IODINE REDUCING SUBSTANCES IN THYMUS GLAND EXTRACTS

by

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Table of Contents

1. Introduction
   A. Embryology of the thymus
   B. Anatomical relations of the thymus
   C. Histology of the thymus
   D. Physiology of the thymus
      (a) Introduction
      (b) Involution
      (c) Clinical observations
      (d) Effect of thymectomy
      (e) Connections with calcification
      (f) Connections with the gonads
      (g) Connections with the adrenals
      (h) Other endocrine relationships
      (i) Relationship with lymphatic system
      (j) As a source of nucleic acid
      (k) Effect of feeding and injecting thymus substance
      (l) The work of Rowntree and his associates.
   E. Thymus preparations and extracts
   F. Effect of some sulphydryl compounds and other reducing substances on growth and development.
   G. Other reports of glutathione and ascorbic acid in the thymus.

2. Purpose of this research
   A. Plan of investigation
B. Review of methods of analysis
   (a) Glutathione
   (b) Cysteine
   (c) Ascorbic acid

3. Experimental work
   A. Preparation of extracts
   B. Determination of total I.R.T.
   C. Analysis for glutathione
   D. Analysis for cysteine
   E. Analysis for ascorbic acid
   F. Analysis of the extract
   G. Determination of nitrogen distribution

4. Discussion

5. Conclusion
IODINE REDUCING SUBSTANCES IN THYMUS GLAND EXTRACT.

Introduction

The thymus was given its name by Galen who named it from the Greek Ἐμὺς since in the sheep it seemed to resemble a bunch of thyme. It is still the enigmatic organ. It remains the last major mass of tissue whose function continues to defy elucidation though it gives hints of playing an important part in the metabolism of the organism. Though it is a lymphoid structure, it differs from other lymph glands both in its embryonic origin and in its histologic structure. In the modern enthusiasm for endocrinology, many have sought to include it among the glands of internal secretion. As shall be seen, however, most questions regarding it are only vaguely answered if at all and have given rise to considerable controversy and contention.
Embryology.

The thymus bodies arise in all vertebrates as one of the several sets of outgrowths from the pharyngial pouches. The number of pairs of thymic outgrowths varies widely both between and in the different groups. In fishes, amphibians, reptiles and birds thymic tissue may arise from the second, third, fourth and fifth pouches (40, 52, 136, 143). The mammalian thymus is derived from the third and fourth pouches. In most species, thymus IV is only a rudimentary and transitory structure, but in some, including the cat, it always develops (7, 53, 143, 293). In all vertebrates the greater portion of the gland arises from entodermal epithelium of the medial and ventral portions of the third pouch. Its origin is thus very similar to that of a number of other organs including the palatine tonsils from the second pouch, parathyroids from the dorsal walls of the third and fourth pouch and the ultimobranchial or lateral thyroid bodies from the fifth. The inferior pair of parathyroids (the parathymus glands) in particular are closely associated with the upper end of the developing thymus. Parathyroid tissue is frequently found embedded in its upper parts.

The mammalian thymus III anlagen first show at the ten somites stage. At first hollow, they soon become solid epithelial strands. The upper ends soon atrophy and undergo adsorption. Early in uterine life, the cells lose their epithelial character and send out processes which anastomose to form a reticulum. At about this stage, the small thymic cells (see below) begin to appear and the gland slowly takes on the lymphoid appearance of later life.
Anatomical Relations

Thymus bodies are found in all vertebrates from cyclostomes to birds and mammals.

In fishes it remains in a primitive condition in that it retains its connection with the epithelium from which it arises. In the angler fish, it forms a flat ovate placoid of epithelial and lymphoid tissue on the wall of the gill chamber (40). In the trout there are three thymic buds above the first, second and third gill arches (52).

In tadpoles according to Speidal (256), the thymus consists of lymphoid tissue interspersed with large blood sinuses. There is no definite arrangement into cortex and medulla. In the frog (Rana pipens), the thymic bodies are a pair of compact masses on the sides of the head just posterior to the tympanic membrane. They show a cortex and medulla. There is no connection with the epithelium, the branchial pouches having disappeared. The glands are said to decrease in size during metamorphosis (221).

The glands vary in shape in the different classes of reptiles. In snakes, the glands are elongated and extend along the carotids. In lizards and crocodiles, they are broad and flat and extend along the carotids from the pericardium (190).

In birds, the thymus proper consists of two rows of nodules lying on either side of the trachea. The number of nodules is variable even in the same species (50). Riddle (214, 217) believed that the bursa Fabricii, a glandular pouch opening into the cloaca, is also composed of thymic tissue and Greenwood (91) found similar tissue elsewhere in the body.

The fully developed human thymus is roughly triangular in
shape with its base resting on the pericardium. It extends from the level of the fourth costal cartilage to the lower tip of the thyroid. Anteriorly it is in contact with the sternum and posteriorly, the trachea. It is composed of two lobes each of which is divided into lobules, 1-2 mm. in diameter, giving the gland a warty appearance (90, 203).

In other mammals, the gland is similar to that of man in most respects. It varies in the number of lobes, the size of the lobules, the relative amounts in the thorax and in the neck and in the frequency with which detached portions of thymic tissue are found (194).

**Histology**

On microscopic examination, the thymus is found to consist of a cortex and a medulla. The cortex is made up of densely packed masses of small cells morphologically identical with the small lymphocytes, supported by a network of reticulo-epithelial cells continuous with a similar network in the medulla. The medulla is marked off from the cortex by the abrupt disappearance of most of the small thymocytes. It also contains the very characteristic structures, the concentric corpuscles of Hassall. The medulla is more vascular than the cortex.

The origin of the reticular cells from the original epithelial tissue can be traced embryologically. Their epithelial nature becomes prominent in transplants and tissue cultures of the gland.

The origin of the small thymocytes on the other hand is not so settled. Most investigators regard them as lymphocytes which have invaded the epithelium since they resemble lymphocytes elsewhere in all their morphological characteristics, in their serological reactions, in their susceptibility to roentgen ray injury and in their general behaviour.
There are however some workers who regard the small thymic cells as derived with all other components of the gland from the original epithelial cells (92, 171, 175, 176).

Hassall's corpuscles are made up of large pearshaped cells arranged concentrically around a central core containing cellular debris, cysts and sometimes calcified material. They are probably spent and degenerating epithelial cells which have grown under unique conditions involving the loss of a surface from which desquamation could take place normally. Most investigators hold this view (58, 148, 171, 176, 293) though other interpretations are not lacking. Jordan et al (137, 138) believe them to be due to occlusion of the lumen of medullary capillaries with a resulting lack of nourishment for the surrounding cells.

Occasionally in the thymus of mammals there are found duct-like spaces lined with columnar or cuboidal epithelium. These when traced are found to end blindly. It has been suggested that they are due to remnants of the branchial pouch epithelium or due to failure of the original thymus tubule to lose its lumen completely.

There are also present in the gland very variable numbers of mononuclear and polymuclear eosinophiles.

The above remarks refer chiefly to the thymus of higher vertebrates. In the fish, the lymphoid character is not so dominant and there are no Hassall's corpuscles (52).

Physiology

The function of the thymus is still unknown. Data obtained from experiments involving thymus extirpation, implantation, feeding, injection of extracts as well as from clinical observations have been
interpreted differently by different observers, with the result that the physiology of the gland is most vague. However, the facts that it undergoes an involution beginning about puberty after increasing in weight up to that time; that it undergoes acute involution in starvation, pregnancy, severe disease or other adverse conditions; that after gonadectomy its normal involution is delayed; and that under other conditions such as Grave's disease, acromegaly and after adrenalectomy it regenerates - all indicate that it plays some important part in metabolism at least up to the period of sexual maturity, even though it is not essential to life.

Involution

One clear property of the thymus is that it is relatively large in infancy but that during maturity it slowly regresses to practically disappear in old age. That it reaches its greatest absolute weight at about the age of puberty is certain. A general average of weights reported for the human thymus was given by Loewenberg (157) as follows: at birth 12 grams; at puberty (12-15 years) 35 grams; at 25 years, 23 grams; at 60 years 12 grams; at 70 years 6 grams. Others (33, 35, 293, 300) reported similar figures. Relative to the weight of the body, the thymus weight is greatest at birth. At all ages there is, moreover, a high individual variation.

After puberty, the gland atrophies, rapidly at first, and then more gradually, so that thymic tissue may be found even in the most aged. This natural atrophy is referred to as "age involution". The same essential picture of growth up to about puberty and atrophy thereafter was the result of more complete and better controlled investigations on various animals, (Paton and Goodal, (199) on guinea pigs, Hatai (113)
and Chiodi (45) on rats and others). Hammett (100, 102) however stated that involution does not begin until early adulthood but Hammar (99) pointed out that this increase after puberty of the whole gland did not exclude reduction of parenchyma.

In the pigeon, Riddle and Fry (216) found that thymic involution began two months before sexual maturity measuring the latter by the beginning of egg laying (at about six months of age). It is also apparent that in birds, it degenerates more slowly than in mammals (186).

There is much difference of opinion as to whether the gland is at its maximum weight before, at, or after the onset of puberty. More careful work will have to be done to determine the exact moment, physiologically, when involution begins (45, 100).

The thymus is a very labile organ. Under adverse body circumstances or when the organism is subjected to unusual stress, it undergoes an "accidental" involution, as opposed to the normal age involution. Malnutrition, starvation, operative shock, trauma, infections, wasting diseases and pregnancy will all cause accidental involution (157, 268). It decreases in size in most illnesses lasting more than twenty-four hours (33). According to Riddle and Fry (216) the reduction of the thymus more speedily tells of disease or adverse conditions in Laboratory animals than the macroscopic examination of any other organ. Regeneration normally follows accidental involution.

There are some histological differences between the two types of involution. In age involution there is a decrease in the number of Hassal's corpuscles, while in accidental involution there is an increase.
Clinical Observations

This aspect has been reviewed by Loewenberg (157) and numerous reports are to be found in medical literature. The thymus may be involved in various abnormal conditions - it may be enlarged or atrophied, it may be the seat of inflammation, abscess or hemorrhage, it may be infected by syphilis, tuberculosis, actinomycosis etc., or it may be the seat of malignant or benign tumors - but there seems to be no disease which can be ascribed to disfunction of the gland itself. It is affected in many endocrinopathies but in all cases apparently only secondarily (92, 300). There have been, however, reports that partial ablation of overlarge thymi has corrected retarded mental and physical development (36).

The term Mors thymica has been applied to instances of sudden death of infants who had previously been in apparently good health and in whom the only abnormal finding has been an enlarged thymus. The cause of such a tragedy has usually been ascribed to either pressure of the gland on trachea or heart or to anaphylactic phenomena.

The most celebrated syndrome involving the thymus is that known as Status thymicolymphaticus. The anomaly is an indefinite one. One of the symptoms is a hyperplasia of the lymphatic structures, including the thymus, but there are also manifest numerous other disturbances involving the gonads, the cardio-vascular system, the gastrointestinal tract etc.. The basal metabolic rate is low, there is a tendency to allergic phenomena and a greater susceptibility to infection.

Most of the pathological relations of the thymus emphasize its similarities with other lymphoid tissues.
Effect of Thymectomy

It was early demonstrated that the thymus was not essential for life. The ill effects, including tetany and abnormal bone development, obtained by some early investigators have been ascribed to infections, malnutrition or to unwitting removal of the parathyroids (194).

By far the greater number of more recent experiments on various animals, including the rat (191), the guinea pig (199), the rabbit (169, 284), the dog (194), the pigeon or chicken (174, 186, 217) have showed that thymectomy was not followed by detectable symptoms in otherwise normal animals.

