CHEMICAL AND PHYSIOLOGICAL STUDIES OF THE PARATHYROID HORMONE

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CHEMICAL AND PHYSIOLOGICAL STUDIES OF THE PARATHYROID HORMONE.

By

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THESIS

submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

McGill University,
Montreal.

April 20, 1931.

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PREFACE

The author wishes to take this occasion to express his sincere gratitude to Dr. J. B. Collip for his suggestions, unfailing interest, and encouragement.

The writer also wishes to acknowledge his debt to the National Research Council and to McGill University for financial aid, also for the material and the apparatus made available for this work.

JOHN ALLARDYCE

INTRODUCTION.

Discovery and Anatomical Arrangement of the Parathyroid Glands.

The parathyroid glands have been recognized in all classes of vertebrates with the exception of fishes (1). As a rule there are four distinct glands so situated with regard to the thyroid gland that they have been referred to as the superior or external and the inferior or internal pairs. Sandstrom (2) was the first to describe the superior pair. For some reason or other they were forgotten until eleven years later - 1891 - they were rediscovered by Gley (3). The inferior pair were described by Kohn (4) in 1895. Accessory parathyroid tissue is sometimes present and may be situated in the thorax nearer to or embedded in the thymus gland (1). The parathyroids develop as thickenings of the endoderm of the third and the fourth branchial clefts. The superior pair of glandules is related to the fourth branchial clefts with reference to development, while the inferior pair is similarly related to the third branchial clefts.

Theories of Parathyroid Function.

Raynard's work (5), in which he removed the thyroid gland from a number of dogs and frequently found that death resulted in the course of a few days, is probably the earliest record made bearing upon the physiology of the parathyroid glands.

Schiff (6), in 1859, removed the thyroid glands from a number of dogs, rabbits, rats, guinea pigs and fowls. Some of his animals survived while others died within a few days.

Following his discovery of the external pair of parathyroid glands in 1891, Gley carried out a series of experiments to demonstrate that the fatal results from thyroidectomy, as obtained by Schiff, were due to the removal of the parathyroids along with the thyroid.

By 1909, although some authors still considered the parathyroid glands as rudiments, or rather that they represented an embryonic condition of the thyroid which may assume the structure and function of the thyroid at times when the latter is overworked, the consensus of opinion favoured the treatment of the parathyroids as characteristic histological structures, and their function as physiologically distinct from that of the thyroid and quite indispensible. From the work of Vassale and Generali (7) and of Berkeley and Beebe (8), such a conclusion obtained strong support. Vassale

and

and Generali removed the external and internal pairs of parathyroids in a number of dogs and cats, disturbing the thyroid in each case as little as possible. Although their animals were all in good condition the day following the operation, they all died within ten days, the majority succumbing in three to five days. Berkeley and Beebe carried out a series of thyroidectomies, parathyroidectomies and thyroparathyroidectomies, and found fatal results only where the parathyroids had been removed.

Two theories have been set up to explain the function of the parathyroid glands, viz: the toxin and the calcium theories. Considerable material has been brought forward by the supporters of each.

Vassale and Generali (7) adopted the view that the thyroid gland stimulated metabolism and that in the course of the metabolic changes a toxin was produced which was greater in amount the more intense the metabolism. This toxin, they claimed, was taken care of by the parathyroid glands. In support of their theory they had observed that, following parathyroidectomy, the symptoms are much less severe in fasting dogs than in dogs receiving food, and further, the severity is greatly increased by feeding meat.

Biedl (9) found he could relieve, at least temporarily, the symptoms of tetany by copious bleeding followed by transfusion of normal blood. In such a case it was thought

that

that relief was obtained by removing much of the toxin in the blood withdrawn. MacCallum and Voegtlin (10), and Berkeley and Beebe (8) also obtained relief by blood-letting and replacement of the blood withdrawn by physiological saline. The latter authors believed the toxin was derived during the course of metabolism from the decomposition of albumin. The fact that a meat diet markedly increased the symptoms of tetany seemed to support their belief. Further support was added when Koch (11) found methyl-guanidine and other nitrogenous bases in the urine following parathyroidectomy. Still more recently the presence of increased amounts of guanidine, apparently as dimethyl-guanidine, in the urine of adults in cases of tetany was demonstrated by Findlay and Sharpe (12) and by Mattrass and Sharpe (13). In 1924, Paton (14) claimed to have produced symptoms identical with those observed in operative and idiopathic tetany by administering guanidine and methyl-guanidine. He found the symptoms of tetania parathyreopriva were markedly aggravated by small doses of these nitrogenous bases, and that the amount of the latter was increased in the blood and the urine in experimental tetany and in the urine in idiopathic tetany.

The data thus far mentioned certainly makes a strong case for the supporters of the toxin theory. However, it has had a strong rival in the calcium theory.

In 1901, Loeb (15) noted that twitchings of the

muscles

muscles of experimental animals following parathyroidectomy could also be induced by the injection into normal animals of any salt that would precipitate calcium. Eight years later MacCallum and Voegtlin (10) examined the calcium content of the body tissues and the blood in cases of parathyroid tetany, and found values as low as fifty per cent of the normal. They also found that almost immediate, but temporary, relief was obtained by intravenous injections of soluble calcium salts. Administration of these salts by other routes was also effective but less dramatic. MacCallum (16) added further information in 1912 to show that the galvanic hyper-excitability of the nerves, a characteristic feature of tetany, was due to some change in the blood. He was able to produce such a condition in the nerves of a normal dog's leg by perfusing the vessels of the leg with blood from a dog in tetany. The substance in the blood causing this greater electrical excitability during tetany MacCallum, Lambert and Voegl (17) definitely proved by dialysis experiments to be calcium.

By administration of calcium salts, Luckhardt and Goldberg (18) succeeded in keeping parathyroidectomized dogs in good health and free from symptoms associated with tetany.

In 1923 Salvesen (19) showed that parathyroid tetany was due to low blood calcium and that the function of the parathyroid glands was to regulate the level of blood calcium.

Greenwald (20) sought in 1924 to obtain concrete evidence of a toxin in the blood of animals in parathyroid tetany, but found such absent.

Collip (21) the next year prepared extracts from the parathyroid glands by means of which he was able to prevent or control parathyroid tetany, and further, noted that this was brought about by restoring the blood serum calcium to normal levels. With larger doses, hypercalcaemia resulted.

Collip (22) expressed the opinion that guanidine intoxication bears no relationship to parathyroid tetany. While he admitted that the supporters of the toxin theory had made out a strong case for such a relationship he felt there was so much evidence not in accord with it that this theory was no longer tenable.

Collip and Clark (23) observed guanidine intoxication in dogs coincident with parathyroid hormone overdosage. There was no antagonistic influence of guanidine upon the parathyroid hormone. Examination of the urea and non-protein nitrogen curves of blood from parathyroidectomized dogs by these authors showed that the curves ran practically parallel without any tendency to indicate accumulation of non-protein nitrogen. Such a finding does not harmonize with the toxin theory, which would call for an increased non-protein nitrogen through accumulation of guanidine. Salvesen (19) also

reported

reported no increase in urea and non-protein nitrogen during tetany. On the other hand, Collip (22) reported that when normal dogs were injected with sufficient guanidine compounds to produce symptoms, there was a marked increase in non-protein nitrogen of the blood. It would seem from this that guanidine poisoning and parathyroid tetany are different phenomena.

In view of the strong evidence brought out favouring the calcium theory, and the failure to demonstrate the presence of a toxin in the blood of parathyroidectomized dogs in a state of tetany, the guanidine theory has been discarded.

Preparation of Active Extracts.

Using horse parathyroids which he extracted with sterile water or glycerin, Moussu (24) injected parathyroidectomized dogs with the equivalent of twelve to fifteen glands and claimed to have obtained relief for post-operative tetany. He also reported success with capsules of fresh parathyroids ground up with milk sugar and given by the oral route.

In 1909

In 1909 Berkeley and Beebe (25) published their results on the preparation of an extract from beef parathyroids which relieved the symptoms of tetany. They removed extraneous tissue from the parathyroid glands, cut them up fine and reduced them to a fine pulp by grinding with sand. This pulp was shaken for two hours at room temperature with six to eight volumes of 0.9 per cent sodium chloride to which had been added two drops of 10 per cent sodium hydroxide. This mixture, after being left in the refrigerator for eighteen to thirty-six hours, was filtered through gauze to remove fat and tissue, and next through moderately thick filter paper. The filtrate was preserved by adding chloroform and keeping in the refrigerator. This extract given hypodermically, Berkeley and Beebe claimed gave relief in ten to fifteen minutes to dogs in tetany, and complete recovery in two hours. Further, they reported their extract as containing nucleoprotein, globulin, and albumin. The nucleoprotein was precipitated with acetic acid, the globulin with half-saturated ammonium sulphate, and the albumin with complete saturation. The nucleoprotein made up the greatest part of the precipitates, the globulin about one-fifth as much as the nucleoprotein, while the amount of albumin was too small to do anything with. The nucleoprotein fraction was the only one to show potency. It was purified further by dissolving in dilute alkali and reprecipitating with acetic acid. This precipitate was thoroughly washed,

dissolved

dissolved, and reprecipitated twice more. It was finally dissolved in dilute alkaline medium and preserved. Berkeley and Beebe found that such a purified preparation while fresh had lost none of its potency in the process of purification. This potency was entirely destroyed by boiling the nucleoprotein or heating it to 80°C for thirty minutes. Tryptic digestion or the action of pepsin and hydrochloric acid for forty-eight hours severely injured but did not completely destroy the activity of the nucleoprotein. These authors also claimed that their nucleoprotein was effective against tetany when given by mouth, but its action was quicker and more certain when given subcutaneously or intraperitoneally.

Berman (26), in 1924, claimed to have obtained from ox parathyroids, by extraction with acidified alcohol, an extract which, after removal of lipins and proteins, was concentrated and yielded a crystalline material. The latter when dissolved in Ringer's solution and injected into the blood stream raised the calcium content of the blood. Unfortunately Berman gave no details of his work in the short note published.

MacCallum (27), writing in 1924, seemed very skeptical as to the merits of parathyroid gland preparations. "Some of them", he said, "seemed to have some effect in restoring calcium balance and the normal excitability of nerves, but at best it is a slight and questionable effect and less satisfactory in

experimental

experimental animals than in the tetany of adults, from which it may probably be assumed that the psychic effect of any treatment plays a part." This view has been justified by more recent work.

In the papers published in 1924 by Hanson (28), although he gave no absolute proof of the presence of the parathyroid hormone in his preparations, it would appear from our present knowledge that his "Hydrochloric X" contained the active principle. After removing as much extraneous fat as possible from beef parathyroids, Hanson ground them up fine and extracted with boiling dilute hydrochloric acid (approximately 0.35%) for two hours. The fat rising to the top in such extracts on cooling was removed by skimming or filtration. From this point he followed either of two methods. One of these consisted of precipitating the active principle with one per cent phosphotungstic acid, and after letting the mixture stand an hour the supernatant liquid was siphoned off. Water was then added to the precipitate and an hour later siphoned off. This was repeated until excess of phosphotungstic acid was removed. The precipitate left was dried on glass plates at 60°, and finally ground in a mortar yielding a grey-brown powder. In the other method, Hanson evaporated the hydrochloric acid extract down and finally obtained by drying at 80° in flat pyrex pans a dark grey powder which was slowly hygroscopic and soluble in boiling water. With either of these preparations he claimed to have freed pregnant women

from

from dental caries, and brought about improvement in cases of duodenal and gastric ulcer, varicose ulcer, tetany, and nervous exhaustion.

Collip (21), in 1925, presented absolute proof that extracts of beef parathyroids obtained by hydrolysis with dilute hydrochloric acid contained the active principle responsible for relieving or preventing tetany in thyroparathyroidectomized dogs. Further, he showed that the probable manner of action of this active principle was through a direct effect on calcium metabolism. Later in the same year Collip and Clark (23) purified this active extract further. Their method (22,29,30) was in principle the same as originally outlined by Collip (21) together with a preliminary purification by salting out with sodium chloride followed by several isoelectric precipitations. Fresh beef parathyroids were ground in a meat chopper, placed in large Pyrex tubes (5 x 45 cm.) and covered with an equal volume of five per cent hydrochloric acid. Three per cent acid was used if the glands had been preserved in dry acetone. The tubes and their contents were then placed in a boiling water bath for thirty minutes to one hour. During the digestion, the mixture was stirred and broken up with a glass rod until most of the material was in solution and the rest quite finely divided. The mixture was then diluted with four parts of hot water. After it had cooled, the fat which had risen to the top and congealed was removed mechanically. Sodium hydroxide was added to the liquid until

alkaline

alkaline (pH 8 to 9). At this pH practically all the suspended material dissolved. To this solution hydrochloric acid was added with constant stirring until considerable precipitation had occurred. The object of this step was to have the liquid at a pH such that as much as possible of the active material was in solution and at the same time rapid filtration was possible. The pH at this point was usually about 5.5 to 5.6, but the proper condition was best determined by trial. To remove any active material left in the precipitate, the latter was redissolved in weak alkali and again treated as before with hydrochloric acid. This recovery process was repeated as long as any active material remained in the filtrates thus obtained. This point was determined by noting whether, upon making the filtrate acid to Congo red and saturating it with sodium chloride, an appreciable amount of precipitate was formed. The active principle was separated from the filtrates by salting-out with sodium chloride, the first filtrate being treated separately from those obtained in the recovery process. The salting-out was done by making the solutions acid to Congo red and saturating with sodium chloride. The substance generally flocked out and rose to the top of the liquid. The precipitate was transferred to a filter, separated from its mother liquor, dissolved in weak sodium hydroxide, centrifuged, and the liquid adjusted to pH 4.8. The isoelectric precipitate which thus separated was either filtered or centrifuged, dissolved by the addition of hydrochloric acid, and the process

repeated

repeated until the mother liquors were quite clear and devoid of colour. The substance was finally dissolved in hydrochloric acid of about pH 3, passed through a Berkefeld filter, standardized, and was then ready for use.

The potency of Collip's extract has been repeatedly confirmed by many workers, Macleod and Taylor (31), Cameron and Moorhouse (32), Fisher and Larson (33), Hjort, Robison and Tendick (34), and others.

Fisher and Larson (33) tried, in addition to Collip's method of preparation, several modifications using as extraction reagents HCl-EtOH in one case and HAc-EtOH in another. They also tried to purify their extract further, after carrying out the HCl-H_2O extraction, by either the addition of three volumes of 95 per cent alcohol or half saturation with ammonium sulphate. They reported all their extracts active but the one prepared similarly to Collip's the most potent.

Hjort, Robison and Tendick (34) also made a comparative study of various extraction reagents. They obtained inactive extracts by using distilled water, 65 per cent alcohol, 0.1 per cent acetic acid in 40 per cent alcohol, 0.5 per cent sodium hydroxide in 65 per cent alcohol, 0.4 per cent sodium hydroxide in 40 per cent alcohol, 0.4 per cent sodium hydroxide, ether, or acetone. Active extracts were prepared at room temperature with 0.5 per cent hydrochloric acid in 65 per cent alcohol, 0.3 per cent hydrochloric acid in 40 per

cent

cent alcohol, or 0.3 per cent hydrochloric acid. They also obtained potent preparations by boiling the glands with 0.1 - 0.5 per cent hydrochloric acid for fifteen minutes to two hours. Apparently, activity was absent in all extracts save those made with acid-water or acid-alcohol. They found more of the active principle could be extracted at 100° than at 15°C. In addition they claimed that acetone-desiccated chloroform-defatted parathyroids yielded, under the same conditions, more of the active principle than acetone-desiccated glands. The fatty tissue of the parathyroids yielded no potent principle while the lipoid-free portion did. In purifying their extracts these authors found that the bulk of the protein could be removed without affecting the potency of the extract.

Tweedy (35) after digesting ox glands with hydrochloric acid and carrying out an isoelectric precipitation, adjusted the filtrate to pH 5.5 - 5.8 and added acetone until the concentration of the latter was 60 to 70 per cent. After letting this stand in the ice box for twelve to fifteen hours, a precipitate formed which was removed by filtration. The filtrate was concentrated at 40° to 45° to dryness in a vacuum, and this residue taken up in 2.5 per cent trichloracetic acid. Gradually a finely divided precipitate formed. After letting it stand twelve to fifteen hours in the ice box the precipitate was separated by centrifuging, or, if still

suspended

suspended, by filtration. This precipitate was dissolved in a strong alcohol solution, and later reprecipitated by addition of anhydrous ether. Tweedy also extracted the active substance with liquid ammonia, ethyl acetate, and ethylbutyrate from the solid residue which he had obtained by concentration of his acetone solution. Quite recently, Tweedy (36) obtained the potent material in dry form by first removing the inert fraction with acetone from a hot hydrochloric acid extract and precipitating the active fraction with trichloracetic acid. This precipitate was then treated with chloroform to remove inert lipoid material. His product was soluble in both water and 0.9 per cent sodium chloride solution. No evidence was presented to show his active material more potent than Collip's nor that it was in a purer form.

Chemistry of the Active Principle.

Berkeley and Beebe (8), in 1909, separated their parathyroid extract into three fractions. The potent principle, they claimed, lay entirely in the nucleoprotein fraction which was readily precipitated with acetic acid. It was soluble in dilute alkali. Its potency was completely destroyed by boiling or heating to 80° for thirty minutes. On the other hand, tryptic digestion or the action of pepsin and hydrochloric acid for forty-eight hours, although severely

injuring

injuring the activity, did not entirely destroy it. Preparations placed in the ice box gradually lost their potency. This instability of their extracts led Berkeley and Beebe to believe that the active principle of the parathyroid glands was an enzyme.

Hanson (28), in 1924, described his desiccated parathyroid preparation, "Hydrochloric X Sicca" as a dark grey powder which was slowly hygroscopic, and soluble in boiling water.

Fisher and Larson (33) the following year reported, in contrast to the finding of Berkeley and Beebe, the active principle as quite stable, losing none of its potency on heating to 70° for five minutes in normal hydrochloric acid or on leaving in the ice box for three months. From this they concluded it was not an enzyme.

Collip and Clark (29) in the same year carried out the first detailed study of the chemical nature of the active principle of the parathyroid glands. They reported it as a light grey amorphous powder, very soluble in water on either side of its isoelectric point, pH 4.8 to 4.9, but only slightly soluble at this point. Although going into solution on the acid side of pH 4.8, yet it was reprecipitated if the concentration of the acid, such as hydrochloric, reached 4 per cent. It was also precipitated from acid solutions by

half

half-saturation with ammonium sulphate, or complete saturation with sodium chloride. It was quite soluble in 80 per cent alcohol, only to an extent of 0.1 per cent in absolute alcohol, and insoluble in ether, acetone and pyridine. The air-dried powder contained 14.5 per cent nitrogen. By drying this powder in a vacuum over sulphuric acid and potassium hydroxide, the nitrogen content was 15.5 per cent, thus indicating the hygroscopic nature of the purified powder. It gave the common protein reactions, namely, the xanthoproteic, Millon's, biuret, ninhydrin, and Hopkins-Cole reactions. The Molisch Carbohydrate reaction and the orcinol-hydrochloric acid tests for pentoses were negative. Sulphur and iron were present while phosphorus was absent. The active principle was removed from solution by norit and by the Folin-Wu tungstic acid reagent. It would not dialyze to any appreciable extent through collodion membranes. The action of both pepsin and trypsin, rendered the principle inert. Boiling for one hour in either 10 per cent hydrochloric acid or 5 per cent sodium hydroxide did likewise. The purified substance as a desiccated solid or weak acid sterile solution was found to be stable.

The above properties, together with the fact that repeated isoelectric precipitations failed to alter the potency or change the nitrogen content of the active material, pointed to it being a fairly well characterized individual of protein nature. As Collip and Clark pointed out, there

is

is the possibility that the active substance may be some fairly simple compound which associates itself with this particular protein-like substance. This, however, seems unlikely in view of the fact that the proteolytic enzymes, pepsin and trypsin, completely inactivate the most potent preparations.

Tweedy (36) last year reported the active principle insoluble in anhydrous acetic acid, appreciably soluble in 98 per cent, and freely soluble in 94 per cent. Although it was insoluble in pyridine, it was not precipitated from a ten per cent acetic acid solution by addition of pyridine gradually up to a concentration of 94 per cent. At 70°, dry phenol or orthocresol dissolved it to the extent of 10 per cent. No loss in activity was noted from heating for seven hours at 70° in phenol, partial loss for one hour at 150°, and complete destruction for one-half hour at 175° in an atmosphere of nitrogen. Complete inactivation also resulted from suspending the active principle in 0.5 per cent hydrochloric acid in absolute alcohol and heating twenty minutes at 70°, or suspending in absolute alcohol for one hour and saturating with hydrogen chloride at 10°.

Physiology of the Parathyroid Hormone.

The first detailed study of the physiology of the

parathyroid

parathyroid hormone was carried out in 1925 by Collip and his associates (21,23, & 37). In their work it was clearly established that the outstanding physiological action of the parathyroid hormone was normally the regulation of calcium metabolism and the maintenance of the blood calcium at a definite level. The other effects resulting from parathyroidectomy or administration of excess parathyroid hormone they believed were secondary to the changes induced in calcium metabolism.

They found that while single small doses of the hormone produced no ill effects, repeated doses at short intervals is another matter. Further, all animals did not respond to the same degree to these injections. Rabbits and hens gave little or no response to repeated injections of the hormone, while dogs proved most sensitive. With single injections in dogs the chief effect was a rise in the blood serum calcium which reached the maximum point of the curve usually in fifteen to eighteen hours after giving the injection, and then descended at about the same rate. Although different dogs responded to different degrees, yet, the response of each was directly proportional to the size of the dose. Collip noted that young dogs responded better than old ones, but found no relationship between degree of response and weight of dog. Macleod and Taylor (31) also observed greater serum calcium rises in lean dogs than in fat ones.

Unless

Unless the size of dose was sufficient to raise the calcium over 15 mgms. per 100 c.c. serum there were no ill effects. If the calcium rose above fifteen, vomiting usually occurred, but this in itself was not serious unless the calcium level was maintained above fifteen.

The effect of a second injection depended on the time interval. If it were given shortly after the first injection, the serum calcium curve was little altered other than it rose higher. On the other hand, if the second injection were given when the calcium was near the peak, a dip in the curve appeared before it started to climb further. Repeated injections at intervals of a few hours caused pyramiding resulting in a maximum calcium around 20 mgms. per 100 c.c. serum. When values above 15 were maintained a train of symptoms began to appear. The dogs had attacks of vomiting followed by diarrhoea. The calcium, after reaching the upper limit, which it held for some hours, began to fall. Coincident with this fall urgent symptoms became manifested. There was a certain degree of respiratory distress, vomiting and passing of blood by bowel followed by a state of collapse and death. About the time the calcium began to fall, inorganic phosphorus in the serum began to rise. Blood urea and non-protein nitrogen also showed a marked rise. There was a decided decrease in blood volume, the blood becoming more viscous and very difficult to draw from the veins.

Parathyroid hormone overdosage also disturbed the acid-base balance, producing first a compensated alkalosis followed by a compensated acidosis and finally an uncompensated acidosis just prior to death.

The kidney practically ceased functioning about the time the serum calcium curve reached its peak. The volume of urine decreased, and the excretion of phosphorus, ammonia, and titratable acid fell off. This failure in kidney function coincided with the rise in the blood of phosphorus, urea, and non-protein nitrogen already referred to.

Post-mortem examination of dogs killed with parathyroid hormone overdosage showed marked congestion of the alimentary canal and blood in the stomach and intestine. There appeared to be no actual rupture of the endothelial walls but apparently a general diapedesis took place. The water content of various tissues following parathyroid hormone overdosage did not change but the calcium content, although showing no change in muscle and brain, and only a slight increase in liver, showed a marked rise in heart and particularly in kidney tissue.

Complete removal of the parathyroids from dogs resulted in the appearance of a typical syndrome beginning the second or third day after the operation. The symptoms recorded were tremors, psychical depression, paresis of the muscles of mastication, trismus, rigidity of the hind limbs, uncertain

and

and spastic gait, muscular weakness and convulsions. There was loss of appetite, vomiting, palpitation, and dyspnoea. Unless these conditions were corrected by injections of the parathyroid hormone, death soon followed. By a careful administration of this hormone such parathyroidectomized dogs were maintained in a normal state of health over a long period, but they tended to show an increasing immunity to the active principle. Lisser and Shepardson(38) noted a similar increased tolerance toward parathyroid hormone in a case of complete parathyroidectomy in a young woman during the course of one year. Parathyroidectomized dogs showed the same degree of response and effects identical with those noted in normal animals for overdosage.

Rabbits originally thought immune to repeated injections of parathyroid hormone were found by Collip to show hypercalcaemia following administration of enormous doses of the hormone, but the inorganic phosphorus of the blood showed no change, nor were any of the signs of overdosage phenomena produced. This led Collip and his associates to conclude that the overdosage phenomena seen in dogs were due to three conditions, namely, hypercalcaemia, hyperphosphatemia, and in the terminal stage uncompensated acidosis. Although rabbits showed a sluggish response in the elevation of serum calcium following injection of parathyroid hormone, yet removal of the parathyroid glands from normal rabbits resulted in the early precipitation of violent tetany with death following

very

very soon thereafter, usually within thirty hours after the operation. Once tetany had set in, administration of the hormone had little effect. In the preterminal stage these animals show an enormous rise in inorganic blood phosphorus, and it was with this that Collip associated the violent and unyielding character of the tetany manifested in rabbits.

In 1925, Cameron and Moorhouse (32) made a study of the concentration of calcium in whole blood, serum, plasma and cerebro-spinal fluid with particular reference to the form in which the calcium existed. In normal dogs the serum calcium values are usually equal to those for plasma. On the other hand, it was shown by Kramer and Tisdall (39) in 1922 that the red corpuscles did not contain any calcium, and this obtained further support from the work of Rona, Petow, and Wittkower (40) in 1924. Cameron and Moorhouse assumed that the calcium in the cerebro-spinal fluid represented the amount of calcium which was capable of diffusing through an animal membrane. Based on this assumption they found the diffusible calcium of normal dog's plasma averaged 53 per cent. of the serum calcium. The diffusible fraction was thought to exist in an inorganic form, while the non-diffusible fraction was a specific organic compound of calcium. Cameron and Moorhouse further suggested that in the process of clotting this organic compound of calcium went over into another organic form which dissociated to a greater extent so that a larger percentage of the serum calcium is diffusible. Following parathyroidectomy

both

both the diffusible and the non-diffusible calcium decreased, but there was an apparent gradual rise in the percentage of diffusible calcium until in acute tetany it tended to become equal to the serum calcium. This increase they considered only apparent since with approach of tetany more and more of the organic calcium compound was drawn into the clot, leaving this serum calcium 3 or 4 mgms. per cent lower than the plasma calcium. This would indicate that the plasma calcium was only slightly reduced in acute tetany while the serum calcium was lowered nearly fifty per cent of its normal value.

