernible. A further period of diminished sodium excretion followed but, in many cases, the events were so timed that the periods of decreased sodium excretion coalesced and that of increased excretion was not evident. On this evidence these authors suggested that the initial oliguria was due to the action of antidiuretic hormone but they produced no evidence whatsoever of the increased liberation of this substance.

It could be argued that the oliguric phase might be responsible for the initial diminished sodium output since this would lead to a marked dilution of the extracellular fluid, as shown by Zimmerman (1951). As a result of this dilution there would be a tendency for electrolytes to be retained in
order to maintain isotonicity, an observation which is in agreement with the experimental findings following the injection of pitressin - Holland and Stead, (1951).

From all the evidence quoted it would appear to be fairly well established that in the immediate post-operative period there is a marked, if transitory, retention of water along with a more prolonged period of salt retention in which two phases may or may not be detectable.

It is now necessary to consider the changes which occur in potassium excretion during the same period.

While it was recognised early in the 1930's that there was a considerable increase in the urinary output of potassium post-operatively (Cuthbertson, 1930) the initial reaction was to dismiss the finding as being due to the oxidative destruction of tissue resulting from starvation. The classical experiments of Benedict (1915) and of Gamble and associates (1923), in which the excretion of potassium was compared with that of nitrogen in persons subjected to starvation, have provided us with the facts that in this condition the ratio of urinary potassium to urinary nitrogen is relatively constant and in the order of 1:10. Cuthbertson (1930) noted that in the post-operation period the amount of potassium excreted was more than was to be expected from the nitrogen
figures. Cuthbertson, McGirr and Robertson (1932) demonstrated that the loss of potassium was also in excess of that produced by the non-use of limbs - Cuthbertson (1929) - and that the response was more generalised than localised. The only other source of available potassium is that contained in the cells and therefore these must suffer depletion. Schmidtmann and Mathes (1927) had shown already that there was an increased permeability in the cells surrounding damaged tissue and that this led to loss of potassium from the cytoplasm.

In the case of the increased potassium excretion an early parallel was drawn to the activity of the adrenal glands since hypopotassae mia was one of the best known changes in the 'alarm reaction' of Selye (Kendall and Ingle, 1937). Opinions differ regarding the variation of the potassium content of blood in trauma but it appears that the marked rise in the levels obtained by the earlier workers - Clark and Cleghorn (1942) - were due to the use of inaccurate methods of estimation. However it was as a result of these observations and the finding of an increased urinary output of potassium that the connection to the 'alarm reaction' was made.

Stewart and Rourke (1942) also noted the difference in the potassium to nitrogen ratio in urine in the post-traumatic period and suggested that there was a possible connection with the trauma
itself. Howard (1943) disagreed with Cuthbertson's findings and, although he had no actual proof for his statement, he maintained that the patients were in a state of negative potassium balance due to starvation. He went as far as to state that if fed a minimal well-rounded diet there was no loss of potassium. Reifenstein (1944) also held this view and suggested that the increased potassium excretion was due to the utilisation of stored glycogen. The fact that there was a marked decrease in the excretion of 17-ketosteroids over the same period was taken as substantiation of this. However it has been shown since then that the fall in the 17-ketosteroid excretion was not due to starvation since patients fed by means of intravenous drip exhibit the same response - Forbes and his co-workers (1947). It is interesting to note that although at this time both Howard and Reifenstein observed the decreased excretion of potassium following the diuresis no attempt was made to correlate this with increased deposition of glycogen.

Limbert et al. (1945) also attributed the potassium diuresis to starvation but, in 1946, Howard pointed out the relationship in time between the water retention and the potassium diuresis and also the fact that the loss of this element was greater in the immediate post-operative than was to be expected from theoretical grounds. Howard's previous
observation (loc. cit.) can be explained by the fact that the diuresis is only immediately following the operation; the net result over the two or three days after operation is a positive potassium balance.

There is now a considerable amount of evidence to show that the main increase in potassium excretion occurs either on the first post-operative day - Blixenkrone-Moller (1949), Wilkinson et al. (1950, A) - or on the second - Elman and his colleagues (1949).

Various suggestions have been made concerning the reasons for the increased excretion and these vary from the effect of saline infusions - Nelson, Friesen and Kremen (1950) - to endocrine factors. One hypothesis which has been accepted widely is that there is a shift in the distribution of electrolytes between the various compartments. There is considerable cell disruption in the traumatised area and this will lead to a localised alteration in the electrolyte concentration of the extracellular fluid. A definite increase in the concentration of potassium results from the release of the large amounts contained in the cells and this is accompanied by a decrease in the concentration of both sodium and chloride. This latter result leads to a withdrawal of sodium and of chloride from undamaged tissue - Ricca, Fink, Steadman and Warren (1945) and Fox and Bier (1947). The passage of this potassium into the urine cannot be due to impaired kidney function because, as pointed out by
Blixenkrone-Moller(1949), the increased excretion occurs at the peak of the water retention and the kidney must effect a considerable concentration of the ion. It has been suggested that one of the main reasons for the oliguria is that the water is retained in order to dilute the end products of catabolism - Silvette and Britton(1933). This could be achieved as a result of transference of water from the blood to tissues due to increased permeability of the blood vessel walls - Gradinescu(1913).

The disturbance in potassium metabolism may be summarised as a phase of increased excretion in the immediate post-operative period followed by a rather longer period of retention which starts on or about the third day after the operation.

3. **NITROGEN METABOLISM.**

Since the end of last century it has been demonstrated that nitrogen is lost to the body as a result of infection, disease and trauma. Apart from one of the earliest observations, that of Bauer(1872) who noted an increase in urinary nitrogen subsequent to blood letting, most of the initial data was concerned with cases of typhoid fever - Shaffer and Coleman(1909), Coleman and DuBois(1915). Following this work similar increases in nitrogen excretion were noted in tuberculosis - McCann(1922) - and other diseases - Barr and DuBois(1918), Cecil and his co-workers(1922). Bauer's
original work was confirmed by Taylor and Lewis (1915). In 1918 Cooke and Whipple carried out a series of interesting experiments in which sterile abscesses were induced in dogs as a result of turpentine injections. Although there was no infection at the site of injection a marked increase in the amount of nitrogen excreted was discernible along with a concomitant increase in the non-protein nitrogen content of the blood. The reactions observed were similar in nature to those obtained by the injection of a toxic proteose - Whipple and Cooke (1917) - and it was suggested that there were two phases in the reaction. The first was due to the local injury set up by the irritant whereas the second was a more general injury to the body protein which was triggered by the liberation of toxic autolytic products absorbed from the site of the local injury. This theory attributing a part of the general response to trauma to the absorption of metabolites produced at the site of the injury was supported by Cannon and Bayliss (1919).

Cuthbertson opened up the field of study of nitrogen metabolism subsequent to trauma when he reported increased urine excretion of nitrogen in patients who had suffered a fracture of a limb. From a study of nitrogen, phosphorus and sulphur excretion he concluded that in these cases the source of these increases was tissue protein. This period of increased oxidative destruction of protein was designated
the 'catabolic phase'. He was able to prove that the increases noted were not due to mere disuse of the limbs and from a detailed study of the nitrogen content of rat limbs he showed that the nitrogen excreted in the 'catabolic phase' was not derived solely from the tissues in the traumatised area, (1932).

The possibility that the 'catabolic phase' was due to a simple starvation phenomenon, since normal feeding was not continued over the period of trauma, suggested itself and Cuthbertson (1932) made a detailed study of this aspect. A factor which tended to confirm this view was that after the 'catabolic phase' had ended, often within seven to ten days, there followed a period of positive nitrogen balance or 'anabolic phase', in which there was presumably a synthesis of new tissue.

The classical experiments of Benedict (1915) and of Gamble and his colleagues (1923) in which the relationship of urinary nitrogen to urinary potassium had been investigated in subjects in a state of starvation, had shown that approximately one gram of potassium is excreted for every ten grams of nitrogen. This is the ratio in which these two elements are present in muscle tissue. Cuthbertson (1930) found that less nitrogen was excreted than was to be expected from the potassium results and also that the period of negative nitrogen balance continued even after the resumption of normal feeding. These two findings
discredit the theory that the changes are due to starvation, especially the latter one since the return to normal feeding after starvation quickly terminates the negative nitrogen balance.

Many reports are to be found in the literature concerning the increased urinary output of nitrogen after shock and these show it to be a phenomenon which has its origin in many other states apart from surgical trauma. While there is fairly general agreement concerning the existence of such phenomena, there is a diversity of opinion as regards the intensity of the response.

In the case of third degree burns, Lucido (1940), reported the excretion of massive amounts of nitrogen in the urine and associated with this there was a rise in the non-protein nitrogen content of the blood. This latter finding was not due to renal insufficiency as throughout the whole period large volumes of urine were excreted. The loss of nitrogen was so great that only heroic attempts at intravenous administration of high protein, high caloric diet maintained even an approximation to nitrogen balance. Substantiation of these facts was readily forthcoming - Clowes and co-workers (1943), Taylor and colleagues (1943). The suggestion was made by Clowes et al. (1943) that there existed a relationship between the area covered by the burn and the amount of nitrogen lost in the urine, a theory that differed from the findings of
Cope (1943). It must be remembered, however, that in the case of Cope's work the main source of nitrogen intake was whole blood and this in itself must have some influence on his results. There is some doubt as to whether the protein in the intact red cell is available for general metabolic use - Madden and Clay (1945) - and it appears to be established with reasonable certainty that serum proteins themselves may act as an efficient nitrogen-sparing agent, even in cases with vigorous protein catabolism - Holman and co-workers (1934), Pommerenke and associates (1935), Daft and colleagues (1938) and Browne, Schlenker and Stevenson (1944).

A marked difference between the findings reported in surgical trauma and those in burning is that whilst in the former case the period of negative nitrogen balance lasts for a relatively short time and is followed by a period of nitrogen retention, in the latter case the negative balance exists for a much longer time. In both types of trauma it has been established that urea and ammonia are the major components of the urinary nitrogen - Cuthbertson (1942). Cuthbertson et al. (1939) were able to show that there was a slight increase in the excretion of creatinine, a result which was fully expected as a result of trauma, and that the excretion of uric acid was only slightly greater than that found in normals. As pointed out by Howard (1945) these findings may be taken
as an indication that the disposition of the nitrogenous portion of the protein catabolised as a result of trauma follows the same pattern as in health, the only difference is therefore in the amount of protein metabolised.

In the case of fractures a reasonable assumption appeared to be that the increase in urinary nitrogen might find its origin in the atrophy of the disused limb. However it would appear that this is not the case and that the actual atrophied tissue donates only a small amount to the nitrogen excreted. Cuthbertson(1942) studied this aspect in rats and by observing the weight difference between the atrophied and the unaffected limb he was able to calculate the difference in nitrogen content, using average figures for the nitrogen content of tissue. This work was confirmed by Howard and colleagues(1944) and was later extended and made more complete by Munro and Cumming (1948) who actually estimated the nitrogen content of the atrophied and the undamaged limbs. While a small difference was found to exist it was not comparable with the amount of nitrogen lost in the urine and thus Cuthbertson's earlier findings were substantiated. The greater part of the nitrogen must come from sources other than the damaged area.

The reason for this increased nitrogen output has been open to wide speculation. In 1930 Cuthbertson suggested that it was due, in all probability, to
the body catabolising its reserves in order to meet the exigencies of repair and maintenance. This view has been held in preference to that in which it was regarded as being due to a clearance of disintegration products from the damaged tissues. It has been demonstrated that the administration of a diet rich in first class protein and of high caloric value modifies considerably the loss of body protein although it does not eliminate it entirely, Cuthbertson(1936). Also the administration of a diet containing 1% methionine has resulted in a decreased urinary loss of nitrogen after burning - Croft and Peters(1945) - and this fact agrees with Cuthbertson's view that the catabolism was a result of a demand by the body for certain key amino acids.

Whilst the view that the increased nitrogen output resulted from protein catabolism was held widely not everyone ascribed to the idea. Another concept was that it was due to an 'anti-anabolic' phase in which ingested protein was not utilised, the amino acids passing through the liver and being excreted by the kidneys - Grossman and colleagues(1945). If this were the case it would be expected that the major part of the increased nitrogen output would consist of amino acids. That this is not the case has been shown by Browne et al.(1944) and by Wilkinson et al. (1950). Both of these groups were able to show that urea was the major nitrogenous constituent of the
urine in the post-operative period and this result proves conclusively that the 'anti-anabolic' theory is invalid. If additional proof should be thought necessary it may be found in the work of Werner (1947) who by the use of a mixture of pure amino acids, and also by the use of casein hydrolysate and high protein-fortified milk orally was able to maintain an approximation to nitrogen balance in patients who had suffered fractures. This further evidence of protein utilisation and also that available in the work of Brunschwig and associates (1942), Co Tui and co-workers (1944), and Levenson and colleagues (1945) may be regarded as substantiation of the previously mentioned work.

The amount of nitrogen excreted after trauma is by no means a constant amount and varies from person to person. Levenson et al. (1945) found that patients who were poorly nourished at the time of trauma excreted less nitrogen than a well-nourished person and this was explained on the basis of a smaller reserve of protein which could be utilised. In cases of gastro-intestinal carcinoma, where the body reserve of protein is usually severely depleted, there is often a diminution in the serum protein level - Ariel, Abels, Pack and Rhoads (1943). This fall in serum protein value is not usually demonstrable in well-nourished people and it possibly owes part of its origin to an impairment in the ability to
synthesise serum protein - Ariel(1950). This work confirmed the results obtained much earlier by Daft et al.(1938) who carried out their observations on dogs. Their method of traumatising the animals was to inject turpentine subcutaneously and thus produce sterile abscesses. It was observed that the increase in urinary nitrogen of a normal non-anaemic dog was very much greater than that of a dog which had been anaemic for some time and in which the source of more readily available protein had suffered depletion. The use of dogs in which anaemia had been evident for only a short period yielded results intermediate between the above. Similar decreased amounts of urinary nitrogen were observed by Madden and co-workers (1937, 1940). Madden and Clay(1945), using similar techniques noted that when a protein depleted dog was given a normal diet after injury nitrogen balance was maintained, or nearly so, and that there was only a slight rise in urinary nitrogen. When a high protein intake was given, however, protein depleted dogs showed strongly positive nitrogen balances while still excreting relatively small amounts of nitrogen.

Cuthbertson concluded from the foregoing work that there must be a source of protein in the body, which is more readily available for catabolism than the structural proteins. This available protein acts as a source of amino acids in the absence of an exogenous source. Whipple and Madden,(1944),
considered that the circulating plasma proteins were the medium of exchange, deriving their supply from the liver and being able to supply protein for synthesis of new cell protein or of haemoglobin.

The view was expressed by Browne and colleagues (1944), and by Peters (1944), that all well-nourished persons suffer protein depletion after injury and that it is a physiological process which should not be interfered with in any manner. With regards to the under-nourished person steps should be taken to supply him with a reasonable reserve, if possible, before operation. They did observe, however, that, except in cases where the depletion was too severe, such people were capable of summoning certain conservative processes that permitted of protein utilisation.

Much work has been done with a view to trying to eliminate the phase of negative nitrogen balance, especially by parental and oral administration of amino acids, protein hydrolysates and protein enriched milk. The work of Cuthbertson (1936) has been mentioned already as well as the efficacy of plasma as a source of nitrogen, vide supra. Co Tui et al. (1944), by the use of intravenous administration of large quantities of both amino acid mixture and protein hydrolysate reported that he was able to maintain positive nitrogen balances in cases of gastrectomy even although the amounts of nitrogen lost in the
urine were very large. Considerable doubt has been cast on the validity of this work since Wilkinson et al. (1950) were unable to confirm the findings. Similar results were obtained by Hirshfeld and co-workers (1945) and Riegel and associates (1947), although the latter group observed that when feeding by tube directly into the stomach was utilised there was a very large variation in the amount of nitrogen excreted in the faeces. It is of interest to note that unless gastro-intestinal lesions are present or occur, it has been proved that there is no abnormality in the intestinal absorption of nitrogen after injury - Howard et al. (1944). This observation contradicts the theory of Werner and colleagues (1949) that the post-operative nitrogen loss could be explained by a simple caloric lack due to malabsorption. Wilkinson et al. (1950) also showed that there is a considerable difference between the response to trauma and that to a simple caloric lack. As a result of a study of control subjects who were submitted to the same dietary restrictions as patients undergoing gastrectomy, it was proved that, whereas in the former cases the level of urinary nitrogen fell rapidly after the restriction of the food intake, in the latter there was an increased nitrogen loss in the urine. Browne et al. (1944) demonstrated that a very large proportion of the protein ingested at the height of the catabolic phase was excreted as urea.
and that the intravenous administration of protein hydrolysates had the same end result. Werner (1947), however, claimed that he had been able to maintain two patients, who had suffered fractures of the femur, in nitrogen balance by means of casein hydrolysates and high protein milk orally. In the case of gastric patients there appeared to be a sparing of nitrogen as compared with non-gastric cases. A point worth remembering is that in the fracture patients the balance experiments were not commenced until two to three days after the injury and hence the initial period of the increased nitrogen excretion would be lost.

A very detailed study of the use of intravenous administration of protein hydrolysates and protein-enriched milk orally was carried out by Wilkinson et al. (1950). They were able to demonstrate that, in cases of partial gastrectomy, the administration of milk by tube or of hydrolysate parentally resulted in a corresponding increase in the urinary nitrogen and did not modify the catabolic phase in any essential way. The only noticeable difference was in a slight prolongation of the catabolic phase due to delay in the recommencement of the usual diet. It was concluded that the post-operative changes were partially due to the usual reduction in food ingestion and more especially due to the catabolic response. Since the administration of protein leads to a
prolonged period of nitrogen excretion it was suggested that the increase in urinary nitrogen is a better means of demonstrating the catabolic phase than the actual negative balance.

This view concerning the effect of the administration of protein during the post-operative period has been modified. It is recognised now that such treatment will result in a diminished negative nitrogen balance, although very large amounts of protein must be administered owing to the increased urinary output - Abbott(1955), Elman(1955) and Stewart(1955). No matter how much protein is given, within reason, the period of negative balance can not be abolished, however. Wilkinson et al.(1950) hold the view that the negative balance is a physiological response and one which should not be interfered with, an opinion that is shared also by Peters(1948), Moore and Ball (1952) and Cuthbertson(1954) but one with which Elman (1955) strongly disagrees.

We are now in possession of the facts that post-operatively there is a period of negative nitrogen balance, which is affected, but not abolished by the administration of protein, and which is made evident by an increase in the excretion of nitrogen in the urine. These observations regarding the nitrogen metabolism could be explained as a result of a general inflammatory reaction at the site of injury. It has been shown by Arey(1936) that the cells are destroyed
at the wound area both by direct injury and as a result of metabolic deficiencies following the injuries to blood vessels and lymphatics. A substance capable of increasing both the capillary permeability and the ease of migration of leucocytes has been isolated by Menkin, (1939, 1940), from traumatised tissue and it could be this substance that is responsible for the increased nitrogen excretion.

The observed metabolic changes in sodium, potassium and nitrogen will now be considered in the light of the knowledge concerning the effects of increased activity of the adrenal glands.

4. THE ADRENAL GLANDS AND THE METABOLIC RESPONSE TO TRAUMA.

While little, if any, direct evidence of the stimulation of the adrenal glands was available when this work was undertaken, there was a considerable number of facts available which indicated that such a stimulation did occur. The main reason for this scarcity of direct evidence was the lack of any readily applicable method of estimating adrenal cortical steroids but, since this work was started many such methods have been devised. The result of this has been a flood of published papers within a very short time, on this subject. However, the indirect evidence will be dealt with first since it was the background to this series of observations.
As early as 1916, it was observed in rats, that after adrenalectomy there was a diminished excretion of nitrogen in the urine along with a marked increase in the blood urea nitrogen (Marshall and Davis,). Since, in this case, the removal of the adrenals would lead to the cessation of cortical steroid production and liberation it can be argued that the increased production of these substances would result in the reverse observations regarding nitrogen metabolism. Silvette and Britton (1933) noted that adrenalectomised rats excreted less water than normal rats under the same fasting conditions and that the treatment of such rats with adrenocortical extract decreased the amount of chloride which they excreted in the urine to the same quantity as excreted by normal rats. Loeb and colleagues (1933), studied the electrolyte balance in dogs following adrenalectomy until the animals died as a result of insufficiency. There was a marked loss of sodium and chloride and it was concluded that the adrenals had a regulating effect upon sodium and chloride metabolism as well as upon renal function. At approximately the same time Harrop and co-workers (1933), found that the injection of adrenal cortical extract into adrenalectomised dogs resulted in a very noticeable retention of sodium and of chloride along with a transitory loss of nitrogen. When the treatment with the extract was withheld there was a great loss of sodium and of chloride
and a retention of nitrogen. This suggested that the adrenal glands play an important part in the regulation of these substances and also that the locus of the regulating function of the hormone(s) lies in the kidney. A possible explanation of the effects of cortical hormone in eliminating the symptoms of insufficiency was proposed by Swingle and associates (1936). The mode of action was the mobilisation and shift of tissue fluids to the extracellular spaces and to the blood stream as a result of altered permeability of the cell membranes. This would allow osmotically active substances which are normally retained to pass outward from the cells thereby raising the osmotic pressure of the hypotonic extracellular environment. It should be stressed that Swingle has not stated that there is increased capillary permeability as a result of cortical hormone administration, as has been attributed to him by many writers. Swingle also found that adrenalectomised rats could be maintained on a diet of high sodium chloride but low potassium content in the absence of cortical hormone. When sodium was eliminated from the diet it was found that massive doses of extract were required to maintain life, (1937). Further evidence that the adrenals were concerned in the regulation of sodium was forthcoming from Swanson and Smith (1936) who observed that when normal animals are kept on a low sodium chloride diet there is an
increase in the size of the gland, initially, and then this is followed by atrophy if the diet is continued. In 1937 McQuarrie, Johnson and Ziegler made a study of the plasma electrolytes in cases of Cushing's syndrome and found that the results were a complete and striking contrast to those observed in Addison's disease. Since Addison's disease was known to be due to hypo-activity of the adrenals these figures indicated that Cushing's syndrome was due to hyper-activity of the adrenal glands. A few years elapsed before it was reported that there is a marked increased excretion of, and a definite negative balance in, nitrogen in the early stages of Cushing's syndrome (Woodyatt). Albright (1943) stated that he had not seen a case of Cushing's syndrome in which a marked negative nitrogen balance was evident, but since he had not had the opportunity of seeing anyone in the early stages of the condition, this could have been due to the loss of protein before the studies were commenced. However, Albright did suggest that the results of Woodyatt indicated a possible connection between the adrenal activation and the response obtained in Selye's 'alarm reaction' since the metabolic changes evident in the early stages of Cushing's syndrome and in the immediate post-operative phase were very similar. The hypertrophy of the adrenal cortices which is evident in response to an alarming stimulus was regarded by Albright as being due to an over-
production of 'S' hormone, now termed the glucocorticoid, and that this was attended by the underproduction of 'N' hormone, now called the adrenal testoid hormone. Both of these adjustments will lead to a decreased synthesis of new tissue with the resultant availability of protein for other metabolic functions. This state of hormone imbalance was evident in the period commencing 12-24 hours after the operation. Albright further suggested that, in the initial period, up to 12-24 hours post-operatively, there was an initial release of both 'N' and 'S' hormones and the transient increase that was often observed in the excretion of 17-ketosteroids was considered as being the result of this compensatory increase in the 'N' hormone production which would offset the pathologically increased 'S' hormone production.