A few investigators have found changes in metabolism after thymectomy. Sandberg, Perla and Holly (232) reported that urinary nitrogen increases from 13% of intake to 40% twelve weeks after the operation, the increase being accounted for by urea. Riddle and Braucher (215) found an erythrocyte count 16% higher than the normal in pigeons from which the thymus and bursa had been removed. Miller (183) reported calcium abnormalities in thymectomized hens but here the probability seems to be that the parathyroids were involved.

In general, the conclusion has been widely drawn that the functions of the thymus may be performed or compensated for by other lymphoid tissues.

It has been shown (89) that remnants of the thymus may hypertrophy after the removal of the major portion of the gland.

Connections with calcification

On the basis of a close embryonic origin, a relationship between the thymus and the parathyroids has been thought likely. This
belief was reinforced by the disturbances in calcium metabolism which sometimes resulted from the extirpation experiments of some early workers. More recently others have associated the thymus with calcification. Riddle (213) found that some pigeons which laid eggs without shells, had atrophied thymus. He believed that there was a hormone acting on the oviduct and named it Thymovidin. He could not reproduce the condition by removing the thymus, blaming this failure on rather hypothetical (see 91) thymic tissue elsewhere in the body. Others have also obtained similar negative results (1, 186).

Glaessner and Hass (82) stated that regeneration of fractured bones was delayed in thymectomized cats but that it may be stimulated by the injection of a thymus extract.

Harris (114), finding that the thymus atrophied in hypervitaminosis D concluded that it had some role in calcium metabolism. He also stated that thymus implants increased the density of the bones. He has made a short review of the evidence linking the thymus and calcification.

Rowntree, Clark and Hanson (233) reported a rise in blood calcium and phosphorus with accelerated bone development in the offspring of rats treated with Hanson's thymus extract (see below).

On the other hand other investigators (193) found no such evidence. Park and McClure (194) in an extensive review and after careful experiments on dogs noted no changes whatever after thymectomy. They attributed previous results to parathyroidectomy, varying degrees of rickets and other disturbances not connected with the thymus. Thomson and Collip (277) state that they remain unconvinced that any definite role of the thymus in calcification has been established.
Connection with the Gonads

The most obvious property of the thymus is that of starting to regress apparently just at the age when sexual glands are beginning to function. This has led many workers to believe that there is an intimate relationship between the thymus and the gonads and to plan experiments accordingly. Many of the results of these have been reviewed by Andersen (5).

A few investigators have reported changes in the gonads following thymectomy. Paton (196, 197, 198), found heavier testes in young operated rats than in controls but no difference in adults. Others have reported a decrease in the size of the gonads but these results appear to have been due to the poor conditions of the animals.

Most workers report no change whatever in the sex organs after removal of the thymus. Hainan and Marshall (98) and Renton (211) with guinea pigs, Pappenheimer (191), Reinhardt (210), Plagge (204), Segaloff and Nelson (241) and Anderson (6) with rats, Masui and Tamura (173) on mice, Park and McClure (194) with dogs, Riddle and Krizecky (217), Achert and Morris (1), Morgan and Grierson (186) on birds and Allen (3) on tadpoles, all obtained negative results. They found no significant deviations from the normal in the factors noted, including size and weight of gonads, spermatogenesis or commencement of ovulation.

It has also been shown that thymectomy had no effect on pregnancy (6, 199, 241).

The conclusion is that extirpation of the thymus has no effect on the development and function of the reproductive organs.

On the other hand extirpation of the gonads has been shown to have a definite effect on the thymus. The normal age involution was
delayed and slowed. This delay was most marked after prepuberal castration but was also measurable in adult castrates. One investigator, only, Paton (197) reported no difference following spaying in guinea pigs. All others (75, 88, 91, 98, 120, 170, 296) in giving their observations on rats, guinea pigs, fowls, heifers agreed that the normal atrophy of the thymus was delayed though not prevented by gonadectomy. Chiodi (45) determined the growth curve of the thymus in normal and in castrated rats. He showed that in animals castrated before puberty this curve was parallel to, though higher, than the normal. He gave as his opinion that since the general shape of the thymus growth curve was not affected by castration that the involution of the thymus was not influenced by the development of the sex glands.

Results obtained by Evans and Simpson (56) showed that the effect of castration was due to the removal of the interstitial cells, since destruction of the germinal epithelium by operative cryptorchidism or by vitamin E deficiency caused no abnormalities in thymus development. Biddelph and Meyer (20) however, reported a delay of thymus involution in the male, but not female, rat on a vitamin E deficient diet. The delay was not great.

Hammar (99) stated that the injection of the serum of mature animals into immature ones of the same species caused thymus involution. This effect was not found on injecting the serum of immature animals.

Moreover, atrophy of the thymus was produced by the injection of sex hormones and of gonadotropic substances into both prepuberal and mature animals. Also the effect of gonadectomy could be overcome by
the injection of sex hormones in suitable amounts.

Ovarian, placental and testicular normones all caused apparently the same effect in animals regardless of sex. Allen in 1928 (4) found that the thymus of monkeys injected with an ovarian extract weighed less than those of controls. Golding and Ramirez (87) injected aqueous extracts of ovary and placenta into rats and obtained a similar result. Extracts of mare's uterus and placenta were also potent (66) as was a testicular hormone obtained from urine (150, 151).

That the effect was actually due to the sex hormone was proved by the use of more or less purified preparations and also of synthetic hormones and substances of closely related structure. Estrone or theelin, estrin, estradiol, testosterone propionate and the "unnatural" androstenedione and androstenediol have caused atrophy of the thymus in normal and gonadectomized rats of both sexes. If administered in suitable amounts some of these hormones have prevented castration hypertrophy of the thymus (45, 76, 150, 151, 152, 154, 202, 296). Only Low (159) reported negative results on injection of theelin.

Butcher and Persike (42) used a pituitary gonadotropic preparation Antuitrin S. This caused thymic regression in normal rats over 17-18 days of age. It had no effect on gonadectomized animals.

These results are very definite and seem to show conclusively that the increase in the sex hormone content of the circulation (corresponding to the onset of puberty) is an important factor in the normal age involution of the thymus.

Many cases have been reported of thymus hyperplasia associated with hypogonadism, cryptorchidism and similar conditions (157) and much has been made of them as pointing towards the function of the thymus.
But in the light of recent research, it would appear that the thymus was only secondarily involved.

It can therefore be said that, to date, no definite relationship between the gonads and the thymus has been demonstrated.

Connection of the Thymus with the Adrenals

Another reported interrelation of the thymus has been with the adrenals. Wiessel (294) in 1905 noted that in status lymphaticus, there are frequently lesions of the adrenals, also the atrophy of the adrenals in Addison's disease is associated with a persistent thymus. Other clinical observations (132, 191, 240) have been along the same lines.

Experimental work has also indicated some sort of mutual antagonism between the two glands. In 1914 Grove and Wislocki (49) obtained a generalized hyperplasia of lymphoid tissue following the removal of the adrenal cortex. Jaffe (134) and Marine, Manley and Baumann (170) obtained the same effect. Richter and Wislocki (212) while investigating the changes produced by hypophysectomy found that while the adrenals were hypoplastic, the thymus and lymph glands of the whole body were markedly enlarged. According to Chiodi (45), in rats which had been castrated prepuberally and adrenalectomized at 80-100 days of age, there was only a slight increase in thymus weight as compared with control castrates.

Selye (242) reported the converse results that enlarged adrenals caused an atrophy in the thymus. The injection of cortin would bring about this atrophy with or without the presence of adrenals. Ingle (133) gave rats cortin in their drinking water and obtained an involution of the thymus. Low (59) however reported negative results from the injection of an adrenal cortical preparation called Eschatin.
The administration of pituitary adrenocorticotrophic hormone was said to cause thymus regression if the adrenals were present (67, 270).

Investigation on this apparent relationship by working from the thymus end, have not been so productive. Here also the removal of the thymus has given little hint as to its function. Park and McClure (194) in their very thorough research got no change in the adrenals after thymectomy. Segaloir and Nelson (240) thought that thymectomy might have an effect on the course of adrenal insufficiency in the rat. They therefore injected cortin into adrenalectomized rats from some of which the thymus was removed also. There was no difference in the growth curves of the two groups.

On the other hand Rowntree (222) wrote that the administration of Hanson's thymus extract (see below) led to hypoplasia of the rat adrenal. Gershon-Cohen et al (80) destroyed much of the thymus by X-ray irradiation and report adrenal enlargement, but this treatment was perhaps a violent one.

From the above it is seen that experiments on this aspect of thymus function, while making it certain that adrenal impairment is associated with hypertrophy or at least regeneration of the thymus, have not shown definitely just what this connection is.

Connection of the Thymus with the Thyroid

Based on the clinical observation that in exophthalmic goiter there is often enlargement of the thymus (181, 168, 290), a relation has been sought with the thyroid. Speidal (256) has compared a hyperplasia of lymphocytes in the thymus of tadpoles which had been administered.
thyroid substance, with the thymus enlargement mentioned above.

It has also been shown that thyroidectomy hastens the involution of the thymus and also prevents the regeneration normally following adrenalectomy (170).

In Hamnett's view, however, these effects may well be due to the general physiological disturbance and not to any specific relationship (100, 101). He stated that the main factor in the growth of the thymus is its close connection with the general bodily condition.

In the opinion of Williamson and Pearce (295), the thymus is essentially a lymph reservoir for the thyroid. Chouke, Whiteshead and Parker (46), however, could find no trace of any such lymphatic system in human cadavers or in dogs. Nor could Weller find any embryological evidence of it (293).

Connections with other Endocrine glands

No definite interrelationships with the other endocrines have been made out. After hypophysectomy it has been reported that the thymus enlarged (212) but it has also been reported to atrophy (253, 67). Pituitary gonadotropic hormone caused thymus involution but presumably through the medium of the sex glands. Low (159) obtained no effect following the injection of an anterior pituitary extract (alpha and beta cell hormones). In this regard, it might be mentioned that Low injected several endocrine extracts into rats and got negative effects in all cases (see above). He admits that his extracts were only "supposed" to contain hormones, however. Some of them were commercial preparations.

Bomshov and Sladovic (32) have made the most recent claim to have demonstrated a thymus hormone. It was obtained from the lipoidal
fraction. Its administration was stated to cause disappearance of glycogen from the liver of rats. They ascribed the stimulating action of the thymus on growth to the mobilization of this carbohydrate. This antagonism to the Islets of Langerhans has been reported earlier by one or two others, but has not been investigated thoroughly (157).

Summary of Endocrine Connections

From the above, it is seen that no definite and specific interrelationship between the thymus and and any of the glands of internal secretion has been established. Though the above review is far from being complete, so voluminous is the literature on the thymus, even if it were more comprehensive the conclusion just stated would be unchanged.

It has not been proved that the thymus itself has any internal secretion. There are some authorities who deny that it has any endocrine properties whatever. It contains no groups of cells which seem likely to be secretory. The greater number of its cells are thymocytes which are closely related to, if not identical with, lymphocytes. The purpose of the reticular cells is, apparently, to provide a support for the thymocytes. The cells of Hassall's corpuscles must be looked upon as degenerating reticular cells. They are hyalinized and may undergo liquifaction or become cystic or calcified. The cystic ducts are found only very occasionally (The greatest frequency is in dog's thymi where it is 20%).