Cameron and Moorhouse consider the constancy of blood calcium due to the presence of an organic calcium compound capable of dissociating to yield calcium ions or of combining with them. That this dissociation can go to completion was shown by the quantitative precipitation of calcium on addition of ammonium oxalate if sufficient time was allowed for dissociation to be complete. Tetany they believe is the result of a decrease in some organic calcium compound in the blood causing, through a disturbed equilibrium, a decrease in inorganic calcium. The action of the parathyroid hormone, they pointed out, was possibly to control the formation of this organic calcium compound.

From a study of the calcium content in serum and cerebrospinal fluid in humans, Greenberg, Ballard, and Dalton (41) concluded in 1930 that the calcium in cerebrospinal

fluid

fluid was a close measure of the diffusible calcium only when the blood calcium is at a normal stable level.

Morgulis and Perley (42) found that ingestion of calcium salts, particularly if accompanied by injection of parathyroid hormone, raised the serum calcium. Intravenoue injection of a modified Ringer's solution containing a large amount of calcium chloride had the same effect. In neither case, however, did the cerebrospinal fluid show any considerable rise in calcium. Similar results were obtained in both normal and parathyroidectomized dogs. As a result, they suggested that some factor other than membrane equilibrium governed the distribution of calcium between plasma and cerebrospinal fluid, and further that the parathyroid hormone has no effect upon this distribution.

Reiche (43) found no relationship between blood calcium and cerebrospinal fluid calcium. On the other hand, he noted that the concentration of calcium in the cerebrospinal fluid varied concomitantly with the pressure of the cerebrospinal fluid. He concluded that the cerebrospinal fluid is secreted by cells constituting the choroid plexus.

The effect of parathyroid hormone on the distribution of calcium in the case of jaundice was studied by Cantarow, Dodek and Gordon (44) in 1927. They considered the tendency to prolonged bleeding in jaundiced patients was due to a functional deficiency of calcium through the combination of

the

the latter with the bile pigments in an effort to counteract their toxicity. Such a combination would likely lessen the calcium available by decreasing its diffusibility and ionization. Parathyroid hormone counteracted this by mobilizing calcium and restoring its functional availability. In 1930, Gunther and Greenberg (45) brought forward evidence to show there was no deficiency in diffusible calcium of the serum of jaundiced patients regardless of the severity of the jaundice or of the absence or presence of bleeding phenomena. Due to low serum albumin in jaundice the non-diffusible calcium may be low. However, the content of diffusible calcium is a more accurate measure of physiologically available calcium than the values of either non-diffusible or total calcium. Hence some factor other than available calcium must be sought to explain abnormal bleeding phenomena of jaundice. This obtained further support from Ravdin, Riegel, and Morrison[46] who found that increasing the concentration of ionized calcium in the plasma did not lessen the coagulation time of normal or jaundiced dogs, except in rare cases.

The effect of diet upon the response to injections of the parathyroid hormone has also been studied by different workers. The fact that dogs were very sensitive to injections of the hormone while rabbits and guinea pigs were resistant suggested that the dietary habits of a species might influence the response to the parathyroid hormone. Collip[22] found

that

that changing the diet from meat to bread had little influence on the response of dogs to parathyroid hormone injection.

Macleod and Taylor[31] placed one dog on a lean meat diet, and another on a diet of porridge, bread and milk. Both animals were given daily injections of parathyroid hormone. The first dog developed all the symptoms of hypercalcaemia while the second remained quite normal. Two weeks later the first dog was put on a carbohydrate diet, and glucose injected. As a result this dog showed a definite improvement although the parathyroid injections were still being given. On placing the same dog back on a lean meat diet, the symptoms of hypercalcaemia were again manifested. Repeating this experiment with two other dogs failed to confirm the above observations; the dog on the meat diet failed to respond to injections of the hormone, while the dog on the carbohydrate diet developed the symptoms associated with hypercalcaemia. Further work showed that lean dogs were more sensitive to parathyroid injections. Fat dogs lost their tolerance to the hormone injection after they had been reduced by fasting to a lean condition. These authors believe that fat, either in the diet or in fat reserves of the body, exerts some protection against the action of the parathyroid hormone. Starving guinea pigs and rabbits, however, did not cause them to lose their tolerance.

Morgan and Garrison[47] observed that young dogs, raised on a diet with a Ca/P ratio = 1.18/1.64 and without

vitamin D

vitamin D, responded only slightly to parathyroid hormone. When cod liver oil, or "Viosterol", was added to the diet the usual response to the hormones was elicited. In the case of a diet low in calcium and phosphorus, the blood calcium rose independently of vitamin D in response to parathyroid hormone injections following administration of large doses of sodium carbonate.

Bodansky, Blair, and Joffe[48] succeeded in getting guinea pigs to respond to parathyroid hormone injections by first starving them for 60 hours or longer. Their tolerance, previous to this fasting, was considered due to the basic nature of the diet. These authors also suggest that an increased rate of excretion may be an even greater factor in explaining the tolerance of some animals.

In 1929, Reiss, Winter and Halpern[49] threw further light on the tolerance factor when they obtained extracts from the thymus which caused a lowering of blood calcium when the experiments were of short duration. In the case of long-continued experiments, their extracts produced a rise in blood calcium and the calcium content of the bones. Extracts from the spleen acted in the same manner, while muscle and liver extracts were ineffective.

Nitschke[50] in the same year presented even more convincing evidence that the thymus can yield a principle

antagonistic

antagonistic to the parathyroid hormone. He prepared two extracts from fresh calves' thymus one of which lowered the blood calcium, producing tetanic convulsions, while the other lowered the blood phosphate and produced convulsions. His calcium-lowering principle was water-soluble and resistant, within limits, to heat, acid and alkali. It appeared to be a relatively simple albuminous substance. The phosphate-lowering principle showed similar stability, but was insoluble in water and behaved like a fatty substance. Both were antagonistic to parathyroid hormone.

Mervish and Bosman (51) by 1927 had prepared an extract from bovine ovaries which caused a lowering of the blood calcium in rabbits and man. They obtained a similar effect with extracts of suprarenal cortex (52). In 1930 Mirvish (53) focused his attention on the effect of cereals on the blood calcium level. He pointed out that E. Mellanby (54) had shown, in 1922, that oatmeal was in some way associated with a pathological phase of calcium metabolism for puppies getting a bare sufficiency of vitamin D could be made to develop rickets by feeding a diet of cereals, particularly oatmeal. In 1923 M. Mellanby (55), working on puppies, had found thst the greater the amount of cereal eaten, other things being equal, the greater the tendency to production of poorly calcified teeth. Holst (56), in 1927, produced rickets by using a cereal diet, and noted this could be prevented by

addition

addition to the diet of calcium salt but not by addition of phosphates. In 1928 Green and Mellanby (57) showed the same interfering action of cereals on calcification of the bones in rats and that the anticalcifying power was counteracted by giving more vitamin D or calcium carbonate. The cereal lost its anticalcifying power when boiled with one per cent hydrochloric acid. Mirvish (53) succeeded in obtaining from oatmeal by extraction at room temperature with 0.5 per cent hydrochloric acid an extract, free from starch, thermostable, dialysable, and soluble in alcohol, which lowered the blood calcium in rabbits 30 per cent in twenty-four to forty-eight hours. This calcium lowering principle he called "Calcovarin".

In cases of osteomalacia Mirvish pointed out that the disturbance in calcium metabolism is usually associated with ovarian function at the time of puberty and pregnancy but is not due to a drainage by the foetus. Further he pointed out that the diet is usually rich in cereal and the disturbance can be corrected by addition of cod-liver oil to the diet or exposure of the individual to sunlight. The disturbance in calcium metabolism may thus be due to either a deficiency of vitamin D or to an excess of "Calcovarin" arising from the ovary of the cereal fed. He considers that "Calcovarin" reacts with or antagonizes the parathyroid secretion and that vitamin D is necessary for the proper functioning of the parathyroid secretion.

Further

Further evidence for the presence in oats of a principle causing a decrease in blood calcium was obtained by Agnoli (58). He observed in the case of rabbits, fed exclusively on greens, an increase in blood calcium of fifteen to sixteen per cent following administration of irradiated ergosterol. On the other hand, rabbits fed on oats showed a decrease of ten to eleven per cent in blood calcium in spite of administration of irradiated ergosterol.

In 1930, Fine (59) presented evidence for the spontaneous cure of rickets on a rachiticogenic wheat diet by the proper adjustment of the Ca/P ratio. With a comparable oat diet there was less tendency towards such spontaneous cures.

Bethke, Kiek and Wilder (60) pointed out that within certain limits the ratio of calcium to phosphorus in the diet is of greater significance in calcification than the actual concentration of these elements. The wider the Ca/P ratio, the greater is the requirement of vitamin D.

The source and disposition of calcium relative to high and low blood calcium values has been the subject of many investigations and considerable controversy.

MacCallum and Voegtlin (10), in 1909, reported an increased excretion of calcium accompanying the lowered blood calcium following parathyroidectomy.

Cooke (61)

Cooke (61), on the other hand, found no change in the amount of calcium excreted following parathyroidectomy.

Greenwald and Gross (62) criticised the conclusions of MacCallum and Voegtlin and of Cooke on this point from the fact that the evidence presented in their data was not conclusive. Greenwald and Gross then proceeded to carry out a series of metabolism experiments in an effort to obtain more definite data. As a result they succeeded in conclusively demonstrating that there is no increase in calcium excretion after removing the parathyroids. Further, they showed that injection of parathyroid hormone not only increased the serum calcium but was followed by an increased excretion of both calcium and phosphorus.

Holt, La Mer, and Chown (63) observed that when serum was shaken with solid $Ca_3(PO_4)_2$ there was a marked reduction of the calcium and phosphorus left in solution. They concluded that the serum is normally supersaturated with $Ca_3(PO_4)_2$, and they suggested the presence in the blood of a substance delaying the precipitation of calcium absorbed from the intestine. No attempt was made to explain how the blood calcium level was maintained on a low calcium diet.

Greenwald and Gross (64) suggested that parathyroid hormone was responsible for holding $Ca_3(PO_4)_2$ in solution in the blood or was necessary for the synthesis of such a

substance.

substance. They pointed out that their experiments showed that this substance not merely retarded the precipitation of $Ca_3(PO_4)_2$ but actually dissolved it, for while dogs got practically no calcium in their food they still showed a rise in serum calcium following injection of parathyroid hormone. This rise they assumed was at the expense of body tissue or bones, As the increased calcium excretion was accompanied by an approximately equivalent excretion of phosphorus they concluded that the bones were the source. Following removal of the parathyroids, $Ca_3(PO_4)_2$ was precipitated and the excretion of phosphorus decreased. The level of serum phosphorus was maintained by metabolism of proteins and phosphatides, but calcium fell unless supplied in large amounts. With excess parathyroid hormones excess $Ca_3(PO_4)_2$ was dissolved from the bones to increase the calcium and phosphorus in the blood. The rise in blood phosphorus, however, was not obtained as the kidney speedily removed it. Calcium and phosphorus were excreted in larger and approximately equivalent amounts. They concluded that hypercalcaemia induced by excess parathyroid hormone was not due to improved assimilation but to increased loss of calcium from the bones. In 1926 Greenwald and Gross (65) noted that smaller doses of parathyroid hormone administered over longer periods maintained an increased excretion of calcium and phosphorus. They concluded the calcium must be derived from the bone for no other tissue contains sufficient to supply the amount excreted. However, they failed to obtain

radiographic

radiographic evidence of any marked change in the bone. They pointed out that the hypothetical calcium-dissolving substance previously referred to may not circulate in the blood at all, but may exist in or in the immediate vicinity of the osteoblasts or other cells that may be responsible for removing calcium from the bone, or that the hormone may liberate calcium from some non-osseous tissue and that by some other mechanism the calcium of the bone is drawn upon to make good the deficiency.

In 1927 Stewart and Percival (66) came to the conclusion that food ingested was not the source of high serum calcium following injection of parathyroid hormone, for they still got such rises in calcium, even after removal of the alimentary tract. Hence body tissues and bone must act as sources of calcium.

From 1928-30 Taylor and Fine published a series of papers (67,68,69) in which they found that there was a small but constant excretion of calcium from the blood through the intestinal wall. Calcium, injected intravenously into normal animals, raised the blood calcium to high levels which were back to normal within three hours. The disappearance of the blood calcium could not be accounted for by increased excretion through the kidneys or intestine. Injection of parathyroid extract in normal animals had no effect upon the excretion of calcium through the intestine. On the other hand, they reported a much increased excretion of calcium through the intestine following parathyroidectomy.

Dixon, Davenport and Ranson (70), in 1929, examined striped muscle of parathyroidectomized dogs in state of tetany and found the calcium content to lie within the range for normal dogs.

Tsai and Hsu (71), in 1930, published their results on bone as the source of calcium mobilized by parathyroid hormone. They pointed out that if the calcium mobilized by the parathyroid hormone comes from bone, and if the distribution of calcium and phosphorus in other tissues is in no way influenced by the hormone, then the ratio Ca/P for the increased fraction in plasma following injection of the hormone should be the same as in bone. Assuming the calcium and phosphorus were present in bone as $Ca_3(PO_4)_2$, the Ca/P ratio should be equal to 1.94. Even though these elements may exist in other forms in bone as suggested by Shear and Kramer (73), the actual determination by these authors (74) showed that in leg bones of normal adult rats the mean ratio of residual calcium (total Ca - Ca in combination with CO_2) to phosphorus was 1.99, a value close to 1.94. Tsai and Hsu injected parathormone intravenously in four dogs and drew samples of blood under heparin at different intervals. The calcium rose at once and reached a maximum in four hours. Phosphorus did not begin to rise during the first two hours but then rose to a maximum at the end of six hours. In recovering, phosphorus was also slower than calcium. Since the rise in calcium and phosphorus took place at different times, Tsai and Hsu compared

the

the changes for the corresponding phases, rapidly rising, maximum elevation, and recovery. They found for three out of the four dogs that the maximum increases of these elements when expressed in percantage were the same, but when expressed in absolute values the ration was approximately 2:1, as in bone. Similar results were obtained for the first phase. In the third phase the ratio was less, possibly due to the relatively slower rate of recovery of phosphorus for which the slower rate of excretion may be responsible.

Hsu and Tsai (72) also studied the muscle as a source of calcium. They reasoned that if muscle serves as an immediate source of mobilized calcium following parathyroid injection, its calcium content should decrease during the rise in blood calcium. On the other hand, if the hormone did not liberate calcium from the muscle, then any increase or decrease of blood calcium through the action of the hormone should alter the muscle calcium in the same direction. They found the calcium content in muscle of parathyroidectomized dogs lower than that of normals. The decrease of muscle calcium was less abrupt and less extensive than the fall in blood calcium. They concluded the decrease of muscle calcium in the parathyroidectomized dogs was due to the fall in blood calcium. In no case did the hormone cause a depression of the muscle calcium. In general the muscle calcium changes appeared to follow the change in serum calcium, and hence muscle was not the source of calcium mobilized by the hormones.

Hypercalcaemia

Hypercalcaemia resulting from excessive doses of irradiated ergosterol has likewise created considerable discussion with reference to the source of mobilized calcium. Jones, Rapoport and Hodes(75), in 1930, criticized much of the earlier work on the ground that many of the so-called calcium-free diets contained sufficient calcium to supply the excess in hypercalcaemia. To overcome this they used a diet with practically no calcium in it. It consisted of lactose, lard, sucrose, cane sugar charcoal and an alcoholic extract of wheat embryo. Such a diet, although incomplete, and unbalanced, contained sufficient vitamin B and supply of energy to prevent excessive destruction of body tissues. This they pointed out was of prime importance in minimizing the liberation of calcium which undoubtedly results from break down of body tissue. Dogs getting this calcium-free diet along with daily injections of irradiated ergosterol over a period of three weeks showed but slight rise in serum calcium. On supplying calcium to this diet these dogs showed a marked rise in serum calcium. Control dogs getting the calcium-free diet plus 0.25 per cent calcium and the same dose of irradiated ergosterol received by the other dogs showed a marked hypercalcaemia within two weeks. From these results Jones, Rapoport and Hodes concluded that the excess calcium of irradiated ergosterol hypercalcaemia came from the intestinal tract and not the body tissues. They made no attempt to determine whether this was due to increased absorption or to decreased elimination.

Bischoff

Bischoff (76) reported in 1930 that while either parathyroid hormone or irradiated ergosterol produce hypercalcaemia, a combination of the two does not result in an additive effect on serum calcium. After getting hypercalcaemia by daily injections of "vigantol", Bischoff injected parathyroid hormone. As a result he obtained after a brief preliminary stage a distinct and protracted lowering of serum calcium. In such cases too, the lethal symptoms of excess hormone appeared sooner and with smaller doses.

Watchorn (77) working with rats on a calcium-free diet found that large doses of irradiated ergosterol did not affect phosphorus metabolism. Further, they gained in weight, while the controls receiving calcium did not. Although the rats gained in weight and were apparently quite healthy, they excreted considerable calcium in the urine, thus creating a marked negative calcium balance. This was taken to mean that calcium was being lost by the bones and tissues and that irradiated ergosterol can exert its effect whether calcium is present in the diet or not.

A study of the effect of excess parathyroid hormone on the condition of the bones has thrown further light on the source of the mobilized calcium. In 1928, Fine and Brown (78) made a radiographic study of the regeneration of bone under the influence of excess parathyroid hormone. From the evidence obtained they concluded that the hormone delays deposition of

calcium

calcium in regenerating bone of young dogs, while in full grown dogs the evidence was not definite as to the potency of the hormone in this or the reverse direction.

Burns (79), on the other hand, reported that injection of parathyroid hormone in young growing rats on a diet considered favorable to calcium retention gave no indication of failure in bone formation.

Barr (80), working from the clinical side, summarized the possible clinical features of parathyroid tumours. These included abnormally high serum calcium, rarefaction of bone, occurrence of multiple cystic bone tumours, muscle weakness and hypotonia, abnormal excretion of calcium in the urine, and the formation of calcium stones.

Wilder (81) noted in a case of osteitis fibrosa the following symptoms: excessive parathyroid activity, progressive weakness, loss of muscle tone, anemia, pain in the bones, decalcification of the skeleton, giant cell tumours, hypercalcaemia, and hypophosphatemia. The condition, in part at least, was successfully combated by treatment with ultraviolet light and a diet rich in vitamin D. He suggested that vitamin D inhibits the activity of the parathyroid glands.

Bodansky, Blair and Joffe (48) found bone lesions in young guinea pigs even after a single injection of parathyroid hormone. They also noted that by starting with small doses

and

and then gradually increasing these until large doses were being administered, that overdosage phenomena were absent. Despite this they often found lesions typical of osteitis fibrosa. Even small doses continued over a sufficiently long period often caused bone resorption and decalcification without hypercalcaemia.

In recent years, ultraviolet rays and irradiated ergosterol have played a very important rôle in the treatment of rickets which is concerned with retarded ossification. It is now believed that any one or more of the following factors may be operative in rickets: calcium deficiency, phosphorus deficiency, improper Ca/P ratio, lack or deficiency of vitamin D, and lack of ultra-violet radiations. Macchi (82), in 1929, carried out two series of experiments on rachitic infants. In the first series, five to six mgms. irradiated ergosterol was administered every other day. In the second series, the babies were exposed to ultra-violet light on alternating days. Every ten days the calcium and phosphorus in both serum and cerebrospinal fluid were determined. At the same time radiological examinations were made. The initial calciums were high while the phosphoruses were low. Normal values were restored following several weeks of either treatment. Parallel with this adjustment, the ossification became normal. Irradiation of serum from rachitic children raised the phosphorus content considerably, while with serum of cured rachitic children there was little or no change in

the

the phosphorus.

Demole and Christ (83) raised the serum calcium in both normal and parathyroidectomized dogs with irradiated ergosterol. In the latter case tetany was prevented by giving the ergosterol before or soon after the operation. Tetany, allowed to develop, was relieved by giving irradiated ergosterol.

In 1930, Taylor, Branion and Kay (84) noted that with complete parathyroidectomy, dogs were unusually resistant to overdosage of "viosterol", the blood calcium remaining low. On the other hand, normal dogs given large doses of "viosterol" died with calciums at sixteen to twenty and haemorrhage into stomach and intestine. They concluded that vitamin D exerted its effect on blood calcium through stimulation of parathyroid function.

Jones, Rapoport and Hodes (85) also found that irradiated ergosterol had the power to prevent or cure tetany resulting from removal of the parathyroids. They succeeded in getting hypercalcaemia in both normal and parathyroidectomized dogs by giving large doses of irradiated ergosterol. Further, they noted that the hypercalcaemia was not accompanied, as in parathyroid hormone overdosage, by dehydration and increased viscosity of the blood, and the dogs never showed the definite symptoms such as weakness, vomiting and diarrhoea. As a result they concluded that the antirachitic factor did not

act

act by simply stimulating the parathyroid, as Taylor, Branion and Kay had suggested.

Irradiated ergosterol seems to contain at least two potent principles, one - the antirachitic factor; the other - a toxic factor. In 1930, Holtz and Schreiber (86) showed that the toxic effect of excessive doses of irradiated ergosterol was not due to the vitamin D factor but to the "calcinosis factor". They found that vitamin D preparations lost most of their antirachitic value by heating above 160°C. while the toxic factor was little affected. Oxidation with oxygen destroyed both simultaneously, while hydrogenation by means of sodium and alcohol destroyed only the antirachitic factor. They were not able to destroy the toxic factor and leave the antirachitic factor. The symptoms attributed to the toxic factor were: calcification of the kidneys and arteries, rise in calcium and phosphorus of the serum, loss in body weight, debility, extreme thirst, vomiting, and hyperemia of internal organs. The severity of the general symptoms did not always run parallel with the rise in serum calcium. They found that the toxic factor were able to overcome tetany resulting from parathyroidectomy, even in the absence of the antirachitic principle. In their preparations of irradiated ergosterol the proportion of toxic and antirachitic factors varied only within narrow limits irrespective of source of ultra-violet rays, solvent used, or length of exposure. Considering this, it is not surprising that both effects have been attributed to the same

factor

factor. In the light of this work, "hypervitaminosis" is probably a misnomer.

From the work of Rosello and Petrillo (87) in 1928 it would appear that there was some relation of sunlight to the physiological action of the parathyroid hormone. They noted that dogs kept in a dark cellar required eight to ten days with repeated injections of parathyroid hormone before their serum calcium reached a higher level, and that such values decreased more rapidly than usual when the injections were stopped. When their treated dogs were exposed to sunlight, the usual symptoms of hypercalcaemia appeared more rapidly.

Higgins and Sheard (88) in the same year observed that light of certain wave lengths appeared to bear a definite relationship to the physiology of the parathyroid glands. In the absence of optimal light factors, the glands showed increased functional activity. The hyperplasia resulting in the absence of the shorter waves of sunlight was partially obviated by addition to the diet of cod-liver oil.

Methods of Assay

Methods of Assay.

The first attempt to estimate the degree of potency of parathyroid preparations was carried out by Vines (89) in 1923. Based on the "Toxin theory", the more potent the preparation the more active it should be in destroying guanidine. White and Cameron (90), in 1925, tried to apply Vines' method of assay to parathyroid extracts of known potency and found it of no value, for the strongest extracts assayed as of negligible activity.

In 1925, Collip and Clark (23) worked out a method of assay for standardizing the potency of parathyroid preparations. Their method is based on the rise in serum calcium in normal dogs following administration of a single injection. Food is withheld for twenty-four hours prior to the test. The variation in degree of response of different dogs was overcome by using at least ten animals and noting the mean rise for a fifteen-hour period. The increase in serum calcium over this period was shown to be almost directly proportional to the size of the dose. No absolute relationship was found between the weight of an animal and its power to mobilize serum calcium in response to parathyroid hormone. These authors defined a unit of potency as one one-hundredth of that amount of extract which produces on an average a 5-mgm. rise in blood serum calcium in 20-kilo dogs over fifteen hours.

In addition to Collip's method of assay given above,

Burn (91), in 1928, gives in his book on "Methods of Biological Assay" an alternative method, using cats instead of dogs. This method of assay is based on the suggestion of Stewart and Percival (66). After anaesthetizing with ether, a tracheotomy is performed. Paraldehyde (1 gm. per kilo body weight) is given by stomach tube. Blood samples are taken from the carotid artery while injections of the parathyroid preparations to be standardized are given by way of the femoral vein. Stewart and Percival claimed to have obtained by this method, with ten units, a rise in serum calcium of 2 mgm. in two hours. This was the maximum point reached in the calcium curve. The work of Herxheimer (92) in 1927 pointed to certain advantages in using the cat as an assay animal in standardizing parathyroid preparations. For dogs he obtained a 3.7 per cent rise in serum calcium in seven to ten minutes with a maximum level in six to eight hours, and this upper level was retained for twenty-four hours or more. On the other hand, he found with cats that the calcium rose much more rapidly, showing a thirty per cent rise in two minutes, followed by a more gradual rise to the maximum in ten to twenty minutes. At the end of five hours, the calcium was still 3 to 11 per cent high. Contrary to the finding of other workers, Herxheimer found a response to injections of the hormone in rabbits. He reported a 14 per cent rise in serum calcium, this maximum being reached in the rabbit in thirty to sixty minutes. The serum calcium remained at this level for four to five hours. In 1928, Thölldte (93)

suggested

suggested that the serum calcium curves following injection of parathyroid hormone are specific for each species. From the data already presented from Herxheimer's work this would certainly appear to be true as far as dogs, cats and rabbits are concerned. However, no corroboration of this work has been given. There is the possibility that the sharp rise in serum calcium obtained for cats was due to asphyxia.

Collip (94), in 1927, investigated the effect of asphyxia on serum calcium in cats, hens, rabbits, dogs, rats and calves, and found an increase in every case, the rise in these animals amounting to from twelve to thirty per cent.

Emerson (95) reported a twenty per cent rise following asphyxia.

In 1928, Hanson (96) claimed a greater uniformity of response with parathyroidectomized dogs than with normal dogs. He eliminated from his assays any dogs which did not show a drop in serum calcium following parathyroidectomy (bulldogs), and any which showed symptoms of tetany within the twenty-four hours after the operation. Control bloods were drawn twenty-four hours after removing the parathyroids, and the hormone preparation injected. The second samples of blood were drawn six hours later, and the rise in serum calcium noted. Hanson defined his clinical unit as one one-hundredth the amount of extract required to produce each milligram rise in serum

calcium

calcium under the experimental condition specified above. His data included results on twelve parathyroidectomized dogs and four normals, and hardly seems sufficient to merit his conclusion, especially in view of the fact that there were differences of over 100 per cent in the degree of response of some of his parathyroidectomized dogs.