A large volume of indirect evidence is to be obtained from a consideration of the results which followed from the injection of adrenal cortical extract into both animals and humans. It was found that when cortin, the name given to such extracts, was injected into persons known to be suffering from Addison's disease a marked retention of sodium and of chloride which ensued was accompanied by a negative balance of potassium. When similar amounts of cortin were injected into normal persons, little, if any, response was evident. Larger doses elicited an invariable response
in the three substances mentioned - Thorn and his group (1937). This work was extended by Thorn and colleagues (1938) to the use of oral doses of the extract when it was found that similar responses were forthcoming. These changes are of the same type as those found in the post-operative state and the indications are that the adrenal glands will be responsible for the post-traumatic changes as well.

Thorn and Engel, (1938), demonstrated that not only the adrenal cortical hormones but others as well, were capable of eliciting the retention of sodium and of chloride and causing an increased excretion of potassium. Such substances as progesterone, oestrone, \( \alpha \)-oestradiol and testosterone propionate were capable of this. The latter three compounds were responsible for a decreased excretion of nitrogen. By carrying out experiments on adrenalectomised dogs it was shown that the effects were not mediated necessarily through the adrenal gland. This is the first inkling that such renal mechanisms as sodium and potassium excretion might be under the control of substances other than the adrenal cortical hormones. Of the four substances tested only one, namely progesterone, was effective in prolonging life in adrenalectomised animals. A further extension of this work was made by Thorn and Engel (1938) in studying the effects of the then known steroids. Whilst the injection of corticosterone, dehydrocorticosterone and desoxycorti-
costerone all produced the response on the electrolytes, desoxycorticosterone was very much more active than the other two. Allopregnan-3:11:17:20:21-pentol (Compound A, Reichstein and Wintersteiner, Compound D, Kendall) was shown to have no sodium or chloride retaining effect as also was allopregnene-3:17:20-triol (Compound J, Reichstein). Δ⁴-pregnen-11:17:21-triol-3:20-dione (Compound M, Reichstein), while not having an effect on sodium and chloride retention, did increase the renal excretion of potassium.

The role of the adrenal glands in the metabolism of protein and carbohydrate was demonstrated clearly by Evans (1936), in a series of experiments on rats. When they were subjected to an atmospheric pressure equal to half that of normal there was an increase in the carbohydrate content of the intact animals with a parallel increase in nitrogen excretion. The increase noted in the nitrogen excretion was sufficient to account for the observed carbohydrate deposition. When adrenalectomised rats were subjected to the same procedure they did not exhibit the same nitrogen response. Similar work was carried out by Katzin and Long (1939) who injected adrenal cortical extract into both normal and adrenalectomised rats and in both cases found an increase in the deposition of liver glycogen along with an increased nitrogen excretion. The stimulation of glucose production from protein in the fasted rat and the
decreased utilisation of glucose in the fed rat following adrenal cortical extract administration was confirmed by Sprague (1940). The injection of anterior pituitary extract, on the other hand, resulted in a fall in the excretion of nitrogen - Harrison and Long, (1939). Long, Katzin and Fry (1940) from a continuance of the work, suggested that one of the main properties of the cortical hormone(s) is the stimulation of protein catabolism with an increase in gluconeogenesis.

While it was observed that the administration of desoxycorticosterone to rats which were subjected then to trauma (fracture of a limb) resulted in a further increase in the urinary nitrogen excretion, the use of anterior pituitary extract was followed by the non-appearance of the negative nitrogen balance - Cuthbertson and co-workers, (1940-41). This latter finding is explicable by the fact that the effect of the extract possibly represents the normal growth response which is superimposed upon and compensates for, the increased catabolism following injury.

It was shown that the administration of desoxycorticosterone (DOC) or its acetate (DOCA) led to a reduction in the concentration of potassium in rat serum - Kuhlman and associates (1939). The use of the amorphous fraction, which is left after the removal of all the crystalline hormones from the beef adrenal, did not alter the distribution of electrolytes
in the serum while still influencing the rate of excretion of these substances in the urine - Wells and Kendall, (1940). Kendall (1940) observed that the evidence of the previous work was that cortin has little effect on gluconeogenesis or on the efficiency of the muscles but that, on a weight basis, it was many times more efficient than any other adrenal cortical product in the maintenance of renal function. Hartman (1942), by repeated injections of the amorphous fraction succeeded in producing a refractoriness in which further injection proved ineffectual in their action on sodium excretion. This supported his previous contentions (1938, 1940) that there existed at least two substances in the fraction. These he termed the sodium factor and cortin respectively. The cortin was capable of producing the other noted effects even after repeated injection.

One of the first direct observations that the urine of post-operative patients contains cortin-like activity was made by Weil and Browne (1939, 1940). They found that the injection of extracts of such urine into adrenalectomised rats afforded the rats considerable protection against the lethal action of cold. Substantiation of this work followed from that of Dorfman and co-workers (1942) and in the subsequent year Venning and colleagues (1943) demonstrated that the cortin-like material thus excreted also possessed gluconeogenic properties, thus adding
support of the view that it was derived, in all probability, from the adrenal cortex. This work was extended by Venning et al. (1944) who proved that individuals exposed to surgical trauma excreted from three to thirty times the amount of cortin-like substances as compared with normal persons. Also, using the cold exposure test of Selye (1937) they were able to show that seven to twelve per cent of the biological activity of adrenal cortical extract administered intravenously was recoverable from the patient's urine.

A transitory rise in the urinary excretion of 17-ketosteroids following operation has been observed widely. Forbes et al. (1947) found that the increase, when it did occur, was evident in the first specimen voided post-operatively and that the excretion fell rapidly after this, reaching a minimum on the fourth or fifth day after the operation. The return to normal values was rather variable but it was shown that the response was not due to a decreased food intake since patients receiving intravenous nourishment also responded in a like manner. The rise in 17-ketosteroid output in women, under such conditions, indicates increased activity of one aspect at least of the adrenal glands since in the female the sole source of 17-ketosteroids is the 'N' hormone, or adrenal testoid as it is now called - Fraser and associates (1941).
The rise in 17-ketosteroid excretion was observed also by Stevenson and colleagues (1944) but since most of his cases were males, the possibility arises that the increase could be due to altered output from the testes. Further information on this aspect was forthcoming from Perry and Gemmell, (1949), Venning and Browne, (1947) and Mason and Engstrom, (1950). Their results were similar in nature to those reported by Hardy and Ravdin (1952) who felt that, while the figures obtained showed an increase only irregularly, they could be taken as an indication of increased activity of the adrenal glands, in one respect at least.

Another line of study which has been considered to throw light on the possible activation of the adrenals following trauma has been the diminution in the number of circulating eosinophils in the post-operative phase. One of the earliest workers in this field was Schwarz (1914) who suspected that there was an inverse relationship between adrenal activity and the number of circulating eosinophils. It was not until 1939 that a direct observation on the effect of stress on the number of eosinophils was made by Dalton and Selye who also found that eosinopenia was related to the release of adrenal cortical hormones. A complete study of the effects of A.C.T.H. administration on the cells was made by Forsham, Thorn, Prunty and Hills (1948). The fact that the administration of
A.C.T.H. led to a diminution of the number of circulating eosinophils was well established and it was shown that a mean depression of approximately 70% below the pre-injection level was a normal response - Thorn and colleagues (1948). Numerous workers have followed the variation in the number of circulating eosinophils before, during and after operation and all have found that a marked eosinopenia results from the trauma. The severity of the operation seems to have a bearing on the intensity of the response - Moore (1950). Roche and co-workers (1950) observed that in some cases the eosinophils disappeared completely within one to two days following operation. The point arises whether all of this response is due to adrenal activity especially when the occurrence of a transitory eosinopenia is known to follow the injection of adrenaline - Hume, (1949). It was thought that the mode of action of the adrenaline was the successive stimulation of the hypothalamus, pituitary and adrenal cortex - Hume, (1949), Hume and Wittenstein, (1950) and Recant and associates, (1950). However, the work of Best and colleagues, (1952) demonstrated that falls of more than 50% in circulating eosinophils were obtainable in adrenalectomised patients, maintained on small amounts of cortisone, after adrenaline injections. Thus adrenaline itself must be capable of eliciting the eosinopenia in the absence of the adrenal glands.
Another aspect of this work which demands attention is that concerned with the excretion of nitrogen by adrenalectomised animals. In 1936 Sandberg and Perla observed that there was a marked increase in the urinary nitrogen as well as in the volume of water excreted in the period following adrenalectomy. Since collection periods of three or four days were employed the initial oliguria was not detected and it was suggested that the results indicated an impairment in the ability of the kidney to concentrate the nitrogen owing to the diuresis. The comparison of the nitrogen excretion of adrenalectomised rats with that of sham operated ones led to the conclusion that since the former were able to produce a considerable increase in nitrogen excretion, although of lesser degree than the latter, the control of nitrogen balance must be situated elsewhere and not solely in the adrenals. That there is a relationship between adrenal cortical activity and protein metabolism has been shown by the well-established effect of C_{11} oxygenated cortical steroids in causing a breakdown of lymphoid and other tissue - Ingle,(1945).

Ingle and co-workers,(1947) extended this work, showing that adrenalectomised rats exhibited a diminished nitrogen excretion on the first post-operative day, as compared with the controls, but then proceeded to excrete much more and developed a very marked negative nitrogen balance. Further when adrenalectomised
rats were subjected to a fracture of a limb fourteen days after operation there was an initial fall in nitrogen excretion, as above, but the usual period of increased excretion did not ensue. When adrenalectomised rats were treated with cortin from immediately after the operation until the end of the experiment, a rise in the nitrogen excretion became evident but it was still less than in the rats not treated with cortin and in the control series, all rats being force-fed over the period of study. When cortin-treated adrenalectomised rats were subjected to fracture of a hind tibia they excreted even more nitrogen than the controls and thus differed from the similar untreated rats. When a more severe injury was effected, both hind limbs and one fore one fractured, both the cortin treated adrenalectomised rats and the control ones excreted about the same amount of nitrogen, although the amount was greater than in the less severe trauma. Ingle et al. (1947) concluded that, although the presence of the adrenal gland was not essential for the appearance of the negative balance, the existence of some adrenal cortical hormone in the body was required. The negative nitrogen balance was not due specifically to the increased secretion of the hormones which occurs as a result of stress. A similar type of experiment by Noble and Toby (1948), in which unilateral adrenalectomy had been performed previously, resulted in an increased
nitrogen excretion which was of the same order as that occurring in animals with two functioning adrenals. When bilaterally adrenalectomised rats were subjected to trauma the nitrogen response was not evolved unless maintenance doses of adrenal cortical extract had been given. The conclusion was that the catabolism was not solely dependent on the adrenal cortex. They also noted that hypophysectomised animals failed to demonstrate the nitrogen response, even in the presence of administered cortical extract.

This work appears to be contradicted by that of Campbell and associates (1953) who were able to show that the implanting of a pellet containing 25 mg. of cortisone in the rat resulted in a response that was very similar to that which follows trauma. Also the effect of implanting two pellets was practically identical to that of one pellet and simultaneously fracturing a limb. While it is doubtful if a definite view may be expressed concerning the effect of injury in causing the liberation of such a quantity of hormone from the adrenal cortex, the observations are consistent with the metabolic findings which occur in spontaneous adrenal cortical hyperactivity.

5. THE PROBLEM.

As may be seen from the foregoing observations a study of the metabolic responses subsequent to operation in relation to the activity of the adrenal glands seems desirable. Although the metabolic
phenomena observed following trauma are very similar to those seen in cases of increased adrenal activity, thus suggesting a connection between the trauma and the secretory activity of the glands, it has been shown also that similar results could be due to a more general tissue reaction. A considerable amount of indirect evidence has been put forward which indicates that there is an increase in adrenal activity as a result of trauma but, when the work reported here began, no direct chemical proof had been forthcoming.

The first part of this work is concerned with the presentation of evidence that there is a general increase in the activity of these glands during the period of altered metabolism following trauma and this has been made possible by the use of a method which has been shown to provide an index of such activity. An attempt is made to correlate the various responses evident, with reference to the timing of such phenomena.

From a consideration of the published work, as well as from the evidence furnished in the first part, it became evident that a purely cellular response must be partly responsible, along with the effects of adrenal hyperactivity, for the metabolic changes. The second part of this study was undertaken in order to investigate this aspect in cases, such as those subjected to adrenalectomy, where increased secretion from the adrenal glands was impossible, and also in
cases where the possibility of direct stimulation of the adrenals by the hypophysis had been eliminated.

**CHOICE OF METHOD FOR STEROID ESTIMATION.**

There are two types of methods available for the estimation of the adrenal cortical steroids and their metabolites. These are, firstly, biological methods, in which the response of animals to urine extract injections is used as the basis of the test, and, secondly, the chemical methods, which are usually dependent on the existence of a definite type of chemical grouping in the molecule.

The biological methods are employed in the initial work on a definite compound and the results so obtained have proved of the greatest value. However, once the chemistry of the compound under investigation has been elucidated fully, a chemical method is evolved, in most cases, at least. The biological methods are usually rather tedious and time consuming and have the disadvantage that many physiological variations are introduced into the estimation.

The following types of biological assay methods have been employed widely in the work on adrenal cortical steroids and all of these have utilised the effects of the injection of urinary extracts on adrenalectomised rats, mice or dogs. One of the earliest was that of Anderson and Haymaker(1938) who used the fact of maintenance of life, following the
injection of the extracts, as the method of estimating the activity of the extract. In 1939, as mentioned previously, Weil and Browne introduced a method whereby the effect of the injected extract in protecting adrenalectomised rats from the lethal action of severe cold was used as the basis of the estimation. Venning et al. (vide supra) had observed that the administration of adrenal cortical steroids to adrenalectomised rats resulted in the deposition of liver glycogen in the fasting animal and these workers adapted this as a method for estimating the activity of the urine extract. Similar methods for the estimation of the mineralocorticoids have been proposed and utilised by such workers as Tait et al. (1952) and Simpson and Tait (1952).

It will be seen from the above that the criticism which may be levelled against all of the biological methods is that each is dependent on one type of response. When one is interested in a single aspect of adrenal activity this type of method may afford very valuable results. However, when considering the more general state of activity of the adrenal glands a method which will indicate a less specific response is required.

The available chemical methods fall into two types, depending on whether or not partition chromatography is applied in the course of the assay. The use of paper chromatography has been used widely in
the characterisation of the various corticosteroids - Burton et al. (1951) and Bush (1952). The application of such a procedure to urinary extracts was initiated by Burton et al. (1951A), Bush et al. (1952) and, later, used by Llaurado (1955) for the estimation of aldosterone. The great advantage of chromatography is that, by its use, the individual fractions of the corticosteroids may be resolved but the problem then presents itself of actually assaying the separate fractions. This is done, usually, by chemical or physical means, where they are available, but often recourse has to be made to biological methods, e.g. aldosterone. The main objection to the methods employing chromatography is that they are still not in a state where they can be adapted readily to the large number of estimations required in the normal course of events. It can be said also that they do not permit of a simple, rapid and comprehensive estimation of the corticosteroids. A further objection to such methods is that the amounts of the substances being dealt with are so small. These minute amounts of material call for very sensitive and accurate methods and, so far, few such methods are available.

When we come to consider the purely chemical methods for estimating these compounds we are struck by the rather frightening fact that a total of twenty-nine compounds have been isolated, so far, from the adrenal gland. At first sight it would appear highly
improbable that such a number of compounds would lend themselves to a relatively simple method of chemical analysis which would include all, or nearly all, of them. However, it has been observed that all of the corticosteroids isolated fall into one of six groups. These groups may be defined as depending on the nature of the substituents, if any, on the carbon atoms in the 17-, 20- and 21- positions of the parent substance, which is allopregnane.

These six types of groupings in these positions are:-
(1) 20:21-glycol.
(2) 20:21-ketol.
(3) 17:20:21-triol.
(4) 17:21-diol-20-one (dihydroxyacetone).
(5) 17:20-glycol.
(6) 17:20-ketol.

It should be noted that this classification does not take into account the stereochemical differences
possible in the molecules.

Since all of the isolated corticosteroids contain such groupings it has been stated that any urinary steroid containing one of these side chains can be assumed to be of adrenal cortical origin.

One type of chemical method which has been used has been that based on the reducing action of some of the steroids. The method suggested by Heard et al. (1946) utilised relatively crude extracts which were made to react with phosmomolybdic acid. The resulting molybdenum blue was estimated photo-electrically. Talbot and co-workers (1945) subjected their extracts to a more thorough purification, including among their steps a partition of the urine extract between benzene and water and the separation of the ketonic and non-ketonic fractions with Girard's reagent T. The final step was the reduction of copper salt solutions by the extract. These two methods estimate compounds which possess the side chains of the types (2) and (4).

The use of periodic acid, that renowned oxidising agent of organic chemistry, has been of the greatest value in the elucidation of the structures of numerous steroids. The oxidation of 17:20-dihydroxysteroids, by the use of periodic acid, to 17-ketosteroids was discovered by Reichstein and Shoppee (1943) but the credit must go to Fieser (1944) for observing that the reaction is relatively specific and reasonably
quantitative. Use was made of this fact by Talbot and Eitingon (1944) who applied the Zimmerman reaction to the oxidised steroids and thus obtained a measure of certain non-ketonic steroids. Dobriner et al. (1945) have proved that this is not a reliable means of estimating such compounds. It should be noted that compounds which possess side chains of the types (3) and (5), will yield 17-ketosteroids when subjected to this type of oxidation.

When the effect of periodic acid oxidation on the other types of side chains is considered it is found that the types (1), (2), (3) and (4) all yield formaldehyde, type (5) yields acetaldehyde whereas type (6) does not react. The discovery of a suitable method for the estimation of the liberated formaldehyde then leads to a means of estimating steroids which possess the groupings (1)-(4). MacFadyen, (1945), applied a colorimetric method for the estimation of formaldehyde to biological materials and thus opened up the field for the estimation of the steroids by this means. The fact that formaldehyde reacts with chromotropic acid (1:8-dihydroxynaphthalene-3:6-disulphonic acid) when heated in a boiling water bath in the presence of sulphuric acid had been discovered by Eegriwe (1937). The observations of this author concerning the specificity of the reaction were confirmed and extended by MacFadyen who showed that, while methanol and formic acid did not react with
chromotropic acid, acetaldehyde did. The product formed by the interaction of formaldehyde and chromotropic acid is characterised by an absorption spectrum which exhibits three maxima at 380, 480 and 570 µm respectively whereas the spectrum of the acetaldehyde product has only one maximum and that is at 400 µm. By using a wavelength of 570 µm it was possible therefore to eliminate interference from acetaldehyde in the estimation. It was shown that a concentration of at least 9M sulphuric acid in the reaction mixture was essential for the rapid development of the colour and also that a concentration of over 12M sulphuric acid led to a high blank resulting from the darkening of the chromotropic acid.

Lowenstein, Corcoran and Page (1946) introduced a method for the estimation of cortical steroids utilising the periodic acid oxidation and also MacFadyen's method for estimating the formaldehyde, which was separated by diffusion. In order to eliminate substances which interfered with the colour reaction Daughaday and co-workers (1948) introduced a partition stage between benzene and water as well as a distillation step, whereby the liberated formaldehyde was distilled into a sulphite solution. They showed that nearly all of the steroids which yield formaldehyde on periodic acid oxidation pass into the water phase, and little is left in the benzene. Corcoran and Page (1948) while using a very similar method discarded
the partition stage because the inclusion of this step yielded very highly coloured extracts when the method was applied to adrenal tissue itself. Another method, that of Lloyd and Lobotsky (1950) was essentially that of Daughaday's with the exception that the use of glacial acetic acid, as a solvent for the periodic acid oxidation, allows the solution of steroids, such as desoxycorticosterone, which are sparingly soluble in water.

The use of sodium bismuthate, in the place of the periodic acid, was suggested by Brooks and Norymberski (1952). This was shown to convert those steroids with the dihydroxyacetone side chain to 17-ketosteroids whereas periodic acid oxidises them only as far as the 17-hydroxy-17-carboxylic acids stage. In 1953 Norymberski and Stubbs described a method in which bismuthate oxidation was employed and the resultant 17-ketosteroids were estimated by the Zimmerman reaction. If the pre-existing 17-ketosteroids are measured before the oxidation the difference between the two results gives an indication of the amount of 17-ketogenic steroids present. The assay appears to yield an index of one particular function of the adrenal gland namely the release of 17α-hydroxycorticosterone. The method was extended by Brooks and Norymberski (1953) to the combined estimation of formaldehydogenic and 17-ketogenic steroids and thus affords a means of differentiating and estimating
three groups of adrenal steroids:
A) Formaldehydogenic, non-ketogenic.
B) Formaldehydogenic, ketogenic.
C) Ketogenic, non-formaldehydogenic.