Relationship to Lymphoid tissues

Though the thymus is of epithelial origin it is changed very early in prenatal life so as to become lymphoid in appearance. It is also a fact that in most of its physiological and pathological reactions it resembles ordinary lymphoid tissue (175).
On the nature of the thymocytes Maximow (176) writes as follows "They are morphologically identical with lymphocytes. Both show the same susceptibility to X-ray injury; both are cytolysed by sera obtained by the injection of thymus cells into rats (192); and both show the same type of ameboid motion. Gregoire has shown that transplants of the thymus consist only of epithelium if lymphocytes are prevented from migrating into them by mechanical means. Further, the transformation of the small thymocytes into plasma cells and eosinophil myelocytes is generally admitted. The mitochondria have the same appearance in both types of cells. However in spite of these similarities, a few authors are unwilling to classify the small thymocytes as lymphocytes because they believe the thymocytes to have an epithelial origin, although most workers believe that they arise from lymphocytes which have wandered into the epithelium" (176).

In ordinary lymph tissues an age involution similar to that of the thymus begins about the age of puberty, lymphatic tissue being normally prominent in children. Regeneration of lymph glands occurs after adrenalectomy (134). Chiodi (45) found that the weights of the submaxillary, cervical and axillary lymphatic ganglia were slightly higher in castrates as compared with controls, paralleling the effect of the same operation on the thymus. In Grave's disease and in acromegaly where there is thymus enlargement there is a similar enlargement of other lymphoid organs, such as lymph glands, tonsils and spleen. In the condition known as Status lymphaticus, though the most prominent symptom is the enlarged thymus, there is hypertrophy of all lymphoid and lymphatic tissues in the body.

These and other resemblances and the fact that the organism
can apparently well get along without its thymus have led to the view that the thymus is primarily part of the lymphatic system. This does not solve the problem of its function since the function of lymphoid tissues is still indefinitely known but it opens up other avenues of approach to the thymus problem.

The functions of lymphoid tissues are generally said to be the production of lymphocytes, filtration and a certain amount of purification of the blood and some close association with the defense reactions of the organism such as the elaboration of antibodies.

Friedlander (74) stated that the thymus produces lymphocytes. Maximow (175) and Marine (171) hold similar opinions.

There has been considerable work done on the role lymphoid tissue plays in resistance to infections and cancer and some experimenters have investigated the thymus along this line. After adrenalectomy when there has been hypertrophy of the thymus, it has been observed that antibodies were formed more rapidly than in normal animals although these were more susceptible to toxins (273). Loewenberg (157) expressed the opinion that the thymus operated to prevent pulmonary tuberculosis in prepuberal children. He pointed out that this disease is rare or mild in childhood. Pearce and van Allen (200) investigated the effect of thymectomy on syphilis in the rabbit. They found, however, that the disease was milder than in normal controls.

Many cases of sudden death have followed the injection of usually safe amounts of antiserum. Antopsy has shown the cause of death to be Status lymphaticus. Persons suffering from this disorder have a tendency towards asthma, allergical reactions and other protein
sensitivity (157).

With these similarities there remains the different origin of the thymus from other lymph organs (the palatine tonsils would appear to have a somewhat similar origin to the thymus). There is also the anatomical difference in the absence of the typical lymph nodules and lymph sinuses possessed by other lymphoid organs (237).

By virtue of the close similarities of the thymus with other lymphatic organs, it is very reasonable to assume that it is primarily part of that system. But it is still not unreasonable, on account of the differences, to expect the gland to display properties different in kind or degree from the other components of the system. The most recent researches however, have failed to indicate definitely what these properties might be.

Role as regards Nucleic Acid

The fact that the thymus contains a greater concentration of thymonucleic acid than other tissues has led to the conjecture that it acts as a nuclein regulator or as a store of nucleic acid (135a). It would appear, though, that the densely packed thymocytes with their small amount of cytoplasm and relatively large nuclei might account for the larger amount of nucleic acid.

Effect of Feeding or Injecting Thymic Substance on Growth and Development.

Among the first attempts to elucidate thymus function by feeding the gland to various animals were those of Gudernatsch with tadpoles. The treated tadpoles, he reported, grew very large and did not metamorphose (95). Uhlenhuth (281, 282, 283) got developmental disturbances
with tadpoles which were fed on an exclusively thymus diet. He later showed that these were due to the inadequacy of the diet since tadpoles fed supplementary plant food developed normally.

Some of the experiments on the thymus feeding of animals also failed for the same reason. Hewer (I25) in 1916 fed rats on thymus and reported an absence of spermatogenesis. In the light of present knowledge this experiment showed that the gland was lacking in Vitamin E. Hoskins tried the same experiment, including other food stuffs in the diet and got rats which were apparently quite normal (I29, I30). Gudernatch (96) however, reported that thymus fed rats grew more rapidly and were more vigorous than his controls. On the other hand, MacKay and Barnes (I65) found that thymus feeding depressed the growth curve considerably. As mentioned earlier Riddle (2I3) corrected the laying of soft shelled eggs by pigeons by feeding bovine thymus. Others (I, I86) have been unable to confirm these results.

On the whole, therefore, the evidence is against the thymus, having any specific effect as a food.

Extracts of the Thymus Gland

Various extracts have been prepared from the thymus but the administration of these to laboratory animals has given widely varying results in different hands.

The first of these extracts was prepared by Asher, who called it Thymocresin (I57). This extract when injected intramuscularly daily in one milligram doses increased the rate of growth of rats and also kept them alive on a vitamin-free diet on which the controls died (8). Segaloff and Nelson (239), however, prepared an extract according to
Asher's directions, having the same qualitative tests. They injected five times the minimum dose reported by Asher and got no results. They continued the treatment over six successive generations and could not find any variations in growth and development.

Another extract was introduced by Temesvary (276). He employed it in strengthening and prolonging the uterine contractions in the early stages of labor. Later this extract was improved by adding pituitrin from the posterior lobe of the pituitary. It has, however, been found that other tissues could replace the thymus (135). This combination of thymus and pituitary extracts is called Thymophysin.

Downs and Eddy (54) injected rats with a saline suspension of desiccated thymus and could find no difference from saline injected controls.

In 1930 Hanson prepared an extract which he used in the alleviation of some cancers (110). The hypothesis behind this is that cancer is caused by an unbalance between growth promoting elements and "antiplastic agencies. Extracts of thymus, spleen and the organs are used to increase these alleged antiplastic agencies. Hanson reported that this extract, which he called Karkinolysin, had been used with good effects on some cancer cases (110,111).

The Work of Rowntree and his Collaborators

We now come to consider the work of Dr. Rowntree and his collaborators at the Philadelphia Institute for Medical Research. Hanson originally supplied Rowntree with a quantity of his extract, the intention being that the Institute would make tests on fresh batches as they were made.

The procedure that Rowntree and his co-workers, Clark and
Steinberg, adopted was to inject 1 cc. daily of the extract intraperitoneally into successive generations of rats. The results they reported were amazing. These results were summed up in the following quotation "Following the continuous administration of thymus extract to successive generations of parents, marked acceleration in the rate of growth and development has been observed during the early life of the offspring, particularly of the third and later generations. Thus the rate of development encountered in the fifth generation of young rats born of four generations of thymus treated forbears is almost beyond belief" (226). Also see (222, 223, 224, 225).

This accelerated development is shown in the following table from (226).

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>F₁</th>
<th>F₂</th>
<th>F₃</th>
<th>F₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average birth weight (grams)</td>
<td>4.6</td>
<td>5.1</td>
<td>5.3</td>
<td>5.3</td>
<td>5.6</td>
</tr>
<tr>
<td>Ear opened (days)</td>
<td>2 3/4-3 1/2</td>
<td>2</td>
<td>1-2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Teeth erupted (days)</td>
<td>8-10</td>
<td>9</td>
<td>I-2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Hair appeared (days)</td>
<td>12-16</td>
<td>3-12</td>
<td>4-6</td>
<td>4-5</td>
<td>2-3</td>
</tr>
<tr>
<td>Eyes opened (days)</td>
<td>14-17</td>
<td>12-14</td>
<td>4-6</td>
<td>4-6</td>
<td>2-3</td>
</tr>
<tr>
<td>Testes descended (days)</td>
<td>35-40</td>
<td>15-29</td>
<td>5-21</td>
<td>5-12</td>
<td>4-5</td>
</tr>
<tr>
<td>Vagina opened (days)</td>
<td>55-62</td>
<td>30-45</td>
<td>23-32</td>
<td>21-27</td>
<td>18-19</td>
</tr>
</tbody>
</table>

The low numbers usually relate to the late litters in the generation and the high numbers to the first litters born.

The growth curves showed an accruing acceleration in weight in each succeeding generation. For example the figures for the F₄ generation at 25 days of age were 37 grams for the controls and 94 grams for the
tests. Giant rats did not result however, the weight curves of tests and controls approaching after the 60th day. Eventually in fully mature rats little difference was apparent.

With the acceleration of physical development there went a psychic precocity also. The \( F_4 \) rats could look after themselves as well at 3 days of age as normal rats of 16 to 20 days. Weaning was possible at 3 days of age.

The effect of the extract was largely confined to the growth and development of the young but Rowntree also reported a somewhat shorter period between litters in thymus treated parents.

The result of omitting the injections for one generation in a series was the entire disappearance of the precocity built up in previous generations.

They also reported that large doses of the extract were toxic, causing death through complete auricular-ventricular heart block.

X-ray studies on the developing skeleton disclosed that the bones lengthened more rapidly than normally and ossification set in much earlier.

This was correlated with an increase in the calcium and phosphorus content of the blood. The control rats had a calcium content of 9 to 11 mg. % while in the tests the serum calcium increased to 13.3 mg% in the \( F_4 \) generation. The corresponding figures for phosphorus were 3-4 mg. % and 7.5 mg. % (222).

Small adrenals, lymphatic hyperplasia and eosinophilic hyperplasia of the pituitary were other effects reported.

Also Buckley reported accelerated development in the nervous
systems of treated rats (38, 39). Normally only scattered myelinated fibers are found at the end of 48 hours after birth in the rat. Buckley, however, reported that he found myelinization in the spinal cords of 6 day old rats which were descended from thymus treated forbears, equivalent to that of 13 day old normal animals. He later repeated this investigation using a new born rat of Rowntree's F11 thymus treated generation. There was myelinization far in advance of controls of the same age.

Although it was reported (227) that the injection experiments were continued through twelve generations with cumulative precocity in each generation, no figures have been published on the development of the last litter which must have been approaching a limit.

A decrease in the average period between casting of litters was reported for the tests. It was irregular, though, and apparently not accruing. No data was given relative to a shortening of the period of gestation which might have been expected.

Einhorn and Rowntree (60) have observed the effect of removal of the thymus at an early age from successive generations of rats. They have noted retardation of development though it was not as striking as the acceleration on administering extract.

The effect of implanting thymi into rats has also been tried (59, 61). Homologous implants were made weekly, twelve being made in each rat. This was continued for four generations. Precocity was not so marked as that following daily injections of 1 cc of Hanson's extract. Some
data is in the following table.

<table>
<thead>
<tr>
<th></th>
<th>Ears</th>
<th>Teeth</th>
<th>Eyes</th>
<th>Testes</th>
<th>Vagina</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Controls</strong></td>
<td>2.9</td>
<td>8.5</td>
<td>15.2</td>
<td>30.2</td>
<td>44.3</td>
</tr>
<tr>
<td><strong>F₄ generation</strong></td>
<td>2</td>
<td>5</td>
<td>7</td>
<td>24.3</td>
<td>36</td>
</tr>
</tbody>
</table>

Time in days.

They also observed the effect of thymus implanting (58) and the injecting of extract (57) on thymectomized rats. In both cases the retarding effect of thymectomy was overcome and was changed to an accruing acceleration. Frequent homologous implants were found to be more effective than the administration of calf thymus extract.