EXPERIMENTAL WORK.

Cat Method of Assay.

According to Stewart and Percival, one can obtain the maximum elevation of serum calcium from injection of parathyroid hormone in two hours using their cat method of assay. In Collip's method, using dogs, the maximum elevation is obtained only after fifteen hours. Herxheimer claimed an even more rapid rise in serum calcium than reported by Stewart and Percival. He obtained the maximum elevation in ten to twenty minutes in the cat.

This possibility of a more rapid method of assay seemed worth investigating, particularly since later work would include many assays of various extracts prepared from parathyroid glands. This method also looked interesting as a possible alternative to Collip's method when dogs were not obtainable, or the facilities for handling the larger animals were lacking.

Twelve cats were tested for their response to parathyroid hormone injections. Food was withheld twenty-four hours previous to the test. One hour before operating, 0.5 c.c. of 0.1 per cent atropine was injected. At the end of the hour the cat was anaesthetized under a bell jar with ether. As soon as the anaesthetic became effective the cat was placed on its back on the operating table and only sufficient ether

given

given by cone to maintain a light anaesthesia. The hair on the ventral surface of the upper part of one hind leg was shaved and the area painted with iodine. A longitudinal incision about one inch in length was made and the femoral artery and vein exposed. Eight c.c. blood was withdrawn from the artery with a hypodermic needle and transferred to a 15 c.c. centrifuge tube. Forty to one hundred units of parathyroid hormone was then injected into the femoral vein. A second, third, and fourth sample of blood was withdrawn ten minutes, one hour, and two hours after giving the hormone.

After the blood samples had stood for one hour, they were centrifuged and the serum calcium determined. For this purpose the Clark-Collip modification (97) of Tisdall's method (98) was used. To 2 c.c. clear serum in a 15 c.c. centrifuge tube were added 2 c.c. distilled water and 1 c.c. 4 per cent ammonium oxalate solution. These were well mixed and allowed to stand one to two hours. Then, after centrifuging ten minutes to pack the precipitate well, the supernatant liquid was carefully poured off and the tube allowed to drain for five minutes with the mouth of the tube resting on a pad of filter paper. The mouth of the tube was wiped dry with a soft cloth. Three c.c. dilute ammonium hydroxide (2 c.c. ammonia and 98 c.c. water) was then directed from a pipette, first on the precipitate to break it up, and then down the wall of the tube. The suspension was centrifuged and drained again as just described. Then 2 c.c. approximately

normal

normal sulphuric acid was directed on the precipitate from a pipette. The tube was placed in boiling water for a minute, after which the hot oxalic acid solution was titrated with 0.01 normal potassium permanganate, using a 10 c.c. microburette. During the titration the temperature of the oxalic acid solution was maintained at 70 to 75°C. For convenience in maintaining this temperature an 800 c.c. pyrex beaker, filled with water, was fitted with a short coil No. 30 gauge chromel wire. By this means the temperature of the bath could be quickly raised to 70° and readily held in that neighbourhood by adjusting the rheostat.

The 0.01 normal potassium permanganate was prepared each day by diluting down sufficient of the stock 0.1 normal solution. The latter was prepared in the usual way, taking precautions to first of all destroy all organic material by boiling gently for five hours, allowing to cool over night, and filtering through asbestos. This stock solution of permanganate was placed in a glass stoppered brown bottle and the top of the bottle protected from dust with an inverted beaker. To standardize this solution, some sodium oxalate was dried at 105°C for two hours, after which 0.67 gm. of the dried oxalate was dissolved in distilled water, 5 c.c. concentrated sulphuric acid added and the solution diluted to 1000 c.c. Using 5 c.c. of this standard oxalate solution, the average of six determinations placed the value of the potassium permanganate at 0.00965 normal. The normality of the permanganate was

redetermined

redetermined two months later and found to be unchanged. The normality was again determined twelve months later and found to be 0.0096 normal.

The Clark-Collip method for calcium in serum has been found to give excellent results so long as the technique is strictly adhered to and the centrifuging is efficient. The maximum deviation of duplicates rarely exceeded two per cent, and the average deviation was one per cent.

The results obtained from twelve cats by injecting forty to one hundred units of parathyroid hormone intravenously are summarized in Table I.

TABLE I.

TABLE I.

Cat	Weight	Sex	Units Given	Control Ca	Ca Obtained at the end of		
	Kg.						
1	3.	Male	40	10.05	10', 10.05; 45', 9.91; 1.5 hrs. 9.86; 2 hrs. 10.36		
					10 mins.	1 hr.	2 hrs.
2	4.40	Male	40	10.49	10.30	10.49	10.49
3	4.78	Male	40	10.59	10.49	10.15	10.35
4	2.90	Female	40	10.8	10.3		
5	3.75	Female	80	10.15	10.15	10.1	10.4
6	2.45	Male	40	10.7	10.3	10.6	11.4
7	3.90	Female	60	10.7	10.4	11.25	11.5
8	3.70	Female	90	10.8	10.1	10.9	10.7
9	4.75	Male	100	11.2	10.7	11.2	11.2
10	4.15	Male	100	10.75	10.45	10.5	10.6
11	3.85	Female	100	9.65	9.65	9.6	9.5
12	3.00	Female	100	10.7	10.35	11.25	11.45

These figures fail to support the claims of either Herxheimer or Stewart and Percival, for the former reported an increase of over 30 per cent, with a maximum elevation in ten to twenty minutes, while the latter reported a maximum in two hours, the serum calcium rising 2 mg. with as small a dose of the parathyroid hormone as ten units.

Blum (99)

Blum (99) has also been unable to corroborate this rapid rise in serum calcium of the cat.

In order to be certain that the commercial extract, used for the work reported in Table I, was potent, 3.75 c.c. of the same lot (75 units) was injected subcutaneously in a dog. At the end of seventeen hours the calcium in its serum had risen 5.1 mgms., thus proving the extract active.

From this evidence, it appears that the cat cannot be used for a more rapid method of assay of the parathyroid hormone.

Effect of Rendering the Acid Extract Alkaline to Phenolphthalein in the Course of Preparation.

In the first attempts of the author to prepare active extracts of the parathyroid hormone from beef parathyroid glands, the latter were ground up fine with silica so that the time for the acid used in the digestion to penetrate the tissue would be reduced to a minimum. This proved not only a slow and tedious operation but was the cause of much annoyance when in endeavouring to remove samples of the digested mixture at various intervals in the process of extraction, small pieces of the silica became lodged in the pipette. This was overcome by digesting the glands in a large test tube (300 c.c.), which gave the silica a chance to settle clear of the solution being withdrawn. At this point in the work a meat mincer became

available

available, thus saving much time and labour. Using this machine, a large batch of beef parathyroid glands was reduced to a fine paste and thoroughly mixed by putting the material through the mincer half a dozen times. The first extracts prepared showed no potency and subsequent work showed this was likely due to failure to concentrate the extract to a sufficient degree. The first potent preparation was obtained as a crude extract by digesting 60 gms. beef parathyroid in 100 c.c. 1.5 per cent hydrochloric acid at 100° for thirty minutes. At the end of the digestion, this mixture was diluted with four volumes of hot water, and set aside to cool. The fat rose to the top, congealed, and was mechanically removed. Sodium hydroxide was added till the solution was alkaline to phenolphthalein and then acid was added till the mixture was just alkaline to Congo red. It was next filtered and the filtrate concentrated under reduced pressure to 40 c.c. Five c.c. of this extract raised the serum calcium 4 mgm., thus showing that the gland material available had not lost its potency.

Collip and Clark noted in the course of preparing their extracts that when, after acid hydrolysis, the digested mixture was made alkaline to phenolphthalein, most of the residue dissolved. Whether making the solution alkaline had anything to do with the potency of the extract was not shown. To get information on this point, 200 gm. of the finely minced

gland

gland material was digested in 1.5 per cent hydrochloric acid at 100° for thirty minutes, at the end of which four volumes of hot water were added. After chilling the digested mixture the congealed fat was removed mechanically. Sodium hydroxide was added until the solution was just alkaline to Congo red. The solution was then filtered and finally concentrated under reduced pressure to approximately 405 c.c. keeping the temperature of the concentrate always below 40°. Four lots of this concentrate, each measuring 100 c.c., were pipetted off and treated as follows:

(1) left alone.

(2) made alkaline to phenolphthalein and 30 seconds later adjusted to original pH, just alkaline to Congo red.

(3) made alkaline to phenolphthalein and left thus for one hour before bringing back to original pH.

(4) made alkaline to phenolphthalein and left at that pH for four hours before adjusting to original acidity.

Sufficient water was then added to bring each to the same volume.

The results obtained from these four lots of extract are summarized in Table II. It is at once evident from these results that the alkaline treatment is not necessary for the activation of the active principle, and that such treatment does not alter the potency of the extracts.

TABLE II.

TABLE II.

5 c.c. Extract No.	No. of Dogs Used	Average Rise in Ca per 100 c.c. Serum
		Mgm.
1	8	3.9
2	6	3.3
3	10	3.4
4	6	3.8

Optimum Length of Hydrolysis and Strength of Acid.

Collip and Clark hydrolyzed the glands from twenty minutes to one hour. They used 5 per cent hydrochloric with fresh glands and 3 per cent with glands which had been preserved in acetone. In the work that follows, the glands were ground in a meat mincer to a paste and thoroughly mixed in order to eliminate as far as possible any variation in contents of each sample and to reduce to a negligible amount any differences due to time required for the acid to penetrate the gland material. Three strengths of hydrochloric acid were used, 1.5 per cent, 3 per cent and 5 per cent. The periods of extraction studied were fifteen, thirty, forty-five, and sixty minutes. The glands used in this series of experiments had been preserved in acetone, and later the acetone

allowed

allowed to evaporate and the glands preserved in a frozen state.

In order that any differences obtained with these extracts might be reasonably assumed due to the strength of acid used and the period of hydrolysis rather than to other factors, the procedure followed was simplified as much as possible and no attempt made to carry out a purification of the active principle. Four lots of glands were digested for fifteen, thirty, forty-five and sixty minutes respectively with each of the three strengths of hydrochloric acid, making twelve extracts in all. In making each of these twelve extracts, 100 grams of the minced gland material was used, and to this was added 170 c.c. of hydrochloric acid of the desired strength. The acid and gland material were thoroughly mixed in the cold in a tall litre beaker and then placed in a boiling water bath and digested the required time. During the process of digestion, the contents of the beaker were kept stirred at a constant rate by a small motor. At the end of the extraction period, the mixture was diluted with hot water to approximately a litre. After chilling the mixture, the fat congealed and was lifted out and adhering liquid washed back into the extract. Most of the undissolved material was removed by centrifuging. This residue was washed and again centrifuged, the washing being added to the extract. The acid present was partly neutralized, the solution being made just alkaline to Congo red. The solution was then concentrated under reduced pressure to a little less than 200 c.c. The temperature of the extract

during

during this concentration was never permitted to reach 40°. Any residue appearing at this stage was filtered off, thoroughly washed, and the filtrate again concentrated as before. This concentrated extract was transferred to a 200 c.c. volumetric flask and diluted to the mark. After allowing this solution to stand a couple of days, it was filtered and the filtrate preserved in the refrigerator till desired, which was within twenty days of its preparation.

In Table III. are given the increases in serum calcium obtained in eight dogs seventeeh hours after injecting 5 c.c. parathyroid extract prepared as just described, using 1.5 per cent hydrochloric acid, the digestion being carried out as indicated for fifteen, thirty, forty-five, or sixty minutes.

TABLE III.

Dog No.	Weight	Sex	Ca rise in mgms. per 100 c.c. serum with 5 c.c. extract, prepared by digesting in 1.5 per cent HCl for			
			15'	30'	45'	60'
	Kg.					
3	9.5	♂	3.4	3.6	5.2	2.3
8	16.6	♀	0.8	1.0	3.7	2.7
11	24.3	♀	4.2	4.8	6.4	4.7
15	15.0	♀	2.7	2.7	5.0	3.5
17	11.6	♀	5.0	6.5	8.3	3.4
18	22.8	♀	0.4	0.5	4.0	3.2
19	17.4	♂	2.3	2.2	3.3	2.5
20	15.4	♀	3.2	4.5	7.0	2.5
Average			2.75	3.2	5.4	3.1

In Table IV. are the corresponding results when the extract was prepared using 3 per cent hydrochloric acid. The same dogs were used with the exception of two, Nos. 15 and 19, which were replaced by Nos. 21 and 22.

TABLE IV.

Dog No.	Weight	Sex	Ca Rise in mgms. per 100 c.c. serum with 5 c.c. extract, prepared by digesting in 3 per cent HCl for			
			15'	30'	45'	60'
	Kg.					
3	9.5	♂	2.7	2.5	3.0	1.5
8	16.6	♀	1.8	2.2	3.2	2.7
11	24.3	♀	4.6	5.1	5.9	1.3
17	11.6	♀	5.3	5.3	6.2	2.1
18	22.8	♀	1.6	2.1	2.5	1.9
20	15.4	♀	2.1	2.7	3.5	1.7
21	19.0	♀	0.8	0.5	1.4	0.6
22	14.0	♂	0.9	1.4	2.8	0.1
Average			2.5	2.7	3.6	1.5

In Table 5

In Table V. are summarized the results obtained with the extract prepared with 5 per cent hydrochloric acid. In this set of experiments, two dogs, Nos. 11 and 22, were replaced by Nos. 23 and 24.

TABLE V.

Dog No.	Weight	Sex	Ca Rise in mgms. per 100 c.c. serum with 5 c.c. extract prepared by digesting in 5 per cent HCl for			
			15'	30'	45'	60'
	Kg.					
3	9.5	♂	0.3	-0.1	-0.1	
8	16.6	♀	0.5	0.	0.2	
17	11.6	♀	0.2	0.	0.	
18	22.8	♀	0.1	-0.2		
20	15.4	♀	0.2	0.1		
21	19.0	♀	0.4	0.1		0.1
23	23.5	♀	0.6	-0.2		-0.2
24	21.0	♀	- 0.1	-0.3		0.1
Average			0.3	0.	0.	0.

It is at once apparent from Table V. that while acetone-preserved glands digested with 5 per cent hydrochloric acid for fifteen minutes yielded an extract containing a very slight activity, longer extractions with the same strength of acid destroyed all activity. With 1.5 per cent and 3 per cent

acid,

acid, forty-five minutes appeared to be the optimum length of hydrolysis, and the maximum potency was obtained with the 1.5 per cent hydrochloric. To bring out more clearly the effect of different lengths of hydrolysis on the potency of the extract, the values in Tables III. and IV. have been plotted in Figures 1 and 2 respectively. In Figure 3 the curves for the averages shown in Figures 1 and 2 have been placed together for comparison.

Figures 1, 2 & 3.

FIGURE 1.

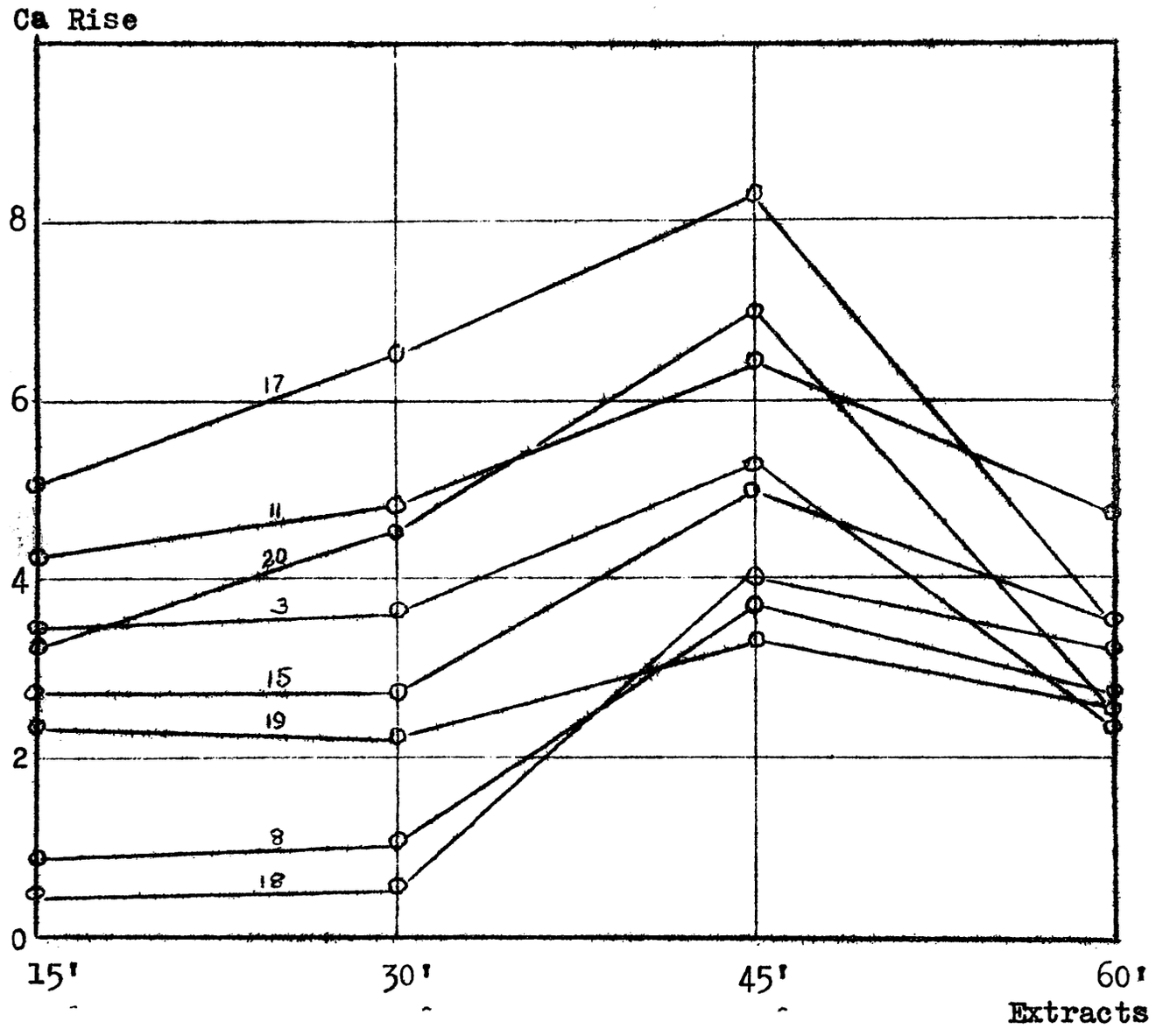


FIGURE 2.

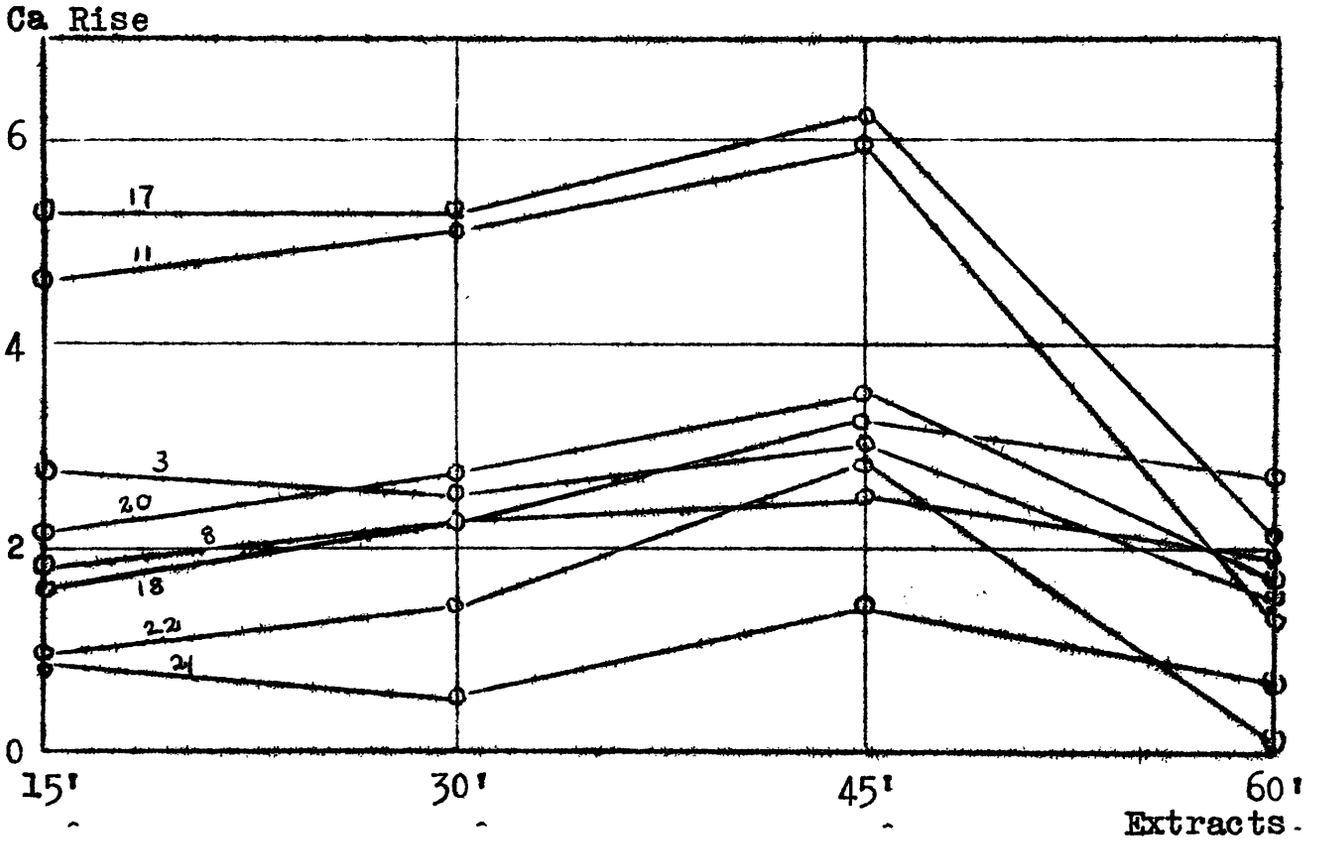
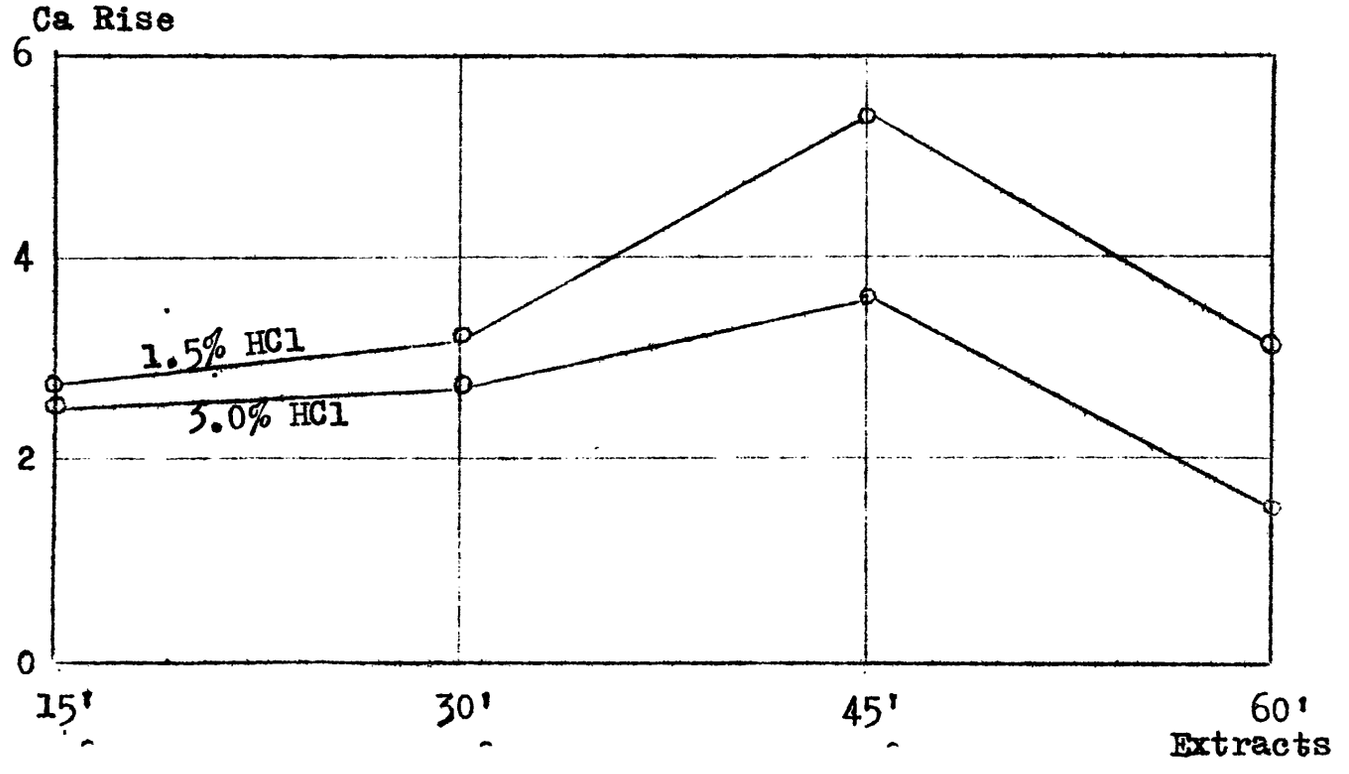


FIGURE 3.



A fresh supply of beef parathyroid glands was obtained from Swift and Co., in Toronto. The glands came packed in "dry ice" and were in excellent condition. They were kept in a frozen state until desired.

In the next series of experiments a little over 1200 gms. of these glands were minced finely and divided into twelve lots, each weighing one hundred grams. Following, with one exception, exactly the same procedure as in the previous series, twelve extracts were made, using either 1.5 per cent, 3 per cent, or 5 per cent hydrochloric acid, and digesting for fifteen, thirty, forty-five, or sixty minutes. The exception referred to above consisted in digesting the glands in an Erlenmeyer flask fitted with a reflux condenser to prevent any change in concentration of the acid by evaporation during the digestion. In this case the flask was shaken every five minutes to keep the contents well mixed. The complete results obtained are shown in Table VI.

TABLE VI.

TABLE VI.