All of the above methods suffer from the disadvantage that, on the whole, they estimate only the free steroids. Tompsett (1953) further extended the periodic acid oxidation method so that it was applicable to the estimation of urinary steroids. It was pointed out that the constitution of the groups attached to the carbon atoms 17, 20 and 21, which is evident in corticosterone and desoxycorticosterone, namely $\text{CH-}\text{CO-CH}_2\text{OH}$, was stable to mild acid hydrolysis. However, the grouping which is characteristic of cortisone, $\text{=C.OH-CO-CH}_2\text{OH}$, is not stable to such treatment and hence the utilisation of such a procedure will exclude steroid metabolites possessing the same type of structure as cortisone and 17-hydroxycortisone while including those resembling corticosterone. Tompsett also expressed the opinion that the grouping $\text{=CH-CHOH-CH}_2\text{OH}$ would be stable to acid hydrolysis.

While these views represent the original ones held by Tompsett they have been modified recently. It was demonstrated that the group $\text{=CH-CO-CH}_2\text{OH}$ is not stable completely to acid hydrolysis and hence steroids of the corticosterone type are not recoverable in 100% yields. Similarly, with the cortisone type of group $\text{=C.OH-CO-CH}_2\text{OH}$, it has been proved that it is not
completely acid-labile and recoveries ranging from 30% to 60% of cortisone added to water and to urine have indicated that A.S.F.S. estimations will include a certain proportion of compounds with a side chain similar to cortisone. However, experiments in which cortisone acetate was administered to patients resulted in recoveries of less than 10% of the given dose and this shows that cortisone itself will contribute little to the result of the A.S.F.S. method. More recently, Tompsett and Smith(1955) have shown that the A.S.F.S. determination is, in all probability, a measure of cortisone metabolites with a 17:20:21-triol side chain, in most cases at least. The use of a reduction step with sodium borohydride, which allows of the inclusion of the 17:21-diol-20-one side chain type of metabolite, in conjunction with the usual A.S.F.S. estimation suggested that, in normal control urines, the greater part of the cortisone metabolites are of the 17:20:21-triol side chain type. Following cortisone acetate administration, orally, the greatest rise appeared to be in the metabolites of the 17:21-diol-20-one side chain type. This thus affords a possible explanation of the slight response in the A.S.F.S. excretion following cortisone acetate administration.

In a personal communication Tompsett(1956) has suggested that the post-operative rise in A.S.F.S. would appear to be associated with an increased
excretion of 17-deoxy steroids related to corticosterone.

It was shown that the possible interference of fatty material, which may be present in urinary extracts, can be minimised if a strict control is kept on the conditions of the distillation. The collection of 9 ml., out of a possible 12 ml., of distillate resulted in figures which were not erroneously high due to interference from the fatty material - Tompsett and Smith, (1954).

While the method of Tompsett measures only a fraction of the total urinary corticosteroid content this is not to be held against it as all the other methods suffer from the same limitation. What is required in the present work is a method which is capable of giving an indication of the state of the activity of the adrenal glands and it is thought that this method fulfils the requirements. Tompsett (1953) has shown that there is no great diurnal variation in the acid stable formaldehydogenic steroid (A.S.F.S.) output and that the daily excretion is higher than normal in hyperadrenalism and lower than normal in Addison's disease. There is also an increased excretion of A.S.F.S. following the administration of ACTH, when there is an increased activity of the adrenal glands. The fact that there is no increased output of A.S.F.S. following the injection of ACTH into patients suffering from Addison's disease sets the
seal upon the method since no increased adrenal activity is possible in such cases.

The results obtained by ACTH administration have been confirmed by Renwick (1955) who was able to show also that the metabolic products of cortisone are largely such as to escape detection by this method. As the metabolic products of desoxycorticosterone excreted in the urine are predominantly of the type which will be detected by the method it was suggested that a substantial increase in A.S.F.S. excretion indicates an increase in the excretion of that group of steroids which includes the hormone or hormones responsible for water and electrolyte metabolism.

As a result of the foregoing observations it was decided that Tompsett's method for estimating A.S.F.S. would serve as a good guide to the state of activity of the adrenal glands during the post-operative period.
METHODS.

Sodium: Barclay Flame Photometer. Internal standard method utilising lithium as the internal standard.

Potassium: Barclay Flame Photometer. Internal standard method utilising lithium as the internal standard.

Nitrogen: Micro-Kjeldahl.


Estimation of acid stable formaldehydogenic steroids in urine.

Reagents:

A.R. Chloroform.

Purified absolute alcohol.

A.R. Sulphuric acid.

A.R. Hydrochloric acid.

15M Sulphuric acid.

13M Sulphuric acid.

10M Sulphuric acid.

1% w/v Sodium sulphite (Na$_2$SO$_3$,7H$_2$O) in water.

10% w/v Stannous chloride (SnCl$_2$,2H$_2$O) in 10% v/v hydrochloric acid.

2N Sodium hydroxide.

A.R. Anhydrous sodium sulphate.

Periodic acid reagent: 0.01M potassium periodate dissolved in 0.15M sulphuric acid.
Chromotropic acid reagent: 300mg. chromotropic acid (Hopkins Cole) dissolved in 2ml. water and made up to 50ml. with 13M sulphuric acid. This solution is prepared freshly for each series of estimations.

Preparation of the pure absolute alcohol:
10g. m-phenylenediamine dihydrochloride were added to 2000ml. absolute alcohol. The mixture was shaken and then left in the dark for seven days during which time it was shaken intermittently. The alcohol was then distilled from the solid, fractions of approximately 300ml. being discarded at the beginning and at the end of the distillation. The purified alcohol was stored in a brown bottle.

PROCEDURE:
20ml. of concentrated hydrochloric acid were added to 100ml. urine in a 250ml. flask and the solution was boiled, under reflux condenser for ten minutes. After cooling, the hydrolysed urine was extracted three times with 50ml. aliquots of chloroform. The chloroform extract was washed with 25ml. of 2N. sodium hydroxide solution and twice with 25ml. portions of distilled water. The extract was dried over anhydrous sodium sulphate and filtered. The extract was evaporated in an all-glass still on a boiling water bath and the last traces of solvent were removed.
under reduced pressure (water pump).

The residue was dissolved in one ml. of absolute alcohol and transferred to a fifty ml. Kjeldahl flask with water washings, (9ml.). 5ml. of periodic acid reagent were added and the well-mixed mixture was allowed to stand at room temperature for 12-18 hours. At the end of this time 1ml. of 10% stannous chloride was added followed by 0.5ml. concentrated sulphuric acid. The mixture was distilled, the first nine ml. being collected in a 10ml. graduated measuring cylinder containing 1ml. of 1% sodium sulphite solution. The cylinder was stoppered and the contents mixed by inversion.

3ml. of the distillate were pipetted into a test tube fitted with a ground glass stopper and then 5ml. of chromotropic acid reagent were added. The contents of the tube were mixed and the stoppered tube was heated in a boiling water bath for thirty minutes. After cooling, 2ml. of 10M sulphuric acid were added, the contents of the tube being well mixed after the addition.

A blank determination was carried out by using 100ml. water in the initial stage and submitting it to the whole procedure.

The optical density of the solution was determined against the blank at 570 mu using a lcm. cell in an Unicam S.P.600 spectrophotometer. A standard graph was obtained by submitting standard alcoholic
solutions of pure desoxycorticosterone acetate to the whole process. The results for the ASFS were expressed as mg. DOC.

Estimation of 17-ketosteroids in urine.

17-ketosteroids were estimated according to Callow et al. (1938) as modified by Zygmuntowicz et al. (1951) and the colour correction was applied - Talbot et al. (1942), M.R.C. Report, (1951). Values were obtained from a graph prepared from standard solutions of dehydro-iso-androsterone.
PROCEDURE.

In the first part of this study, that in which the state of activation of the adrenal glands in relation to the other known responses was being studied, the subjects were four men suffering from chronic duodenal ulcers. Each patient underwent a partial gastrectomy and as a result the intake of food ceased for four days after the operation. There was the usual return to a very restricted diet after this. No fluid was administered by any route during the first two post-operative days and no attempt was made to maintain a constant sodium chloride intake. In fact no sodium chloride was given during the first four post-operative days. As far as medication was concerned the patients underwent the usual pre-operative treatment, receiving an enema and sedative before anaesthesia was induced by means of sodium pentothal.

Urine was collected in six hour periods, the urine being passed freely and catheterisation was not employed. No attempt was made to collect faeces passed during the period of observation since it has been shown that, unless gastro-intestinal lesions are present or occur, there is no great variation in the amounts of the relevant substances excreted in the stools. A point worth mentioning is that, in the immediate post-operative periods, no stools were passed and this fact greatly simplified the interpretation of the results.
Since it was impossible to obtain data by which the various changes could be represented as balances the results are expressed as weights excreted in unit time, the unit of time being the minute. This method has been shown by Wilkinson et al. (1950) to be of the greatest value in interpreting the results and in indicating any definite trend in the pattern.

In the second part of the work the patients studied were four women who were suffering from metastatic carcinoma of the breast and who were subjected either to adrenalectomy or to hypophysectomy. These patients received their food from the dietetic kitchen of the hospital, the aim being to maintain a constant level of nitrogen and potassium intake along with a low dietary sodium content. One of the main sources of potassium was fruit juice and since different batches of juice were found to contain different amounts of potassium, it was decided to utilise only one batch of juice throughout each experiment. This proved very satisfactory as the potassium content of each sample obtained from any one batch proved, on analysis, to be relatively constant.

As a result of this control a relatively constant intake of nitrogen and of potassium was obtained, in any one patient. It will be seen, however, that the potassium and nitrogen intakes varied from patient to patient and this was due to the different capacities that the patients had for food.
Use was made of a diet which was practically devoid of sodium chloride, the intake being 200-400 mg. per day as shown by actual analyses. In order to maintain a relatively constant intake of sodium chloride throughout the whole period of observation cachets of this substance were given to be taken orally and the ingestion of these throughout the day resulted in a controlled intake of approximately 4.5 g. sodium chloride per day. This constant intake of salt was maintained in the immediate post-operative period by the infusion of five hundred ml. of isotonic sodium chloride solution since the oral administration was not feasible. It should be noted that, in no case, was this quantity of fluid sufficient to cause any clinical signs of water intolerance. The return to the oral ingestion of salt was possible usually on the first post-operative day.

It will be noticed that there was an increased sodium intake on the day of operation, in all cases, and this was due to the fact that blood was administered in this period and the sodium content of the plasma as well as that of the citrate anticoagulant has been taken into account in the balance data. The potassium content of the cells has not been included since it has been demonstrated that such material is not readily available to the body as long as the cells remain intact, as will be the case in these relatively short term experiments - Moore and Ball (1953).
In this second group of experiments it will be evident that the potassium intake was re-commenced on the first day after the operation and this was achieved by using the fruit juice as the source of the potassium. On the next day further amounts of potassium were obtained from the fruit juice as well as from the limited intake of food that was permitted. By the third day nearly full dietary conditions were approximated.

Apart from the usual pre-operation treatment mentioned above three of these patients received constant amounts of cortisone before and after the operation. In the case of the patients subjected to adrenalectomy oral doses of 200 mg. of cortisone acetate per day were given, the treatment commencing at least four days before the operation. In one case subjected to hypophysectomy oral doses of 75 mg. of cortisone acetate per day were administered whereas in the other no such treatment was given.

The urine collections in these observations were made in periods of twelve hours and once more no attempt was made to collect any faeces passed during the observations, for the reasons mentioned already. In every case the actual chemical analyses were carried out as soon as possible after the specimens had been received and all the determinations were made in duplicate. The estimations were carried out on the separate twelve hour collections but the results have
been tabulated only for the twenty-four hour periods since the intake may be calculated on these periods only. While the actual contents of the diet were calculated from tables the results were checked by the analyses of duplicate diets at least once in each case.

RESULTS.

The results of the experiments will be presented in two parts, the first dealing with the positive proof of increased adrenal activity as one of the causes of the post-operative metabolic changes and the second with the results obtained in the cases of adrenalectomy and of hypophysectomy.

1). Effect of General Surgical Trauma.

The results obtained in the initial part of this study may be seen presented in graphic form in Figures 1, 2, 3 and 4 and, as already stated, since balance experiments were not possible the results are expressed as amounts excreted in unit time. The results shown are those for each six hour period.

The usual responses in the excretion of both sodium and potassium in the post-operative period were evident. In every case the urinary excretion of sodium fell rapidly and remained at the low level for three or four days, if not longer. It was observed that in one case only was there any evidence at all of a period of
Figure 1.
Excretion of sodium, potassium and A.S.F.S. in the pre- and post-operative periods. The results are expressed as amounts excreted per minute.
Figure 2.
Excretion of sodium, potassium and A.S.F.S. in the pre- and post-operative periods. The results are expressed as amounts excreted per minute. The patient died on the ninth post-operative day.
Figure 3.
Excretion of sodium, potassium and A.S.F.S. in the pre- and post-operative periods. The results are expressed as amounts excreted per minute.
Figure 4.

Excretion of sodium, potassium and A.S.F.S. in the pre- and post-operative periods. The results are expressed as amounts excreted per minute.
increased sodium excretion in the period 18-24 hours after the operation. In all the other cases the diminished sodium output was maintained without interruption.

With reference to the potassium excretion the results were not quite so definite as those evident in the case of the sodium excretion. While there was a definite continuation in the excretion of potassium even during the period when the ion was not being ingested, thus indicating a period of negative balance, there was an increased excretion in only two cases. In these two patients, however, the period of increased excretion was very transitory, being evident only during the period 12-18 hours after the operation. The difference between these two responses and the other two was, therefore, one of degree. While the period of increased potassium excretion was of short duration in three of the cases it was seen that in one, (see Fig. 2.), a second period followed starting on the fourth post-operative day and continuing for two or three days. This difference was due, in all possibility, to the fact that the patient succumbed on the eighth post-operative day.

In every case the rate of water excretion fell during the immediate post-operative period but it soon rose and approximated to the
pre-operative levels.

When the results for the A.S.F.S. were considered it was seen that with the exception of one case, there was an immediate rise in the excretion rate and that the maximum value was obtained during the first post-operative day. This high rate of excretion was not maintained for more than a short period but it will be seen that while the rate fell it did not reach the pre-operative levels. There was another rise in the excretion rate on the third day after which the levels tended to fall to the pre-operative levels. There was a gradual rise in the rate of A.S.F.S. excretion in one case, (see Fig.2), until the maximum was reached on the second day, after which a decrease was evident. However, following this there was a further period of increased output, the maximum level attained being more than double that in the initial response. There then followed a steady decrease in the excretion until death terminated the observations.

2). Discussion.

Evidence has been presented already which indicated that the metabolic changes, which follow operations, could be explained on the basis of the actual cell damage initiating a complex series of reactions. The main objection to the purely local inflammatory reaction theory
lies in the dissociation in time between the various events. The transitory increased excretion of potassium could be due to the cellular changes, since it occurred in the period immediately subsequent to the trauma and also since it ended rather abruptly, as was to be expected. However, the changes in the excretion of both sodium and nitrogen lasted for a much longer period. These differences in time relationship suggested that there were two responses to the trauma, the first or immediate one being due to the reactions produced as a result of the cellular damage. The primary response was followed by a secondary one which could have been initiated as a direct consequence of the cellular damage or could have been due to the effects produced as a result of this damage. It appeared extremely unlikely that these would be two separate and discrete phenomena as regards time and it was thought that they would overlap to some considerable and variable extent. These observations appeared to substantiate those of Le Quesne and Lewis (though not their interpretation), who distinguished an early retention of both sodium and water occurring in the first twenty-four hours post-operatively, and a late sodium retention, commencing twenty-four to forty-eight hours after the operation and lasting
for several days. The overlapping of the two responses would explain the absence, in many cases, of the two distinct phases.

When the results of the A.S.F.S excretion in the immediate post-operative period were considered it was seen that there was a very large increase in the rate of excretion during the first day after the operation. This increase coincided with the period of diminished water excretion as well as with that of diminished sodium excretion. This elevated rate of A.S.F.S excretion also coincided with the period of increased potassium excretion. This evidence was taken as indicating that there was an increase in the activity of the adrenal glands in this period and that this was responsible for, at least, part of the initial responses which were evident.

It was noticed that the period of increased A.S.F.S. excretion lasted for one day, at the most, and after this the level fell somewhat, remaining at this lower level for two or three days. The rate of excretion then fell to the pre-operative level. During the second period there was little evidence of any increased potassium output but the rate of sodium excretion was greatly reduced. Diminished sodium excretion was also evident after the A.S.F.S. levels had reached the pre-operative values and this was
taken as further proof that the increased adrenal activity was responsible for only part of the response.

Further evidence to substantiate this theory was forthcoming from a consideration of the results shown in Figure 2, in which a different response to the trauma was evident, as far as A.S.F.S. and potassium excretions were concerned. The response, as shown by the sodium excretion, was identical in all respects to that exhibited in the other three cases. The fact that the very great increase in the A.S.F.S. excretion which occurred on the fifth post-operative day coincided with a period of increased potassium excretion was suggestive that part of the potassium excretion might have been due to the increased adrenal secretion. However it was observed that in this period there was no evidence of a diminished rate of sodium excretion, the level having regained the pre-operative value, or nearly so, and there was no complete elimination of sodium from the urine as occurred in the earlier response.

The conclusion to be drawn from these observations was that in the period following trauma there were two factors which either separately or together influenced the metabolic activity of the body. These factors were increased adrenal activity, as indicated by the elevated excretion
rate of acid-stable formaldehyrogenic steroids, and an inflammatory reaction which resulted from the actual damage to the cells in the traumatised area.

This observation that there was an increased activity of the adrenal glands was in agreement with the evidence presented by other workers. Thorn and colleagues (1953), using a method designed to measure both free and conjugated 17-hydroxy steroids, showed that there was an increase in such material following major surgical operations. They also made the important observation that minor operations and also fairly mild environmental disturbances did not have the same effect. Increases in A.S.F.S. excretion similar to those reported here have been observed by Tompsett and Smith (1954, 1955) in the post-operative period. Other evidence, of the same nature, has been produced by Cope and Hurlock (1954). Increases in the level of blood corticoids following operation have been reported by Franksson and colleagues (1953, 1954) and from Samuels' laboratories - Sandberg et al. (1954) and Tyler et al. (1954). These latter two groups, working in Samuels' laboratory, carried out a very careful study and were able to demonstrate that the increase in the 17-hydroxy corticoid excretion was roughly proportional to the severity of the operation. They
carried out experiments with both ACTH and bromsulphonephthalein in order to identify the cause of this increase. While it was shown that the response was partly due to the increase in adrenal secretion, part was due also to impaired hepatic metabolism as shown by the ability to excrete bromsulphonephthalein.

The post-operative increases in the A.S.F.S. excretion reported here were, however, far greater than were found in a few cases of liver disease with marked functional impairment. This was taken as an indication that the increases observed really represented an increased secretion from the adrenal gland.

While the work detailed above has tended to illustrate that there was a general increase in the activity of the adrenal gland, other evidence has been published dealing with the increased secretion of one specific steroid under the same conditions. Recently Llaurado (1955) has reported that there was an increase in the quantities of aldosterone excreted in the urine following operations. Aldosterone is accepted, at present, as being the hormone which is of the greatest importance in the control of electrolytes. However, the evidence presented, so far, to substantiate the claim that there was an increased excretion of aldosterone following operation has not
been very convincing. It has been shown also that ACTH is not the specific stimulus to aldosterone production - Cope and Llaurado (1954) - and this is an indication that the adrenal gland may not be the main source of the steroid. As a result of these considerations it was felt that the use of a method which reflected a general increase in the secretion from the adrenal glands would be of at least equal value in furthering the knowledge in this field of surgical trauma.

3). Summary.

It has been confirmed that, in the immediate post-operative period, there was a marked disturbance in the metabolism of sodium and potassium and that this was accompanied by an alteration in adrenal activity. There was a period of marked diminution in the excretion of sodium in the urine and, in the cases reported here, this state lasted for at least three or four days. The existence of a transitory negative balance in potassium, occurring essentially in the initial stages of the post-operative period, has been confirmed also. The period of negative potassium balance has been shown to coincide with a phase of increased excretion of A.S.F.S. This increase in A.S.F.S. excretion has been taken as an indication of increased adrenal activity following the trauma. However, while this increased activity
has been deduced to be responsible for part of the metabolic disturbances, a consideration of the time relationships of the various changes led to the conclusion that some other, possibly cellular, effect was involved also.

4). Effect of Adrenalectomy or Hypophysectomy.

In these circumstances the next step which seemed logical was to observe the changes which occur in the metabolism of sodium, potassium and nitrogen following operations which involve the removal of the adrenal glands or of the hypophysis. By this means it was thought that an insight into the role of these two glands in the metabolic responses which follow surgical trauma would be obtained. A two stage bilateral adrenalectomy appeared of the greatest value, in this respect, as it would offer a direct comparison between the responses where increased adrenal activity was possible and where the possibility of any such activity had been eliminated. In the case of the removal of the hypophysis, no activation of the adrenal glands by increased ACTH production could occur.

It was thought that the results of such experiments would be of great value in furthering the present knowledge concerning the role of these two glands during the post-operative phase.
A). Adrenalectomy.

The results throughout are presented according to the method suggested by Reifenstein and colleagues (1945). The intake was plotted downwards from the balance line and the urinary excretions were plotted upwards from the intake line towards the balance line. The hatched areas thus represent the urinary excretions. Negative balances are represented by the hatched areas above the balance line and positive balances are shown by the clear areas measured down from the balance line to the beginning of the hatched areas. The results are those based on twenty-four hour urine collections and dietary intakes, although twelve hour urine collections were analysed in every case.

The results shown in Figure 5 are those obtained from a case in which a second stage of a bilateral adrenalectomy was being carried out. A.S.F.S. excretion figures were plotted in this case and, as was to be expected, there was no evidence of an increase in the excretion following the removal of the second adrenal.

There was a period of marked diminution in the urinary excretion of sodium and, since sodium chloride was being administered, this amounted to a marked positive balance of sodium. The initial period of positive balance lasted for
Figure 5.
Sodium, potassium and nitrogen balances and A.S.F.S. excretion before and after the second stage of a bilateral adrenalectomy.
two days after which there was evidence of a smaller positive balance.