These very distinctive and most remarkable findings required confirmation. Other investigators however, have been unable to reproduce them. Chiodi in 1938 (44) and Segaloff and Nelson in 1940 (241) have all tried the effect of thymectomy at an early age on rats over five or six successive generations. The last named removed the thymus at the age of 21 days over six generations. The rate of growth of both males and females was unaltered, and there was no departure from the normal in any of the developmental events studied. Putzu (205) reported that no alteration in the somatic or psychic development occurred in the progeny of five consecutive generations of thymectomized rabbits.

Injection of extracts of thymus also produced only negative results. Chiodi (43) made up an extract weekly according to Hanson's method and kept it frozen until it was to be used. The weight curves up to 40 days of age did not vary from those of the controls in the five generations he treated; neither was there any significant difference in
development. Smith and Jones (254) also could detect no variations from the normal in the growth of mice over 5 generations. There was, however, a marked lowering of fertility in the treated offspring of treated mice.

In particular, Miss. Ursula Dale of the Department of Zoology of this University in an attempt to reproduce Rowntree's results, could find no evidence of any acceleration of growth or development at the end of five generations. Further, she was able to detect no difference in size, structure or histological appearance in the pituitary, pineal, thyroid, parathyroids, thymus, pancreas, adrenals, testes or ovaries of the treated rats (51,2).

Burrill and Ivy (41) worked on the theory that treatment during pregnancy might have speeded up the metabolic rates of the fetus and that, after birth, it might have continued to obtain the stimulating principle through the milk of the mother (though Rowntree (225,226) stated that it was not transferred in this way). This altered metabolic rate might be retained and the fetuses of the next generation would be subject to a double effect. Rowntree (230) had stated that treatment of the males was unnecessary. Burrill and Ivy therefore tested to determine whether the reported effects could be produced by daily injections of ground thymus tissue into females during pregnancy only. The experiment was continued for four generations. Normal litters resulted and no significant differences over the controls were reported.

The continued lack of success in thymus experiments along Rowntree's line, especially with regard to the effects of thymectomy, where the difficulties connected with the lability of the extract (see below) would not arise, have cast considerable doubt on the validity of his findings.
Hanson, however, stated that he felt assured that the results were all that had been claimed (III). Loewenberg reported that he had seen the original rat and also the members of twelve successive treated generations (I57). He was "truly astonished" at the precocity of members of the twelfth generation. It was also recorded that Rowntree, Clark and Steinberg gave a motion picture demonstration of their work to the 48th Annual meeting of the American Physiological Society at Washington D.C. in March 1936 (228). Buckley (38) observed in the process of birth of an FIII thymus treated rat in which he found myelinated fibers on examining it immediately thereafter.

Properties of the extracts used by Rowntree.

The extract used by Rowntree in his early experiments was one prepared by Hanson in 1930, whose method was as follows (I10).

Fresh thymi from the neck of calves between 2-6 weeks of age were ground to a thick paste. This was added to 1% HCl in the proportion of 0.6 gr. gland to 1 cc HCl. The mixture was stirred and slowly heated so that it took 1½ hours to reach a temperature of 94°C. It was then cooled and filtered. The filtrate was made up to 990 cc by pouring hot distilled water over the residue. 10 cc. of 3% menthol in 95% ethyl alcohol was added as a preservative and the extract was bottled in glass vials. The pH was about 5.5.

Now the first batch of extract supplied to Rowntree remained biologically potent for four years. It was, moreover, the only batch of Hanson's extract to be reported as anything but very unstable. Hanson himself wrote that he was at a loss to account for its stability (II2).
Early in 1934, a new preparation was received from Hanson. The results obtained with this were entirely negative, no precocity being noted in the third generation (225, 226).

Later the Philadelphia workers prepared their own extract after Hanson's method. They obtained the glands from milk-fed calves and made up the extract immediately. This was done weekly.

The first batch of extract contained oxidised and reduced sulfur compounds to the amount of 15mg.% calculated as glutathione. The second, impotent extract contained practically none. Hanson reported his fresh product to have an iodine reducing titre of over 100 mg.% glutathione. It contained 2-4 % protein.

The fact that reducing substances, especially sulphydryl, were to be found in active extracts but not in inactive, became an important factor in directing the course of Rowntree's experiments (229). It was believed that there was a positive correlation between a high iodine reducing titre (i.r.t.) and the efficiency of collection.

With this in mind Steinberg, one of Rowntree's coworkers, so modified Hanson's method that the conditions were more favorable for getting a high i.r.t.. Steinberg's method involved heating fresh ground thymus glands with 1% HCl rapidly with stirring to 68°C. It was filtered and bottled. 0.2% chlorbutanol was used as the preservative. The proportion of gland to acid was 1.5 gr. to 1.0 cc.. This extract had an i.r.t. of between 200-400 mg.% glutathione. It had a negligible protein content (258).

An analysis of Steinberg's extract gave values for the principal reducing substances, to wit. glutathione, ascorbic acid and
cysteine as about 65, 30 and 5 mg. % respectively. These three accounted for practically all the reducing titre. Ergothioneine was absent (233).

**Effect of Some Sulphydryl Compounds and Other Reducing Substances on Growth and Development.**

There was some basis for Rowntree's belief that the constant presence of -SH in his potent extracts was more than merely adventitious. For several investigators had shown that sulphydryl compounds stimulated cell division (12, 103, 104, 105, 106, 108, 109, 166, 209, 244, 286). Evidence was given that sulphydryl increased mitosis in Paramoecium and Amoeba, that it increased the rate of healing of skin wounds and that, in plants, it was concentrated in meristematic regions. Gregory and Davis (93) found that three consistently related factors with regard to rabbits were, the adult racial size, the rate of segmentation of the eggs and the concentration of glutathione in the new born young.

With this background and with the knowledge that thymus extract contained such reducing substances as glutathione, cysteine and ascorbic acid, Rowntree et al. investigated the effect of administering these substances to rats (230). They injected freshly prepared solutions of the pure chemicals in hydrochloric acid of the same strength of the extract (pH 4.6) in daily 1cc. intraperitoneal doses.

With glutathione alone it was found necessary to inject 2mg. daily. But this amount produced very definite precocity even in the F₂ generation. During the first forty days, the rate of growth increased 20% over the controls. The figures they report are as follows.
A dose of 2 mg. per day of ascorbic acid was also found necessary—no changes being produced by 0.25 mg. or 1.0 mg. daily. In the second generation no increase in rate of growth was observed but somatic development was greater than in the glutathione group. It was most marked in the third generation, the teeth appearing in 6 days, the eyes opening in 10, while gonadal development was over 50% ahead of the controls.

Cysteine was found to be toxic in doses of over 1 mg. daily. In the offspring of treated rats, though weight was down owing to the toxicity of the solution, yet somatic development was increased appreciably.

When glutathione and ascorbic acid were administered together, 1 mg. of each per day, the offspring of the treated rats were markedly precocious. The figures are, for teeth, 6.2 days; for eyes, 9.6 days; for testes, 16 days; and for vagina, 23 days.

Though the effects of these substances did not exactly parallel those of the thymus extracts, yet Rowntree was quite justified in ascribing a large part of the latter to glutathione, cysteine and ascorbic acid.

Another investigator, Lee, getting his hint from Rowntree's
first work, dosed rats with 0.5 to 1.0 mg. of glutathione per day (156). He got unmistakable precocity in the fourth generation, the ears opening at \(1\frac{1}{2}\) days of age, the eyes at 6-7 days while for the gonads, the times are 15-19 days for the descent of the testes and 28 days for the opening of the vagina.

The above experiments do not seem to have been repeated by any other worker.

Other Reports of the Presence of Glutathione and Ascorbic Acid in the Thymus.

The presence of glutathione in the thymus gland has been reported by others (23,187,280). Schaffer and Ziegler (234) have reported the isolation of a small quantity from the thymus.

Ascorbic acid has also been found in the gland (23,64,36,180). The amounts reported seem to vary considerably.

The Purpose of this Research

When it became apparent that the attempt by Miss. Dale (see above) to repeat Rowntree's results was not succeeding, attention was directed towards possible reasons for the impotency of the extract.

The chief of these seemed to be the fact that Miss. Dale prepared her extracts from the thymus of calves considerably older than those by the Philadelphia workers. The latter had available apparently a continuous supply of thymus from calves of 2 to 6 weeks old (258). Hanson wrote that (III) "the first and vital part of the whole process is to secure healthy thymus from calves under six weeks of age". (At this point it might be mentioned that in spite of this insistence on the glands of
very young calves, the glands of correspondingly young rats were not used by Einhorn and Rowntree (59) in their experiments on homologous thymus implants where they obtained definite precocity. The implants were obtained from rats 20 to 50 days old. The figures published show that puberty occurs in their untreated rats between 40 and 60 days of age.)

Miss. Dale, however, was not able to secure a regular supply of glands of under three months of age. The glands were obtained through the courtesy of Burns and Co. Since it is illegal in this province to sell meat from calves under this age, younger glands could not be procured.

The research reported on below, was undertaken to endeavour to check on any differences in extracts prepared from glands of different ages and to compare the results obtained with factors mentioned by Rowntree and his associates.

Plan of Investigation
The work was divided into the following parts.
1. Determination of the iodine reducing titre of extracts prepared by Steinberg's method (258) from glands of different ages.
2. Analysis of the extract for glutathione, cysteine and ascorbic acid.
3. Determination of the nitrogen distribution in the extract.

Methods of analysis for glutathione, cysteine and ascorbic acid

Glutathione

Glutathione was first isolated by Hopkins (126) in 1921. He showed that it contained sulfur and at first believed it to be a dipeptide of cysteine and glutamic acid. Hunter and Eagles (131) threw some doubt on
this structure. A reinvestigation by Hopkins (I27) in which he obtained pure crystalline glutathione showed that it was really a tripeptide containing cysteine, glutamic acid, and glycine. This was soon confirmed by Kendal, McKenzie and Mason (I45).

Glutathione exists in two forms, an oxidized and reduced form, which have the same relation to each other as cystine to cysteine, being the disulphide and sulphydryl forms respectively. In slightly acid, neutral or basic solution, especially in the presence of heavy metals, the reduced form rapidly absorbs oxygen becoming converted to the oxidized form. This reaction is very slow in strongly acid solutions.

The first method put forward for determining the amount of glutathione in biological fluids was Tunncliffe's iodine titration method (279). He used nitroprusside as an external indicator. This procedure was soon modified (79, I72, 201) by the addition of potassium iodide so that starch could be used as an internal indicator. There have been other variations of the iodine method involving potassium iodate, ferricyanide, etc. to release iodine from potassium iodide (77, I22, I89, 298). Mason (I72) however pointed out that the method which is excellent for the pure substance may be invalidated by other tissue constituents.

Mason, therefore, suggested a new method based upon the oxidation of glutathione by potassium ferricyanide. An equivalent amount of ferrocyanide is formed which is estimated colorimetrically as Prussian blue (I72). This method, if the pH was carefully controlled, was said to be more specific than the iodine method.

Another method of analysis measured the red color which develops when sodium nitroprusside and alkali react with GSH. Bierich and Rosenbohm
(22) reported lower values than the iodine method gives. Maloef (167) stated that of all tissue constituents only glutathione and cysteine give the reaction but Hamzaett and Chapman (107) found that the method was unreliable for quantitative estimations.

In 1927 Hunter and Eagles (132) applied a modifed form of Folin and Looneys cystine determination (72) to the estimation of glutathione in pure solution. The method involved comparing the blue color formed by reducing the special phosphotungstic acid reagent in the presence of sodium hydroxide with a cystine standard. The reagent was phospho-18-tungstic acid (ordinary phosphotungstic acid is phospho-24-tungstic acid). Shinohara and Padis in a further study of this method found that glutathione hydrolysed slowly giving cysteine and thus caused discrepancies. Glutathione may therefore be estimated as cysteine, but the two cannot be differentiated by this method when they are mixed in solution (251). The method is thus of limited value in dealing with tissue fluids or extracts.