HCl	Dog No.	Weight	Sex	Ca per 100 c.c. serum				Ca per 100 c.c. serum				Ca per 100 c.c. serum				Ca per 100 c.c. serum			
				Control	15' Ext.	Rise	Aver. Rise	Control	30' Ext.	Rise	Aver. Rise	Control	45' Ext.	Rise	Aver. Rise	Control	60' Ext.	Rise	Aver. Rise
%		Kg.																	
	25	15	♀	10.6	14.8	4.2		11.0	15.6	4.6		10.9	14.7	3.8		11.3	14.3	3.0	
	26	10.5	♀	10.4	15.0	4.6		10.4	15.7	5.3		10.5	15.1	4.6		10.7	15.1	4.4	
1.5	27	16	♂	10.2	13.8	3.6		10.3	14.1	3.8		10.2	12.4	2.2		10.4	12.6	2.2	
	28	20.5	♂	10.2	13.4	3.2	3.9	10.4	13.9	3.5	4.3	10.3	13.3	3.0	3.4	10.5	13.1	2.6	3.05
	29	20	♂	10.9	12.1	1.2		10.8	14.3	3.5		10.8	10.8	0		10.9	10.9	0	
	30	14.5	♀	9.7	12.5	2.8		9.7	14.1	4.4		9.7	9.7	0		9.8	9.7	0	
3	31	21	♀	9.7	11.7	2.0		9.8	12.4	2.6		9.9	9.8	0		9.9	9.7	0	
	32	13.5	♀	10.2	12.2	2.0	2.0	10.5	12.9	2.4	3.2	10.4	10.7	0.3	0.1	10.4	10.4	0	0
	33	15	♂	10.1	10.3	0.2		10.1	10.1	0		10.1	10.0	0		10.1	10.2	0	
	34	22	♂	10.0	10.1	0.1		10.0	9.9	0		10.0	10.0	0		10.2	10.1	0	
5	35	19.5	♂	10.1	10.4	0.3		10.2	10.1	0		10.1	10.1	0		10.3	10.3	0	
	36	19	♂	10.6	11.0	0.4	0.25	10.7	10.7	0	0	10.9	10.9	0	0	10.9	10.8	0	0

As in the previous series, the glands digested with 5 per cent hydrochloric acid for fifteen minutes yielded an extract containing a very slight activity, while those digested for longer periods with the same strength of acid showed no activity. As before, the maximum potency was obtained using 1.5 per cent acid. The optimum length of hydrolysis appeared to be thirty minutes, as compared with forty-five minutes in the first series. For more ready comparison, the results shown in table VI. for extraction with 1.5 and 3 per cent acid have been plotted in Figures 4 and 5 respectively, and the averages of these placed together in Figure 6. The three curves in Figure 7 represent the average values obtained from all the extracts prepared with 1.5, 3, and 5 per cent hydrochloric acid. A discussion of these curves will be taken up later.

FIGURE 4.

FIGURE 4.

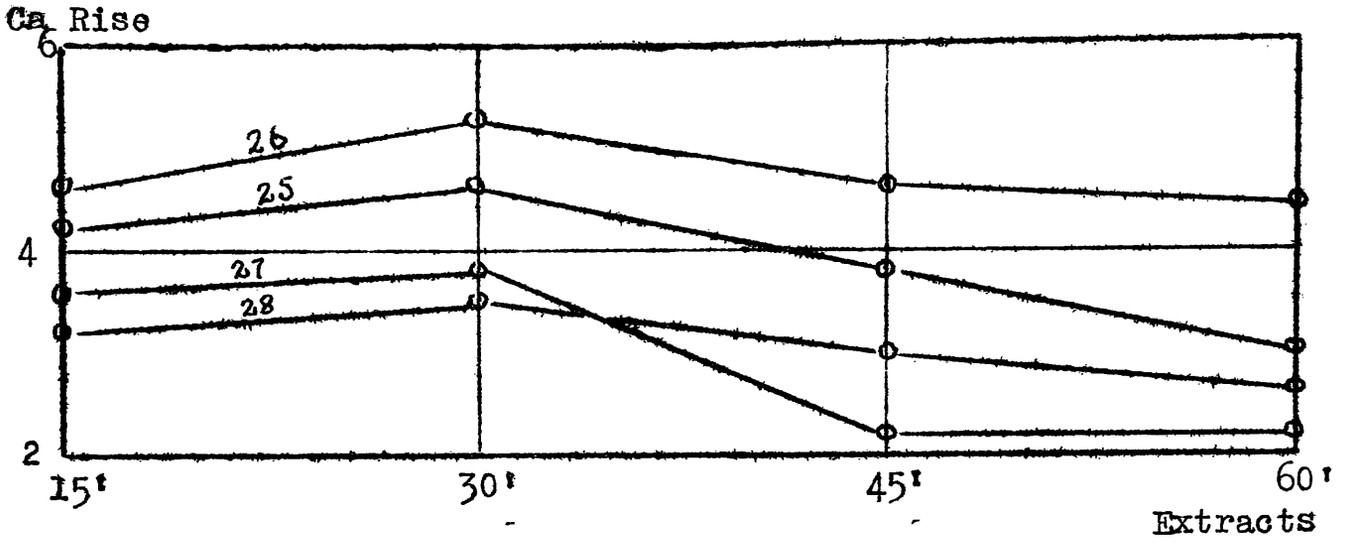


FIGURE 5.

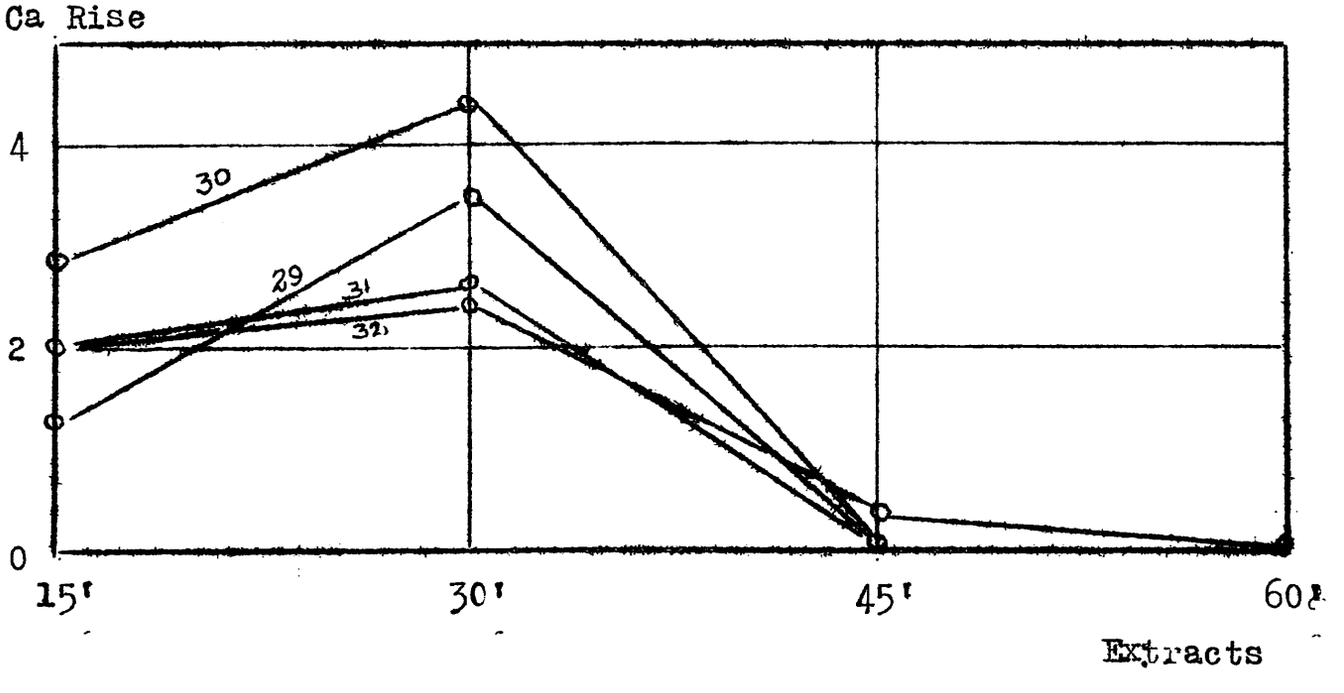


FIGURE 6.

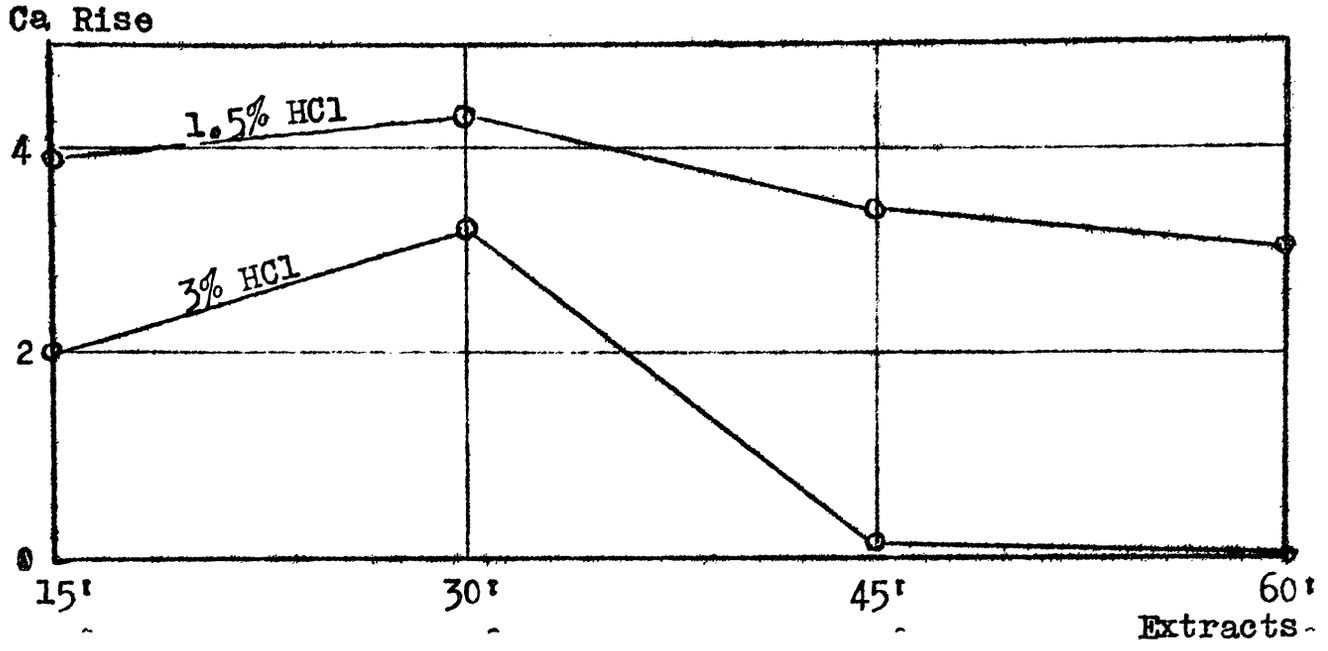
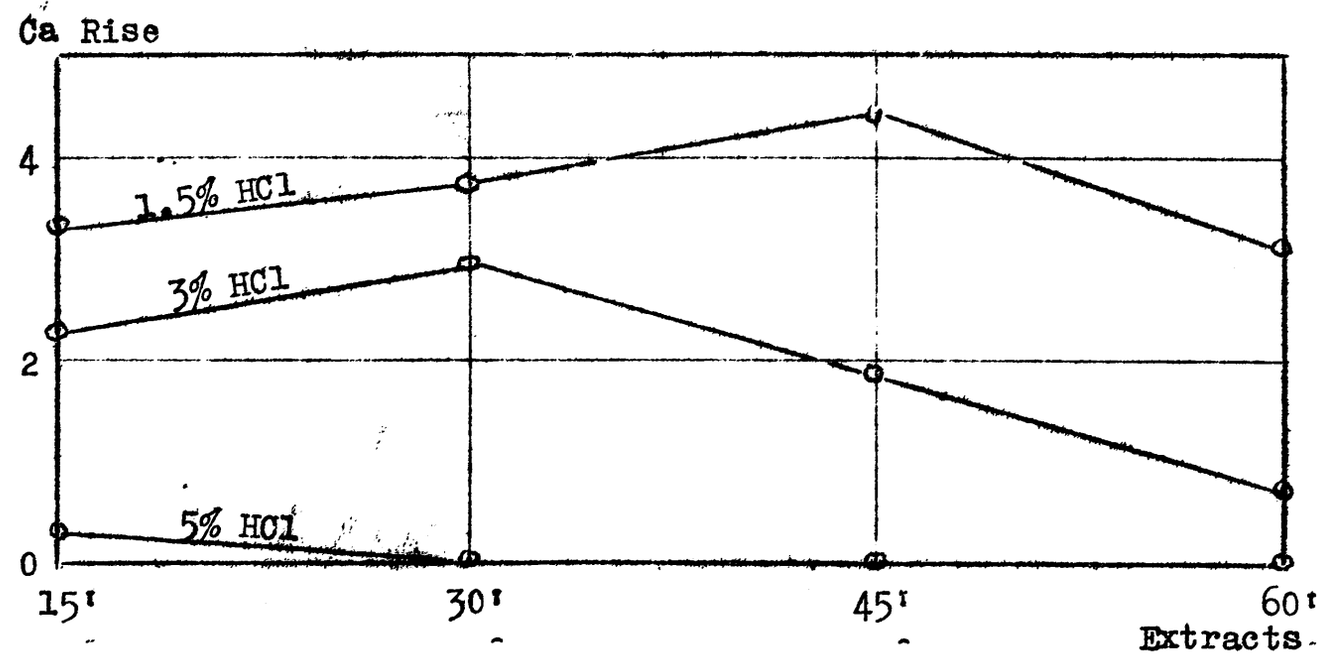


FIGURE 7.



Effect of Incubation at 35°C at Definite pH on the Active Principle.

It is clear that, while digestion of the parathyroid glands with hydrochloric acid ffees some of the active principle, too long heating or using too strong acid destroys some if not all of it. From this it seemed worth while investigating the effect on potency of extracts prepared by digesting at a known pH at a lower temperature and over a longer period.

As this work would involve numerous determinations of pH, a series of buffer solutions was prepared according to the technique of Clark and Lubs (100). This involved the making up of four stock solutions consisting of 0.2 M potassium acid phthalate, 0.2 N hydrochloric acid, 0.2 M potassium acid phosphate, and 0.2 N sodium hydroxide.

The potassium acid phthalate was recrystallized from distilled water and dried at 112° for 2 days. 40.828 gms. of this purified material was dissolved in water and the volume made up to a litre.

The hydrochloric acid was standardized gravimetrically with silver nitrate.

The potassium acid phosphate was recrystallized from water and dried at 112° for two days. 27.232 gms. of this purified salt was dissolved in water and diluted to a litre.

In preparing the sodium hydroxide solution, 100 grams was dissolved in 100 c.c. of water in a Pyrex Erlenmeyer flask.

The

The mouth of the flask was covered with tin foil and the solution allowed to stand till the carbonate had settled. A hardened filter paper, cut to fit a Buchner funnel, was treated with warm, strong (1:1) sodium hydroxide, after which the soda was removed by decantation, and the filter paper washed, first with absolute alcohol, then with dilute alcohol, and finally with distilled water. The greater part of the water was removed from the filter paper by placing the latter in a Buchner funnel and applying gentle suction. The concentrated alkali was then poured on the prepared filter and suction applied. The clear filtrate obtained was diluted at once with cold distilled water, which had previously been boiled to remove carbon dioxide, till the solution was approximately normal. It was then further diluted to nearly 0.2 normal, and finally standardized against the 0.2 N hydrochloric acid and diluted till exactly 0.2 N.

The volume of these stock solutions used to prepare the buffer solutions follow:

(a) To 50 c.c. 0.2 M potassium acid phthalate was added the indicated number of c.c. of 0.2 N hydrochloric acid and the solution diluted to 200 c.c. Brom-phenol-blue was used as indicator.

pH	HCl	pH	HCl	pH	HCl
3.0	20.32	3.4	9.90	3.8	2.63
3.2	14.70	3.6	5.97		

(b) To

(b) To 50 c.c. 0.2 M potassium acid phthalate was added the indicated number of c.c. 0.2 N sodium hydroxide, and the solution diluted to 200 c.c. Brom-phenol-blue was used as indicator from pH 4.0 to 4.6, while methyl red was used from pH 4.6 to 6.0.

pH	NaOH	pH	NaOH	pH	NaOH	pH	NaOH
4.0	0.40	4.6	12.15	5.2	29.95	5.8	43.00
4.2	3.70	4.8	17.70	5.4	35.45	6.0	45.45
4.4	7.50	5.0	23.85	5.6	39.85		

(c) To 50 c.c. 0.2 M potassium acid phosphate was added the indicated number of c.c. 0.2 N sodium hydroxide, and the solution diluted to 200 c.c. Brom-thymol-blue was used as indicator for the pH range 6.0 to 7.0, while phenol-red was used in the range 7.0 to 8.0.

pH	NaOH	pH	NaOH	pH	NaOH	pH	NaOH
6.0	5.70	6.6	17.80	7.2	35.00	7.8	45.20
6.2	8.60	6.8	23.65	7.4	39.50	8.0	46.80
6.4	12.60	7.0	29.63	7.6	42.80		

These buffer solutions were kept in paraffined bottles in the refrigerator.

In order

In order to get an idea of the amount of buffering that would be required to maintain a definite pH in tissue left at 35°, 150 grams very finely divided beef muscle was placed in a flask, and to this was added 260 c.c. 0.9 per cent sodium chloride, together with a preservative in the form of 15 c.c. chloroform and 15 c.c. toluene. After thoroughly mixing, a 10 c.c. portion was centrifuged and the pH determined colorimetrically, using the series of buffers prepared. The pH found was 6.2. 100 c.c. of the mixture was pipetted into each of four flasks, A, B, C and D. To A, B, and C were added 5, 10 and 15 c.c. respectively of a buffer solution of pH 7.0. Sufficient 0.9 per cent sodium chloride was then added to each of the four to bring the volumes to the same value. The flasks were placed in an incubator at 35°. From time to time, as shown in Table VII, 10 c.c. samples were withdrawn, centrifuged, and the pH determined.

TABLE VII.

Time	pH A	pH B	pH C	pH D
2 hours	6.0	6.2	6.2	6.0
1 day	6.1	6.2	6.2	6.0
3 days	6.1	6.2	6.3	6.0
5 days	6.2	6.2	6.3	6.1

From the

From the above results, it is seen that there was only a slight increase in acidity during incubation, even in the case where no buffer had been added. Further, although the buffer added had some effect in maintaining the pH existing before incubation, it did not have sufficient buffering power to raise the pH beyond that value.

In the above experiment the buffer used had been prepared from 0.2 M potassium acid phosphate and 0.2 N sodium hydroxide in the proportions already indicated for pH 7.0. A new buffer solution was made up using the same substances but in concentrations ten times as great. The proportions taken were 50 c.c. 2 M potassium acid phosphate and 42.80 c.c. 2 N sodium hydroxide, and this was diluted to 200 c.c. With these proportions the pH of the buffer should have been 7.6. This was found to be correct when checked against the buffer standards.

According to Dernby (101) and Bradley (102) fresh tissues removed and allowed to stand become more acid, the pH dropping from 7.4 to 6.5 in 24 hours. As the beef used in the previous experiment had been obtained from a local merchant, it was certainly more than twenty-four hours old. In order to get a fresher sample of tissue, a dog which had been used on the operating table was killed and some of the leg muscle quickly removed, washed with 0.9 per cent sodium chloride, and finely minced. Fifty grams of this finely

minced

minced muscle tissue was placed in each of four flasks, A, B, C and D. To each of these were added 0.9 per cent sodium chloride, the concentrated buffer of pH 7.6, and preservatives in the amounts indicated in Table VIII. The flasks were placed in an incubator at 35°. At the end of the first twenty-four hours additional buffer was added. The resulting pH was determined.

TABLE VIII.

Flask	A	B	C	D
Grams muscle,	50	50	50	50
c.c. 0.9 per cent NaCl,	100	95	90	85
c.c. Buffer pH 7.6,	0	5	10	15
Preservative $CHCl_3$ $C_6H_5CH_3$ (1:1)	20	20	20	20
pH before incubation,	6.2	6.6	6.9	7.0
pH after 24 hours incubation	6.2	6.6	6.9	6.9
More buffer added, new total,	35	20	25	30
pH 1 hour after additional buffer,	7.6	7.2	7.2	7.4
pH after two days at 35°,	7.4	7.2	7.2	7.4
pH after eight days at 35°	7.2	7.1	7.2	7.2
pH after sixteen days at 35°	7.4	7.3	7.3	7.4

Since the amount of buffer required to bring the tissue mixture near the desired pH was such as would more than likely introduce an interfering factor when the solution was

later

later injected, it was decided to attempt adjusting the pH by addition of dilute alkali. From the large amount of buffer required to shift the pH, it seemed that the tissues were already highly buffered and this should make the adjustment of pH by titration with alkali possible. Further, the presence of these buffers in the tissue would probably suffice to prevent any rapid spontaneous change in pH thereafter.

Another 50 gram sample of finely minced beef was taken, and to it were added 100 c.c. 0.9 per cent sodium chloride, 10 c.c. chloroform, and 10 c.c. toluene. After shaking this mixture thoroughly for an hour it was centrifuged. Six samples (10 c.c. each) of the supernatant liquid were diluted and titrated with N sodium hydroxide. From time to time during the titration five drops of the solution were removed and the pH determined by comparison with the standard buffer solutions. The titration was carried out to pH 7. The first sample required 0.4 c.c. N sodium hydroxide. The other five each took 0.36 c.c., the amount being less as fewer pH determinations were made in reaching the end-point. Since the original mixture contained 100 c.c. sodium chloride, which would be separated from the beef residues, chloroform and toluene, on centrifuging, each 10 c.c. sample used above contained approximately one-tenth the total acid that must be neutralized to bring the mixture to pH 7.0.

Three more samples, A, B and C, of the finely ground beef, each weighing 50 grams, were placed in flasks. To each

was

was added as before 100 c.c. 0.9 per cent sodium chloride and 20 c.c. preservative. After adding 3.1 c.c. N sodium hydroxide to A, the pH of A was 6.8. 4.1 c.c. of the alkali raised B to pH 7.2, and 5.1 c.c. raised C to pH 7.6. These three samples were then placed in the incubator at 35° for three days. At the end of this period, the pH of each was redetermined and found to have remained constant.

Attention was then turned back to the parathyroid glands. Four lots, finely ground and each weighing 50 grams, were placed in flasks E, F, G and H, to which 100 c.c. 0.9 per cent sodium chloride and 20 c.c. preservative were added. The contents were well mixed. The pH of this mixture at the end of fifteen minutes, and again at the end of an hour, was 6.8.

The contents of F were left at 6.8. The addition of 0.4 c.c. N sodium hydroxide raised the pH of G to 7.2, while 0.8 c.c. alkali brought H up to pH 7.6. E was adjusted to pH 6.4 by adding 0.8 c.c. 0.5 N hydrochloric acid. These four lots of parathyroid glands at the pH indicated were placed in the incubator at 35°, and the pH controlled for a period of twenty-five days, as shown in Table IX, by the addition of acid or alkali.

TABLE IX.

TABLE IX.

Sample	E.		F.		G.		H.	
	pH	0.5 N HCl	pH	N NaOH	pH	N NaOH	pH	N NaOH
Before incubation	6.4		6.8		7.2		7.6	
24 Hrs. at 35°C	6.5		6.8		7.1		7.3	
Correction, c.c.		0.2				0.1		0.3
Redetermined	6.4				7.2		7.6	
2 days at 35°C	6.4		6.8		7.0		7.4	
Correction, c.c.						0.2		0.2
Redetermined					7.2		7.7	
3 days at 35°C	6.5		6.8		7.2		7.4	
Correction, c.c.		0.2						0.15
Redetermined	6.4						7.8	
4 days at 35°C	6.4		6.8		7.2		7.7	
6 days at 35°C	6.6		6.8		7.1		7.5	
Correction, c.c.		0.3				0.1		0.1
Redetermined	6.4				7.2		7.6	
8 days at 35°C	6.5		6.8		7.2		7.6	
10 days at 35°	6.5		6.8		7.2		7.5	
Correction, c.c.		0.2						0.1
Redetermined	6.4						7.6	
13 days at 35°	6.4		6.8		7.2		7.5	
Correction, c.c.								0.1
18 days at 35°	6.4		6.8		7.2		7.6	
25 days at 35°	6.4		6.8		7.0		7.5	

At the end of thirteen days incubation, samples of E, F, G and H were withdrawn and centrifuged. 5 c.c. of the supernatant fluid was injected intramuscularly, the rest of

the

the fluid, together with the precipitate, was returned to its respective flask and incubation continued. The results obtained from these injections are shown in Table X.

TABLE X.

Dog	45	46	51	52
Control serum Ca	10.1	10.3	11.3	11.3
Injection 5 c.c.	E	F	G	H
Serum Ca in 15 hours	10.4	10.1	11.4	11.1
Change in Ca Content	0.3	-0.2	0.1	-0.2

These injections were repeated at the end of twenty days incubation at 35°. The results on this occasion are shown in Table XI.

TABLE XI.

Dog	45	46	51	52
Control serum Ca	10.0	10.4	11.1	11.1
Injection 5 c.c.	E	F	G	H
Serum Ca in 15 hours	10.3	10.5	11.1	10.9
Change in Ca Content	0.3	0.1	0	-0.2

At the end of twenty-five days incubation this was again repeated, but no definite rise in serum calcium was obtained.

obtained. The results are shown in Table XII.

TABLE XII.

Dog	45	46	51	52
Control serum Ca	10.1	10.3	11.1	11.2
Injection 5 c.c.	E	F	G	H
Serum Ca in 15 hours	10.3	10.1	11.0	11.2
Change in Ca Content	0.2	-0.2	-0.1	0

Of the samples of parathyroid glands incubated at 35° over a period of twenty-five days at pH 6.4, 6.8, 7.2 and 7.6, the only one to give even the slightest indication of yielding an active extract was the one kept at pH 6.4. A rise in the serum calcium was obtained from this extract when injected on three different occasions, namely, after incubation at 35° for thirteen, twenty, and twenty-five days. However, the rise obtained at these three times was so small as to be possibly due to experimental error.

In an effort to clear up this last point and to ascertain the effect of incubation of the gland material at still smaller pH, four more lots of glands, J, K, L and M, were prepared in the same manner as before, and the pH adjusted to 3, 4, 5 and 6, respectively, by the addition of hydrochloric acid. At the end of five days incubation at these pH values, a sample of each was centrifuged and 5 c.c. of the

supernatant

supernatant liquid injected intramuscularly. The results obtained are shown in Table XIII.

TABLE XIII.