In the case of potassium there was a period of two days during which no potassium was ingested and since this ion was being excreted a negative balance resulted. Following the recommencement of a dietary intake of potassium even larger amounts were excreted in the urine and the period of negative balance was continued for three days after the resumption of the intake. The negative balance was followed by a short period of 'rebound' retention of the ion.

The pattern of the nitrogen excretion was very similar to that of potassium, although the period of negative balance lasted for a slightly longer time. In this case also there was definite evidence of a negative balance even although there was a rapid return to a full diet.

The results obtained in the two stage bilateral adrenalectomy are shown in Figure 6. These may be summarised as a period of sodium retention lasting for eight days which was followed by one day of slight negative balance. There was a further period of sodium retention and equilibrium was attained only on the day prior to the second operation. The second stage of the operation was followed by a further period of sodium retention but it was evident that on this occasion the
Figure 6.
Sodium, potassium and nitrogen balances and A.S.F.S. excretion before and after both the first and second stages of a bilateral adrenalectomy.

![Graph showing sodium, potassium, nitrogen, A.S.F.S., and water balances over a period of days during control and post-operation periods.](image-url)
amount of sodium excreted was very much greater, over the whole period. The inclusion of further results in this case was not thought to be permissible since on the fifth day after the removal of the second adrenal gland the cortisone dose was decreased in accordance with the usual surgical practice.

The results for the potassium excretion showed that there was an initial period of negative balance lasting for two days after the first operation. A condition of near equilibrium was then maintained, with the exception of one day when a marked loss of potassium was evident. After the second operation there was a negative balance for two days being most marked on the first day, and then there was evidence of the 'rebound' retention.

In the case of the nitrogen there was a period of marked negative balance, even although the intake of nitrogen was restarted on the second post-operative day, and this lasted for ten days. The intake and excretion of nitrogen had regained a state of equilibrium before the second stage of the operation was undertaken. Following the removal of the second adrenal gland there was a similar period of negative balance but this time it was of a much shorter duration.

A consideration of the steroid excretions showed that, in the case of the A.S.F.S., there
was a fall on the first post-operative day but this was followed by an increased excretion lasting for four days. A return to pre-operative levels then ensued. After the removal of the second gland there was no marked alteration in the steroid excretion until the fifth post-operative day, the day on which the dose of cortisone was greatly reduced. The pattern of 17-ketosteroid excretion did not show any great alteration after either of the operations.

A discussion of the foregoing results will follow the presentation of the results which were obtained in the two cases where subjects underwent operation for the removal of the hypophysis.

B). Hypophysectomy.

The results of these two cases are readily summarised and are shown in graphic form in Figures 7 and 8. Figure 7 represents the results for the case in which daily oral doses of 75 mg. cortisone acetate were given whereas Figure 8 demonstrates those for the case where no hormone treatment was given.

In both cases the response may be stated to be a period of marked positive sodium balance lasting for approximately seven days followed by a period of negative balance. There was also an initial increased excretion of potassium which was soon replaced by a 'rebound' phase of positive
Figure 7.
Sodium, potassium and nitrogen balances and A.S.F.S. excretion before and after hypophysectomy. The patient received 75 mg. cortisone acetate orally per day.

![Graph showing sodium, potassium, nitrogen, 17-ketosteroids, and A.S.F.S. balances before and after hypophysectomy.](image)
Figure 8.
Sodium, potassium and nitrogen balances and A.S.F.S. excretion before and after hypophysectomy. The patient did not receive any cortisone acetate.
balance. The period of negative nitrogen balance lasted for four to five days, even although there was an appreciable intake of nitrogen over the same period. In both cases there was a large increase in the excretion of A.S.F.S., the maximum excretion being evident on the first post-operative day in one of the cases. It was extremely unfortunate that the immediate post-operative specimens were lost in the case where no cortisone was being administered but it was thought that the results obtained were positive enough to warrant their inclusion in this work.

In one case there was an increase in the 17-ketosteroid excretion for a period of two days whereas in the other there was no evidence of any definite alteration in the amount excreted.

5). Discussion.

While the main reason for undertaking this part of the study was to observe to what extent the body was capable of producing the usual metabolic responses to trauma either in the absence of the adrenal glands or in the absence of direct stimulation of these glands by the action of the hypophysis, it was of interest to observe the changes which followed the first stage of a bilateral adrenalectomy. When the results were set out graphically in the form of the rate of excretion and then compared with those obtained in the
initial part of this work it was seen that the two types were practically identical (see Figures 1 and 9). The inference to be drawn was that the one adrenal gland was capable of producing a response similar to that obtained in the presence of two such glands and this supported the view of Sandberg and colleagues (1954) that the adrenals were stimulated only submaximally as a result of operative trauma.

When the second stage of the adrenalectomy was considered, the first obvious difference in the responses was the absence of the increased A.S.F.S. excretion in this case. While no prolonged increase was to be expected after the removal of the remaining gland, it did seem possible that a slight increase might have occurred as a result of adrenal stimulation during the initial stages of the operation. However, this did not happen and it may be concluded, therefore, that the changes in metabolism which were evident in the immediate post-operative period were not due to an outpouring of adrenal hormones immediately prior to the removal of the gland. It may be said that in these cases marked changes in the metabolism of sodium, potassium and nitrogen, of a type similar to those which occurred in the presence of the adrenal glands, were evident but that the response was not due to an increased
Excretion of sodium, potassium and A.S.F.S. before and after the first stage of a bilateral adrenalectomy. The results are expressed as amounts excreted per minute.
secretion of the gland. This substantiated the view, based on the indirect evidence, that the metabolic changes were due partially to some other, possibly cellular, phenomena.

The metabolic changes were not due to the effects of the cortisone acetate treatment. The doses were of such magnitude that the secretions of the glands themselves were swamped in the control period. The continuation of the same dose throughout the whole experiment did not allow of any alteration in the concentration of circulating adrenal steroids. It is of interest to note that while the high dose of cortisone acetate was maintained after the operation the rate of A.S.F.S. excretion remained relatively constant and that when the dose was decreased the amount of A.S.F.S. excreted fell correspondingly. In the cases reported here the percentage recovery of the administered cortisone acetate was less than 4% of the dose, a fact that was in agreement with the findings of Renwick (1955) and Tompsett (1955), who found that similar doses of cortisone acetate resulted in increased A.S.F.S. excretions which were equal to less than 10% of the amount of steroid given. This was further proof that while cortisone-like substances will be detected by the method used to estimate A.S.F.S., the administration of cortisone acetate will lead to
only a small increase in A.S.F.S. levels in the urine.

In the case of the 17-ketosteroid excretion it was noticed that there was a decrease in the amounts excreted after the second stage of the adrenalectomy. This result was to be expected since both the patients were women and it has been shown that in females the adrenal glands are the sole source of such steroids. Of the two cases where hypophysectomy was effected only one showed any marked alteration in the amounts of 17-ketosteroids excreted. This fact, coupled with the observation that there was only a very limited response in 17-ketosteroid excretion following the first stage of the adrenalectomy, was regarded as further evidence that the excretion of this type of steroid is not a reliable index of adrenal activity.

It was noticed that the changes in the metabolism of nitrogen and potassium after the second stage of the adrenalectomy were of approximately the same order as those obtained in operations which did not involve surgery of the adrenal glands. As far as sodium was concerned, however, it did appear that, while there was an initial positive balance, this was of a much shorter duration. Amounts comparable with those excreted in the pre-operative period were excreted from the
third day onwards. This could be taken as evidence that the adrenal steroids played a part in the continued retention of sodium.

The two stage bilateral adrenalectomy afforded a better opportunity of comparing the effects of the two types of operations, namely, those in which adrenal secretion was possible and those in which the glands had been removed. In the case of potassium it was observed that the magnitude of the negative balance was of approximately the same order in both cases. However, the period of increased urinary excretion of potassium occurred earlier in the second stage than in the first and was practically abolished when there was a return to an intake of the ion.

It must be remembered that the two stages of the operation involved surgical procedures of approximately equal severity and also that, in the second stage, increased adrenal activity was not possible. These observations would appear to substantiate the view that the observed potassium loss was due, to a large extent, to the changes which occurred as a result of tissue damage. The outpouring of the cellular potassium would explain the fact that the loss of this ion was of relatively short duration and also the fact that nearly equal amounts were excreted in the two cases. The observations that the period
of potassium loss was shorter after the second operation, where no adrenal activity was possible suggested that the potassium response was due partially to the effects of adrenal stimulation.

As far as the nitrogen metabolism was concerned it was evident that the response following the first operation was of greater magnitude, and of longer duration, than that after the second stage. This could be taken as substantiation of the observation that the degree of nitrogen loss varied with the state of the body supply of readily available nitrogen in the form of protein. The fact that the two operations followed closely one upon the other suggested that the differences observed in the response might have been due to the depletion of the body reserves following the first operation. This point will be dealt with later. It was interesting to note that while the magnitude of the negative potassium balances was approximately the same after each operation there was a marked difference in that of the nitrogen balances. This was another indication that the phenomenon could not have been due to a straightforward catabolism of protein following food deprivation and that it must have involved some changes such as the cellular one and the adrenal response which were mentioned previously.

The difference between the two types of
response was most marked with regard to the sodium excretion. A period of positive balance was evident after both stages but the response after the second was not of the same order of magnitude as that which followed the first. There was also a very marked difference in the amount of sodium excreted in the urine. While the amount excreted after the first stage fell rapidly to a very low value, in the second stage a large amount was excreted on the first post-operative day and on the second and third days the amount in the urine was still higher than in the previous case. Since the period of increased A.S.F.S. excretion occurred at the same time as the sodium retention after the first operation and since there was a great alteration in the sodium response in the absence of increased adrenal activity it appeared that the main role of the adrenal gland in the post-operative response was in the control of the body sodium content. This suggested the liberation of a steroid of the same type as aldosterone, if not aldosterone itself, and indicated that the estimation of aldosterone in post-operative urine samples would be of great value in adding a further chapter to the story. While the use of a chemical method for estimating this compound is not feasible valuable evidence might be obtained by the use
of biological assay methods in pilot experiments.

It has been shown that if two operations were carried out on the same patient within ten to fourteen days the metabolic changes evident after the second operation were of a much lesser degree than those after the first - Moore and Ball (1953). The reason for this was thought to be the depletion of the body reserves. However, in the case of the bilateral adrenalectomy reported here the difference in the metabolic changes did not appear to be due to such a phenomenon since there was an excellent dietary intake during the period between the operations. It was evident that as far as potassium was concerned more of this ion had been retained than excreted after the first operation. This was taken as a further indication that the adrenal glands were responsible for part of the post-operative metabolic changes.

However, the most interesting conclusion arrived at from a consideration of the findings was that the observed post-operative metabolic changes after adrenalectomy were very similar to those evident in the presence of increased adrenal secretion.

While the results from the operation involving the removal of the hypophysis substantiated those found in the earlier experiments
they also indicated that the adrenal glands were not under the influence of the hypophysis, as far as their response to trauma was concerned. The response was the same in the two cases and the one in which the secretions of the hypophysis had been eliminated or greatly reduced by the administration of 75 mg. cortisone acetate daily, were of interest. The use of such doses led to the atrophy of the gland and thus increased hypophyseal secretion was not possible during the phase leading to the removal of the gland, as might have been the case where no cortisone acetate was being administered. However, even if such stimulation had occurred before the removal of the gland it would have led to only a transitory increase in the output of A.S.F.S. and not to a rise lasting for two to three days as was the case where no cortisone was given. The fact that the response occurred in the absence of direct hypophyseal stimulation of the adrenal glands suggested, initially, that the active agent might be aldosterone since ACTH is not the specific stimulus to the secretion of this substance. However, other steroids must have been liberated also since aldosterone is acid-labile and would not be detected by the procedure used to estimate A.S.F.S.

It is of interest to recall that the type of
response which has been observed to follow surgical trauma was the same as that which had been found to result from adrenal hyperactivity or from the injection of certain steroids, such as ACTH. In fact, this very type of response has been utilised as a means of assaying adrenal steroids in order to give an indication of the state of activity of the glands. The deduction that increased adrenal glandular activity was responsible for part of the metabolic changes subsequent to trauma which was made on these observations has been substantiated by the results reported here. These showed that there was an increased excretion of A.S.F.S., which is an index of adrenal activity, in the post-operative period.

DISCUSSION.

While it has been indicated that part of the response elicited by trauma was due to increased adrenal secretion, which did not appear to be under the direct influence of the hypophysis, it was evident that a large part of the response must have been due to other causes. In the case of the second stage of a bilateral adrenalectomy where no increase in adrenal activity was possible the changes in the metabolism of sodium, potassium and nitrogen were still evident. These changes must have resulted from the direct effects of the cellular damage in the traumatised area. There
was a considerable amount of disruption of the cells which would lead to an alteration in the electrolyte concentration in the extracellular fluid. Apart from this direct effect it was shown that there was a marked increase in the capillary permeability following operations - Viale and Bruno (1927) and Cope and co-workers (1942). This would lead to the escape of protein to the extracellular compartment with a resultant alteration in the osmotic balance. The disturbance of the osmotic balance would produce a decrease in the intracellular fluid and an increase in the extracellular volume, a fact that could lead to the appearance of a generalised clinical oedema. Mention has been made already of the increase of potassium in the extracellular fluid which resulted from the disruption of the cells and this potassium would find its way into the urine. The decreased concentration of potassium in the tissues surrounding a traumatised area has been demonstrated by Schilling and colleagues (1953) who also demonstrated that there was an increase in the sodium and chloride concentrations in the same area. It has been suggested that there might be an exchange of sodium from the extracellular fluid to replace the potassium that had been lost from the cells - Fox and Baer, (1947) - and, since calculations have indicated that such a transfer of sodium can occur, the retention of sodium could be explained partly on this basis.

It has been suggested that the disruption of the
cells in the traumatised area was not due entirely to the injury itself but mainly to metabolic deficiencies which resulted from damage to the blood vessels and lymphatics - Arey,(1936). The possible liberation of toxic metabolites as a result of this disruption has been mentioned already - Cooke and Whipple,(1918).

Evidence was presented by Duthie and Chain (1939) and by Spector (1951) that peptides were produced at the site of trauma and that these substances, on injection, were capable of producing many of the post-operative phenomena. Similar results have been obtained by the injection of adenosine triphosphate which Green and co-workers (1950) have shown to be the active group of "myotoxin". The similarity between the symptoms of the "alarm" reaction and those of histamine toxicosis was pointed out by Selye (1936) who suggested that the liberation of large amounts of histamine or of a pharmacologically related substance from the tissues could play an important part in the reaction. An interesting point in this connection was the observation of Perla and co-workers (1940) that a combination of sodium chloride and DOCA was effective for the protection against histamine reaction. While this may be part of the cause of the retention of sodium and of the increased excretion of the adrenal steroids no evidence was available which permitted the inclusion of this as a definite statement of fact.

The conclusions which were drawn from the above
work were that the metabolic responses which followed surgical operations were in part due to a series of reactions which resulted from the direct damage done to the tissues and in part to the increased activity of the adrenal glands, as evidenced by the increased excretion of acid-stable formaldehydogenic steroids. More definite proof of these facts could be obtained from the study of the response of an adrenalectomised person subjected to a further operation. However, such cases do not present themselves at very frequent intervals and it was felt that the continuation of this work must be a very slow process.

While it appeared that the adrenal glands were not under the control of the hypophysis, as far as their response to trauma was concerned, this fact would have to be tested by observing the response of a hypophysectomised patient to further operation if the opportunity ever arose.

SUMMARY.
1). It has been confirmed that, in the immediate post-operative period, there was a marked diminution in the amount of sodium excreted in the urine and that this lasted for at least three or four days. A period of potassium loss was also evident and while this appeared to be rather transitory it occurred at the same time as an increased excretion of A.S.F.S., a fact which was regarded
as indicating increased adrenal activity. The alteration in A.S.F.S. excretion was usually of longer duration than that in potassium excretion and was rather more marked. From a consideration of the timing of these responses, as well as from the published data, it has been concluded that the metabolic changes subsequent to trauma were due in part to the effects of hyperactivity of the adrenal glands and in part to the results of cellular damage in the traumatised area.

2). From a study of the cases where adrenalectomy was effected it was evident that the observed alterations in the metabolism following trauma could not be due entirely to increased activity of the adrenal glands. In these cases, where no increased secretion was possible from the adrenal glands, it was observed that the pattern of the changes which occurred in the metabolism of sodium, potassium and nitrogen was similar to that which was evident following general surgical trauma. However, the indications were that the changes which followed the second stage of the adrenalectomy were less marked than those which ensued after the first. These facts indicated that, while the presence of the adrenal glands was not essential for the appearance of the metabolic changes, thus indicating the existence of a cellular reaction, increased secretion from
these glands resulted in an increase in the magnitude of the changes. Here again there was evidence that the metabolic changes which follow trauma were due partially to the effects of increased activity of the adrenal glands. However the purely cellular effect has been shown to play a considerable part in producing the metabolic changes observed.

3) While the removal of the hypophysis eliminated the possibility of direct stimulation of the adrenal glands by means of ACTH liberation, it has been demonstrated that the usual metabolic changes with regard to sodium, potassium and nitrogen were evident and that there was evidence of increased adrenal activity. This was taken as indicating that the adrenal glands were not solely under the control of the hypophysis, as far as their action in response to trauma was concerned.

4) A possible means of continuing the study has been suggested. This would involve the use of methods for the estimation of aldosterone, as well as those which indicate the more general activity of the gland, in cases where either adrenalectomy or hypophysectomy had been carried out and where the patient was being subjected to further operative treatment.
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II. OBSERVATIONS ON THE METABOLISM OF ASCORBIC ACID IN MAN.
# Contents

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INTRODUCTION.

Among the many and varied observations that have been made during the study of the various aspects of ascorbic acid metabolism one which does not appear to have led to any prolonged investigation is that concerning the surprisingly low vitamin C excretion in such conditions as rheumatoid arthritis. Some investigations were carried out, in the period before 1939, on the relationship of the ascorbic acid content of the body to the severity of the condition in cases of rheumatism but, since then, only scattered references to the subject are to be found.

In 1933 Rinehart and Mettler first suggested that a deficiency of ascorbic acid was part of the etiology of rheumatic fever. By means of a comparison of the joint symptomatology and pathology observed in scurvy and in rheumatic fever, it was suggested that a condition of near scurvy might be an important factor in the genesis of the joint conditions evident in the latter disorder. It was suggested also that this near scurvy state might be partially responsible for the more chronic disability of the closely allied condition of rheumatoid arthritis. A survey of the social, age and seasonal incidence of rheumatic fever was thought to lend strength to the theory. Further, the almost complete absence of this disease in the tropics, where the dietary conditions were such that cases of true clinical scurvy were almost unknown,
added support to this view.

Although these observations did not involve the actual biochemical investigation of the state of vitamin C nutrition of the body it may be said that they gave an impetus to such a line of study. Rinehart, Connor and Mettier (1934) demonstrated that there was a lower plasma ascorbic acid concentration in subjects suffering from acute rheumatic fever than in 'normals'. Further evidence was produced by Rinehart (1935, 1936) to substantiate this.

It is of interest to digress for a moment to note that the use of a raw vegetable diet in the treatment of rheumatic conditions had been advocated by Hare (1936). While the primary reason for the use of such diets was the low sodium content it was realised later that the observed clinical improvement might have resulted from the increase in the vitamin C intake.

A slightly different approach to the problem was made by Harris and his group. These workers concentrated on the aspect dealing with the excretion of the vitamin in the urine. Abbasy et al. (1936) observed that children suffering from rheumatism excreted less ascorbic acid than did control subjects who received the same diet. Also, when such patients were given relatively large test doses of vitamin C orally, the output in the urine was again less than controls treated similarly. An interesting point was that,
while patients suffering from osteomyelitis exhibited a diminished excretion of ascorbic acid in the urine, healed osteomyelitis patients excreted amounts which were comparable with those excreted by control subjects. This was in contrast with people convalescing from acute rheumatism in whom the excretion of the vitamin remained subnormal. Since a diminished excretion of ascorbic acid had been noted in persons suffering from many types of infection this subnormal excretion of the vitamin was regarded as being an indication that there was still a latent infective process present.

Further proof that such patients excreted less ascorbic acid was forthcoming from the work of Abbasy, Harris and Ellman (1937) who demonstrated that when patients suffering from rheumatoid arthritis were maintained for four weeks on a strict diet, with a supplement of approximately thirty-five mg. ascorbic acid per day, the urinary excretion fell well below that observed in control subjects under the same conditions. Similar results have been reported by Schultzer (1936) and the fact that such decreased excretions of the vitamin were not limited to this condition was evident from the work of Schroeder (1935) and Harde and co-workers (1935). The latter workers observed low ascorbic acid excretion in cases of pneumonia and the former investigator found that, in a number of infectious diseases, the amount of the
vitamin excreted was well below that evident in 'normals'.

Rinehart, Greenberg and Baker (1936) confirmed that the blood plasma ascorbic acid concentration in people with active rheumatoid arthritis was regularly low if the individual had not been maintained on a diet with a high vitamin C supplement. There was usually an increase in the plasma ascorbic acid concentration when the vitamin was administered but such an increase was not evident in every case. The results indicated that, in most cases, the intake required to maintain an adequate plasma content was very much above the average requirement of 'normals'.

A further paper from Rinehart's laboratories (1936A), confirming the previous observations, noted that the concept of the inter-relationship of ascorbic acid content of the plasma and the condition of rheumatoid arthritis was being questioned by such workers as Schultz and colleagues (1935) and Perry (1935).

Hare and Williams (1938), continuing Hare's original work on the administration of raw vegetable diets to patients suffering from rheumatoid arthritis, confirmed the results obtained by the earlier workers. They observed that most of these subjects were obviously 'unsaturated' with respect to vitamin C, and also that there appeared to be some factor other than the antecedent deficient diet causing this effect. A great delay was observed in obtaining a rise in both
the plasma ascorbic acid level and the degree of saturation and it was found that the severity of the condition played a large part in determining the length of this delay period. A great difference was found between the response of persons with rheumatoid arthritis and of 'normals' who received the same dose. While the results suggested that the rheumatic conditions were related to a state of ascorbic acid unsaturation, it was stated that there was no evidence of a relation between the observed clinical improvement, resulting from the vegetable diet, and the rise in the plasma ascorbic acid concentration.