Benedict and Gottschall (14) used an arsenophosphotungstic acid reagent which was reduced by glutathione with the formation of a blue color.

In 1934 Binet and Weller put forward a very convenient method in which reduced glutathione was precipitated by cadmium lactate at a definite pH. The mercaptide so formed was soluble in acid whereas the GSH was set free and was estimated by a simple iodometric titration. Careful pH control was necessary, the precipitation not being complete at a lower pH than 6.8 while an alkaline medium caused a rapid oxidation of the glutathione. Oxidised glutathione could be determined by first reducing it with cyanide. Cysteine interfered with the test. It was,
however, precipitated completely at pH 6.4 and thus could be removed prior to the glutathione determination (24,25,26,27).

An extremely specific micromethod for the estimation of glutathione depends upon a function of the latter as the "activator" for the enzyme, glyoxalase, which converts methylglyoxal to lactic acid. As a matter of fact the substrate of the enzyme is not methylglyoxal but a compound formed from methylglyoxal and glutathione, the latter being set free as a result of the enzymic reaction. The amount of "activation" is dependent, within a certain range, on the concentration of glutathione. The range is of low concentrations of glutathione. The enzymic activity was determined by measuring the rate at which carbon dioxide was evolved from carbonate by the formation of lactic acid (Woodward 297). A later modification was to titrate the unchanged methylglyoxal with bisulfite (236). Disadvantages of the method would appear to be the difficulty of getting the pure enzyme (a preparation of washed yeast was used), the liability of the enzyme to poisoning (233) and the difficulty of preparing methylglyoxal. An enzymic method is not one which arouses confidence.

Quensel and Wachholder put forward an indirect method of estimating glutathione. The total iodine reducing titre of the solution was determined before and after destroying glutathione with dilute formaldehyde (206,287). This procedure was originally derived for making a correction for ascorbic acid. The presence of cysteine in the solution would cause a discrepancy since it, too, reacts with formaldehyde (124, 164,207).

Another method was based on the reaction

$$2 \text{GSH} + S \rightarrow \text{GSSG} + \text{H}_2\text{S}$$
The GSH containing extract was digested with elemental sulfur in the presence of hydrocyanic acid. The hydrogen sulfide formed was determined iodometrically. Any other substance having a free sulphydryl group (notably cysteine) interfered. Ergothionine did not interfere. Results obtained by this method on potatoes and liver tissue were lower than those obtained by simple iodometric titration and were in good agreement with those obtained using the Binet and Weller cadmium lactate precipitation method (97).

The foregoing review reveals that only one of the methods suggested for the analysis of glutathione is specific for that compound. That method is Woodward's glyoxalase activation method. The other methods depend on the reducing power or some other chemical property similar to that possessed by other substances likely to be present in biological fluids.

Cysteine

Until Mueller isolated methionine in 1922, cysteine with its reversibly oxidised form, cystine, were the only known naturally occurring sulfur containing amino-acids.

As with glutathione, the earliest method of estimating the concentration of cysteine in solutions depended on its ability to reduce iodine (189, II, I60, I23, I55). But aside from its obvious non-specificity, Buckford and Schoetzow (37) showed that cysteine reacted with iodine in other ways than to form cystine as had been previously assumed.

The formation of a reddish color with sodium nitroprusside has been widely used for detecting sulphydryl compounds, but in quantitat-
The best and most specific color test for cysteine is that discovered by Sullivan in 1926 (261). On the addition of sodium I, 2-naphthoquinone-4-sulfonate to a strongly alkaline solution containing cysteine, and in the presence of cyanide and sulfite, a reddish brown color develops. This is converted to a purer red by the addition of sodium hyposulfite (Na$_2$S$_2$O$_4$) while any color given by other substances is discharged. A large number of other compounds have been tested and found to be negative while cysteine alone gives a positive reaction (262,264,267, I24). According to Sullivan and Hess (263) the three unsubstituted groups -SH, -NH$_2$ and -COOH are necessary. This condition is found only in cysteine and compounds of the same configuration in the aliphatic series. The latter are not known to occur in animal or vegetable tissue.

A modification of the method was suggested by Lugg (163) who, to maintain the same pH in assay and standard, swamped both with considerable amounts of glycine. The original Sullivan procedure, however, called for the addition of enough sodium hydroxide to raise the pH very high and Lugg's modification was said to be much less sensitive (266).

Based on the discovery that cystine would reduce the uric acid reagent of Folin and Denis (71) to form a blue solution, Folin and Looney developed a quantitative test for cystine in protein hydrolysates (72). The reagent is phospho-I$_8$-tungstic acid. Cystine itself is unaffected by the reagent but in the presence of sodium sulfite is reduced to cysteine which gives the reaction.

Many modifications of this procedure have been made. The
method has been studied by Lugw (161,162) and especially by Shinohara (245,246,247,248,249,251,252). The latter with his associates thoroughly investigated the nature of the color reaction and developed procedures for independently determining cystine, cysteine and ascorbic acid. They were unable to include glutathione in the scheme (as has already been stated) and this substance interfered if present. The methods they devised were accurate though tedious. A photometric method has also been used (141).

Besides the above there have been other procedures put forward from time to time. None have come into widespread use. Fleming (69) found that cysteine reacted with dimethyl-p-phenylene-diamine hydrochloride when heated in the presence of a small amount of ferric chloride to give a stable deep blue color.

Dyer and Baudisch (55,13) reported a degree of specificity in the reaction between cysteine and o-benzoquinone, similar to that with Sullivan’s naphthoquinone. When an aqueous solution of cysteine was shaken with a chloroform solution of o-benzoquinone, a deep red color developed in the chloroform layer. There are, however, other substances which give the reaction or inhibit the color, including glutathione (265).

Shinohara and Kilpatrick described a test in which a cobalt complex of cysteine was formed and its brownish yellow color measured in a colorimeter (250).

Cysteine has been precipitated from a tissue extract with cuprous mercaptide. After removing the copper, the cysteine was determined by Sullivan’s method (231).

As outlined above, it is easily seen that of all the
methods put forward for the analysis of cysteine, that of Sullivan is the most specific and has the widest application. It is for these very reasons that its use has been far more widespread than that of any other suggested procedure.

Ascorbic acid

The isolation of the antiscorbutic vitamin C was first reported by Waugh and King (291,292) in 1932. They identified it with the "hexuronic acid" isolated in 1928 by Szent-Gyorgyi as a tissue respiratory factor (270). By the above and many other investigators it had been recognized that the vitamin was easily destroyed by mild oxidation.

Its structure was worked out by 1933 and shown to be

\[
\begin{align*}
\text{C} & \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \quad \text{C} \\
\text{O} & \text{OH} \quad \text{OH} \quad \text{OH} \quad \text{OH}
\end{align*}
\]

(65,121,140,182). But before this was established it had been synthesized by Reichstein (203). The early isolation, synthesis and development of practical methods of assay have greatly stimulated the study of vitamin C so that the literature dealing with it is very large.

Before its relationship with the known sugars had been worked out Haworth and Szent-Gyorgyi suggested the name ascorbic acid. The name ceventamic acid, has also been used.

The first reagent for estimating the antiscorbutic potency of foodstuffs was phosphotungstomolybdic acid, \((\text{MoO}_3(\text{WO}_3)\text{P}_2\text{O}_5\text{H}_2\text{O})_{24})\) introduced by Bezssonoff (18). It is unsatisfactory in some cases (142) and gives higher values than some later methods (238). Many other reducing substances also give the color.

In 1927 it was reported by Zilva that antiscorbutic
solutions would rapidly reduce phenolindophenol to its leucobase (301,362). He considered that the reducing agent was not the antiscorbutic factor but that the latter was stabilized by the former. He was misled in this connection by the fact that the reversibly oxidised form of ascorbic acid, dehydroascorbic acid, is capable of preventing scurvy but does not reduce indophenol.

That the reduction of a derivative of phenolindophenol, 2,6-dichlorophenolindophenol, appeared to run parallel to the vitamin C content as determined by biological assay, was first reported by Tillmans, Hirsch and Hirsch in 1932 (278). It was confirmed by Harris and his associates (30,117) and by Bessey and King (17). The use of this reagent for the analysis of ascorbic acid has subsequently been very widespread (16).

The reduction of the indicator by ascorbic acid is represented in the following equation.

Since this method involves the reduction of the indophenol dye by ascorbic acid, many other substances having a lower reducing potential than the reagent are possible sources of interference. It has been found, however, that most of such substances found naturally react at a much-slower rate than ascorbic acid which reacts practically instantaneously (9,17,19,184,288,303). Reduction by phenols, tannins, glutathione and many other substances was eliminated by titrating in acid.
solutions of about pH 3 (23). Cysteine however reduces the dye appreciably even at this acidity, though it reacts slower than does ascorbic acid.

Unknown reducing materials are reported to interfere with the test in nerve tissue (2I,299), in the eye lens and aqueous humor (29) and in cancer tissues (34,56,II6,144). Various substances formed in heating alkaline sugar solutions such as reductone and reductic acid (17) reduce the dye as rapidly as ascorbic acid. Beer, malt and yeast also contain substances which seriously interfere with the test (73,II5). Emmerie and van Eekelen (62,63) found that some interfering substances including cysteine, ergothionine and thiosulfate could be removed with mercuric acetate. It was necessary to remove mercury with hydrogen sulfide and then to eliminate excess HgS. The extra steps involved uncertainty and there was danger of losing some ascorbic acid.

In spite of the weaknesses reviewed above and the consideration, important from the nutritional standpoint, that some of the vitamin might be in reversibly oxidised form and thus undetectable by indophenol but available physio-logically, this method has been almost universally adopted. Other suggested methods have been less specific or less straight forward.

The indophenol method has been extended to such a micro scale by Glick and Biskind using the Linderstrom Lang technique that the ascorbic acid content of a single cell may be estimated (33,84,85).

A convenient and accurate modification of the method has been the use of a photo-electric colorimeter to measure the decrease in color intensity of an excess of the dye on adding the ascorbic acid solution (15,68,185). Since the reaction of ascorbic acid with the dye is
almost instantaneous, a correction could be made for other reducers by extrapolating the reading curve to zero time. Turbid and colored solutions could also be analysed.

Melville and Richardson (179) suggested using the oxazine dye, prune, as the indicator. Theoretically, it should be better than indophenol, since it represents the limit to which the oxidising agent may be weakened and still permit successful titration. King (147) however, reported that it was not as satisfactory as indophenol.

Another alternative method is based on the decoloration of methylene blue (78) by ascorbic acid but this method has not been adopted to any extent.

Folin’s uric acid reagent, phospho-15-tungstic acid (71) has also been used for the estimation of ascorbic acid (177,251,252). The interference of thio/ compounds (cysteine, glutathione etc.) was prevented by the use of formaldehyde (172,177) or mercuric chloride (251). This test was similar to that of Bezssonoff.

Sodium tungstate has also been used, a sky-blue color developing on its reduction (283), but this method has been severely criticised (285).

The action of formaldehyde in destroying interfering sulphydryl compounds in an iodometric titration has been used (289).

Tauber and Kleiner (274) introduced a method in which ferricyanide was reduced and the extent of reduction estimated by adding ferric gum ghatti reagent and determining the Prussian blue so formed colorimetrically. The color was stable and the reagents were easily prepared. Glutathione did not reduce the ferricyanide if the pH is less
than 2 but tannins and other phenols and cysteine did reduce it.

Spent-Gyorgyi (270) found that the adrenal cortex reduced silver nitrate and ascribed this property to its vitamin C content.