Dog	45	46	51	52
Control serum Ca	10.1	10.4	11.4	10.8
Injection 5 c.c.	J	K	L	M
Serum Ca in 15 hours	10.4	10.1	11.5	10.6
Change in Ca Content	0.3	-0.3	0.1	-0.2

Apparently no active material had been set free from the glands during the five-day incubation period, unless it had been freed and subsequently inactivated during this time. To check this last point, another 50 gram lot of the glands was prepared as before and the pH adjusted to 3, as in lot J, and the mixture placed in the incubator at 35°, after first warming in a water bath to this temperature. Samples were withdrawn at the end of 1, 2, 3, 5, 10, and 24 hours, and also at the end of 2 and 4 days. Each sample was centrifuged and 5 c.c. of the supernatant solution placed in the refrigerator until used for injection. The rest of the liquid, together with the precipitate, was returned to the flask at once. The results obtained from injecting these samples are given in Tables XIV and XV, and show that no active material has been freed from the glands by this treatment.

TABLE XIV.

TABLE XIV.

Dog	45	46	51	52
Control serum Ca	10.0	9.9	10.9	10.3
Injections 5 c.c. extract, incubated	1 hr.	2 hrs.	3 hrs.	5 hrs.
Serum Ca in 15 hours	10.2	10.0	10.9	10.1
Change in Ca content	0.2	0.1	0	-0.2

TABLE XV.

Dog	45	46	51	52
Control serum Ca	10.1	10.0	10.9	10.4
Injection 5 c.c. extract, incubated	10 hrs.	1 day	2 days	4 days
Serum Ca in 15 hours	10.3	9.9	10.8	10.4
Change in Ca content	0.2	-0.1	-0.1	0

In the meantime the four lots of gland material, J, K, L, and M, at pH 3, 4, 5, and 6, were kept at 35°. Samples of these were removed and prepared for injection, as before, at the end of twelve days incubation and again after nineteen days. The results are given in Tables XVI and XVII, and show that none of the active principle was freed by this treatment.

TABLE XVI.

TABLE XVI.

Dog	47	48	49	50
Control serum Ca	10.7	10.8	10.4	11.1
5 c.c. injection, 12 day incubation	J	K	L	M
Serum Ca in 15 hours	10.7	10.5	10.6	10.9
Change in Ca Content	0	-0.3	0.2	-0.2

TABLE XVII.

Dog	45	46	51	52
Control serum Ca	10.0	9.9	10.9	10.2
5 c.c. injection, 19 days incubation	J	K	L	M
Serum Ca in 15 hours	10.0	10.0	10.8	10.2
Change in Ca Content	0	0.1	-0.1	0

Destruction of

Destruction of Active Principle by Tissue Enzymes.

Since the active principle of the parathyroids was not freed by keeping the glands at 35° over periods of one hour to twenty-five days, and at pH ranging from 3 to 7.6, it must still be present in the undissolved residues or have been destroyed during the treatment.

Further information on this point was obtained by taking the residues from E, F, G and H, which had been kept at 35° and at pH 6.4, 6.8, 7.2 and 7.6 for twenty-five days, and extracting them in the usual manner for preparing an active extract. In brief, the residue from each of these samples was dried in vacuo and digested at 100° with 100 c.c. 1.5 per cent hydrochloric acid for forty minutes. At the end of the forty minutes, the digestion mixture was poured into 500 c.c. hot water and set aside to cool. The congealed fat was mechanically removed from the chilled mixture. The pH of the solution was adjusted till it was slightly alkaline to Congo red. Undissolved residues were removed by filtration, washed, and the washings and filtrate concentrated in vacuo, and finally made up to 100 c.c. When 5 c.c. of these four extracts was injected, no rise in the serum calcium was obtained, as shown in Table XVIII. It would appear, therefore, that the active principle had been inactivated during the incubation at 35°.

TABLE XVIII.

TABLE XVIII.

Dog	47	48	49	50
Control serum Ca	10.7	10.7	11.1	10.9
5 c.c. injection HCl ext. of residue	E (pH 6.4)	F (pH 6.8)	G (pH 7.2)	H (pH 7.6)
Serum Ca in 15 hours	10.5	11.0	11.1	11.0
Change in Ca Content	-0.2	0.3	0	0.1

The possibility that enzymes in the tissues were responsible for this inactivation seemed not at all unreasonable. In order to destroy such enzymes, 100 c.c. 0.9 per cent sodium chloride was brought to the boil and added to 50 grams of finely ground parathyroid glands. The resultant temperature, 80°, was maintained for five minutes. The temperature was then allowed to drop. At the end of fifteen minutes it was 72°. The mixture was then cooled to 35°, and the hydrogen-ion concentration adjusted to pH 6. Chloroform and toluene were added as preservatives and the mixture set in the incubator at 35°. Samples of this mixture were removed at the end of four and eleven days' incubation. These were centrifuged and 5 c.c. of the supernatant liquid injected intramuscularly. No rise in the serum calcium was observed. The residue was separated from most of the liquid by filtering with suction. This residue was then digested at 100° for

forty

forty minutes with 100 c.c. 1.5 per cent hydrochloric acid, after which the digestion mixture was poured into 500 c.c. hot water, cooled, and the fat congealing on the surface removed. The pH was adjusted till just alkaline to Congo red, and the mixture filtered, washed, and concentrated in vacuo to 100 c.c. When 5 c.c. of this extract was injected, the serum calcium rose 2 mgm. in one case and 2.2 mgms. in the other. From these results it is evident that the higher temperature is necessary both to destroy the tissue enzymes and to free the active principle from the tissues. These results are summarized in Table XIX.

TABLE XIX.

Extract injected Enzymes destroyed	Dog	Serum Ca		
		Control	Resultant	Change
Incubated at 35°, 4 days	52	10.0	9.8	- 0.2
" at 35°, 11 "	50	11.0	10.8	- 0.2
Residues from Incubation	48	10.9	13.1	2.2
Digested in 1.5% HCl 40'	50	10.8	12.8	2.0

Three more lots of glands, R, S and T, each weighing fifty grams, were treated in the usual way with 1.5 per cent hydrochloric acid for forty minutes, except that in S the digestion was carried out at 80° instead of 100° as in R, and

in the

in the case of T, the digestion temperature was 60°. In diluting S and T after the digestion, the water used was at 80° and 60° respectively. In other respects these three extracts were prepared in identically the same manner. R and S yielded active extracts while T showed no activity. This is shown in Table XX.

TABLE XX.

Extract	Dog	Serum Ca		
		Control	Resultant	Change
R - 100°	54	11.0	11.8	0.8
"	54	11.1	12.5	1.4
"	53	10.9	12.0	1.1
S - 80°	54	11.1	12.9	1.8
"	53	10.6	11.7	1.1
T - 60°	54	10.9	10.9	0.
"	53	10.7	11.0	0.3
"	53	10.7	10.6	- 0.1

That the hormone is stable to a temperature such as was used in the incubator in some of the above experiments, provided it is kept under sterile conditions, is shown by the fact that a sterile tube of "Parathormone" containing 100 units, incubated at 35° for thirty days, showed at the end of this time a similar degree of potency to a second tube kept in the refrigerator. A third tube which had been treated as the first, except that its contents had been exposed to the

air,

air, showed no activity at the end of the thirty days.

The process of extraction of the hormone appears to be not merely one of dissolving out the active principle, but would seem to involve some chemical reaction such as hydrolysis, for extracting the glands with 0.9 per cent sodium chloride at 100° for forty-five minutes did not yield an active extract.

Attempts to Further Purify the Parathyroid Preparation.

(a) Harrington's Insulin Method: The difficulties encountered in purifying the parathyroid hormone are of much the same type as those met with in the purification of insulin. In both instances the hormone is an ampholyte present in very small amount in a solution of other ampholytes whose solubility and precipitation coefficients lie very close to one another. Abel (103), in 1926, published his successful results on the purification of insulin. In brief, his method consisted in dissolving the crude insulin in dilute acetic acid and removing the impurities from this solution by addition of a solution of brucine in acetic acid. Any insulin lost at this point was recovered by treating the precipitate with dilute acetic acid and repeating the brucine precipitation. After centrifuging off the impurities, pyridine was added and the solution again centrifuged and set aside to permit the formation of a precipitate of the insulin,

which

which was largely crystalline. Pyridin and acetic acid were removed by washing with water. The insulin precipitate at this point was further purified by dissolving in disodium hydrogen phosphate and then precipitating it by the slow addition of acetic acid until a slight turbidity persisted. Leaving the solution at this stage over night yielded insulin in a highly refractive crystalline form.

Collip (22) makes reference in one of his papers to attempts by workers in his laboratory to apply this method of purification to the parathyroid preparation, but without success.

In 1929, Harrington, Scott, Marks, and Trevan (104) presented further work on Abel's method of purifying insulin and in addition a new method based on the use of saponin. They at first thought Abel's success was due to the efficient buffering by brucine acetate and pyridine, which permitted a slow and exact adjusting of pH in the insulin solution to the isoelectric point. However, attempts to substitute the brucine acetate and pyridine by disodium phosphate and then gradually adjusting the pH by slowly adding carbon dioxide was not successful. This led them to believe that the brucine acetate and pyridine exerted other influences in addition to their buffering effect, such as lowering the surface tension. The action of saponin on the precipitation of insulin was studied. They found that certain saponins not only lowered the surface

tension

tension, but also inhibited precipitation in general and sharpened the isoelectric point. As to the quality of the saponin for this purpose, little relationship was found between the frothing and haemolytic powers of the samples, although in general those with the higher frothing power were better. The haemolytic power of the saponin was considered a better index. This was expressed as the minimum concentration necessary for complete haemolysis of a 5 per cent suspension of rabbit's washed red blood corpuscles in 0.9 per cent sodium chloride at 37° within two hours. Saponins with a haemolytic index less than 1:8000 were considered useless. They noted that the more saponin used, the greater the degree of supersaturation and hence the smaller yield of crystals. On the other hand, if too little saponin was used, crystallization failed, due to the fact that a separation of the different isoelectric points was not effected. Surface relationship was also considered of prime importance in the first separation carried out in the preparation of insulin crystals, but not so important in subsequent separations. In this regard success was obtained when, for a volume of 9 c.c., the surface area was 25 sq. cm.

In brief, Harrington's method for purifying insulin consisted in taking a solution of crude insulin in dilute acetic acid and containing one per cent saponin, warming to 35° and adding dilute ammonia gradually till a definite turbidity occurred. By the end of thirty minutes a flocculent precipitate separated and was removed by centrifuging. This

precipitate

precipitate contained about thirty per cent, by weight, of the original material, and fifteen to twenty per cent of its activity. The solution was then rapidly adjusted with ammonia to pH 5.6 and set aside over night. The microcrystals obtained in this way were collected by centrifuging, redissolved in dilute acetic acid and the precipitation in the presence of saponin repeated. This second precipitate was more crystalline than the first. Finally, this precipitate was dissolved in dilute hydrochloric acid, a phosphate buffer added and the pH adjusted to 5.6. A fully crystalline precipitate separated in a few hours.

While the application of Abel's method for preparing insulin in crystalline form to the purification of the parathyroid hormone had not met with success, it was thought that the more recent method of Harrington might prove successful. Accordingly, a sample of saponin was obtained and its haemolytic index determined. For this purpose some rabbit's blood was obtained, oxalated, and the cells removed by centrifuging. The red blood corpuscles were then washed with 0.9 per cent sodium chloride, and a ten per cent suspension in 0.9 per cent sodium chloride made. The saponin was dissolved in 0.9 per cent sodium chloride and then diluted with this solvent to give the following concentrations:

A - 1/2000;	D - 1/8000;
B - 1/4000;	E - 1/12000;
C - 1/6000;	F - 1/16,000.

Seven

Seven small tubes were taken, and to each was added 2 c.c. of the ten per cent suspension of red blood corpuscles. To the first six was added 2 c.c. of A, B, C, D, E and F respectively. To the seventh was added 2 c.c. 0.9 per cent sodium chloride as a control tube. These seven tubes were then placed in the incubator at 37° for two hours. At the end of this time the control tube remained unchanged. In A and B the haemolysis was complete, in C it was practically complete, while in the other three there was considerable but incomplete haemolysis. The haemolytic index was placed at 1/10,000. From the data given by Harrington on the quality of saponin required, this sample was considered satisfactory for the purpose.

Two 15 c.c. centrifuge tubes, J and K, were taken. To each was added 5 c.c. "Parathormone". On running 1 c.c. 6 per cent saponin into J, considerable amorphous precipitate was thrown down, which readily dissolved on adding 1 c.c. 10 per cent acetic acid. The solution was then warmed to 35° and 0.85 per cent ammonia slowly added till a distinct turbidity occurred, which took place after 0.6 c.c. had been added. After keeping J at 35° for thirty minutes, it was centrifuged. As the supernatant liquid was not clear, 0.2 c.c. of ammonia was added and the tube allowed to stand over night. K was treated in much the same manner, except that 1 c.c. 10 per cent acetic acid was added before the addition of saponin. With the adding of 0.8 c.c. ammonia, there resulted a more marked turbidity than in J. Centrifuged at the end of thirty minutes,

K showed

K showed about three times as much precipitate as was obtained from J before the 0.2 c.c. ammonia was added. K was set aside over night with J. Examination of the precipitates next morning showed them to be amorphous.

The precipitates were centrifuged. J was redissolved in hydrochloric acid and K in acetic. 2 c.c. 6 per cent saponin was added to J and 0.5 c.c. to K. Both tubes were warmed to 35° and ammonia slowly added till a distinct turbidity appeared. After keeping them at 35° for thirty minutes, they were centrifuged, yielding a fair amount of precipitate. The supernatant liquid was rapidly and accurately adjusted to pH 5.6. A very fine precipitate began to appear as this point was approached. After permitting both tubes to stand over night, the precipitate was examined for crystals but was found to be entirely amorphous.

To each of four more 15 c.c. centrifuge tubes, L, M, O and P, were added 5 c.c. "Parathormone" and 2 c.c. 10 per cent acetic acid. 0.5, 1, 2, and 3 c.c. 6 per cent saponin was added to L, M, O and P, respectively. 0.85 per cent ammonia was next added to each till a distinct turbidity appeared. This required 2.9, 1.95, 0.84 and 0.47 c.c., the less being required the more saponin present. After keeping at 35° for thirty minutes, the tubes were centrifuged. The precipitates, L_1, M_1, O_1 and P_1, on examination, showed complete absence of any crystalline material. The solutions decanted

from

from these were adjusted to pH 5.6 and set in the refrigerator for two days. A second series of precipitates, L_2, M_2, O_2, and P_2, was obtained, but these were also amorphous. O_2 was redissolved in 2.5 c.c. 10 per cent acetic acid and made up to 12.5 c.c. with water. 2.5 c.c. 6 per cent saponin was added and the pH adjusted to 5.6. Three days later the precipitate formed was examined and found amorphous.

Precipitates L_1, L_2, M_1, M_2, P_1, and P_2, after being centrifuged, were drained by inverting the tubes and letting them stand thus for three hours. The tubes and precipitates were weighed. Then the precipitates were dissolved in 3 c.c. 0.1 N hydrochloric acid, the solutions transferred, and the centrifuge tubes inverted and allowed to dry as before and then weighed. This enabled one to obtain an approximate estimate of the size of each precipitate. The results obtained are shown in Table XXI.

TABLE XXI.

	L_1	L_2	L_1+L_2	M_1	M_2	M_1+M_2	P_1	P_2	P_1+P_2
Tube and Ppts.	11.9	10.9		11.71	11.31		10.56	10.74	
Tube	11.47	10.62		11.26	11.015		10.155	10.385	
Ppts.	0.43	0.28	0.71	0.45	0.295	0.745	0.405	0.355	0.76

Although the above figures can only be approximate, it is of interest to note the tendency to a larger precipitate the more saponin used.

The

The solution of the parathyroid hormone obtained by dissolving precipitates M_1, M_2, P_1 and P_2 in 0.1 N hydrochloric acid were injected intramuscularly into dogs. No rise in serum calcium was obtained and the injections caused considerable swelling in the legs injected and general ill effects in the dogs.

From these results it would appear that saponin not only failed to be of use in attempting to apply Harrington's insulin method to a purification of the parathyroid hormone, but it in some way destroyed what potency was already present.

(b) Slow Adjustment to the Isoelectric Point with Carbon Dioxide:

In the first part of this experiment, a series of ten tubes, A, B, C, D, E, F, G, H, I and J, was set up and 5 c.c. "Parathormone" containing one hundred units of the active principle placed in each. In order to concentrate the hormone further, the active principle was precipitated with 0.12 c.c. of 0.5 N sodium bicarbonate. After centrifuging, the addition of more bicarbonate failed to bring down any further precipitate. The supernatant liquid was decanted off and the precipitate dissolved in 0.4 c.c. 0.1 N hydrochloric acid. Absolute ethyl alcohol was then added to A, B, C, D, E, F, G, H, I and J in such amounts as to make the alcohol concentration 10, 20, 30, 40, 50, 60, 70, 80, 90 and 95 per cent respectively. After leaving these acid-alcoholic

solutions

solutions of the parathyroid hormone in the refrigerator over night, examination the next day revealed no signs of a precipitate. To each of them was added 2 c.c. of a buffer solution (pH = 4.8), and in the case of A, B, and C, where no turbidity appeared, 0.1 N sodium bicarbonate was added till a slight turbidity was apparent. This required 0.5, 0.37, and 0.25 c.c. respectively. The pH of each of the ten solutions was determined and the following values found:

A = 4.8,	D = 4.6,	H = 4.7
B = 4.8,	E = 4.5,	I = 4.7
C = 4.7,	F = 4.5,	J = 4.9.
	G = 4.6,	

All the tubes were placed in the refrigerator for two days, at the end of which time they were examined for crystals. While a precipitate had formed in each case, it was strictly amorphous, and the addition of bicarbonate did not bring down any further precipitate, thus showing that all the active material was in the amorphous precipitate. Apparently, the slow adjustment of the pH to the isoelectric point of the hormone did not permit any separation of active and inactive material.

Inasmuch as approaching the isoelectric point from the acid side had not been of any avail, it was approached from the alkaline side by slowly adding carbon dioxide. For this purpose the precipitates obtained above were centrifuged

and,

and, after decanting the liquid, dissolved in 0.1 c.c. 1 N sodium hydroxide, with the exception of J, which had been precipitated from 95 per cent alcohol, and which appeared to be insoluble under this treatment. 3 c.c. water was added to each.

From this point each received a slightly different treatment. To A was added 1 c.c. of a buffer solution with pH = 4.8. This produced a small precipitate which dissolved on shaking. Carbon dioxide was passed into this solution. After about thirty seconds a milkiness appeared in the solution rather suddenly. B received the same treatment, except that it was warmed to 55° before passing in the carbon dioxide. This did not prevent the formation of the precipitate, nor did a further heating for ten minutes dissolve the precipitate. C was treated in the same manner as B, with the same results. In D, no buffer was added, and no heat applied. A precipitate similar in appearance to the others was formed by the carbon dioxide. In E, carbon dioxide was passed into the alkaline solution of the hormone only till the first trace of precipitate could be noticed. When this was shaken, more precipitate formed which was not dissolved by warming to 55°. To F still less carbon dioxide was added, the addition of the gas being stopped even before a precipitate could be seen. However, when this solution was shaken a precipitate gradually formed. G was a repetition of F, except that the treatment was carried out in the presence of the buffer with pH 4.8,

and the

and the solution warmed to 55° before adding the carbon dioxide. In H not enough carbon dioxide was added to give a precipitate. In I, the solution was warmed and a large excess of the gas added. The pH of each was determined and the following values found:

A = 6.4,	D = 6.7,	G = 8.0,
B = 6.6,	E = 6.6,	H = 8.4,
C = 7.5,	F = 7.0,	I = 6.5.

Precipitates of approximately the same size were formed in all of these with the exception of G and H. When more carbon dioxide was passed into G and H, precipitates were formed and the solutions showed a pH 6.7 and 6.2. At this point it will be recalled that J was left containing a precipitate obtained when to the acidified 95 per cent alcohol solution containing parathyroid hormone was added 2 c.c. of the buffer pH 4.8. This precipitate did not dissolve like the rest when 0.1 c.c. N sodium hydroxide was added. Examination after it had been left in the refrigerator in this condition for three days revealed a few needle-like crystals mixed with the amorphous material. Attempts to seed out some of these crystals were not successful. The precipitates formed in the other tubes were examined after being in the refrigerator three days and found to be entirely amorphous.

All the above precipitates were again centrifuged, separated from the supernatant liquid, and dissolved in 0.1 c.c.

N sodium

N sodium hydroxide. To each of these solutions was added 2 c.c. of a buffer, and carbon dioxide passed in till a precipitate began to appear. The pH of the solution at this point was noted. The results will be clear from the data in Table XXII.

TABLE XXII.

Tube	pH Buffer Added	Treatment	pH Resulting Solutions
A	5.2	CO_2 ⟶ precipitate	6.6
B	5.8	⟶ precipitate without CO_2	7.8
G	5.8	"	7.8
C	6.4	CO_2 ⟶ precipitate	6.9
D	7.0	"	6.8
E	7.6	"	6.8
F	8.0	"	6.7
H	5.2	pH > 8. CO_2 ⟶ precipitate	6.4
I	None	CO_2 ⟶ precipitate	6.5

The precipitate obtained from B without adding any carbon dioxide did not appear to be as great as in A. However, the addition of carbon dioxide did not increase the size of precipitate. The pH dropped to 6.2. This was repeated with a second sample, G, which gave identical results. The tubes were all placed in the refrigerator for twelve hours. On

close

close examination under the microscope the precipitates were found to be entirely amorphous.

The precipitates referred to in Table XXII were treated as follows:-

A: Set aside in refrigerator for three days to allow further opportunity for any crystals to form. No crystals appeared so the precipitate was dissolved in 0.2 c.c. N sodium hydroxide after centrifuging. To this alkaline solution 1 c.c. absolute ethyl alcohol was added. This produced a faint turbidity. At the end of twenty-four hours a precipitate had settled out which contained a few fine crystals mixed with amorphous material.

B: Dissolved in 0.2 c.c. N sodium hydroxide. Addition of absolute alcohol to this solution produced a turbidity which disappeared on shaking. More alcohol was added till the turbidity persisted on shaking. By twenty-four hours, a precipitate had been thrown down which was largely amorphous, but with a few crystals beginning to appear. Two days later, the tube contained quite a number of crystals. At the end of another two days, it was again examined and appeared to be mostly crystalline. An attempt was made to recrystallize this material. The crystals were redissolved in 0.8 c.c. 0.1 N sodium hydroxide, and 1.5 c.c. absolute alcohol added to produce a faint turbidity. A few crystals separated by the next day. These were dissolved in 1 c.c. 0.1 N hydrochloric

acid

acid.

C: Dissolved in 0.1 c.c. N sodium hydroxide, and diluted to 65 c.c. Carbon dioxide was passed into this solution till the first cloudiness appeared. The pH at this point was 6. The flask was corked and set in the cooler for three days. The precipitate formed was entirely amorphous. After centrifuging, it was dissolved in 0.2 c.c. N sodium hydroxide and 1 c.c. absolute alcohol added. The next day the precipitate formed contained a few crystals. The following day they were separated from the liquid, dissolved in alkali, and reprecipitated with alcohol. The crystals separated from this solution on standing were dissolved in 1 c.c. 0.1 N HCl.

D: Dissolved in 0.1 c.c. N sodium hydroxide and diluted to 50 c.c. Carbon dioxide was passed into the solution for thirty seconds, when a faint turbidity appeared at pH 6. The flask was corked and placed in cooler for three days. The precipitate formed was amorphous. It was dissolved in 0.2 c.c. N sodium hydroxide and 2 c.c. absolute alcohol added. By the following day quite a few crystals had formed which appeared to be insoluble in 0.8 c.c. N sodium hydroxide, but readily soluble in 0.1 N hydrochloric acid.

E: Dissolved in alkali, diluted, and treated with carbon dioxide in the same manner as was C. As before, the precipitate thrown down was amorphous. It was redissolved in alkali and then reprecipitated with alcohol. This procedure

yielded

yielded some crystals along with an amorphous material. The precipitate was dissolved in dilute ammonium hydroxide and then reprecipitated with alcohol. This precipitate was partly crystalline. It was dissolved in 1 c.c. 0.1 N hydrochloric acid.

F: Dissolved in 0.4 c.c. 0.1 N hydrochloric acid and diluted to 3 c.c.

G: Dissolved in 0.2 c.c. N sodium hydroxide, diluted to 10 c.c. with water, and to this added 10 c.c. absolute alcohol. No precipitate was formed till carbon dioxide was passed into the solution. A turbidity appeared at pH 6.5. The precipitate formed on standing was amorphous. When this amorphous material was dissolved in alkali and reprecipitated with alcohol, crystals were formed. These were dissolved in 1 c.c. 0.1 N hydrochloric acid.

H. Dissolved in 0.1 c.c. N sodium hydroxide. Added 7 c.c. absolute alcohol. The precipitate thrown down by this excess of alcohol was amorphous. It was redissolved in 0.8 c.c. of the alkali, and 1.5 c.c. of the alcohol added. Any precipitate formed by this addition redissolved on shaking. Although the solution was clear, yet it would take very little more alcohol to throw down a precipitate. This solution was placed in the cooler. Two hours later a precipitate was present and it was almost entirely crystalline. This was

dissolved

dissolved in alkali and recrystallized with alcohol. The recrystallized material was dissolved in 1 c.c. 0.1 N hydrochloric acid.

I. Dissolved in 0.4 c.c. 0.1 N hydrochloric acid, and the solution diluted with absolute alcohol until the concentration of the latter was 95 per cent. As in the case of J, this produced no precipitate until 2 c.c. of the buffer pH 4.8 was added, when a cloudy precipitate separated out. There was no tendency for this to become crystalline on standing. As with J, attempts to dissolve it in 0.1 c.c. N sodium hydroxide failed. However, after leaving this alkaline mixture in the cooler for two days, some crystals appeared.

The amorphous precipitate obtained from F with carbon dioxide, as already stated, and later dissolved in hydrochloric acid was injected in dog 47, but produced little or no rise in the serum calcium, the calcium values found being: Control - 10.5; 15 hour sample - 10.7.

The crystalline material obtained from B, E and H, and redissolved in acid, was injected in dog 47, and that from C, D and G was taken up in acid and injected in dog 48. Blood samples were drawn before the injection and six and sixteen hours afterwards. The values for serum calcium found in these bloods were:

Dog	Injected	Serum Calcium		
		Control	6 Hours	16 Hours
47	B, E, H	10.5	10.4	10.4
48	C, D, G.	10.6	10.5	10.5

This

This would indicate that the crystalline material had no activity in so far as its effect on the serum calcium was concerned. This was checked by preparing four more samples of crystals. In each of these the active principle was precipitated with sodium bicarbonate, centrifuged, and dissolved in 0.2 c.c. N sodium hydroxide. Sufficient absolute alcohol was then added till a very faint turbidity appeared. The crystals which separated from these on standing were dissolved in 0.1 N hydrochloric acid. When the first of these four preparations failed to give any rise in the serum calcium after being injected in dog 47, the other three were combined and injected in dog 48. Again there was no response.