Similar results indicating a low ascorbic acid content of blood plasma in rheumatoid arthritis were reported by Hall, Darling and Taylor (1939). Since all of the patients were receiving a diet which contained eighty mg. of the vitamin per day this study would appear to indicate that there was an increased requirement of the substance. It was noted that when extra supplements of the vitamin were given there was no clinical improvement which could be attributed to this treatment. While Rinehart (1939) agreed with the finding that there was an increased requirement, he differed in his views regarding the efficacy of the treatment in alleviating the symptoms. Many papers have appeared which tend to support the view held by Hall et al. (1939) and among these may be mentioned those by Jacques (1940), Secher (1940), Freyberg
Thus it is evident that, in people suffering from rheumatoid arthritis, there is a diminished concentration of ascorbic acid in the blood plasma and also that the amount of this substance excreted, either normally or after the administration of extra amounts, is less than that excreted by 'normal' persons. So far, no indication has been given as to the reason for the existence of these phenomena. Another fact which has not been explained is that which has been observed frequently and which was noted first by Johnson and Zilva (1934). This is the fate of the large quantities of ascorbic acid which are not accounted for in the urine when massive doses are ingested.

A large amount of work has been carried out which has detailed the changes in the concentrations of ascorbic acid, dehydroascorbic acid and diketogulonic acid in the blood plasma under various conditions and many observations have been made on the alteration in ascorbic acid content of urine under similar conditions. However, very few reports have been forthcoming regarding the alteration in the oxalate content of either urine or blood following the ingestion of large amounts of ascorbic acid.

The fact that ascorbic acid on oxidation can give rise to oxalic acid has been demonstrated widely.

When the constitution of ascorbic acid was being
elucidated it was discovered that oxalic acid was one of the products of *in vitro* oxidation of the vitamin - Herbert and co-workers (1933). Oxidation with aqueous iodine was shown to lead, initially, to the formation of a reducing substance and this, on further oxidation, yielded oxalic acid and a trihydroxybutyric acid.

![Chemical Structure](image)

The series of changes were thought to be highly complex but it was demonstrated that the yield of oxalic acid, following iodine oxidation, was 95\% of the theoretical.

Similar observations were made by Borsook and colleagues (1936) but they considered that the route taken by the oxidation was slightly different. They showed that the 2:3-diketo-L-gulonic acid, which is formed by the opening of the lactone ring of dehydro-ascorbic acid, was converted into some intermediary compound which eventually broke down to yield a mixture of oxalic and L-threonic acids. This route was held to be more likely than the one involving the direct oxidation of the dehydroascorbic acid to these products.
Other observations which confirmed the production of oxalic acid by the *in vitro* oxidation of ascorbic acid and its derivatives also deserve mention. Among these are those of Ghosh and Rakshit (1938), Jurist and Christiansen (1939) and Rosenfeld (1943).

With these facts in mind it is of interest to note that the synthesis of D-ascorbic acid from ethyl glyoxalate and D-threose was effected by Helferich and Peters (1937). The preparation of the laevo-isomer was made difficult by the scarcity of the L-threose starting material. Hence the synthesis of ascorbic acid from, and the breakdown into, a molecule containing two carbon atoms and one with a four carbon atom chain had been shown to occur *in vitro*.

The first suggestion that ascorbic acid gave rise to part of the oxalic acid content of urine was put forward by Flaschenträger and Müller (1938). They considered that this substance would result from the further oxidation of dehydroascorbic acid, possibly in a manner similar to that which occurred *in vitro*. Following this suggestion Scheinkman (1940) proved that when guinea pigs were given relatively large doses of ascorbic acid there was a definite increase in the amount of oxalic acid excreted in the urine.

A very important paper by Rosenfeld (1943) brought to light the fact that *in vitro*, at pH 7.0 and in the presence of phosphate buffer, dehydroascorbic acid did not suffer oxidation through diketogulonic acid
but rather by a direct fission of the lactone molecule. This resulted in the formation of oxalic acid and a four carbon chain compound. It appeared that the presence of oxygen led to the formation of oxalylthreonate as the first product of oxidation and that in the absence of phosphate the dehydroascorbic acid was converted into another compound with a chain of six carbon atoms. In a report published at this time Barrett (1943) stated that when ascorbic acid was added to human blood, in vitro, there was an increase in the oxalic acid content. However, the author pointed out that the results, which were obtained by using the method of Barber and Gallimore (1940), were not of the highest order of accuracy and it was not certain if all the oxalate found had pre-existed in the blood.

While Rosenfeld (loc. cit.) had deduced that the daily output of 27 mg. of oxalate reported by Dodds and Gallimore (1932) corresponded to the destruction of approximately fifty milligrams of ascorbic acid, it must not be thought that other sources of oxalic acid are not available. As a result of experiments in which C$^{14}$-labelled glucose was used, Burns, Burch and King (1951) confirmed that glucose could act as a source of oxalic acid in guinea pigs. These workers also proved that urinary oxalate was derived, at least partially, from dietary ascorbic acid. When the vitamin, labelled with C$^{14}$ in the 1-C atom position, was fed to both 'normal' and scorbutic guinea pigs
it was observed that approximately two per cent of the label appeared in the urine in the form of oxalate. While this indicated the response to added ascorbic acid it was noted also that approximately eight per cent of the daily intake of the vitamin was excreted as oxalate.

A large portion (approximately twenty-five per cent) of the label appeared in the respiratory carbon dioxide of the guinea pigs but it has been proved recently, by Hellman and Burns (1955) that this was not the case in man. Utilising L-ascorbic acid, labelled with C\textsuperscript{14} in the 1-C position, these authors showed that essentially all of the label was eliminated as either ascorbic acid, dehydroascorbic acid, diketogulonic acid or oxalic acid and only three per cent was to be found in the respiratory carbon dioxide. This difference was pointed out also by Burns and co-workers (1956).

A study of the excretion of oxalate in the urine was made by Lamden and Chrystowski (1954) following the ingestion of varying amounts of ascorbic acid, in 'normal' subjects. It was concluded that the ingestion of less than four grams of the vitamin daily resulted in a negligible increase in the oxalate content of the urine.

It has been established, therefore, that ascorbic acid is one source of the oxalic acid found in human urine and also that in cases of infection, including
11.

rheumatoid arthritis, there is a decreased excretion of the vitamin as compared with 'normal' subjects. The suggestion has been made that this difference in excretion is due to an increased destruction of the vitamin and not to a deficiency in the diet. The possibility presents itself therefore, that, if this latter statement is correct, there will be an increased output of oxalate in the urine of such patients.

OBJECT.

In these circumstances it was thought desirable to investigate, in 'normals', the response in urinary oxalate following the ingestion of large amounts of ascorbic acid orally and then to compare the results thus obtained with those found in patients, suffering from active rheumatoid arthritis, subjected to similar treatment. The determination of the ascorbic acid content of the blood plasma was thought to be essential in order to give an indication of the degree of saturation with regard to the vitamin.

PROCEDURE.

The cases studied may be divided into two classes, namely rheumatoid and non-rheumatoid. The admittance of three cases of definite scurvy, which exhibited all the clinical signs of the condition, to one of the wards proved to be of the greatest interest and benefit to this study.
All of the patients received the usual ward diet with the exception of fruit and vegetables and they were advised that the ingestion of fruit or fruit juices was not permissible during the period of the test. In this way the subjects were maintained on a diet from which ascorbic acid was lacking and which contained little, if any, oxalate. The removal of fruit and vegetables from the diet was effected several days before the urine collections began and this was found to result in urinary oxalate levels which were relatively constant.

When the ascorbic acid was administered orally, the subjects received a total of ten grams of the vitamin on each of two successive days. The ascorbic acid was given in the form of five hundred milligram tablets, which were taken at intervals throughout the day but no attempt was made to institute a strict schedule. In every case, however, the vitamin was ingested between the hours of 9 a.m. and 10 p.m. Twenty-four hour urine samples were collected in brown Winchesters, each of which contained 225 ml. of a preservative consisting of metaphosphoric acid and thiourea. Each bottle contained 45 g. of metaphosphoric acid and 7.5 g. thiourea, and as will be confirmed later, this prevents the autoxidation of voided ascorbic acid to oxalate, in vitro. The urine collections began three or four days before the vitamin C was given and were continued for several days after
the dose.

In the latter experiments, when ascorbic acid was being given intravenously, each subject received five grams of the vitamin (Roche ampoules for intravenous use) in a total volume of one hundred ml. of sterile saline. In order to standardise the conditions of the injection a total of five minutes was spent, in each case, in giving the dose. Twelve hour urine collections were made in these cases and again each specimen was collected in a brown bottle containing the metaphosphoric acid-thiourea preservative. However, only half the amount of preservative used in the twenty-four collections was added in these cases.

A point which must be emphasised strongly is that every subject was informed that all the urine samples had to be passed directly into the preservative or, if this was not possible, great care had to be taken to ensure that the specimen was poured into the bottle with the minimum of delay. In this manner the possibility of any in vitro oxidation of ascorbic acid to oxalate was minimised, as far as was possible.

An estimation of the ascorbic acid content of the blood plasma was made, in every case, prior to the administration of the vitamin. The amount of ascorbic acid excreted in the urine was determined in all samples.
14.

METHODS.

1). Urinary oxalate.

The method used in this study was essentially that proposed by Powers and Levatin (1944). The main difference, which is one of technique, was that while these authors suggested the use of an electric hot-plate as a source of heat for the ether distillation a hot water bath was used in these experiments. The use of a hot-plate proved to involve too great a risk of fire.

A modified Clausen continuous extraction apparatus similar to that used by Powers and Levatin was employed (see Fig. 1.). The substitution of a 300 by 25 mm. test-tube for the Kjeldahl flask permitted the use of smaller volumes of ether. The extractor consisted of a 25 ml. rimmed test-tube into which was placed a funnel-shaped tube made from half of a 25ml. bulb pipette. The over-all length of this tube was about 200 mm. and four holes had been punched, with a hot wire, in the sealed bottom end. A small hole was drilled near the top of the rimmed test-tube to allow the removal of this tube by means of a wire hooked at one end. The rimmed test-tube was supported by shoulders approximately 75 mm. from the top of the outer tube. A "cold finger" type of condenser was attached to the top of the outer tube so that the tip of the finger was situated
in the mouth of the funnel part of the extractor.

A special type of centrifuge tube was used (see Fig.1.). This consisted of the usual type of 15 ml. capacity, the bottom of which had been drawn out to give an internal diameter of about 1 mm. The use of such a tube was necessary in the handling of the very small precipitates encountered.

Reagents:-
Ether, A.R.
95% v/v alcohol.
2% v/v acetic acid.
10% w/v calcium chloride solution.
Acid-alcohol solution. This was prepared by mixing 60 ml. of 95% alcohol, 10 ml. of 2% acetic acid and 20 ml. water.
20% v/v sulphuric acid.
1% w/v manganese sulphate.
0.01N potassium permanganate solution.
0.01N sodium thiosulphate solution.
10% w/v potassium iodide solution.
1% w/v starch solution. This solution was prepared freshly each day.

Procedure:-
One millilitre of concentrated hydrochloric acid was added to 25 ml. of the urine sample and,
after thorough mixing, the solution was heated in a boiling water bath for half an hour. This step has been shown to be essential to ensure the complete hydrolysis of the oxaluric acid present. 10 ml. of the cooled urine were measured into the inner test-tube and 2 ml. of water and 25 ml. ether were added to the outer one. The extractor was connected up and placed in position in the water bath. The level of the bottom of the extractor was adjusted so that it was just in contact with the hot water and the tube was agitated until steady ebullition of the ether had been attained. The depth of the extractor in the water was varied until the rate of evaporation of the ether was such that approximately 100 to 110 drops of ether were formed on the condenser per minute.

The extraction was continued, without interruption, for six hours and any loss of ether which occurred during this time was made good. At the end of the extraction period, the inner tube and extractor were removed and the sides of this tube were washed down with 2 ml. of 95% alcohol. After the addition of 1 ml. of 2% acetic acid the ether was evaporated off from the aqueous layer. For this purpose a water bath maintained at about 70°C was employed and great care was taken to ensure that the tube was
shaken continuously to mix the ether and aqueous phases. While it has been proved that a dilute solution of oxalic acid in ether is volatile, the presence of water has been shown to result in the retention of the acid in the aqueous phase - Powers and Levatin (1944).

The aqueous solution remaining in the tube was transferred to the special centrifuge tube by means of the usual type of semi-micro transfer tube, which had an internal diameter of approximately 1 mm., and a water pump was used as the source of the reduced pressure. The sides of the outer tube were washed twice with two ml. portions of 95% alcohol and these washings were transferred to the centrifuge tube.

0.5 ml. of a 10% calcium chloride solution was added to the tube and, after thorough mixing with an air current passed through a capillary, the solution was overlayed with 2 ml. of the acid-alcohol solution. This prevented the formation of any precipitate on the surface of the solution and thus eliminated a possible source of error. After standing overnight, the solution was centrifuged, the supernatant liquid was poured off and the tube inverted to allow drainage of the alcohol. 2 ml. of acid-alcohol were added and the precipitate was stirred up. After the addition of a further 3 ml. of acid-alcohol the tube was
centrifuged and the supernatant liquid decanted off. The precipitate was allowed to drain thoroughly and the remaining alcohol was evaporated off by placing the tube in a hot air oven for a few minutes.

The precipitate was dissolved, with the aid of heat, in 1 ml. of 20% sulphuric acid, 0.5 ml. of 1% manganese sulphate solution was added and this was followed by 3 ml. of 0.01N potassium permanganate solution. The thoroughly mixed solution was allowed to stand for eight to ten minutes, care being taken that the temperature of the solution was not above 35°C at any time after the addition of the permanganate, and then 2 ml. of 10% potassium iodide were added. The liberated iodine was titrated in the usual manner with 0.01N sodium thiosulphate, using starch as indicator.

A blank estimation was carried out using distilled water in place of the urine.

Calculation.

From the value of exactly 0.01N potassium permanganate used to oxidise the oxalic acid the reagent blank was subtracted and the result multiplied by 0.45. This figure was the amount of oxalic acid, expressed as milligrams, extracted from 10 ml. of the diluted urine. In order to
allow for the dilution of the urine by the hydrochloric acid this figure was multiplied by a factor, 1.04. A further multiplication by 10 yielded the amount of oxalate, expressed as oxalic acid, extractable from 100 ml. of urine.

2). Ascorbic acid.
A). Urine: The colorimetric method of Roe and Kuether (1943), which gives a measure of the total ascorbic acid content, was used.

B). Plasma: 1). Total ascorbic acid content was measured by the colorimetric method of Roe and Kuether (1943).

2). Reduced ascorbic acid was measured by the method of Stewart, Horn and Robson (1953) which utilises the reduction of a solution of 2:6-dichlorophenolindophenol by ascorbic acid.

Accuracy of oxalate method.
A short investigation of the accuracy of the oxalate method was undertaken before the main part of the study was commenced. Since many estimations were to be carried out the experiments were so arranged that an indication of the reproducibility of the method could be obtained.
also. For this purpose a series of recovery experiments was set up in which known amounts of oxalic acid were added to urine samples before the determinations were made. Eight estimations were made at each level of added oxalic acid. By this means both the accuracy and the reproducibility of the method were determined.

The results shown in Table 1 are those obtained in two such experiments. The amounts of oxalic acid which were added corresponded to the upper and lower levels which were expected to be found in the subsequent experiments.

It will be seen that at the higher level, in which additions of 3 mg. oxalic acid per 100 ml. of urine were made, the mean recovery was 97.7%, the range being from 93.3% to 101.0%. At the lower level, in which 1 mg. oxalic acid was added, the recovery was slightly less, being 95.8% with a range from 93.0% to 107.0%. The standard deviation of the method was found to be ± 0.09 at the higher level and ± 0.10 at the lower, both of these figures being expressed as milligrams per 100 ml. urine.

From these figures it was evident that the method was of sufficient accuracy for the purpose for which it was to be used. A point worth noting is that, while these recovery experiments are of great value, they do not prove that the
<table>
<thead>
<tr>
<th>Oxalate content of urine mg./100 ml.</th>
<th>Amount of oxalic acid Added mg./100 ml.</th>
<th>Recovered mg./100 ml.</th>
<th>% Recovery.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.88.</td>
<td>0.00.</td>
<td>0.00.</td>
<td>0.00.</td>
</tr>
<tr>
<td>4.87.</td>
<td>3.00.</td>
<td>2.99.</td>
<td>99.6.</td>
</tr>
<tr>
<td>4.73.</td>
<td>3.00.</td>
<td>2.85.</td>
<td>95.0.</td>
</tr>
<tr>
<td>4.68.</td>
<td>3.00.</td>
<td>2.80.</td>
<td>93.3.</td>
</tr>
<tr>
<td>4.77.</td>
<td>3.00.</td>
<td>2.89.</td>
<td>96.3.</td>
</tr>
<tr>
<td>4.87.</td>
<td>3.00.</td>
<td>2.99.</td>
<td>99.6.</td>
</tr>
<tr>
<td>4.77.</td>
<td>3.00.</td>
<td>2.89.</td>
<td>96.3.</td>
</tr>
<tr>
<td>4.91.</td>
<td>3.00.</td>
<td>3.03.</td>
<td>101.0.</td>
</tr>
<tr>
<td>4.91.</td>
<td>3.00.</td>
<td>3.03.</td>
<td>101.0.</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>97.7.</td>
</tr>
<tr>
<td>2.90.</td>
<td>1.00.</td>
<td>1.02.</td>
<td>102.0.</td>
</tr>
<tr>
<td>2.81.</td>
<td>1.00.</td>
<td>0.93.</td>
<td>93.0.</td>
</tr>
<tr>
<td>2.81.</td>
<td>1.00.</td>
<td>0.93.</td>
<td>93.0.</td>
</tr>
<tr>
<td>2.71.</td>
<td>1.00.</td>
<td>0.83.</td>
<td>83.0.</td>
</tr>
<tr>
<td>2.90.</td>
<td>1.00.</td>
<td>1.02.</td>
<td>102.0.</td>
</tr>
<tr>
<td>2.95.</td>
<td>1.00.</td>
<td>1.07.</td>
<td>107.0.</td>
</tr>
<tr>
<td>2.81.</td>
<td>1.00.</td>
<td>0.93.</td>
<td>93.0.</td>
</tr>
<tr>
<td>2.81.</td>
<td>1.00.</td>
<td>0.93.</td>
<td>93.0.</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>95.8.</td>
</tr>
<tr>
<td>Mean of 16 estimations:</td>
<td></td>
<td></td>
<td>96.7.</td>
</tr>
</tbody>
</table>
The substance initially present in the urine is oxalate or oxalic acid. However, since the main interest centred in the alteration of the amount of oxalate excreted, the fact that a recovery of 96.7% of added oxalic acid was possible indicated that any increased excretion would be detected by the method.

Another point which was investigated was that concerning the efficacy of the metaphosphoric acid - thiocurea preservative in preventing the \textit{in vitro} oxidation of ascorbic acid to oxalic acid. For this purpose five grams of ascorbic acid were added to a twenty-four urine sample, the "oxalate" content of which was known, and which contained the usual amount of preservative. This amount of ascorbic acid proved to be greater than that found in any of the subsequent urine samples, even after the ingestion of ten grams of the vitamin. It was found that, after standing for twenty-four hours at room temperature, there was no detectable increase in the "oxalate" content, a result in agreement with that of Powers and Levatin (1954).

\textbf{RESULTS.}

Before considering the results obtained after the administration of large amounts of ascorbic acid it is of interest to note those which were obtained
for the level of oxalate excretion in the control periods prior to the vitamin treatment.

In the case of the non-rheumatoid subjects it was found that the average excretion per day was thirty-seven milligrams, expressed as oxalic acid. The standard deviation of the results was found to be ± 7.6 mg. A lower excretion of oxalate was observed in the cases suffering from active rheumatoid arthritis, the level in these cases being 32.2 mg. per day with a standard deviation of ± 8.4 mg., the results again being expressed as oxalic acid. By means of "Student's" t test it was shown that the difference between these two sets of figures is statistically significant.

Effects of oral administration of ascorbic acid.

The type of response that was obtained following the administration of ten grams of ascorbic acid on each of two successive days is indicated in Figure 2. It will be seen that, in the subjects not suffering from rheumatoid arthritis, there was usually a rise in the oxalate excretion on the first day of the treatment, although the increase was rather variable. While most of the patients exhibited a maximum oxalate excretion on the second day, in one case this maximum was not evident until a day later. The duration of the period of increased excretion appears to be rather variable but it may be said that by the fourth day
Figure 2.
Excretion of oxalate, expressed as oxalic acid, following the administration of ten grams of ascorbic acid orally on each of two successive days.
after the end of the treatment the excretion had regained the pre-treatment levels.

The patients suffering from rheumatoid arthritis exhibited an entirely different type of response to the same treatment. There did not appear to be any set pattern for the excretion of oxalate following the ingestion of the vitamin. Although there was a tendency for the amount of oxalate excreted in the urine to increase the levels attained did not approximate to those evident in the non-rheumatoid subjects. The total increase in the oxalate excretion was very much less than it was in the former cases.

As far as the excretion of ascorbic acid, as measured by the method of Roe and Kuether, was concerned it was found that there was a large amount to be found in the urine on each of the two days during which the vitamin was being ingested. However, the amounts excreted fell off rapidly after the administration had been stopped, reaching the pre-treatment levels by the third day. This type of change in ascorbic acid output was found in both sets of subjects and the application of "Student's" t test to the results indicated that there was no significant difference in the amounts excreted by the two groups.

**DISCUSSION.**

The first point which was evident from the results was that the subjects who were suffering from
rheumatoid arthritis excreted less oxalate in the urine than did the non-rheumatoid ones. This difference could not be due, in these cases, to a dietary phenomenon since both sets of patients were receiving the same type of diet, namely one deficient in ascorbic acid and one from which sources of exogenous oxalate had been removed. The mean values for the excretions were 37.0 ± 7.6 mg. per day for the non-rheumatoid subjects (a result based on thirty-eight different twenty-four hour collections from seven different cases) and 32.2 ± 8.4 mg. per day from the rheumatoids (a result based on thirty-seven, twenty-four urine collections, from five different cases). The application of "Student's" t test to these results produced a value of 2.60 for t and this corresponded to a probability which was less than 0.01. This was an indication that the difference between the two sets of results was highly significant, statistically.