Harde (II3) developed an analytic method on this reaction but its value has been questioned, some investigators considering it useless (31,269, 285). Giroud and Leblond (81) found that a positive test was very specific for ascorbic acid, but that a negative test did not prove its absence owing to the presence of factors inhibiting the reduction.

The dark violet color of a ferrous-ascorbic acid complex has been used in a colorimetric test by Szent-Gyorgyi (272). The formation of the complex was said to depend on the -HOC : COH- grouping.

Scudi and Ratish (238) based a method on the reduction of diazotized sulfanilamide in acid solution. They claimed that cysteine did not interfere.

It has been reported by Sevine (263) that the reduction in the cold of an acidified solution of sodium selenite to free selenium was a specific reaction of ascorbic acid.

Roe (219,220) has described a method whereby ascorbic acid was converted by boiling with hydrochloric acid into furfural. The latter was determined with aniline. It is doubtful if this procedure can be standardized to give reproducible results.

In 1930 Szent-Gyorgyi (271) obtained an enzyme from cabbage capable of oxidising "hexuronic acid". Tauber, Kleiner and Mishkind (275) prepared a similar oxidative enzyme from the pericarp of squash which they claimed was specific for ascorbic acid. Others have also prepared this enzyme (153,257), which has been called ascorbic acid oxidase. Its
Its application to the analysis of ascorbic acid involved iodine or indophenol titrations before and after treating a solution with the enzyme (257,275). Doubt, however, has been thrown on the specificity of the enzyme (137,255). The enzyme has not been obtained in the pure form and it has been claimed that the oxidation of ascorbic acid was due to the catalytic action of traces of copper (42,128,146,259,260).

A spectroscopic method involved measuring the intensity of the absorption band of ascorbic acid at 265 μm. The extent of interference was estimated by destroying the acid by the addition of copper salts and remeasuring the band (218).

Another physical method for use in cases where extraneous reducers were absent was the addition of copper ions to a solution in which ascorbic acid was being electrolysed using a dropping mercury anode. A polarographic "wave" appeared in the current voltage curve at -1.60 volts (from a calomel zero)(149).

All the methods proposed for the determination of ascorbic acid have various disadvantages. Of the methods for which specificity was claimed, the enzymic procedure is uncertain, the spectroscopic and polarographic require expensive equipment. In the other, chemical tests, at least cysteine, if not other substances also, interferes by reducing the reagents used. It is probable that a completely specific method cannot be based on the reducing powers of ascorbic acid. But of those which are so based, and which, as has been seen, includes practically all, there is none so straight forward and so simple as Tillman's 2, 6-dichlorophenolindophenol method. It is rather strange that in a decade of research, no other method has been devised which can compare with regard to ease and accuracy with a test introduced early in vitamin C research before the
vitamin had been isolated or any of its physical or chemical properties established.
EXPERIMENTAL WORK

I. Preparation of the Extract

Thymus glands of calves were obtained as soon as possible after killing and were placed on "dry ice" until ready to be extracted. They were then allowed to partly thaw out, were cut into small pieces with a chisel and put through a meat grinder. The tissue was weighed and placed in a 1 liter round-bottomed flask. An amount of 1% hydrochloric acid was poured over it so that there was 1.0 cc. of acid for each 1.5 gr. of gland.

The mixture was stirred in the cold for about 10 minutes after which the temperature was raised rapidly to 68°C, stirring the while with a stirrer run by a small electric motor. The mixture was then cooled with running water. The flask and its contents were left overnight in the cooler to allow the greater part of the sediment settle on the bottom. The extract was filtered off through a Buchner funnel using suction.

It was kept in pyrex rubber-stoppered flasks in the cooler, 0.2% chlorbutanol being added as a preservative.

Determination of the total I.R.T.

A 10cc. portion of extract was acidified with 0.5cc. concentrated hydrochloric acid, after which 10cc. of 10% phosphotungstic acid were added dropwise with stirring. The mixture was cooled for 10-15 minutes. The precipitate was filtered off and washed thoroughly with 1% hydrochloric acid, the washings being caught in the filtrate.

1cc. of fresh 5% KI solution and 6 drops of 5% starch solution
were added to the filtrate. 0.001 N \( \text{KI}_3 \) solution was added in excess from a burette. The blue color so formed was discharged with standard 0.001 N \( \text{Na}_2\text{S}_2\text{O}_3 \) solution and further \( \text{KI}_3 \) was run in to the reappearance of a blue tint, stable for one minute.

The i.r.t. was calculated as mg.% glutathione, by the following formula,

\[
\text{I.R.T.} = \frac{V \times N \times M \times 100}{E}
\]

where \( V \) is the total volume of \( \text{KI}_3 \) used less the equivalent of the \( \text{Na}_2\text{S}_2\text{O}_3 \) added,

\( N \) is the normality of the \( \text{KI}_3 \),

\( M \) is the molecular weight of glutathione (307),

and \( E \) is the volume of extract used.

The I.R.T.s of extracts prepared from glands of calves of varying ages were measured and are tabulated below.(page 48a)

2. Analysis for Glutathione

I. Formaldehyde method

Griffin (94) tentatively used Wachholder's method which involved destruction of glutathione by formaldehyde, and comparing the iodine reducing value of the solution with a sample not so treated with formaldehyde. The applicability of this method was checked by determining its effect on cysteine and ascorbic acid.

Cysteine was destroyed completely after standing one hour in a solution containing about 4% formaldehyde.

The loss of ascorbic acid under similar conditions was found to be 31.7% as compared with a control titration.
### TABLE SHOWING VARIATION IN I.R.T. IN EXTRACTS FROM GLANDS OF DIFFERENT AGES

<table>
<thead>
<tr>
<th>AGE</th>
<th>FETAL 2-8 months</th>
<th>WEEKS</th>
<th>MONTHS</th>
<th>MONTHS</th>
<th>MONTHS</th>
<th>YEARS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 5 6</td>
<td>2 3 4</td>
<td>6 8 10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>I.R.T. Mg%</td>
<td>114</td>
<td>245 180#</td>
<td>150# 145#</td>
<td>224 169#</td>
<td>180##</td>
<td></td>
</tr>
<tr>
<td>GSH</td>
<td>165</td>
<td>205 242</td>
<td>243</td>
<td>269</td>
<td>206##</td>
<td></td>
</tr>
<tr>
<td></td>
<td>158</td>
<td>230</td>
<td>209</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>156</td>
<td></td>
<td>198</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>181</td>
<td></td>
<td>219</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average I.R.T.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in the different age groups</td>
<td>155</td>
<td>219</td>
<td>202</td>
<td>199</td>
<td>193</td>
<td></td>
</tr>
</tbody>
</table>

*This series of extracts with low i.r.t. values were made up at one time and had been treated similarly.*

*These glands were much involuted. The thymic tissue had to be separated from much fat and connective tissue.*
The method outlined for determining the total iodine reducing titre could not be employed because of the reaction between thiosulfate and formaldehyde. The end-point was therefore the appearance of the blue color on running in the standard KI0₃ solution the first time.

2. Cadmium lactate precipitation method

The Binet and Weller method which precipitated glutathione as a cadmium lactate mercaptide was employed.

Reagents

- Trichloracetic acid: a 10% solution
- Metaphosphoric acid: a 5% freshly prepared solution
- Cadmium lactate: a saturated solution. The cadmium lactate was prepared by dissolving cadmium hydroxide (from cadmium sulfate and sodium hydroxide) in lactic acid. The solution was evaporated and cooled to crystallize out the cadmium lactate. It was dissolved in water and recrystallized once. The crystals were then thoroughly washed with acetone until the washing liquid was neutral.
  - Sodium hydroxide: 0.5N and 0.1N solutions
  - Phosphoric acid: a 10% solution

Procedure

To 5cc. of extract, 5cc. of 10% trichloracetic acid was added. After stirring and cooling, the protein precipitate was centrifuged down.

One cc. of 5% metaphosphoric acid was added, followed by 2cc. of cadmium lactate solution (#).

# If total glutathione (oxidised as well as reduced) was to be determined

1cc. of fresh 5% potassium cyanide solution was added to the deproteinized
The solution was neutralized to pH 6.8 (not over 7.0) and allowed to stand overnight. A white precipitate formed immediately.

This precipitate was centrifuged down, the supernatent was discarded, the precipitate was washed with alcohol and dissolved in 10% phosphoric acid. The glutathione content was determined by iodometric titration in the manner earlier described.

**Interference of cysteine**

Binet and Weller removed cysteine by a preliminary precipitation at pH 6.4 in spite of the fact that glutathione had started to precipitate at this point.

The loss of glutathione after three hours standing at pH 6.4 was estimated by comparing the iodine reducing titre of the precipitate, calculated as cysteine, with the cysteine content of the extract as determined by Sullivan's method (see page 51).

Table showing cysteine content if extract estimated by different methods.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Cysteine content by Sullivan's Method Mg% (Average)</th>
<th>Cysteine Content by Cd Lactate Method Mg% (Average)</th>
<th>Reduced Glutathione Content by Cd Lactate Method after ppting. cysteine. Mg% (Average)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>5.4</td>
<td>7.0</td>
<td>19.2</td>
</tr>
<tr>
<td>2.</td>
<td>7.0</td>
<td>7.8</td>
<td>12.9</td>
</tr>
<tr>
<td>3.</td>
<td>5.2</td>
<td>6.4</td>
<td>14.4</td>
</tr>
</tbody>
</table>

extract which was then allowed to stand for thirty minutes before adding metaphosphoric acid and proceeding with the determination.
Because of this loss of glutathione in the cysteine precipitate, both substances were brought down together at a pH of 6.3. To get the glutathione content of the extract the equivalent of the cysteine content found was subtracted from the total value obtained.

Since pure glutathione was not available or easily prepared, the method has not been checked.

3. Analysis for Cysteine

The procedure adopted was the specific Sullivan colorimetric method. The standardized procedure closely follows that proposed by Sullivan.

Apparatus

Colorimeter The instrument used was a Klett-Summerson photoelectric colorimeter, made by the Klett Manufacturing Company of New York.

Reagents

Sodium cyanide. A 1% solution in 0.8N sodium hydroxide. This solution is stable for several weeks.

Sodium sulfite. A 10% solution (anhydrous salt) in 0.5N sodium hydroxide. This solution is stable for months.

Sodium hyposulfite (or hydrosulfite) (Na₂S₂O₄·2H₂O). A 2% solution in 0.5N sodium hydroxide. This solution must be freshly prepared a few minutes before using.

Sodium hydroxide. A 5N solution.

I, 2-Naphthoquinone-4-sodium sulfonate. A 1% aqueous solution, freshly prepared.
Procedure:

To 5cc. of the test solution in a colorimeter tube the following reagents were added in order. The contents of the tube were mixed after each addition by inverting the tube.

First, 2c.c. of sodium cyanide solution, followed by 1c.c. of the naphthoquinone reagent and finally 5c.c. of sodium sulfite solution. The tube was then allowed to stand for thirty minutes at a temperature of 20-25°C.

In the case of pure solutions of cysteine, the solution was then diluted with 2c.c. water, and in the case of the extract, with 2c.c. 5M sodium hydroxide to get a high alkalinity in the highly buffered solution.

At the end of the thirty minute period, 1c.c. of sodium hyposulfite solution was added and mixed. The tube was left to stand for ten minutes before the color intensity was measured in the colorimeter. This ten minute period was found necessary to permit the color to become fairly stable.

The cysteine content of the solution was read off a color intensity-concentration graph prepared using solutions of known concentrations of cysteine hydrochloride in 1% hydrochloric acid.

The graph is herewith included.