Further light was thrown on the nature of these crystals when on heating them no charring occurred. As crystals had been formed in the absence of prepared buffers, the latter could be excluded when one considered what the crystalline material might be. The long needle-like shape excluded sodium chloride. This left sodium bicarbonate, which was introduced into the solution either at the beginning, when it was used to precipitate the active principle from acid solution, or later when carbon dioxide was passed into the sodium hydroxide solution to lower the pH and bring about precipitation. In both these cases the amount of sodium bicarbonate that could be present would be small, for the precipitates were centrifuged and the liquid decanted. Evidently sufficient bicarbonate remained adsorbed on the precipitate that was thrown down later in the

procedure

procedure when alcohol was added, for by the action of acid on the crystals, and by comparison with crystals of sodium bicarbonate formed from very dilute solutions under conditions parallel to those in the experiment, little doubt was left as to their identity.

The isoelectric point of the active principle has been shown by Collip to be in the neighbourhood of pH 4.8. This was verified when the pH of the solutions were determined after precipitating the active principle from acid solutions, either with sodium hydroxide or sodium bicarbonate. On the other hand, it was found that when the active principle was thrown down from an alkaline solution on passing in carbon dioxide, the pH was on the alkaline side of the original isoelectric point, the new pH being around 6.8, as has already been pointed out. In order to be certain that this precipitate thrown down by carbon dioxide contained the active principle and not some inert fraction, two samples, S and T, of "Parathormone", each containing one hundred units, were taken and 0.1 c.c. 0.5 N sodium bicarbonate added. After centrifuging, more bicarbonate was added, but no further precipitation occurred. The pH of the solutions at this point was 4.8. The liquid was decanted and the precipitates dissolved in 0.8 c.c. 0.1 N sodium hydroxide. Into these alkaline solutions carbon dioxide was passed and a precipitate was thrown down. The precipitates were removed by centrifuging and the pH of the solutions determined. It was 6.8. Addition

of more

of more carbon dioxide brought down a very slight precipitate at the same pH. The precipitates were washed with distilled water and then dissolved in 0.4 c.c. 0.1 N hydrochloric acid and the solution made up to 3 c.c. For convenience, these solutions will be designated S_p and T_p, and the filtrates obtained from the carbon dioxide precipitation S_f and T_f. The effect of these on the serum calcium when injected intramuscularly is shown below in Table XXIII.

TABLE XXIII.

Dog	Solution	Serum Calcium		
		Control	15 Hours later	Rise
49	S_p	10.1	12.8	2.7
50	S_f	11.0	10.8	0
51	T_p	11.5	15.9	4.4
52	T_f	11.0	11.2	0.2

The above results leave no doubt as to the carbon dioxide precipitate containing the active principle, and it would appear that the carbon dioxide removes all the active substance from solution.

(c) Continuous Extraction with Ethyl Alcohol: Collip and Clark have reported that the purest form of the parathyroid hormone obtained by several

several ioselectric precipitations was soluble to the extent of 0.1 per cent in absolute alcohol. This suggested the possibility of the small soluble fraction containing the hormone in purer form.

To test this possibility 500 grams of finely minced beef parathyroid glands was covered with an equal volume of 1.5 per cent hydrochloric acid and hydrolyzed in a water bath at 100° for forty-five minutes, at the end of which time four volumes of hot water were added. After chilling, the fat congealed and was removed from the surface and the solution made alkaline to phenolphthalein with sodium hydroxide. Hydrochloric acid was then added till considerable precipitation occurred, which at the same time permitted rapid filtration. After filtering, the precipitate on the filter paper was dissolved in weak alkali and again treated with acid as before. This process was repeated three times. By this means most of the active principle was saved. The hormone was then salted out by making the filtrates acid to Congo red and saturating with sodium chloride. The substance flocked out, rose to the top, and was filtered off. The precipitate was dissolved in weak sodium hydroxide, centrifuged, and the pH adjusted to 4.8. The isoelectric precipitate separating at this point was centrifuged off and then dissolved in hydrochloric acid. After the fourth isoelectric precipitation, the mother liquor was clear. The product was dried in a vacuum over calcium chloride for five days, after which it was placed in an

extraction

extraction thimble and subjected to continuous extraction with absolute ethyl alcohol under reduced pressure. The extraction was carried out in a special piece of apparatus made for this purpose. It is illustrated in Figure 8.

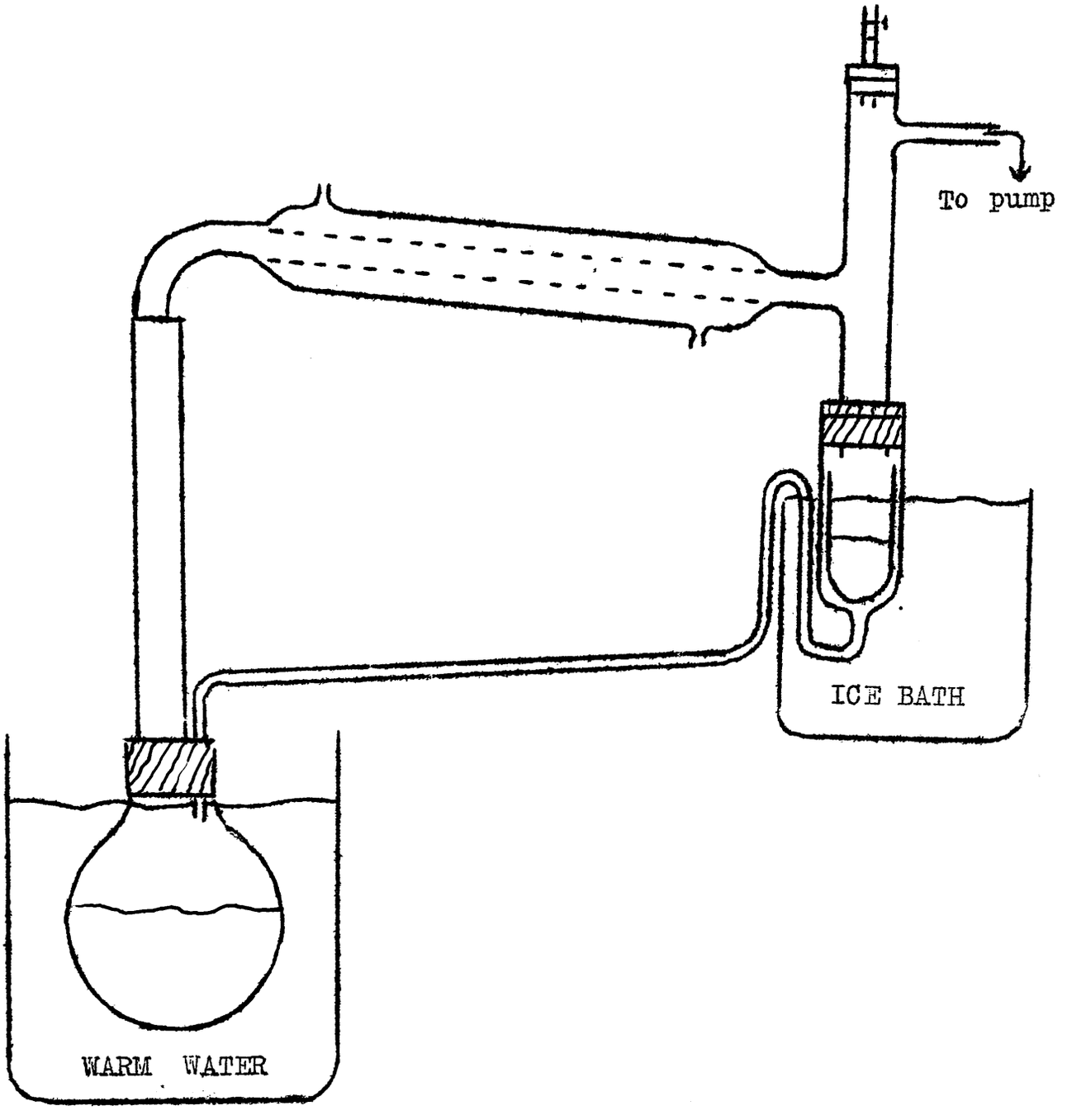
As the extraction continued, a fine white amorphous powder was thrown down in the boiling flask. The extraction was continued for forty-eight hours. A portion of the alcoholic extract on dilution with water became milky, and the addition of acid did not seem to affect this milky appearance. When sodium hydroxide was added the milkiness was replaced by a fine flocculation which was readily soluble in ether, which also dissolved the fine white precipitate which had settled out on the wall of the flask. Certainly the solubilities here noted do not correspond to those observed for the active principle as represented in the isoelectric precipitate. In this particular case, the fraction obtained by alcohol extraction appears to be of lipoid nature.

The alcohol was evaporated off, the residue suspended in 0.9 per cent sodium chloride, and injected intramuscularly in a dog. In addition to a control sample of blood, others were drawn at 17, 24, 41 and 48 hours. The values obtained for the serum calcium were:

Control	17 Hrs.	24 Hrs.	41 Hrs.	48 Hrs.
10.9	10.9	10.9	11.2	11.1

It is

FIGURE 8.



It is at once evident that the fraction separated in the absolute alcohol contained little or no active material.

Inasmuch as no attempt had been made to determine the potency of the isoelectric precipitate before extraction, there was no means of ascertaining whether the residue was more or less potent than the original precipitate. However, when a portion of the residue was dissolved in 3 c.c. 0.1 N hydrochloric acid and injected, the serum calcium rose 3 mgms., thus showing that the residue contained active parathyroid hormone. When the total nitrogen in the balance of the residue was determined it gave values of 14.3 per cent and 14.6 per cent, which is approximately that observed by Collip and Clark for their isoelectric precipitate.

From the above findings it seemed reasonable to assume that the potency of the parathyroid hormone had not been altered by continuous extraction with absolute ethyl alcohol.

(d) Continuous Extraction with Butyl Alcohol: In 1920, Dakin (105) suggested a method for separating the amino acids in proteins. After hydrolysis of the protein with acid, he neutralized the acid with baryta and then concentrated the aqueous solution till the semi-crystalline mass of amino acids began to appear. The latter was subjected to extraction with normal butyl alcohol

first

first and later with a saturated aqueous butyl alcohol solution.

While it was not desired to break up the isoelectric precipitate of the parathyroid hormone to such an extent as to destroy its potency, it was thought that extraction with butyl alcohol might remove an inert fraction and leave the hormone in purer form.

The same apparatus was used in this experiment as in the previous one. The isoelectric precipitate of the parathyroid hormone was in this case obtained by adding slowly a few drops of dilute sodium hydroxide to 20 c.c. "Parathormone" containing 20 units per c.c. The precipitate thus obtained was transferred to a small filter. After testing the filtrate, to ensure that all the active principle had been thrown down, the precipitate was washed several times with ether and then allowed to dry in air for two days. It was then subjected to continuous extraction with normal butyl alcohol (B. Pt. 117°C) for twelve hours. After this treatment the insoluble residue was washed several times with ether and then, after the ether had evaporated, dissolved in 5 c.c. 0.1 N hydrochloric acid. When this was injected in dog 53 the serum calcium rose at the end of fifteen hours from 10.4 to 13.4 per 100 c.c. The butyl alcohol extract was evaporated to dryness under reduced pressure and the residue taken up in 5 c.c. 0.1 N hydrochloric acid. This acid solution was injected in dog 57. It did not produce any rise in serum calcium, the values found being 10.6

before

before and 10.4 after the injection.

Another isoelectric precipitate of the hormone was prepared in the same manner from 20 c.c. "Parathormone", and subjected to extraction with normal butyl alcohol. The residue obtained was thoroughly washed with ether and dried in air for five days. At the end of this time the total nitrogen of the residue was determined and found to be 14.3 per cent and 14.1 per cent, showing that the extraction had not altered the nitrogen content of the active material.

A third lot of the isoelectric precipitate was similarly prepared and then extracted with saturated aqueous butyl alcohol for nine hours. The residue was washed with ether to remove the butyl alcohol and taken up in 5 c.c. 0.1 N hydrochloric acid. This, when injected in dog 53, raised the serum calcium from 10.5 to 13.7. On the other hand, when the aqueous butyl alcohol was removed from the extract and the residue dissolved in 5 c.c. 0.1 N hydrochloric acid and injected in dog 57, the calcium remained constant, within experimental error, the values being 10.6 and 10.8.

A fourth lot of the isoelectric precipitate was extracted with saturated aqueous butyl alcohol in order to determine the total nitrogen content of the residue and thus ascertain whether it had been altered by this treatment. No change in the nitrogen content was found.

(e) Precipitation ..

(e) Precipitation with Potassium Acid Phthalate: Collip (106) while working with his placental extract, observed that a large inactive precipitate could be thrown down from his A-P-L fraction by saturating with potassium acid phthalate, and he suggested that the action of this salt on the parathyroid extract might be worth trying.

Just enough dilute sodium hydroxide was added to 5 c.c. "Parathormone" to throw down the active principle and then redissolve it. The addition of dilute potassium acid phthalate to this solution caused no precipitation till four drops of 0.2 N hydrochloric acid was added, when a very slight precipitate resulted. This was allowed to stand for two hours, after which it was centrifuged and the precipitate separated from the liquid, which still showed some turbidity as though the precipitation had not been complete. The liquid was set aside in the cooler for four days. In the meantime the first precipitate obtained with the potassium acid phthalate was dissolved in 3 c.c. 0.1 N hydrochloric acid and injected in dog 53. The serum calcium before the injection was 10.7, and fifteen hours later it was 11.0. The filtrate from the first precipitate was taken from the cooler and examined. No further precipitation had occurred. The addition of four drops 0.2 N hydrochloric acid brought down further precipitate, which was removed by centrifuging. When this second precipitate was dissolved in 3 c.c. 0.1 N hydrochloric acid and injected in

dog 53,

dog 53, the calcium rose from 10.6 to 11.2. The filtrate from this second precipitate was injected in dog 57 and had no effect on the serum calcium.

When dilute potassium acid phthalate was added directly to a second 5 c.c. portion of "Parathormone" nothing but a very faint turbidity appeared, which did not become more pronounced on standing for seven days in the cooler. Dilute alkali was added at the end of this time in just sufficient amount to give a small precipitate which was removed by centrifuging, dissolved in hydrochloric acid, and injected in dog 53. The serum calcium found in this case was 10.5 before and 10.8 after the injection. The filtrate was taken and a few drops of dilute alkali added. This brought down a further and larger precipitate, which was dissolved in acid and injected in dog 57. The serum calcium, as a result, rose from 11.2 to 12.9. The filtrate from this second precipitate did not alter the serum calcium level in dog 53.

From the above it appeared that the potassium acid phthalate, under a suitable pH range, was capable of throwing down the active principle more or less completely depending on the amount of phthalate added.

A third 5 c.c. portion of "Parathormone" was saturated with potassium acid phthalate, and the amorphous precipitate separated from the liquid by centrifuging. When this precipitate was dissolved in acid and injected in dog 53, the serum calcium

rose

rose from 10.5 to 11.5. On the other hand, the filtrate produced no change in the serum calcium level in dog 57. Repetition of this experiment on the same dogs a week later produced in dog 53, after injecting the dissolved precipitate, a change in serum calcium from 10.6 to 13.8, while the filtrate showed no activity when injected in dog 57.

Factors in the Degree of Response to Parathyroid Hormone Injection.

The degree of response to parathyroid hormone injection not only varies with the species, but different individuals in the same species may show a marked difference in the degree of response. Dogs were chosen by Collip for his method of assaying parathyroid extracts because of their sensitiveness to this hormone in comparison with the response elicited in other animals, and because of the ease with which frequent blood examinations can be made. By using ten or more dogs and noting the average serum calcium rise, Collip has obtained a value for the commercial product which agrees remarkably well with the value found by Eli Lilly and Co., who prepare and standardize "Parathormone".

In the course of the present work there were occasions when the number of dogs desired for such assays was not available. In an effort to overcome this difficulty, an attempt was made to standardize four dogs by noting the serum calcium rise obtained from injecting fifty units of "Parathormone" intramuscularly at intervals of not less than three days. This, in

nearly

nearly all cases, was sufficient time to permit the calcium level to return to normal. It was hoped that, although the degree of response from one dog to the other might differ considerably, each would give a constant serum calcium elevation with repeated injections, and thus permit one to interpret such increases in terms of potency of the parathyroid extract. With this idea in mind, four dogs were injected twenty-eight times over a period lasting from October 27th to March 2nd. On each occasion fifty units of "Parathormone" were given intramuscularly. Two samples of blood (10 c.c. each) were drawn, one just before the injection and the other sixteen hours later. During this sixteen hour interval food and water were withheld, and no food was given during the twenty-four hours prior to the test. The values found for the control and sixteen-hour blood samples, together with any rise in serum calcium noted, are shown in Tables XXIV to XXVII, and are expressed as milligrams per 100 c.c. serum. In Table XXIV are included the results obtained from October 27th to December 8th.

TABLE XXIV.

TABLE XXIV.

Dog	47			48			49			50			Average Rise
Weight	15.7 K.			26.2 K.			23.7 K.			17.3 K.			
Sex	Female			Male			Male			Female			
Date	Control	16 Hours	Rise	Control	16 Hours	Rise	Control	16 Hours	Rise	Control	16 Hours	Rise	
Oct. 27	10.6	14.8	4.2	11.2	13.3	2.1	11.2	11.2	0	10.9	11.9	1.0	1.8
" 30	10.8	15.3	4.5	11.6	15.1	3.5	11.1	15.6	4.5	10.8	13.6	2.8	3.8
Nov. 3	11.0	15.4	4.4	11.0	13.8	2.8	10.9	14.3	3.4	11.0	13.2	2.2	3.2
" 6	10.6	15.1	4.5	11.0	13.9	2.9	11.8	14.6	2.8	12.1	13.5	1.4	2.9
" 10				10.5	13.9	3.4	10.7	13.3	2.6	11.0	13.5	2.5	2.8
" 27	10.5	14.2	3.7	10.2	16.1	5.9	10.3	12.4	2.1	10.7	14.0	3.3	3.7
Dec. 4	9.9	10.6	0.7	10.6	14.0	3.4	10.2	10.3	0.1	10.4	13.8	3.4	1.9
" 8	9.9	11.2	1.3	10.2	15.4	5.2	11.0	11.2	0.2				2.2
Average	10.5	13.8	3.3	10.8	14.4	3.6	10.9	12.9	2.0	11.0	13.4	2.4	2.8

From the data in Table XXIV. it is quite evident that the degree of response, elicited in the same dog with the same amount of the parathyroid hormone injected on different occasions, is not constant. Dog 47 showed a remarkably close agreement in the response obtained for the first four injections, and dog 48 showed a fairly constant response for the same occasions. Outside of this, these four dogs showed little or no uniformity in the degree of response. Another point of interest brought out in these results is the apparent increased tolerance shown by Nos. 47 and 49 with repeated injection, while on the other hand Nos. 48 and 50 tend to show a decreased tolerance. The failure of dog 49 to show any rise in serum calcium with the first injection of the hormone, while with the second injection the serum calcium rose 4.5 mgm., is worth noting. From the first result one would have been inclined to discard this dog as a non-reactor. Of the sixty-two dogs used by the experimenter, none could be classified as failing to respond to injection of the parathyroid hormone, although two of them showed no response to the first injection and several others showed less than 0.5 mgm. rise in serum calcium. In this connection it is interesting to note that the response to a second injection of the hormone has almost invariably been greater than that from the first, irrespective of the degree of response from the first. It is also observed from Table XXIV. that despite the marked variations in response, the average of the thirty observations made was 2.8, which closely approximates the theoretical value, 2.5.

Inasmuch

Inasmuch as many consider a sufficiency of vitamin D essential for the proper functioning of the parathyroid hormone, and since the dogs, cooped as they were in the animal house during the winter months, received little or no sunshine, it was thought that lack of sufficient vitamin D might be responsible for the variations in responses obtained. Accordingly, from December 9th to January 12th vitamin D, in the form of one dessertspoonful of cod-liver oil, was added to the daily ration of rolled oats and beef heart. The results obtained over the period December 9th to December 29th are given in Table XXV.

The addition of cod-liver oil appears to have had no effect either on the serum calcium level or on the response to parathyroid hormone injections. The average of the sixteen responses observed was 2.2, or 0.6 mgms. lower than the average found in the first series, reported in Table XXIV. This lowering is due to the increased tolerance shown by dogs 47 and 49.

Since

TABLE XXV.

Dog	47			48			49			50			Average Rise
Date	Control	16 Hrs.	Rise	Control	16 Hrs.	Rise	Control	16 Hrs.	Rise	Control	16 Hrs.	Rise	
Dec. 15	9.6	11.7	2.1	10.6	14.9	4.3	11.0	11.9	0.9	11.0	13.0	2.0	2.3
" 19	9.9	12.8	2.8	11.1	16.8	5.7	10.6	13.3	2.7	10.9	13.1	2.2	3.3
" 22	10.2	10.5	0.3	10.7	13.4	2.7	10.2	11.0	0.8	10.4	12.0	1.6	1.3
" 29	9.6	10.7	1.1	10.4	13.2	2.8	10.9	11.4	0.5	9.9	13.0	3.1	1.9
Average	9.8	11.2	1.4	10.7	14.6	3.9	10.7	11.9	1.2	10.6	12.8	2.2	2.2

Since injection of parathyroid hormone not only raises the serum calcium but, according to Greenwald and Gross (65), also increases the amount of calcium excreted, the source of calcium must become smaller with repeated injections unless sufficient calcium is supplied in the diet to make up for this loss. Further, inasmuch as the serum calcium starts to rise soon after the injection, reaching a maximum in the case of the dog in fifteen hours, when the hormone was given either subcutaneously or intramuscularly, and in about half this time, as will be shown later, when given intravenously, there must be some source of readily available calcium. If this readily available calcium has been depleted, the hormone would not be able to exert its full effect, that is the degree of response may depend not only on the strength of the extract used but also on the amount of readily available calcium. If this is true, then the lack of uniformity of response to parathyroid hormone with repeated injections of the same number of units may be due to the variation of the amount of readily available calcium, and an increase in the latter should give greater and more uniform responses. The effect in this direction of adding a soluble calcium salt to the diet was tried. On January 3rd 2 grams of calcium lactate was added daily to the present diet, which consisted of rolled oats, beef heart and cod-liver oil. The results are shown in Table XXVI, and while there appears to be no increase in the degree of response there is possibly a little better uniformity. Continued feeding of calcium lactate over a longer period might bring this out more clearly.

TABLE XXVI.

TABLE XXVI.

Dog	47			48			49			50			Average Rise
Date	Control	16 Hrs.	Rise	Control	16 Hrs.	Rise	Control	16 Hrs.	Rise	Control	16 Hrs.	Rise	
Jan. 5	10.0	11.6	1.6	10.6	15.0	4.4	11.1	11.9	0.8	10.7	13.8	3.1	2.5
" 8	9.9	12.9	3.0	10.7	13.3	2.6	11.0	11.9	0.9	10.8	13.6	2.8	2.3
" 12	10.0	11.0	1.0	10.6	12.8	2.2	10.7	11.2	0.5	10.9	12.5	1.6	1.3
Average	10.0	11.8	1.8	10.6	13.7	3.1	10.9	11.7	0.8	10.8	13.3	2.5	2.0

A closer examination of the diet of these dogs, together with a consideration of the earlier works of Mellanby and of Mirvish upon cereals with reference to the presence, particularly in oatmeal, of an anti-vitamin D factor, suggested that the large amount of oatmeal in the diet, together with a variable amount of meat, might be a factor in the irregularity of responses obtained. The rolled oats was made up into a thick porridge. Into this was stirred pieces of beef heart. After the porridge was cooked, it was stirred and ladled out into bowls. Each dog got about 1500 gms. of this mixture daily. Roughly, the proportion of porridge to meat was about nine to one, but it is easy to see that the amount of meat present might vary considerably.

On January 13th, the diet was changed. The dogs were given beef heart only. This was continued until January 20th, when cod-liver oil was added. On the 27th, the cod-liver oil was replaced by 2 grams of calcium lactate. On the 30th, the calcium lactate was withdrawn. From February 3rd to 10th, the dogs were placed on a diet of rolled oats only. On the 10th, 2 grams of calcium lactate was added to this oatmeal ration. From February 13th to March 2nd, beef heart only was fed. The results from this series of experiments are summarized in Table XXVII.

TABLE XXVII.

TABLE XXVII.

Diet	Dog	47			48			49			50			
	Date	Control	16 Hrs.	Rise	Control	16 Hrs.	Rise	Control	16 Hrs.	Rise	Control	16 Hrs.	Rise	Av.
Meat	Jan.15	10.3	12.3	2.0	11.0	15.0	4.0	11.3	11.6	0.3	10.9	12.8	1.9	2.0
	" 19	10.0	11.5	1.5	10.6	15.3	4.7	10.7	11.5	0.8	10.4	11.2	0.8	1.9
Meat & Cod-liver oil	Jan.22	10.1	11.1	1.0	10.9	15.4	4.5	10.7	11.5	0.8	10.5	12.2	1.7	2.0
	" 26	10.0	10.8	0.8	11.0	15.7	4.7	10.7	11.3	0.6	10.5	12.3	1.8	2.0
Meat & Ca lactate.	Jan.29	10.0	10.5	0.5	10.4	16.1	5.7	10.0	11.6	1.6	10.4	12.1	1.7	2.4
Meat	Feb. 2	10.2	10.7	0.5	10.6	14.9	4.3	9.9	10.9	1.0	10.4	11.8	1.4	1.8
Rolled Oats	" 5	9.9	10.4	0.5	10.1	13.6	3.5	10.2	10.0	0	10.3	11.1	0.8	1.2
	" 9	10.0	10.5	0.5	10.6	13.0	2.4	10.3	10.3	0	10.5	10.7	0.2	0.8
Rolled Oats & Ca.	Feb.12	9.9	10.2	0.3	10.0	13.9	3.9	10.5	10.3	0	10.3	10.7	0.4	1.1
Meat	Feb.16	10.1	10.5	0.4	10.6	13.4	2.8	10.6	11.0	0.4	10.1	11.5	1.4	1.2
	" 19	10.2	10.9	0.7	10.4	13.9	3.5	10.5	11.0	0.5	10.3	11.5	1.2	1.5
	" 23	10.2	10.9	0.7	10.5	14.0	3.5	10.5	11.0	0.5	10.3	12.0	1.7	1.6
	Mar. 2	10.8	11.9	1.1	10.9	14.6	3.7	10.9	11.0	0.1				1.6

It is at once evident from these results that dogs on a meat diet give a more uniform response than when placed on a diet containing either rolled oats or a mixture of rolled oats and meat. The addition of cod-liver oil did not appear to have any effect on the response to parathyroid hormone, while calcium lactate increased the average response slightly. On the rolled oats diet, the response was less. That this was not due merely to an increasing tolerance that would have occurred even on a meat diet is shown by the fact that when the diet was changed back to meat the response gradually rose again.