A similar difference between the amounts of oxalate excreted by the two groups was evident following the ingestion of twenty grams of ascorbic acid. The results, to be found in Table 2, demonstrated that while the average increase in the oxalate excretion, expressed in terms of oxalic acid, was forty-six milligrams in the rheumatic subjects, the average increase for the non-rheumatoids was one hundred and two milligrams. When these figures were converted
<table>
<thead>
<tr>
<th>Subject</th>
<th>Increase in urine oxalate excretion expressed as;</th>
<th>Amount of A.A. 'retained'</th>
<th>% of 'retained' A.A. excreted as oxalate (in terms of A.A.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxalic acid.</td>
<td>A.A.</td>
<td></td>
</tr>
<tr>
<td>1. N-R.</td>
<td>166 mg.</td>
<td>325 mg.</td>
<td>16.6 g.</td>
</tr>
<tr>
<td>2. N-R.</td>
<td>102 mg.</td>
<td>200 mg.</td>
<td>17.0 g.</td>
</tr>
<tr>
<td>3. N-R.</td>
<td>132 mg.</td>
<td>259 mg.</td>
<td>12.4 g.</td>
</tr>
<tr>
<td>4. N-R.</td>
<td>93 mg.</td>
<td>182 mg.</td>
<td>15.0 g.</td>
</tr>
<tr>
<td>5. S.</td>
<td>A) 128 mg.</td>
<td>251 mg.</td>
<td>15.6 g.</td>
</tr>
<tr>
<td></td>
<td>B) 148 mg.</td>
<td>290 mg.</td>
<td>13.5 g.</td>
</tr>
<tr>
<td>6. S.</td>
<td>98 mg.</td>
<td>192 mg.</td>
<td>16.0 g.</td>
</tr>
<tr>
<td>7. S.</td>
<td>93 mg.</td>
<td>182 mg.</td>
<td>16.3 g.</td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td>120 mg.</td>
<td>235 mg.</td>
<td>15.3 g.</td>
</tr>
<tr>
<td>8. R.</td>
<td>A) 34 mg.</td>
<td>67 mg.</td>
<td>17.3 g.</td>
</tr>
<tr>
<td></td>
<td>B) 15 mg.</td>
<td>29 mg.</td>
<td>16.9 g.</td>
</tr>
<tr>
<td>9. R.</td>
<td>A) 86 mg.</td>
<td>169 mg.</td>
<td>14.0 g.</td>
</tr>
<tr>
<td></td>
<td>B) 66 mg.</td>
<td>129 mg.</td>
<td>12.0 g.</td>
</tr>
<tr>
<td>10. R.</td>
<td>86 mg.</td>
<td>169 mg.</td>
<td>16.6 g.</td>
</tr>
<tr>
<td>11. R.</td>
<td>A) 15 mg.</td>
<td>29 mg.</td>
<td>16.1 g.</td>
</tr>
<tr>
<td></td>
<td>B) 19 mg.</td>
<td>37 mg.</td>
<td>14.6 g.</td>
</tr>
<tr>
<td><strong>MEAN</strong></td>
<td>46 mg.</td>
<td>90 mg.</td>
<td>15.4 g.</td>
</tr>
</tbody>
</table>

N-R. represents non-rheumatoid.

R. represents rheumatoid.

S. represents scurvy.

A) & B) indicate two tests made on the same subject.

'Retained' A.A. represents the amount of ascorbic acid not excreted as such in the urine.
into amounts of ascorbic acid oxidised it was seen that they corresponded to ninety and two hundred and thirty-five milligrams respectively.

When the amounts of the ascorbic acid doses not accounted for in the urine were considered it was found that there was no statistical difference between the two series (Table 2). This proved that the difference in the oxalate excretion was not due to a difference in the amount of the vitamin eliminated from the body in the urine.

If the assumption is made, for the present, that there was no difference in the absorption, in the intestinal tract, of the ascorbic acid then the difference between the amount of the vitamin ingested and that excreted in the urine will give an indication of the amount retained by the body. When the percentage of the 'retained' vitamin which was converted into oxalate, expressed in terms of ascorbic acid, was considered, (see Table 2), it was seen that the difference between the two groups was significant. This was confirmed by a statistical treatment which showed that the probability was less than 0.01.

It has been shown thus that the excretion of oxalate was less in persons with active rheumatoid arthritis than in non-rheumatoids and that the difference was made more marked by the ingestion of large doses of the vitamin. It would appear that the difference was not due to a differing degree of
Table 2.
Oral Dose of Ascorbic Acid.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Plasma concentration mg./100 ml.</th>
<th>% of 'retained' dose excreted as oxalate (in terms of A.A.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A.A.</td>
<td>T.A.A.</td>
</tr>
<tr>
<td>1.N-R.</td>
<td>0.20</td>
<td>0.25</td>
</tr>
<tr>
<td>2.N-R.</td>
<td>0.20</td>
<td>0.30</td>
</tr>
<tr>
<td>3.N-R.</td>
<td>0.10</td>
<td>0.15</td>
</tr>
<tr>
<td>4.N-R.</td>
<td>0.35</td>
<td>0.40</td>
</tr>
<tr>
<td>5. S.</td>
<td>A)0.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>B)0.40</td>
<td>0.48</td>
</tr>
<tr>
<td>6. S.</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>7. S.</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>8. R.</td>
<td>A)0.00</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>B)0.25</td>
<td>0.30</td>
</tr>
<tr>
<td>9. R.</td>
<td>A)0.10</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td>B)1.30</td>
<td>1.40</td>
</tr>
<tr>
<td>10. R.</td>
<td>0.00</td>
<td>0.04</td>
</tr>
<tr>
<td>11. R.</td>
<td>A)0.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>B)0.40</td>
<td>0.48</td>
</tr>
</tbody>
</table>

N-R. represents non-rheumatoid subjects.
R. represents rheumatoid subjects.
S. represents scurvy subjects.

'Retained' dose represents the amount of the vitamin not excreted as such in the urine.
saturation with respect to the vitamin. This was clearly demonstrated as a result of the investigations which were carried out on the scorbutic subjects. These persons exhibited the same type of response as the other non-rheumatoid subjects following the ingestion of large amounts of ascorbic acid and differed markedly from the rheumatoids in this respect. The results in Table 3, in which the plasma concentrations of the vitamin are given along with the percentages of the 'retained' dose converted to oxalate, showed that, if the plasma level was regarded as an indication of the state of saturation of the body, there was no relation between the two factors. It will be seen that, with the exception of the one high plasma level evident in case 9, the plasma figures for the two groups correspond for the oral dose.

Since there was no significant difference in the amount of ascorbic acid excreted in the urine by the two groups the possibility suggested itself that there might be a possible difference in the amount of the vitamin absorbed in the intestinal tract. In order to study this aspect the use of intravenous administration of the dose was introduced.

A preliminary investigation of the use of the intravenous administration of ascorbic acid showed that there was a very wide scatter in the results for both rheumatoid and normal subjects and that the response appeared to depend on the previous treatment
which the rheumatoid persons had received. It became evident that a very large group of subjects would have to be studied to allow a statistical treatment of the results. Unfortunately, the number of untreated rheumatoids available in this hospital was too small to permit such an investigation although it is hoped that a sufficient number will be made available in time. A search of the literature failed to produce any reference to the amount of orally administered ascorbic acid which is excreted in the faeces. Hellman and Burns (1955), however, found that less than 1% of the administered radioactivity appeared in the faeces after the intravenous administration of the vitamin labelled with C\(^{14}\) in the 1-position but, there is the possibility that a much larger proportion would be excreted after an oral dose.

Therefore, the problem as to whether the absorption of ascorbic acid in the intestine plays any part in producing the decreased amounts of oxalate in the urine of rheumatoids remains unsolved for the present although it is hoped that an opportunity will be given to amass the required data in due course.

While the solution to this problem has not been found it is interesting to observe that the results obtained by the oral administration of ascorbic acid agree with those reported by Lamden and Chrystowski (1954) and yield further evidence that oxalic acid is
a metabolite of vitamin C in humans. The average figure (1.57%) for the percentage conversion of the ascorbic acid to oxalate which was obtained in this work was practically identical with that (1.50%) which was calculated from the figures presented by these workers. When the results of Hellman and Burns (1955) are considered it is seen that approximately 5% of a tracer dose of 30 mg. of L-ascorbic-\(^{14}\)C acid, administered intravenously, was excreted as oxalate. It could be argued from these results that only a small portion of ascorbic acid is metabolised by a pathway which involves the formation of oxalic acid and that the higher percentage conversion obtained by the intravenous administration is explicable on the basis of a more efficient utilisation of the small dose. However there is always the possibility that the oxalic acid formed is either metabolised further or retained in the body.

As far as the further metabolism of the oxalate is concerned the indications are that this substance is metabolically inert. Curtin and King (1955) showed that when \(^{14}\)C labelled oxalic acid was injected into rats over 50% of the label was to be found in the urine as unchanged oxalic acid or oxalate. There was no evidence of any conversion of the oxalate to other compounds and less than 0.4% of the label appeared in the respiratory carbon dioxide. A relatively high proportion of the label was accounted for as
oxalate retained in the tissues and it was found that the highest concentration of the acid was in the bones.

It is felt that the investigation of the metabolism of oxalate in the human subject would be of great interest and, possibly, value and would indicate if this phenomenon was responsible for the difference in oxalate excretion noted between rheumatoid and non-rheumatoid subjects.

Conclusion.

It has been demonstrated that there was a difference in the amounts of oxalate excreted by non-rheumatoids and by subjects suffering from the disease both on low ascorbic acid diets and after the ingestion of twenty grams of the vitamin. This difference did not appear to be related to the degree of saturation of the body, as indicated by the plasma ascorbic acid concentration, nor to the amount of the vitamin excreted in the urine. While it was possible that the amount of ascorbic acid absorbed in the intestinal tract might be different in the two groups, the use of the intravenous administration of the vitamin did not prove of any value in deciding this point owing to the limited number of rheumatoid subjects available in this hospital.

While the results would appear to indicate that only a small fraction of ascorbic acid is metabolised by a route which involves the formation of oxalic
acid it is felt that a further study of the metabolism of oxalate itself is required before such a statement may be made with any certainty. Such a study in both non-rheumatoid and rheumatoid subjects would also determine if the difference in the amounts of oxalate excreted by the two groups was due to an alteration in the metabolism of this substance.

It would appear, therefore, that the surprisingly low excretion of ascorbic acid which has been shown to exist in persons suffering from rheumatoid arthritis does not result from an increased destruction of the vitamin, involving the formation of oxalate, unless the oxalate so formed is either retained in the tissues and bones, or metabolised further.

The fact that the rheumatoid subjects excreted less oxalate than the non-rheumatoid controls, under the same conditions, provides a further indication that there is a difference in the metabolism of ascorbic acid between the two groups. In order to decide if there is also a difference in the metabolism of oxalate in the two groups further investigation is required.
SUMMARY.

1). It has been confirmed that when ascorbic acid is given, in large oral doses, there is an increase in the amount of oxalate excreted in the urine.

2). It has been shown that the level of oxalate excretion is lower in persons suffering from rheumatoid arthritis than that in non-rheumatoid subjects. The administration of oral doses of vitamin C has been demonstrated to result in a difference in the amount of oxalate excreted in the urine of the two groups. The rheumatoid subjects excreted approximately half the amount that the other group did.

3). It has been shown that the degree of saturation with respect to the vitamin, as indicated by the plasma concentration, does not bear any relation to the percentage of the orally administered ascorbic acid which is converted to oxalate.

4). A possible extension of the work, involving the study of the metabolism of oxalate itself, has been suggested.
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The Effect of Cortisone and Adrenocorticotropic Hormone on the Dehydroascorbic Acid of Human Plasma

BY C. P. STEWART, D. B. HORN AND J. S. ROBSON
Department of Clinical Chemistry, University of Edinburgh

(Received 30 May 1952)

It is well known that ascorbic acid in aqueous solution is easily and reversibly oxidized to dehydroascorbic acid, and it is believed that above pH 4 the latter can undergo irreversible change, possibly to diketogulonic acid, as a preliminary to further oxidation. The possibility of the existence of dehydroascorbic acid or diketogulonic acid in blood plasma, however, has been generally ignored since the time of Borsook, Davenport, Jeffreys & Warner (1937) who, with van Eekelen (1935), Kellie & Zilva (1936), Farmer & Abt (1936) and Ralli & Sherry (1941), claimed that vitamin C exists in plasma in the reduced state as ascorbic acid.

The brief statement that dehydroascorbic acid exists in human blood has recently been made by Chen & Scheuck (1950) but no supporting evidence was given. Apart from its academic interest, the relation between dehydroascorbic acid and experimental diabetes (Patterson, 1950) and the close association of ascorbic acid with the adrenal cortex and stress (Selye, 1946) suggest that the form in which vitamin C exists in blood is worthy of investigation. The experiments described in this paper establish the facts that a significant part of the total vitamin C present in normal human plasma exists as dehydroascorbic acid, that diketogulonic acid does not exist in plasma in significant quantities, and that the ratio between dehydroascorbic acid and ascorbic acid can be consistently influenced by adrenocortical steroids.

EXPERIMENTAL AND RESULTS

The existence of dehydroascorbic acid in human plasma

Chemical methods. Plasma from normal human subjects was submitted to the technique of Roe & Keuther (1943) and to a procedure employing 2,6-dichlorophenolindophenol. The former involves preliminary oxidation of ascorbic acid with activated charcoal and the colorimetric estimation of the reaction product with 2,4-dinitrophenylhydrazine under defined conditions. It is claimed to estimate the dehydroascorbic acid produced by oxidation of ascorbic acid plus any preformed dehydroascorbic acid and diketogulonic acid (i.e. total ascorbic acid1). The procedure involving 2,6-dichlorophenolindophenol was applied to a sample of the same plasma in order to estimate ascorbic acid itself. For this purpose 3.0 ml. of 3% (w/v) metaphosphoric acid were added to 2.0 ml. of plasma, and the precipitated proteins separated by centrifugation and filtration. A known excess of standard solution of the indophenol was added to a sample of the filtrate, and the residual colour was measured in a spectrophotometer (Unicam SP 500) at a wavelength of 480 mμ, 30 sec. from the time of addition of the indophenol. The method is similar in principle to that of Mindlin & Butler (1937–8), except that it was found possible to dispense with the buffer, and greater accuracy was obtained by constructing a standard curve instead of using a single standard solution.

The validity of the procedure was first tested by measuring the rate of fading of the indophenol solution under varying conditions, and the results are shown in Fig. 1. The indophenol was descolorized rather slowly in the presence of aqueous metaphosphoric acid (curve A) and more rapidly in the presence of trichloroacetic acid (curve B) at the same pH. Ascorbic acid added to the metaphosphoric acid solution (curve C) completely stabilized the indophenol colour after the initial reduction due to oxidation of the ascorbic acid. Unfortunately, it was impossible to obtain readings earlier than 30 sec. after the addition of the indophenol, but the reduction of the dye by ascorbic acid is certainly completed within 30 sec. When the indophenol was added to a metaphosphoric acid solution containing a trace of H2S (as an example of a substance oxidized slowly by the indophenol) fading continued to occur after 30 sec. and for as long as determinations were made (curve D). A similar slow reduction of the dye occurred in the presence of metaphosphoric acid and Na2S2O3 (curve E) in a concentration comparable with that in plasma; the curve, however, can be superimposed on that for metaphosphoric acid alone, and it can be concluded that thiosulphate had no appreciable effect. It is of interest to note also that stabilization of the indophenol colour did not occur when ascorbic acid was added to trichloroacetic acid (curve F). Similar results were obtained when the indophenol was added to metaphosphoric acid filtrates of plasma. With normal plasma the reaction with the dye was complete in 30 sec. (curve G) and this also occurred when the plasma was treated with H2S which was subsequently removed by Na2S2O3 (curve H). This curve shows that the H2S, used in this way, does not interfere with the determination of ascorbic acid, although if the removal of H2S is incomplete (curve J) stabilization of colour is delayed and estimation of ascorbic acid is impossible. The general conclusion is that with spectrophotometer readings taken 30–60 sec. after addition of the dye, with metaphosphoric acid but not trichloroacetic acid as the protein precipitant and with no residual H2S, the readings represent ascorbic acid concentrations, and that the presence of interfering substances is indicated by a steady change of spectrophotometer readings during the chosen period.
To test the reproducibility of results, twenty determinations were made on a solution of pure ascorbic acid; the mean result was 1.81 ± 0.025 mg./100 ml., the range being 1.77–1.83; similarly, a sample of plasma with added ascorbic acid gave twenty results between 2.09 and 2.13 mg./100 ml., with a mean of 2.11 and s.d. of 0.019. At a lower ascorbic acid concentration the figures for twenty replicate determinations were: mean, 0.43 mg./100 ml.; s.d., 0.023; range, 0.40–0.48.

![Graph](image)

Fig. 1. The effect of time on the transmission at 480 mμ of 1.0 ml. of the indophenol solution (4 mg./100 ml.) in the presence of 2.0 ml. of various reagents. A, metaphosphoric acid 3% (w/v); B, trichloroacetic acid 5% (w/v); C, HPO4, 3% (w/v) plus ascorbic acid, 1 mg./100 ml.; D, HPO4, 3% (w/v) + H2S; E, HPO4, 3% (w/v) + Na2S2O3, 8 mg./100 ml.; F, trichloroacetic acid 5% (w/v) + ascorbic acid, 1 mg./100 ml.; G, 2 ml. plasma + 3 ml. HPO4, 3% (w/v); H, as G, after treatment with H2S and removal of excess H2S by N2; J, as H, but with H2S incompletely removed.

The results of recovery experiments when pure ascorbic acid was added to plasma are shown in Table 1. Recoveries were always within 0.05 mg. ascorbic acid/100 ml.

<table>
<thead>
<tr>
<th>Added</th>
<th>Found</th>
<th>Calculated</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.37</td>
<td>0.4</td>
<td>-0.03</td>
</tr>
<tr>
<td>0.5</td>
<td>0.64</td>
<td>0.65</td>
<td>-0.01</td>
</tr>
<tr>
<td>1.0</td>
<td>1.17</td>
<td>1.15</td>
<td>+0.02</td>
</tr>
<tr>
<td>1.5</td>
<td>1.70</td>
<td>1.65</td>
<td>+0.05</td>
</tr>
<tr>
<td>2.0</td>
<td>2.20</td>
<td>2.15</td>
<td>+0.05</td>
</tr>
</tbody>
</table>

Invariably the results obtained by the method of Roe & Keuther (1943) were higher than those by the method using the indophenol, although both methods were standardized against the same original solution of pure ascorbic acid. A similar difference was found in the plasma from subjects who had been given a diet rich in vitamin C for a few weeks. These results are shown in columns 4 and 5 of Tables 2 and 3. In the normal fasting subjects on an ordinary diet, the mean ratio of plasma ascorbic acid determined by the indophenol method to the 'total ascorbic acid' determined by the method of Roe & Keuther (1943) was 0.68 ± 0.15 (Table 2, column 6). After a period of diet rich in vitamin C, the ratio became 0.82 ± 0.06 (Table 3, column 6), the absolute differences remaining about the same.

There are several possible explanations of this phenomenon other than the obvious one that the difference represents dehydroascorbic acid and/or diketogulonic acid existing in the circulating plasma. Oxidation of ascorbic acid to dehydroascorbic acid and subsequent change to diketogulonic acid may occur spontaneously after withdrawal of blood or during the manipulation involved in the indophenol method prior to the estimation. Alternatively, interfering substances may be reacting with 2,4-dinitrophenylhydrazine in the method of Roe & Keuther (1943), giving a falsely high figure for the concentration of total ascorbic acid. Indeed, both possibilities may be involved.

In regard to the first of these suggestions, the stability of ascorbic acid was investigated in blood, plasma and plasma filtrate. For this purpose, venous blood was withdrawn into an oiled syringe; part of the sample was allowed to stand at room temperature with occasional mixing, the remainder being used for the immediate determination of plasma total ascorbic acid and of ascorbic acid by the method of Roe & Keuther (1943), and by the indophenol method respectively. After about 60 and 120 min., further determinations were made by both methods. Similar experiments were carried out on plasma separated immediately after the withdrawal of blood. A third series was done on plasma filtrate prepared as quickly as possible after the withdrawal of the blood. The earliest determination possible in any of these experiments was about 30 min. after withdrawal. Typical results of these experiments are shown in Fig. 2, where it is seen that from the time of the first determination there is a very slow fall in the apparent ascorbic acid con-
such recoveries are complete. Blood concentrations of ascorbic acid were determined during the separation of plasma filtrate under these conditions. The total ascorbic acid does not alter during the period of these experiments or, indeed, for several hours afterwards.

If the decay curve before the time of the first determination is the same as it is afterwards, extrapolation to zero time should provide an estimate of ascorbic acid at that time. It is evident from Fig. 2 that the plasma concentrations of ascorbic acid at zero time obtained by such extrapolation are very little different from the concentrations 30 min. later and are still lower than those of total ascorbic acid by the method of Roe & Keuther (1943). The validity of the assumption that the rate of loss of ascorbic acid between the withdrawal time (t=0) and the earliest determination (t=30 min.) follows the same simple decay curve as it does subsequently is established by the recovery of ascorbic acid and dehydroascorbic acid added to blood plasma or plasma filtrate. Table 1 shows that such recoveries are complete. It is concluded from these experiments that the differences between the plasma concentrations of total ascorbic acid as determined by the method of Roe & Keuther (1943) and those determined by the indophenol method are not due to the oxidation of ascorbic acid following the withdrawal of blood and during the indophenol procedure.

In regard to the second possibility, Roe & Keuther (1943) have satisfactorily excluded many potential interfering substances, either because the colour with 2,4-dinitrophenylhydrazine does not develop under the strongly acid conditions of the estimation, or because they (e.g. glucose) are not present in plasma in sufficient concentrations to interfere significantly with the results.

The nature of the substance or substances responsible for the difference obtained by the two methods was investigated by means of hydrogen sulphide.