When the naphthoquinone was added, the solution unless it was a pure solution of cysteine, turned a dirty brown or black color. The addition of sodium hyposulfite removed this dark color leaving the solution a pure red color, or orange in very dilute solutions of cysteine.

Extent of interference by ascorbic acid

To determine whether or no ascorbic acid interfered with the
development of the color, varying amounts of it were added to solutions of known cysteine content, which were then treated by Sullivan's method.

A brown solution resulted on the addition of naphthoquinone. This was apparently completely cleared by the hyposulfite leaving only the bright red caused by cysteine.

Table showing recovery of cysteine from solutions containing ascorbic acid.

<table>
<thead>
<tr>
<th>Cysteine Content Mg./5 c.c.</th>
<th>Ascorbic Acid Added Mg./5 c.c.</th>
<th>Cysteine determined Mg./5 c.c. (Average)</th>
<th>Percentage Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15</td>
<td>0.5</td>
<td>0.17</td>
<td>113</td>
</tr>
<tr>
<td>0.15</td>
<td>1.0</td>
<td>0.18</td>
<td>120</td>
</tr>
<tr>
<td>0.15</td>
<td>1.5</td>
<td>0.18</td>
<td>120</td>
</tr>
<tr>
<td>0.15</td>
<td>2.0</td>
<td>0.17</td>
<td>113</td>
</tr>
<tr>
<td>0.15</td>
<td>2.5</td>
<td>0.175</td>
<td>116</td>
</tr>
<tr>
<td>0.15</td>
<td>3.0</td>
<td>0.145</td>
<td>96</td>
</tr>
<tr>
<td>0.25</td>
<td>0.5</td>
<td>0.24</td>
<td>96</td>
</tr>
<tr>
<td>0.25</td>
<td>1.0</td>
<td>0.24</td>
<td>96</td>
</tr>
<tr>
<td>0.25</td>
<td>1.5</td>
<td>0.26</td>
<td>104</td>
</tr>
<tr>
<td>0.25</td>
<td>2.0</td>
<td>0.25</td>
<td>100</td>
</tr>
<tr>
<td>0.25</td>
<td>2.5</td>
<td>0.24</td>
<td>96</td>
</tr>
<tr>
<td>0.25</td>
<td>3.0</td>
<td>0.23</td>
<td>92</td>
</tr>
</tbody>
</table>

Recovery of cysteine added to extract

In order to test the applicability of the method to thymus extract, cysteine was added to portions of the extract. Its cysteine content was determined before and after such addition.
Table showing recovery of cysteine added to thymus extract.

<table>
<thead>
<tr>
<th>Cysteine content of extract</th>
<th>Cysteine added Mg./5c.c. (Average)</th>
<th>Colorimeter Reading</th>
<th>Cysteine Determined Mg./5c.c.</th>
<th>Recovery of added cysteine Mg./5c.c.</th>
<th>Percentage Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.11</td>
<td>0.06</td>
<td>80</td>
<td>0.165</td>
<td>0.055</td>
<td>92</td>
</tr>
<tr>
<td>0.11</td>
<td>0.06</td>
<td>82</td>
<td>0.175</td>
<td>0.065</td>
<td>108</td>
</tr>
<tr>
<td>0.10</td>
<td>0.11</td>
<td>93</td>
<td>0.225</td>
<td>0.125</td>
<td>114</td>
</tr>
<tr>
<td>0.10</td>
<td>0.11</td>
<td>92</td>
<td>0.22</td>
<td>0.12</td>
<td>109</td>
</tr>
</tbody>
</table>

4. Analysis for Ascorbic Acid

The direct titration of an ascorbic acid solution with a standard solution of 2, 6 dichlorophenolindophenol or, conversely, the titration of a measured quantity of the dye with the ascorbic acid solution both of which methods have been widely used, was found to be unsatisfactory from the point of view of accurate estimation.

In the first place, the end-point was very vague owing to the weak color and to the formation of brownish substances as a result of the reaction between the indophenol and the acid. No concentration of dye or of ascorbic acid would give a satisfactory end-point. In the second place, the time taken for the titration would increase the effect of interfering substances, especially cysteine, if they were present, as would be the case in titration of the extract.

Recourse was therefore made to a procedure involving the use of a photoelectric colorimeter. In this method, visual titration was replaced by an objective photoelectric measurement of the amount of dye decolorized when a measured amount of an ascorbic acid solution reacted with an excess
of dye, the initial depth of color of the dye being measured before hand.
Since greater dilutions were used, the brown reaction products did not
become troublesome and, in fact, could not be detected. Also the
determination could be effected in ten to fifteen seconds, thus minimizing
errors due to the presence of other dye reducing substances.

Since strongly acid solutions decolorize the dye to a slight
extent, the readings were made in a sodium acetate buffer so that the pH
was 3.0 to 3.5. This pH was not low enough to cause significant fading of
the dye and yet was not so high as to increase seriously the rate at which
other reducing substances react with the dye.

**Apparatus**

The photoelectric colorimeter used was the Klett previously
described.

A 9c.c. rapid-delivery pipette made by cutting the lower end
off a 10c.c. pipette and recalibrating to deliver 9c.c.

**Reagents**

2, 6-dichlorophenolindophenol solution, approximately 0.05 gr.
per 100 c.c. of water. This solution was filtered. It would keep several
weeks if stored in a dark glass bottle. Dilution of this solution 1:10
registered about 150 on the colorimeter in a blank determination using 1c.c.
of 1% hydrochloric acid.

Sodium acetate. A solution of 10 gr. of NaC\textsubscript{2}H\textsubscript{3}O\textsubscript{2}·2H\textsubscript{2}O in 100 c.c. of water
was made up. A few drops of toluene were added to ensure its keeping.

Dye-acetate solution. The indophenol so diluted and mixed that 9c.c. of
the dye-acetate solution and 1c.c. of an acid solution containing 0.5% HCl and 2.5% HPO₃ gave a reading of about 150 on the colorimeter and had a pH of 3.0-3.5.

Ascorbic acid solutions of known concentration in 1% HCl.
Metaphosphoric acid. A freshly prepared 5% solution.
Hydrochloric acid. A 1% solution.

Procedure

The galvanometer of the colorimeter was set at zero using a tube filled with distilled water.

The initial optical density of the dye was determined by placing 1 c.c. of an acid mixture of 0.5% HCl and 2.5% HPO₃ in a colorimeter tube, running in 9c.c. of the dye-acetate solution, inverting the tube once, immediately placing the tube in the colorimeter and taking the reading.

The colorimeter must have been switched on and allowed to warm up not less than an hour before making the determination. It must also be protected from draughts, bunsen burners etc., since a temperature change would affect the reading.

The rapid addition of 9c.c. of the dye-acetate solution to 1c.c. of test solution was supposed to ensure complete mixing. Some difficulties experienced at first were found to arise from incomplete mixture, variations in the color, often hardly distinguishable, occurring from one part of the tube to another. The final procedure therefore called for inversion of the tube prior to taking the reading.
A tube containing 10 c.c. of test solution and 90 c.c. distilled water was placed in the colorimeter and the light filter was adjusted for any color or turbidity in the solution, the galvanometer needle being brought back to zero.

The final optical density was then determined in the same manner as above, the reading being made as soon as possible.

The relationship between the amount of dye decolorized and the concentration of ascorbic acid was given by the following equation.

\[ X = K \left( L_1 - L_2 \right) \]

where \( X \) is the concentration of ascorbic acid

\( L_1 \) is the initial optical density of the dye

\( L_2 \) is the final optical density of the dye, and

\( K \) is a constant, depending on the type of colorimeter and the quantities of reagents used.

**Determination of **\( K \)**

The ascorbic acid solutions in \( \frac{1}{10} \) HCl were diluted with an equal volume of \( \frac{5}{7} \) HPO\(_3\). This final concentration of ascorbic acid is \( X \). (see table page 58).

**Application of the method to the analysis of thymus extract.**

1. Precipitation of protein.

The following protein precipitants were investigated, trichloracetic acid, tannic acid, metaphosphoric acid and phosphotungstic acid.

(i) Trichloracetic acid slowly faded the dye and, at concentrations necessary for protein precipitation, about \( \frac{5}{7} \), was found to destroy ascorbic acid.
Table showing values of K determined

<table>
<thead>
<tr>
<th>K in mg. %</th>
<th>L₁ (ό)</th>
<th>L₂ (ό)</th>
<th>K</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.955</td>
<td>151</td>
<td>125</td>
<td>0.15</td>
</tr>
<tr>
<td>6.59</td>
<td>151</td>
<td>106</td>
<td>0.15</td>
</tr>
<tr>
<td>7.325</td>
<td>151</td>
<td>103</td>
<td>0.15</td>
</tr>
<tr>
<td>8.225</td>
<td>144</td>
<td>90</td>
<td>0.15</td>
</tr>
<tr>
<td>10.99</td>
<td>151</td>
<td>77</td>
<td>0.14</td>
</tr>
<tr>
<td>13.56</td>
<td>144</td>
<td>39</td>
<td>0.13</td>
</tr>
<tr>
<td>14.65</td>
<td>151</td>
<td>34</td>
<td>0.125</td>
</tr>
<tr>
<td>15.40</td>
<td>130</td>
<td>17</td>
<td>0.14</td>
</tr>
<tr>
<td>16.45</td>
<td>144</td>
<td>7</td>
<td>0.12</td>
</tr>
</tbody>
</table>

These values are averages of not less than three determinations.

Average value for K is 0.14.

(ii) Tannic acid reduced the dye very rapidly.

(iii) Metaphosphoric acid did not affect either the dye or the ascorbic acid. It has, in fact, been recommended for stabilizing the latter (188).

The ordinary commercial metaphosphoric acid was found to be a very inefficient protein precipitant, so it was treated after Folin (70) as follows:

200 grams of commercial metaphosphoric were fused with 2 grams of potassium nitrate in a crucible. Heating was continued until all water had been driven off and fumes of phosphorus pentoxide began to appear. The slag was skimmed off, and the melt was poured into tin plates (It stuck fast to porcelain) and when cool was broken into pieces and stored in a
tightly stoppered bottle. The glassy product had to be ground very fine since it dissolved with difficulty.

(iv) Phosphotungstic acid did not affect the dye and appeared not to affect the ascorbic acid though a blue color developed in the solution when it was mixed with the latter.

(3) Recovery of ascorbic acid added to extract

A portion of extract was deproteinized by adding dropwise an equal volume of either 10% phosphotungstic acid or 5% metaphosphoric acid. After stirring and cooling the precipitate was centrifuged down. One c.c. portions of the supernatant solution were taken for testing.

To another portion of the same extract, ascorbic acid was added. It was then deproteinized like the foregoing.

Table showing recovery of ascorbic acid added to extract

<table>
<thead>
<tr>
<th>Conc. in Extract Mg.% (Average)</th>
<th>Am't added Mg.%</th>
<th>Protein Pptant Used</th>
<th>Am't. Recovered Mg.% (Average)</th>
<th>Percentage Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2</td>
<td>35.6</td>
<td>Phosphotungstic</td>
<td>40.3</td>
<td>101.4%</td>
</tr>
<tr>
<td>15.6</td>
<td>22.4</td>
<td>Metaphosphoric</td>
<td>37.6</td>
<td>97.5%</td>
</tr>
<tr>
<td>15.6</td>
<td>13.9</td>
<td>&quot;</td>
<td>30.7</td>
<td>105.1%</td>
</tr>
</tbody>
</table>

(2) Extent of interference by cysteine

Cysteine also reduces the indophenol reagent, and there is a small amount of cysteine in the extract. Cysteine hydrochloride was therefore added to a deproteinized extract to determine the extent to which it would interfere under the conditions of this procedure.
Table showing recovery of ascorbic acid from cysteine-enriched extract

<table>
<thead>
<tr>
<th>Ascorbic Acid Content before adding cysteine Mg.% (Average)</th>
<th>Cysteine added Mg.%</th>
<th>Ascorbic Acid Recovered Mg.%</th>
<th>Percentage Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.5</td>
<td>5.0</td>
<td>8.3</td>
<td>97.6%</td>
</tr>
<tr>
<td>8.5</td>
<td>9.5</td>
<td>7.3</td>
<td>85.7%</td>
</tr>
</tbody>
</table>

The amounts of cysteine added were equal or greater than the amounts probably already in the solution. Errors caused by the reduction of the dye by cysteine are not serious in this procedure.