Before leaving this series of experiments Dogs 47 and 49 were injected with two hundred units (four times the dose given before) of parathyroid hormone. If the small responses elicited by fifty units throughout the latter part of the experiment were due to an acquired tolerance rather than a depleted source of readily available calcium, then the two hundred units should give a response of the order of four times that obtained with fifty units. On the other hand, if it were due to a depletion of the calcium available, the response would be of a much smaller order. The values obtained were:

Dog	Control	16 Hr.	Rise
47	10.8	15.3	4.5
49	10.6	13.3	2.7

These

These results point to an acquired tolerance rather than a depletion of available calcium as the chief factor responsible for the decrease in response.

From the results obtained with the dogs on the oatmeal diet, it appeared that the cereal contained some substance which interfered with the response of the animal to parathyroid hormone. As has already been mentioned, Mirvish (53) has prepared an extract from oatmeal which he found, in a number of cases, lowered the serum calcium in rabbits 30 per cent in twenty-four to forty-eight hours. He called the active principle of his extract "Calcovarin".

A number of extracts of "Calcovarin" were prepared and these injected in dogs, either separately or along with a definite amount of "Parathormone", and the effect on the serum calcium observed. In preparing these extracts, finely ground rolled oats was placed in a canvas bag and suspended in 0.6 per cent hydrochloric acid in water for forty-eight hours. At the end of this time, the whole thing was heated in a water bath for three hours, after which the liquid was poured off and the bags allowed to drain without squeezing. The cereal was then extracted a second time in fresh acid for another forty-eight hours, heated for three hours, and allowed to drain as before. After neutralizing most of the acid present and leaving the extract just acid to litmus, it was concentrated in a vacuum to one-quarter its original volume, filtered through

glass

glass wool and then further concentrated to about 100 c.c. The thick solution thus obtained was placed in collodion membranes and dialyzed against water for forty-eight hours. The yellow dialysate obtained was further concentrated till precipitation threatened. The first extract of "Calcovarin" was used in this form. In subsequent preparations, the concentration was carried further till only a brown paste was left. The latter was extracted with hot 95 per cent alcohol for two hours, after which the alcoholic extract was removed, the alcohol distilled off, and the residue dissolved in water and injected.

As stated above, the first extract prepared was not purified to the same degree as subsequent ones. This crude extract was injected subcutaneously in dogs 58 and 59. They each received the equivalent of 250 grams of rolled oats. At the same time dog 58 was also given fifty units "Parathormone" intramuscularly. The serum calcium changes noted are shown in Table XXVIII.

TABLE XXVIII.

Dog	Units "Parathormone"	"Calcovarin" Equivalents	Serum Calcium			
			Control	16 Hours	24 Hours	48 Hours
58	50	250 gm. R.O.	10.1	14.1	11.8	10.5
59		250 gm. R.O.	11.8	10.9	10.8	11.1

The 4 mgm.

The 4 mgm. rise observed from dog 58 would at first sight appear to preclude the possibility of any antagonistic action of the "Calcovarin" on the fifty units of "Parathormone" injected. However, this dog appeared to be remarkably sensitive to the parathyroid hormone, for when fifty units of the hormone were given on two occasions, four and eight days later, the serum calcium rose 6.1 and 5.8 mgms. respectively. Three days previous to the "Calcovarin" injection this dog received his first injection of the parathyroid hormone and gave a rise of only 4.4 mgms. for fifty units. From analogies, one would expect the response to the second injection to be greater than the first and equal to or greater than the third. If this applies here, then the "Calcovarin" has antagonized to some extent the effect of the parathyroid hormone.

It will be noted that the control calcium of dog 59 is rather high. However, this value is considered correct since, when the dog was first tested for response three days earlier, the serum calcium values found both before and after injection of fifty units of "Parathormone" were 11.8, thus agreeing with this value. The drop in the calcium level noted following the "Calcovarin" injection, although not large, is greater than the experimental error usually encountered, and hence it would appear that the extract prepared from rolled oats was capable of lowering the serum calcium.

A second extract of the oatmeal was prepared and on

this

Parathyroid Hormone, Cont.

this occasion the alcoholic extraction for further purification was applied. This second extract was divided into three equal portions (each equivalent to 300 gms. of rolled oats). One portion was injected intravenously in dog 56. A second portion was injected similarly in dog 57, which was also given intramuscularly, at the same time, fifty units of "Parathormone". The third portion was injected intravenously in dog 58, which also received, at the same time, and by the same route, fifty units of "Parathormone". The results of these injections on the serum calcium are shown in Table XXIX.

TABLE XXIX.

Dog	Units "Parathormone"	"Calcovarin"		Serum Calcium								
		Equivalents Gm.R.O.	Route	Control	2 Hrs.	4 Hrs.	6 Hrs.	8 Hrs.	12 Hrs.	15 Hrs.	24 Hrs.	48 Hrs.
56		300	Intrav.	10.5						10.5	10.2	10.5
57	50, Intramusc.	300	"	10.9						14.3	13.5	11.1
58	50, Intrav.	300	"	13.8	14.3	14.5	14.9	15.2	15.9	15.3	14.7	14.2

According to Mirvish's work on rabbits, the maximum lowering of serum calcium was obtained in the twenty-four or forty-eight hour samples. In dog 56 there may have been a slight lowering, but it was not sufficient to exclude that due to experimental error. When the effect of the double injection in dog 57 is considered, the net result seen is a 3.4 mgm. rise. This would appear to be the full normal response of this dog to

fifty units

fifty units of "Parathormone", for on the three previous occasions, January 13th, February 12th, and February 16th, the calcium elevations obtained with fifty units were 2.1, 4.8 and 2.9 respectively. The result reported in Table XXIX was obtained on February 19th. In the case of dog 58, the maximum elevation of serum calcium was 2.1 mgms., obtained in twelve hours. It will be recalled that this animal on previous occasions showed elevations of 4.4, 6.1 and 5.8 mgms. for the same dose of parathyroid hormone. Such figures would tend to lead one to believe the extract from rolled oats had an antagonistic action on the parathyroid hormone injected. However, it will be noticed that the control calcium is already well up at the start of this experiment and consequently the response to the parathyroid hormone injected will be somewhat less. Consequently, one cannot be certain whether or not the extract from rolled oats had any antagonistic action on the parathyroid hormone in dog 58.

A third lot of the extract was prepared and divided in three portions (each equivalent to 500 gms. of rolled oats). One portion was injected intravenously in dog 53. A second portion was given in like manner to dog 59. Dog 57 was given the third portion, together with fifty units of "Parathormone". The effect of these extracts on the serum calcium is shown in Table XXX.

TABLE XXX.

TABLE XXX.

Dog	Units "Parathormone"	"Calcovarin"		Serum Calcium								
		Equivalent	Route	Control	2 Hrs.	4 Hrs.	6 Hrs.	8 Hrs.	12 Hrs.	15 Hrs.	24 Hrs.	48 Hrs.
53		Rolled Oats 500 gms.	Intrav.	10.1						10.1	10.3	10.2
57	50, Intrav.	500 "	"	11.0	11.7	12.9	13.2	13.0	13.4	12.3	11.2	11.0
59		500 "	"	9.8						10.0	10.0	9.9

From the data in Table XXX, the oatmeal extract evidently had no lowering effect on the serum calcium in dogs 53 and 59. When this extract was injected in dog 57, along with fifty units of "Parathormone", the serum calcium rose 2.4 mgms. Under similar treatment (except that the dose of "Calcovarin" was smaller), as shown in Table XXIX, the serum calcium rose 3.4 mgms. Whether this decrease in response resulted from the larger dose of "Calcovarin" given, or was merely due to the natural variation in response that has been noted before, can only be shown by further injections (See Table XXXI).

A fourth lot of extract was prepared and purified by alcoholic extraction as before. This extract was divided into three equal portions, each equivalent to 500 grams of rolled oats. One portion was injected subcutaneously in dog 53. A second portion, together with fifty units of "Parathormone", was given intravenously to dog 56. The third

portion

portion was injected subcutaneously in dog 57, which also received fifty units of "Parathormone" by the same route. The results obtained are shown in Table XXXI.

TABLE XXXI.

Dog	Units "Parathormone"	"Calcovarin"		Serum Calcium								
		Equivalent	Route	Control	2 Hrs.	4 Hrs.	6 Hrs.	8 Hrs.	12 Hrs.	15 Hrs.	24 Hrs.	48 Hrs.
53		Rolled Oats 500 gms.	Subcu.	10.7						10.7	10.9	
56	50, Intrav.	500 "	Intrav.	10.8	11.3	12.2	11.5	11.1	1.0	10.4	10.7	10.6
57	50, Subcu.	500 "	Subcu.	11.0						15.	13.3	

On a previous occasion, with the same amount of "Calcovarin", but given intravenously rather than subcutaneously, dog 53 showed no lowering in serum calcium. At that time it was thought that failure of the serum calcium to fall might be due to the "Calcovarin" being too quickly removed from the system when injected directly into the blood stream. However, the subcutaneous injection, which would spread any effect over a longer period, did not appear to have any influence either on the serum calcium level. In the case of dog 56 the maximum response obtained was a 1.4 mgm. rise in the serum calcium, and this came at the end of the first four hours. Since this dog had previously given responses of 4.0 and 3.1 when injected intramuscularly with fifty units of "Parathormone", the "Calcovarin" may have had some antagonistic action, unless the response to the parathyroid extract is smaller when the hormone is given intravenously. As

most

most injections of the parathyroid extract have been given intramuscularly or subcutaneously, there are not many figures available on intravenous injections. If the "Calcovarin" has not antagonized the "Parathormone" injected intravenously in this work, it would appear that the response from intravenous injections is smaller and that the maximum elevation of serum calcium may come sooner than when the hormone is given either subcutaneously or intramuscularly. As to dog 57, little need be said, for the response to the "Parathormone" injected appears to be quite normal despite the "Calcovarin" present, thus lending further support to the data already given above that "Calcovarin" does not lower the serum calcium in dogs.

In order to be certain that the substance, claimed by Mirvish to cause a lowering of serum calcium in rabbits, was not being lost by failure to dissolve it in the treatment with alcohol, the residue from this treatment in preparing the last extract was dissolved in water, concentrated to 50 c.c., and injected in dogs 60 and 61. Dog 60 was given 10 c.c. subcutaneously, while dog 61 was injected with 40 c.c. intravenously. No change in the serum calcium level was noted in either dog, as shown below, in Table XXXII.

TABLE XXXII.

Dog	Water Solution of Alcohol Insoluble Residue		Serum Calcium		
	Equivalents	Route	Control	15 Hrs.	24 Hrs.
60	300 gms. R. O.	Subcu.	11.3	11.1	11.5
61	1200 " "	Intrav.	10.7	10.9	10.8

The data presented in Tables XXVIII. to XXXII. force one to conclude the absence in the oatmeal extracts of any substance in sufficient quantity to lower the serum calcium in the dog. If larger doses were given, or the injections repeated over a period of time, such extracts might affect the calcium level, but the present data obtained on dogs does not support Mirvish's contention.

The smaller responses obtained from dogs 58, 57, and 56, as shown in Tables XXIX, XXX and XXI, when the "Parathormone" was given intravenously, made it seem advisable to ascertain whether this was due to the manner in which the hormone was given, or whether it was due to the presence of the extract from the oatmeal. Accordingly, four dogs whose responses to the parathyroid hormone were pretty well known were each, on separate occasions, given fifty units of "Parathormone" intravenously, and the blood serum calcium followed closely. The results obtained are shown in Table XXXIII, and are accompanied, for comparison, with the most recent fifteen hour elevations previously found for the same dose given intramuscularly.

TABLE XXXIII.

TABLE XXXIII.

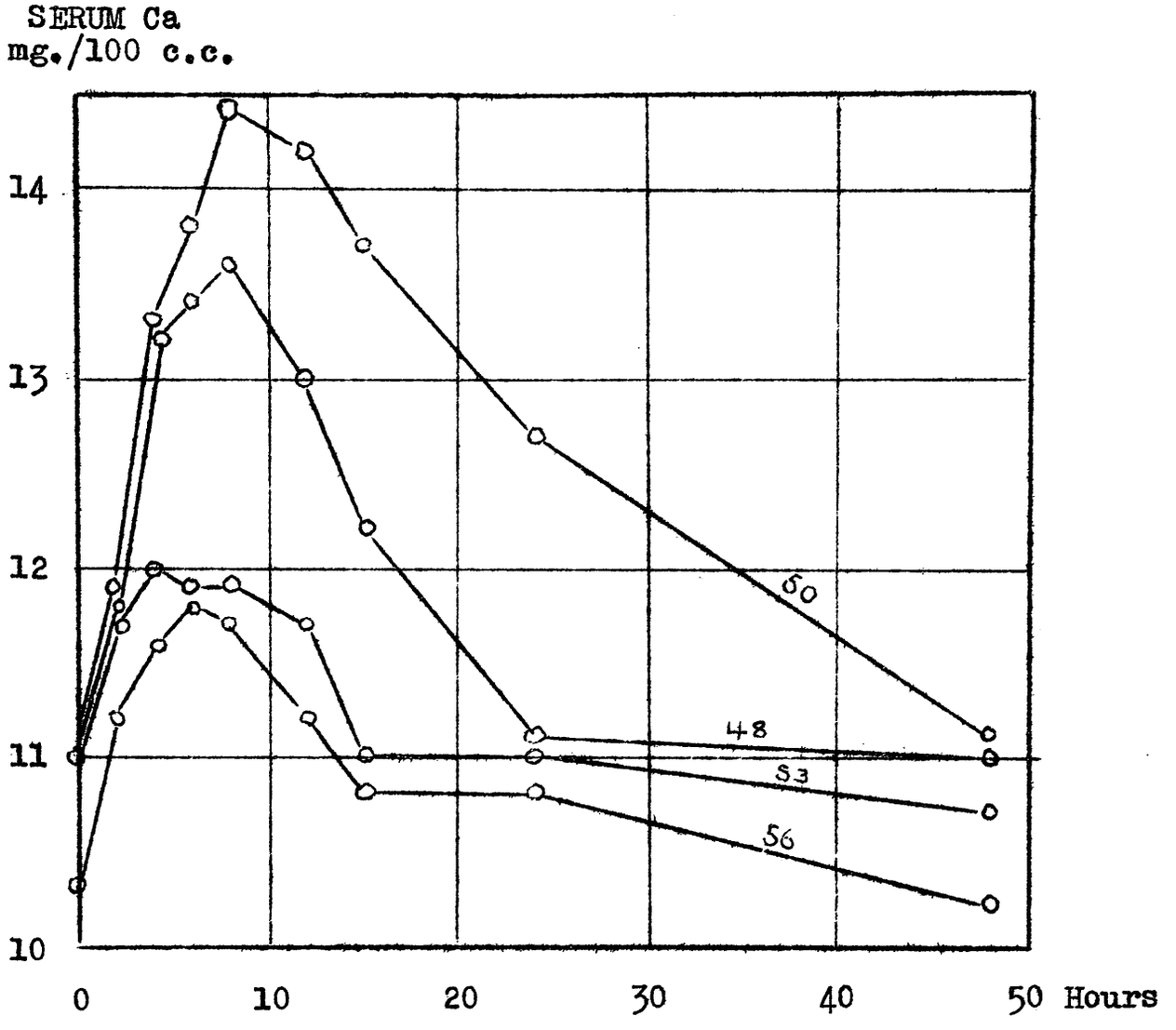
Dog		Serum Calcium								
	15 Hrs. Intramusc.	Control	2 Hrs.	4 Hrs.	6 Hrs.	8 Hrs.	12 Hrs.	15 Hrs.	24 Hrs.	48 Hrs.
53	2.2	11.0	11.7	12.0	11.9	11.9	11.7	11.0	11.0	10.7
56	3.1	10.3	11.2	11.6	11.8	11.7	11.2	10.8	10.8	10.3
48	3.6	11.0	11.9	13.3	13.4	13.6	13.0	12.2	11.1	11.0
50	5.8 (?)	11.0	11.8	13.2	13.8	14.4	14.2	13.7	12.7	11.1

In each of the above cases the maximum serum calcium value was reached in four to eight hours rather than in fifteen hours as found by Collip when the hormone was given either subcutaneously or intramuscularly. Further, the serum calcium elevation resulting from intravenous injection was in each case less, being in two of these only 50 per cent of that obtained by intramuscular injection. The value 5.8 for dog 50 has been questioned since it is much above that animal's usual response. However, it is the last elevation noted in this dog previous to the intravenous injection which was given one week later. At the time this 5.8 value was obtained it was noted that the dog had had a hard vomiting spell just previous to receiving the injection. Whether this disturbance was responsible for the greater response than usual is not known, and is mentioned here only as an observation and possible cause.

To show more clearly the path taken by the serum calcium following intravenous injection, the values shown in Table XXXIII have been plotted in Figure 9.

FIGURE 9.

FIGURE 9.



Although considerable work has been done on the raising of serum calcium with irradiated ergosterol and with parathyroid hormone, the result obtained by Bischoff (76), on using a combination of the two, was rather surprising and seemed to warrant further study. Accordingly, two dogs, Nos. 55 and 62, were injected intravenously with a preparation of irradiated ergosterol, kindly supplied by H. Jephcott, of the Glaxo Laboratories, London, and stated to contain a million "Ostellin" units per c.c. It was planned to obtain hypercalcaemia in this manner and then, stopping the injections of irradiated ergosterol, to give one hundred units of parathyroid hormone to one of the dogs and follow the serum calcium curves in both till they returned to the original normal level. By following this procedure, and making a comparison of the calcium curves obtained, the nature of the effect of parathyroid hormone injection under these conditions could be studied. Three possibilities were considered. The hormone might have an additive effect, no effect, or it might depress the serum calcium level.

Beginning with 100,000 "Ostelin" units, the daily dose of irradiated ergosterol was increased up to two million units. The changes in the calcium and inorganic phosphate of the serum were followed. In both dogs, the serum calcium first showed a slight fall, reaching a minimum on the fourth day. It regained its original level about the sixth day, and from this point began to gradually rise above the control value. On the tenth day the calcium of dog 55 began to climb more rapidly, while that of dog 62 continued rising at a much slower rate. At

this

this point the ergosterol injections were stopped. Each dog had received 10,200,000 "Ostelin" units. On the eleventh day dog 55 had a calcium value of 16.5 and six hours later it was 17.6. One hundred units of parathyroid hormone were injected intramuscularly in dog 55 at this time. Up to this point the animal showed none of the symptoms generally associated with hypercalcaemia. On the morning of the twelfth day (eighteen hours later) the serum calcium had risen to 22.8 and the dog showed the usual symptoms accompanying parathyroid hormone overdosage . Twenty-four hours after the hormone had been injected the calcium had fallen to 20.5. The blood was rather viscous at this time, and vomiting of blood had occurred. The dog was almost in a state of collapse. In an attempt to save the animal, saline injections were given intravenously, 275 c.c. being administered the first time. This caused an immediate and marked improvement in the dog's condition. On the thirteenth day the dog did not appear so well. Two more injections of saline were given, 300 c.c. in the morning and 500 c.c. in the afternoon. Before giving the afternoon injection, a blood sample was drawn. The calcium content was 17.3. By the fourteenth day it had fallen to 16.3. Another 500 c.c. of saline was administered on the morning of the fourteenth. On the morning of the fifteenth day the animal appeared to be much worse, had been vomiting blood, exhibited marked dyspnoea, and was almost in a state of collapse. Attempts to collect a blood sample by vein puncture were not successful, due to the viscosity of the blood. The dog was killed and a blood sample obtained immediately from the vena cava. The

calcium

calcium content was 13.6. On opening up the stomach of this dog, the typical chocolate-coloured mucosa, previously noted by Collip in cases of dogs killed by parathyroid hormone overdosage, was found.

The phosphorus curve followed the same general direction as the calcium curve, except that the rise was more gradual and less marked. The maximum level in dog 55 was 8.2, which was reached twenty-four hours before the dog was killed. The highest phosphorus value obtained for dog 62 was 7.6, and for its calcium 12.7.

During this experiment dog 62 appeared to remain in good health. On the other hand, dog 55 showed many signs of trouble. The urine secretion of the latter began to rise sharply on the tenth day, reaching a maximum on the eleventh day, when the volume was four to five times that normally secreted. Following the injection of parathyroid hormone, the urine secretion dropped to about twice the normal volume, rose again the next day to four times, and then dropped a little on the fourteenth day, with a still further drop on the fifteenth and last day, although the volume collected for this day was still above the normal secretion. Pugsley (107) found that the urinary calcium for dog 55 began to rise sharply on the tenth day, reaching a maximum on the eleventh day, when it was approximately nineteen times the normal value for this dog. It remained high throughout the rest of the experiment,

varying directly with the volume of urine secreted. The inorganic phosphate excreted was also followed by Pugsley. In the case of dog 55 it rose on the eleventh day to one and a half times the normal level, and then dropped back on the twelfth day to normal. The last three days of the experiment it was below the normal level. In the case of dog 62, there was no increase of calcium excretion, but, if anything, a slight decrease, while the phosphorus excretion remained at practically the normal level.

By the fifteenth day the serum calcium and phosphorus of dog 62 had fallen to 12.3 and 6.5 respectively, and were still there on the seventeenth day. On this and the next four days dog 62 was given a daily dose of two million "Ostelin" units. In contrast to the first injections of irradiated ergosterol given to this dog, these second injections produced within twenty-four hours a rise in both serum calcium and phosphorus which although only slight at the end of the first twenty-four hours rose more rapidly thereafter. In this instance, the serum calcium rose to 23.7 by the twenty-fourth day and then gradually fell again to 11.7 by the thirty-fifth day. The serum inorganic phosphate reached its highest level (7.7) on the twentieth day. Reference to Figure 10 shows the general tendency of the phosphorus in the serum to gradually return to normal after the twenty-second day, and even drop below normal.

Pugsley has

Pugsley has reported this urinary calcium and inorganic phosphate as showing increases similar to those noted for dog 55 in the first half of this experiment.

At the beginning of the experiment, dog 62 weighed 16.5 k. and at the end of the first three weeks weighed approximately the same (16.2 K.). On the twenty-third day, with its calcium at 22,5, dog 62 ate very little, and from here till the thirty-fifth day had a very poor appetite and lost weight steadily. On the thirty-third day it weighed 13.6 K. From the thirty-fifth day on, its appetite was much improved, and all the food given was eaten. Its weight on the thirty-seventh day was 13.8 K., and on the fortieth and last day of the experiment, 13.9 K.

The changes in calcium and inorganic phosphate of the serum for dogs 55 and 62 following the injections of irradiated ergosterol have been summarized in Tables XXXIII. and XXXIV, and graphically represented in Figure 10.

TABLE XXXIII.

TABLE XXXIII.

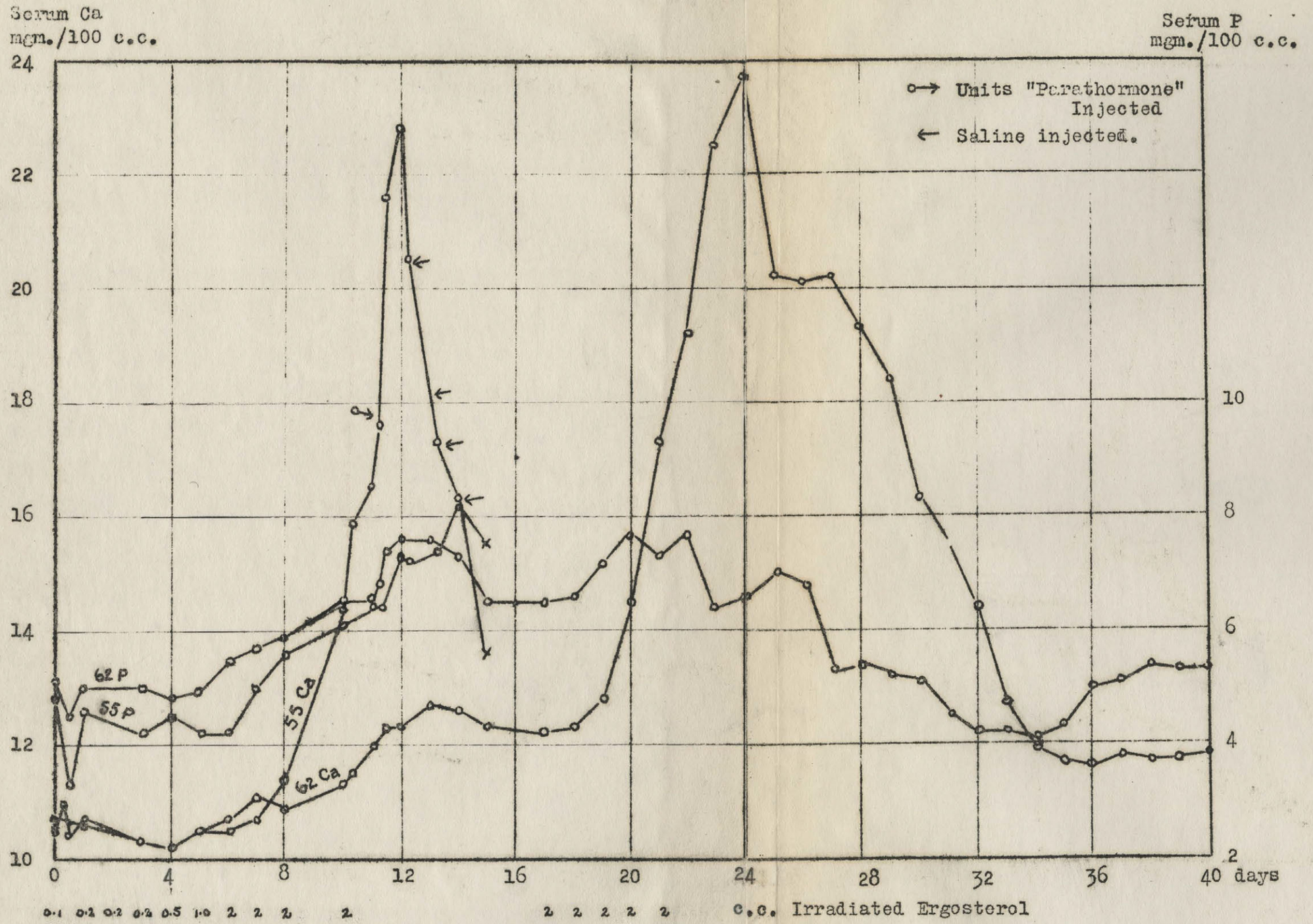
Time	Dog 55 Female, 17.5 K.		Dog 62 Female, 16.5 K.	
	Ca	P	Ca	P
Control	10.5	4.8	10.7	5.1
6 hours	11.	4.0	10.7	
12 "	10.4	3.3	10.6	4.5
1 day	10.7	4.6	10.6	5.0
3 days	10.3	4.2	10.3	5.0
4 "	10.2	4.5	10.2	4.8
5 "	10.5	4.2	10.5	4.9
6 "	10.5	4.2	10.7	5.5
7 "	10.7	5.0	11.1	5.7
8 "	11.4	5.6	10.9	5.9
10 "	14.4	6.1	11.3	6.5
+ 6 hours	15.9		11.5	
11 days	16.5	6.4	12.0	6.5
+ 6 hours	17.6	6.4	11.8	6.8
+12 "	21.6		12.3	7.4
12 days	22.8	7.3	12.3	7.6
+ 6 hours	20.5	7.2		
13 days			12.7	7.6
+ 6 hours	17.3	7.4		
14 days	16.3	8.2	12.6	7.3
15 "	13.6	7.5	12.3	6.5

TABLE XXXIV.