Pure H,S, prepared from Sb2S5, was passed for 10 min. through samples of filtrate prepared with metaphosphoric acid from plasma in which determinations by the two methods had already been carried out (i.e. subjects shown in Tables 2 and 3). Excess of H,S was then removed by blowing

---

### Table 2. Concentration of vitamin C in the plasma of normal fasting subjects taking an ordinary diet

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Sex</th>
<th>Age (yr.)</th>
<th>Indophenol method</th>
<th>Roe &amp; Keuther method</th>
<th>Ratio: Indophenol method</th>
<th>Ratio: Roe &amp; Keuther method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ascorbic acid (mg./100 ml.)</td>
<td>'Total ascorbic acid' (mg./100 ml.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>M.</td>
<td>24</td>
<td>0.30</td>
<td>0.70</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>M.</td>
<td>24</td>
<td>0.30</td>
<td>0.60</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M.</td>
<td>30</td>
<td>0.78</td>
<td>0.85</td>
<td>0.84</td>
<td>0.84</td>
</tr>
<tr>
<td>4</td>
<td>F.</td>
<td>21</td>
<td>0.30</td>
<td>0.55</td>
<td>0.55</td>
<td>1.04</td>
</tr>
<tr>
<td>5</td>
<td>M.</td>
<td>23</td>
<td>0.14</td>
<td>0.40</td>
<td>0.35</td>
<td>1.08</td>
</tr>
<tr>
<td>6</td>
<td>F.</td>
<td>28</td>
<td>0.35</td>
<td>0.50</td>
<td>0.70</td>
<td>1.04</td>
</tr>
<tr>
<td>7</td>
<td>F.</td>
<td>35</td>
<td>1.0</td>
<td>1.30</td>
<td>0.77</td>
<td>1.00</td>
</tr>
<tr>
<td>8</td>
<td>F.</td>
<td>21</td>
<td>0.32</td>
<td>0.30</td>
<td>0.64</td>
<td>1.00</td>
</tr>
<tr>
<td>9</td>
<td>F.</td>
<td>21</td>
<td>1.0</td>
<td>1.0</td>
<td>0.75</td>
<td>1.00</td>
</tr>
<tr>
<td>10</td>
<td>F.</td>
<td>23</td>
<td>0.50</td>
<td>0.70</td>
<td>0.71</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.68</td>
<td>1.01</td>
</tr>
<tr>
<td>s.d.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>±0.15</td>
<td>±0.03</td>
</tr>
</tbody>
</table>

---

### Table 3. Concentration of vitamin C in the plasma of normal fasting subjects taking a diet rich in vitamin C

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Sex</th>
<th>Age (yr.)</th>
<th>Indophenol method</th>
<th>Roe &amp; Keuther method</th>
<th>Ratio: Indophenol method</th>
<th>Ratio: Roe &amp; Keuther method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ascorbic acid (mg./100 ml.)</td>
<td>'Total ascorbic acid' (mg./100 ml.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>F.</td>
<td>28</td>
<td>0.84</td>
<td>1.30</td>
<td>0.85</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>M.</td>
<td>33</td>
<td>0.44</td>
<td>0.60</td>
<td>0.84</td>
<td>1.00</td>
</tr>
<tr>
<td>3</td>
<td>M.</td>
<td>30</td>
<td>1.0</td>
<td>1.10</td>
<td>0.84</td>
<td>1.00</td>
</tr>
<tr>
<td>4</td>
<td>F.</td>
<td>25</td>
<td>0.94</td>
<td>1.10</td>
<td>0.85</td>
<td>1.00</td>
</tr>
<tr>
<td>5</td>
<td>F.</td>
<td>20</td>
<td>1.55</td>
<td>1.40</td>
<td>0.81</td>
<td>1.05</td>
</tr>
<tr>
<td>6</td>
<td>F.</td>
<td>21</td>
<td>0.94</td>
<td>1.10</td>
<td>0.85</td>
<td>1.00</td>
</tr>
<tr>
<td>7</td>
<td>M.</td>
<td>24</td>
<td>1.57</td>
<td>2.22</td>
<td>0.88</td>
<td>0.99</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.82</td>
<td>1.01</td>
</tr>
<tr>
<td>s.d.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>±0.06</td>
<td>±0.03</td>
</tr>
</tbody>
</table>
pure N₂ saturated with water vapour through the solution for 2-3 hr., by which time the nitroprusside and lead acetate reactions were negative.

The indophenol method was again applied (the stability of the colour showing absence of interference by hydrogen sulphide), and the ratios of these determinations to the original determinations by the method of Roe & Keuther (1943) are given in column 7 of Tables 2 and 3. In every case, the indophenol-reducing substance has increased to the value given by the Roe & Keuther (1943) method for total ascorbic acid, so that the mean ratio rises from 0.68 or 0.82 to 1.0 ± 0.03 irrespective of the original value. Control experiments showed that when a trichloroacetic acid filtrate of plasma, as used for the Roe & Keuther (1943) method, was treated with hydrogen sulphide and nitrogen prior to oxidation with charcoal, the result did not differ from that obtained by the usual procedure.

![Figure 2](image)

**Fig. 2.** The effect of time on the apparent ascorbic acid content of plasma. ○—○, plasma separated immediately after blood withdrawal, samples used for ascorbic acid determination by the indophenol method. (Plasma separated and immediately treated with metaphosphoric acid gave results indistinguishable from these.) △—△, plasma allowed to stand in contact with red cells until required (indophenol method). x—x, plasma separated immediately after blood withdrawal; 'total ascorbic acid' determined by Roe & Keuther method.

Thus the substance in plasma filtrate, which is responsible for the difference between the ascorbic acid content measured by the indophenol method and the 'total ascorbic acid' measured by the 2,4-dinitrophenylhydrazine method of Roe & Keuther (1943), (a) reacts with dinitrophenylhydrazine but not with the indophenol, (b) is reducible by hydrogen sulphide to a substance which reacts with the indophenol as rapidly as does ascorbic acid, and (c) when calculated as ascorbic acid, accounts precisely for the observed difference. Taking into account the specificities of the two reactions used in the analyses, it is difficult to imagine such behaviour being due to anything except dehydroascorbic acid. Diketogulonic acid also reacts with dinitrophenylhydrazine under the conditions of Roe & Keuther (1943), but may be ruled out since it is not reduced by hydrogen sulphide in acid solution. It may be concluded, with a high degree of probability, that the observed differences are due to the presence of dehydroascorbic acid in the filtrates prepared from plasma by addition of metaphosphoric acid. The extension of this conclusion to native plasma rests partly on the time relationships discussed earlier and partly on the well established fact that in weakly acid solution at room temperature equilibrium between dehydroascorbic acid and diketogulonic acid (with the latter forming about 80% of the equilibrium mixture) is reached only in about 70 hr. Hence if diketogulonic acid were originally present in the plasma it should still be detectable in the acidified plasma filtrate 30 min. later (Herbert, Hirst, Percival, Reynolds & Smith, 1933). The distinction may be of some importance, since at the slightly alkaline reaction of plasma some conversion of dehydroascorbic acid to diketogulonic acid might be expected. The results of the experiments just described suggest, however, that plasma itself contains dehydroascorbic acid but no appreciable amount of diketogulonic acid.

**Changes in the plasma concentration of ascorbic acid and dehydroascorbic acid following intramuscular administration of adrenocorticotropic hormone (ACTH)**

The known relationship of vitamin C to the adrenal cortex suggested that an investigation of the ratio of dehydroascorbic acid to total ascorbic acid in plasma following administration of ACTH and compound E would be of interest. Further, a consistent alteration in the ratio brought about by these means would strengthen the conclusion that dehydroascorbic acid exists in plasma.

ACTH ('Aether', Armour Laboratories, Armour and Co., Chicago) was given by intramuscular injection in doses of 100 mg. to four normal fasting and resting subjects. Prior to the injection one or two blood samples were withdrawn. Following the injection, samples of blood were withdrawn at hourly intervals for 4-7 hr., and during this period the subjects were allowed to drink weak tea or coffee which was shown to have no effect upon the concentration of ascorbic acid or dehydroascorbic acid. The same subjects were used as controls about 1 week later, at which time samples were taken at hourly intervals for 4-6 hr. for analysis without hormone administration but otherwise under the same conditions. The activity of the ACTH used was confirmed in all cases by eosinopenic responses of at least 50% within 2-3 hr. after the injection. In addition, H₂S was passed through a sample of the metaphosphoric acid filtrate for 10 min., after which excess H₂S was removed by N₂ and a further estimation of the indophenol-reducing power was made.
Table 4. Changes in the concentration of plasma ascorbic acid and dehydroascorbic acid following 100 mg. ACTH given by intramuscular injection

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Plasma ascorbic acid (Δ mg./100 ml.)</th>
<th>Plasma dehydroascorbic acid (Δ mg./100 ml.)</th>
<th>Plasma 'total ascorbic acid' (Δ mg./100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Δ %)</td>
<td>(Δ %)</td>
<td>(Δ %)</td>
</tr>
<tr>
<td>1 B.S.</td>
<td>+0.42</td>
<td>-0.17</td>
<td>+0.25</td>
</tr>
<tr>
<td>2 J.R.</td>
<td>+0.25</td>
<td>-0.13</td>
<td>+0.15</td>
</tr>
<tr>
<td>3 M.K.</td>
<td>+0.25</td>
<td>-0.18</td>
<td>+0.65</td>
</tr>
<tr>
<td>4 E.B.</td>
<td>+0.23</td>
<td>-0.13</td>
<td>+0.25</td>
</tr>
<tr>
<td>Mean</td>
<td>—</td>
<td>+0.42</td>
<td>+14</td>
</tr>
</tbody>
</table>

* No fasting dehydroascorbic acid present.

Table 5. The effect of treatment with hydrogen sulphide on the ratio of ascorbic acid (indophenol method) to 'total ascorbic acid' (Roe & Keuther method) after administration of ACTH

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Roe &amp; Keuther method</td>
<td>Roe &amp; Keuther method</td>
<td>Roe &amp; Keuther method</td>
<td>Roe &amp; Keuther method</td>
</tr>
<tr>
<td>Fasting</td>
<td>0.76</td>
<td>0.98</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>ACTH 1 hr. previously</td>
<td>0.86</td>
<td>0.98</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>ACTH 2 hr. previously</td>
<td>0.86</td>
<td>1.01</td>
<td>1.01</td>
<td>1.01</td>
</tr>
<tr>
<td>ACTH 3 hr. previously</td>
<td>0.87</td>
<td>0.95</td>
<td>0.95</td>
<td>0.95</td>
</tr>
<tr>
<td>ACTH 4 hr. previously</td>
<td>0.92</td>
<td>0.85</td>
<td>0.92</td>
<td>0.87</td>
</tr>
<tr>
<td>ACTH 5 hr. previously</td>
<td>0.98</td>
<td>0.99</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mean</td>
<td>—</td>
<td>0.98</td>
<td>—</td>
<td>0.99</td>
</tr>
</tbody>
</table>

The results are summarized in Table 4 and one case is shown graphically in Fig. 3. Following the administration of ACTH, a rise in total ascorbic acid occurred in all four subjects as determined by the method of Roe & Keuther (1943). The rise started within 1 hr. of the injection and reached a maximum in 2–4 hr., corresponding approximately in time to the maximum eosinopenia. The mean percentage change in total ascorbic acid concentration (ascorbic acid + dehydroascorbic acid) was +14, absolute elevations varying from 0.05 to 0.25 mg./100 ml. plasma. Ascorbic acid itself, as determined by indophenol reduction, also showed increases of from 13 to 76% of the fasting level, and these increases were maximal at the time corresponding to the peak of total ascorbic acid concentration. The absolute increments in ascorbic acid concentration varied from 0.25 to 0.41 mg./100 ml. plasma. The larger increments in ascorbic acid as compared with total ascorbic acid (ascorbic acid + dehydroascorbic acid) resulted in a diminution in the absolute concentration of dehydroascorbic acid amounting to 0.13–0.18 mg./100 ml. in three subjects (50–100%). In the fourth subject no fasting dehydroascorbic acid existed prior to the injection and none appeared following it. The control experiments in which no ACTH was given showed only very slight irregular alterations in the plasma concentration of total and reduced ascorbic acid. The results obtained following the passage of hydrogen sulphide through the metaphosphoric acid plasma filtrate are shown in Table 5, which gives the ratios of ascorbic acid to total vitamin C before and after injection of ACTH for subjects B.S. and M.K. The disappearance of dehydroascorbic acid in these subjects is shown by the rise in the ratio from 0.76 to 0.98 and from 0.65 to 1.0 respectively; the reappearance of dehydroascorbic acid in M.K. is evidenced by the gradual fall of the ratio to 0.85, 4 hr. after the injection. Hydrogen sulphide had the effect of making the ratio very nearly 1.0 whatever its value in the original plasma. It is important to note that when the original ratio is 1.0, hydrogen sulphide does not increase it.
Changes in the plasma concentration of ascorbic acid and dehydroascorbic acid following oral administration of cortisone acetate

The changes in plasma ascorbic acid and dehydroascorbic acid following administration of ACTH are not easily interpreted. ACTH is known to diminish the concentration of ascorbic acid in the adrenal gland (e.g. Sayers, Sayers, Liang & Long, 1946). This might explain the increase in the plasma total ascorbic acid, but does not by itself account for the decrease in dehydroascorbic acid. In addition, however, the increased secretion of adrenocortical hormones, acting peripherally, might produce effects upon vitamin C metabolism capable of accounting for the latter phenomenon. In order to dissociate such central from peripheral effects, the changes in blood concentration following the oral administration of 350 mg. of cortisone acetate were investigated in five normal subjects, one of whom (J.R.) had already been examined following ACTH administration.

As before, the subjects were fasting and at rest, though weak tea or coffee was allowed. One or two samples of blood were withdrawn prior to giving the cortisone, after which samples were taken into oiled syringes at 30 min., 1 hr., and then at hourly intervals for 4-6 hr. The chemical determinations were made as before, and eosinophils were counted.

The results are shown graphically for two subjects in Fig. 4 and in Table 6 they are summarized for the five subjects in terms of the maximum changes occurring in the plasma concentration of ascorbic and dehydroascorbic acids. These occurred between 0-5 and 2 hr. after administration of cortisone and corresponded in time to the maximal eosinopenia (i.e., 50%). The mean increase in plasma ascorbic acid was 38%, corresponding to an absolute rise in concentration ranging from 0-1 to 0-34 mg./100 ml. of plasma. There was no rise in the concentration of total vitamin C in any of the subjects and the maximum rise in concentration of ascorbic acid exactly equalled the original concentration of dehydroascorbic acid, so that all the dehydroascorbic acid in the plasma temporarily disappeared. The ratio of ascorbic acid to total vitamin C for subjects J.R. and M.McI. at various times following administration of the cortisone is given in Table 7.

Table 6. Changes in concentration of plasma ascorbic acid and dehydroascorbic acid following 350 mg. cortisone given orally

<table>
<thead>
<tr>
<th>Subject no.</th>
<th>Plasma ascorbic acid</th>
<th>Plasma dehydroascorbic acid</th>
<th>Plasma 'total ascorbic acid'</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Δmg./100 ml.)</td>
<td>(Δ%)</td>
<td>(Δmg./100 ml.)</td>
</tr>
<tr>
<td>1 H.L.</td>
<td>+0.54</td>
<td>+44.0</td>
<td>—</td>
</tr>
<tr>
<td>2 J.R.</td>
<td>+0.20</td>
<td>+26.0</td>
<td>—</td>
</tr>
<tr>
<td>3 M.McI.</td>
<td>+0.10</td>
<td>+22.0</td>
<td>—</td>
</tr>
<tr>
<td>4 H.A.</td>
<td>+0.17</td>
<td>+33.0</td>
<td>—</td>
</tr>
<tr>
<td>5 R.C.</td>
<td>+0.10</td>
<td>+55.0</td>
<td>—</td>
</tr>
<tr>
<td>Mean</td>
<td>—</td>
<td>+38.0</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 7. The effect of treatment with hydrogen sulphide on the ratio of ascorbic acid (indophenol method) to 'total ascorbic acid' (Roe & Keuther method) after administration of 350 mg. cortisone given orally

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Roe &amp; Keuther method</td>
<td>Roe &amp; Keuther method</td>
<td>Roe &amp; Keuther method</td>
<td>Roe &amp; Keuther method</td>
</tr>
<tr>
<td>Fasting</td>
<td>0.74</td>
<td>1.00</td>
<td>0.82</td>
<td>1.02</td>
</tr>
<tr>
<td>Cortisone, 1 hr. previously</td>
<td>1.00</td>
<td>0.99</td>
<td>1.00</td>
<td>0.98</td>
</tr>
<tr>
<td>Cortisone, 2 hr. previously</td>
<td>0.83</td>
<td>1.0</td>
<td>0.95</td>
<td>1.04</td>
</tr>
<tr>
<td>Cortisone, 3 hr. previously</td>
<td>0.87</td>
<td>1.01</td>
<td>0.94</td>
<td>1.02</td>
</tr>
<tr>
<td>Cortisone, 4 hr. previously</td>
<td>0.74</td>
<td>0.98</td>
<td>0.92</td>
<td>1.02</td>
</tr>
<tr>
<td>Mean</td>
<td>—</td>
<td>1.0</td>
<td>—</td>
<td>1.02</td>
</tr>
</tbody>
</table>

Fig. 4. The effect on the ascorbic acid (black columns) and dehydroascorbic acid (white columns) of human plasma of oral administration of 350 mg. of cortisone acetate given immediately after withdrawal of the control blood sample. Two cases.
which also shows the effect of hydrogen sulphide upon this ratio. Without hydrogen sulphide treatment the ratio rises to 1-0 about 1 hr. after administration of cortisone (i.e. dehydroascorbic acid has disappeared) and subsequently falls as plasma dehydroascorbic acid reappears. Treatment with hydrogen sulphide gives a ratio approximately 1-0 (but never significantly greater), irrespective of the original value, so that the changes are legitimately interpreted as indicating disappearance of dehydroascorbic acid.

**DISCUSSION**

The possibility of the existence of significant concentrations of dehydroascorbic acid in blood or tissues has received little attention in the past. The bare statement that both dehydroascorbic acid and diketogulonic acid occur in plasma and urine was made by Chen & Schuck (1950). Nyden (1948) studied alterations in ascorbic and dehydroascorbic acids in rat tissues and plasma following their injection with *Trypanosoma hippicum*. She used the method of Roe & Oesterling (1944), devised originally for the estimation of dehydroascorbic acid in plants, to estimate tissue and plasma dehydroascorbic acid concentrations. In our hands this method proved unsuitable for the determination of plasma dehydroascorbic acid, since reduction of dehydroascorbic acid occurred in the presence of all samples of thiourea (1 %, w/v) available to us, and oxidation occurred when thiourea was omitted. The concentration of thiourea required to maintain the naturally occurring ratio between the reduced and oxidized forms of the vitamin was unpredictable. The almost complete absence in the literature of a simple comparative study of the method of Roe & Keuther (1943) and one estimating the vitamin in the reduced form (e.g. by indophenol or methylene blue) is surprising. Lowry, Lopez & Bossey (1945) compared the micro-indophenol method of Mindlin & Butler (1937–8) with their own micro-adaptation of the method of Roe & Keuther (1943), using 0-01 ml. of serum but without making any direct comparison with the micromethod of Roe & Keuther (1943). They found no significant difference between the results of the two techniques when applied to serum, but it is questionable whether their micromodification was sensitive enough to detect the small differences reported in this paper. In addition, it has been established by Friedman, Rumin & Kees (1938), Farmer & Abt (1938) and by Cushman & Butler (1938) that, as we also have found, potassium cyanide employed in the method of Mindlin & Butler (1937–8) invalidates the results; this compound was presumably used by Lowry et al. (1945). As long ago as 1935, Martini, Bonsignine & Pinotte claimed that dehydroascorbic acid existed in significant concentration in guinea pig tissues, but the use of trichloroacetic acid in tissue extraction may invalidate these results, at least partly, since trichloroacetic acid itself appears to decolorize indophenol.

The source of the increment in total plasma vitamin C following intramuscular ACTH is unknown. Vogt (1948) was unable to detect significantly increased amounts of vitamin C in venous blood leaving the adrenal cortex of dogs when the gland was directly stimulated. It can be said, however, that the amount of ascorbic acid reputed to disappear from the adrenal cortex after ACTH administration is sufficient, if added to the extracellular body fluids, to increase the ascorbic acid concentration by the amounts observed.

The temporary diminution or disappearance of plasma dehydroascorbic acid is an effect common both to intramuscular ACTH and oral cortisone. The fact that total plasma vitamin C concentration does not alter significantly following cortisone administration indicates that one of the essential responses to this adrenocortical hormone is an activation of the mechanism for the reduction of dehydroascorbic acid.

Simple *in vitro* experiments in which cortisone and ACTH are incubated with dehydroascorbic acid either in solution or in plasma were carried out in the course of this work but have not been reported in detail in the experimental part. They showed clearly that the observed apparent reduction of dehydroascorbic acid is not a direct chemical effect of the circulating hormone. Borsook et al. (1937) have shown that normal human plasma does not possess the power of reducing dehydroascorbic acid. We have confirmed this and shown further that the power of such reduction is not acquired by plasma following ACTH or cortisone administration. The activation is, therefore, likely to occur within the cells either of the blood or of tissues and it is possible that the mechanism involves glutathione. Experiments to test this possibility are now in progress.

**SUMMARY**

1. A comparison of the method of Roe & Keuther (1943) with a procedure using 2,6-dichlorophenol-indophenol for determination of "total ascorbic acid" and ascorbic acid respectively provides results which suggest that a significant part of the vitamin C in human plasma is present as dehydroascorbic acid.

2. This possibility is further substantiated by the fact that the differences obtained in the concentrations of plasma vitamin C by the two methods are completely removed by treating plasma filtrate with hydrogen sulphide prior to using the indophenol method, irrespective of the magnitude of the difference which existed before such treatment.
3. Taking into account the specificities of the two methods and the reactions they involve, it is difficult to explain such results except as being due to the presence of dehydroascorbic acid. These procedures do not suggest the presence of diketogulonic acid in human plasma.