Conclusion

The method outlined above for the estimation of ascorbic acid would seem to give results of fair accuracy when applied to thymus extract.

5. Analysis of Thymus Extract

Two portions of the extract were deproteinized, each with an equal volume, in one case, of 5% metaphosphoric acid, and in the other, of 10% trichloracetic acid. Ascorbic acid, cysteine and the total I.R.T. were determined in the metaphosphoric acid filtrate and glutathione in the trichloracetic acid filtrate. The results are tabulated in the following table, (page 61)

6. Determination of the Nitrogen distribution

The protein content was determined by a Kjeldahl method, the nitrogen content being measured before and after precipitating the protein (II9).
<table>
<thead>
<tr>
<th>Extract</th>
<th>Total I.R.T. Mg % GSH</th>
<th>Ascorbic Acid</th>
<th>Glutathione</th>
<th>Cysteine</th>
<th>Sum of Reduction of three Constituents %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Concentration Mg%</td>
<td>Percent of Total Reduction</td>
<td>Concentration Mg%</td>
<td>Percent of Total Reduction</td>
</tr>
<tr>
<td>1</td>
<td>208</td>
<td>37.2</td>
<td>62.6</td>
<td>51.9</td>
<td>24.95</td>
</tr>
<tr>
<td>2</td>
<td>204</td>
<td>39.2</td>
<td>67.25</td>
<td>62.3</td>
<td>30.5</td>
</tr>
<tr>
<td>3</td>
<td>174</td>
<td>28.8</td>
<td>57.9</td>
<td>47.9</td>
<td>27.5</td>
</tr>
<tr>
<td>4</td>
<td>178</td>
<td>27.4</td>
<td>53.9</td>
<td>67.3</td>
<td>37.8</td>
</tr>
<tr>
<td>5</td>
<td>180</td>
<td>34.2</td>
<td>58.6</td>
<td>71.35</td>
<td>34.1</td>
</tr>
<tr>
<td>6</td>
<td>198</td>
<td>26.8</td>
<td>47.4</td>
<td>60.75</td>
<td>30.7</td>
</tr>
<tr>
<td>7</td>
<td>219</td>
<td>36.8</td>
<td>58.7</td>
<td>70.5</td>
<td>32.2</td>
</tr>
<tr>
<td>Average</td>
<td>198.6</td>
<td>32.9</td>
<td>57.9</td>
<td>61.7</td>
<td>31.1</td>
</tr>
</tbody>
</table>
To 10c.c. of extract or filtrate in a Kjeldahl flask, 20c.c. concentrated sulfuric acid and 0.2 gr. copper sulfate were added. The mixture was heated until it became a light green, the fumes being removed by suction through a funnel over the mouth of the flask.

After allowing the flask to cool, 200 c.c. of water were added, followed by concentrated sodium hydroxide until the mixture was alkaline. The flask was cooled during this addition. Several pieces of granular zinc were added to prevent bumping, and a small piece of paraffin to check foaming.

The contents of the flask were distilled over into standard sulfuric acid, a Kjeldahl trap being inserted to prevent splashing over of any alkali. Excess sulfuric acid was titrated with standard sodium hydroxide solution using methyl orange as indicator.

The protein precipitants used were metaphosphoric acid and phosphotungstic acid.

All determinations were done in duplicate and average values were taken.

Table showing distribution of Nitrogen in Thymus extract.

<table>
<thead>
<tr>
<th></th>
<th>0.31 Gr. /100c.c. Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Nitrogen</td>
<td></td>
</tr>
<tr>
<td>Non Protein Nitrogen</td>
<td>0.29</td>
</tr>
<tr>
<td>HPO₃ Filtrate</td>
<td></td>
</tr>
<tr>
<td>Protein Nitrogen</td>
<td>0.02</td>
</tr>
<tr>
<td>Nitrogen in Phosphotungstic Acid Filtrate</td>
<td>0.07</td>
</tr>
<tr>
<td>Nitrogen in Phosphotungstic Acid Precipitate</td>
<td>0.24</td>
</tr>
</tbody>
</table>
DISCUSSION

This investigation was for the purpose of checking on any chemical difference between Miss Dale's extract and that prepared by Rowntree and his associates, which might account for the difference in biological effects. Unfortunately there were but few criteria to use in making the comparison. There were as follows, (1) the iodine reducing power of the extract, (2) the absolute content of, and the distribution of the reducing power between, the three reducing substances, glutathione, ascorbic acid and cysteine, and (3) the total nitrogen and the protein nitrogen content of the extract.

Before discussion these factors I would emphasize the fact that extracts made up by somewhat different methods have been reported as biologically active. Hanson, like ourselves, made use of solid carbon dioxide to preserve the glands from autolysis until the extract was to be made up. Rowntree, on the other hand, immediately ground the fresh gland into cold 1% acid which was then transported to the laboratory. With regard to the actual extraction, Hanson's original method involved a slow heating to 94-96°C while Steinberg's called for a rapid heating to 68°C. Hanson at first used menthol as a preservative. He wrote in 1938 that Rowntree used no preservative at all. Steinberg's recipe included the addition of chlorbutanol.

The only variation used by us and not reported as used by others was to leave the extraction mixture in the ice box overnight in order to facilitate filtration.

The only essential difference between our extracts and those of the other workers would appear to be the age of the calves used,
and this difference on first sight would not appear to be important. For calves at 3-4 months of age are still well removed from sexual maturity (over 12 months). Their thymi (to judge by analogy with human beings and rats) are probably still growing though at a slower rate relative to the growth of the animal than those of 2-6 weeks old calves. (Here one may recall the observation made above concerning the age of rats from which thymi were taken for the implanting experiments). However there was the possibility that the difference in age might show itself in some chemical difference in the extract.

A series of extracts was therefore made up from glands of varying ages and the results are tabulated on page 48a. Inspection of these reveals that there is a slight decrease in I. R. T. with an increase in the age of the gland. It may be questioned whether the variations are significant and in my opinion they are not so. It is seen that there is great variation in the values in each age group. Though the 4-6 weeks group had the highest average, the 6-10 months group (average about 9% less that 4-6 weeks) contained the highest value recorded. The titre of the full grown steers is of similar magnitude to the rest. Its slightly lower average is probably due to the time consumed in freeing remaining glandular tissue from large amounts of connective tissue and fat. It is probable that the I. R. T. of any animal's thymus is not a function of its age.

The second criterion was the amounts of the three reducing constituents. The results of my analyses of extracts of 3-4 months old thymi are in the table on page 61. These results with their comparative constancy and with the closeness to which the sum of the percentages of the total I. R. T. of the determined amounts of glutathione, cysteine and
ascorbic acid approach 100% are very gratifying. They are also similar to values reported from 2-6 weeks-old-thymus extracts by Schaffer, Ziegler and Rowntree.

For convenience in comparing the two sets of results, the following table is included (page 66). It gives the average values found by myself and the averages given in each of Schaffer, Ziegler and Rowntree's two tables. Since the latter include the values found in extracts which had aged up to 20 days, there are also tabulated the values found in four extracts which are more comparable with mine, whose analyses were complete within four days of the making up of the extracts.

Bearing in mind, great variations which may occur in the I. R. T. of extracts prepared in identical manner from different glands considerable variations in the content of the substances responsible for that variation would be expected. But the significant fact is that the percentage of the total I. R. T. of any one of the three substances determined lies within the same range both in Schaffer's results and in mine. This would indicate that the composition of the gland at the two ages is, in regard to iodine reducing substances capable of being set free by the conditions of extraction, very similar. I do not believe that any other conclusions may be drawn.

It is of great interest to note that Miss Dale's rats were receiving somewhat over half a milligram of glutathione daily. Now this is the amount of glutathione, with which Lee, as reported earlier, obtained an unmistakeable precocity of growth and development in the fourth consecutive generation of injected rats. It will also be remembered, however, that Rowntree could get similar precocity only with 2 mg. per day,
<table>
<thead>
<tr>
<th>Extract</th>
<th>Total I.R.T. Mg % GSH</th>
<th>Ascorbic Acid</th>
<th>Glutathione</th>
<th>Cysteine</th>
<th>Sum of Reduction of three Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Concentration Mg %</td>
<td>Percent of Total Reduction %</td>
<td>Concentration Mg %</td>
<td>Percent of Total Reduction %</td>
</tr>
<tr>
<td>Average (Smith)</td>
<td>198.6</td>
<td>32.9</td>
<td>57.9</td>
<td>61.7</td>
<td>31.1</td>
</tr>
<tr>
<td>(a) Average (S.,Z.&amp;R.)</td>
<td>149</td>
<td>25.0</td>
<td>57.8</td>
<td>43.3</td>
<td>28.9</td>
</tr>
<tr>
<td>(b) Average (S.,Z.&amp;R.)</td>
<td>132</td>
<td>21.3</td>
<td>51.9</td>
<td>50.8</td>
<td>40.7</td>
</tr>
<tr>
<td>Extracts (S.,Z.&amp;R.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. (1 day)</td>
<td>179</td>
<td>30.3</td>
<td>57.7</td>
<td>65.7</td>
<td>36.7</td>
</tr>
<tr>
<td>2. (9 days)</td>
<td>109</td>
<td>18.5</td>
<td>58.3</td>
<td>26.3</td>
<td>24.2</td>
</tr>
<tr>
<td>3. (1 day)</td>
<td>188</td>
<td>31.0</td>
<td>54.1</td>
<td>70.9</td>
<td>37.7</td>
</tr>
<tr>
<td>4. (6 days)</td>
<td>171</td>
<td>27.1</td>
<td>52.0</td>
<td>58.7</td>
<td>34.3</td>
</tr>
</tbody>
</table>
a result which may have been connected with the purity of his preparation. However when 1 mg. of ascorbic acid was also given, 1 mg. of glutathione was all that was required. Miss Dale’s rats received somewhat less than a third of a milligram of ascorbic acid daily. The failure of Miss Dale’s rats to respond even in the fifth generation increases the doubt with which one contemplates the results claimed by the group of investigators working around Rowntree.

With regard to the nitrogen content, Steinberg reported his to contain 0.43 grams per 100 cc. The protein content was practically negligible. My figures show (see page 62) that though the total nitrogen was only 0.31 grams %, 0.02 grams % were precipitated by metaphosphoric acid and are tabulated as protein nitrogen. This larger protein content may have been due to the practice of leaving the extract in contact with the tissue for up to twenty four hours. This larger amount of protein is the only difference of any magnitude between the factors reported of Rowntree’s extracts and of those used here.

The very large percentage of the nitrogen precipitated by phosphotungstic acid shows that a large part of it exists as various degradation products of proteins.
CONCLUSIONS

1. The I. R. T's. of thymus extracts have been determined, the content in these extracts of glutathione, ascorbic acid and cysteine have been estimated and the nitrogen distribution in the extracts has been indicated.

2. The values so obtained have been compared with values reported by L. G. Rowntree and his co-workers for the extracts they used. No differences in the composition of the extracts could be found which would account for the impotency of the one and the potency of the other to cause accumulative increase in the growth and development of succeeding generations of rats.
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