Time	Dog 62		Time	Dog 62	
	Ca	P		Ca	P
17 days	12.2	6.5	29 days	18.4	5.2
18 "	12.3	6.6	30 "	16.3	5.1
19 "	12.8	7.2	31 "	15.5	4.5
20 "	14.5	7.7	32 "	14.4	4.2
21 "	17.3	7.3	33 "	12.7	4.2
222 "	19.2	7.7	34 "	11.9	4.1
23 "	22.5	6.4	35 "	11.7	4.3
24 "	23.7	6.6	36 "	11.6	5.0
25 "	20.2	7.0	37 "	11.8	5.1
26 "	20.1	6.8	38 "	11.7	5.4
27 "	20.2	5.3	39 "	11.7	5.3
28 "	19.3	5.4	40 "	11.8	5.3

Figure 10.

FIGURE 10.


Serum Ca
mgm./100 c.c.
Serum P
mgm./100 c.c.
24
22
20
18
16
14
12
10
10
8
6
4
2
Units "Parathormone" Injected
Saline injected.
62 P
55 P
55 Ca
62 Ca
0
4
8
12
16
20
24
28
32
36
40 days
0.1 0.1 0.2 0.2 0.5 1.0 2 2 2 2
2 2 2 2 2
c.c. Irradiated Ergosterol

DISCUSSION.

The twelve cats injected intravenously with forty to one hundred units of parathyroid hormone showed no rise in serum calcium in ten minutes, and little or no rise at the end of the first or second hour. Inasmuch as the amount of parathyroid hormone given was from four to ten times that used by Stewart and Percival, there seems little likelihood that the cat can be used in assaying extracts of the hormone by noting the effect of the latter on the serum calcium over periods of two hours or less. Further, since the assay using cats has to be carried out under anaesthesia, there is always the danger of elevating the serum calcium by asphyxia, which may be the cause of the calcium elevations reported by Herxheimer, and by Stewart and Percival.

By simplifying, as far as possible, the procedure for the preparation of active extracts of the parathyroid hormone, it has been possible to make a comparative study of the factors influencing the potency of these extracts. It was found that making the extract alkaline, following acid hydrolysis, improved the rate of filtration, but such treatment had no effect on the potency of the extracts prepared. Inasmuch as making the acid hydrolysate alkaline to phenolphthalein and leaving it thus for periods of thirty seconds, one hour, or four hours, before restoring the pH to 5.6, did not alter the potency from that shown by extracts which had

been

been adjusted to pH 5.6 without making them alkaline, it appeared that the acid hydrolysis had freed the active principle and that the latter was not affected, as far as potency was concerned, by rendering the solution alkaline.

Having ascertained that the alkaline treatment was not necessary for obtaining a potent parathyroid extract, this step was omitted in preparing extracts for a comparative study of potency. From Figures 1 to 7 it is at once clear that the strength of acid and length of hydrolysis are important factors in the degree of potency obtained. Of the three strengths of acid used, 1.5, 3, and 5 per cent, the weakest of these yielded the extract with greatest potency. With five per cent. acid, the hydrolysate obtained by fifteen minutes extraction showed only slight activity, and those prepared by longer periods of extraction showed no activity. Using acetone-preserved glands the optimum period of hydrolysis with 1.5 and 3 per cent acid was forty-five minutes, while with glands preserved in "dry ice" the optimum period was thirty minutes. When the results obtained from the extracts of both acetone-preserved glands and those preserved in "dry ice" were combined and the averages plotted, as shown in figure 7, it was seen that as the strength of acid employed became greater, the optimum period of hydrolysis became shorter. From this, it would seem that while acid hydrolysis freed the active principle from the glands, too long hydrolysis destroyed it, and the rate of destruction was greater the stronger the acid. With the three strengths of acid used in the four

periods

periods of hydrolysis the greatest potency was obtained with forty-five minutes extraction, using 1.5 per cent acid.

From the data given in Table IX it will be seen that little trouble was experienced in maintaining a mixture of finely ground parathyroid glands in saline at definite pH. The original pH of such a mixture was 6.8, and this remained constant over the full period of observation (25 days). This and the fact that it required considerable buffer solution to alter the pH suggested that the gland tissue was already highly buffered. Where the gland mixtures were made alkaline, the new pH was maintained by addition from time to time of small amounts of alkali. Where a more acid pH was set up, a small amount of acid was required from time to time to retain this acidity. While the lowering of an alkaline pH might be assumed due to the formation of acids by autolysis, such reasoning cannot be applied when one considers the mixtures set up at pH 6.8 and pH 6.4, since the one at pH 6.8 retained this value, and the other at pH 6.4 tended to become less acid. The constancy of the original pH and the tendency of those mixtures adjusted to the acid or alkaline side of this point to return to the original pH suggested that this was due to a strong buffering action possessed by the glands.

Since incubation of the parathyroid glands in physiological saline at 35° for periods of one hour to twenty-five days, and at pH values ranging from 3 to 7.6, did not free

the active

the active principle, it must still be present in the undissolved residues, or have been inactivated during the incubation. The latter proved to be the case for extraction of the residues in the usual way at 100° for forty minutes with 1.5 per cent acid failed to yield an extract showing any potency. The cause of this inactivation has been traced to the presence of enzymes in the tissue. Incubation at 35° in physiological saline adjusted to pH 6, even after destruction of these enzymes, did not free the active principle. However, the active principle was shown to be still present in the gland material for subsequent digestion of the residue with 1.5 per cent acid at 100° gave a potent extract. From these results it would appear that the higher temperature is necessary both to destroy the enzymes, which would otherwise inactivate the hormone, and to free the latter from the tissues. While active extracts were obtained when the acid digestion was carried out at 80° instead of 100°, no activity was obtained by extracting at 60°. The failure of this 60° extract to be potent may be due to the active principle not being freed at this temperature, or to the survival of the inactivating enzymes. The possibility of the inactivation being due to instability of the active principle, when kept at 35° for too long a period, was found to have no support since a sterile preparation of the parathyroid extract held at 35° for thirty days appeared to have lost none of its activity. Further, since extraction with physiological saline at 100° failed to yield any potency, acid was considered

essential

essential in preparing active extracts.

Attempts to apply Harrington's saponin method for the purification of Insulin to further purify the active principle of the parathyroid glands yielded precipitates which were both amorphous and inactive. That the saponin exerted some buffering effect seemed reasonable since the appearance of the precipitate in its presence was gradual and not abrupt as would have been the case had the isoelectric point been approached in similar manner in the absence of a buffer. However, inasmuch as the activity which had been present was destroyed by the saponin, it appeared that the latter had reacted with the active principle to form an inactive substance.

When a buffer was added to a series of acid solutions of the active principle containing alcohol in concentrations from 10 to 95 per cent, an amorphous precipitate gradually formed in each case. Further examination after leaving the material in the ice box for forty-eight hours indicated that precipitation was complete and the nature of the precipitate entirely amorphous. From this it appeared that slowly approaching the isoelectric point from the acid side did not bring about a separation of active and inactive fractions.

The isoelectric point was then approached from the alkaline side by adding carbon dioxide both in the presence and in the absence of buffers, and at different temperatures. The precipitates obtained in this manner were always amorphous.

Heating

Heating to drive off carbon dioxide did not cause the precipitate to go back into solution. The peculiar point about the carbon dioxide precipitations was the pH at which they took place. It will be noted that they occurred as high as pH 7.8 and the lowest level was 6.4. Despite this, the precipitate was shown to contain the active principle and if sufficient carbon dioxide had been added, the filtrate was free of the active substance. When these precipitates were dissolved in alkali and alcohol added, the precipitate thrown down was later found to contain fine needle-like crystals. Further work, however, showed these crystals to be inactive, and they were later identified as crystals of sodium bicarbonate.

Continuous extraction of the isoelectric precipitate with absolute ethyl alcohol did not alter its nitrogen content nor destroy its potency. This, together with the fact that the alcoholic extract did not exhibit any definite activity, was taken to indicate that such extraction did not alter the composition of the isoelectric precipitate, and hence could not be used to split the active principle into smaller units.

While in Dakin's method of protein anyalysis butyl alcohol has been used only to separate various amino acids after hydrolysis of the protein, it was thought that extraction with butyl alcohol might split off an active or inactive fraction from the protein-like principle of the parathyroid glands. Neither the absolute nor the saturated aqueous butyl

alcohol

alcohol extracts contained any activity. The residues remained potent and their nitrogen content constant. From this it was inferred that the composition of the isoelectric precipitate had not been altered by the butyl alcohol extractions.

When sufficient potassium acid phthalate was added to a solution of the parathyroid hormone in a suitable pH range, all of the active principle was removed from the solution. With smaller amounts of the phthalate, some of the active principle was left behind in the solution, this amount being smaller the more phthalate added. No inactive fraction was separated.

The effect of diet on the degree of response to parathyroid hormone has been followed in four dogs for a period of a little over four months. During this period each dog received twenty-eight injections of the hormone in doses of fifty units. On a diet of rolled oats and beef heart (roughly 9:1), there was considerable variation in the degree of response in each dog from time to time. Although occasionally consecutive values obtained for the serum calcium elevation were close, no uniformity in response could be counted on. Of these four dogs, two showed a tendency towards an increase in tolerance and two towards a decrease. In each case the second injection of the hormone elicited a greater response than the first. Addition of cod-liver oil to the diet did not alter the degree of response nor make it more uniform, and hence the lack of uniformity of response in the same dog on successive

occasions

occasions could not be attributed to a deficiency of vitamin D. Addition of calcium lactate to this diet, in order to increase the readily available calcium, did not bring about any definite improvement. When the oatmeal was removed from the diet and meat alone fed, a much more uniform response was obtained. This became more evident when the average response of the four dogs from time to time was compared. Still further improvement was found when cod-liver oil was added to this meat diet. Addition of calcium lactate appeared, if anything, to lessen this uniformity. Replacing the meat diet by one containing only oatmeal lessened both the degree and uniformity of response. Reverting once more to the meat diet brought about an increased and more uniform response. From this it would appear that dogs respond better and more uniformly on a meat diet, and this uniformity is further improved by addition of cod-liver oil.

From the poorer and less uniform responses obtained on the oatmeal diet it would appear that the oatmeal might contain some substance antagonistic to the action of the parathyroid hormone. Attempts to obtain definite evidence of this, in the way of an extract from the oatmeal which would lower the serum calcium in dogs or reduce the effect of "Parathormone", were not successful. From this it would seem that further work will be necessary before one can decide whether the oatmeal contains a substance interfering with calcium metabolism, or whether there is something lacking in the oatmeal necessary for normal calcium metabolism.

The

The reaction of dogs 47 and 49 to a large dose of "Parathormone" after they had shown a gradually decreasing response to smaller doses appeared to place the cause of the decrease in response to an acquired tolerance rather than to a depletion of the available calcium.

A comparison of the responses obtained when "Parathormone" was given intravenously with those given intramuscularly or subcutaneously showed that by injecting directly into the blood stream, the maximum elevation in serum calcium was obtained in four to eight hours instead of fifteen to eighteen hours, and further the degree of response was less.

The first effect noted from injecting intravenously irradiated ergosterol was a depression in both the calcium and inorganic phosphate of the serum. It took about a week of repeated injections of large doses of the irradiated ergosterol before these constituents of the serum began to rise above the normal level. This was followed a few days later by a very rapid rise in the one dog but not in the other. This latent period was attributed to the fact that both dogs had been kept indoors all winter and were therefore low in vitamin D stores which apparently had to be built up by the irradiated ergosterol before the latter could elevate the calcium and inorganic phosphate in the serum. This seems to be borne out by the fact that dog 62, which had had a litter of seven pups about six weeks previous, showed but a slight rise in serum calcium following the first series of injections of irradiated ergosterol,

but

but with the second series of injections of the same amount of ergosterol showed an elevation in serum calcium similar to that observed in dog 55.

No antagonistic action of the parathyroid hormone to the calcium-raising power of irradiated ergosterol was found. Instead, the serum calcium rose still higher, and the dog showed all the symptoms of parathyroid hormone overdosage.

In contrast to the well known symptoms of parathyroid hormone overdosage, dog 62, whose serum calcium had been raised to 23.7 with irradiated ergosterol alone, remained alert and active despite the fact that the serum calcium stayed above 20 for five days. During the whole experimental period this dog remained alert and active and showed none of the depression assoviated with parathyroid hormone overdosage. Attacks of vomiting and diarrhoea were also absent. There was no respiratory distress. The phosphorus curve did not follow the course usually taken in parathyroid hormone overdosage, but tended to follow the general direction of the calcium curve.

No difficulty was experienced in drawing the blood samples, even when the serum calcium had been over the twenty-milligram level for five days. There appeared to be no increase in viscosity, and the blood was much slower in coagulating.

SUMMARY.

SUMMARY.

1. Cats have been found to show little or no rise in serum calcium within the first two hours after injecting parathyroid hormone intravenously, even when as much as one hundred units were given, and hence cannot be used, as suggested by Stewart and Percival, in assaying extracts of the parathyroid hormone.

2. The potency of the active principle obtained by acid hydrolysis was not altered by making the hydrolysate alkaline to phenolphthalein.

3. Acid hydrolysis freed the active principle of the parathyroid glands, but too long hydrolysis destroyed it. Using 1.5, 3, and 5 per cent hydrochloric acid, the rate of destruction increased with the strength of acid used. With these strengths of hydrochloric acid, the most potent extract was obtained by hydrolysing for forty-five minutes with 1.5 per cent acid.

4. The parathyroid glands exhibited a marked buffering action.

5. In the course of preparing active extracts, heating at 80 to 100° was found necessary in order both to free the active principle and to destroy enzymes which would otherwise inactivate the hormone.

6. Acid

6. Acid was also found essential for the freeing of the active principle from the glands.

7. Saponin, applied as in Harrington's method for purifying insulin, did not separate active and inactive fractions from "Parathormone", but destroyed all activity originally present.

8. Gradually approaching the isoelectric point from the acid side yielded an amorphous precipitate without a separation into active and inactive fractions.

9. Saturation of an alkaline solution with carbon dioxide removed all the activity in an amorphous precipitate which came down about pH 6.8. Smaller amounts of carbon dioxide failed to yield an inactive fraction.

10. The crystals, obtained when the active carbon dioxide precipitate was dissolved in alkali and alcohol added, were shown to be inactive and later were identified as sodium bicarbonate.

11. Continuous extraction with absolute ethyl alcohol did not alter the composition of the isoelectric precipitate.

12. Continuous extraction, first with absolute normal butyl alcohol, and then with saturated aqueous butyl alcohol, did not alter the composition of the isoelectric

precipitate

precipitate.

13. Addition of potassium acid phthalate to a solution of the active principle did not remove an inactive fraction.

14. Dogs responded better and more uniformly on a meat diet than they did on an oatmeal diet. This uniformity was further improved by the addition of cod-liver oil. Increasing the readily available calcium by feeding calcium lactate did not cause a more constant elevation of the serum calcium.

15. Extracts prepared from oatmeal gave no evidence for the presence in oatmeal of an interfering or antagonistic factor to the parathyroid hormone.

16. Decrease in response with repeated injections was found to be due to an increased tolerance rather than a depletion of readily available calcium.

17. Intravenous injections of the parathyroid hormone gave the maximum elevation of the serum calcium in four to eight hours, and the response was always less than when the hormone was given either subcutaneously or intramuscularly.

18. The rate and the degree which irradiated ergosterol raised the calcium and inorganic phosphate of the

serum

serum appeared to be greatly influenced by the store of calcium, phosphorus, and vitamin D in the body.

19. The parathyroid hormone in the presence of irradiated ergosterol did not depress the serum calcium, as Bischoff claimed to have found, but gave an additive effect.

20. The symptoms exhibited with hypercalcaemia due to irradiated ergosterol were quite different from those accompanying hypercalcaemia due to parathyroid hormone.

BIBLIOGRAPHY.

1. Cowdry: Endocrinology and Metabolism, New York and London, 1922, i, 501.

2. Sandström: Upsala Läkareför. forb., 1880, XV, 44.

3. Gley: Compt. rend. soc. de biol., Paris, 1891, III, 551, 841 and 843.

4. Kohn: Arch. f. mikv. Anat., Bonn, 1895, XLIV, 366.

5. Raynard: Compt. rend. des travaux de l'école royale vétérinaire de Lyon pendant l'année scolaire, 1834-35. Rec. de med. vét. Lyon, 1836, XIII, 8. Quoted from Sutherland Simpson, Endocrinology and Metabolism, 1922, I, 510.

6. Schiff: (a) Untersuchungen über die Zuckerbildung auf die Erzeugung, Würtzburg, 1895, Stohel VI, 156.

(b) Aub. f. exper. Path. u. Pharm., Leipzig, 1884, XVIII, 25.

(c) Rev. med. de la Suisse rom., Geneva, 1884.

Quoted from Endocrinology and Metabolism, I, 425.

7. Vassale and Generali: Arch. ital. de Biol., 1900, XXXIII, 154.

8. Berkeley and Beebe: J. Med. Research, Boston, 1909, XX, 149.

9. Biedl: The Internal Secretory Organs, New York, 1913, 56.

10.

10. MacCallum and Voegtlin: Jour. Exper. Med., 1909, XI, 118.

11. Koch: (a) Jour. Biol. Chem., 1912-13, XII, 313.
(b) " " " 1913, XV, 43.

12. Findlay and Sharpe: Quart. Jour. Med., 1920, XLIII, 433.

13. Nattrass and Sharpe: Brit. Med. Jour., 1921, II, 28.

14. Paton: Edinburgh Med. Jour., 1924, 541.

15. Loeb: Amer. Jour. Physiol., 1901, V, 352.

16. MacCallum: Jour. Amer. Med. Assoc., 1912, LIX, 319.

17. MacCallum, Lambert and Voegl: Jour. Exper. Med., 1914, XX, 149.

18. Luckhardt and Goldberg: Jour. Amer. Med. Assoc., 1923, LXXX, 79.

19. Salvesen: Jour. Biol. Chem., 1923, LVI, 443.

20. Greenwald: Jour. Biol. Chem., 1924, LXI, 33.

21. Collip: Jour. Biol. Chem., 1925, LXIII, 395.

22. Collip: Harvey Lectures of Harvey Soc., 1925-6, p.113.

23. Collip and Clark: Jour. Biol. Chem., 1925, LXIV, 485.

24. Moussu: Compt. rend. soc. de biol., Paris, 1898, 10 s. V, 867.

25. Berkeley and Beebe: Jour. Med. Research, Boston, 1909, XX, 149.

26. Berman: Proc. Soc. Exper. Biol. and Med., 1924, XXI, 465.

27. MacCallum: Medicine, 1924, III, 137.

28. Hanson: Mil. Surg., 1924, LIV, 78 and 218.

29. Collip and Clark: Jour. Biol. Chem., 1925, LXVI, 133.

30. Collip: Handbuch der biol. Arbeitsmethoden, 1928, Abt. V, Teil 3/B, S.803.

31. Macleod and Taylor: Trans. Roy. Soc. Canada, 1925, XIX, V, 27.

32. Cameron and Moorhouse: (a) Jour. Biol. Chem., 1925, LXIII, 687.

(b) Trans. Roy. Soc. Canada, 1925, XIX, V, 39.

33. Fisher and Larson: Amer. Jour. Physiol., 1925, LXXV, 93.

34. Hjort, Robison and Tendick: Jour. Biol. Chem., 1925, LXV, 117.

35. Tweedy: Proc. Soc. Exper. Biol. & Med., 1926, 24, 147.

36. Tweedy: Jour. Biol. Chem., 1930, LXXXVIII, 649.

37. Collip, Clark and Scott: Jour. Biol. Chem., 1925, LXIII, 439.

38. Lisser and Shepardson: Endocr., 1929, 13, 3, 427.

39. Kramer and Tisdall: Jour. Biol. Chem., 1922, LIII, 241.

40. Rona, Petow and Wittkower: Biochem. Z., 1924, CL, 468.

41. Greenberg, Ballard and Dalton: Proc. Soc. Exptl. Biol. & Med., 1930, 27, 514.

42. Morgulis and Perley: Jour. Biol. Chem., 1930, 88, 169.

43. Reiche: Klin. Wochschr., 1930, 9, 1209.

44. Cantarow, Dodek and Gordon: Tr. College Physicians, 1927, XLIX, 132.

45. Gunther and Greenberg: Arch. Internal Med., 1930, XLV, 983.

46. Ravdin, Riegel and Morrison: Ann. Surgery, 1930, XCI, 801.

47. Morgan and Garrison: Jour. Biol. Chem., 1930, LXXXV, 678.

48. Bodansky, Blair and Jaffe: Jour. Biol. Chem., 1930, LXXXVIII, 629.

49. Reiss, Winter and Halpern: Endokrinologie, 1929, V, 230.

50. Nitschke: Z. ges. expl.Med., 1929, LXV, 637.

51. Mirvish and Bosman: Quart. Jour. Exper. Physiol., 1927, XVIII, 11 and 29.

52. Mirvish and Bosman: Brit. Jour. Exp. Biol., 1929, VI, 4, p.350.

53. Mirvish: Biochem. Jour., 1930, XXIV, 2, p.233.

54. Mellanby, E.: Brit. Med. Jour., 1922, 2, p.849.

55. Mellanby, M.: Brit. Dent. J., 1923, XLIV, 1031.

56. Holst: Jour. Hyg., 1927, XXVI, 437.

57. Green and Mellanby: Biochem. J., 1928, XXII, 102.

58. Agnoli: Boll. soc. ital. biol. sper., 1930, V, 543.

59. Fine: Cereal Chemistry, 1930, VII, 456.

60. Bethke, Kiek and Wilder: Ohio Agr. Exper. Sta., 48th An. Rept. Bull. 1930, 446, 147.

61. Cooke: Jour. Exp. Med., 1910, XII, 45.

62. Greenwald and Gross: Jour. Biol. Chem., 1925, LXVI, 185.

63. Holt, LaMer and Chown: Jour. Biol. Chem., 1925, LXIV, 567.

64. Greenwald and Gross: Jour. Biol. Chem., 1925, LXVI, 217.

65. Greenwald and Gross: Jour. Biol. Chem., 1926, LVIII, 325.

66. Stewart and Percival: Biochem. J., 1927, XXI, 301.

67. Fine and Taylor: Am. Jour. Physiol., 1928, LXXXV, 368.

68. Taylor and Fine: Amer. Jour. Physiol., 1929, XC, 539.

69. Taylor and Fine: Am. Jour. Physiol., 1930, XCIII, 544.

70. Dixon, Davenport and Ranson: Jour. Biol. Chem., 1929, LXXXIII, 3, 737.

71. Tsai and Hsu: Chinese Jour. Physiol., 1930, IV, 3, 265.

72. Hsu and Tsai: " " " 1930, IV, 4, 423.

73. Shear

73. Shear and Kramer: Jour. Biol. Chem., 1928, LXXIX, 125.

74. Kramer and Shear: Jour. Biol. Chem., 1928, LXXIX, 147.

75. Jones, Rapoport and Hodes: Jour. Biol. Chem., 1930, LXXXIX, 647.

76. Bischoff: Z. physiol. Chem., 1930, 188, 247.

77. Watchorn: Biochem. J., 1930, XXIV, 5, 1561.

78. Fine and Brown: New Eng. J. Med., 1928, 198, 932.

79. Burns: Biochem. J., 1929, XXIII, 840.

80. Barr: M. Press, 1929, 127, 347.

81. Wilder: Endocrinology, 1929, XIII, 231.

82. Macchi: Pediatria Rivista, 1929, XXXVII, 1175.

83. Demole and Christ: Arch. expl. Path. u. Pharmakol., 1929, 146, 361.

84. Taylor, Bramon and Kay: J. Physiol., 1930, XXXV, 69.

85. Jones, Rapoport and Hodes: Jour. Biol. Chem., 1930, LXXXVI, 267.

86. Holtz and Schreiber: Z. physiol. Chem., 1930, 191, 1.

87. Rosello and Petrillo: Endocr., 1928, XII, 797.

88. Higgins and Sheard: Science, 1928, LXVII, 536.

89. Vines: Brit. Med. Jour., 1923, 2, 559.

90. White and Cameron: Trans. Roy. Soc. Canada, 1925, XIX, V, 45.

91. Burn: "Methods of Biological Assay", Oxford University Press, 1928.

92. Herxheimer: Klin. Woch., 1927, VI, 2268.

93. Thölldte: Krankheits forsch., 1928, VI, 397.

94. Collip: Trans. Roy. Soc. Canada, 1927, XXI, V, 151.

95. Emerson: Jour. Lab. Clin. Med., 1928, XIV, 195.

96. Hanson: Jour. Am. Med. Assn., 1928, XC, 747.

97. Clark and Collip: Jour. Biol. Chem., 1925, LXIII, 461.

98. Tisdall: Jour. Biol. Chem., 1923, LVI, 439.

99. Blum: Mett. Grenzab. Med. Chirurg., 1927, XLI, 569.

100. Clark and Lubs: Jour. Bacteriol., 1917, 2, 1, 109 & 191.

101. Dernby: Jour. Biol. Chem., 1918, XXXV, 179.

102. Bradley: Proc. Amer. Soc. Biol. Chem., 1921, V, 15.

103. Abel: Proc. Nat. Acad. Sc., 1926, XII, 132.

104. Harrington, Scott, Marks and Trevan: Biochem. J., 1929, XXIII, 3, 384.

105. Dakin: Biochem. J., 1918, XII, 290.
Jour. Biol. Chem., 1920, XLIV, 499.

106. Collip: In press.

107. Pugsley: Private Communication.