4. The administration of adrenocorticotropin hormone to human subjects resulted in a rise in the concentration of plasma total ascorbic acid and of plasma ascorbic acid with a diminution in the concentration of plasma dehydroascorbic acid 2–4 hr. after intramuscular injection.

5. The oral administration of cortisone to human subjects resulted in no rise in the concentration of plasma total ascorbic acid, but in a rise in ascorbic acid of such a magnitude that dehydroascorbic acid temporarily disappeared from the plasma 0.5–2 hr. after administration.

6. The significance of these alterations is discussed both with respect to the origin of the increment of total ascorbic acid appearing in the plasma and to the mechanisms involved in the reduction of dehydroascorbic acid to ascorbic acid.

We wish to thank the Medical Research Council for a grant which helped to defray the cost of materials and technical assistance as well as for a supply of hormones.

*Note added in proof.* In a few experiments it has now been found that, following the administration of cortisone or ACTH and coincident with the resulting diminution of the plasma dehydroascorbic acid concentration, there is a decrease in the reduced glutathione concentration of the whole blood. When, later, the original dehydroascorbic acid concentration is restored, the reduced glutathione of the blood also increases. The sum of the reduced and oxidized glutathione remains constant throughout.

REFERENCES


Dehydroascorbic Acid in Human Blood Plasma

By C. P. STEWART, D. B. HORN and J. S. ROBSON, Clinical Laboratory, Royal Infirmary, Edinburgh

The existence of dehydroascorbic acid in human plasma

A year ago we (Stewart, Horn & Robson, 1952, 1953) showed that human blood plasma contains quite considerable amounts of dehydroascorbic acid. Previously it had been generally believed that this substance was not present in blood plasma or was present only in negligibly small concentration. The evidence on which we founded our statement was as follows.

In the first place, a colorimetric method of determining ascorbic acid by its power of reducing indophenol gave results consistently lower than those obtained, in the same plasma, by the method of Roe & Kuether (1943) which measures dehydroascorbic acid and diketogulonic acid in addition to ascorbic acid. Control experiments with the two methods suggested that the difference was real, and observations on the stability of ascorbic acid in plasma negated the idea that oxidation of ascorbic acid might be occurring in the plasma (or whole blood) after its withdrawal or during the actual determination with indophenol. Further, treatment of plasma or plasma filtrates with pure hydrogen sulphide produced an increase in the ascorbic acid measured by indophenol exactly equal to the dehydroascorbic acid calculated as being present originally. Not only did these experiments very strongly suggest the presence of dehydroascorbic acid in human plasma—in amounts varying from below 0.1 to 0.36 mg/100 ml.—but they suggested, equally strongly, the virtual absence of diketogulonic acid, since this substance is not reduced by hydrogen sulphide but reacts with dinitrophenylhydrazine under the conditions used by Roe & Kuether.

Confirmatory evidence of a similar character was obtained during experiments in which the apparent ratio of ascorbic acid to dehydroascorbic acid was altered, but since our original publication we have sought confirmation by using a different reducing agent in place of the non-specific hydrogen sulphide. Dr Mapson kindly gave us a culture of the Bacterium coli strain which he and Ingram (Mapson & Ingram, 1951) had shown to be capable of reducing dehydroascorbic acid quantitatively to ascorbic acid. Suspensions of this organism, shown to be fully active by control experiments with aqueous solutions freshly prepared to contain dehydroascorbic acid, were incubated with plasma under the conditions of pH and anaerobiosis described by Mapson & Ingram. In each experiment the preformed ascorbic
acid was measured by the indophenol method, the 'total' was measured by the Roe & Kuether method, and the indophenol-reducing power was measured after treatment with hydrogen sulphide and also after incubation with the bacteria. The behaviour of the final solution after addition of indophenol showed—the colour density remaining stable over the period of observation—that it was ascorbic acid that was being measured. The results are shown in Table 1. Evidently the substance in the original plasma which reacts with dinitrophenylhydrazine but not with indophenol is reduced to an indophenol-reducing substance (resembling ascorbic acid in its rapidity of reaction with indophenol) both by hydrogen sulphide and by Bact. coli. Calculated as ascorbic acid, the increase in indophenol-reducing substance produced by both agents accounts, within experimental error, for the difference between the figures given for the original plasma by the indophenol and the Roe & Kuether methods.

Incidentally, it may be remarked, dehydroascorbic acid has been demonstrated by the same methods, in the plasma of the rabbit and of the rat.

Table 1. Effect of Bact. coli on the ascorbic-acid content of plasma (mg/100 ml.)

<table>
<thead>
<tr>
<th>Plasma</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid (by indophenol-titration)</td>
<td>0.25</td>
<td>0.33</td>
<td>0.61</td>
<td>0.15</td>
<td>1.23</td>
</tr>
<tr>
<td>Ascorbic acid after H&lt;sub&gt;2&lt;/sub&gt;S treatment</td>
<td>0.40</td>
<td>0.42</td>
<td>0.68</td>
<td>0.28</td>
<td>1.35</td>
</tr>
<tr>
<td>Ascorbic acid after treatment with Bact. coli</td>
<td>0.42</td>
<td>0.49</td>
<td>0.69</td>
<td>0.29</td>
<td>1.37</td>
</tr>
<tr>
<td>Total ascorbic acid (method of Roe &amp; Kuether, 1943)</td>
<td>0.44</td>
<td>0.50</td>
<td></td>
<td>0.28</td>
<td></td>
</tr>
</tbody>
</table>

Effect of various agents on the dehydroascorbic acid content of plasma

Cortisone

Because of the known relationship between vitamin C and the adrenal cortex, the effect of cortisone and of ACTH on the plasma ascorbic acid was studied. Cortisone, given orally as a suspension of the acetate, was tested in normal subjects, fasting and at rest. The dose used was 350 mg, the plasma ascorbic acid and dehydroascorbic acid being determined before and at intervals afterwards. (Control observations over a similar period of time without cortisone administration showed virtual constancy of both). Whilst the 'total' ascorbic acid measured by the Roe & Kuether method was unaltered, the ascorbic acid itself (indophenol method) rose so that, temporarily, the dehydroascorbic acid almost or quite disappeared from the plasma, this disappearance coinciding in time with the maximum eosinopenia. Moreover, in all these samples treatment of plasma with hydrogen sulphide (and removal of the excess by nitrogen) produced an ascorbic-acid value equal to the 'total'; when none of the substance believed to be dehydroascorbic acid was present, hydrogen sulphide did not increase the indophenol-reducing power (Table 2).

Control experiments showed that the cortisone acetate was itself unable to reduce dehydroascorbic acid in vitro, and that plasma, whether 'normal' or collected

* These experiments were made by Miss Christina Thompson.
Table 2. Effect of cortisone on the plasma ascorbic-acid concentration (mg/100 ml.).

300 mg cortisone acetate given orally at time 0

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ascorbic acid</th>
<th>Ascorbic acid after H&lt;sub&gt;2&lt;/sub&gt;S treatment</th>
<th>'Total' ascorbic acid (method of Roe &amp; Kuether, 1943)</th>
<th>Dehydroascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.56</td>
<td>0.75</td>
<td>0.75</td>
<td>0.19</td>
</tr>
<tr>
<td>1</td>
<td>0.76</td>
<td>0.76</td>
<td>0.75</td>
<td>0.13</td>
</tr>
<tr>
<td>2</td>
<td>0.62</td>
<td>0.75</td>
<td>0.75</td>
<td>0.21</td>
</tr>
<tr>
<td>3</td>
<td>0.65</td>
<td>0.75</td>
<td>0.76</td>
<td>0.19</td>
</tr>
<tr>
<td>5</td>
<td>0.56</td>
<td>0.71</td>
<td>0.75</td>
<td>0.13</td>
</tr>
</tbody>
</table>

1 h after cortisone administration, was also without reducing power for added dehydroascorbic acid.

It is concluded that, as a result of some cellular activity, cortisone causes the rapid but temporary disappearance of dehydroascorbic acid from the plasma. Indirect evidence in support of this conclusion has recently been published by Loxton & LeVay (1953) who found a fall in the redox potential of the peripheral tissues 1–2 h after the oral administration of cortisone.

Adrenocorticotropic hormone

ACTH ('Acthar', Armour & Co. Ltd., The Armour Laboratories, London) was given intramuscularly, the dose being 100 mg under conditions and with measurements similar to those for the cortisone experiments. The results with respect to disappearance of dehydroascorbic acid were rather like those found with cortisone (except that a little dehydroascorbic acid usually remained). There was, however, an important difference—the 'total' ascorbic acid rose significantly after ACTH whereas it did not after cortisone. Whether this represents, besides the replacement of dehydroascorbic acid by ascorbic acid, an actual withdrawal of ascorbic acid from the adrenal gland as a result of hormonal stimulation, cannot be determined by these experiments; the quantities involved, however, are such as to make such an explanation possible.

As with cortisone, the changes after ACTH were shown, by control experiments, to involve cellular intervention. Also, as before, hydrogen sulphide always reduced exactly any dehydroascorbic acid present (Table 3). These changes following administration of cortisone and ACTH, with the effects of hydrogen sulphide, constitute, in fact, an important contribution to the sum of the evidence on which rests the identification of dehydroascorbic acid in plasma.

Table 3. Effect of ACTH on the plasma ascorbic-acid concentration (mg/100 ml.).

100 mg ACTH given intramuscularly at time 0

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Ascorbic acid</th>
<th>Ascorbic acid after H&lt;sub&gt;2&lt;/sub&gt;S treatment</th>
<th>'Total' ascorbic acid (method of Roe &amp; Kuether, 1943)</th>
<th>Dehydroascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.00</td>
<td>1.30</td>
<td>1.30</td>
<td>0.20</td>
</tr>
<tr>
<td>1</td>
<td>1.21</td>
<td>1.36</td>
<td>1.35</td>
<td>0.14</td>
</tr>
<tr>
<td>2</td>
<td>1.15</td>
<td>1.36</td>
<td>1.35</td>
<td>0.20</td>
</tr>
<tr>
<td>3</td>
<td>1.30</td>
<td>1.45</td>
<td>1.50</td>
<td>0.20</td>
</tr>
<tr>
<td>4</td>
<td>1.42</td>
<td>1.50</td>
<td>1.55</td>
<td>0.13</td>
</tr>
<tr>
<td>7</td>
<td>1.22</td>
<td>–</td>
<td>1.50</td>
<td>0.28</td>
</tr>
<tr>
<td>9</td>
<td>1.20</td>
<td>–</td>
<td>1.45</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Sodium salicylate

Large oral doses of sodium salicylate (7 g) produce a rapid effect on the plasma ascorbic acid similar to that of ACTH—an increase in the ‘total’ ascorbic acid combined with a reduction and virtual disappearance of the dehydroascorbic acid. This was, in our experiments, associated with some degree of eosinopenia, but it was noteworthy that dehydroascorbic acid reappeared in the plasma whilst the salicylate concentration was still high. The results of one such experiment are shown in Table 4. It is tempting to think that the changes in plasma ascorbic acid indicate a stimulation of adrenal activity—either specifically by the drug or by

Table 4. Effect of 7 g sodium salicylate, given orally as a single dose, on the ascorbic-acid concentration of the plasma

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Salicylate</th>
<th>Ascorbic acid</th>
<th>'Total' ascorbic acid</th>
<th>Dehydroascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0.85</td>
<td>1.10</td>
<td>0.20</td>
</tr>
<tr>
<td>0.5</td>
<td>17</td>
<td>0.80</td>
<td>1.09</td>
<td>0.16</td>
</tr>
<tr>
<td>1</td>
<td>28</td>
<td>0.85</td>
<td>1.05</td>
<td>0.19</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>1.15</td>
<td>1.12</td>
<td>0.01</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
<td>-</td>
<td>1.25</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>31.5</td>
<td>0.26</td>
<td>1.30</td>
<td>0.20</td>
</tr>
<tr>
<td>5</td>
<td>29</td>
<td>0.85</td>
<td>1.30</td>
<td>0.45</td>
</tr>
<tr>
<td>6</td>
<td>27.5</td>
<td>0.88</td>
<td>1.30</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Table 5. Effect of daily administration of 7 g sodium salicylate in divided doses on the ascorbic-acid concentration of the plasma

<table>
<thead>
<tr>
<th>Day</th>
<th>Salicylate</th>
<th>Ascorbic acid</th>
<th>'Total' ascorbic acid</th>
<th>Dehydroascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>0.46</td>
<td>0.85</td>
<td>0.39</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.40</td>
<td>0.60</td>
<td>0.20</td>
</tr>
<tr>
<td>3*</td>
<td></td>
<td>0.40</td>
<td>0.85</td>
<td>0.45</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>0.50</td>
<td>0.50</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0.50</td>
<td>0.55</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.34</td>
<td>0.50</td>
<td>0</td>
</tr>
<tr>
<td>7*</td>
<td></td>
<td>0.50</td>
<td>0.50</td>
<td>0</td>
</tr>
<tr>
<td>8*</td>
<td>18</td>
<td>0.40</td>
<td>0.40</td>
<td>0</td>
</tr>
<tr>
<td>9*</td>
<td></td>
<td>0.28</td>
<td>0.30</td>
<td>0.02</td>
</tr>
<tr>
<td>10*</td>
<td></td>
<td>0.29</td>
<td>0.29</td>
<td>0.01</td>
</tr>
<tr>
<td>11*</td>
<td></td>
<td>0.29</td>
<td>0.29</td>
<td>0.01</td>
</tr>
<tr>
<td>12*</td>
<td></td>
<td>0.26</td>
<td>0.26</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* The salicylate was given on the days marked by an asterisk.
dose of sodium salicylate was given prematurely and produced the temporary disappearance of dehydroascorbic acid already mentioned. When, 4 days later, regular administration of sodium salicylate (7 g/day in five doses) began, the dehydroascorbic acid vanished; in this instance, though not always, the total ascorbic-acid concentration of the plasma slowly fell.

**Relationship between the concentrations of plasma dehydroascorbic acid and blood glutathione**

We have not yet accumulated sufficient data to determine whether or not there is a correlation between the concentration of dehydroascorbic acid in the plasma and that of glutathione or oxidized glutathione in the blood cells. However, in one or two experiments with cortisone and ACTH it has been found that, coincident with the disappearance of dehydroascorbic acid from the plasma, there was a disappearance of glutathione (by conversion to the disulphide form) from the whole blood. The close parallelism illustrated in Table 6, however, was not a regular occurrence, and sometimes the oxidized glutathione increased throughout the time of observation and particularly at a time when dehydroascorbic acid was returning to the plasma. These observations must be regarded as preliminary, to be extended with use of a method more specific than that of Woodward & Fry (1932) hitherto employed. Nevertheless they do indicate some relationship between dehydroascorbic acid and cellular glutathione—as is to be expected—only partially detected, however, when only the blood cells are examined. Mr Bhattacharya, working in this laboratory, has detected a similar tendency to increase in the oxidized glutathione concentration of the blood in rats given injections of dehydroascorbic acid with the object of producing a diabetic state, the effect being manifest some

---

**Table 6. Effect of ACTH or cortisone on plasma ascorbic acid and blood glutathione (mg/100 ml.)**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Plasma</th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ascorbic acid</td>
<td>Dehydroascorbic acid</td>
</tr>
<tr>
<td>0</td>
<td>1.12</td>
<td>0.08</td>
</tr>
<tr>
<td>0.5</td>
<td>1.12</td>
<td>0.08</td>
</tr>
<tr>
<td>1</td>
<td>1.19</td>
<td>0.01</td>
</tr>
<tr>
<td>2</td>
<td>1.30</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1.33</td>
<td>0.07</td>
</tr>
<tr>
<td>4</td>
<td>1.33</td>
<td>0.07</td>
</tr>
<tr>
<td>5</td>
<td>1.33</td>
<td>0.03</td>
</tr>
<tr>
<td>6</td>
<td>1.05</td>
<td>0.15</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>(b) 350 mg cortisone acetate given orally at time 0</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.45</td>
</tr>
<tr>
<td>0.5</td>
<td>0.45</td>
</tr>
<tr>
<td>1</td>
<td>0.52</td>
</tr>
<tr>
<td>1.5</td>
<td>0.60</td>
</tr>
<tr>
<td>2.5</td>
<td>0.44</td>
</tr>
<tr>
<td>3.5</td>
<td>0.43</td>
</tr>
<tr>
<td>7</td>
<td>0.42</td>
</tr>
</tbody>
</table>

of observation and particularly at a time when dehydroascorbic acid was returning to the plasma.
days after the last injection. This tendency also must still be treated with some reserve since it may be merely residual from a direct oxidation of glutathione by the enormous amounts of dehydroascorbic acid previously in the circulation.

Discussion

It seems that dehydroascorbic acid exists in the plasma in equilibrium with ascorbic acid, that the relative amounts of the two substances can be altered by certain agents, ACTH, cortisone and salicylate. These three all reduce the dehydroascorbic acid. The effect appears to be cellular and to be associated with changes in the soluble sulphydryl compounds.

One of the fundamentally important effects of ACTH has been considered to be the reduction it causes in the amount of ascorbic acid in the adrenal glands; the results reported here suggest that this is only a part of the effect, another part of which is an alteration of the relative amounts of ascorbic acid and dehydroascorbic acid in the periphery under the influence of adrenocortical hormones.

Whatever the ultimate explanation may prove to be, the demonstration of dehydroascorbic acid in the plasma indicates a metabolic importance of the vitamin which was by no means self-evident when it was believed to exist solely in the reduced form.

REFERENCES

Use of $p$-Chloromercuribenzoic Acid in the Determination of Ascorbic Acid in the Presence of Sulphydryl Compounds

In certain circumstances hydrogen sulfide, cysteine and related sulphydryl compounds react with $2:6$-dichlorophenol-indophenol (indophenol) and thus may interfere with the determination of ascorbic acid using this reagent. In view of the known ability of $p$-chloromercuribenzoic acid to combine with sulphydryl compounds, the possibility of using this reagent to suppress such interference has been examined.

Results indicate that this reagent does not itself react with indophenol nor does it affect the determination of ascorbic acid in pure solution, using a colorimetric procedure based on the decolorization of excess indophenol. Under similar conditions the addition of $p$-chloromercuribenzoic acid has been found to suppress completely the decolorization of indophenol by hydrogen sulfide, cysteine and glutathione respectively.

The reagent has been used in the following manner:

1 ml. of $p$-chloromercuribenzoic acid solution (200 mgm./100 ml. in 0.05 N sodium hydroxide) was added to 3 ml. of standard ascorbic acid solution (0.0-2.0 mgm./100 ml.), or test solution, in 1.8 per cent (w/v) metaphosphoric acid solution. Because of the low solubility of $p$-chloromercuribenzoic acid at this pH (about 1.5) most of the excess reagent was precipitated. The solution containing the precipitate was allowed to stand for 5 min. and then centrifuged. To 2 ml. of the supernatant fluid was added 0.5 ml. sodium citrate solution followed by 1 ml. indophenol solution (4 mgm./100 ml.) and the optical density of the resulting colour determined 30 sec. after the addition of the dye using a Unicam spectrophotometer S.P. 600 at a wave-length of 520 mp. The concentration of the sodium citrate used was such as to bring the final $p$H to 3.5. With solutions of ascorbic acid in 1.8 per cent (w/v) metaphosphoric acid a concentration of 7 per cent (w/v) was required.

It is suggested that $p$-chloromercuribenzoic acid may be of value in increasing the specificity of the indophenol reaction applied to the determination of ascorbic acid in biological material containing sulphydryl compounds. It offers advantages over mercuric acetate for the removal of cysteine and certain other sulphydryl compounds in that subsequent
treatment with hydrogen sulphide, to remove the excess mercuric ions and to convert the dehydro-ascorbic acid formed to ascorbic acid, is not required. It thus avoids a procedure which itself introduces several sources of error and does not permit the determination of ascorbic acid in the presence of dehydroascorbic acid.

The effect of p-chloromercuribenzoic acid on the determination of the 'ascorbic acid' content of human plasma is being studied and it is hoped to publish results shortly.

We are indebted to Dr. A. B. Roy for the gift of the p-chloromercuribenzoic acid and for suggestions which led to its use described here.

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METABOLIC RESPONSE TO ADRENALECTOMY

After surgical operation or other injury, retention of water and sodium, together with a loss of potassium and nitrogen, is usually demonstrable. Several groups of investigators have shown, in man, an increase in the urinary excretion of adrenocorticosteroids during the postoperative period; and an increase in the concentration in the blood has also been demonstrated. These observations have been taken to support the view that trauma produces an increase in the secretory activity of the adrenal cortex, which, in turn, is responsible for the alterations in excretion of water, electrolytes, and nitrogen. On the other hand, the findings of Ingle et al. on adrenalectomised animals maintained on a constant dose of cortin are inconsistent with this simple hypothesis.

An opportunity to test the hypothesis in a human subject was afforded us by an operation for the removal of the second adrenal gland of a patient suffering from malignant hypertension. Large amounts of cortisone acetate were given, a dose of 200 mg. per day being given for three days before the operation and continued for several days after the removal of the gland.

The findings in 24-hr. collections of urine are shown in the accompanying figure; no stools were passed during the first five days after operation. Although the intake of sodium chloride was kept virtually constant, there was a postoperative decrease in the amount excreted in the urine, with the result that there was considerable retention of sodium and chloride for several days.

As in operations not involving the adrenal gland, there was also a period of negative nitrogen balance. This

was not due to starvation, for it reached its maximum three or four days after operation, when normal feeding had been resumed. Similarly, the urinary excretion of potassium was maintained after operation, leading to a marked negative balance when the intake was reduced to nil on the first two postoperative days, and actually increasing when the intake reached preoperative levels from the third to the fifth postoperative day. The urinary excretion of acid-stable formaldehydoneogenic steroids, which usually increases after operation, was constant throughout the period of observations.

In fine, the postoperative metabolic changes involving sodium, potassium, and nitrogen were similar to those following operations on patients with intact functioning suprarenal glands. It is quite obvious that in this case the postoperative metabolic response was not due to increased secretion by the adrenal cortex. This conclusion is in agreement with the statement lately made by Dr. Stuart Mason in a discussion at the Royal Society of Medicine.

Our thanks are due to Sir James Learmonth, who gave us the opportunity to study this case.

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